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REVIEW ARTICLE

Regulating the regulators: role of phosphorylation in modulating the function of the GBF1/BIG family of Sec7 ARF-GEFs

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Membrane traffic between secretory and endosomal compartments is vesicle-mediated and must be tightly balanced to maintain a physiological compartment size. Vesicle formation is initiated by guanine nucleotide exchange factors (GEFs) that activate the ARF family of small GTPases. Regulatory mechanisms, including reversible phosphorylation, allow ARF-GEFs to support vesicle formation only at the right time and place in response to cellular needs. Here, we review current knowledge of how the Golgi-specific brefeldin A-resistance factor 1 (GBF1)/brefeldin A-inhibited guanine nucleotide exchange protein (BIG) family of ARF-GEFs is influenced by phosphorylation and use predictive paradigms to propose new regulatory paradigms. We describe a conserved cluster of phosphorylation sites within the N-terminal domains of the GBF1/BIG ARF-GEFs and suggest that these sites may respond to homeostatic signals related to cell growth and division. In the C-terminal region, GBF1 shows phosphorylation sites clustered differently as compared with the similar configuration found in both BIG1 and BIG2. Despite this similarity, BIG1 and BIG2 phosphorylation patterns are divergent in other domains. The different clustering of phosphorylation sites suggests that the nonconserved sites may represent distinct regulatory nodes and specify the function of GBF1, BIG1, and BIG2.

Keywords: ARF; BIG1; BIG2; GBF1; GEF; membrane traffic; phosphorylation; Sec7

Vesicular traffic requires the regulated activity of the ADP-ribosylation factor (ARF) superfamily of GTPases (reviewed in [1]). ARFs alternate between inactive (GDP-bound) and active (GTP-bound) states. Activation requires the release of bound GDP from the ARF, thus allowing the binding of the activating GTP. Spontaneous release of GDP is the rate-limiting step in the activation process and requires the action of guanine nucleotide exchange factors (GEFs) to promote the release of GDP through a conserved,

Abbreviations
ALK, anaplastic lymphoma kinase; AMPK, adenosine monophosphate-activated protein kinase; AP-1, adaptor protein 1; ARF, ADP-ribosylation factor; ARL, ARF-like protein; BFA, brefeldin A; BIG, brefeldin A-inhibited guanine nucleotide exchange protein; cAMP, cyclic adenosine monophosphate; CDK, cyclin-dependent kinase; CHK, CSK-homologous kinase; CK2, casein kinase 2; COSMIC, Catalogue of Somatic Mutations in Cancer; DCB, dimerization and cyclophilin binding; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GABAβ1L, gamma-aminobutyric acid receptor beta subunit 1L; GBF1, Golgi-specific brefeldin A-resistance factor 1; GEF, guanine nucleotide exchange factor; GGA, Golgi-associated gamma adaptin ARF-interacting protein; GM130, Golgi marker 130; HDS, homology downstream of Sec7; HUS, homology upstream of Sec7; kDa, kilodalton; NLS, nuclear localization signal; PKA, protein kinase A; PLK, polo-like kinase; PM, plasma membrane; PSD, postsynaptic density; PTM, post-translational modification; TGN, *trans*-Golgi network.

catalytic Sec7 domain (Sec7d) (reviewed in [2]). There are 16 mammalian ARF-GEFs, and they are divided into two major groups based on their sensitivity to the drug brefeldin A (BFA): the BFA-*resistant* cytohesins, brefeldin-resistant ARF-GEF/IQSec proteins, exchange factor for ARF6/postsynaptic density proteins, and F-Box GEFs; and the BFA-*sensitive* Golgi-specific brefeldin A-resistance factor 1 (GBF1), and the brefeldin A-inhibited guanine nucleotide exchange protein 1 and 2 (BIG1 and BIG2) [3–5]. A BIG1/2-related protein, named BIG3, has been identified but is predicted to lack GEF activity since it misses a conserved motif that includes the catalytic glutamic acid residue [6].

The three BFA-sensitive GEFs facilitate vesicle formation within the secretory (GBF1) and endocytic (BIG1/2) pathways [7–15]. All three are cytosolic proteins that associate with cellular membranes at specific times and sites to impart spatiotemporal restriction on ARF signaling. One of the key questions in understanding ARF activation and membrane traffic is how the initiating GEFs are themselves regulated. Many enzymes are controlled by reversible post-translational modifications (PTMs), and in this review, we combine data from low- and high-throughput phosphoproteomic analyses, predictive computational tools, and current knowledge of GBF1/BIG1/BIG2 phosphorylation to posit hypotheses as to the possible regulatory inputs that may govern GEF functions in cells.

Domain structure and conservation of the large GEFs

The BFA-sensitive GBF1 and BIG1/2 are also known as large ARF-GEFs, based on their > 200 kilodalton (kDa) molecular weight. They share a common domain structure, with an N-terminal dimerization and cyclophilin binding (DCB) domain followed by a homology upstream of Sec7 (HUS), a centrally located catalytic Sec7d, and C-terminal homology downstream of Sec7 (HDS) domains (Fig. 1) (reviewed in [2,16-18]). The DCB, HUS, and HDS domains (including HDS4) are unique to the large GEFs and are not present in other proteins, while the Sec7d is present in all 16 ARF-GEFs. Sequences of the DCB, HUS, and HDS domains are highly conserved within the GBF1 or the BIG orthologs across the phylogenetic tree (reviewed in [19] and [20]).

Comparison of sequence conservation between the large GEFs shows a high degree of similarity between BIG1 and BIG2 in all their domains, with ~ 85% similarity in DCB, ~ 90% in HUS, ~ 95% in Sec7d, ~ 93% in HDS1, ~ 95% in HDS2, ~ 94% in HDS3, and ~ 91% in HDS4 (Fig. 1A). The level of conservation is lower between the BIGs and GBF1, with ~ 37/40% similarity in DCB between BIG1/BIG2 and GBF1, ~ 44/45% in HUS, ~ 63/62% in Sec7d, ~ 38/42% in HDS1, ~ 39/38% in HDS2, and ~ 38% in HDS3 (Fig. 1A).

Each domain in the large GEFs is connected to its downstream or upstream neighbor through linker regions of variable length. Unlike the domains, the linker regions show limited sequence conservation. Within the BIGs, the four longest linkers range in sequence similarity: ~ 33% in the DCB-HUS linker, ~ 75% in the HUS-Sec7d linker, ~ 89% in the HDS2–HDS3 linker, and ~ 24% in the HDS3–HDS4 linker (Fig. 1B). It is perhaps noteworthy that the linkers closest to the catalytic Sec7d (i.e., HUS-Sec7d and HDS2–HDS3) are significantly more conserved than the more distal linkers, raising the possibility that these linkers may perform functions that coordinate with the neighboring Sec7d.

An even poorer linker sequence conservation is observed between the BIGs and GBF1, with ~ 10/22% similarity in the DCB-HUS linker between BIG1/BIG2 and GBF1, ~ 28% in the HUS-Sec7d linker, and ~ 5/6% in the HDS2–HDS3 linker (Fig. 1B). As for the BIGs, the linker closest to the Sec7d (HUS-Sec7d) is the most highly conserved, perhaps supporting the notion that it may coordinate with the neighboring domain.

The level of conservation within the domains and the linkers may support a few speculations about possible modes of regulation of the large GEFs. First, the conserved domain organization and sequences of the three GEFs despite the fact that each exhibits distinct localization and has a distinct interactome (see below) may suggest that these domains are involved in the overall parameters of protein folding, rather than particular interactions or regulation. This notion is supported by the high sequence conservation within the Sec7d, where structural crystallographic data have shown analogous folding among different GEFs [21-25]. If the conserved domains are in fact a major determinant of large GEF folding, it may follow that PTMs within these domains may globally alter the conformation of the GEFs.

Second, the presence of the highly conserved HDS4 exclusively in the BIGs may suggest that HDS4 imparts an additional regulatory regime on the overall folding of the BIGs that is absent in GBF1. This may suggest that all large GEFs have a similar folding backbone defined by the conserved DCB/HUS/Sec7/HDS1–3 domains, but that the folding of the BIGs is further informed by the HDS4. Furthermore, it might be speculated that PTMs in HDS4 may alter the

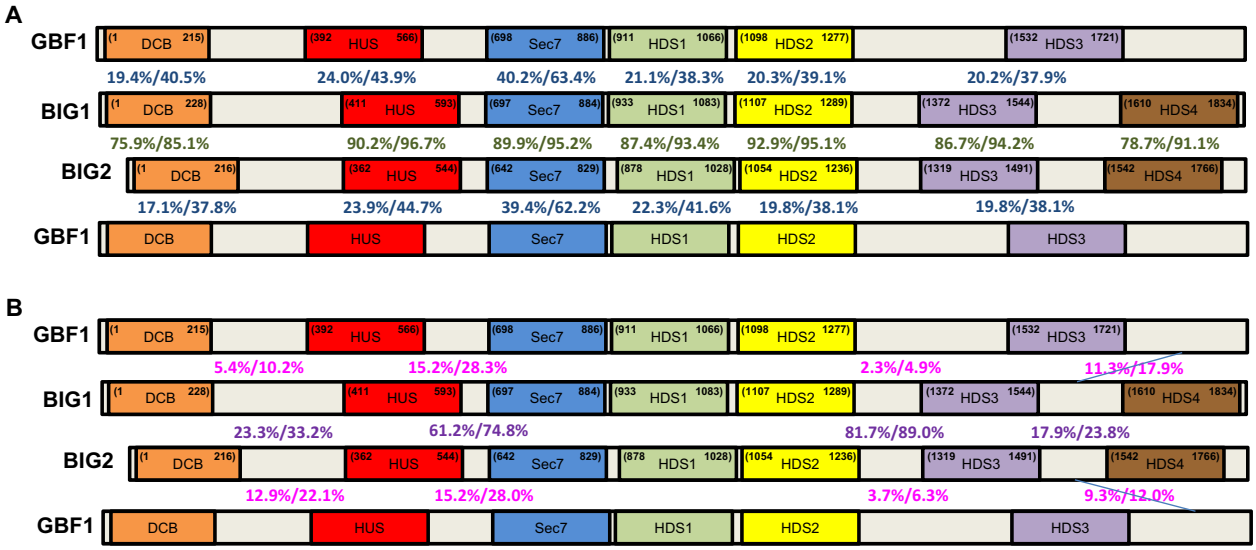

Fig. 1. Domain arrangement and homology in the large GEFs. Domain organization shows the N-terminal DCB and HUS domains, the central catalytic Sec7 domain (Sec7d), and the C-terminal HDS domain. The level of similarity was obtained after sequence alignments done with the EMBOSS Needle alignment algorithm. (A) Similarity between domains of the GEFs. (B) Similarity between linkers of the GEFs.

folding of the BIGs through a mechanism that is impossible for GBF1.

Third, the limited conservation between GBF1 and the BIGs within the linkers may suggest that the linkers are largely responsible for the differential localization, interactome, and regulation of the three GEFs. Furthermore, the surprisingly low conservation between the BIGs in some linkers (~ 24% similarity in HDS3–HDS4 as opposed to > 75% similarity in HUS-Sec7) may suggest that these low similarity linkers may mediate PTM inputs to differentially regulate BIG1 and BIG2.

Phosphorylation sites within the large GEFs

We used the proteomic databases PhosphoSite, UniProt, and ExPASy to mine information on GEF phosphorylation [26–30]. We first present the overall phosphorylation patterns for the three GEFs and then discuss how specific phosphorylations of each GEF may impact its function.

Golgi-specific brefeldin A-resistance factor 1 is reported to be phosphorylated on 86 different serine, threonine, and tyrosine residues (Table S1), and our mass spectrometry studies on recombinant GBF1 expressed in HeLa cells confirmed four phosphorylated residues: S1318, S1320, S1773, and S1784 (unpublished data). BIG1 is reported to be phosphorylated on 45 different serine, threonine, and tyrosine residues (Table S2), and BIG2 is reported to be phosphorylated on 56 different serine, threonine, and tyrosine residues (Table S3). However, many of the reported sites in each GEF have been detected in only a single analysis, making it difficult to ascertain the robustness of such findings. We arbitrarily set a bar of three independent analyses detecting the same phosphorylated residue as our threshold for representation and diagram the phosphorylated residues in each GEF (35 sites in GBF1, 25 in BIG1, and 25 in BIG2) in Fig. 2. A number of potentially important inferences can be drawn from the distributions of these refined phosphorylated (P) sites.

First, if the assumption that the domains facilitate overall folding of the GEFs is accurate, phosphorylation of the DCB site (S174) and/or the HUS site (Y515) could regulate GBF1 conformation (Fig. 2). Similarly, phosphorylations of the three DCB sites in BIG1 (S31, T48, and S52) may alter BIG1 conformation. It is possible that PTMs of the DCB (or HUS) may interfere with the known interactions between these domains to affect GEF dimerization [31]. That correctly folded DCB capable of binding HUS is important for GEF functionality has been shown by the fact that mutations in GBF1 that prevent DCB-HUS interaction (E130A) cause premature degradation of GBF1 [32], while mutations in BIG2 that prevent DCB-HUS interaction (E209K) cause periventricular heterotopia with microcephaly [33–35] and other disorders [36,37]. In addition, in BIG1, phosphorylation of a cluster within HDS1 (Y1068, T1072, S1079, T1081, and T1083) or a P site in HDS4 (S1704) may impact

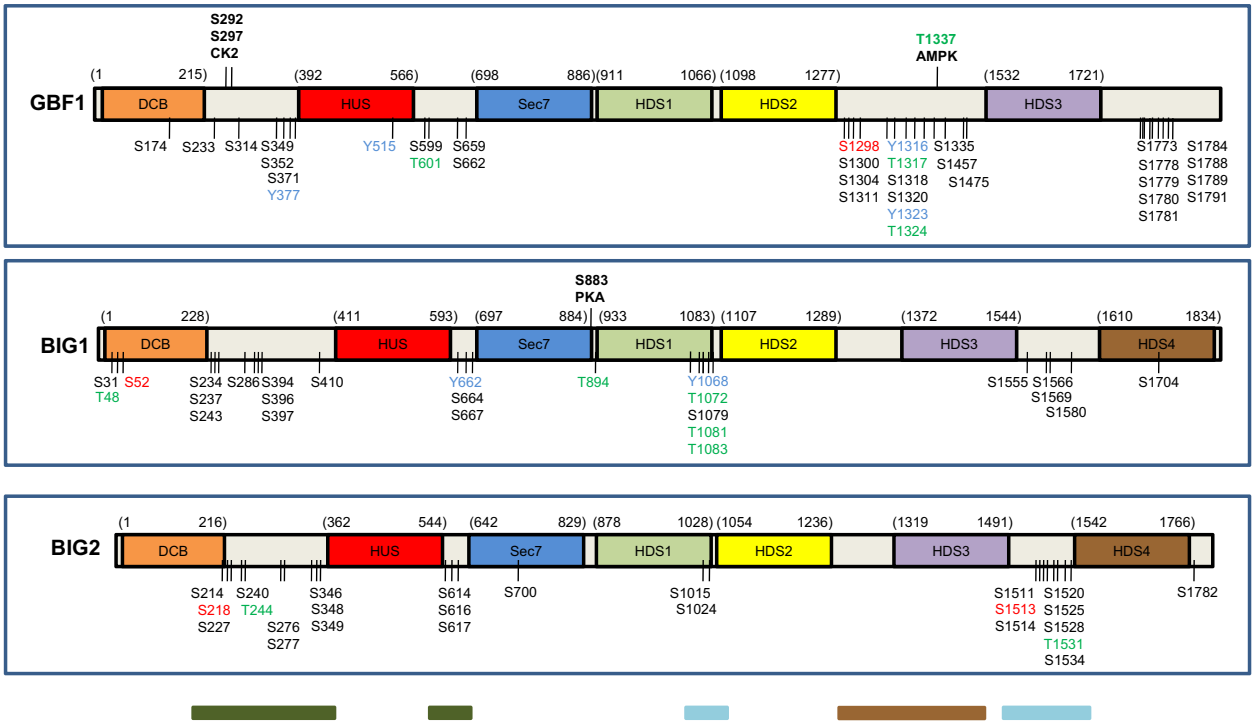

Fig. 2. Phosphorylation sites in the large GEFs. The phosphorylation sites detected in more than three independent reports are selected from data in Tables S1–S3. Serines are in black, tyrosines are in blue, and threonines are in green. Bolded residues mark phosphorylation sites in published reports and involved in a process listed in the corresponding Tables S1–S3. Residues in red are linked to cancer in the COSMIC database. Thick green lines at the bottom mark regions with clustered P sites shared between the three GEFs. Thick brown line marks region with clustered P sites found exclusively within GBF1. Thick blue lines mark regions with clustered P sites found exclusively within the BIGs.

BIG1 folding (Fig. 2). Within BIG2, phosphorylation of P sites within HDS1 (S1015 and S1024) might control BIG2 folding.

Second, the vast majority of P sites in every GEF lie within linker regions, with a large cluster of P sites in the DCB-HUS and the HUS-Sec7d linkers, suggesting that some of these sites might undergo similar regimes of PTMs to affect similar changes in the proteins.

Third, GBF1 has a large cluster of P sites within the HDS2-HDS3 linker that is absent in the BIGs, and PTM of these residues may affect GBF1-specific localization and/or interactome. GBF1 also has a cluster of P sites within its tail region that is absent in the BIGs, and those sites may be modified in response to unique regulatory inputs to control GBF1-specific effects. However, it might be argued that the tail of GBF1 is analogous to the HDS3-HDS4 linker in the BIGs (~18% similarity exists between the GBF1 tail and BIG1 HDS3-HDS4 linker, and ~ 12% between GBF1 and BIG2; Fig. 1B) and could serve an analogous regulatory role.

Fourth, both BIGs have multiple phosphorylation sites within HDS1 that are absent in GBF1, perhaps suggestive of a regulatory regime that is shared only between these endosomal GEFs. Both BIGs have a large cluster of P sites in the HDS3-HDS4 linker that is not present in GBF1 (with the caveat that this region might be analogous to the tail of GBF1), suggesting that PTM of these residues may alter the BIGs' behavior in a manner that is unique to these endosomal GEFs.

Additional hypotheses about possible regulation might be suggested by the relative distribution/position of phosphorylated S (in black), T (in green), and Y (in blue) sites within each GEF (Fig. 2). A number of phosphoproteomic analyses have defined the 'usual' distribution of phosphorylated residues in the total proteome as ~ 84–88% S, ~ 11–20% T, and only ~ 0.3–1.5% Y (ex: [38–40]). In general agreement, the vast majority of phosphorylated sites within the three GEFs are serines (78%), with threonine phosphorylation also within norms (15%), albeit in BIG1, threonine phosphorylation accounts for 25% of the total. The biggest difference between total proteome phosphorylation versus that of GEFs is in tyrosine phosphorylation: In GBF1, four out of 35 total

phosphorylated sites (~ 11%) are on tyrosine, and in BIG1, two out of 25 phosphorylated sites (8%) are on tyrosines, while BIG2 does not appear to be phosphorylated on Y. Tyrosine kinases are usually silent in cells and become activated in response to a signal, making Y phosphorylation primarily a regulatory PTM. The frequent Y sites in GBF1 and BIG1 may suggest that these GEFs are modified by mitogen-stimulated kinases, perhaps to modulate their function during cell growth, possibly in response to the increased need for membrane trafficking. If our hypothesis that the domains control overall folding of GEFs is correct, we would predict that phosphorylation of Y515 in GBF1 and Y1068 in BIG1 might alter their conformation. Perhaps the different Y distribution (HUS in GBF1 versus HDS1 in BIG1) might suggest that the regulatory mechanisms that govern the folding of these GEFs differ.

Surprisingly, BIG2 appears to lack high-confidence phosphorylated Y sites (phosphorylated Y797, Y1540, and Y1632 have been detected only in single reports). However, this will need to be further explored by direct analyses, as it might be expected that an endosomal GEF might respond to growth factor stimulation.

We used the MusiteDeep framework tool to assess the confidence of the reported P sites within the three GEFs. Those with confidence values of at least 0.6 in Tables S1–S3 are considered highly predictive. The majority of sites reported as phosphorylated in databases also have high probability scores, raising the confidence that they might be phosphorylated *in vivo*. However, there are also outliers, with some sites (ex: S174 in GBF1, T48 in BIG1, and T616 in BIG2) being reported as phosphorylated in at least three independent proteomic screens and yet having a relatively low predictive score. The opposite is also true, with some sites (ex: S347 in GBF1, S595 in BIG1, and S279 in BIG2) having a very high predictive score and yet not being detected in at least three proteomic screens. Such sites might represent very transient and/or rare phosphorylations and could be functionally important.

To further refine the map of P sites most likely involved in the regulation of the large GEFs, we assessed the conservation of the highly ranked sites (those reported in at least three publications and with a confidence value above 0.6) across species. The *Homo sapiens* sequence was compared to that of the mouse *Mus musculus,* the zebrafish *Danio rerio*, and the fruit fly *Drosophila melanogaster*. Conservation was marked with a plus sign in Tables S1–S3, and phosphorylated sites conserved in at least human, mouse, and zebrafish orthologs are depicted in Fig. 3.

The majority of most probable GBF1 phosphosites were conserved across species, except for P sites within the HUS-Sec7d linker and the tail. This may suggest that the poorly conserved P sites are involved in species-specific interactions and/or functions. Interestingly, all four phosphorylated Y are conserved across the vertebrates and also in *D. melanogaster* (with the exception of Y1323), suggesting that modification of Ys may regulate key parameters of GBF1 function.

The majority of P sites within BIG1 and BIG2 also were conserved, and the refinement did not alter the overall distribution of P sites in the different regions of these GEFs. As for GBF1, all Ys were conserved in BIG1. The conservation refinement removed S700 in the Sec7d of BIG2, which eliminated the single phosphosite within the catalytic domain of any of the GEFs.

Currently, we posit that the conserved P sites in Fig. 3 may represent the most functionally important PTMs. However, we should not assume that the unique sites are less critical, as they may in fact represent species-specific modifications reflecting the particular developmental or physiological demand of that organism. Since we currently lack robust data on those residues, additional experimental evidence will be needed to assess their importance.

To gain insight into the possible signaling inputs that may regulate the large GEFs, we used the group-based prediction system 3.0, KinasePhos, and PhosphoNET predictive tools to compile lists of kinases likely to phosphorylate the sites shown in Fig. 3, that is, P sites detected in at least three publications and conserved between human, mouse, and zebrafish (Table S4). Analysis of the results showed a low level of correspondence between the different predictive tools, with the majority of kinases predicted by only a single algorithm. Kinases predicted by at least two programs are marked in brown and may represent the most likely candidates for regulating the cognate GEF, although it remains possible that other kinases may also modify these proteins. However, the lack of strong correspondence prevents robust conclusions or predictions about how the GEFs are regulated by specific kinases.

Phosphorylation in GEF function—facts and possibilities

Multiple scenarios for regulating GEF functionality through phosphorylation can be envisioned: (a) Large GEFs are soluble within the cytosol and must be recruited to membranes to function in ARF activation, indicating that membrane recruitment could represent

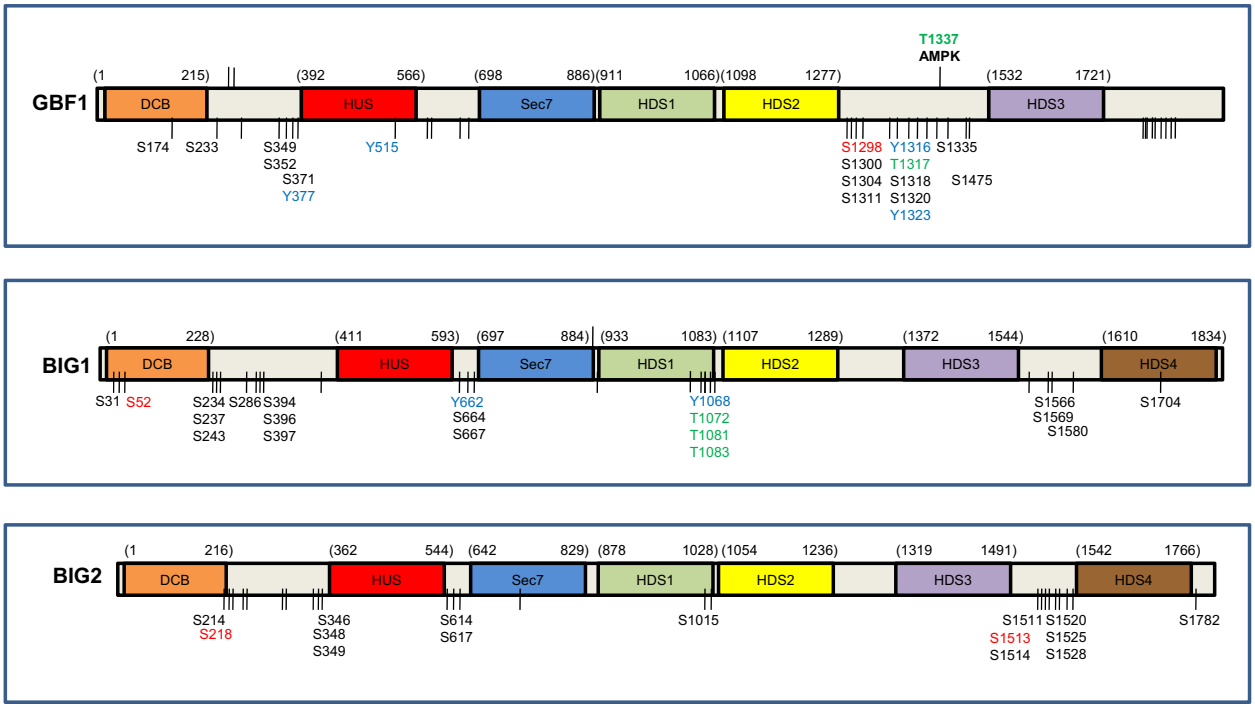

Fig. 3. Conserved phosphorylation sites in the large GEFs. The phosphorylation sites shown in Fig. 2 were assessed for conservation between the human, mouse, zebrafish, and fruit fly orthologs using the NCBI Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, U.S. National Library of Medicine, Besthesda, MD, USA) alignment tool. P sites conserved in human, mouse, and zebrafish are marked with numbers, while nonconserved sites lack numbers. Serines are in black, tyrosines are in blue, and threonines are in green. Bolded residues mark phosphorylation sites in published reports and involved in a process listed in the corresponding Tables S1–S3. Residues in red are linked to cancer in the COSMIC database.

a regulatory point; (b) ARF activation requires the engagement of the catalytic Sec7d, and phosphorylation may regulate the accessibility or the activity of the Sec7d; (c) GEFs interact with numerous proteins, and phosphorylations that either stimulate or inhibit such interactions could impact GEF functionality; and (d) GEF stability and degradation might be regulated through phosphorylation. Below, we describe known examples of phosphorylation altering a GEF's cellular localization, catalytic activity, and/or stability. We correlate the known functional consequences of phosphorylating specific sites in each GEF with the information in Figs 2 and 3 to help generate plausible models for the role of phosphorylation in regulating each GEF.

GBF1

Golgi-specific brefeldin A-resistance factor 1 is composed of 1859 amino acids and cycles between the cytosol and the membranes of the endoplasmic reticulum–Golgi intermediate compartment (ERGIC), the Golgi, and the *trans*-Golgi network (TGN). At the ERGIC–Golgi interface, GBF1 activates ARFs to support COPI vesicle formation, while at the TGN, GBF1 indirectly facilitates AP-coated vesicle biogenesis [41,42]. In addition, GBF1 has been detected at the plasma membrane (PM) in glioblastoma cells [43] and in chemotaxing neutrophils responding to *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) stimulation [44]. Moreover, in mitotic cells, phosphorylated GBF1 has been shown to localize to the spindle [45]. The multiple localizations raise the intriguing question of whether GBF1 targeting to distinct cellular sites might be under PTM control. Indeed, the elegant work of the Guardavaccaro group clearly established a role of phosphorylation in GBF1 localization [45]: GBF1 is transiently phosphorylated on S292 and S297 during late metaphase, and this correlates with GBF1 association with the spindle. This work strongly suggests that the addition of P groups to particular serines generates a targeting signal that directs GBF1 to the spindle.

S292/S297 phosphorylation is mediated by casein kinase-2 (CK2) [45], an acidic kinase whose activity is increased by cyclin-dependent kinase 1 (CDK1)-mediated phosphorylation of neighboring P sites [46]. This may suggest that during mitosis, CDK1 may

phosphorylate GBF1 on serines adjacent to S292 and S297, and indeed, GBF1 is phosphorylated by CDK1 during mitosis, although the relevant amino acid(s) were not identified in that study [47].

Based on the paradigm that GBF1 phosphorylation on S292/S297 regulates its targeting to the spindle, we tentatively posit that the fMLP-induced relocation of GBF1 to the cell surface also may be regulated by an as yet uncharacterized signaling cascade downstream from a G protein-coupled receptor and members of the mitogen-activated protein kinase family. Future studies are needed to probe the phosphorylation status of PM-localized GBF1 in fMLP-stimulated neutrophils to shed light on which P sites could be involved in targeting GBF1 to the PM.

Localization of GBF1 also is controlled through the phosphorylation of T1337 by AMP-activated protein kinase (AMPK) during the G2/M transition, and this PTM correlates with GBF1 dissociation from the membranes (Table S1). Phosphorylation of GBF1 by AMPK is necessary for mitosis because overexpression of a GBF1/T1337A mutant causes cells to arrest at the G2/M transition [48]. The AMPK-mediated phosphorylation of GBF1 at T1337 also can be induced by 2-deoxyglucose and also results in GBF1 dissociation from the membrane [49]. In addition, phosphorylation of GBF1 by AMPK, possibly at T1337, regulates its function in facilitating the trafficking of proteins such as von Willebrand factor in interphase cells, but the mechanism is unclear [50]. Furthermore, CDK1-mediated phosphorylation causes GBF1 to dissociate from Golgi membranes [47], albeit the modified P site was not identified in that study.

In addition to defining GBF1 localization, phosphorylation also regulates its degradation. Phosphorylation of S292/S297 results in its degradation, an event essential for cytokinesis, as the expression of the S292A/S297A mutant blocks cytokinesis [45]. S292/S297 is one of three putative degrons in GBF1, the other being S1300/S1304 and S1481/S1486 [45]. Only S1300/S1304 is reported to be phosphorylated and may play a role in GBF1 stability. Interestingly, S1300 and S1304 roughly fit consensus motifs for CDK and are adjacent to S1298, which contains a putative CK2 motif, suggesting that CDK-mediated phosphorylation of S1300 may stimulate the CK2-mediated phosphorylation of S1298 during mitosis. Importantly, the Catalogue of Somatic Mutations in Cancer (COSMIC), an online database that tracks amino acid mutations that correlate with specific cancers [51], lists the S1298P mutation as present in renal cell carcinoma (Fig. 2, red residue). As cancer cells are mitotically more active than nontransformed cells, this may suggest that preventing GBF1 phosphorylation on S1298 supports mitosis.

Other than the above-described phosphorylation of T1337 and S292/S297 affecting GBF1 localization and stability, how phosphorylation of other P sites may regulate GBF1 is unknown. The lack of information precludes the generation of strong models, but perhaps some preliminary predictions can be made. It is possible that phosphorylation may affect the GBF1 interactome: Phosphorylation of the DCB-HUS linker may regulate interactions with Rab1b (shown to bind to amino acids 1–384 of GBF1 [52]), phosphorylation of the DCB-HUS linker and/or HUS-Sec7d linker may affect GBF1 interactions with Golgi-associated gamma adaptin ARF-interacting protein (GGA; shown to bind to amino acids 1–692 of GBF1 [53]) and γ-coat protein (shown to bind to amino acids 1–662 of GBF1 [54]), and phosphorylation of the tail may affect GBF1 interactions with p115 (shown to bind to amino acids 1762–1859 of GBF1 [55]). Experimental analyses will be needed to test the effects of phosphorylations on the GBF1 interactome and the possible functional consequences of each PTM.

In an unpublished work, we examined the effect of mutating S1773 on GBF1 function by expressing the GBF1/S1773D mimetic of constitutively phosphorylated GBF1. HeLa cells were transfected with either GBF1 or the GBF1/S1773D mutant (both constructs are GFP-tagged and also contain the A795E substitution that makes them resistant to BFA) for 48 h. Cells were then treated with BFA for 2 h. BFA inhibits the catalytic activity of GBF1 in normal cells and causes Golgi fragmentation ([55-57]). Cells were subsequently fixed and processed for immunofluorescence to detect the construct (green) and Golgi marker 130 (GM130) (red). As shown in Fig. 4A, untransfected cells have disrupted Golgi, while cells expressing exogenous BFA-resistant GBF1 have intact Golgi (arrows). Importantly, all transfected cells have normal-looking nuclei. A different phenotype is observed in cells expressing the BFA-resistant GBF1/S1773D mutant (Fig. 4B). While this mutant also appears to support normal Golgi architecture (arrows), the transfected cells exhibit dramatic nuclear alterations. Additional analyses will be needed to identify the molecular underpinnings of this phenotype.

Two highly conserved high probability P sites within GBF1 domains (not linkers) at S174 and Y515 may regulate the overall folding of GBF1 and/or dimerization (Fig. 3). Both are conserved in mammals, zebrafish, and fruit fly, perhaps reflecting their functional importance (Table S1). Y515 phosphorylation is predicted to involve anaplastic lymphoma kinase (ALK;

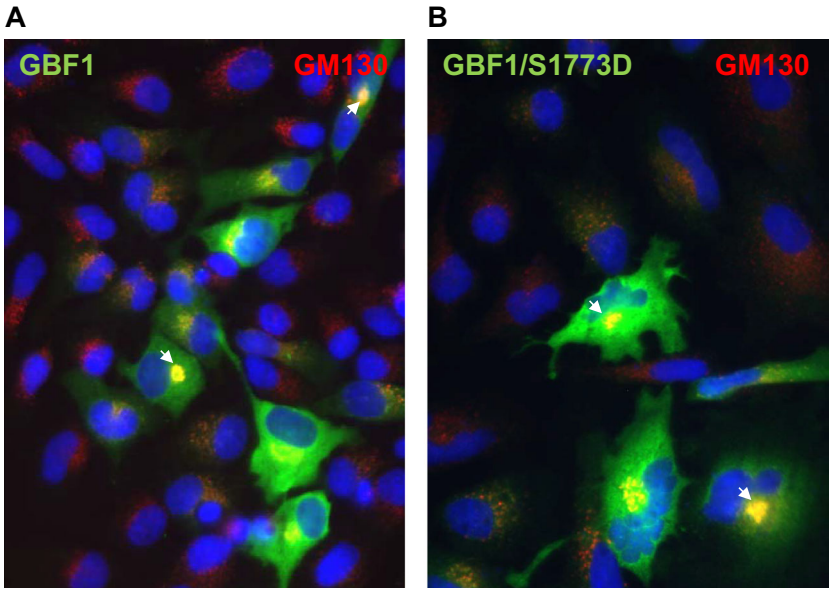

Fig. 4. Mutation of GBF1 phosphorylation site causes nuclear alterations. BFA-resistant and GFP-tagged GBF1 (A) and GBF1/S1773D (B) constructs were transfected into HeLa cells. Forty-eight hours after transfection, cells were treated with 0.5 $\mu g \cdot mL^{-1}$ BFA for 2 h, and then fixed and processed for immunofluorescence using anti-GFP (green) to detect the constructs and anti-GM130 (red) to monitor Golgi architecture. Nuclei were stained with 4′,6-diamidino-2-phenylindole. Untransfected cells have dispersed Golgi, while cells overexpressing either construct have intact Golgi (arrows). Importantly, cells expressing the GBF1/S1773D mutant show dramatic nuclear phenotypes.

Table S4), a surface receptor tyrosine kinase belonging to the insulin receptor superfamily often rearranged, mutated, or amplified in tumors such as anaplastic large-cell lymphomas, neuroblastoma, and non-small-cell lung cancer [56,57]. Based on the surface localization of ALK, it is possible that it may modify GBF1 on Y515 in cells in which GBF1 localizes to the cell surface. However, it remains possible that Y515 is modified by a soluble tyrosine kinase such as a member of the Src family. The possible involvement of Src in cargo-stimulated traffic within the secretory pathway has been previously proposed [58-62]. Experimental evidence will be needed to determine which kinase phosphorylates Y515 and what functional effect that has on GBF1 localization, activity, and/or stability.

BIG1

BIG1 is a 209 kDa protein composed of 1849 amino acids that localizes primarily at the TGN [12] where it activates ARF1 and ARF3 (purified BIG1 increases GTP binding of ARF1 and ARF3 in a dose-dependent manner [63]). ARF1/3 regulates the traffic of proteins from the TGN to the PM and the endolysosomal pathway by interacting with effectors to form transport vesicles [17,64–66].

Phosphorylation has been shown to regulate BIG1 localization, and phosphorylation by the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) at S883 causes its translocation into the nucleus [67] (Fig. 2). A mutation of S883 that prevents phosphorylation also prevents nuclear accumulation of BIG1 even in cells with increasing cAMP [67]. BIG1 translocation into the nucleus also occurs in response to stress such as overnight incubation with serum-free media [68] and also could be due to PKA because PKA activity has been linked to stress response signaling pathways [69]. BIG1 has been reported to contain a nuclear localization signal (NLS) encoded in amino acids 711–715 (KKPKR) in the Sec7d, raising the possibility that S883 phosphorylation may expose the NLS and/or promote BIG1 interaction with nuclear import factors. Irrespective of the molecular mechanism, it is relevant to consider the parallel between GBF1 and BIG1 phosphorylation regulating their subcellular localization. Currently, the role BIG1 may play within the nucleus is unknown.

BIG1 phosphorylation may also control its localization to the TGN, a process regulated by the small GTPases ARF-like protein 1 (ARL1) [70,71] and ARF4/5 [72]. The interaction between BIG1 and ARL1 is mediated by three Huntingtin, elongation factor 3, protein phosphatase 2A, and yeast kinase target of rapamycin kinase 1 repeat domains within the DCB domain. These repeats (α helices 4, 6, and 8) form a short arc to bind ARL1, with Y109 located in α helix 4 of the DCB domain. The binding of BIG1 and ARL1 is diminished by the Y109D mutation [73]. Interestingly, Y109 was detected in one proteomic report (Table S2; note that this P site has a low predictive probability score), suggesting that phosphorylation of Y109 could modulate the recruitment of BIG1 to the Golgi by impacting its association with ARL1. It is currently unknown whether Y109 phosphorylation could affect the interaction between BIG1 and ARF4/5.

It can be postulated that BIG1 recruitment to the TGN also might be regulated through PTMs of a large cluster of T sites within the HDS1 domain, adjacent to Y1068. This model is based on the HDS1 domain facilitating the association of Sec7p, the yeast ortholog of BIG1/2, with the TGN membrane in a process regulated by a positive feedback loop and increased by Sec7p-activated ARF1 [74,75]. Experimental studies will be needed to assess whether this mechanism is conserved within the mammalian BIG1.

Phosphorylation also regulates BIG1 enzymatic activity: Incubation of immunoprecipitated BIG1 with recombinant PKA significantly decreased its GEF activity [76], presumably by phosphorylation of S883 (PKA is known to phosphorylate S883), albeit the actual site was not identified in that study. The decreased catalytic activity could be due to the fact that S883 lies within the loop after helix-J in the Sec7d of BIG1, a loop shown to be required for the binding of the ARF substrate in the highly conserved BIG2 [21]. Thus, the introduction of the phosphate group onto S883 might prevent the critical contact of the ARF substrate with the loop after helix-J, preventing ARF activation. However, it remains possible that additional sites were phosphorylated by PKA, and more work is needed to correlate specific PTMs with changes in catalytic activity.

Catalytic activity might also be impacted by phosphorylation of the HDS4 domain, as studies of Sec7p show that HDS4, along with HDS2 and HDS3, exert an autoinhibitory effect on the catalytic activity in the yeast GEF [75]. HDS4 appears to be the primary factor in this autoinhibition because a Sec7p construct lacking HDS4 has similar activity to a Sec7p construct lacking HDS2, HDS3, and HDS4. A model has been proposed in which the HDS2–4 domains fold back on the N-terminal region of Sec7p to prevent ARF1 activation. The autoinhibition by HDS4 is relieved by the binding of the yeast Rab GTPases Ypt1, Ypt31, and Ypt32 to HDS4 [74-75,77-79]. HDS4 also seems to be important for the dimerization of Sec7p [74,78,79]. The possible regulatory function of PTMs of the HDS4 appears unique for BIG1 and BIG2, as GBF1 lacks HDS4 and must be regulated through a distinct mechanism.

BIG1 phosphorylation on S52 appears important in BIG1 function, as COSMIC data correlate the S52N mutation with esophageal cancer (Fig. 3). As with GBF1 above, the cancer-associated mutation prevents BIG1 phosphorylation, but the consequence of S52 PTM to BIG1 activity or stability is unknown. Interestingly, S52 is not conserved in the closely related BIG2, suggesting that this residue might respond to BIG1-specific regulatory inputs.

Phosphorylation also may influence the BIG1 interactome. A cluster of P sites within the HDS3-HDS4 linker may regulate BIG1 association with myosin IXb, as myosin IXb has been shown to bind to amino acids 1305–1849 of BIG1 [80]. Modification of those sites also may impact BIG1 binding to kinesin family member 21A, shown to bind to amino acids 885–1849 of BIG1 [81]. It is currently impossible to evaluate whether and, if so, which P site might regulate BIG1 interactions with its other binding partners, nucleolin [68] and KN motif and ankyrin repeat domain-containing protein 1 [82], as no binding motifs for those proteins have been identified within BIG1.

BIG2

BIG2 is a 202 kDa soluble cytoplasmic protein composed of 1785 amino acids. BIG2, like BIG1, predominantly localizes to the TGN [12], but also can be found at endosomes, neuronal axons, and synaptic membranes [13,83-85]. BIG2-mediated ARF activation regulates the recruitment of adaptor protein 1 (AP-1) and GGA1 to the TGN to facilitate the formation of transport vesicles [13,14]. AP-1 and GGA regulate protein trafficking between the TGN and endosomes (reviewed in [86,87]).

BIG2 catalytic activity has been shown to be regulated by phosphorylation: Incubation of immunoprecipitated BIG2 with PKA inhibited its GEF activity [76], suggesting a regulatory regime through cAMP levels. However, the relevant P site was not identified in that study.

BIG2 phosphorylation on S218 and S1513 may have a functional role, as suggested by the COSMIC database, which shows the S218F mutation in colon cancer and squamous cell carcinoma [88,89] and the S1513N mutation in malignant melanoma [90] (Fig. 2). As for GBF1 and BIG1, the cancer-associated mutations prevent BIG2 phosphorylation. In addition, the phosphorylation of S1528 is strongly upregulated during anaphase/telophase [91], but the functional consequences of this finding remain to be determined.

Currently, there are no functional consequences ascribed to phosphorylation of any specific P site in BIG2, and caution must be taken to propose models of PTM regulation. Yet, we venture to consider that PTMs of specific sites may alter the BIG2 interactome. Specifically, phosphorylation of the DCB-HUS linker could regulate binding to interactors such as exocyst complex protein of 70 kDa (shown to associate with amino acids 1–643 of BIG2 [92]), nonmuscle myosin 2 (shown to associate with amino acids 1–250 [93]), and alpha-amylase 1 (shown to associate with amino acids

284–301 [94]). Phosphorylation of the unique P site in the tail of BIG2 (S1782) might perhaps regulate the binding of gamma-aminobutyric acid receptor beta subunit 1L, which interacts with residues 1682–1785 of BIG2 [83]. Whether any of these interactions are regulated by PTMs is currently unknown and will need to be tested experimentally.

It is noteworthy that despite the similar clustering of P sites within BIG1 and BIG2, these proteins appear to be regulated through distinct signaling cascades. Only one P site in the HUS-Sec7d linker of BIG1 and BIG2 (S664 in BIG1 and S614 and S617 in BIG2) is predicted to be modified by the same kinases [C-terminal src kinase-homologous kinase (CHK), polo-like kinase, and ribosomal s6 kinase; Table S4]. All other analogous P sites within the N termini of BIG1 and BIG2 are predicted to be phosphorylated by different kinases. Thus, despite the similarities in the overall distribution of the N-terminal P sites between the GEFs, it is likely that the kinases and the upstream signaling cascades that initiate the phosphorylation of each GEF differ significantly to reflect distinct regulatory regimes.

Summary

The enzymes that initiate ARF activation and vesicle formation, the large GEFs GBF1, BIG1, and BIG2, must contain inherent mechanisms to respond to a cellular need for activated ARF, and it is likely that their functionality is constrained by reversible site-specific phosphorylations. Indeed, phosphorylation of specific P sites has been shown to regulate GEF intracellular localization (for GBF1 and BIG1), enhance degradation (for GBF1), and inhibit enzymatic activity (for BIG1 and BIG2). However, these limited examples represent a minuscule proportion of total P sites within the three GEFs, and future work is needed to correlate phosphorylation of a particular site with phenotypic and/or functional consequences.

We hypothesize that phosphomics data combined with predictive tools may provide insight into potential regulatory regimes in the large GEFs. Our preliminary analyses suggest that the three GEFs are most conserved in their P site distribution within their N termini, consistent with the highest sequence conservation within those regions. It could be postulated that PTM of these sites may have similar effects on each GEF. The kinases predicted to modify 'analogous' N-terminal sites differ between the three GEFs, suggesting that a similar mechanistic response might be elicited by different signaling inputs. In contrast, the distribution of P sites within the C termini of GBF1 and BIG1/2 is poorly conserved, suggesting that the effects caused by PTM of these sites may be mediated through distinct molecular mechanisms. This is especially evident in P site distribution within the HDS1 domain, suggesting that the BIGs are regulated differently than GBF1.

Surprisingly, despite the overall similarity in P site distribution in BIG1 and BIG2, the kinases predicted to modify those sites differ extensively, suggesting that these highly related proteins nevertheless might be regulated through distinct mechanisms and in response to distinct signaling pathways. The kinases responsible for site-specific phosphorylation of BIG1 and BIG2 remain to be identified and may shed light on the signaling cascades that modulate the behavior of these GEFs.

Although proteomic databases and computer-based tools are useful to generate models of PTM regulation of a GEF's functionality, experimental work is needed to answer many key remaining questions. A concerted effort from numerous laboratories is needed to uncover the regulatory modalities that transmit information from the cellular milieu to the GEFs to 'tell' them when and where to initiate ARF activation. Such knowledge is essential to build a systems-level understanding of how ARF activation is coordinated in a cell to regulate the multitude of developmental and physiological changes necessary for cellular homeostasis.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Table S1. Phosphorylation sites in GBF1.
Table S2. Phosphorylation sites in BIG1.
Table S3. Phosphorylation sites in BIG2.
Table S4. Kinases predicted to phosphorylate the large GEFs.