Distinct Functions for Arf Guanine Nucleotide Exchange Factors at the Golgi Complex: GBF1 and BIGs Are Required for Assembly and Maintenance of the Golgi Stack and *trans*-Golgi Network, Respectively

Florin Manolea,* Alejandro Claude,[†] Justin Chun,* Javier Rosas,[†] and Paul Melançon*

*Department of Cell Biology, University of Alberta, Edmonton, AB, T6G 2H7 Canada; and [†]Instituto de Bioquímica, Universidad Austral de Chile, Campus Isla Teja, Casilla 567, Valdivia, Chile

Submitted May 1, 2007; Revised October 31, 2007; Accepted November 6, 2007
Monitoring Editor: Benjamin Glick

We examined the relative function of the two classes of guanine nucleotide exchange factors (GEFs) for ADP-ribosylation factors that regulate recruitment of coat proteins on the Golgi complex. Complementary overexpression and RNA-based knockdown approaches established that GBF1 regulates COPI recruitment on *cis*-Golgi compartments, whereas BIGs appear specialized for adaptor proteins on the *trans*-Golgi. Knockdown of GBF1 and/or COPI did not prevent export of VSVGtsO45 from the endoplasmic reticulum (ER), but caused its accumulation into peripheral vesiculotubular clusters. In contrast, knockdown of BIG1 and BIG2 caused loss of clathrin adaptor proteins and redistribution of several TGN markers, but had no impact on COPI and several Golgi markers. Surprisingly, brefeldin A–inhibited guanine nucleotide exchange factors (BIGs) knockdown prevented neither traffic of VSVGtsO45 to the plasma membrane nor assembly of a polarized Golgi stack. Our observations indicate that COPII is the only coat required for sorting and export from the ER exit sites, whereas GBF1 but not BIGs, is required for COPI recruitment, Golgi subcompartmentalization, and cargo progression to the cell surface.

INTRODUCTION

The Golgi complex, the central sorting station of the secretory pathway, exhibits a very intricate organization that likely reflects the complexity of trafficking and sorting events that take place within this organelle (Rambourg and Clermont, 1990; Mogelsvang *et al.*, 2004). It comprises three main structural elements: two extensive tubular-reticular networks flanking the *cis*- and *trans*-sides of a central stack of flat disk-shaped cisternae. The flat cisternae that form the core of the Golgi complex (compact zones) appear interconnected by lateral tubular networks (noncompact zones) to form a continuous membrane ribbon (Rambourg and Clermont, 1990; Mogelsvang *et al.*, 2004). Subcellular fractionation and immunocytochemical analysis further established

that *cis*-, medial, and *trans*-Golgi elements contain different sets of resident enzymes and that the Golgi complex is therefore functionally compartmentalized (Farquhar and Palade, 1998; Polishchuk and Mironov, 2004). For example, the early acting enzyme mannosidase I localizes to *cis*-Golgi membranes, whereas later acting ones such as mannosidase II (ManII) and sialyltransferase concentrate in medial and *trans* compartments, respectively.

Imaging of live cells revealed that the Golgi complex is not static as initially assumed from its intricate structure, but rather surprisingly dynamic and linked to several other organelles by active bidirectional transport routes (Bonifacino and Glick, 2004). In animal cells, cargo initially translocated into the endoplasmic reticulum (ER) is selected for transport from specialized ER exit sites (ERES) to vesiculotubular clusters (VTCs; Fromme and Schekman, 2005; Tang *et al.*, 2005). Shortly after their formation, these pleiomorphic carriers are transported on microtubules toward the Golgi complex (Presley *et al.*, 1997; Scales *et al.*, 1997) where they collect, fuse into a network, and subsequently become a flattened *cis*-Golgi cisterna (Bonifacino and Glick, 2004); peripheral VTCs and those accumulated near the Golgi are collectively called ER-Golgi intermediate compartment or (ERGIC; Appenzeller-Herzog and Hauri, 2006). Current evidence suggests that cargo molecules then progress through the Golgi stack by a process termed cisternal maturation whereby newly formed *cis*-cisternae containing cargo progressively move toward the *trans* side as they lose early acting Golgi enzymes and acquire late acting ones (Puthenveedu and Linstedt, 2005; Losev *et al.*, 2006; Matsuura-Tokita *et al.*, 2006). At the *trans*-Golgi network (TGN), cargo is then

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07-04-0394>) on November 14, 2007.

Address correspondence to: Paul Melançon (Paul.Melancon@UAlberta.ca).

Abbreviations used: AP, adaptor protein; Arf, ADP-ribosylation factor; BFA, brefeldin A; BIG, brefeldin A–inhibited guanine nucleotide exchange factor; COP, coat protein; ERES, ER exit sites; ERGIC, ER-Golgi intermediate compartment; GBF, Golgi-specific brefeldin A resistance factor; GEF, guanine nucleotide exchange factor; GGA, gamma ear Golgi-localized Arf-binding protein; IF, immunofluorescence; ManII, mannosidase II; KD, knockdown; PM, plasma membrane; shRNA, short hairpin RNA; siRNA, small inhibitory RNA; TGN, *trans*-Golgi Network; VSVG, vesicular stomatitis virus glycoprotein; VTC, vesiculotubular cluster.

sorted to destinations that include the endosome, plasma membrane (PM), lysosomes, or secretory granules (Rodriguez-Boulant and Musch, 2005).

Formation of cargo carriers depends on the spatially and temporally regulated membrane recruitment of specific coat proteins (COPs) from the cytoplasm. At the membrane, COPs select cargo and serve as a scaffold for membrane deformation and vesicle budding (Bonifacino and Glick, 2004; Rabouille and Klumperman, 2005). Transport between the ER and the Golgi complex involves two types of COPs. The COPI coat, first identified *in situ* at the periphery of the Golgi (Orci *et al.*, 1986), has been implicated in both anterograde and retrograde traffic between the Golgi and VTCs (Duden, 2003). COPII-coated structures on the other hand, form at ERES and mediate export of cargo from the ER (Barlowe, 2003; Tang *et al.*, 2005). Packaging of endosome-targeted cargo at the TGN involves clathrin and several adaptor proteins (AP), including the multimeric AP-1, AP-3, and AP-4 and the monomeric gamma ear Golgi-localized Arf-binding protein (GGAs; Bonifacino, 2004; Robinson, 2004). Current evidence suggests a multistep process in which all three GGAs act in concert at the TGN to concentrate their ligands in coated regions for eventual transfer to AP-1 (Ghosh and Kornfeld, 2004). The recently identified exomer may play similar function for specialized endosomal cargo (Wang *et al.*, 2006).

The recruitment of COPs and their adaptors is controlled by small GTPases, which in turn are regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (D'Souza-Schorey and Chavrier, 2006; Nie and Randazzo, 2006). Sar1 initiates recruitment of COPII (Sato and Nakano, 2007) whereas ADP-ribosylation factors (Arfs) regulate assembly of not only COPI, but also AP-1, -3, and -4 and the GGAs (D'Souza-Schorey and Chavrier, 2006). Arfs have been divided into three classes based on sequence similarity. Arf1 and Arf3, members of the class I (Arf1-3), as well as the class II Arf5, have been localized to the Golgi complex; in contrast, Arf6, the only class III Arf, associates primarily with the plasma membrane and endosomes (Donaldson and Honda, 2005). Several Arf-GEFs have been characterized, all of which contain a conserved Sec7 domain responsible for Arf activation (Cox *et al.*, 2004; Mouratou *et al.*, 2005). Two Arf-GEF subfamilies called GBF1 and BIGs appear to regulate Arf activation for coat recruitment on the Golgi complex. Both of these GEFs are inhibited by the fungal metabolite brefeldin A (BFA; Mansour *et al.*, 1999; Togawa *et al.*, 1999; Niu *et al.*, 2005; Zhao *et al.*, 2006). GBF1 and BIGs localize to *cis*- and *trans*-compartments of the Golgi complex, respectively, where they have been proposed to facilitate recruitment of the COPI coat and clathrin adaptors (Claude *et al.*, 1999; Kawamoto *et al.*, 2002; Shinotsuka *et al.*, 2002a; Zhao *et al.*, 2002, 2006; Garcia-Mata *et al.*, 2003).

Cell-free assays with *Saccharomyces cerevisiae* extracts unambiguously established that Sar1-dependent recruitment of COPII drives cargo sorting, as well as budding and release of carriers targeted to the Golgi complex (Barlowe, 2003; Fromme and Schekman, 2005). No such general agreement over the mechanism of cargo export from the ER exists for animal cells, however. In these cells, treatment with BFA or expression of a GDP-arrested Arf mutant blocks export of anterograde cargo from the ER and interferes with its concentration at ERES (Ward *et al.*, 2001; Barzilay *et al.*, 2005). Such observations suggest that formation and release of Golgi carriers in animal cells is more complex and likely involves a two-step process that depends on sequential action of both Sar1 and Arfs (Garcia-Mata *et al.*, 2003; Altan-Bonnet *et al.*, 2004). In this two-step model, Sar1 initially

recruits COPII, concentrates cargo, and organizes ER export domains by recruiting additional peripheral proteins such as SNAREs and rab1 and its effector p115 (Moyer *et al.*, 2001; Weide *et al.*, 2001). Subsequently, recruitment of GBF1, possibly through its interaction with p115 (Garcia-Mata and Sztul, 2003) leads to Arf activation and the recruitment of numerous effectors that will mature the ER export domains into ERGIC membranes before their release as separate carriers bound for the Golgi complex (Altan-Bonnet *et al.*, 2004). This model is consistent with the observation that expression of a dominant negative mutant of rab1b causes dispersal of the Golgi as observed with BFA, possibly by preventing sequential recruitment of p115 and GBF1 (Alvarez *et al.*, 2003). The two-step model is further supported by the fact that several enteroviral 3A proteins that target GBF1 and block Arf activation, also prevent export from the ER and trap cargo in ERES rather than peripheral VTCs (Wessels *et al.*, 2006a,b).

Here, we report complementary overexpression and (KD) knockdown studies that probe in more detail the role of GBF1 during cargo export from the ER and examine the relative functions of the two Golgi-localized Arf-GEFs in the maintenance and function of the Golgi complex.

MATERIALS AND METHODS

Tissue Culture and Reagents

Media and culture reagents were purchased from Invitrogen (Carlsbad, CA). Disposable plastic ware and culture six-well plates were purchased from Falcon Plastics (Oxnard, CA). Monolayers of HeLa, A-549, and BHK-21 cells were maintained in DMEM supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 100 μ g/ml penicillin G, 100 μ g/ml streptomycin, and 2 mM glutamine. For incubations at 32°C, cells were shifted into CO₂-independent media (Invitrogen, Great Island, NY). For the experiment described in Figures 6 and 8, we switched to HeLa cells obtained from ECACC (Sigma-Aldrich, 93021013) were used because they displayed better morphology and clear separation of *cis*- and *trans*-Golgi compartments. BFA and monensin were purchased from Sigma-Aldrich (St. Louis, MO). FuGENE 6 and complete protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Indianapolis, IN). All other chemicals were reagent grade and unless otherwise indicated were purchased from Sigma-Aldrich.

Antibodies

9D4 serum was raised against Sec7d-encompassing recombinant proteins containing residues 513–873 of human GBF1. Sera raised against GBF1 (9D2; 1:200) and BIG1 (9D3; 1:500) were described previously (Zhao *et al.*, 2002; Claude *et al.*, 2003). The following additional antibodies were used: anti-BIG2 (a kind gift from Dr. K. Nakayama, Kyoto University, Kyoto, Japan) at 1:100; anti-GFP (Dr. G. Eitzen, University of Alberta, Edmonton, AB, Canada) at 1:2000; anti-AP-1 (clone 88, BD Biosciences Pharmingen) at 1:600; anti-TGN46 (AbD Serotec, Kidlington, Oxford, United Kingdom) at 1:500. Only for immunoblot analysis we used anti-BIG2 (Bethyl Laboratories, Montgomery, TX) and anti-calnexin (Stressgen Biotechnologies, Victoria, BC, Canada). Other antibodies raised against Sec31, giantin, p115 (7D1), ManII, ERGIC-53 (G1/95), and β COP (M3A5) were used as previously described (Claude *et al.*, 1999; Zhao *et al.*, 2006). Secondary antibodies for this study were as follows: ALEXA488-, ALEXA594-, or ALEXA660-conjugated goat or donkey anti-rabbit and anti-mouse antibodies, as well as Alexa555-conjugated donkey anti-sheep antibody (Molecular Probes, Eugene, OR) at 1:600.

Preparation of Cell Extracts and Analysis by RT-PCR and Immunoblots

For RT-PCR analysis, 1–2 $\times$ 10⁶ cells grown in each well of a six-well plate were trypsinized, washed, and processed using the RNeasy kit according to manufacturer's instructions (Qiagen, Chatsworth, CA). Recovered RNA was analyzed using Qiagen one-step RT-PCR kit according to manufacturer's instructions using gene-specific primers. PCR conditions and cycle numbers were optimized for each primer pair to yield single products of expected size whose level varied in proportion to the amount of RNA added.

Immunoblot analysis of detergent extracts prepared from control and KD cells were carried out essentially as described previously (Zhao *et al.*, 2006). For determination of GBF1 and BIGs KD efficiency and specificity, HeLa cells transfected as described with the appropriate amounts of small interfering RNAs (siRNAs) were lysed after 72 h. Seventy-five-micrograms of each sample were transferred to a nitrocellulose membrane and probed with

antisera for GBF1 (9D4, 1:2500), BIG1 (9D3, 1:1000), BIG2 (1:1000), TGN46 (1:2000), AP-1 (1:5000), calnexin (1:20000), β -COP (M3A5, 1:3000), and GGA3 (1:5000) and detected with HRP-conjugated goat anti-rabbit (Bio-Rad Laboratories, Hercules, CA).

Overexpression of VSVG-tsO45 and Other cDNAs

The vesicular stomatitis virus glycoprotein (VSVG)-green fluorescent protein (GFP)-encoding plasmid was a kind gift from Dr. John F. Presley (McGill University, Montreal, QC, Canada). A VSVG-tsO45 virus stock was obtained from Dr. William Balch (Scripps Institute, La Jolla, CA) and grown into a working stock by infection of BHK cells at low multiplicity of infection. Measurement of VSVG traffic in siRNA-treated cells involved separate transfection steps and various combinations of temperature shifts, as illustrated in Figure 3. Briefly, HeLa cells plated at ~15% confluency were transfected with the appropriate siRNA duplexes 24 h later. For VSVG-tsO45-GFP expression, KD cells were washed 50 h after RNA transfection, transfected again with 1 μ g of VSVG-encoding plasmid, and returned to a 37°C incubator for a further 18 h. Transfected cells were then transferred to a 40°C water-jacketed CO₂ incubator for 4 h, followed by shift to the permissive temperature 32°C for various lengths of time. This shortened incubation at 40°C minimized cellular stress and proved sufficient to accumulate newly synthesized VSVG-protein in the ER. The 32°C incubation was performed in a water bath and required the use of CO₂ independent media. Cells were fixed at different time points after shift to 32°C, as specified. For experiments shown in Figure 3B, cells were transferred directly from 37 to 32°C to bypass the temperature shift to 40°C. For experiments involving live virus, cells were infected with VSV-tsO45 1 h before being shifted to 40°C as described (Zhao *et al.*, 2006). VSVG-GFP was detected either directly using intrinsic GFP fluorescence or by immunofluorescence (IF) using a combination of antibody raised against GFP and ALEXA488-conjugated secondary antibody. The latter method yielded stronger and more stable signal that permitted analysis of cells with low to moderate levels of VSVG in order to avoid artifacts due to overloading the ER with unfolded proteins. Both methods of detection gave similar results. Experiments involving live virus or plasmid-driven expression of VSVG-GFP yielded identical results.

For the monensin treatment experiments, HeLa cells grown to ~60% density were transfected with 2 μ g plasmid encoding GalT-GFP per 60-mm plate. After 24 h, cells were replated on glass coverslips at ~15% confluency and transfected with the appropriate siRNA duplexes 24 h later. Seventy-two hours after siRNA transfection, cells were treated with either 4 μ M monensin or equivalent volume of methanol for the periods of time specified and then fixed and processed for IF.

For Arf-GEF overexpression studies (Supplementary Figure 1), BHK cells grown on glass coverslips to ~50% density were transfected with 1 μ g of purified pCEP4 vector plasmid encoding either GBF1 (Claude *et al.*, 1999) or BIG1 (Mansour *et al.*, 1999), using FuGENE 6 according to the manufacturer's instructions. For exogenous expression of Golgi markers, HeLa cells were cotransfected with 1 μ g of plasmid encoding HA-furin that was obtained Dr. J. Bonifacino (Cell Biology and Metabolism Branch, NIH, Bethesda, MD). Twenty-four hours after transfection, cells were treated with 10 μ M BFA or an equivalent volume of DMSO, fixed, and processed for IF using the indicated antibodies.

Construction of the Anti-GBF1, -BIG1, and -BIG2 short hairpin RNA-expressing pSUPER-tet Plasmids

To create an inducible form of pSUPER (OligoEngine, Seattle, WA, USA) under the control of the *tet* repressor, we replaced the promoter sequence of this plasmid with the promoter of the pTER plasmid (Clontech, Mountain View, CA). Briefly, a 2498-base pair fragment between the HindIII and BamHI sites from pTER was first amplified by PCR and cloned into the pGEM-T Easy vector (Promega, Madison, WI) using overhanging 3' deoxyadenine residues. A 1537-base pair fragment containing the promoter with the *tet* repressor sequence was then liberated with HindIII and AflIII and used to replace the pSUPER promoter using the same restriction sites. The resulting pSUPER-tet plasmid was verified by sequencing.

To generate Arf-GEF-targeting plasmids, pSUPER-tet was linearized by double digestion with HindIII and BglII and synthetic 60-base pair oligomers encoding the desired short hairpin RNA (shRNA) sequences were ultimately inserted using these restriction sites. The double-stranded oligo sequences were designed with cohesive BglII and HindIII sites: (5') GATCCCTTCAA-GAGAYYYYYYYYYYYYYTTTTTA (3') where the 19-nucleotide X and Y residues corresponded to the target sense and antisense sequences identified by Dharmacon (Lafayette, CO) as likely candidates for siRNA-mediated silencing of GBF1, BIG1, and BIG2 expression. All constructions were verified by sequencing. Plasmids were transfected into HeLa cells using 1 μ g of plasmid per well of a six-well dish, and cells were fixed after 96 h to allow an extra 24 h for expression and processing of shRNAs.

siRNA Methods

Pools and individual siRNAs targeting different regions of human (h) GBF1 (MU-019783), hBIG1 (MU-012207), hBIG2 (MQ-012208), and h β -COP (MQ-

017940) were purchased from Dharmacon. We followed the Oligofectamine (Invitrogen) transfection protocol for HeLa cells as described (Harborth *et al.*, 2001). Different combinations of targeting duplexes, time points, and siRNA concentrations were assessed to optimize conditions for most effective KD. RT-PCR of cell extracts established that Arf-GEF mRNA levels dropped below 10% by 30 h and remained knocked down for as long as 72 h. Immunoblot analysis of cell extracts was consistent with effective and specific KD (Supplementary Figure 2). Interestingly, IF analysis showed that GBF1 was effectively knocked down in 50–60% of the cells after 72 h after siRNA transfection; in contrast, BIG1 appeared less stable and was undetectable by IF in greater than 70% of the cells as early as 48 h after transfection.

For the experiments presented here, HeLa cells were incubated with a pool of siRNAs targeting sequences 2 and 3 for GBF1, each at a concentration of 100 nM. For BIGs KD, we used a pool of siRNAs targeting sequences 2 and 3 for BIG1 (75 nM each) and 1–4 for BIG2 (50 nM each). For β -COP KD we used sequence 2 at 200 nM. Note that lower siRNA concentrations (50 nM instead of 75 nM for BIG1 and 25 nM instead of 50 nM for BIG2) were sufficient for effective BIGs KD (loss of BIG1 and redistribution of AP-1). As control, cells were exposed to matching concentrations (200–300 nM) of a nonspecific GL2 luciferase siRNA designed as described (Elbashir *et al.*, 2002).

IF Microscopy and Image Analysis

Cells grown on glass coverslips were washed in phosphate-buffered saline (PBS) and fixed with 3% paraformaldehyde in PBS at room temperature for 20 min. Labeling of cells with different antibodies was carried out as described previously (Zhao *et al.*, 2002). Unless otherwise indicated, images were obtained by standard IF with an Axioskop II microscope (Carl Zeiss, Thornwood, NY) using a 63 $\times$ objective (Plan-APOCHROMAT; NA 1.4) and equipped with a CoolSNAP HQ Photometrics camera (Tucson, AZ). Confocal images (see Figures 4, 6, and 8) were acquired using an LSM 510 (Carl Zeiss) equipped with a similar 63 $\times$ objective. When two markers were imaged in the same cells, each fluorophore was excited and detected sequentially (multi-track mode) to avoid channel bleed-through. Laser intensity and filters were adjusted to give maximum signal and avoid saturation. Unless otherwise indicated, a single focal plane (0.8–1 μ m) was analyzed.

For Figure 8, the line profile analysis was performed using Image-ProPlus Software (Media Cybernetics, Silver Spring, MD). To examine signal distribution within a single stack, we selected red and green structures of similar intensity that clearly appeared contiguous and in close proximity (less than 0.8 μ m). Quantification of signal overlap was performed using MetaMorph (Universal Imaging, West Chester, PA).

RESULTS

GBF1 and BIG1 Regulate the Recruitment of Different Coat Proteins on the Golgi Complex

Several overexpression studies have largely supported functional and selective links between the *cis*-Golgi localized GBF1 and the COPI coat and on the other hand the *trans*-Golgi localized BIGs and the clathrin adaptor AP-1. For example, Nakayama and colleagues established that overexpression of GBF1 protects Arfs and COPI against BFA-induced redistribution (Kawamoto *et al.*, 2002). This protective effect appears specific because gross overexpression of GBF1 did not prevent release of the clathrin adaptor AP-1 (Supplementary Figure 1B), whereas even moderate GBF1 overexpression was sufficient to abrogate the effects of BFA on COPI and the medial Golgi marker Man II (Supplementary Figure 1, A and C). As expected from this specificity, overexpression of GBF1, although it protects the Golgi stack (Claude *et al.*, 1999; Kawamoto *et al.*, 2002; Niu *et al.*, 2005; Zhao *et al.*, 2006; see also Supplementary Figure 1C), did not prevent redistribution of the TGN detected with exogenous hemagglutinin (HA)-furin (Supplementary Figure 1D).

Overexpression of BIGs has effects opposite to GBF1 on stabilization of COPI and clathrin adaptors. Previous work demonstrated that BIG2 overexpression prevented release of the clathrin adaptor AP-1 but not that of COPI (Shinotsuka *et al.*, 2002b). Similarly, BIG1 overexpression had no protective effect on COPI (Supplementary Figure 1A) and the Golgi stack (Supplementary Figure 1C), but did prevent the effects of BFA on the membrane recruitment of the clathrin adaptor AP-1 (Supplementary Figure 1B). Altogether, these results

confirm that the two Arf-GEF subfamilies regulate the recruitment of distinct coat proteins on the Golgi complex.

Knockdown of GBF1 Confirms Its Role in Regulating Assembly of the COPI Coat

To examine in more detail the relative function of GBF1 and BIGs in the Golgi complex, we turned to complementary siRNA-based methods to knockdown their expression. RT-PCR analysis established that pools of RNA duplex oligonucleotides targeted to GBF1, BIG1, or BIG2 effectively and selectively knocked down mRNA levels by more than 90% within 30 h of transfection and maintained low mRNA levels for at least 72 h (unpublished data). Analysis of cell extracts by immunoblotting established that GBF1 KD efficiently reduced GBF1 levels with no detectable effect on either β -COP, calnexin, BIGs, or other the TGN markers examined. Similarly, BIGs KD reduced BIGs levels with no detectable effect on AP-1, GGA3, TGN46, β -COP, GBF1, or calnexin (Supplementary Figure 2). To further establish specificity of the KD, we individually tested several RNA duplexes targeting different regions of the GEFs. With the exception of BIG1 1, all individual duplexes yielded KD comparable to the pools; nevertheless, two each of the GBF1 (2 and 3), BIG1 (2 and 3) and BIG2 (1 and 3) targeted RNA duplexes appeared most effective (unpublished data) and were selected for further analysis. Vectors encoding both GFP and shRNAs corresponding to those sequences were constructed. Analysis of transfectants readily identified in the GFP channel confirmed that each RNA sequence effectively suppressed expression of the targeted Arf-GEF (Figure 1).

The availability of duplexes that effectively and selectively KD Golgi-localized Arf-GEFs allowed us to examine their function in more detail. As shown in Figure 2, A and B, GBF1 KD abrogated juxta-nuclear localization of COPI in the vast majority of RNA-treated cells. Quantitative analysis confirmed that treatment of monolayers with GBF1-targeted

Figure 1. Specific and effective KD of GBF1, BIG1, and BIG2 using siRNA sequences expressed from pSUPER vector. HeLa cells were transfected with 1 μ g of GFP-encoding pSUPER plasmids modified with a *tet* repressor regulated cassette driving expression of either nothing (Ctl) or various individual shRNAs targeting GBF1, BIG1, or BIG2 (see *Materials and Methods* for construction details). After 96 h, cells were fixed and stained for the markers indicated on the left. Outlines mark GFP-positive transformed cells that were identified in the green channel (shown in inset). Images shown are representative of at least three separate experiments. Bar, 20 μ m.

RNA duplexes for 72 h eliminated juxta-nuclear staining for COPI in more than 90% of transfected cells (110 cells counted in three separate experiments). In some cases, particularly at shorter times after RNA-transfection, GBF1 KD was partial and resulted in appearance of several dispersed Golgi fragments showing weak GBF1 staining and some residual COPI (not shown). For this reason, complete loss of detectable membrane-associated COPI was used routinely as a more sensitive measure of effective GBF1 KD.

Previous observations with BFA- or GDP-arrested mutant forms of Arf1 (Dascher and Balch, 1994) predict that lack of COPI recruitment should not impact the function of the COPII coat at ERES but eventually lead to loss of detectable Golgi structures. As expected, GBF1 KD (confirmed by loss of GBF1/COPI staining, insets) caused no significant change in the overall number and distribution of the ERES marker Sec31 (Figure 2C). In contrast, loss of GBF1 led to complete redistribution of juxta-nuclear signal for the well-characterized *medial*-Golgi marker ManII (Figure 2D). GBF1 KD also caused redistribution the *cis*-Golgi markers p115 and giantin (Figure 2, E and F). Interestingly, as previously observed after BFA treatment (Seelig *et al.*, 1994; Nelson *et al.*, 1998; Seemann *et al.*, 2000), a significant fraction of these proteins accumulated in peripheral puncta, confirming that these proteins efficiently traffic and accumulate into post-ERES structures in absence of a functional COPI coat. We expected disruption of Golgi traffic to affect the TGN because this organelle receives material from both endosomes and the Golgi stack. Indeed, as shown in Figure 2, G and H, loss of the Golgi complex led to redistribution of both BIG1 and the TGN marker TGN46 (Figure 2, G and H). These results confirm that GBF1 is required for the membrane recruitment of COPI and assembly of the Golgi complex.

GBF1 Knockdown Does Not Prevent Export from the ER But Blocks Cargo in Post-ERES Peripheral VTC Structures

It has been argued that cargo export from the ER requires a functional COPI system (Altan-Bonnet *et al.*, 2004). This conclusion is based on the observation that BFA blocks export of anterograde cargo molecules such as VSVG (Ward *et al.*, 2001). The identification of KD conditions that effectively reduce GBF1 levels allowed us to test if a functional COPI system and the activity of GBF1 was necessary for cargo traffic out of ERES. To measure cargo traffic out of the ER, we took advantage of a thermosensitive mutant of VSVG (VSVG-tsO45) that can be accumulated in the ER at the nonpermissive temperature (40°C) and released synchronously upon shift to a permissive temperature (32°C). Cells were transfected with a plasmid encoding a GFP-tagged form of VSVG-tsO45 50 h after RNA-transfection with either luciferase- (Mock), or GBF1-targeted RNA duplexes. As illustrated in Figure 3A, cells were shifted to the nonpermissive temperature to accumulate VSVG in the ER and analyzed at various times after a shift to the permissive temperature.

GBF1 KD completely blocked traffic of VSVG to the cell surface, but to our surprise did not prevent VSVG accumulation in peripheral punctate structures. The images shown in Figure 3A, confirmed that in cells treated with irrelevant RNA, a large fraction of VSVG present in the ER at the beginning of the temperature shift ($t = 0$) trafficked to the cell surface within 2 h. In sharp contrast, VSVG completely cleared the ER but never accumulated in a juxta-nuclear structure or reached the cell surface in cells transfected with GBF1-targeted siRNA (Figure 3A), even 120 min after temperature shift. Instead, VSVG accumulated in small bright

Figure 2. Knockdown of GBF1 prevents assembly of the Golgi complex. HeLa cells were transfected with an equimolar mixture of GBF1-targeting siRNA duplexes 2 and 3 as described in *Materials and Methods*. After 72 h, cells were fixed and double-stained for the markers indicated. Top panels show the pattern observed with GBF1/COPI double-staining. The remaining panels display the distribution of various ERES (Sec31), Golgi (ManII, p115, giantin), and TGN (BIG1, TGN46) markers, with the inset showing the pattern obtained with the second marker (GBF1 or COPI). Cells with effective KD (loss/redistribution of GBF1/COPI) were outlined. Images shown are representative of at least three separate experiments. Bar, 20 μ m.

puncta in the cell periphery. Importantly, accumulation of VSVG in peripheral structures was not caused by cellular stress or aggregation of misfolded proteins at the nonpermissive temperature (40°C) because similar puncta accumulation was observed when cells were shifted directly to 32°C, bypassing incubation at 40°C (Figure 3B).

To obtain independent confirmation that the effect of GBF1 KD resulted from lack of COPI recruitment, we tested in parallel the impact of reducing levels of the β -subunit of COPI on Golgi assembly and function. This subunit plays a critical role in several COPI function such as Arf binding

(Zhao *et al.*, 1997) and cargo recruitment (Eugster *et al.*, 2004), and its KD was expected to result in the effective loss of COPI activity. As described in *Materials and Methods*, four β -COP-targeted RNA duplexes were tested for their ability to disperse Golgi markers (unpublished data) and the most effective one selected for further analysis. Treatment with this RNA duplex effectively caused ManII redistribution (unpublished data) and led to similar accumulation of VSVG cargo into peripheral puncta (Figure 3, A and B). To ascertain that export from ERES was not due to residual COPI activity, we reexamined cargo transport in cells subjected to double KD. As shown in Figure 3, A and B (bottom panels), loss of both GBF1 and COPI did not prevent complete clearing of VSVG from the ER and its accumulation in peripheral puncta.

The accumulation of cargo in puncta observed in GBF1 and COPI KD initially appeared inconsistent with the results previously reported with BFA. This apparent discrepancy prompted us to examine the impact of BFA treatment on cargo accumulation in GBF1 KD cells. Interestingly, treatment with BFA before the shift to permissive temperature prevented accumulation of VSV-G in puncta and yielded the previously reported (Ward *et al.*, 2001), largely reticular pattern in both mock and GBF1 KD cells (Figure 3C). The observation that BFA treatment prevents accumulation of cargo in peripheral VTCs, even in GBF1 KD cells, suggests that the drug affects not only GBF1 activity but additional steps critical to cargo export.

Further analysis of the VSVG positive peripheral puncta identified them as post-ERES structures. Images shown in Figure 4 (top panels) first confirmed that they lack COPI, as expected. Furthermore, the VSVG-positive peripheral structures stain for ERGIC53, but not for Sec31, and therefore likely correspond to VTCs (Figure 4, middle and bottom panels). Quantitative analysis of these and five similar images confirmed that greater than $87 \pm 6\%$ of the VSVG positive puncta ($n = 287$) also contain ERGIC53 whereas fewer than $15 \pm 3\%$ overlapped with Sec31 ($n = 161$). Altogether, these results demonstrate that cargo sorting from ERES and transport to peripheral VTCs can take place even in the absence of GBF1/COPI.

BIGs Knockdown Blocks Recruitment of Clathrin Adaptors But Does Not Prevent COPI Recruitment or Maintenance of the Golgi Stack

Arf activation at the TGN has been implicated in the recruitment of several clathrin adaptor molecules such as AP-1 and GGAs (Bonifacino and Traub, 2003), a process likely controlled by the TGN-localized BIGs. Because BIG1 and BIG2 form heterodimers and probably perform redundant function at the TGN (Yamaji *et al.*, 2000), KD of both BIGs was necessary to examine their function. To eliminate BIGs, we selected a combination of BIG1- and BIG2-targeted RNA duplexes that as mentioned above proved effective in reducing levels of both BIG1 and BIG2 in several immunoblot (see Supplementary Figure 2) and IF experiments.

To confirm the involvement of BIGs in clathrin adaptor recruitment, we first examined the impact of BIGs KD on the distribution of endogenous AP-1 and GGA3. As shown in Figure 5, A and B, BIGs KD eliminated bright juxta-nuclear staining for AP-1, yielding a weaker and more dispersed punctate pattern. Quantitative analysis of these and similar images from three separate experiments confirmed that BIGs KD caused AP-1 redistribution in greater than 85% of transfected cells ($n = 61$). BIGs KD similarly caused redistribution of endogenous GGA3 from a compact juxtanuclear structure to a diffuse pattern (Figure

Figure 3. GBF1 and/or COPI KD block VSVG traffic and cause cargo accumulation in peripheral puncta. (A and B) HeLa cells were transfected with siRNA duplexes targeting luciferase (Mock), GBF1, and/or β COP, as indicated on the left. Fifty hours after transfection, cells were transfected again with a plasmid encoding VSVGtO45-GFP and then temperature-shifted and fixed, as illustrated. For panel A cells were shifted to the nonpermissive temperature for 4 h before the shift to permissive temperature, whereas in B cells were shifted directly to from 37°C to permissive temperature. Cells were double-stained for COPI and GFP. Images reveal the VSVG-GFP pattern observed at the indicated times after shift to permissive temperature. Knockdown was confirmed by redistribution of COPI (not shown). Images shown are representative of at least three separate experiments. Bar, 20 μ m. (C) HeLa cells were transfected with siRNA duplexes targeting luciferase (Mock) or GBF1, as indicated on the left. Fifty hours after transfection, cells were transfected again with a plasmid encoding VSVGtO45-GFP, then temperature shifted, treated with DMSO/(10 μ g/ml) BFA, and fixed, as illustrated. Cells were double-stained for COPI and GFP. Images in the bottom panel reveal the GFP-VSVG pattern. Knockdown was confirmed by redistribution of COPI (not shown). Images shown are representative of at least two separate experiments. Bar, 20 μ m.

5C). We observed identical AP-1 redistribution whether performing KD treatment for 2 or 3 d, which led us to conclude that residual membrane association of clathrin adaptors likely reflects Arf activation by endosome associated Arf-GEFs of the ARNOs and EFA6 subfamilies (D'Souza-Schorey and Chavrier, 2006).

The effects of BIGs KD appear specific for clathrin adaptors because loss of BIGs had no measurable impact on the recruitment of the COPII (unpublished data) or COPI coat (Figure 5D). As expected from the presence of COPI on juxtanuclear structures, loss of BIGs had no impact on the localization of *cis*- or medial Golgi markers such as GBF1 (unpublished data), p115 or ManII (Figure 5, E and F). Retention of Golgi structures cannot reflect partial BIGs KD because a 2 d treatment with siRNA duplexes was sufficient to eliminate BIG1 staining and extending BIGs KD to 3 d did not cause detectable reduction or fragmentation of Golgi signal in any of the transfectants examined.

BIGs KD Disrupts Assembly of the TGN

BIGs KD and/or consequent loss of adaptor recruitment in the Golgi region leads to loss of a detectable TGN. The first indication that BIGs may be essential for TGN assembly came from examination of the well-characterized TGN membrane marker TGN46. Effective BIGs KD, as measured

by AP-1 redistribution (inset), caused dispersal of TGN46 (Figure 5G). This marker did not accumulate at the cell surface but rather relocalized to weak puncta scattered throughout the cytoplasm, as previously observed in GBF1 KD cells (Figure 2H). To further probe the impact of BIGs KD, we examined the distribution of cargo receptors that cycle between the TGN and endosomes to transport lysosomal hydrolases and whose function rely on GGAs (Bonifacino and Traub, 2003; Ni *et al.*, 2006). These sorting receptors include not only canonical mannose 6-phosphate receptors, but also the more recently described sortilin, a member of the Vps10p family. Several experiments established that the cation independent mannose 6-phosphate receptor retained the characteristic punctate pattern of a late endosomal marker in BIGs KD cells (unpublished data). In sharp contrast, BIGs KD reproducibly prevented normal localization of sortilin; this marker redistributed to a weak dispersed pattern and did not appear to accumulate at the cell surface (Figure 5H).

To examine whether the redistribution of TGN markers resulted from a defect in assembly and maintenance of a functional TGN, we took advantage of the fact that treatment with the proton ionophore monensin traps a subset of Golgi enzymes such as GalT into dispersed vacuoles derived from the TGN (Borsig *et al.*, 1999; Puri *et al.*, 2002; Schaub *et*

Figure 4. GBF1 KD traps cargo in ERGIC53-positive VTCs that are separate from Sec31-positive ERES. HeLa cells were transfected first with siRNA duplexes targeting GBF1 and 50 h later with a plasmid encoding VSVGtsO45-GFP as described in *Materials and Methods*. After 18 h, cells were shifted to 40°C for 4 h and then to 32°C for 120 min, as illustrated in Figure 3A. Fixed cells were double-stained for the indicated markers. Single-slice confocal images are shown. Insets display threefold magnification of boxed areas of merged images shown in center panels. Images shown are representative of at least two separate experiments. Bar, 20 μ m.

al., 2006). GalT resides primarily in *trans*-Golgi cisternae but does cycle to and from the TGN where it becomes trapped in monensin-treated cells (Schaub *et al.*, 2006). Any residual trafficking to the TGN in BIGs KD cells should therefore be detectable by accumulation of GalT in remaining TGN structures after treatment with monensin. We first verified that BIGs KD had no impact on the Golgi localization of GalT and its codistribution with the medial Golgi marker ManII (Figure 6, top panels). Second, we confirmed that in control cells a brief 15 min treatment with monensin was sufficient to cause accumulation of GalT in vacuoles clearly separate from the Golgi ribbon (Figure 6, middle panels). As shown in Figure 6 (bottom panels), monensin did not change the GalT juxta-nuclear distribution in BIGs KD cells, even when the treatment was lengthened from 15 to 30 min. Quantitative analysis revealed that GalT redistributed to dispersed vacuoles distinct from ManII positive Golgi ribbons in more than $91 \pm 4\%$ of control cells ($n = 117$), whereas this occurred in fewer than $14 \pm 10\%$ of KD cells ($n = 46$). The lack of GalT redistribution in the majority of BIGs KD cells demonstrates that effective KD was achieved and strongly suggests that BIGs play a critical role in assembly of the TGN.

BIGs KD Does Not Prevent Cargo Traffic to the Cell Surface or Assembly of a Polarized Golgi Stack

To determine whether the structures detected with several Golgi markers in BIGs KD cells remained functional we first examined traffic of VSVG-tsO45-GFP. Expression of BIGs was knocked down in HeLa cells as before, and these cells were subjected to a VSVG expression and temperature shift protocol similar to that described in Figure 3A. As shown in Figure 7, BIGs KD did not prevent VSVG transport to the juxta-nuclear region or its appearance at the cell surface. VSVG reached the juxta-nuclear region 20 min after shift to

Figure 5. BIGs KD blocks recruitment of TGN-specific coats, re-distributes TGN markers, but does not prevent assembly the Golgi stack. HeLa cells were transfected with pools of siRNA duplexes targeting both BIG1 (2 and 3) and BIG2 (1–4) as described in *Materials and Methods*. Fixed cells were double-stained for the indicated markers. (A and B) The pattern observed with BIG1/AP-1 double-staining. (C–H) The distribution of various Golgi (COPI, p115, ManII) and TGN (GGA3, TGN46, sortilin) markers with the inset showing pattern obtained with the second marker (BIG1 or AP-1). Cells with effective KD (loss/redistribution of BIG1/AP-1) were outlined. Images shown are representative of at least three separate experiments. BIGs KD for 3 d did not cause detectable reduction or fragmentation of Golgi signal (Man II, p115, and COPI) in any of the transfectants examined ($n = 30, 142$, and 39, respectively). Bar, 20 μ m.

the permissive temperature and clearly accumulated at the PM within 2 h. Furthermore, loss of BIGs appeared not to affect the kinetics of VSVG traffic because, at all time points examined, the fraction of cells with VSVG at the Golgi or the PM were nearly identical in mock and BIGs KD cells (Figure 7B). These results suggest that BIGs and a functional TGN are not required for efficient sorting of some cargo molecules to the PM of nonpolarized cells.

Figure 6. BIGs KD prevents monensin-induced redistribution of GalT-GFP. HeLa cells were transfected with a plasmid encoding GalT-GFP and a pool of siRNA duplexes targeting either luciferase (Mock), or BIG1 and BIG2 (BIGs) as described in *Materials and Methods*. Seventy-two hours after siRNA transfection, cells were treated with either methanol or 4 μ M monensin for the indicated periods of time. Cells were then fixed and stained for ManII and AP-1. Cells with effective BIGs KD were identified by loss of AP-1 juxtanuclear staining. Single-slice confocal images of the ManII (red) and GFP signal are shown. Merged images shown on the right. Images shown are representative of at least three separate experiments. Bar, 20 μ m.

The observation that BIGs KD does not prevent cargo traffic to the cell surface suggests that the GBF1/COPI coat machinery may be sufficient to drive assembly a Golgi stack that cannot only produce carriers but may have retained its characteristic polarized organization. We tested this possibility by examining the impact of BIGs KD on the relative

distribution of several markers with limited distribution within the Golgi stack. HeLa cells were subjected to BIGs KD and examined by IF using a triple labeling protocol. As shown in Figure 8, BIGs KD did not prevent assembly of a polarized Golgi stack in which *cis*-Golgi markers such as p115 and GBF1 remain well resolved from a *trans*-cisterna

Figure 7. BIGs KD does not block traffic of VSVG to the cell surface. HeLa cells were transfected with siRNA duplexes targeting either luciferase (Mock) or BIG1 and BIG2 (BIGs), as indicated on the left. Fifty hours after transfection, cells were transfected again with a plasmid encoding VSVGtO45-GFP, then temperature shifted, and fixed, as illustrated in Figure 3A. (A) Cells fixed at the indicated times after shift-down to 32°C were double-stained for BIG1 and GFP. Knockdown was confirmed by redistribution of BIG1 (not shown). Images reveal the GFP-VSVG pattern observed at the times shown above the panels. Arrowheads mark plasma membrane (PM) where VSVG accumulates. Cells with effective KD (loss of BIG1) were marked by asterisks. Images shown are representative of at least six separate experiments. Bar, 20 μ m. (B) At each time point after release for both mock (● and ▲, solid line) and BIGs KD (○ and △, dashed line) cells, a minimum of 25 cells were scored for presence of VSVG at Golgi only (○, ●), or at Golgi and PM (△, ▲). The fraction of cells with the indicated pattern was expressed as percentage and is shown as a function of time after shift-down to 32°C.

Figure 8. BIGs KD does not prevent assembly of a polarized Golgi stack. HeLa cells were transfected with a pool of siRNA duplexes targeting either luciferase (Mock), or BIG1 and BIG2 (BIGs). After 48 h, cells were transfected again with a plasmid encoding GalT-GFP. Cells were fixed 24 h later and double-stained for either BIG1/p115 or AP-1/GBF1. Single-slice confocal images are shown. Merged images display the red and GFP signal from the enlarged boxed areas. Graphs in the right column report pixel intensity profiles in all three channels along the white bar shown in the merged panels. Images shown are representative of at least three separate experiments. Bar, 20 μ m.

marker such as GalT-GFP. Effective BIGs KD was confirmed using BIG1 and AP-1 as markers (blue channel). Rightmost panels show an image of an enlarged area in which the red (*cis*) and green (*trans*) signals have been merged. Normal separation of *cis*- and *trans*-markers was observed in all transfectants examined (68 cells from three separate experiments).

To better illustrate the spatial resolution of the Golgi markers, we measured signal intensity along the white line shown in the merged image and reported values for each marker in the graphs on the right. The graphs confirmed good overlap of BIG1 and AP-1 with GalT-GFP in mock-treated cells and clear separation of *cis*- and *trans*-markers in both mock-treated and BIGs KD cells. Quantitative analysis of the average distance between *cis*- and *trans*-Golgi markers in several regions ($n > 6$) of cells from two separate experiments established that the average distance between p115/GalT or GBF1/GalT peaks were nearly identical in control and BIGs KD cells. Furthermore, quantitative analysis of fluorescence signal overlap in several images similar to those shown in Figure 8 confirmed that both pairs of *cis*/*trans* markers remained well resolved in BIGs KD cells (Table 1). Altogether, these results strongly suggest that GBF1/

COPI, but not BIGs/clathrin, is essential to drive assembly and maintenance of a polarized Golgi stack.

DISCUSSION

We exploited complementary overexpression and KD approaches to examine the relative functions of GBF1 and BIGs at the Golgi complex. As expected from their distinct local-

Table 1. BIGs KD does not affect separation of *cis*- and *trans*-Golgi markers^a

	p115 vs. GalT (%)	GBF1 vs. GalT (%)
Mock	29 $\pm$ 5% (n = 6)	36 $\pm$ 9% (n = 8)
BIGs KD	27 $\pm$ 9% (n = 19)	30 $\pm$ 10% (n = 23)

^a The extent of signal overlap in percentages between the two indicated markers was measured as described in *Materials and Methods*. Shown is the average $\pm$ SD obtained for indicated number of samples.

ization, overexpression of GBF1 and BIG1 had opposite effects on the sensitivity of the COPI and AP-1 coats to BFA. GBF1 stabilized COPI and protected *cis*-compartments but not AP-1 or HA-furin positive *trans*-Golgi membranes; in contrast BIG1 protected AP-1 but not COPI. Knockdown with several specific RNA duplexes or shRNAs confirmed that GBF1 and BIGs perform distinct functions in the Golgi complex. GBF1 KD caused redistribution of COPI and most Golgi markers to a diffuse pattern, whereas tethering factors such as p115 and giantin redistributed into puncta and reticular structures. Contrary to expectation, KD of GBF1 and COPI, singly or in combination, did not prevent export of VSVGsO45 from the ER, but caused its accumulation into ERGIC53-positive puncta separate from Sec31-positive ERES. On the other hand, BIGs KD had no impact on the COPI machinery or several Golgi markers but caused loss of clathrin adaptor proteins as well as redistribution of TGN46 and the sorting receptor sortilin. Further tests with monensin confirmed loss of detectable sorting of GalT to the TGN. Despite these clear effects on the TGN, BIGs KD prevented neither traffic of VSVGsO45 to the PM, nor assembly of a polarized Golgi stack. Our observations indicate that COPII is the only coat required for sorting and export from ERES. Furthermore, GBF1 but not BIGs appears necessary for COPI recruitment and cargo progression from VTCs to cell surface. The maintenance of a polarized Golgi stack in absence of BIGs suggests that the GBF1-COPI machinery is sufficient to drive the maturation process responsible for Golgi organization.

Effectiveness and Selectivity of Golgi ArfGEFs KD

Several experiments established that the KD methods used in our study led to selective and effective loss of the targeted proteins. Multiple observations confirmed the selectivity of the KD effects. These include the fact that similar KD effects were observed using any one of several sequences targeting different regions of the mRNA, and whether sequences were delivered by direct transfection of duplexes or by plasmid-driven synthesis of short hairpins RNA. The observation that targeting either β -COP or GBF1 had the same impact on ER export and the Golgi complex further supports our conclusion that KDs were selective.

The extent of KD varied within any given cell population, and depended on the nature of the target and length of treatment. These variations allowed us to identify with confidence cells displaying effective KD of targeted proteins. For example, we could readily recognize cells with partial KD for GBF1 at shorter treatment times (48 h); these cells lacked or showed little detectable GBF1 but still displayed a fragmented Golgi complex stained weakly with COPI. By lengthening treatment time to 72 h and selecting cells with no remaining COPI staining, we could ensure that all cells analyzed had effective GBF1 KD. In the case of BIGs, short treatment (48 h) proved effective at eliminating BIG and dispersing AP-1 IF signal. The fact that lengthening treatment to 72 h had no further impact on the distribution of TGN and Golgi markers established that effective KD had been achieved.

GBF1 Is Essential for COPI Recruitment and Assembly of the Golgi Complex, But Is Not Required for Cargo Concentration and Export from ERES

Results obtained through our combined use of overexpression and RNA-dependent silencing established that GBF1, but not BIGs, is required to activate Arfs for recruitment of COPI. The localization of GBF1 to early compartments of the

Golgi complex first suggested a functional link between GBF1 and COPI (Kawamoto *et al.*, 2002; Zhao *et al.*, 2002). Subsequent studies revealed that overexpression of the charge-reversal dominant negative mutant GBF1[E794K] (Garcia-Mata *et al.*, 2003) or microinjection of neutralizing GBF1 antibodies (Zhao *et al.*, 2006) caused loss of COPI recruitment. The loss of COPI recruitment and Golgi structure after GBF1 KD (Figure 2), as well as the coat and compartment-specific protection conferred by GBF1 overexpression (Supplementary Figure 1), extend these observations and confirm this functional link.

The availability of tools for the effective KD of GBF1 and COPI allowed us to test the two-step model which proposes that Arfs and COPI are required for traffic out of ERES. Contrary to predictions from this model, we observed that the GBF1/COPI machinery was not required for the concentration and export of VSVG cargo from ERES. These results are consistent with a wealth of information derived from yeast cell-free assays (Barlowe, 2003; Sato and Nakano, 2007) and the recent identification of COPII carriers in animal cells (Zeuschner *et al.*, 2006). These results are also consistent with our previous demonstration that GBF1 does not associate with ERES, but rather with VTCs that are close but physically separate from ERES (Zhao *et al.*, 2006).

To explain the apparent block in cargo export by BFA or Arf mutants (Ward *et al.*, 2001), we propose that these treatments do not block ER export, but rather prevent their retention in VTCs by promoting retrograde traffic from VTCs to ER. This model is based on our recent demonstration that BFA causes VTCs to lose their cargo to the ER through a microtubule-dependent mechanism (Zhao *et al.*, 2006). This could occur if the inactive GBF1 trapped on membranes by BFA (Niu *et al.*, 2005; Szul *et al.*, 2005; Zhao *et al.*, 2006) somehow interfered with protein sorting at ERES and/or VTCs. Alternatively, BFA could affect additional targets such as BARS, which has been implicated in membrane scission of COPI vesicles (Yang *et al.*, 2005) and whose activity is inhibited by BFA-induced ADP-ribosylation (Weigert *et al.*, 1999).

As initially suggested by the impact of the GBF1[E794K] mutant (Garcia-Mata *et al.*, 2003), GBF1 KD prevented the formation of motile transport competent carriers necessary for assembly and maintenance of the Golgi complex. In GBF1 KD cells, VSVG cargo accumulated in VTCs that contained several tethering factors such as p115 and giantin, but failed to either mature into Golgi resident enzyme-containing structures or to associate with microtubules and migrate to the cell center. The fact that VSVG but not Golgi resident enzymes accumulated in VTCs likely reflects the presence in VSVG of a di-acidic sorting signal efficiently recognized by COPII (Nishimura *et al.*, 1999; Sato and Nakano, 2007). The reason for the lack of movement to the cell center remains unknown but, as proposed by Szul and colleagues (Garcia-Mata *et al.*, 2003), it may be related to the absence of COPI-driven active protein sorting that normally drives formation of membrane domains critical for recruitment of other proteins such as rabs and motors/accessory proteins (Short *et al.*, 2005).

BIGs Are Required for Recruitment of Clathrin Adaptor and Maintenance of the TGN

Previous work established that BIGs localize at the TGN and overlap with clathrin, suggesting that BIGs regulate Arf activation for recruitment of GGAs and other adaptor proteins such as AP-1 (Yamaji *et al.*, 2000; Zhao *et al.*, 2002). We confirmed here the functional link between BIG1 and AP-1 by showing that BIG1 overexpression stabilizes AP-1 but not

COPI against dispersal after short BFA treatment. Nakayama and colleagues confirmed a similar link between BIG2 and AP-1 using related approaches (Shinotsuka *et al.*, 2002b). As predicted, BIGs KD caused loss of both AP-1 and GGA3 from the juxta-nuclear region. This redistribution of AP-1 is similar to that reported after BFA treatment of several cell lines in which the Golgi complex is either naturally resistant to BFA or acquired resistance after mutagenesis. For example, the Golgi stack of MDCK and PtK1 cells (Robinson and Kreis, 1992) or mutagenized CHO-K1 cells (Torii *et al.*, 1995) remains unperturbed after BFA treatment, whereas some AP-1 localizes to disperse puncta. In all cases, residual membrane association of clathrin adaptors likely results from Arf activation by endosome-associated BFA resistant Arf-GEFs of the ARNO and EFA6 subfamilies (D'Souza-Schorey and Chavrier, 2006).

Previous attempts to test the model that GGAs and APs recognize sorting signals in endosomal-targeted cargo and drive maturation of the TGN, focused on blocking the function of either AP1, -3, and -4 or GGAs (Bonifacino and Traub, 2003; Gleeson *et al.*, 2004). However, expression of dominant negative mutants or silencing of either types of adaptors led to variable outcomes ranging from tubulation of the Golgi, accumulation of endosomal cargo in the TGN or its dispersal to peripheral endosome structures (Puertollano *et al.*, 2001a,b; Ghosh *et al.*, 2003). These apparent discrepancies should not be surprising, however, because current evidence suggests that adaptors function in both anterograde and retrograde traffic and at the TGN participate in a multistep process involving both GGAs and APs (Ghosh and Kornfeld, 2004); loss of only a subset of the adaptors would imbalance this process, with complex consequences. Effective KD of BIGs circumvented this problem by preventing recruitment of both APs and GGAs to the Golgi complex. Under these conditions, we could readily detect loss of a recognizable juxta-nuclear TGN structure stained by TGN46 or sortilin. Disruption of sorting to the TGN was confirmed by clear loss of GalT accumulation in dispersed vacuoles after monensin treatment. We predict that simultaneous silencing of all GGAs and several APs will be required to observe effects similar to those we report for BIGs KD.

GBF1, But Not BIGs, Is Required for Assembly of a Polarized Golgi Stack

One of the more unexpected results of our study was the observation that BIGs KD did not prevent assembly of a polarized Golgi stack in mammalian cells; these stacks not only retained a degree of polarization similar to that of control cells, but also efficiently trafficked VSVG to the cell surface (Figures 7 and 8). These observations were surprising because previous work in *S. cerevisiae* had established that loss of Sec7p, the single orthologue of BIGs, completely alters Golgi morphology and blocks protein secretion. For example, Sec7 temperature-sensitive mutants accumulate large numbers of stacked Golgi membranes (Berkeley bodies) with concomitant block in traffic to the vacuole and cell surface (Novick *et al.*, 1980; Esmon *et al.*, 1981; Franzusoff and Schekman, 1989; Rambourg *et al.*, 1993; Deitz *et al.*, 2000). Although undetectable levels of remaining BIGs could account for those observations, we consider this possibility extremely unlikely as argued above. We propose instead that the apparent discrepancy between the impact of Sec7 inactivation and BIGs KD may reflect differences in the organization of the secretory pathways of *S. cerevisiae* and animals.

Yeast Golgi elements appear as fine or coarse nodular networks that are neither cisternal nor arranged in stacks

(Morin-Ganet *et al.*, 2000; Rambourg *et al.*, 2001; Kepes *et al.*, 2005). These morphogenetic studies revealed a gradual transformation of Golgi elements after their initial assembly from ER-derived vesicles: from tubular clusters, they become a network of fine tubules linked by nodes that transform into a thicker nodular network that eventually releases secretory granules by rupture of tubular areas. The presence of mixed forms containing two networks of different calibers (Morin-Ganet *et al.*, 2000) actually suggests that the gradual transformation from early to late elements involves concerted action of both the COPI and clathrin coats within a continuous network. This view is consistent with recent description of Golgi maturation in live yeast (Losev *et al.*, 2006; Matsuura-Tokita *et al.*, 2006) and may explain why loss of Sec7 would prevent maturation of the nodular network and cause accumulation of membranes unable to release cargo carriers.

In contrast, the Golgi complex of animal cells occurs as a structured stack of discrete flattened cisternae with varying extent of fenestration that is flanked by extensive tubular-reticular networks (Rambourg and Clermont, 1990; Thorne-Tjomsland *et al.*, 1998; Mogelsvang *et al.*, 2004). Our results suggest that the GBF1-dependent recruitment of the COPI coat is sufficient to promote the formation of specialized membrane domains and cargo carriers that can move cargo from VTCs, assemble onto the Golgi matrix and subsequently drive the maturation process to yield a polarized stack. The fact that one can eliminate BIGs and maintain much of this organization suggests that these mechanisms are robust and that stacking of separate cisternae may permit the observed uncoupling of *cis*- and *trans*-acting coats.

The observation that VSVG trafficked normally to the cell surface in BIGs KD cells remains consistent with the well-established role of the TGN in sorting of cargo to various destinations in both nonpolarized and polarized cells (Bonifacino and Traub, 2003; Rodriguez-Boulant *et al.*, 2005; Rodriguez-Boulant and Musch, 2005; Bard and Malhotra, 2006). Maturation by the COPI coat in animal cells may allow the creation of membrane domains on the *trans*-side that become enriched in anterograde cargo proteins and eventually peel off the stack to carry some or all of its content to the cell surface. Under normal conditions such intermediates would likely be absorbed in the TGN from which cargo would be then sorted to its various destinations. Our results establish that whatever mechanisms normally drive traffic of VSVG from the TGN remain operational in the absence of BIGs/clathrin-dependent sorting. The absence of a detectable TGN in BIGs KD cells raises one additional issue: why so few, if any, of the membranes released from the Golgi stack remain in this area. Further work will clearly be required to establish when and how the BIGs machinery is recruited to late cisternae to facilitate membrane retention, formation of the TGN, and sorting of endosomal cargo.

ACKNOWLEDGMENTS

We thank L. Channon for maintenance of cultured cells and RT-PCR analysis, Z. Shapovalova for construction of pSUPER vectors targeting BIG2, and F. Mast for advice on image quantitation as well as H. Chan for technical help with confocal microscopy. We thank Drs. K. Nakayama and H.-W. Shin (Kyoto University) for the BIG2 antibody and Dr. J. Rohrer (University of Zurich) for advice on the monensin experiments. Finally, we thank Drs. T. Hobman and Z. Wang (University of Alberta) for helpful discussions. This study was initiated with funds from the Alberta Heritage Foundation for Medical Research and supported by grants (P.M.) from the Canadian Institutes of Health Research and the Human Frontier Science Program. A.C. and J.R. were supported by the Fondecyt Grant 1030346.

REFERENCES

- Altan-Bonnet, N., Sougrat, R., and Lippincott-Schwartz, J. (2004). Molecular basis for Golgi maintenance and biogenesis. *Curr. Opin. Cell Biol.* 16, 364–372.
- Alvarez, C., Garcia-Mata, R., Brandon, E., and Sztul, E. (2003). COPI recruitment is modulated by a Rab1b-dependent mechanism. *Mol. Biol. Cell* 14, 2116–2127.
- Appenzeller-Herzog, C., and Hauri, H. P. (2006). The ER-Golgi intermediate compartment (ERGIC): in search of its identity and function. *J. Cell Sci.* 119, 2173–2183.
- Bard, F., and Malhotra, V. (2006). The formation of TGN-to-plasma-membrane transport carriers. *Annu. Rev. Cell Dev. Biol.* 22, 439–455.
- Barlowe, C. (2003). Signals for COPII-dependent export from the ER: what's the ticket out? *Trends Cell Biol.* 13, 295–300.
- Barzilay, E., Ben-Califa, N., Hirschberg, K., and Neumann, D. (2005). Uncoupling of brefeldin A-mediated coatamer protein complex-I dissociation from Golgi redistribution. *Traffic* 6, 794–802.
- Bonifacio, J. S. (2004). The GGA proteins: adaptors on the move. *Nat. Rev. Mol. Cell Biol.* 5, 23–32.
- Bonifacio, J. S., and Glick, B. S. (2004). The mechanisms of vesicle budding and fusion. *Cell* 116, 153–166.
- Bonifacio, J. S., and Traub, L. M. (2003). Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu. Rev. Biochem.* 72, 395–447.
- Borsig, L., Imbach, T., Hochli, M., and Berger, E. G. (1999). alpha1,3Fucosyltransferase VI is expressed in HepG2 cells and codistributed with beta1,4galactosyltransferase I in the golgi apparatus and monensin-induced swollen vesicles. *Glycobiology* 9, 1273–1280.
- Claude, A., Zhao, B. P., Kuziemy, C. E., Dahan, S., Berger, S. J., Yan, J. P., Armold, A. D., Sullivan, E. M., and Melancon, P. (1999). GBF1, a novel Golgi-associated BFA-resistant guanine nucleotide exchange factor that displays specificity for ADP-ribosylation factor 5. *J. Cell Biol.* 146, 71–84.
- Claude, A., Zhao, B. P., and Melancon, P. (2003). Characterization of alternatively spliced and truncated forms of the Arf guanine nucleotide exchange factor GBF1 defines regions important for activity. *Biochem. Biophys. Res. Commun.* 303, 160–169.
- Cox, R., Mason-Gamer, R. J., Jackson, C. L., and Segev, N. (2004). Phylogenetic analysis of Sec7-domain-containing Arf nucleotide exchangers. *Mol. Biol. Cell* 15, 1487–1505.
- D'Souza-Schorey, C., and Chavrier, P. (2006). ARF proteins: roles in membrane traffic and beyond. *Nat. Rev. Mol. Cell Biol.* 7, 347–358.
- Dascher, C., and Balch, W. E. (1994). Dominant inhibitory mutants of ARF1 block endoplasmic reticulum to Golgi transport and trigger disassembly of the Golgi apparatus. *J. Biol. Chem.* 269, 1437–1448.
- Deitz, S. B., Rambourg, A., Kepes, F., and Franzusoff, A. (2000). Sec7p directs the transitions required for yeast Golgi biogenesis. *Traffic* 1, 172–183.
- Donaldson, J. G., and Honda, A. (2005). Localization and function of Arf family GTPases. *Biochem. Soc. Trans.* 33, 639–642.
- Duden, R. (2003). ER-to-Golgi transport: COP I and COP II function (Review). *Mol. Membr. Biol.* 20, 197–207.
- Elbashir, S. M., Harborth, J., Weber, K., and Tuschl, T. (2002). Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* 26, 199–213.
- Esmon, B., Novick, P., and Schekman, R. (1981). Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. *Cell* 25, 451–460.
- Eugster, A., Frigerio, G., Dale, M., and Duden, R. (2004). The alpha- and beta'-COP WD40 domains mediate cargo-selective interactions with distinct di-lysine motifs. *Mol. Biol. Cell* 15, 1011–1023.
- Farquhar, M. G., and Palade, G. E. (1998). The Golgi apparatus: 100 years of progress and controversy. *Trends Cell Biol.* 8, 2–10.
- Franzusoff, A., and Schekman, R. (1989). Functional compartments of the yeast Golgi apparatus are defined by the sec7 mutation. *EMBO J.* 8, 2695–2702.
- Fromme, J. C., and Schekman, R. (2005). COPII-coated vesicles: flexible enough for large cargo? *Curr. Opin. Cell Biol.* 17, 345–352.
- Garcia-Mata, R., and Sztul, E. (2003). The membrane-tethering protein p115 interacts with GBF1, an ARF guanine-nucleotide-exchange factor. *EMBO Rep.* 4, 320–325.
- Garcia-Mata, R., Sztul, T., Alvarez, C., and Sztul, E. (2003). ADP-ribosylation factor/COPI-dependent events at the endoplasmic reticulum-Golgi interface are regulated by the guanine nucleotide exchange factor GBF1. *Mol. Biol. Cell* 14, 2250–2261.
- Ghosh, P., Griffith, J., Geuze, H. J., and Kornfeld, S. (2003). Mammalian GGAs act together to sort mannose 6-phosphate receptors. *J. Cell Biol.* 163, 755–766.
- Ghosh, P., and Kornfeld, S. (2004). The GGA proteins: key players in protein sorting at the trans-Golgi network. *Eur. J. Cell Biol.* 83, 257–262.
- Gleeson, P. A., Lock, J. G., Luke, M. R., and Stow, J. L. (2004). Domains of the TGN: coats, tethers and G proteins. *Traffic* 5, 315–326.
- Harborth, J., Elbashir, S. M., Bechert, K., Tuschl, T. C., and Weber, K. (2001). Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J. Cell Science* 114, 4557–4565.
- Kawamoto, K., Yoshida, Y., Tamaki, H., Torii, S., Shinotsuka, C., Yamashina, S., and Nakayama, K. (2002). GBF1, a guanine nucleotide exchange factor for ADP-ribosylation factors, is localized to the cis-Golgi and involved in membrane association of the COPI coat. *Traffic* 3, 483–495.
- Kepes, F., Rambourg, A., and Satiat-Jeunemaitre, B. (2005). Morphodynamics of the secretory pathway. *Int. Rev. Cytol.* 242, 55–120.
- Losev, E., Reinke, C. A., Jellen, J., Strongin, D. E., Bevis, B. J., and Glick, B. S. (2006). Golgi maturation visualized in living yeast. *Nature* 441, 1002–1006.
- Mansour, S. J., Skaug, J., Zhao, X. H., Giordano, J., Scherer, S. W., and Melancon, P. (1999). p200 ARF-GEP1, a Golgi-localized guanine nucleotide exchange protein whose Sec7 domain is targeted by the drug brefeldin A. *Proc. Natl. Acad. Sci. USA* 96, 7968–7973.
- Matsuura-Tokita, K., Takeuchi, M., Ichihara, A., Mikuriya, K., and Nakano, A. (2006). Live imaging of yeast Golgi cisternal maturation. *Nature* 441, 1007–1010.
- Mogelsvang, S., Marsh, B. J., Ladinsky, M. S., and Howell, K. E. (2004). Predicting function from structure: 3D structure studies of the mammalian Golgi complex. *Traffic* 5, 338–345.
- Morin-Ganet, M. N., Rambourg, A., Deitz, S. B., Franzusoff, A., and Kepes, F. (2000). Morphogenesis and dynamics of the yeast Golgi apparatus. *Traffic* 1, 56–68.
- Mouratou, B., Biou, V., Joubert, A., Cohen, J., Shields, D. J., Geldner, N., Jurgens, G., Melancon, P., and Cherfils, J. (2005). The domain architecture of large guanine nucleotide exchange factors for the small GTP-binding protein Arf. *BMC Genomics* 6, 20.
- Moyer, B. D., Allan, B. B., and Balch, W. E. (2001). Rab1 interaction with a GM130 effector complex regulates COPII vesicle cis-Golgi tethering. *Traffic* 2, 268–276.
- Nelson, D. S., Alvarez, C., Gao, Y. S., Garcia-Mata, R., Fialkowski, E., and Sztul, E. (1998). The membrane transport factor TAP/p115 cycles between the Golgi and earlier secretory compartments and contains distinct domains required for its localization and function. *J. Cell Biol.* 143, 319–331.
- Ni, X., Canuel, M., and Morales, C. R. (2006). The sorting and trafficking of lysosomal proteins. *Histol. Histopathol.* 21, 899–913.
- Nie, Z., and Randazzo, P. A. (2006). Arf GAPs and membrane traffic. *J. Cell Sci.* 119, 1203–1211.
- Nishimura, N., Bannykh, S., Slabough, S., Matteson, J., Altschuler, Y., Hahn, K., and Balch, W. E. (1999). A di-acidic (DXE) code directs concentration of cargo during export from the endoplasmic reticulum. *J. Biol. Chem.* 274, 15937–15946.
- Niu, T. K., Pfeifer, A. C., Lippincott-Schwartz, J., and Jackson, C. L. (2005). Dynamics of GBF1, a Brefeldin A-sensitive Arf1 exchange factor at the Golgi. *Mol. Biol. Cell* 16, 1213–1222.
- Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* 21, 205–215.
- Orci, L., Glick, B. S., and Rothman, J. E. (1986). A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. *Cell* 46, 171–184.
- Polishchuk, R. S., and Mironov, A. A. (2004). Structural aspects of Golgi function. *Cell Mol. Life Sci.* 61, 146–158.
- Presley, J. F., Cole, N. B., Schroer, T. A., Hirschberg, K., Zaal, K. J., and Lippincott-Schwartz, J. (1997). ER-to-Golgi transport visualized in living cells [see comments]. *Nature* 389, 81–85.
- Puertollano, R., Aguilar, R. C., Gorshkova, I., Crouch, R. J., and Bonifacio, J. S. (2001a). Sorting of mannose 6-phosphate receptors mediated by the GGAs. *Science* 292, 1712–1716.
- Puertollano, R., Randazzo, P. A., Presley, J. F., Hartnell, L. M., and Bonifacio, J. S. (2001b). The GGAs promote ARF-dependent recruitment of clathrin to the TGN. *Cell* 105, 93–102.

- Puri, S., Bachert, C., Fimmel, C. J., and Linstedt, A. D. (2002). Cycling of early Golgi proteins via the cell surface and endosomes upon luminal pH disruption. *Traffic* 3, 641–653.
- Puttenveedu, M. A., and Linstedt, A. D. (2005). Subcompartmentalizing the Golgi apparatus. *Curr. Opin. Cell Biol.* 17, 369–375.
- Rabouille, C., and Klumperman, J. (2005). Opinion: The maturing role of COPI vesicles in intra-Golgi transport. *Nat. Rev. Mol. Cell Biol.* 6, 812–817.
- Rambourg, A., and Clermont, Y. (1990). Three-dimensional electron microscopy: structure of the Golgi apparatus. *Eur. J. Cell Biol.* 51, 189–200.
- Rambourg, A., Clermont, Y., and Kepes, F. (1993). Modulation of the Golgi apparatus in *Saccharomyces cerevisiae* sec7 mutants as seen by three-dimensional electron microscopy. *Anat. Rec.* 237, 441–452.
- Rambourg, A., Jackson, C. L., and Clermont, Y. (2001). Three dimensional configuration of the secretory pathway and segregation of secretion granules in the yeast *Saccharomyces cerevisiae*. *J. Cell Sci.* 114, 2231–2239.
- Robinson, M. S. (2004). Adaptable adaptors for coated vesicles. *Trends Cell Biol.* 14, 167–174.
- Robinson, M. S., and Kreis, T. E. (1992). Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of brefeldin A and G protein activators. *Cell* 69, 129–138.
- Rodriguez-Boulán, E., Kreitzer, G., and Musch, A. (2005). Organization of vesicular trafficking in epithelia. *Nat. Rev. Mol. Cell Biol.* 6, 233–247.
- Rodriguez-Boulán, E., and Musch, A. (2005). Protein sorting in the Golgi complex: shifting paradigms. *Biochim. Biophys. Acta* 1744, 455–464.
- Sato, K., and Nakano, A. (2007). Mechanisms of COPII vesicle formation and protein sorting. *FEBS Lett.* 581, 2076–2082.
- Scales, S. J., Pepperkok, R., and Kreis, T. E. (1997). Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI. *Cell* 90, 1137–1148.
- Schaub, B. E., Berger, B., Berger, E. G., and Rohrer, J. (2006). Transition of galactosyltransferase 1 from trans-Golgi cisterna to the trans-Golgi network is signal mediated. *Mol. Biol. Cell* 17, 5153–5162.
- Seelig, H. P., Schranz, P., Schroter, H., Wiemann, C., and Renz, M. (1994). Macrogolgin—a new 376 kD Golgi complex outer membrane protein as target of antibodies in patients with rheumatic diseases and HIV infections. *J. Autoimmun.* 7, 67–91.
- Seemann, J., Jokitalo, E., Pypaert, M., and Warren, G. (2000). Matrix proteins can generate the higher order architecture of the Golgi apparatus. *Nature* 407, 1022–1026.
- Shinotsuka, C., Waguri, S., Wakasugi, M., Uchiyama, Y., and Nakayama, K. (2002a). Dominant-negative mutant of BIG2, an ARF-guanine nucleotide exchange factor, specifically affects membrane trafficking from the trans-Golgi network through inhibiting membrane association of AP-1 and GGA coat proteins. *Biochem. Biophys. Res. Commun.* 294, 254–260.
- Shinotsuka, C., Yoshida, Y., Kawamoto, K., Takatsu, H., and Nakayama, K. (2002b). Overexpression of an ADP-ribosylation factor-guanine nucleotide exchange factor, BIG2, uncouples brefeldin A-induced adaptor protein-1 coat dissociation and membrane tubulation. *J. Biol. Chem.* 277, 9468–9473.
- Short, B., Haas, A., and Barr, F. A. (2005). Golgins and GTPases, giving identity and structure to the Golgi apparatus. *Biochim. Biophys. Acta* 1744, 383–395.
- Szul, T., Garcia-Mata, R., Brandon, E., Shestopal, S., Alvarez, C., and Sztul, E. (2005). Dissection of membrane dynamics of the ARF-guanine nucleotide exchange factor GBF1. *Traffic* 6, 374–385.
- Tang, B. L., Wang, Y., Ong, Y. S., and Hong, W. (2005). COPII and exit from the endoplasmic reticulum. *Biochim. Biophys. Acta* 1744, 293–303.
- Thorne-Tjomsland, G., Dumontier, M., and Jamieson, J. C. (1998). 3D topography of noncompact zone Golgi tubules in rat spermatids: a computer-assisted serial section reconstruction study. *Anat. Rec.* 250, 381–396.
- Togawa, A., Morinaga, N., Ogasawara, M., Moss, J., and Vaughan, M. (1999). Purification and cloning of a brefeldin A-inhibited guanine nucleotide-exchange protein for ADP-ribosylation factors. *J. Biol. Chem.* 274, 12308–12315.
- Torii, S., Banno, T., Watanabe, T., Ikehara, Y., Murakami, K., and Nakayama, K. (1995). Cytotoxicity of brefeldin A correlates with its inhibitory effect on membrane binding of COP coat proteins. *J. Biol. Chem.* 270, 11574–11580.
- Wang, C. W., Hamamoto, S., Orci, L., and Schekman, R. (2006). Exomer: a coat complex for transport of select membrane proteins from the trans-Golgi network to the plasma membrane in yeast. *J. Cell Biol.* 174, 973–983.
- Ward, T. H., Polishchuk, R. S., Caplan, S., Hirschberg, K., and Lippincott-Schwartz, J. (2001). Maintenance of Golgi structure and function depends on the integrity of ER export. *J. Cell Biol.* 155, 557–570.
- Weide, T., Bayer, M., Koster, M., Siebrasse, J. P., Peters, R., and Barnekow, A. (2001). The Golgi matrix protein GM 130, a specific interacting partner of the small GTPase rab1b. *EMBO Rep.* 2, 336–341.
- Weigert, R. *et al.* (1999). CtBP/BARS induces fission of Golgi membranes by acylating lysophosphatidic acid. *Nature* 402, 429–433.
- Wessels, E., Duijsings, D., Lanke, K. H., van Dooren, S. H., Jackson, C. L., Melchers, W. J., and van Kuppeveld, F. J. (2006a). Effects of picornavirus 3A proteins on protein transport and GBF1-dependent COP-I recruitment. *J. Virol.* 80, 11852–11860.
- Wessels, E. *et al.* (2006b). A viral protein that blocks Arf1-mediated COP-I assembly by inhibiting the guanine nucleotide exchange factor GBF1. *Dev. Cell* 11, 191–201.
- Yamaji, R., Adamik, R., Takeda, K., Togawa, A., Pacheco-Rodriguez, G., Ferrans, V. J., Moss, J., and Vaughan, M. (2000). Identification and localization of two brefeldin A-inhibited guanine nucleotide-exchange proteins for ADP-ribosylation factors in a macromolecular complex. *Proc. Natl. Acad. Sci. USA* 97, 2567–2572.
- Yang, J. S., Lee, S. Y., Spano, S., Gad, H., Zhang, L., Nie, Z., Bonazzi, M., Corda, D., Luini, A., and Hsu, V. W. (2005). A role for BARS at the fission step of COPI vesicle formation from Golgi membrane. *EMBO J.* 24, 4133–4143.
- Zeuschner, D., Geerts, W. J., van Donselaar, E., Humbel, B. M., Slot, J. W., Koster, A. J., and Klumperman, J. (2006). Immuno-electron tomography of ER exit sites reveals the existence of free COPII-coated transport carriers. *Nat. Cell Biol.* 8, 377–383.
- Zhao, L., Helms, J. B., Brugger, B., Harter, C., Martoglio, B., Graf, R., Brunner, J., and Wieland, F. T. (1997). Direct and GTP-dependent interaction of ADP ribosylation factor 1 with coatomer subunit beta. *Proc. Natl. Acad. Sci. USA* 94, 4418–4423.
- Zhao, X., Claude, A., Chun, J., Shields, D. J., Presley, J. F., and Melancon, P. (2006). GBF1, a cis-Golgi and VTCs-localized ARF-GEF, is implicated in ER-to-Golgi protein traffic. *J. Cell Sci.* 119, 3743–3753.
- Zhao, X., Lasell, T. K., and Melancon, P. (2002). Localization of large ADP-ribosylation factor-guanine nucleotide exchange factors to different Golgi compartments: evidence for distinct functions in protein traffic. *Mol. Biol. Cell* 13, 119–133.