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

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ADP ribosylation factor 1 (Arf1) plays a critical role in regulating secretory traffic and membrane transport within the Golgi of eukaryotic cells. Arf1 is activated by guanine nucleotide exchange factors (ArfGEFs), which confer spatial and temporal specificity to vesicular transport. We describe here the discovery and characterization of golgicide A, a potent, highly specific, reversible inhibitor of the *cis*-Golgi ArfGEF GBF1. Inhibition of GBF1 function resulted in rapid dissociation of COPI vesicle coat from Golgi membranes and subsequent disassembly of the Golgi and *trans*-Golgi network. Secretion of soluble and membrane-associated proteins was arrested at the endoplasmic reticulum–Golgi intermediate compartment, whereas endocytosis and recycling of transferrin were unaffected by GBF1 inhibition. Internalized shiga toxin was arrested within the endocytic compartment and was unable to reach the dispersed *trans*-Golgi network. Collectively, these results highlight the central role for GBF1 in coordinating bidirectional transport and maintaining structural integrity of the Golgi.

ADP ribosylation factor proteins, or Arf proteins, are members of the Ras superfamily of small guanosine triphosphatases (GTPases) that mediate vesicular transport in the secretory and endocytic pathways. The Golgi-localized Arf1 is present in all eukaryotic cells and regulates both anterograde and retrograde traffic[1]. As expected for members of the Ras GTPase family, Arf1 cycles between its cytosolic GDP-bound form and its membrane-associated GTP-bound form. In its GTP-bound state, Arf1 recruits adaptor and vesicle coat proteins to initiate the formation and release of transport vesicles. More specifically, Arf1 recruits the heptameric coatomer complex at the *cis*-Golgi face, resulting in assembly of COPI coated vesicles, while clathrin adaptor proteins are recruited to the *trans*-Golgi network (TGN) and endosomes.

The Arf activity cycle is initiated by the interaction of Arf1 with guanine nucleotide exchange factors (GEFs), which exchange GDP for GTP and allow for spatiotemporal activation of Arf1. The ArfGEFS for Arf1 are divided into two families: the large brefeldin A (BFA; **1**)-susceptible molecules, which localize to the Golgi and TGN, and the smaller BFA-resistant ARNO-family GEFs, which predominantly localize to endosomes[2,3]. In mammalian cells, the BFA-susceptible ArfGEFs include GBF1 (Golgi BFA resistance factor 1), a *cis*-Golgi–localized GEF that assists in the recruitment of coat protein COPI[4–6], and BIG1 and BIG2, two functionally similar TGN-localized GEFs that facilitate recruitment of clathrin coat protein[7,8]. After activation by GBF1, Arf1-mediated COPI coat recruitment enables vesicle transport between the Golgi and endoplasmic reticulum[9]. Activation by BIG1 and BIG2 results in Arf1 recruitment of adaptor proteins (AP-1, AP-3 and AP-4), which mediate transport between endosomes and either the TGN or lysosomes, and Golgi-associated γ-adaptin ear-containing Arf-binding proteins (GGA1, GGA2 and GGA3), monomeric proteins involved in trafficking from the TGN and within the endosomal compartment[10–15].

Recently, the function of individual ArfGEFs has been explored. Among the ArfGEFs, GBF1 has been studied most intensively, either by small interfering RNA (siRNA)-mediated silencing[4,16–18] or by the expression of dominant-negative forms[5,19–21]. The phenotypic and functional effects of these perturbations have not been in complete agreement, but nevertheless they indicate that GBF1 is essential to intra-Golgi transport. We describe here the discovery and characterization of a potent, highly specific, rapidly reversible small-molecule inhibitor of GBF1 function. This compound, which we called golgicide A (GCA; **2**), reveals diverse and distinct roles for GBF1 in maintaining structure and bidirectional transport through the Golgi and *trans*-Golgi network.

RESULTS

Golgicide A is an inhibitor of shiga toxin cytotoxicity

From a high-throughput screen for small molecules that inhibit the effect of bacterial toxins on host cells[22], we identified a compound that potently and effectively protected Vero cells from shiga toxin. This compound, which we named golgicide A (**Fig. 1a**), inhibited the effect of shiga toxin on protein synthesis with a half-maximal inhibitory

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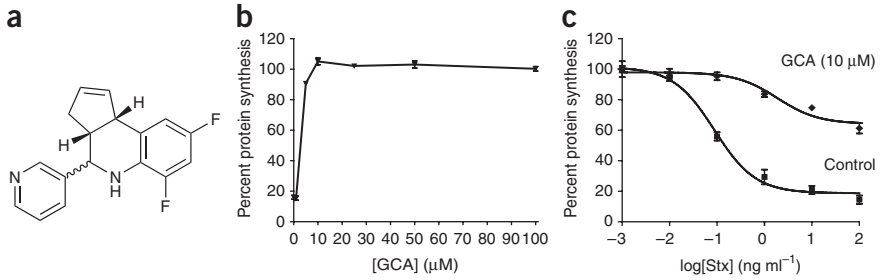

Figure 1 Discovery of GCA as a potent and effective inhibitor of Stx susceptibility. (**a**) Structure of GCA. (**b**) Vero cells were pretreated for 30 min at 37 °C with varying concentrations of GCA, followed by incubation with Stx (1 ng ml^{-1}) for 4 h at 37 °C. Percent protein synthesis indicates the amount of radioactive amino acid incorporation in GCA-treated cells as a percentage of radioactive amino acid incorporation in cells lacking Stx treatment (control). Protein synthesis levels and compound-response curves were determined as described in the Methods. (**c**) Protein synthesis levels for control (squares; no compound) and GCA-treated (triangles; 10 μM) Vero cells were determined using the radioactive amino acid incorporation assay as described in **b**. Percent protein synthesis is expressed as the amount of radioactive amino acid incorporation in untreated or GCA-treated cells at a given toxin concentration as a percentage of radioactive amino acid incorporation in cells lacking Stx treatment. Toxin IC_{50} values for GCA-treated cells were significantly increased over control cells ($P < 0.01$; see Methods). For **b** and **c**, data points (mean ± s.d.) represent triplicate data at the indicated compound or toxin concentrations, respectively, from one representative experiment. Stx, shiga toxin; GCA, golgicide A.

concentration (IC_{50}) of 3.3 μM (**Fig. 1b**). When treated at a concentration of 10 μM, Vero cells where highly protected against the effects of shiga toxin (**Fig. 1c**).

GCA causes Golgi and TGN dissociation and dispersal

Other compounds identified in this screen acted through the inhibition of intracellular toxin transport[22], leading us to examine the effect

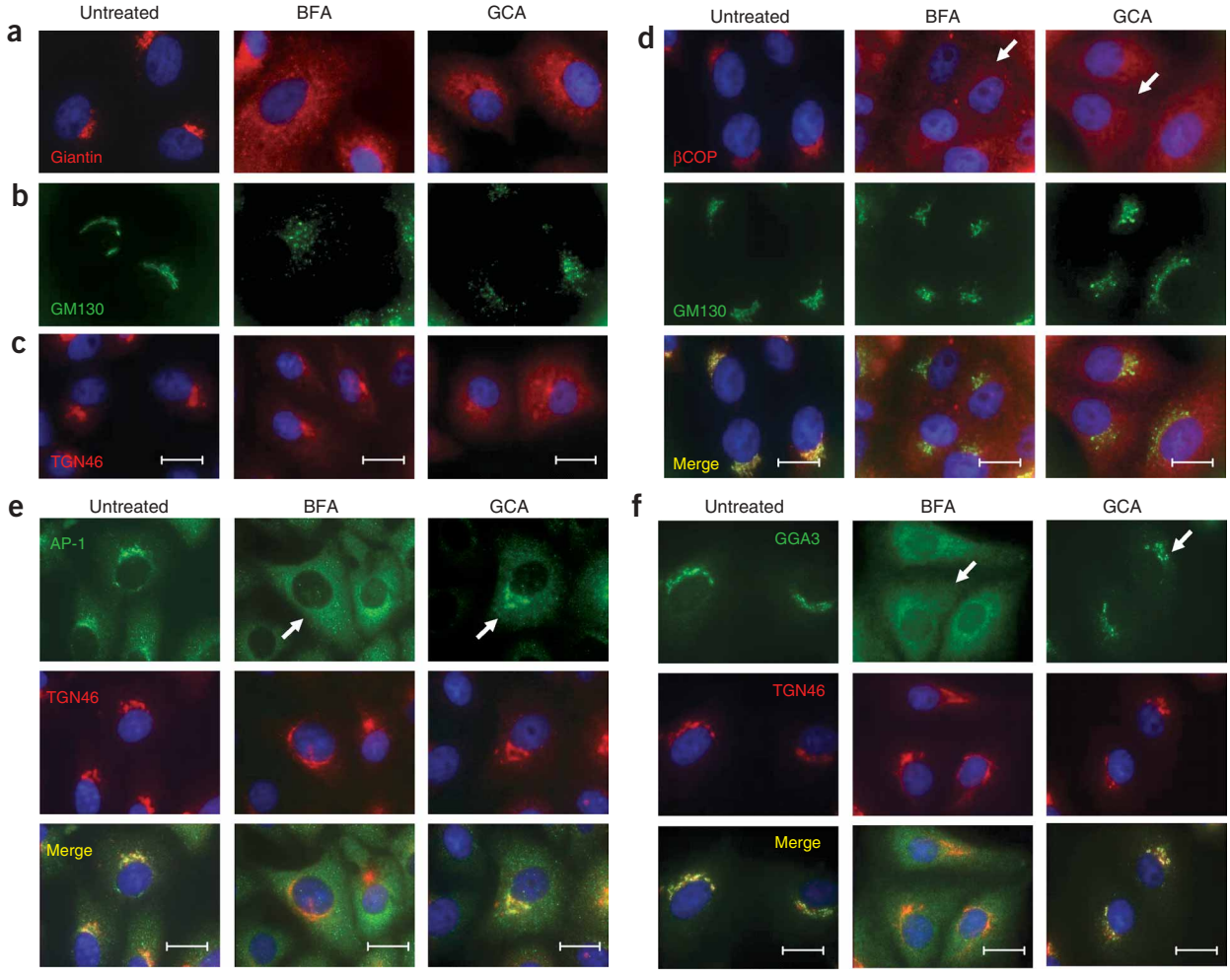

Figure 2 GCA disperses *medial*- and *cis*-Golgi, inhibits COPI recruitment and maintains localization of AP-1 and GGA3 to TGN. (**a**–**c**) Vero cells were either left untreated or treated with BFA (10 μg ml^{-1}) or GCA (10 μM) for 1 h, and the localization of giantin (*medial*-Golgi) (**a**), GM130 (*cis*-Golgi) (**b**) and TGN46 (TGN) (**c**) was determined (see Methods). The distribution of *medial*- and *cis*-Golgi was similar in BFA- and GCA-treated cells, while GCA did not induce tubulation of the TGN as observed in BFA-treated cells. (**d**) Vero cells treated for 5 min with BFA (10 μg ml^{-1}) or GCA (10 μM) show dispersed COPI staining (arrows) that does not colocalize with the Golgi (green) as in untreated cells. (**e**,**f**) Unlike BFA, GCA maintains AP-1 (**e**) and GGA3 (**f**) association with the TGN after 5 min of treatment (see arrows). BFA, brefeldin A; blue, nuclei. White scale bars, 20 μm.

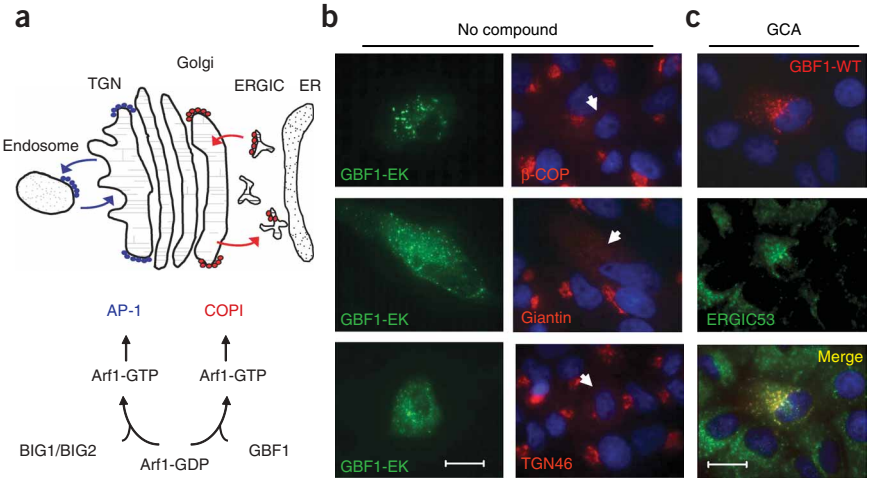

Figure 3 The effects of GCA are similar to expression of inactive GBF1-E794K. (**a**) Schematic diagram of the roles of GBF1, BIG1 and BIG2 in Arf1 activation. Arf1 in the inactive (GDP-bound) form is localized to the cytoplasm. Exchange of GDP for GTP by either BIG1/BIG2 or GBF1 results in local Arf1 activation, membrane localization and subsequent recruitment of vesicle coat proteins such as AP-1 or COPI. (**b**) Vero cells expressing the GBF1-EK mutant show dispersal of βCOP, the *medial*-Golgi (giantin) and the *trans*-Golgi network (TGN46). Arrows indicate the effect of GBF1-E794K expression on giantin, βCOP or TGN46 localization. Blue, nuclei. (**c**) GBF1-WT is predominantly membrane associated in GCA-treated cells and is distributed to punctate structures that colabel with anti-ERGIC53. White scale bars, 20 μm.

of GCA on toxin trafficking and on intracellular organelle morphology. Immunofluorescence experiments demonstrated substantial effects of GCA on the Golgi and TGN. Whereas the Golgi remains as a tightly organized perinuclear ribbon in untreated cells, GCA caused complete dispersal of the *medial*-Golgi marker giantin (**Fig. 2a**) and the *cis*-Golgi marker GM130 (**Fig. 2b**). These morphological effects on the *cis*- and *medial*-Golgi were highly reminiscent of those caused by BFA (refs. 23,24). Time-course experiments revealed that the Golgi initially became extensively tubulated before its complete dispersal (**Supplementary Fig. 1a** online), which is similar to the effects of BFA on *cis*- and *medial*-Golgi. Treatment with GCA resulted in a diffuse and punctate distribution of the *medial*-Golgi marker giantin. The punctate structures were in contact with Sec31-positive foci, which is indicative of their association with endoplasmic reticulum exit sites (ERES; **Supplementary Fig. 1b**). These findings were very similar to observations of the effects of BFA, which causes Golgi matrix proteins to concentrate at ERES (ref. 25). The effects of these compounds on the TGN, however, were subtly different. Whereas BFA induced the formation of tubules derived from TGN and endosomes, GCA caused the TGN to disperse into small vesicles that subsequently disseminated throughout the cell (**Fig. 2c** and **Supplementary Fig. 2** online).

The morphologic effects of GCA did not result from disruption of microtubules or actin cytoskeleton (**Supplementary Fig. 3** online), and GCA did not interfere with transit of transferrin through the endocytic and recycling pathways (**Supplementary Fig. 4a**,**b** online). Finally, the effects of GCA were found to be rapidly reversible. Within 15 min of removing the compound, the Golgi and TGN began to reassemble (**Supplementary Fig. 5a** online). The effects of GCA on protein secretion (**Supplementary Fig. 5b**) were likewise found to be completely reversible within 1 h of compound removal.

GCA disperses COPI but not AP1 or GGA3 from Golgi

BFA inhibits Arf1 activation, resulting in the rapid dispersal of Golgi-associated vesicle coat proteins COPI and AP-1 at the Golgi and TGN, respectively[26–28]. Because the effects of GCA on the Golgi were similar to those of BFA, we compared the effects of these compounds on COPI, AP-1 and GGA3 localization. GCA treatment resulted in a rapid redistribution of COPI from the Golgi that was evident within 5 min and occurred before morphologic changes to Golgi structure, which is similar to the effect of BFA (**Fig. 2d**). In contrast, AP-1 (**Fig. 2e**) and GGA3 (**Fig. 2f**) remained associated with the TGN until the Golgi and TGN began to disperse. These results were distinct from those observed with BFA, which caused rapid dispersal of AP-1 and GGA3 to a diffuse cytoplasmic distribution within minutes of BFA addition.

In summary, GCA and BFA have similar phenotypic effects on *medial*- and *cis*-Golgi that correlate with rapid dispersal of COPI from Golgi membranes. In contrast, these two compounds have differing morphologic effects on the TGN and have differing effects on AP-1 and GGA3 localization.

Effects of GCA are similar to dominant-inactive GBF1

The observation that GCA rapidly dispersed βCOP (a component of the COPI coat) but not AP-1 or GGA3 from Golgi membranes suggested that GCA may specifically target GBF1, the ArfGEF responsible for Arf1 activation and COPI recruitment to *cis*-Golgi membranes, and not affect BIG1 or BIG2, the ArfGEFs responsible for Arf1 activation and vesicle coat recruitment at endosomes and TGN (**Fig. 3a**). Consistent with the possibility that GCA inhibited GBF1 activity, we found that expression of GBF1-E794K, a dominant-inactive GBF1 (ref. 21), resulted in dispersal of βCOP from Golgi membranes and disruption of TGN and *medial*-Golgi structure (**Fig. 3b**), much like the effects of GCA. The dominant-inactive GBF1 was previously shown to localize to the endoplasmic reticulum–Golgi intermediate compartment (ERGIC)[21], and we found that upon GCA treatment, wild-type GBF1 was largely colocalized with ERGIC-53, a marker for this compartment (**Fig. 3c**). Therefore, treatment with GCA phenotypically resembled expression of dominant-inactive GBF1, thereby further supporting the possibility that GBF1 is the target of GCA.

We next examined the effect of BIG1 overexpression on susceptibility to BFA and GCA. Overexpression of BIG1 was recently shown to partially rescue the effects of BFA on the TGN (ref. 16). We confirmed that BIG1 transfection was partially protective against BFA effects on the TGN (**Supplementary Fig. 6a** online) and had no protective effect at the Golgi (**Supplementary Fig. 6b**). By comparison, expression of BIG1 had no protective effect against GCA on either the TGN or Golgi (**Supplementary Fig. 6a**,**b**).

GCA is selective for GBF1

In the course of examining the effect of GCA on various cell types, we found that MDCK cells were resistant to GCA as evidenced by a lack of effect of this compound on Golgi morphology and βCOP localization (**Supplementary Fig. 7a** online). The Golgi apparatus of MDCK cells has also been reported to be resistant to the effects of BFA (refs. 29–31). Because BFA is known to bind within the highly

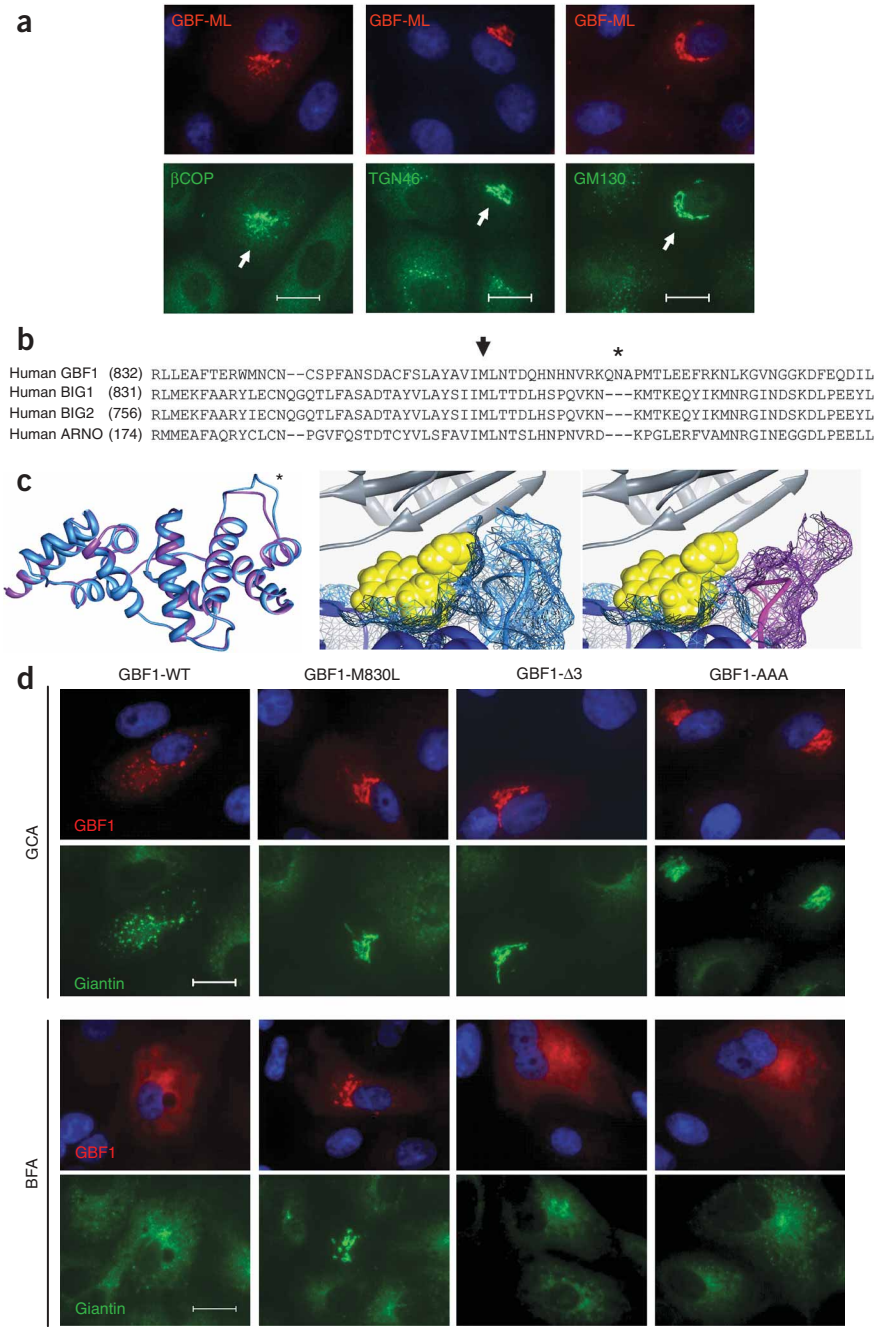

Figure 4 GCA is selective for GBF1. (**a**) Expression of GBF1-M832L (GBF1-ML) results in protection from the effects of GCA. Vero cells were transiently transfected with GBF1-ML and exposed to 100 μM GCA. βCOP, TGN46 and GM130 localizations were resistant to the effects of GCA. Arrows indicate cells expressing GBF1-ML. White scale bars, 20 μm. (**b**) Comparison of the amino acid sequence of Sec7 domains reveals considerable divergence between BIG1, BIG2 and GBF1. Particularly notable was the presence of three extra residues in the GBF1 sequence (asterisk). Arrows indicate cells expressing GBF1-ML. (**c**) The GBF1 Sec7 domain (residues 693 to 887) was thread on the previously reported ARNO–Arf1–BFA complex. Only the Sec7 domains of GBF1 (blue) and ARNO (purple) are shown (left panel). An asterisk indicates a tripeptide insertion in GBF1 sequence. GCA was docked into the BFA binding pocket (middle panel, light blue). The corresponding region of ARNO, BIG1 and BIG2 lacks the tripeptide (right panel, purple) and does not contact GCA. In these images, GCA is yellow and Arf1 ribbon diagram is gray. (**d**) Mutagenesis of the tripeptide results in resistance to GCA but not BFA. Cells were transiently transfected either with GBF1-WT, GBF1-ML, GBF1-Δ3 (QNV deleted) or GBF1-AAA (QNV to AAA) and then exposed to GCA (10 μM; top panel) or BFA (10 μg ml^{-1}; bottom panel) for 1 h and labeled with antibodies against hemagglutinin and giantin. White scale bars, 20 μm.

conserved Sec7 domain of Golgi-associated ArfGEFs, and because we suspected GBF1 to be the target of GCA, we examined the possibility that differences in the canine GBF1 Sec7 domain accounted for MDCK cell resistance to both compounds. We amplified, cloned and sequenced the canine GBF1 Sec7 domain. Analysis of several independent clones revealed a leucine substitution for methionine at residue 832 of the full-length protein (numbering corresponds to the human GBF1 sequence). Comparison with all other mammalian GBF1 homologs revealed this substitution to be unique to the canine gene (**Supplementary Fig. 7b**). Notably, mutagenesis of the corresponding methionine to leucine in the yeast ArfGEF homolog Gea1 and the human GBF1 resulted in resistance to BFA (refs. 32,33).

The M832L substitution was introduced into the hamster GBF1 and the effect of its expression on GCA susceptibility was examined in transfected cells. Vero cells expressing this mutant were highly resistant to GCA, as evidenced by maintenance of βCOP localization, TGN morphology and *cis*-Golgi structure, even at compound concentrations as high as 100 μM (**Fig. 4a**). As described later, expression of GBF1-M832L rescued the functional effects of GCA on secretion and on retrograde toxin transport. The ability of this mutant to fully protect Vero cells against the phenotypic and functional effects of this compound indicates that GCA specifically targets GBF1 and does not have evident off-target effects.

The mechanism of GCA specificity for GBF1 was investigated by molecular modeling and site-directed mutagenesis. The GBF1 Sec7 domain was modeled in complex with Arf1 using the published structure of the Arf1–ARNO–BFA complex[34]. Although there is considerable sequence divergence between GBF1 and BIG1 and BIG2 (**Fig. 4b**), the predicted tertiary structure of the GBF1 Sec7 domain is very similar to that of ARNO (**Fig. 4c**). When the ARNO-Arf1 and predicted GBF1-Arf1 complexes were compared, the BFA-interacting regions of the interfacial cleft were virtually identical. Nevertheless, the observation that the GBF1-M832L mutant was resistant to both BFA and GCA suggested that these compounds may bind within the same GBF1-Arf1 interfacial region. When GCA was docked into this pocket, it was found to extend past the BFA binding region to contact a tripeptide loop that exists in GBF1 and is lacking in other ArfGEFS, including ARNO, BIG1 and BIG2 (**Fig. 4c**).

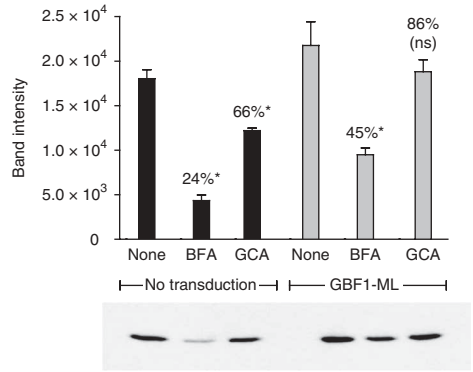

Figure 5 GCA causes a decrease in GBF1-dependent Arf1 activation. Vero cells transduced with Arf1-V5 alone or Arf1-V5 plus GBF1-M832L-HA were exposed either to no compound (none), BFA (10 μg ml^{-1}) or GCA (10 μM) for 1 h. The cells were then lysed and the extracts were incubated with immobilized GST-GGA3. Bound proteins were released and separated by SDS-PAGE. Arf1-V5 was detected by western blot and band intensity was determined using ImageJ software (http://rsb.info.nih.gov/ij/index.html). Statistical analysis was performed on duplicate experiments. Asterisk, statistically significant difference from untreated sample ($P = 0.05$); ns, not significantly different from untreated sample. Error bars, s.d.

The contribution of the GBF1 tripeptide extension to GCA susceptibility was investigated by mutagenesis. Deletion of residues 845 to 848 or substitution of alanines for all three residues resulted in resistance to GCA, as indicated by the ability of these mutants to maintain Golgi morphology in the presence of the compound (**Fig. 4d**). However, as expected, these mutants remained susceptible to BFA (**Fig. 4d**), as this loop lies outside the BFA binding pocket and would not be expected to contribute to BFA susceptibility (**Supplementary Fig. 8a** online). Further mutagenesis of individual residues revealed that substitution of alanine for Arg843, Glu845 or Asp846 resulted in loss of susceptibility to GCA, whereas none of these residues were required for BFA susceptibility (**Supplementary Fig. 8b**). Lys844, which protrudes from the opposite side of the GBF1 loop, did not affect GCA susceptibility. In summary, these results reveal that GCA susceptibility is dependent on residues within a tripeptide found within the GBF1 Sec7 domain.

GCA causes a decrease in GBF1-mediated Arf1 activation

GBF1 facilitates the exchange of GDP for GTP on Arf1 (refs. 5,6). To determine whether GCA affected GBF1-mediated Arf1 activation, cells were treated with this compound or with BFA and Arf1-GTP was isolated from cellular extracts[35]. GCA caused a consistent and statistically significant ($P < 0.05$) decrease of 34% in Arf1 activation *in vivo* (**Fig. 5**). BFA caused a greater decrease in Arf1-GTP (approximately 75% compared with untreated control), which was expected given this compound's more promiscuous effects on ArfGEFs. To directly determine whether the effect of GCA on Arf1 activation was due to inhibition of GBF1 function, cells were transduced with the GCA- and BFA-resistant GBF1-M832L mutant and Arf1-GTP levels were assessed in cells treated with these compounds. Whereas GBF1-M832L expression restored Arf1-GTP to approximately 45% of control in BFA-treated cells, Arf1 activation was increased to 86% of control in GCA-treated cells (**Fig. 5**). Notably, expression of GBF1-M832L resulted in increased cellular Arf1-GTP levels in untreated cells.

In summary, GCA caused a decrease in Arf1 activation that was attenuated in cells expressing the GCA-resistant mutant. The inability of GBF1-M832L overexpression to completely restore Arf1-GTP levels in GCA-treated cells to the levels of untreated cells was likely due to the fact that GBF1-M832L expression was not observed in all of the cells (**Supplementary Fig. 9** online). BFA caused a much larger decrease in cellular Arf1-GTP, and the effect was only partially reversed by the expression of GBF1-M832L. Both of these observations are explained by BFA effects on ArfGEFs other than GBF1. Indeed, these data provide a rough estimate of the relative contribution of GBF1 and other ArfGEFs to cellular Arf1 activation. These data suggest that in Vero cells growing under tissue culture conditions, GBF1 accounts for approximately 30% of cellular Arf1 activation, BIG1 and BIG2 account for approximately 45%, and BFA-resistant Arf1GEFS such as ARNO account for the remainder.

Inhibition of GBF1 function arrests protein secretion

Having demonstrated that GCA is a specific inhibitor of GBF1 function, we used this compound to examine the role of GBF1 in secretory transport. Previous studies with a dominant-inactive mutant indicate that GBF1 function is required for maturation of endoplasmic reticulum–Golgi intermediate vesicles to a transport-competent state[4,5]. Recent studies with siRNA-mediated inhibition suggest that GBF1 is required for anterograde transport of membrane-anchored cargo but is not required for secretion of soluble molecules[4].

To assess the role of GBF1 in secretion of membrane-anchored proteins, we examined the effect of GCA on transport of a green fluorescent protein (GFP)-tagged, temperature-sensitive VSV-G (vesicular stomatitis virus G protein) mutant (tsVSVG-GFP)[36]. At the nonpermissive temperature of 40 °C, this protein is retained and accumulates in the endoplasmic reticulum. Following a shift to the permissive temperature (32 °C), tsVSVG-GFP transits through the ERGIC to the Golgi and ultimately to the plasma membrane. Cells were either transfected with tsVSVG-GFP alone or cotransfected with plasmid encoding GBF1-WT or GBF1-M832L, and the fate of tsVSV-G was followed in GCA-treated cells. In untreated cells, tsVSVG-GFP was transported from the endoplasmic reticulum to the Golgi within 60 min and was located predominantly at the plasma membrane by 4 h (**Fig. 6a**). GCA treatment, however, caused tsVSVG-GFP to be partially retained both in a reticular, endoplasmic reticulum–like distribution and in diffuse punctate structures (**Fig. 6b**). GBF1-WT overexpression did not overcome the block in tsVSVG-GFP secretion in GCA-treated cells, whereas cotransfection with GBF1-M832L restored tsVSVG-GFP transport to the plasma membrane (**Fig. 6c**). Expression of the GCA-resistant GBF1 loop mutants also restored transport of tsVSVG-GFP to the plasma membrane in the presence of GCA (**Supplementary Fig. 10** online).

GCA treatment did not completely block tsVSVG-GFP transport from the endoplasmic reticulum, as this protein was also found in peripheral punctate structures after 60 min incubation. These structures were identified as the endoplasmic reticulum–Golgi intermediate compartment by their labeling with anti-ERGIC53, which indicates that tsVSVG-GFP was capable of transport from the endoplasmic reticulum to the ERGIC in cells lacking GBF1 function (**Fig. 6d**).

To monitor the secretion of soluble cargo proteins, we expressed GFP bearing a neuropeptide Y secretion signal (NPY-GFP)[37]. This protein is secreted from Vero cells with a half-life of approximately 60 min, as judged by pulse chase experiments[22]. Vero cells expressing NPY-GFP demonstrated markedly decreased GFP secretion in the presence of GCA. If inhibition of NPY-GFP secretion in GCA-treated cells was solely due to the inhibition of GBF1 function, then expression of the GCA-resistant GBF1-M832L mutant should restore protein secretion to levels seen with untreated cells. Therefore, the effect of expressing GBF1-WT or GBF1-M832L on GFP secretion was assessed

in GCA-treated cells. Whereas cells transduced with GBF1-WT failed to transport NPY-GFP to the plasma membrane in the presence of GCA, the expression of GBF1-M832L restored of NPY-GFP transport through the Golgi to the plasma membrane (**Fig. 6e**). Together, these findings reveal that GBF1 function is required for secretion of both soluble and membrane-associated model proteins.

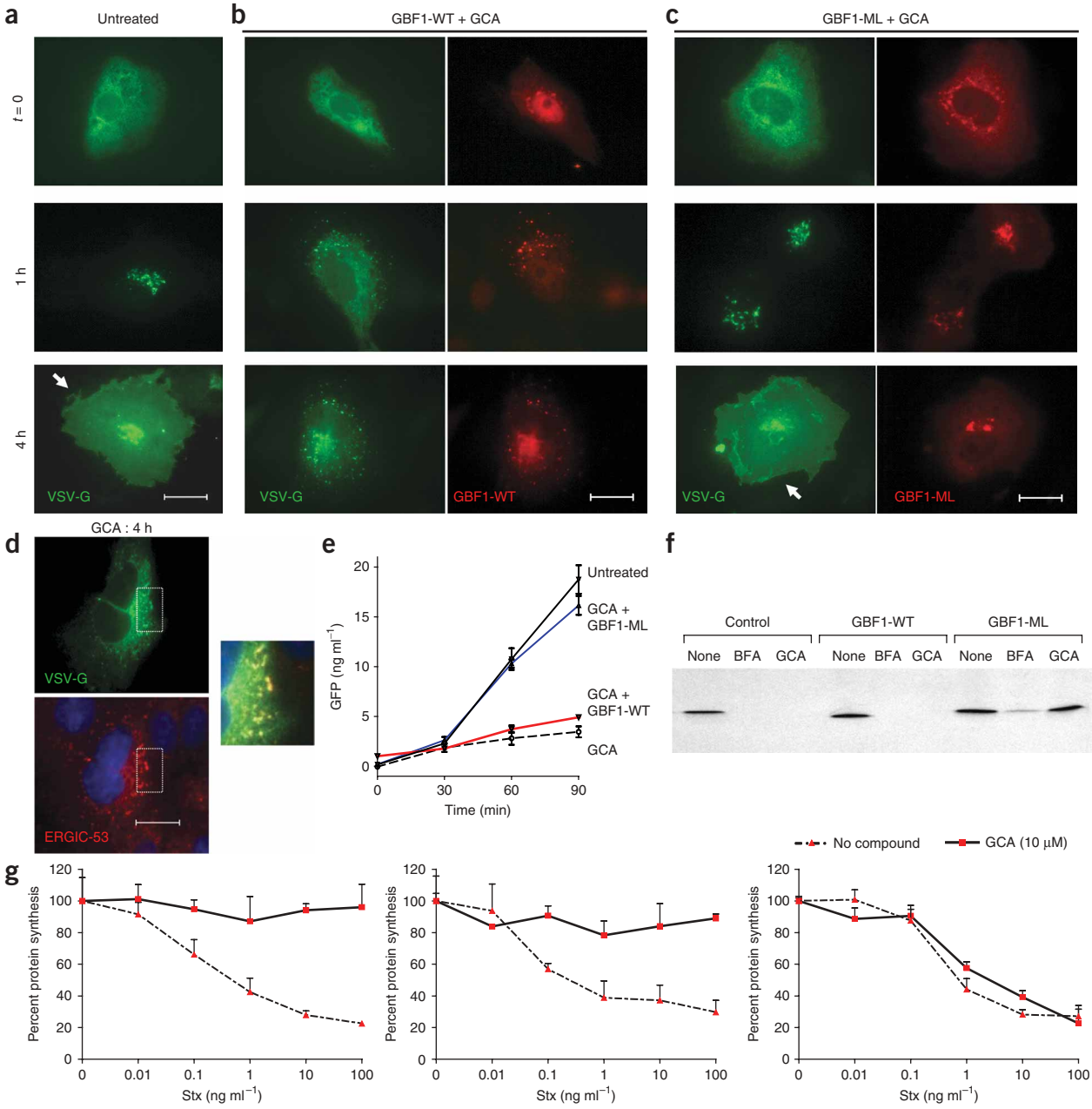

Figure 6 GBF1 inhibition arrests secretion of membrane-anchored and soluble proteins. (**a**–**c**) Vero cells were cotransfected with tsVSVG-GFP and either no additional plasmid (**a**), wild-type GBF1 (GBF1-WT) (**b**) or the GBF1-M832L mutant (GBF1-ML) (**c**). Transport of tsVSVG-GFP was assessed in the presence of either no compound (**a**) or GCA (10 μM) (**b**,**c**) as described in **Supplementary Methods**. Expression of the GBF1-ML mutant restored tsVSVG-GFP transit to the plasma membrane (arrow). White scale bars, 20 μm. (**d**) tsVSVG-GFP colocalizes with ERGIC53 in GCA-treated cells. Vero cells transfected with tsVSVG-GFP were incubated at 40 °C before a shift to 32 °C for 4 h in the presence of GCA (10 μM). The cells were fixed and labeled with an anti-ERGIC53 antibody (red). Inset shows magnification of boxed area and colocalization of VSVG with the ERGIC (yellow). White scale bar, 20 μm. (**e**) GCA inhibits secretion of soluble cargo. Cells were either transduced with adenovirus expressing NPY-GFP alone or cotransduced with NPY-GFP plus adenovirus expressing GBF1-WT or GBF1-ML. The cells were left untreated or were exposed to GCA (10 μM), and GFP concentration was assessed by ELISA (**Supplementary Methods**). Error bars, s.d. (**f**) GCA inhibits toxin transport to the TGN. Control Vero cells, or those transduced with GBF1-WT or GBF1-ML, were preincubated with no compound, BFA (10 μg ml^{-1}) or GCA (10 μM), then StxB-SS-His was added and its transit to the TGN was assessed by measuring toxin sulfation as described in **Supplementary Methods**. (**g**) GCA inhibits shiga toxicity and is completely reversed in cells expressing GBF1-ML. Cells were either left untransduced or transduced with adenovirus expressing GBF1-WT or GBF1-ML and were exposed either to GCA (solid line) or to no compound (dotted line) before treatment with increasing concentrations of Stx. After 4 h of incubation, protein synthesis was assessed as described in **Supplementary Methods**. Error bars, s.d.

Inhibition of GBF1 impairs retrograde toxin transport

We next assessed the role of GBF1 in endocytic and retrograde transport pathways by tracking the intracellular fate of cholera toxin (Ctx), shiga toxin (Stx) and transferrin (Tfn). These ligands bind to receptors at the plasma membrane and are transported in retrograde direction to recycling endosomes. From there, Tfn recycles back to the plasma membrane, whereas Stx and Ctx are transported from endosomes to the TGN, eventually reaching the endoplasmic reticulum via the Golgi. As described earlier, GCA treatment did not affect the transport of Ctx or Tfn to perinuclear recycling endosomes, nor did it affect the rate of Tfn recycling to the plasma membrane. These results indicate that GBF1 function is not required for transport through endocytic transport pathways.

We next examined the effect of GCA on transport of Stx from endosomes to the TGN using a Stx B subunit that bears overlapping tyrosine sulfation sites (StxB-SS)[22]. During its transport through the retrograde transport pathway, this protein is transported from endosomes to the TGN, where it is sulfated by resident tyrosyl-protein sulfotransferases[38,39]. However, treatment with BFA and GCA resulted in marked attenuation of toxin sulfation. Expression of GBF1-WT failed to rescue the transport of StxB-SS to the TGN in BFA- and GCA-treated cells. Expression of GBF1-M832L only partially restored toxin sulfation to control levels in BFA-treated cells, presumably because this compound also inhibits BIG1 and BIG2. However, expression of GBF1-M832L completely restored toxin sulfation to control levels in GCA-treated cells (**Fig. 6f**). These results indicate that GBF1 function is required for toxin transport from endosomes to the TGN. Whether GBF1 functions directly in endosome-to-TGN transport, or indirectly through its effects on maintaining Golgi structure, is currently unknown.

GCA was identified in a high-throughput screen for its ability to inhibit the effects of shiga toxin on mammalian cells. The data presented thus far indicate that retrograde toxin transport was arrested within the endocytic compartment. To determine whether the effects of GCA on toxin transport were due solely to GBF1 inhibition, we examined the ability of GBF1-M832L to restore toxin susceptibility to GCA-treated cells. Cells were transduced with the appropriate GBF1 constructs and treated with GCA. Toxin susceptibility was highly attenuated in control cells and those transduced with GBF1-WT. In contrast, Stx susceptibility was fully restored in GCA-treated cells expressing GBF1-M832L, which indicates that the effects of GCA on toxin susceptibility were solely due to GBF1 inhibition (**Fig. 6g**). Together, these results indicate that GBF1 function is not required for transport of bacterial toxins to recycling endosomes, but this ArfGEF is required for retrograde transport of Stx from endosomes to the TGN and Golgi.

DISCUSSION

Phenotypic screens aimed at identifying inhibitors of endocytic and secretory transport have uncovered small molecules that are highly useful probes of intracellular transport. Exo1 (**3**) (ref. 40) and Exo2 (**4**) (ref. 41) were uncovered from an image-based screen for inhibitors of secretory transport and exhibited Golgi-disruptive effects. Studies with Exo2 revealed that treatment with this compound ablated the Golgi but maintained TGN integrity and suggested the existence of an alternative pathway for cholera toxin transport. Exo1 exhibited BFA-like effects on Arf1 dissociation from Golgi membranes, but unlike BFA, it did not interfere with ArfGEF activity. A high-throughput screen for inhibitors of dynamin GTPase activity discovered dynasore (**5**), a particularly useful probe for studying the dynamics of dynamin-mediated clathrin coat formation[42,43]. Similarly, secramine (**6**) was identified as a new tool for dissecting the functions of Cdc42 and possibly other RhoGTPases[44]. Finally, SecinH3 (**7**), an inhibitor of the cytoadhesins, was recently identified; its use revealed a role for these BFA-insensitive ArfGEFs in insulin signaling[45]. Together, these studies underscore the utility of small molecules as biological probes for dynamic intracellular processes.

Herein we describe the identification and characterization of GCA, a potent, highly effective, rapidly reversible inhibitor of GBF1. Mutagenesis and molecular modeling revealed that GCA binds within an interfacial cleft formed between Arf1 and the GBF1 Sec7 domain. Notably, BFA binds within this cleft[46,47], and substitution of Met832 to leucine results in resistance to BFA (refs. 32,33), and to GCA. However, GCA extends beyond the BFA binding cleft to contact a tripeptide loop present in GBF1 but lacking from all other known Arf1GEFS, thereby accounting for its selectivity.

Having demonstrated the highly selective nature of GCA activity, we used the compound to delineate the role of GBF1 in anterograde and retrograde transport through the Golgi. We found that transport of tsVSVG-GFP was arrested in the endoplasmic reticulum–Golgi intermediate compartment, which indicates that GBF1 function was not required for exit of cargo from the endoplasmic reticulum, but was required for transport past the ERGIC. These results are similar to those found with expression of a dominant-negative GBF1 and by siRNA-mediated inhibition of GBF1 (ref. 48). Inhibition of GBF1 function with GCA also blocked secretion of GFP bearing a secretion signal. This finding differs from those of RNA interference–based studies, which found relatively mild effects of GBF1 inhibition on Golgi morphology and which suggested GBF1 was not required for secretion of soluble cargo[4]. The relatively mild effects of GBF1 inhibition found by RNAi likely resulted from less than complete inhibition of GBF1 expression, as functional inhibition by GCA resulted in marked disruption of Golgi morphology and an absolute block in secretion of soluble and transmembrane proteins.

The role of GBF1 in retrograde transport to the Golgi has not previously been investigated. We demonstrate that endocytic cargo is transported normally through recycling endosomes in the presence of GCA, which indicates that GBF1 function is not required for these pathways. However, upon inhibition of GBF1 function, bacterial toxins are trapped in the endocytic compartment and are unable to reach the dispersed TGN, which indicates that GBF1 function is required to maintain communication between endosomes and the TGN.

In summary, GCA revealed essential roles for GBF1 in maintaining Golgi structure and enabling anterograde and retrograde traffic through the Golgi and TGN. Given its specificity, efficacy and reversibility, GCA should be a unique and powerful tool for further elucidating the mechanisms underlying assembly and transport within the Golgi.

METHODS

Chemical characterization of GCA. GCA was identified from a commercial compound library available from ChemDiv. Analysis of the screening DMSO solution by reverse-phase LC-MS revealed the compound to be present as a 10:1 diastereomeric mixture. The compound was resynthesized using a published procedure (**Supplementary Methods** online), which also resulted in a 10:1 diastereomeric mixture that was determined to be identical to that obtained from the screening library by ^{1}H NMR and LC-MS. Purification of the major isomer was achieved by recrystallization from acetonitrile and water. The biological activity of this purified major isomer was identical to that of the material obtained from the library DMSO solution. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.78 (d, J = 1.5 Hz, 1H), 8.62 (dd, J = 1.2, 3.8 Hz, 1H), 8.03 (d, J = 7.9 Hz, 1H), 7.52 (m, 1H), 6.63 (m, 2H), 5.83 (m, 1H), 5.69 (m, 1H),

4.70 (d, $J = 2.9$ Hz, 1H), 4.10 (d, $J = 8.5$ Hz, 1H), 3.95 (s, 1H), 3.00 (m, 1H), 2.59 (m, 1H), 1.82 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 147.7, 147.2, 138.7, 135.8, 133.0, 132.2, 131.1, 124.3, 110.4, 110.3, 101.3, 101.2, 101.1, 55.6, 46.2, 45.6, 31.3; MS (*m/z*): [M+1] calcd. for $C_{17}H_{14}F_2N_2$, 284.3; found, 285.19.

Radioactive protein synthesis assay. The luciferase-based high-throughput screen to identify inhibitors of Stx trafficking has previously been described[22,49]. Confirmation of positive hits from the ChemDiv4 library screen was assessed by a previously described radioactive assay[22] and is described in **Supplementary Methods**.

Immunofluorescence. For all immunofluorescence experiments, cells were fixed in 4% (v/v) paraformaldehyde in cold phosphate-buffered saline (PBS, 4 °C), permeabilized with 0.1% Triton X-100 (v/v) in PBS, blocked, then probed with primary and secondary (Alexa Fluor 488–labeled or Alexa Fluor 594–labeled donkey anti-IgG) antibodies diluted in blocking buffer (DMEM containing 10% fetal calf serum (v/v) plus 1 mg ml^{-1} bovine serum albumin). Cells were rinsed thoroughly in PBS before mounting in SlowFade Gold reagent containing DAPI. Fluorescence imaging used epifluorescence (Zeiss) microscopy.

Cloning, sequence analysis, site-directed mutagenesis and generation of adenoviral expression constructs. The hamster GBF1 complementary DNA (a gift from P. Melançon, University of Alberta) was used as template for constructing hemagglutinin-tagged wild-type and mutant cDNA. Methods and primer sequences for cloning of canine GBF1 Sec7 domain, mutagenesis of GBF1 and generation of adenoviral constructs are described in **Supplementary Methods**.

Arf1-GTP pulldown assay. This pulldown assay was performed with human Arf1 and the VHS and GAT domains of human GGA3, cloned and purified as described in **Supplementary Methods**. The pulldown assay followed a published protocol[35] with modifications as described in **Supplementary Methods**.

Molecular modeling of the GBF1–Arf1–GCA complex. The GBF1 homology model was built using Prime 2.0 software from FirstDiscovery suite (Schrodinger, LLC) and ARNO coordinates from the ARNO-Arf1 complex (PDB ID 1R8Q) as a template[34]. Fully flexible ligand docking was performed with Glide 5.0 (Schrodinger, LLC). The protein was prepared for grid generation and subsequent docking using the Protein Preparation Wizard tool from FirstDiscovery suite. The default settings for grid calculations and docking were used. Images were created with UCSF Chimera version 1.2540 (ref. 50).

Toxin and transferrin internalization. For CtxB and transferrin trafficking experiments, Vero cells grown in chamber slides (2.5×10^4 cells per chamber) were treated with serum-free medium containing DMSO, GCA or BFA at the indicated concentrations and times at 37 °C. Following the binding of toxin and transferrin at 4 °C, cells were shifted to 19 °C for 1 h to allow for toxin internalization. Cells were processed for immunofluorescence as described above.

Trafficking of tsVSVG-GFP, NPY-GFP secretion and StxB-SS-His sulfation assays. Transport of tsVSVG-GFP (ref. 36), secretion of NPY-GFP (ref. 22) and sulfation of StxB-SS-His (ref. 22) were performed using published protocols with modifications as described in **Supplementary Methods**.

Accession codes. Protein Data Bank: The ARNO-Arf1 complex was deposited as part of a previous study under accession code 1R8Q (ref. 34).

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

J.B.S., W.J.S. and J.L. performed experiments. J.B.S. and W.J.S. assisted in manuscript preparation. B.B. performed docking and molecular modeling computations. J.W.C. synthesized and analyzed GCA. N.S.G. designed chemical synthesis, analyzed GCA and assisted in manuscript preparation. D.B.H. designed, performed and analyzed experiments and prepared the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturechemicalbiology/.

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