Promiscuity of the catalytic Sec7 domain within the guanine nucleotide exchange factor GBF1 in ARF activation, Golgi homeostasis, and effector recruitment

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ABSTRACT **The integrity of the Golgi and *trans*-Golgi network (TGN) is disrupted by brefeldin A (BFA), which inhibits the Golgi-localized BFA-sensitive factor (GBF1) and brefeldin A–inhibited guanine nucleotide-exchange factors (BIG1 and BIG2). Using a cellular replacement assay to assess GBF1 functionality without interference from the BIGs, we show that GBF1 alone maintains Golgi architecture; facilitates secretion; activates ADP-ribosylation factor (ARF)1, 3, 4, and 5; and recruits ARF effectors to Golgi membranes. Unexpectedly, GBF1 also supports TGN integrity and recruits numerous TGN-localized ARF effectors. The impact of the catalytic Sec7 domain (Sec7d) on GBF1 functionality was assessed by swapping it with the Sec7d from ARF nucleotide-binding site opener (ARNO)/cytohesin-2, a plasma membrane GEF reported to activate all ARFs. The resulting chimera (GBF1-ARNO-GBF1 [GARG]) targets like GBF1, supports Golgi/TGN architecture, and facilitates secretion. However, unlike GBF1, GARG activates all ARFs (including ARF6) at the Golgi/TGN and recruits additional ARF effectors to the Golgi/TGN. Our results have general implications: 1) GEF's targeting is independent of Sec7d, but Sec7d influence the GEF substrate specificity and downstream effector events; 2) all ARFs have access to all membranes, but are restricted in their distribution by the localization of their activating GEFs; and 3) effector association with membranes requires the coincidental presence of activated ARFs and specific membrane identifiers.**

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Abbreviations used: AP1, adaptor protein 1; APP, Alzheimer's precursor protein; ARF, ADP-ribosylation factor; ARNO, ARF nucleotide-binding site opener; BFA, Brefeldin A; BIG1 and BIG2, BFA-inhibited guanine nucleotide-exchange factors; COPI, coat protein complex I; FAPP2, Golgi-associated four-phosphate adaptor protein 2; GARG, GBF1-ARNO-GBF1; GBF1, Golgi-localized BFA-sensitive factor; GCA, Golgicide A; GDP, guanosine diphosphate; GEF, guanine nucleotide exchange factor; GGA, Golgi-localized gamma-adaptin ear homology domain ARF-binding protein; GLuc, *Gaussia* luciferase; GTP, guanosine triphosphate; PBS, phosphate-buffered saline; TGN, *trans*-Golgi network.

INTRODUCTION

Compartment integrity and vesicular traffic require the recruitment of numerous proteins from the cytosol to restricted membrane sites to facilitate cargo sorting, vesicle formation, microtubule-based motility, and lipid metabolism (van Vliet *et al*., 2003). Many such proteins are recruited to membranes by binding to activated forms of small GTPases of the ADP-ribosylation factor (ARF) superfamily. In primate cells, the five ARFs belonging to class I (ARF1 and ARF3), class II (ARF4 and ARF5), and class III (ARF6) localize to different compartments and appear to perform different functions (Hosaka *et al*., 1996). ARF1 localizes to and presumably functions at multiple compartments, including ERGIC, Golgi, *trans*-Golgi network (TGN), endosomes, and plasma membrane (Hosaka *et al*., 1996). ARF3 appears more restricted and is concentrated at the TGN (Padilla *et al*., 2004; Shin *et al*., 2004). ARF 4 and ARF5 preferentially localize at the ERGIC and the Golgi, but ARF4 also has been reported to

function at the endosome (Volpicelli-Daley *et al.*, 2005; Chun *et al.*, 2008; Duijsings *et al.*, 2009). ARF6 is the most restricted in distribution and localizes and functions predominantly at the plasma membrane (and perhaps to some degree within endosomal compartments) (Cavenagh *et al.*, 1996; Cohen *et al.*, 2007). However, how the specific distribution of the different ARFs is achieved and how the different ARFs inform the recruitment of distinct effectors at each site remain unclear.

Like all GTPases, ARFs function by cycling between an inactive guanosine diphosphate (GDP)-bound state and an active guanosine triphosphate (GTP)-bound form. ARF activation is mediated by a family of guanine nucleotide exchange factors (GEFs) characterized by a conserved catalytic Sec7d, which governs the displacement of GDP from the ARF and thus promotes the binding of the activating GTP. GEFs can be subdivided into the Golgi-localized Brefeldin A (BFA)-sensitive factor (GBF1); BFA-inhibited guanine nucleotide-exchange factors (BIG1 and BIG2) and the BFA-resistant cytohesins, BRAGs, EFA6; and F-box proteins (Bui *et al.*, 2009; Wright *et al.*, 2014). ARF activation is essential for compartmental integrity and vesicular traffic at the endoplasmic reticulum (ER)–Golgi interface, within the Golgi, and from the TGN to the plasma membrane and requires BFA-sensitive GEFs, as treatment of cells with BFA causes Golgi/TGN collapse and blocks secretion (Oda *et al.*, 1990; Orci *et al.*, 1991).

The relative contributions of the three BFA-sensitive GEFs to the maintenance of intracellular compartments remain unclear. GBF1 appears to be the only GEF acting within the early secretory pathway since GBF1 is the only GEF localizing to the ERGIC and early Golgi (the BIGs localize to the TGN and endosomal compartments), and depleting GBF1 by small interfering RNA (siRNA) or inhibiting it with selective drugs such as Golgicide A (GCA) or LG186 that do not inhibit the BIGs cause the collapse of the Golgi and block secretion (Mansour *et al.*, 1998; Claude *et al.*, 1999; Kawamoto *et al.*, 2002; Garcia-Mata *et al.*, 2003; Szul *et al.*, 2007; Saenz *et al.*, 2009; Boal *et al.*, 2010). GBF1 depletion or inhibition blocks the membrane recruitment of numerous effectors including the heptameric coat protein complex I (COPI) necessary for the formation of recycling COPI vesicles (Aridor *et al.*, 1995; Griffiths *et al.*, 1995; Lowe and Kreis, 1996, 1998), GMAP210 is required for Golgi integrity and positioning (Infante *et al.*, 1999; Pernet-Gallay *et al.*, 2002; Rios *et al.*, 2004), golgin-160 is necessary for the Golgi transit of select cargo (Hicks *et al.*, 2006; Williams *et al.*, 2006), and Golgi-associated four-phosphate adaptor protein 2 (FAPP2) is crucial for sphingolipids transfer and metabolism (Godi *et al.*, 2004; D'Angelo *et al.*, 2007). Despite its preferential concentration at the *cis*-Golgi, GBF1 is also detected at the TGN, and the extent to which GBF1 acts at the TGN remains largely unknown (Lowery *et al.*, 2013).

BIG1 predominantly localizes to the TGN, but relocates to the nucleus under certain cellular stresses such as serum starvation (Padilla *et al.*, 2004). How BIG1 functions in the nucleus and whether that function is mediated through its nucleotide exchange on ARFs are unknown. BIG1 also appears to perform some function(s) at the *medial*-Golgi since depletion of BIG1 causes an impairment in the glycosylation of some (ICAM-1 and β-integrin) but not all (E-cadherin) cargo proteins at a step prior to the activity of α1–6 *N*-acetyl-glucosaminyl-transferase V, an enzyme localized to the *trans*-Golgi (Shen *et al.*, 2007). In addition, the Golgi membranes in BIG1 siRNA-treated cells appear less compact and sharply defined, with more vesicles present around the abnormal Golgi/TGN than in mock siRNA-treated cells and partial Golgi fragmentation (Boal and Stephens, 2010). In contrast, other reports indicate that depleting BIG1 has no effect on Golgi architecture (as defined by the characteristic localization of giantin and normal COPI distribution [Shen *et al.*, 2006]) or the TGN (as defined by normal localization of golgin-245 [Ishizaki *et al.*, 2008]). Thus, the exact functions of BIG1-mediated ARF activation remain unclear. It also remains unclear whether the lack of strong phenotypes after BIG1 depletion reflect the ability of the related BIG2 to support such functions. The level of functional overlap is difficult to tease out, but BIG1 also performs functions distinct from those of BIG2, as Schwann cell-specific deletion of BIG1 impaired myelination in mice (Miyamoto *et al.*, 2018), showcasing the inability of other GEFs to complement the BIG1 deficit.

BIG2 localizes to the TGN where it colocalizes extensively with BIG1 and also to endosomal compartments (Pacheco-Rodriguez *et al.*, 2002; Shin *et al.*, 2004). BIG2-mediated ARF activation appears essential for the recruitment of the AP1 adaptor protein, since: 1) overexpression of BIG2 in cells where other GEFs are inactivated with BFA maintained AP1 (but not COPI) recruitment to membranes; 2) overexpression of the dominant inactive BIG2/E738K caused the dissociation of AP1 (and Golgi-localized gamma-adaptin ear homology domain ARF-binding protein [GGA1]), but did not affect membrane recruitment of COPI (Shinotsuka *et al.*, 2002a,b); and 3) depletion of BIG2 caused AP1 dissociation (Ishizaki *et al.*, 2008). In addition to AP1, BIG2 also seems to regulate membrane recruitment of GGA3 and golgin-97 since high level expression of the inactive BIG2/E738K results in their dissociation (Shin *et al.*, 2004, 2005). However, conflicting results from Ishizaki *et al.* (2008) suggest that BIG2 doesn't regulate GGA3 and golgin-245 recruitment since both remained membrane-associated in cells from which BIG1/2 were depleted individually or together (Ishizaki *et al.*, 2008). Instead, BIG2 may function at the Golgi since the expression of the inactive BIG2/E738K caused the arrest of E-cadherin traffic in the Golgi (Shen *et al.*, 2007, 2012). Thus, although BIG2 involvement in AP1 recruitment is confirmed, the exact role BIG2 plays in the recruitment of other effectors at the TGN remains uncertain. Equally unclear is the functional overlap between BIG2 and BIG1, even though BIG2 regulates at least some specific functions since mutations in BIG2 cause periventricular heterotopia with microcephaly (Sheen *et al.*, 2004), whereas deletion of BIG2 in mice is embryonic lethal (Grzmil *et al.*, 2010), indicating the inability of other GEFs to compensate for the lack of functional BIG2. In addition to their roles at the TGN, both BIG1 and BIG2 localize to recycling endosomes and function to regulate the endosomal cycling of the transferrin receptor (Shen *et al.*, 2006), furin, and TGN46 (Ishizaki *et al.*, 2008). BIGs appear essential for the integrity of the recycling endosomes, as inactivation of BIG1/2 with BFA causes the tubulation of Rab11-positive endosomes (Shin *et al.*, 2004).

Although we increased our understanding of GEF functions through RNA interference (RNAi)-mediated or genetic depletion, drug-mediated inhibition, and expression of dominant inactive forms of GEFs, such approaches have limitations. First, they do not assess whether a particular GEF is sufficient to support a certain event, only that it is required. Second, they do not test whether a GEF supports multiple events, since the inhibition of the first step will by default preclude the analysis of subsequent steps. Here, we utilize a cellular replacement assay to explore the activity of one of the BFA-sensitive GEFs, GBF1, by inhibiting all endogenous GEFs with BFA and then putting back only GBF1 made BFA-resistant by the A795E mutation (GBF1/795).

The Sec7d in GEFs is believed to define its selectivity for ARF substrates, and thereby impact the recruitment of specific effectors by the activated ARFs. To determine how the Sec7d can alter GEF function, we substituted the Sec7d of GBF1 with that of ARF nucleotide-binding site opener (ARNO)/cytohesin-2, a GEF localized to

the plasma membrane (Macia *et al.*, 2001; Cohen *et al.*, 2007). The resulting chimera (GARG: GBF1-ARNO-GBF1) was then used in the cellular replacement assay to assess its behavior.

RESULTS

GBF1/795 supports Golgi architecture and recruits ARF effectors to the Golgi

Golgi biogenesis and maintenance and the integrity of the secretory pathway require GBF1-mediated ARFs activation, as shown by Golgi disruption and block in secretion in cells depleted of GBF1 (Kawamoto *et al.*, 2002; Zhao *et al.*, 2002; Garcia-Mata and Sztul, 2003), or in which the catalytic activity of GBF1 is inhibited by BFA (Saenz *et al.*, 2009; Boal *et al.*, 2010). BFA intercalates into an interface formed by the Sec7d of GBF1 and ARF-GDP and prevents GDP expulsion by the catalytic glutamic acid at position 794 within the Sec7d (Beraud-Dufour *et al.*, 1998; Goldberg, 1998; Renault *et al.*, 2003). However, introducing the A795E substitution immediately adjacent to the glutamic acid (to generate GBF1/795) prevents BFA entry into the binding pocket and makes GBF1/795 resistant to BFA. Cells expressing GBF1/795 and treated with BFA maintain Golgi architecture and support secretion (Pocognoni *et al.*, 2018). In agreement, we show that cells expressing GBF1/795 have morphologically recognizable Golgi (labeled with the GM130 Golgi marker), whereas untransfected cells show dispersed Golgi (Figure 1A). The ability of GBF1/795 to activate ARF in the presence of BFA was assessed by monitoring the recruitment of known ARF effectors to the ERGIC/Golgi. We show that GBF1/795 sustains the recruitment of 1) the β-COP component of the heptameric COPI coat that facilitates COPI vesicle formation at the ERGIC and the Golgi (Presley *et al.*, 2002; Elsner *et al.*, 2003; Faini *et al.*, 2012) (Figure 1B); 2) the GMAP210 coiled–coil golgin with a role in maintaining Golgi ribbon integrity and position and containing an ARF1-binding GRAB (grip-related Arf-binding domain that aids its localization to the *cis*-Golgi (Infante *et al.*, 1999; Pernet-Gallay *et al.*, 2002; Rios *et al.*, 2004) (Figure 1C); 3) the golgin-160 required for the transport of the β1-adrenergic receptor and the GLUT4 glucose transporter through the Golgi (Hicks *et al.*, 2006; Williams *et al.*, 2006) and which localizes to *cis*- and *medial*-Golgi (Hicks and Machamer, 2002) (Figure 1D); and 4) the FAPP2 sphingolipid transfer protein essential for proper lipid transport and metabolism that preferentially localizes to *cis*-Golgi (D'Angelo *et al.*, 2007) (Figure 1E).

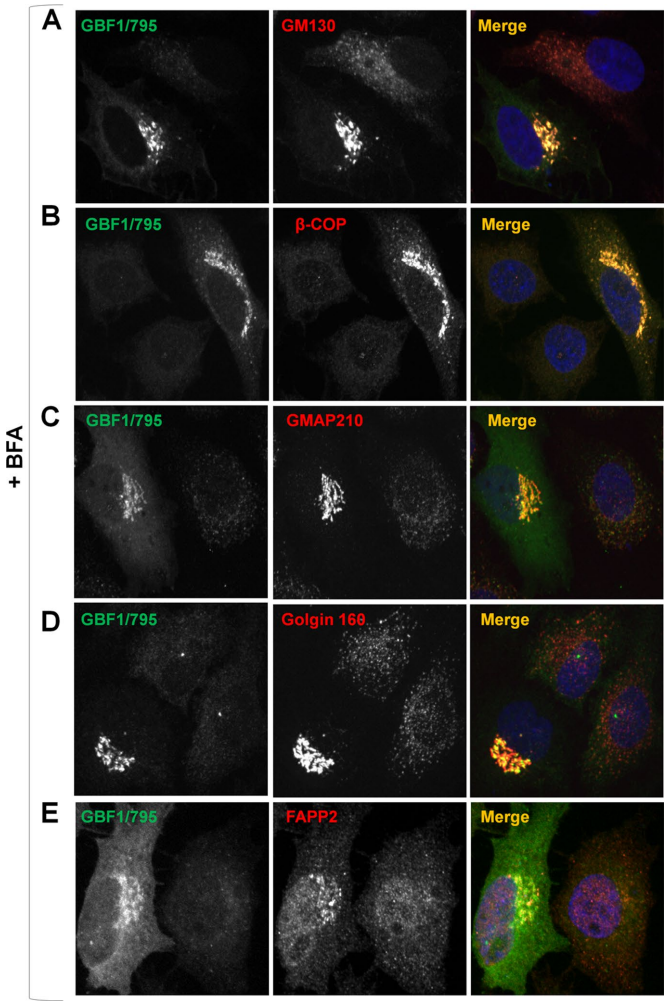

FIGURE 1: GBF1/795 supports Golgi architecture and recruits ARF effectors to the Golgi. HeLa cells were transfected with GFP-tagged GBF1/795 for 24 h, treated with BFA (0.5 μg/ml) for 30 min, and processed for IF with antibodies to GFP and the indicated markers. GBF1/795-expressing cells, but not untransfected cells contain morphologically recognizable Golgi and recruit ARF effectors to the Golgi.

GBF1/795 supports TGN integrity and recruits ARF effectors to the TGN

That GBF1/795 supports Golgi homeostasis was expected since GBF1 localizes to the Golgi and functions therein (Zhao *et al.*, 2002, 2006; Garcia-Mata *et al.*, 2003). However, GBF1 is also detected at more distal TGN compartments (Lowery *et al.*, 2013), raising the possibility that GBF1 may impact the biogenesis and/or maintenance of the TGN. Three golgins, golgin-245, golgin-97, and GCC88 (Lu and Hong, 2003; Luke *et al.*, 2003; Yoshino *et al.*, 2003; Derby *et al.*, 2004) that localize to distinct subdomains of the TGN, were used to monitor the ability of GBF1/795 to sustain TGN architecture in BFA-treated cells. Morphologically recognizable TGN structures containing golgin-245 (Figure 2A), golgin-97 (Figure 2B), or GCC88 (Figure 2C) were observed in cells expressing GBF1/795 but not in untransfected cell. Thus, GBF1/795 can support the architecture of at least the TGN subdomains defined by golgin-245, golgin-97, and GCC88 in cells in which the TGN-localized BIG1 and BIG2 are inactivated with BFA.

We have shown previously that BIG1 and BIG2 are ARF effectors since they preferentially bind the active GTP-bound forms of ARF4 and ARF5 to localize to the TGN (Lowery *et al.*, 2013). To assess whether BIG1 and BIG2 recruitment to TGN membranes is mediated by GBF1, we examined BIG1 and BIG2 localization in cells expressing GBF1/795 and treated with BFA. Both, BIG1 (Figure 2D) and BIG2 (Figure 2E) were recruited to morphologically recognizable TGN in cells expressing GBF1/795, but not in untransfected cells. BIG1 and BIG2 in untransfected cells showed a fine punctate distribution, suggesting that BIG1/2 may remain membrane bound in the presence of BFA, as would be expected since BFA stabilizes the membrane-associated complex of GEFs with their ARF-GDP substrates (Robineau *et al.*, 2000; Mossessova *et al.*, 2003; Niu *et al.*, 2005; Szul *et al.*, 2005).

ARF activation at the TGN is required for the recruitment of the AP1 and the three GGA1-3 adaptors involved in cargo sorting and delivery from the TGN to compartments of the endosomal system, but it remains unclear which GEF(s) are involved (Robinson, 2004, 2015; Hirst *et al.*, 2012). In addition, the MINT3 adaptor involved in the sorting and trafficking of the furin protease (Han *et al.*, 2008) and the Alzheimer's precursor protein (APP) (Shrivastava-Ranjan *et al.*, 2008; Caster and Kahn, 2013) between the TGN and endosomes

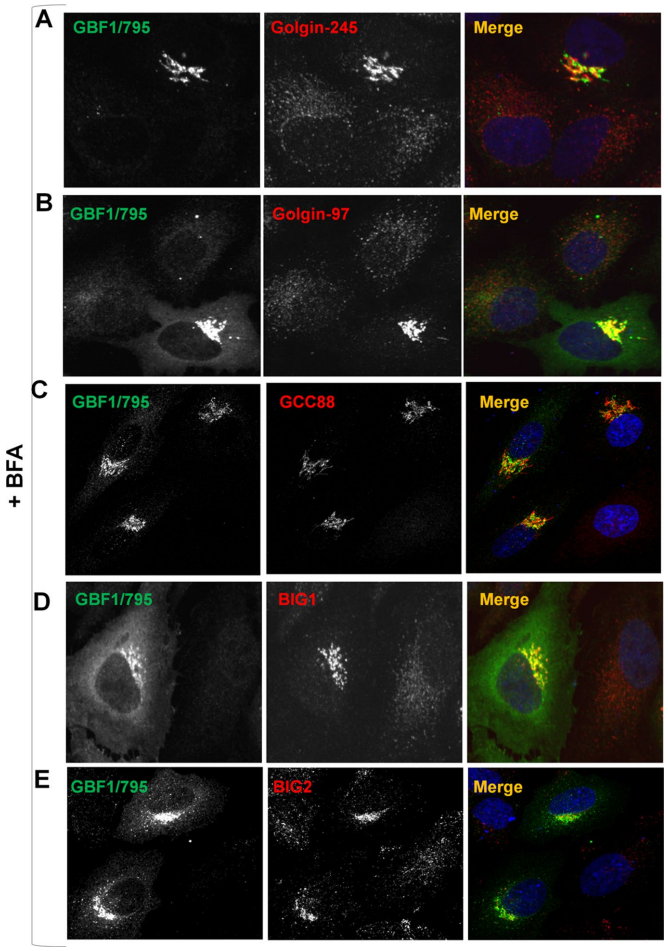

FIGURE 2: GBF1/795 maintains TGN architecture and recruits ARF effectors to the TGN. HeLa cells were transfected with GFP-tagged GBF1/795 for 24 h, treated with BFA (0.5 μg/ml) for 30 min, and processed for IF with antibodies to GFP and the indicated markers. GBF1/795-expressing cells, but not untransfected cells, contain morphologically recognizable TGN and recruit ARF effectors to the TGN.

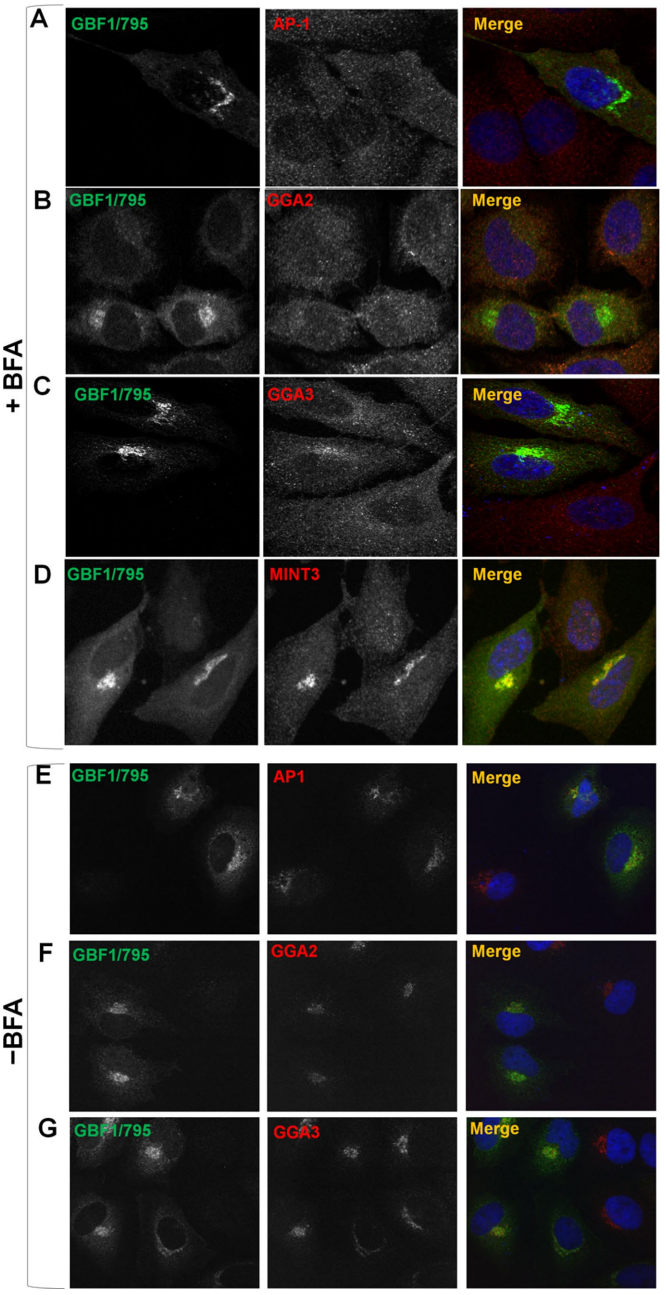

FIGURE 3: GBF1/795 shows selectivity for ARF effector recruitment. HeLa cells were transfected with GFP-tagged GBF1/795 for 24 h, treated with BFA (0.5 μg/ml) for 30 min (A–D) or left untreated (E–G), and processed for IF with antibodies to GFP and the indicated markers. BFA-treated GBF1/795-expressing cells, but not untransfected cells, contain morphologically recognizable Golgi/TGN that recruit MINT3, but do not recruit AP1, GGA2, or GGA3 adaptors (A–D). Cells not treated with BFA contain TGN-localized AP1, GGA2, and GGA3 irrespective of expressing GBF1/795 (E–G).

also is recruited by activated ARF, but the GEF that activates the relevant ARFs is unknown. We show that BFA-treated cells expressing GBF1/795 have dispersed AP1 (Figure 3A), GGA2 (Figure 3B), and GGA3 (Figure 3C), but membrane-localized MINT3 (Figure 3D). To ensure that the lack of TGN staining is not due to technical issues, we show that using the same experimental protocol, we can detect the TGN-localized AP1, GGA2, and GGA3 in cells not treated with BFA (Figure 3, E–G). Thus, GBF1/795-mediated ARF activation supports the architecture of at least some TGN subcompartments and mediates the membrane recruitment of some (MINT3), but not all (AP-1, GGA2, GGA3), TGN-localized ARF effectors.

GARG (GBF1 containing Sec7d from ARNO/cytohesin-2) supports Golgi architecture and recruits ARF effectors to the Golgi

The role of the catalytic Sec7 domain in influencing the functional output of GBF1 was assessed by substituting the Sec7d of GBF1 with the Sec7d from ARNO (also called cytohesin-2) to generate the GBF1-ARNO-GBF1 chimera called GARG (Figure 4A). ARNO localizes to the plasma membrane where it regulates actin dynamics and endosomal traffic by activating ARF1 and possibly ARF6 (Frank *et al.*, 1998a,b; Santy *et al.*, 2001). ARNO is not known to activate ARF3 or class II ARFs in vivo. ARNO is intrinsically BFA resistant due to an amino acid sequence in its Sec7d. Since GARG contains a BFA-resistant Sec7d, it is BFA resistant and its cellular functions can be tested without interference from endogenous GBF1/BIG1/BIG2 in cells treated with BFA.

We first tested whether overexpressing ARNO can support Golgi/TGN homeostasis in cells treated with BFA. Cells expressing

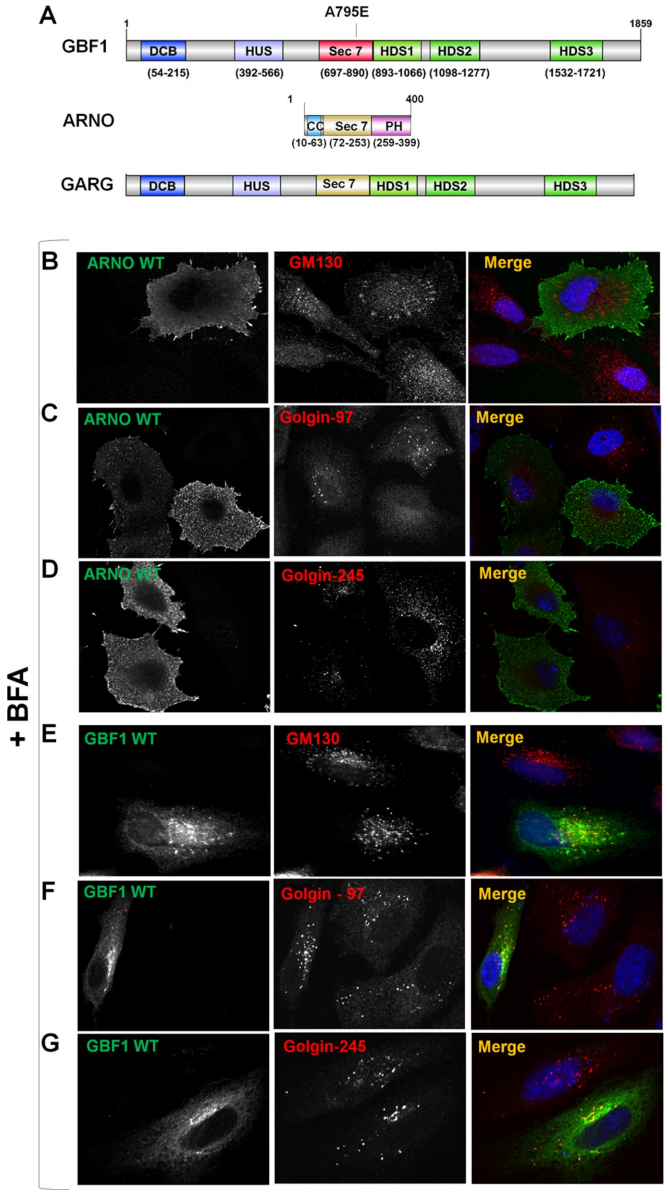

FIGURE 4: Schematic of GARG. (A) Domain organization of GBF1, ARNO, and GARG. The A795E mutation within the Sec7d that confers BFA resistance to GBF1/795 is marked. (B–G) HeLa cells were transfected with Flag-tagged wild-type ARNO (B–D) or GFP-tagged wild-type GBF1 (E–G) for 24 h, treated with BFA (0.5 μg/ml) for 30 min, and processed for IF with antibodies to Flag (B–D) or GFP (E–G) and the indicated markers. Neither ARNO nor wild-type GBF1 maintain Golgi/TGN architecture or recruit ARF effectors to the TGN in BFA-treated cells.

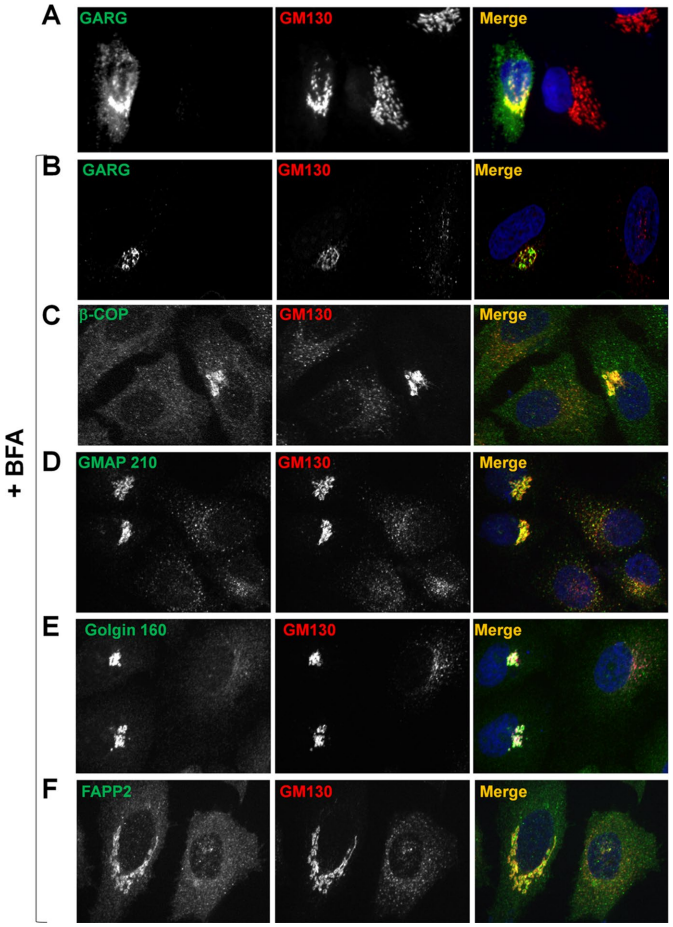

FIGURE 5: GARG supports Golgi architecture and recruits ARF effectors to the Golgi. (A, B) HeLa cells were transfected with GFP-tagged GARG for 24 h and either left untreated (A) or treated with BFA (0.5 μg/ml) for 30 min (B), and processed for IF with antibodies to GFP and GM130. GARG targets to the Golgi in untreated cells (A), and maintains Golgi integrity in BFA-treated cells (B). (C–F) HeLa cells were transfected with Myc-tagged GARG for 24 h, treated with BFA (0.5 μg/ml) for 30 min, and then processed for IF with antibodies to GM130 (to detect cells with intact Golgi, i.e., those expressing GARG) and the indicated markers. Cells containing intact Golgi (i.e., expressing GARG) recruit ARF effectors to the Golgi.

ARNO do not maintain the Golgi or the TGN, as shown by the dispersed distribution of GM130 (Figure 4B), golgin-97 (Figure 4C), and golgin-245 (Figure 4D). A similar pattern of disruption is observed in cells expressing the BFA-sensitive wild-type GBF1 (Figure 4, E–G). Thus, neither the BFA-resistant ARNO nor the BFA-sensitive wild-type GBF1 can maintain Golgi/TGN in cells treated with BFA.

We then assessed whether GARG can support Golgi/TGN homeostasis. When expressed in HeLa cells, GARG colocalizes with the GM130 Golgi marker, but doesn't target to the plasma membrane (Figure 5A), indicating targeting analogous to that of GBF1, but not ARNO. This implies that the targeting of GARG to the Golgi is governed by domains within the protein other than the changed Sec7d. GARG is fully functional in sustaining Golgi architecture in cells treated with BFA, as indicated by the characteristic localization of the GM130 Golgi marker in GARG expressing cells but not in untransfected cells (Figure 5B). GARG appears to function in a manner analogous to that of GBF1/795 since it supports the recruitment of the ARF effectors β-COP (Figure 5C; in these panels, cells expressing GARG are identified by the intact pattern of GM130), GMAP210 (Figure 5D), golgin-160 (Figure 5E), and FAPP2 (Figure 5F).

GARG (GBF1 containing Sec7d from ARNO) supports TGN architecture and recruits ARF effectors to the TGN

Because GBF1/795 supports the architecture of the TGN and recruits ARF effectors to the TGN, we examined whether GARG can mediate analogous ARF activation. In these studies, cells expressing GARG are identified by their content of a morphologically recognizable Golgi labeled with the GM130 Golgi marker. We show that BFA-treated cells containing Golgi-localized GM130 (i.e., expressing

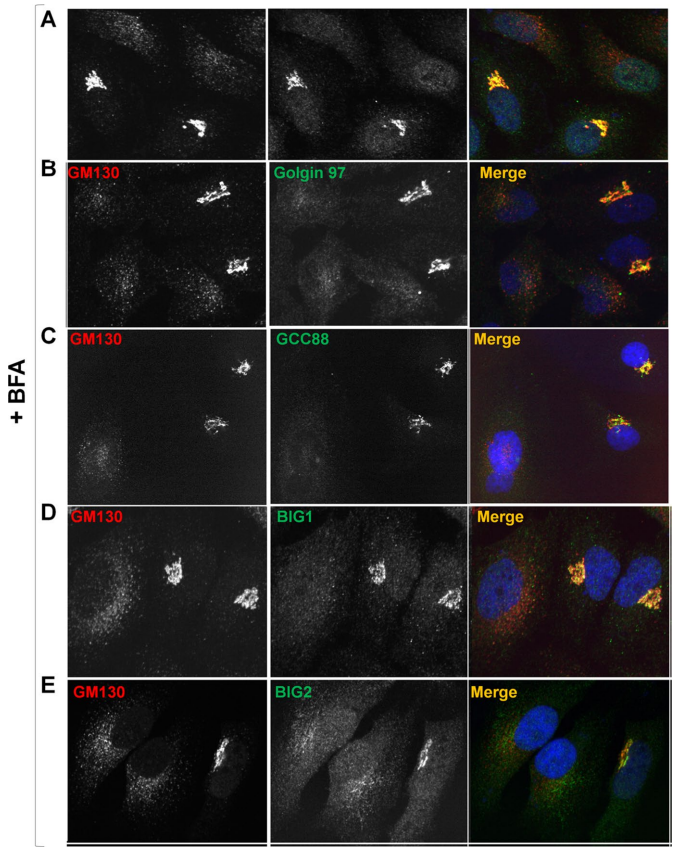

FIGURE 6: GARG supports TGN integrity and recruits ARF effectors to the TGN. HeLa cells were transfected with Myc-tagged GARG for 24 h, treated with BFA (0.5 μg/ml) for 30 min, and then processed for IF with antibodies to GM130 (to detect cells with intact Golgi, i.e., those expressing GARG) and the indicated markers. Cells containing morphologically recognizable Golgi (i.e., expressing GARG) recruit ARF effectors to the TGN.

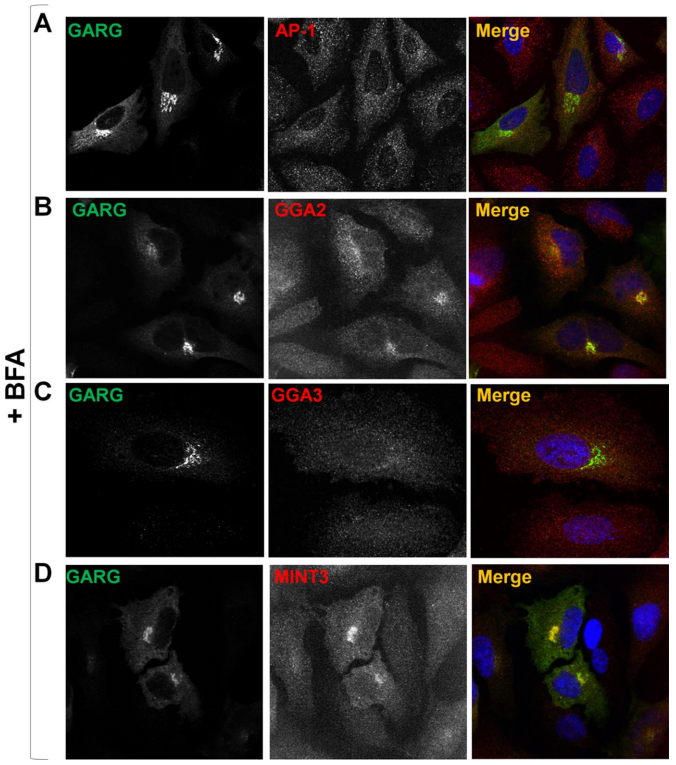

FIGURE 7: GARG shows selectivity for ARF effector recruitment. HeLa cells were transfected with GFP-tagged GARG for 24 h, treated with BFA (0.5 μg/ml) for 30 min and processed for IF with antibodies to GFP and the indicated markers. BFA-treated GARG-expressing cells, but not untransfected cells, contain morphologically recognizable Golgi/TGN that recruit GGA2 and MINT3, but do not recruit AP1 or GGA2 adaptors.

GARG) have TGN-localized golgin-245 (Figure 6A), golgin-97 (Figure 6B), and GCC88 (Figure 6C), whereas untransfected cells show dispersed patterns for these proteins. Like GBF1/795, GARG facilitates the recruitment of the ARF effectors BIG1 (Figure 6D) and BIG2 (Figure 6E) to the TGN in BFA-treated cells. This suggests that GARG activates ARF4 and ARF5 at the TGN since those ARFs have been shown to be necessary for BIG1/2 membrane association (Lowery *et al.*, 2013). Examination of additional TGN-localized ARF effectors indicates that like GBF1/795, GARG does not support the recruitment of AP1 (Figure 7A) or GGA3 (Figure 7C), but does support the recruitment of GGA2 (Figure 7B) and MINT3 (Figure 7D). Thus, GARG appears to support the recruitment of GGA2 to membranes, a function not performed by GBF1/795.

GBF1 and GARG support secretion

GBF1-mediated ARF activation is required for protein secretion and cells treated with BFA or the GBF1-specific Golgicide A (GCA), or depleted of GBF1 do not support cargo release (Szul *et al.*, 2007; Saenz *et al.*, 2009). To explore whether GBF1/795 and GARG are sufficient to sustain secretion, we utilized a *Gaussia* luciferase-based secretion assay and compared secretion from cells transfected with GBF1/795, GARG, or an empty vector in the presence of increasing concentrations of BFA. As shown in Figure 8, cells expressing an empty vector are impaired in secretion, with less than 30% secretion at the lowest BFA concentration and less than 10% at the highest. In contrast, cells expressing GBF1/795 support robust secretion, with more than 75% secretion even at the highest BFA concentration used. Similarly, cells expressing GARG support secretion with an efficiency similar to that of GBF1/795, indicating that GARG can perform all the ARF activation events normally performed by GBF1 within the secretory pathway.

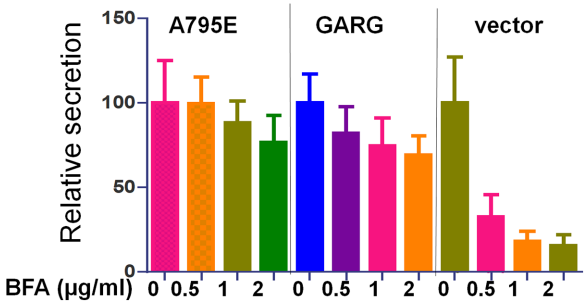

FIGURE 8: GBF1/795 and GARG support protein secretion. HeLa cells were cotransfected with plasmids encoding *Gaussia* luciferase and GBF1/795, GARG, or empty vector for 24 h, the medium was removed, and the cells were incubated in fresh medium supplemented with the indicated amount of BFA. After a 4-h incubation, the amount of secreted luciferase was measured. GBF1/795 and GARG support secretion in the presence of BFA, whereas cells transfected with empty vector are inhibited.

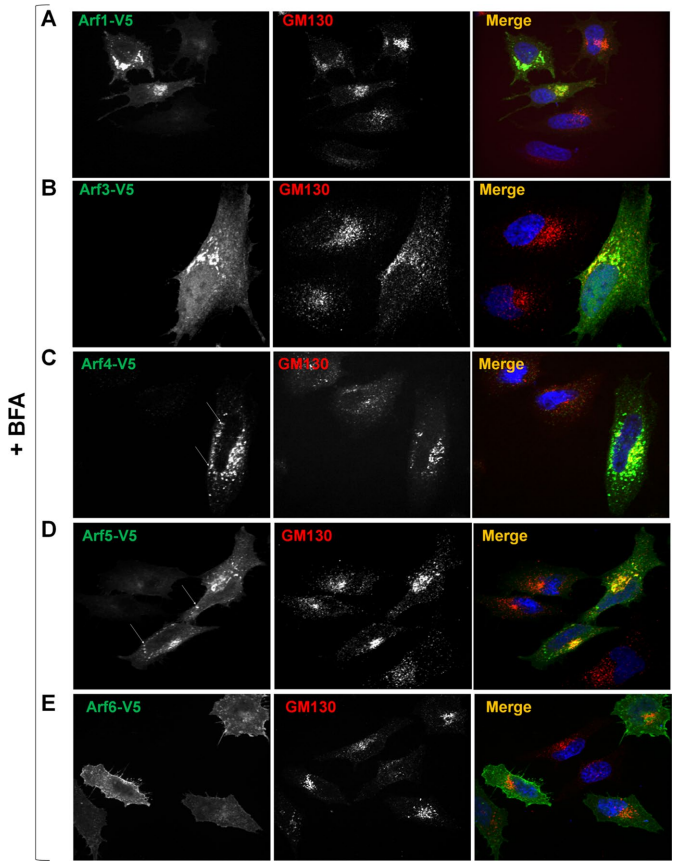

FIGURE 9: GBF1/795 recruits class I and class II ARFs to the Golgi/TGN. HeLa cells were cotransfected with Myc-tagged GBF1/795 and V5-tagged ARFs for 24 h, treated with BFA (0.5 µg/ml) for 30 min, and then processed for IF with antibodies to GM130 (to detect cells with intact Golgi, i.e., those expressing GBF1/795) and V5. All ARFs except ARF6 are recruited to perinuclear morphologically recognizable Golgi/TGN. ARF4 and ARF5 are also detected on more peripheral elements (arrows).

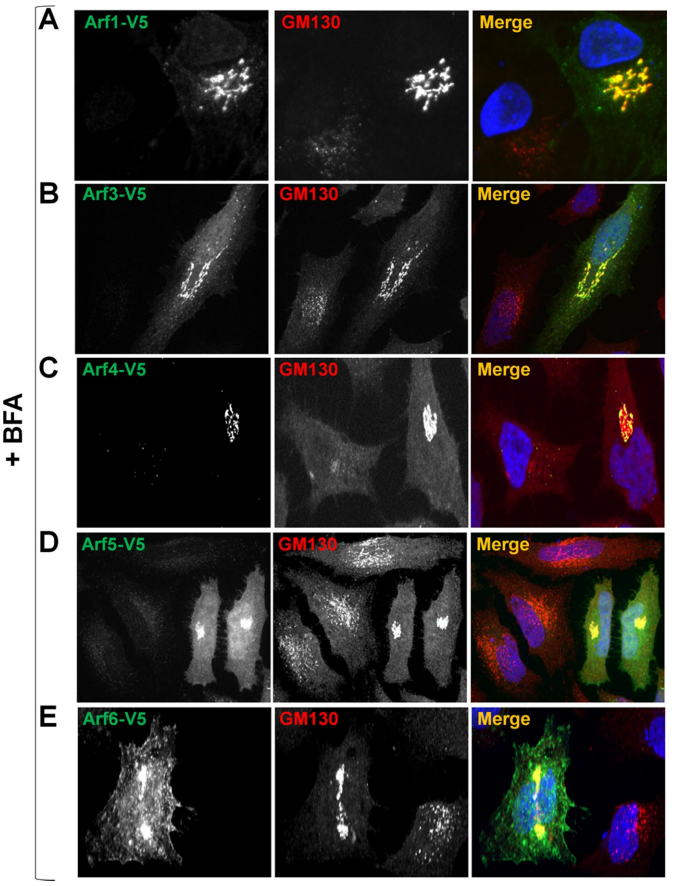

FIGURE 10: GARG recruits all ARFs to the Golgi/TGN. HeLa cells were cotransfected with Myc-tagged GARG and V5-tagged ARFs for 24 h, treated with BFA (0.5 µg/ml) for 30 min, and then processed for IF with antibodies to GM130 (to detect cells with intact Golgi, i.e., those expressing GARG) and V5. All ARFs, including ARF6, are recruited to perinuclear morphologically recognizable Golgi/TGN.

GBF1 activates a subset of ARFs at the Golgi/TGN

Four of the five human ARFs (ARF1, 3, 4, and 5) localize to the ERGIC/Golgi/TGN and could be activated by GBF1, BIG1, and/or BIG2. To identify the ARFs activated by GBF1, we cotransfected cells with GBF1/795 and each of the five human ARFs; treated cells with BFA to inactivate the endogenous GBF1, BIG1, and BIG2; and monitored the membrane-recruitment of the ARFs as a measure of activation status. Cells coexpressing GBF1/795 and a specific ARF were identified by the presence of the ARF and the presence of intact GM130-labeled Golgi (we routinely observe more than 90% cotransfection with these plasmids). We observed ARF1 (Figure 9A), ARF3 (Figure 9B), ARF4 (Figure 9C), and ARF5 (Figure 9D) in morphologically recognizable Golgi/TGN structures in transfected cells. ARF4 (Figure 9C, arrows) and ARF5 (Figure 9D, arrows) were also present in peripheral punctate structures. In contrast, ARF6 was detected only on the plasma membrane (in agreement with being recruited there by BFA-resistant GEFs [Macia *et al.*, 2001]) and was not recruited to the Golgi/TGN. Thus, the Golgi/TGN-localized GBF1/795 appears to activate ARF1, 3, 4, and 5, but not ARF6.

GARG (GBF1 containing Sec7d from ARNO) activates all ARFs, including ARF6 at the Golgi/TGN

GARG supports all tested functions performed by GBF1/795, implying that GARG activates all the ARFs normally activated by GBF1/795. In agreement, in BFA-treated cells expressing GARG (identified by an intact GM130-labeled Golgi), we detected ARF1 (Figure 10A), ARF3 (Figure 10B), ARF4 (Figure 10C), and ARF5 (Figure 10D) associated with Golgi/TGN membranes. GARG contains the Sec7d from ARNO, which is known to activate ARF6, raising the question of whether GARG could also activate ARF6 on Golgi/TGN membranes. We show that in BFA-treated cells expressing GARG, ARF6 is recruited to the Golgi/TGN, in addition to its normal plasma membrane localization (Figure 10E). Thus, unlike GBF1/795, the Golgi/TGN-localized GARG can recruit ARF6 to Golgi/TGN membranes.

GARG does not recruit ARF6 effectors to the Golgi/TGN

GARG activates ARF6 at the Golgi/TGN, raising the possibility that the activated ARF6 can facilitate the recruitment of ARF6 effectors to the Golgi/TGN. We first examined the AP2 adaptor protein component of clathrin-coated pits and vesicles (McMahon and Boucrot, 2011; Mettlen *et al.*, 2018). In both untransfected cells and GARG-expressing cells, AP2 was detected in a punctate pattern consistent with its usual PM localization without recruitment to the Golgi/TGN-localized GARG (Figure 11A). Similar punctate distribution and lack of Golgi/TGN recruitment was observed for JIP3, ARF6 effector regulating the dynamics of endosomal tubules and impacting neurite morphogenesis of cortical neurons (Suzuki *et al.*, 2010; Montagnac *et al.*, 2011; Marchesin *et al.*, 2015).

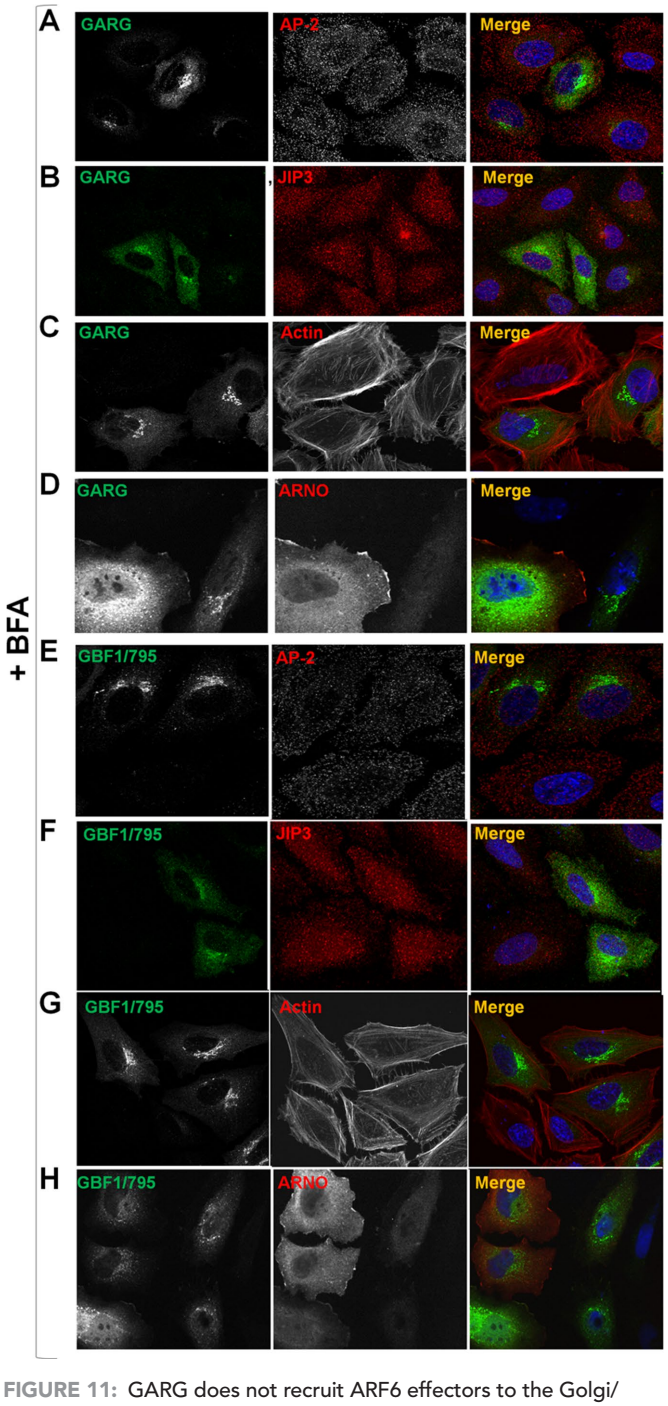

FIGURE 11: GARG does not recruit ARF6 effectors to the Golgi/TGN. (A–C, E–G) HeLa cells were transfected with Venus-tagged GARG (A–C) or GFP-tagged GBF1/795 (E–G) for 24 h, treated with BFA (0.5 μg/ml) for 30 min, and then processed for IF with antibodies to GFP and either indicated markers or stained with phalloidin. Cells expressing GARG or GBF1/795 do not show Golgi/TGN-localized AP-2, JIP3 or actin. (D, H) HeLa cells were cotransfected with Myc-tagged ARNO and either Venus-tagged GARG (D) or GFP-tagged GBF1/795 (H) for 24 h, treated with BFA (0.5 μg/ml) for 30 min, and then processed for IF with antibodies to Flag and GFP. Cells expressing GARG or GBF1/795 do not show Golgi-localized ARNO. ARNO expression causes partial Golgi/TGN disruption.

Activated ARF6 is known to stimulate PLD to increase the production of PIP_2 and the subsequent recruitment of actin (Schafer *et al.*, 2000; Santy and Casanova, 2001; Li, Shome *et al.*, 2003; Hiroyama and Exton, 2005). Examination of actin architecture in GARG-expressing cells shows stress fibers and a subcortical pattern that appears analogous to that in untransfected cells, without preferential recruitment of actin to the Golgi/TGN (Figure 11C). We also examined the behavior of the ARF6 effector ARNO (Santy and Casanova, 2001; Cohen *et al.*, 2007) by cotransfecting GARG and ARNO into cells and assessing ARNO recruitment to the Golgi/TGN. We show that ARNO localizes to the PM, but is not recruited to GARG-containing structures (Figure 11D). ARNO expression has been shown to cause Golgi disruption (Monier *et al.*, 1998) and we also observe a less compacted Golgi in ARNO-expressing cells. Together, our results suggest that GARG-mediated recruitment of ARF6 to the Golgi/TGN does not automatically result in the recruitment of ARF6 effectors (at least the ones tested here) to the Golgi/TGN. An analogous lack of ARF6 effector recruitment is seen in cells expressing the GBF1/795 construct (Figure 11, E–H).

DISCUSSION

Activated ARFs recruit essential cytosolic effectors to the membranes of the ERGIC/Golgi/TGN to facilitate cargo selection and vesicle biogenesis. BFA disrupts this process by inhibiting the catalytic activity of GBF1, BIG1, and BIG2. The specificity of these GEFs in activating one or more ARF in a spatially restricted manner to facilitate recruitment of specific effectors remains poorly defined. To tease apart the functionality of the three GEFs, we used a cell replacement assay in which we inhibit the endogenous GBF1/BIG1/2 with BFA and then put back only GBF1/795 that is insensitive to BFA to probe GBF1 functions without the interference from BIG1/2.

GBF1 functions at the Golgi and the TGN

GBF1 is concentrated at ERGIC and *cis*-Golgi and functions therein (Zhao *et al.*, 2002; Garcia-Mata *et al.*, 2003). GBF1 is also detected at the TGN, but whether GBF1 controls TGN integrity and the recruitment of ARF effectors at the TGN is less clear since BIG1 and BIG2 have been localized to the TGN (Zhao *et al.*, 2002) and are assumed to act there. Here, we dissected GBF1 cellular function away from BIG1/2 activities.

As expected, GBF1/795 supports Golgi morphology and recruits ARF effectors to the Golgi, including the heptameric COPI coat essential for Golgi homeostasis (Styers *et al.*, 2008); GMAP210, a golgin required for Golgi integrity and positioning relative to the MTOC (Rios *et al.*, 2004; Cardenas *et al.*, 2009); golgin-160, a transport factor required for the trafficking of select transmembrane cargos through the Golgi (Hicks *et al.*, 2006; Williams *et al.*, 2006); and the FAPP2, a sphingolipid transfer protein required for Golgi to PM transport (Godi *et al.*, 2004; Tuuf and Mattjus, 2014). GBF/795 alone supports the secretion of soluble proteins, indicating that it is the sole BFA-sensitive GEF required for this process.

Unexpectedly, GBF1/795 also supports the architecture of TGN. The TGN is a complex organelle composed of multiple subdomains containing distinct marker proteins (reviewed in Kjer-Nielsen *et al.*, 1999; Luke *et al.*, 2003; Derby *et al.*, 2004, 2007; Gleeson *et al.*, 2004; Spang, 2016). For example, golgin-245 and golgin-97 localize to a distinct domain than the GCC88 and GCC185 golgins, as shown by the amplification of distinct, nonoverlapping golgin-positive structures in cells simultaneously expressing GCC88 and golgin-97 (Derby *et al.*, 2004). We show that cells expressing GBF1/795 contain TGN-localized golgin-245, golgin-97, and GCC88 while they are dispersed in untransfected cells treated with BFA. Thus, at least some subdomains of the TGN are maintained by GBF1/795 without the participation of the BIGs.

Additional evidence for GBF1 function at the TGN was shown by the recruitment of BIG1 and BIG2 (effectors of ARF4 and ARF5; Lowery *et al.*, 2013) and of MINT3, an effector of an as yet uncharacterized ARF (Shrivastava-Ranjan *et al.*, 2008; Caster and Kahn, 2013). In contrast, GBF1/795 did not support the recruitment of AP1, GGA2, and GGA3, suggesting that this function is normally performed by other GEFs, perhaps BIG1/2. The lack of AP1 recruitment by GBF1 is consistent with previous studies implicating BIG1/2 in AP1 recruitment (Ishizaki *et al.*, 2008). The regulation of GGA1 and GGA3 recruitment is unclear: whereas overexpression of the inactive BIG2/E738K causes GGA1 (Shinotsuka *et al.*, 2002a) and GGA3 (Shin *et al.*, 2005) to dissociate from membranes, those findings contradict other studies showing that the depletion of BIG2 has no visible impact on GGA3 membrane association (Ishizaki *et al.*, 2008). To further complicate matters, GGA1-3 have been shown to bind GBF1 (Lefrancois and McCormick, 2007), raising the possibility that GBF1 might mediate the ARF activation that recruits GGAs to the TGN. In support: 1) GGA3 was recruited to membranes in BFA-treated MDCK cells in which only GBF1 (but not BIG1/2) is BFA resistant due to an altered Sec7d sequence in the canine GBF1 (Lefrancois and McCormick, 2007); 2) RNAi-mediated GBF1 depletion caused GGA3 dissociation from membranes (Boal and Stephens, 2010); and 3) the expression of the dominant, inactive GBF1/E794K caused the dissociation of all three GGA1-3 from membranes (Lefrancois and McCormick, 2007). However, a contradictory result showing that selectively inactivating only GBF1 with GCA maintains GGA3 on the membranes (Boal *et al.*, 2010) underscores the difficulty in assigning a role to a particular GEF at the TGN. Our definitive results confirm that GBF1 alone is unable to support GGA2 or GGA3 recruitment. Thus, it appears that all three GGAs are recruited to the TGN through ARFs activated by the BIGs.

Together, our results suggest that GBF1 functions at multiple compartments of the secretory pathway to support the recruitment of distinct ARF effectors to the ERGIC (i.e., GMAP-210), the Golgi (i.e., golgin-160) and the TGN (i.e., MINT3). The spatially differential recruitment of effectors implies that factors in addition to the GEF and the ARF activated by that GEF govern effector localization and thereby control the downstream events.

GBF1 is promiscuous and activates all class I and class II, but not class III, ARFs

Four of the five human ARFs (ARF1, 3, 4, and 5) localize to the Golgi/TGN, with ARF4 and ARF5 also associating with the ERGIC (Cavenagh *et al.*, 1996; Hosaka *et al.*, 1996; Lowe *et al.*, 1996). All are activated by BFA-sensitive GEFs since all four quickly redistribute from the membranes in BFA-treated cells, albeit some ARF4/5 dissociates more slowly from ERGIC membranes (Chun *et al.*, 2008). The single class III ARF6 predominantly localizes to the plasma membrane and is activated by BFA-insensitive GEFs, as it remains membrane-bound in BFA-treated cells (Cavenagh *et al.*, 1996; Aikawa and Martin, 2005; Cohen *et al.*, 2007).

The ARF specificities of the three BFA-sensitive GEFs remain poorly defined. For example, analyses of GBF1 show that 1) recombinant GBF1 activates ARF5, but doesn't activate ARF1 or ARF3 (Claude *et al.*, 1999); 2) endogenous GBF1 coprecipitates with ARF1 and ARF4, but not with ARF3 in BFA-treated cells in which GBF1 is predicted to be trapped in a complex with its substrates (Szul *et al.*, 2007); 3) GBF1 cycling time on the membrane is altered by ARF1-GDP, indicating that it functions as a substrate (Niu *et al.*, 2005; Szul *et al.*, 2005); and 4) the removal of ARF1 and ARF4 (but no other single or double ARFs) causes Golgi tubulation analogous to that resulting from GBF1 depletion (Volpicelli-Daley *et al.*, 2005). The conclusion most consistent with these findings suggests that GBF1 activates ARF1 and 4, but perhaps not the other ARFs.

BIG1 purified from bovine brain shows activity toward ARF1 and ARF3 (Morinaga *et al.*, 1996), whereas analyses of BIG2 provide contradictory results and show that 1) recombinant BIG2 catalyzes guanine–nucleotide exchange on ARF1 and ARF5 in vitro (Togawa *et al.*, 1999); 2) overexpression of BIG2 in cells causes an increase in activated ARF1 and ARF3 (Shin *et al.*, 2005); and 3) only the removal of the ARF1 and ARF3 pair alters transferrin cycling, a function regulated by BIG2 (Volpicelli-Daley *et al.*, 2005). The most consistent findings suggest that BIG1 and BIG2 both activate ARF1 and 3, but probably not the other ARFs.

Using BFA-treated cells expressing GBF1/795, we show that GBF1/795 recruits ARF1, 3, 4, and 5 to the Golgi. ARF 1/4/5 are predicted to be activated by GBF1 based on their localization to the ERGIC/*cis*-Golgi. Unexpectedly, GBF1/795 also recruited ARF3, previously assumed to be activated by the BIGs at the TGN. Thus, the catalytic Sec7d of GBF1 can utilize all human class I and class II ARFs as substrates. In contrast, ARF6 was not recruited to the Golgi in cells expressing GBF1/795, indicating that the Sec7d of GBF1 discriminates against the single class III ARF.

GARG supports all functions of GBF1/795, but also performs additional functions

It is unknown to what degree the Sec7d of a GEF influences the behavior of that GEF. We explored this for GBF1 by swapping its Sec7d with the Sec7d from ARNO/cytohesin-2, a GEF localized at the plasma membrane where it regulates actin dynamics and endosomal traffic by activating ARF1 and possibly ARF6 (Frank *et al.*, 1998a,b; Caumont *et al.*, 2000; Turner and Brown, 2001). The resulting GARG chimera localizes in a manner analogous to endogenous GBF1, indicating that targeting of GBF1 is independent of its Sec7d. The Sec7d might be the only domain within GBF1 not involved in its localization, since GBF1 association with membranes has been shown to require an intact N-terminus (Monetta *et al.*, 2007), HDS1 (Meissner *et al.*, 2018; Quilty *et al.*, 2018), HDS2 (Pocognoni *et al.*, 2018), as well as the C-terminus (Garcia-Mata and Sztul, 2003). GARG does not target to the plasma membrane, the localization of endogenous ARNO/cytohesin-2, indicating that the Sec7d has limited input into GEF targeting and that GEF's selectivity for membranes is governed through interactions other than with their substrate ARFs. It seems that the catalytic Sec7d functions autonomously and in fact is a mere "worker" positioned at select sites through interactions defined by the rest of GBF1. It is likely that this is a general paradigm for GEFs.

We assessed whether GARG can perform the functions supported by GBF1/795. We show that like GBF1/795, GARG supports Golgi and TGN integrity, facilitates secretion of cargo, and mediates the recruitment of all the Golgi/TGN-localized ARF effectors that GBF1/795 recruits. Unexpectedly, GARG also supports the recruitment of GGA2, a function that GBF1/795 does not perform. This suggests that GARG activates an ARF in addition to those activated by GBF1/795, and that it is this additional ARF that recruits GGA2 (see below).

GBF1 containing the Sec7d of ARNO/cytohesin-2 activates all ARFs, including ARF6 at the Golgi/TGN

The substrate specificity of ARNO/cytohesin-2 is unclear. Purified ARNO/cytohesin-2 has the highest activity on ARF1, 80% less activity on ARF5, and 95% less activity on ARF6 (Frank *et al.*, 1998b; Macia *et al.*, 2001; Zeeh *et al.*, 2006). A more recent study shows that purified ARNO/cytohesin-2 artificially tethered to

lipid membranes has the highest specific activity on ARF4 and ARF5, lower and equivalent activity on ARF1 and ARF3, and lowest activity on ARF6 (Peurois *et al.*, 2017). In vivo studies show that ARNO/cytohesin-2 activates ARF1 in cells, but whether it activates other ARFs was not explored in those works (Cohen *et al.*, 2007).

Our finding that GARG can support all processes supported by GBF1/795 suggested that GARG activates all the ARFs that GBF1/795 activates. In agreement, we show that GARG recruits ARF1, ARF3, ARF4, and ARF5 to the Golgi/TGN, a finding consistent with the ability of purified ARNO to activate these ARFs. Furthermore, GARG also recruits ARF6 at the Golgi/TGN, a process not supported by GBF1/795 but consistent with the ability of the ARNO Sec7d to activate ARF6.

These results have a number of implications: 1) the ability of the Golgi/TGN-localized GARG to activate ARF3, ARF4 and ARF5 suggests that the Sec7d within the endogenous ARNO/cytohesin-2 localized to the plasma membrane can activate the same ARFs. Yet ARF3, ARF4 and ARF5 are not found on the plasma membrane, indicating that the presence of a GEF is insufficient to define the distribution of activated ARFs. 2) The ability of GARG to activate ARF6 indicates that the Sec7d controls the ARF selectivity of the GEF since the Sec7d from ARNO enabled the "mostly GBF1" GARG to activate ARF6. This also implies that the selectivity of the Sec7d impacts the recruitment of effectors and controls the downstream signaling events. 3) The ability of GARG to recruit all ARFs including ARF6 to Golgi/TGN indicates that all ARFs, including ARF6, have access to all cellular membranes. ARF6 has been assumed to be always membrane bound, based on findings showing that the ARF6/T27N mutant (assumed to represent the GDP-bound form) is found on membrane structures resembling endosomes and is recovered exclusively in the membrane fraction after fractionation of transfected cells (Peters *et al.*, 1995; Radhakrishna *et al.*, 1996; D'Souza-Schorey *et al.*, 1998). However, more recent studies reported that the ARF6/T27N mutant is prone to aggregation (Macia *et al.*, 2004). This group generated the ARF6/T44N mutant (based on the GDP-GTP structural cycle of ARF6 [Menetrey *et al.*, 2000; Pasqualato *et al.*, 2001]) and showed that the ARF6/T44N and wild-type ARF6 are recovered in the membrane and the cytosolic fractions of transfected cells. These findings suggest that endogenous ARF6 in the GDP-bound state may cycle between membranes and the cytosol and thereby have access to all cellular membranes. This implies that the selective subcellular distribution of ARFs is informed by their activation through spatially restricted GEFs but is incompletely governed by it (see point 1 above). 4) Although GARG recruited all ARFs to the Golgi/TGN, not all ARF effectors were recruited to Golgi/TGN. Specifically, AP1 and GGA3 were not recruited, implying that effectors associate with membranes not just by the virtue of activated ARFs but also require specific membrane identifiers.

MATERIALS AND METHODS

Antibodies

Polyclonal anti-GFP (ab290), mouse monoclonal anti-β COP (ab6323), and rabbit monoclonal FAPP2 (ab235300) antibody were purchased from Abcam (Cambridge, MA). Monoclonal anti-GBF1 (612116), GGA2 (BD612612), GGA3 (BD612310), GM130 (BD610823), GMAP210 (611713), golgin-245 (BD611280), and polyclonal anti-MINT3 (BD611380) were obtained from BD Bioscience (San Jose, CA). Polyclonal MINT3 antibodies were also a gift from Richard Kahn (Emory University, Atlanta, GA). Monoclonal anti-V5 (R960-25), golgin-97(A21270), polyclonal anti-GM130 (PA1-077), and anti-JIP3 (PA5-59728) were purchased from Thermo Scientific (Rockford, IL). Monoclonal anti-GFP (A11120), anti-BIG1 (MABS1247) raised against 311 amino acid fragment (residues 212–522) of human BIG1, anti-BIG2 (MABS1246) against 249 amino acids from the N-terminal region of human BIG2, anti-AP1 (A4200), and polyclonal anti-Flag (F7425) and anti-golgin-160 (HPA040044) were from MilliporeSigma (Burlington, MA). Polyclonal anti-GCC88 (GTX120148) were from GeneTex (Irvine, CA). Secondary goat anti-rabbit antibodies conjugated with Alexa 488 (A11034) or Alexa 594 (A11012), goat anti-mouse conjugated with Alexa 488 (A11029) or Alexa 594 (A11042), and phalloidin conjugated with Alexa 594 (A12381) were from Thermo Scientific (Hanover Park, IL).

Plasmids

GBF1/795 has been described previously (Pocognoni *et al.*, 2018). Replacement of GBF1 Sec7d with Sec7d from ARNO/cytohesin-2 in full-length GBF1 was performed in multiple PCRs, including three overlapping PCRs. Primers used were 5′-gaccaagctgtgagatagtagatgg-3′ (GBF1-F) and 5′-ccatgttgaaatctggcaggagacagg-3′ (GBF1/ARNO-R) to amplify N-terminus of GBF1, before GBF1 Sec7; 5′-cctgccagatttcaacatggacccca-3′ (ARNO/GBF1-F) and 5′-gttctcccggtcattcccgtcatcc-3′ (ARNO/GBF1-R) to amplify ARNO Sec7; 5′-cgggaatgaccgggagaactatgtgtg-3′ (GBF1/ARNO-F), and 5′-cacgatgaaggacagcgattcc-3′ (GBF1-R) to amplify C-terminus of GBF1, after GBF1 Sec7. The GBF1 sequence was amplified from wild-type GBF1 sequence in pcDNA4-myc/his vector (Invitrogen), and the Sec7d was amplified from FLAG-tagged ARNO/cytohesin-2 that was a gift from James Casanova (University of Virginia, Charlottesville, VA). The used primers had incorporated restriction sites for cloning the GARG chimera into the pcDNA4 vector. V5-tagged ARF constructs were a gift from Richard Kahn (Emory University, Atlanta, GA).

Mammalian cell culture and transfection

HeLa cells were grown in MEM supplemented with glucose and glutamine and 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 1 mM sodium pyruvate; all of these reagents were purchased from Cellgro (Manassas, VA). Cells were grown at 37°C in 5% CO_2 until ~75% confluent and were transfected with Mirus TransIT-LT1 transfection reagent (Mirus Bio Corp., Madison, WI), according to the manufacturer's instructions. After transfection, cells were grown overnight and used for experiments.

Immunofluorescence microscopy

In some experiments, cells were incubated with 5 μg/ml BFA for 30 min before processing by immunofluorescence. Cells were processed for immunofluorescence as follows: cells were washed three times in phosphate-buffered saline (PBS), fixed in 3% paraformaldehyde in PBS for 10 min, and quenched with 10 mM ammonium chloride in PBS for another 10 min. Subsequently, cells were permeabilized in 0.1% Triton X-100 in PBS for 7 min. The coverslips were then washed in PBS, blocked in PBS containing 2.5% goat serum and 0.2% Tween 20 for 5 min, and in PBS, 0.4% fish skin gelatin and 0.2% Tween-20 for another 5 min. Cells were incubated with primary antibody diluted in 0.4% fish skin gelatin for 1 h at room temperature, washed in PBS–0.2% Tween 20, and blocked as described above. Then, cells were incubated with secondary antibodies diluted in 2.5% goat serum for 45 min at room temperature. Nuclei were stained using Hoechst, and coverslips were washed with PBS–0.2% Tween 20 and mounted on slides in ProLong Gold antifade reagent (Invitrogen, Madison WI). Cells were visualized with a Leitz Wetlzar microscope with epifluorescence and Hoffman Modulation Contrast optics from Chroma Technology. Images were captured with a 12-bit CCD camera from Q imaging using iVision-Mac software.

Confocal microscopy

Confocal imaging was with a PerkinElmer Ultraview ERS 6FE spinning disk confocal attached to a Nikon TE 2000-U microscope equipped with laser and filter sets for FITC, TRITC, and DAPI fluorescence. Images were captured using a Hamamatsu C9100-50 EM-CCD camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and 60× or 100× Plan APO oil-immersion objectives. The imaging system was controlled by Volocity 6.2 software (PerkinElmer, Shelton, CT).

Secretion assay

HeLa cells were cotransfected in 96-well plates with plasmids encoding *Gaussia* luciferase (GLuc) and the GBF1/795 or GARG construct (1:9 mass ratio). An empty vector was used as a negative control. The next day, the medium was removed, and the cells were washed to remove secreted luciferase and incubated in 25 µl of fresh medium supplemented with the indicated amount of BFA. After 4 h of incubation, the medium was transferred into another 96-well plate, and the amount of secreted luciferase was measured with BioLux *Gaussia* Luciferase Assay Kit (New England BioLabs) according to the manufacturer's recommendations.

ACKNOWLEDGMENTS

We regret the many omissions of citations of outstanding work in the field that could not be cited or discussed in greater detail due to space limitations. This work was supported by grants from the National Institutes of Health (R01GM122802 to E.S., R01AI125561 to G.A.B. and E.S., and R01DK110292 to S.R.) and the National Science Foundation (MCB-1615607 to E.S.).

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