Specific inhibition of GPCR-independent G protein signaling by a rationally engineered protein

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Activation of heterotrimeric G proteins by cytoplasmic nonreceptor proteins is an alternative to the classical mechanism via G protein-coupled receptors (GPCRs). A subset of nonreceptor G protein activators is characterized by a conserved sequence named the Gα-binding and activating (GBA) motif, which confers guanine nucleotide exchange factor (GEF) activity in vitro and promotes G protein-dependent signaling in cells. GBA proteins have important roles in physiology and disease but remain greatly understudied. This is due, in part, to the lack of efficient tools that specifically disrupt GBA motif function in the context of the large multifunctional proteins in which they are embedded. This hindrance to the study of alternative mechanisms of G protein activation contrasts with the wealth of convenient chemical and genetic tools to manipulate GPCR-dependent activation. Here, we describe the rational design and implementation of a genetically encoded protein that specifically inhibits GBA motifs: GBA inhibitor (GBAi). GBAi was engineered by introducing modifications in Gai that preclude coupling to every known major binding partner [GPCRs, Gαi, effectors, guanine nucleotide dissociation inhibitors (GDIs), GTPase-activating proteins (GAPs), or the chaperone/GEF Ric-8A], while favoring high-affinity binding to all known GBA motifs. We demonstrate that GBAi does not interfere with canonical GPCR-G protein signaling but blocks GBA-dependent signaling in cancer cells. Furthermore, by implementing GBAi in vivo, we show that GBA-dependent signaling modulates phenotypes during Xenopus laevis embryonic development. In summary, GBAi is a selective, efficient, and convenient tool to dissect the biological processes controlled by a GPCR-independent mechanism of G protein activation mediated by cytoplasmic factors.

Significance

The core mechanism of heterotrimeric G protein regulation consists of activation by G protein-coupled receptors (GPCRs) via nucleotide exchange (1). GPCRs are guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP for GTP on Gα subunits, which, in turn, leads to the dissociation of GαGβγ heterotrimers. Subsequently, both Gα-GTP and “free” Gβγ subunits modulate the activity of downstream effectors. However, there is a complex network of accessory proteins that modulate G protein signaling beyond the action of GPCRs. This includes guanine nucleotide dissociation inhibitors (GDIs) (2, 3), which bind Gα and lock it in an inactive GDP-bound state, and GTPase-activating proteins (GAPs) (4), which accelerate the rate of GTP hydrolysis by Gα. Ric-8 proteins are also G protein binding partners that facilitate Gα folding and stability while also possessing GEF activity in vitro (5). More recently, we and others have characterized a group of nonreceptor proteins that possess GEF activity for Gai proteins in vitro and promote G protein signaling in cells (6–9) as determined by measurements that reflect either the formation of GTP-bound Gai (e.g., CAMP reduction, Gαi-GTP specific antibodies) (6, 10–12) or free Gβγ [e.g., bioluminescence resonance energy transfer (BRET)/FRET biosensors or PI3K-Akt signaling] (6, 7, 11, 13, 14). This family of nonreceptor GEFs is characterized by containing the Gα-binding and activating (GBA) motif (6–9), a 30- to 35-residue-long sequence that is necessary and sufficient for Gai binding and activation. The GBA motif has been found in proteins from worms to humans (15), suggesting that G protein activation by GBA motif-containing proteins is an ancient mechanism of signaling regulation that appeared at least 300 Mya.

The GBA motif has been identified in four mammalian proteins to date: Gαi-interacting vesicle-associated protein (GIV; also known as Gridin), Dishevelled-associating protein with high frequency of leucines (DAPLE), Calnuc, and NUCB2 (6, 7, 9). Although there is evidence that the GBA motif of Calnuc and NUCB2 binds and activates Gαi in vitro (9), the biological consequences of this have not yet been elucidated. For example, Calnuc binds to Gai on the surface of the Golgi apparatus (16) and modulates intracellular trafficking (17), but it is unclear whether one is a consequence of the other or whether the function of the GBA motif plays a role. On the other hand, the biological functions of the GBA motifs of GIV and DAPLE have been characterized more extensively. Both GIV and DAPLE regulate signal transduction via G protein activation downstream of membrane receptors that are not necessarily GPCRs, like


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receptor tyrosine kinases or integrins (6, 8, 18). As a consequence, they impact a variety of cellular processes (6, 8, 18), like cell motility, proliferation, or autophagy, and their dysregulation is associated with human diseases, such as cancer, liver fibrosis, nephrotic syndrome, and insulin resistance (8, 19). However, it is unclear if other functions previously described for GIV and DAPLE are GBA-dependent. For example, it is not known if the role of GIV in memory and angiogenesis (20–22), or the possible role of DAPLE in embryonic development (23–25), is mediated via the GBA motif.

The development of tools that modulate or interfere with different steps of G protein signaling is closely intertwined with advances in the molecular understanding of this signaling mechanism and the development of novel therapeutics. This is well exemplified by the fact that >25% of US Food and Drug Administration-approved drugs are direct modulators of GPCRs. More recently, there has also been success in the identification of small-molecule inhibitors that target Gq or Gγ subunits directly (26), or even some of their modulators, like regulator of G protein signaling (RGS) proteins (GAPs) (27) or GoLoco motif proteins (GDIs) (28). In addition to small molecules, an important approach to study G protein signaling has relied on the use of toxins, like pertussis, cholera, or Pasteurella multocida toxins (29). Pertussis toxin has been particularly useful to define and study G protein signaling mechanisms. This toxin ADP ribosylates α-subunits of the Gi/o family and specifically precludes their coupling to GPCRs. For over three decades, sensitivity to pertussis toxin has been used as an operational definition to mark molecular mechanisms or biological processes that are mediated by GPCR-Gi/o signaling. Another approach has been the use of genetically encoded tools, like the C-terminal domain of GRK2 (30). This domain, originally termed “beta-adrenergic receptor kinase 1 carboxy terminus (βARKct),” binds with high affinity to free Gβγ subunits and occludes the surface normally used by the G protein to bind and activate its effectors. Analogous to the generic use of pertussis toxin for GPCR-Gi/o coupling, βARKct has been widely used for decades to define and study Gβγ-dependent events. Other peptides or protein domains that preclude Gα or Gβγ binding to effectors have also been described and validated in applications ranging from in vitro biochemistry to animal models (26, 31, 32).

In contrast to this wealth of tools to manipulate other components of the G protein regulatory machinery, the study of GBA-mediated G protein regulation have been dampened by the lack of experimental tools. There is no small molecule or biological agent that specifically inhibits this signaling mechanism. Because the GBA motif is embedded in multifunctional proteins, assessing the role of its G protein regulatory function in different biological processes has been limited to multistep genetic manipulations. To date, the most convincing approach for this has been to genetically blunt the expression of the endogenous GBA protein and replace it with a version in which the G protein regulatory function has been disabled by mutagenesis. This is not only cumbersome and labor-intensive but has numerous technical limitations, like the difficulty in manipulating and delivering (e.g., via viral particles) large genes (e.g., ~6 kb for GIV and DAPLE) efficiently, or limited control over varying levels of expression of the proteins of interest. In addition to these limitations that hamper the clear interpretation of results or preclude experimental design in some settings (like whole animals), this approach does not account for possible compensatory effects mediated by other GBA proteins that are not targeted simultaneously in the same system. To overcome some of these limitations and expand the repertoire of tools to investigate the unknown functions of GBA proteins, we set out to develop a genetically encoded synthetic protein that functions as a generic inhibitor of GBA motifs. All GBA motifs described to date are located within structurally disordered regions well separated from other functional domains of the proteins in which they are embedded (33), so we reasoned that they could be specifically blocked if they bound to a protein that precluded their interaction with the target G protein. We chose to achieve this goal by rationally modifying Gai itself instead of another synthetic scaffold for several reasons. By definition, Gai binds generically to all GBA motifs, and the structural basis for this interaction has been recently characterized in detail by NMR spectroscopy, computational modeling, and biochemistry (33). We leveraged this information, along with a wealth of prior structural and biochemical information on other G protein regulatory interactions, to engineer modifications in Gai that favor GBA association and preclude its binding to other interacting partners. Here, we show a comprehensive biochemical characterization of the resulting “GBA inhibitor” (GBAi) and its validation in two different biological systems (i.e., cancer cells in culture, vertebrate embryos). Our results establish GBAi as a selective and efficient tool to dissect the biological processes controlled by a GPCR-independent mechanism of G protein activation mediated by cytoplasmic factors.

Results and Discussion

Rational Design of GBAi. We envisioned the design of a genetically encoded protein to inhibit GBA proteins (GBAi) based on Gα3 as the starting template. We sought to engineer two main properties to achieve efficient and specific inhibition of GBA motifs. One was that GBAi remains bound to GBA motifs even after exchange of GDP for GTP (Fig. 1L), which is known to cause disengagement of GBA motifs from Gαi proteins (6, 8, 9, 15). This property would favor the constitutive association with GBA motifs in cells. The second property was that GBAi does not interact with Gai binding partners that are not GαB proteins (Fig. 1B). This would make the action of GBAi specific for GBA motifs. To achieve these goals, we introduced a number of modifications in Gα3 based on prior knowledge from the literature (Fig. 1C).

Mutation of Gly203 to Ala (G203A) is known to permit nucleotide binding and hydrolysis with properties similar to those of Gai wild-type (wt) at physiological concentrations of Mg2+ (~1 mM), but disrupts a change in conformation of the Switch II region (SwII) that occurs in the wt protein upon GTP binding (34, 35). This change in conformation in Gai wt leads to its disengagement from G protein binding partners that bind to inactive, GDP-bound Gα (e.g., Gβγ motifs, Gαi motifs) and promotes GDP release from GTP-bound, active Gαi (45–47). Based on this, we reasoned that introducing the G203A mutation would preclude GBA disengagement from GBAi even upon loading of GTP. At the same time, this mutation would also preclude binding to effectors and RGS GAPs, which bind to GTP-bound active conformations of Gα by making direct contacts with the SwII (37–39). To disrupt GBAi binding to GoLoco GDIs, another major group of Gai binding partners, we mutated Asn149 to Ile (N149I), which is known to disrupt Gai binding to all GoLoco motifs without affecting other properties of the G protein (40). We deleted the last nine C-terminal residues (ΔC) with the dual purpose of disrupting binding to GPCR (41–43) and the chaperone/GEF Ric-8A (44). The first 25 N-terminal residues (ΔN) were also deleted to preclude binding to Gβγ subunits based on the facts that the N terminus is one of the major Gβγ contacts in the heterotrimeric structure and that previous reports have shown that deletion of the N terminus of Gα3 (45) or other Gα subunits (46–48) is sufficient to abolish Gβγ binding. Neither ΔC nor ΔN has significant effects on Gα nucleotide binding or hydrolysis (33, 49). G203A, N149I, ΔC, or ΔN is known or predicted to have no effect on GBAi binding based on structural insights and/or experimental data (33) (Fig. 1C).

GPCRs, Gβγ effectors, RGS GAPs, GoLoco GDIs, and Ric-8A are, to our knowledge, all of the Gai binding partners for which a biological function has been clearly established. Thus, the set of mutations described above is expected to preserve binding to GBA motifs without interfering with the function of
other Gαi binding partners. To further rule out possible off-target effects of GBAi, we designed a mutated version in which Trp211 is replaced by Ala (W211A) to be used as a negative control in all of our experiments (Fig. 1C). This mutation has been shown to disrupt binding of Gαi3 to every GBA motif described to date (6, 7, 9), but it has no significant effect on G protein structural integrity or the ability to bind and hydrolyze nucleotides (50). Thus, disruption of GBAi-mediated effects by the W211A mutation will increase confidence in its specificity for inhibiting GBA motifs. We next proceeded to validate that the
properties predicted from the effect of individual mutations described above hold true when present simultaneously in GBAi.

**GBAi Is Properly Folded and Binds Nucleotides.** First, we assessed the structural integrity of purified GBAi. For this, we monitored its thermal denaturation by differential scanning fluorimetry assays carried out in the presence or absence of an excess of nucleotides (Fig. S1). This assay is equivalent to that recently established by Sun et al. (51) to assess the overall stability and nucleotide binding ability of a Goi1 mutants. The GBAi melting temperature ($T_m$) in this assay was comparable to that of wt Goi3 in the absence of added nucleotides ($\sim$40 °C). Addition of an excess amount of GDP (250 μM) increased the $T_m$ of both GBAi and Goi3 only moderately ($\sim$4 °C), whereas addition of the same concentration of the nonhydrolyzable GTP analog guanosine 5’-O-γ-thio-triphosphate (GTPγS) had a more marked effect ($\sim$+20 °C; Fig. S1). The larger extent of the stabilization upon GTPγS binding compared with GDP binding is consistent with previous observations with Goi1 (51). In contrast, addition of GTPγS to Goi3 S47R, a recently characterized GTP-binding deficient mutant (52), did not recapitulate the marked thermal stabilization observed for GBAi or Goi3 (Fig. S1). Taken together, these findings indicate that GBAi is folded properly and retains the ability to bind nucleotides.

**GBAi Binds to GBA Motifs and Does Not Dissociate upon GTP Binding.** To start characterizing its biochemical properties, we compared the binding of GBAi to GBA motifs with that of Goi3 and examined the effect of GTP mimetics on the binding. For this, GBAi was purified from bacteria and binding to a peptide corresponding to the GBA motif of GIV (residues 1,671–1,701) was measured using a fluorescence polarization (FP) assay. GIV is the prototypical protein with a GBA motif, and we have previously shown that peptides corresponding to the GBA motif fully recapitulate the binding properties of the protein (33, 53). GBAi binds to the GIV-derived peptide with an affinity similar to that of Goi3 in the presence of GDP (Fig. 1D and Table S1). The equilibrium dissociation constants ($K_d$) were $\sim$0.5 μM, which is consistent with previously reported values using different methods (6, 13). As expected, binding to Goi3 was almost abolished in the presence of GDP-AlF$_4^-$ (which mimics the GTP-bound transition state) (39) (Fig. 1D). In contrast, GBAi binding affinity was the same in the presence of GDP or GDP-AlF$_4^-$. The GBAi W211A mutant did not bind GIV in the presence of either GDP or GDP-AlF$_4^-$, demonstrating that it is a bona fide negative control for GBAi binding. Equivalent results were obtained when Goi3 and GBAi preloaded with the nonhydrolyzable GTP analog GTPγS (instead of GDP-AlF$_2^-$) were compared with GDP in the same FP assay (Fig. S2). We also confirmed that binding of GBAi to full-length GIV, instead of to the GBA motif peptide used in FP assays, is not disrupted by GDP-AlF$_4^-$ or GTPγS in pulldown assays (Fig. 1E). These results indicate that GBAi binds to GIV with an affinity comparable to that of Goi3-GDP and that, contrary to what occurs with Goi3, GBAi binding is not disrupted upon GTP binding.

Next, we investigated the properties of the interaction of GBAi with other GBA motifs. For this, we carried out FP experiments as above with peptides derived from the three other mammalian proteins with a GBA motif described to date (i.e., DAPLE, Calnuc, and NUCB2) (Fig. S34). The results were analogous to those obtained with GIV, indicating that GBAi binds to each one of the GBA motifs with the same affinity as Goi3-GDP and that the binding is not affected by GTP mimetics but can be abolished by the W211A mutation (Fig. S3 B and C and Table S1). Similar observations were made when the interactions of full-length DAPLE, Calnuc, and NUCB2 were tested using a protein–protein binding assay different from FP (i.e., GST pulldowns) (Fig. S3D), which further validates our conclusions on the properties of GBAi binding. Finally, we also tested if the binding mode of GBA motifs was conserved between GBAi and Goi3. Previous structural and biochemical studies have shed light onto how GBA motifs engage G proteins. A critical feature is that a set of hydrophobic residues in the GBA motif is used to make contacts with a groove formed by the SwI and o3 helix of Goi3 (6–9, 15, 54). Mutation of a Phe conserved across all GBA motifs (Fig. S34) to Ala (F→A) has been previously shown to markedly reduce their binding to Goi3 (6–9, 15). We found that the same occurs with GBAi (Fig. S4), supporting that GBA motifs bind to GBAi and Goi3 in a similar manner. Taken together, these findings show that GBAi binds efficiently to all known GBA motifs, and does not disengage from the GBA motifs upon GTP binding, and that the W211A mutation disrupts its binding to GBA motifs.

**GBAi Does Not Bind to Gβγ.** Next, we investigated if GBAi fulfills its second desired property that was part of our original design (Fig. 1 A and B); that is, it does not interact with Gai binding partners that are not GBA proteins. We focused our initial efforts on Gβγ because it binds constitutively to Go subunits with high affinity and is the predominant binding partner in cells. An initial concern was that, according to one report (55), Gβγ subunits can interact with Goi (α–transducin) even if the N terminus is deleted. However, the observations were based on assays carried out in vitro using supraphysiological concentrations of G proteins (55) and are in discrepancy with several other reports showing that deletion of the N terminus of Goi3 or other Go subunits disrupts Gβγ binding under more physiological conditions (45–48). To directly compare binding of GBAi and Goi3 to Gβγ, we used a previously described (56, 57) BRET assay in HEK293T cells. In this assay, BRET results from the interaction between free Gβγ and its effector GRK3 (Fig. 2A), which can be inhibited by Goi binding to Gβγ due to competition for an overlapping interaction region with GRK3. As expected, expression of Gβγ alone in the absence of exogenous Go resulted in high levels of BRET that were quenched upon expression of increasing amounts of Goi3 in a dose-dependent manner (Fig. 2A). In contrast, expression of comparable amounts of GBAi did not reduce the BRET signal, indicating that GBAi does not associate with Gβγ in cells. To further validate this conclusion, we carried out immunoprecipitation experiments in MCF-7 cells (Fig. S5). Equal amounts of epitope-tagged Gβγ and GBAi (wt or W211A) were coexpressed in Dulbecco’s Modified Eagle Medium (DMEM) with Gαi3 and Gβγ subunits not expressing either GBAi or Gαi3:Gβγ or other Gα subunits not expressing either GBAi or Gαi3:Gβγ. Equal amounts of epitope-tagged Gβγ and GBAi (wt or W211A) were coexpressed with exogenous Gβγ and immunoprecipitated. Exogenous and endogenous Gβγ was present in Goi3 immunoprecipitates, whereas Gβγ was not detected in the GBAi immunoprecipitates (Fig. S5). These results indicate that GBAi does not bind to Gβγ in cells.

**GBAi Does Not Interfere with GPCR-Mediated G Protein Signaling.** The C-terminal tail of Gα subunits and their association with Gβγ subunits are obligatory requirements for coupling to GPCRs (41–43). To confirm that GBAi, which lacks these two features, does not couple to GPCRs, we used the BRET-based assay described above. In cells expressing Goi3 and the GPCR adenosine 1 receptor (in addition to the BRET donor and acceptor), adenosine led to a rapid increase in BRET, which reflects the dissociation of Goi3:Gβγ heterotrimers upon activation (Fig. 2B). On the contrary, in cells expressing GBAi instead of Goi3, no significant BRET increase was observed in response to adenosine (Fig. 2B). In agreement with Fig. 2A, the basal BRET signal before adenosine stimulation was already as high as in cells not expressing either GBAi or Goi3, which reflects the lack of association of GBAi with Gβγ (Fig. 2B) and also explains the insensitivity to GPCR stimulation. To further validate that GBAi does not interfere with GPCR-mediated signaling, we tested the effect of expressing GBAi on the activation of Goi3:Gβγ heterotrimers using the same BRET-based assay (Fig. 2C). We found that the BRET response caused by the dissociation of Goi3:Gβγ

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heterotrimerics upon adenosine stimulation was not affected by coexpression of GBAi (wt or W211A) with Gui3. Together, these observations show that GBAi does not couple to GPCRs and does not interfere with GPCR-mediated activation of G proteins.

To further validate that GBAi does not interfere with GPCR-mediated signaling, we investigated its effect on downstream G protein-dependent readouts. First, we monitored cAMP levels in HEK293T cells using a previously described BRET-based biosensor (“nanoluc-exchange protein directly activated by cAMP-VenusVenus,” Nluc-EPAC-VV) (56) in which BRET efficiency is inversely proportional to cAMP levels. Upon GPCR stimulation, Gαβγ heterotrimer dissociates and V-Gαi3 competitively displaces V-Gαi3 plus endogenous Gβ1, Gα2, and Gα3, whereas the lower band corresponds to GBAi. (B) GBAi does not couple to GPCRs in mammalian cells as determined by BRET. (Top) Schematic depicting the BRET assay used to determine the coupling of Gui3 or GBAi to GPCRs. Under resting conditions, V-Gαi3 associates with Gui3 and BRET is low. Upon GPCR stimulation, the Gui3-V-Gαi3 heterotrimer dissociates and V-Gαi3 interacts with mas-GRK3ct-NLuc, leading to an increase in BRET. (Middle) HEK293T cells were transfected with plasmids encoding for Gαβγi, GBAi wt, and GABABRs (0.2 μg) along with plasmids for the BRET donor and acceptor (mas-GRK3ct-NLuc and V-Gαi3), as well as for the adenosine 1 receptor. BRET was measured every second. After 30 s of measurement under resting conditions, cells were stimulated with adenosine (10 μM). One representative experiment of three is shown. (Bottom) Protein expression of Gui3, GBAi wt, and G-V-Gαi3 was assessed by immunoblotting (IB) with the indicated antibodies. (C) GBAi does not interfere with GPCR-mediated activation of Gui3 as determined by BRET. (Top) Schematic depicting the BRET experiment used to assess the possible interference of GBAi with Gui3 coupling to GPCRs. The experimental design is as in B except that GBAi is coexpressed, along with Gui3 and the rest of the BRET assay components, to test if it could impair the G protein-dependent BRET increase observed upon GPCR stimulation. (Middle) Experiments were carried out exactly as in B except that GBAi (wt or W211A, 2 μg) and Gui3 (0.5 μg) were expressed simultaneously in the same cells. One representative experiment of three is shown. (Bottom) Protein expression of Gui3, GBAi wt, and G-V-Gαi3 was assessed by IB with the indicated antibodies. (D) GBAi does not interfere with GPCR-mediated regulation of cAMP by Gαi3. (Top Left) Schematic depicting the experiment used to assess the possible interference of GBAi with GPCR-mediated regulation of cAMP levels. (Top Right) Protein expression of Gαi3, GBAi wt, and G-V-Gαi3 was assessed by IB with the indicated antibodies. (Bottom) HEK293T cells were transfected with plasmids encoding for Nluc-EPAC-VV (0.05 μg) and GABARsR (0.2 μg) in the presence (+GBAi) or absence (control) of GBAi wt (2 μg). BRET was measured every 4 s. Forskolin (Fsk, 1 μM) and GABA (1 μM) were added (sequentially) at the indicated times. The blue trace corresponds to unstimulated control cells and is duplicated in both panels as a visual reference of the baseline. Results from three independent experiments are expressed as mean ± SEM. (E) GBAi does not interfere with GPCR-mediated regulation of ERK1/2 by Gαi3. (Top) Schematic depicting the experiment used to assess the possible interference of GBAi with GPCR-mediated regulation of ERK1/2. (Bottom) HEK293T cells were transfected with plasmids encoding for α2A-AR (0.2 μg) in the presence (+GBAi) or absence (control) of GBAi wt (2 μg). Cells were serum-starved overnight and then stimulated with brimonidine (5 μM) for the indicated times. Cell lysates were immunoblotted with the indicated antibodies. One representative experiment of three is shown.
His-Gα to activation (as determined by its phosphorylation) in response mediated by GABABR (Fig. S6). Next, we investigated if GBAi forskolin-mediated cAMP elevation was inhibited by prestimulation of the GPCR-mediated regulation of G protein signaling.

GBAi Does Not Interact with Gα Effectors and the Gα Regulators RGS GAPs, GoLoco GDIs, and Ric-8A. In addition to GPCRs and Gβγ subunits, Gα proteins bind to effectors and various regulators. An effector of Gα subunits can be defined as a protein whose function is modulated upon specific binding of the G protein in its GTP-bound active conformation. Our results in Fig. 1D and Figs. S2 and S3 suggest that GBAi cannot adopt an active conformation because it does not disengage from GBA motifs in the presence of GTP mimetics, presumably due to the properties associated with the G203A mutation. This is further supported by the fact that GBAi does not interfere with Gα regulation of its effector adenylyl cyclase in cells (Fig. 2D). Next, we tested directly if GBAi binds to KB-1753, a synthetic peptide that binds to Gα subunits like an effector (37). KB-1753 is the only effector-like molecule that has been crystalized in complex with Gα to date, and its biochemical properties have been extensively characterized (37). Using a fluorescently labeled KB-1753 peptide in FP assays, we found that, as expected, it bound to Gα3 preferentially when preincubated with GTPγS or GDP-AlF4− versus GDP, whereas no binding to GBAi was detected in any of the three conditions (Fig. 3A). The same lack of GBAi binding was observed when binding was assessed in pulldown assays (Fig. S7A). Similar experiments were carried out with GST-fused GAIP (also known as RGS19), a representative member of the RGS GAP family. RGS GAPs also bind to GTP-loaded active conformations of Gα proteins, with a preference for the transition state mimicked by GDP-AlF4− (39). As for KB-1753, GAIP bound to Gα3, but not GBAi, in the GDP-AlF4−

![Fig. 3. GBAi does not bind to Gα effectors or the Gα regulators RGS GAPs, GoLoco GDIs, and Ric-8A. (A) GBAi does not bind to the effector-like peptide KB-1753. Binding of a fluorescently labeled KB-1753 peptide to the indicated concentrations of purified His-tagged Gα3 (black), GBAi wt (blue), or GBAi W211A (red) was determined by FP in the presence of GDP, GTPγS, or GDP-AlF4− as indicated. Data were normalized to maximal binding and fitted to a one-site binding model (solid lines) to calculate the indicated Kd. KB-1753 binds Gα3 with high affinity in the presence of GDP-AlF4− or GTPγS and low affinity in the presence of GDP, whereas GBAi does not bind in any of the three conditions. Results from three independent experiments are expressed as mean ± SEM. (B) GBAi does not bind to the RGS GAP protein GAIP. Purified GST or GST-GAIP was immobilized on glutathione-agarose beads and incubated with purified His-Gα3 or His-GBAi in the presence of GDP, GTPγS, or GDP-AlF4− as indicated. Resin-bound proteins were eluted, separated by SDS/PAGE, and analyzed by Ponceau S-staining and immunoblotting (IB) as indicated. GST-GAIP binds His-Gα3 in the presence of GDP-AlF4−, but not GDP, whereas it does not bind to GBAi either in the presence of GDP or GDP-AlF4−. One representative experiment of three is shown. (C) GBAi does not bind to the GoLoco GDI motif of RGS12 (GoLoco R12). Binding of a fluorescently labeled peptide corresponding to GoLoco R12 (RGS12 residues 1,185–1,221) to the indicated concentrations of purified His-tagged Gα3 (black), GBAi wt (blue), or GBAi W211A (red) was determined by FP in the presence of GDP (Left), GTPγS (Center), or GDP-AlF4− (Right). Data were normalized to maximal binding and fitted to a one-site binding model (solid lines) to calculate the indicated Kd. GoLoco R12 binds Gα3 with high affinity in the presence of GDP and low affinity in the presence of GTPγS or GDP-AlF4−, whereas GBAi binding is almost or completely absent under the same conditions. Results from three independent experiments are expressed as mean ± SEM. (D) GBAi does not bind to the chaperone/GEF protein Ric-8A. Experiments were carried out exactly as in B except that GST–Ric-8A was used instead of GST–KB-1753 and all conditions were tested in the presence of GDP. GST–Ric-8A binds to His-Gα3 but not to His-GBAi. One representative experiment of three is shown.]
(Fig. 3B) or GTPyS (Fig. S7B) condition. We also confirmed the lack of GBAi binding to GAIp in cell-based assays by carrying out communoprecipitations after expression of the proteins of interest in HEK293T cells (Fig. S8A). Taken together with the results shown in Fig. 1 and Figs. S2 and S3, these findings indicate that GBAi cannot adopt an active conformation capable of engaging effectors or other regulators that bind to Goa-GTP like RGS proteins.

Next, we investigated if GBAi binds to GoLoco GDIs, another major family of Goi regulators (3). Much like proteins with a GBA motif, GoLoco GDIs bind to GDP-bound Gai with a marked preference over GTP-bound Gai (2, 3). Because the N149I mutation in Gai has been previously shown to abolish binding to all GoLoco motif-containing proteins (40), we reasoned that GBAi would not bind to this class of G protein regulators. For this, we compared the binding of Gai3 and GBAi to the GoLoco motif of RGS12 (GoLoco R12) as a representative example using an FP assay analogous to that used in Fig. 1D. Consistent with previous reports (60), GoLoco R12 bound to Gai3-GDP with high affinity (K<sub>D</sub> below 100 nM) (Fig. 3C). In contrast GBAi binding was nearly absent and even weaker than the binding of GoLoco R12 to active (GDP-AlF<sub>4</sub>)<sup>-</sup> Gai3 (Fig. 3C). We also found that GBAi expressed in mammalian cells does not bind to a truncated form of RGS12 containing its GoLoco motif but lacking its RGS domain (Fig. S8B).

Finally, we tested if GBAi interacts with the nonreceptor GEF/chaperone Ric-5A and found that it does not interact with either when using purified proteins in vitro (Fig. 3D) or by communoprecipitation in mammalian cells (Fig. S8C). This is not surprising because it has been previously reported that a deletion of the last nine residues of Gai, like the one present in GBAi (Fig. 1C), completely abolishes Ric-8A binding (44).

**GBAi Inhibits GIV-Mediated Enhancement of PI3K-Akt Signaling in Response to Integrin Stimulation.** The results presented so far demonstrate that GBAi displays all of the desired properties described in Fig. 1 to behave as an efficient and specific inhibitor of GBA proteins. To provide proof-of-principle evidence that GBAi can indeed function as an inhibitor of the biological function of GBA motifs, we tested it in a well-characterized system. We have recently described that GIV promotes integrin signaling in cancer cells by activating G proteins via its GBA motif (18, 61). Others have arrived at a similar conclusion independently (62). The mechanism by which GIV promotes integrin signaling was elucidated using loss-of-function and gain-of-function genetic approaches, along with pharmacological manipulations, and was corroborated in multiple cell lines (18, 61). Thus, this is a robust system for testing GBAi. In brief, GIV is recruited to active integrins, leading to G protein activation via its GBA motif and subsequent enhancement of G<sub>p</sub>PI3K-Akt signaling (Fig. 4A). To interrogate this mechanism with GBAi, we used MCF-7 cells expressing a control plasmid or a plasmid encoding for GIV (Fig. 4B and C). MCF-7 cells are poorly invasive breast cancer cells that naturally express very low levels of GIV (18, 61). Upon exogenous expression of GIV, MCF-7 cells gain proinvasive traits (7, 18, 61), including enhanced integrin-Akt signaling. Consistent with our previous observations (18, 61), GIV expression in MCF-7 cells did not affect Akt activation (as determined by the levels of Akt phosphorylated at S473) in unstimulated cells (time 0 in the figures) (Fig. S9), but potentiated it in response to collagen I stimulation compared with control cells (Fig. 4B and C, compare black lines/symbols in Fig. 4C). Importantly, expression of GBAi wt inhibited the enhancement of Akt activation in GIV-expressing cells (compare blue with black lines/symbols in Fig. 4C, Right) to the levels of activation observed in control cells (not expressing exogenous GIV and in the absence of GBAi) (Fig. 4B and C, black lines/symbols in Fig. 4C, Left). On the other hand, GBAi wt had no effect on the activation of Akt in control cells not expressing exogenous GIV (compare blue and black lines/symbols in Fig. 4C, Left), suggesting that GBAi specifically inhibits the GIV-dependent response. Moreover, the inhibition mediated by GBAi wt was not reproduced by the GBA binding-deficient mutant W211A (Fig. 4B and C, compare red with black lines/symbols in Fig. 4C, Left), which indicates that the action of GBAi is due to inhibition of GIV’s GBA motif and not to some other spurious effect. These results demonstrate that GBAi functions as a specific inhibitor of GBA-mediated signaling of GIV in cells.

**GBAi Inhibits DAPLE-Mediated Gastrulation and Convergent Extension Defects in Xenopus laevis Embryos.** The inhibition of GIV-mediated enhancement of integrin signaling in cancer cells is an important validation benchmark for GBAi. Next, we simultaneously interrogated if GBAi possesses two important features: (i) usefulness in revealing uncharacterized biological activities of GBA motifs and (ii) effectiveness in blocking...
Fig. 5. GBAi inhibits DAPLE-mediated convergent extension defects in *X. laevis* embryos. (A and B) GBAi wt or the F1675A mutation in DAPLE inhibits developmental defects induced by DAPLE. (A) Schematic depicting the assay to assess DAPLE-induced developmental defects. mRNAs are injected equatorially in both dorsal blastomeres of two- to four-cell embryos [stage (st) 2–3], and phenotypes are assessed at st 30. Representative phenotypes are shown: normal or dorsally bent embryos (mild or severe). (B, Left) DAPLE (500 pg), GBAi wt (1 ng), or GBAi W211A (1 ng) mRNAs were injected as indicated, and the frequency of phenotypes is assessed at st 30. The total number of embryos analyzed from three independent experiments for each group is indicated on the top of the graph. (Bottom) Protein expression of DAPLE and GBAi was assessed by immunoblotting (IB) with the indicated antibodies. (B, Right) DAPLE wt or DAPLE FA mRNAs (250 pg or 500 pg) were injected, and phenotypes were analyzed as in the left graph. (Bottom) Protein expression of DAPLE wt and DAPLE FA (250 pg of mRNA) was assessed by IB with the indicated antibodies. (C–F) GBAi wt, but not GBAi W211A, blocks DAPLE-mediated inhibition of convergent extension movements. (C) DAPLE (500 pg) and GBAi (wt or W211A, 1 ng) were coinjected into the animal hemisphere of both blastomeres of two-cell embryos. Animal caps were dissected at st 8, treated (or not treated) with activin to induce elongation, and analyzed for elongation at st 15. Representative pictures of activin-treated versus untreated caps injected with the indicated mRNAs (D) and the frequency of different elongation phenotypes [E, none, +, mild, or ++, strong elongation] are shown. The total number of embryos analyzed from three independent experiments for each group is indicated on the top of the graph. (F) RT-PCR of activin-mediated gene induction from caps at st 10.5 injected with the indicated mRNAs and treated or not treated with activin. Whole embryos (WE) with (+RT) or without (−RT) the reverse transcriptase reaction are shown on the left lanes as positive and negative controls, respectively. ***P < 0.001, χ² test.
GBA-mediated processes in whole organisms in vivo. For this, we investigated the role of the GBA motif of DAPLE in embryogenesis using *Xenopus laevis* as a model of vertebrate development.

Noncanonical Wnt signaling is crucial for the orchestration of cell movements during gastrulation, and Dishevelled is a core component of this pathway (63). DAPLE has been previously shown to play a role in embryonic development, and it is believed to do so, at least in part, by modulating noncanonical Wnt signaling via direct interaction with Dishevelled (23, 24). On the other hand, we have recently shown that the GBA motif of DAPLE is also required for efficient noncanonical Wnt signaling in isolated cells (6). However, it is unknown if the GBA motif is important for the effects of DAPLE on embryo development. Consistent with previous reports (23, 24), we found that dorsal injection of DAPLE mRNA (encoding residues 1,217–2,028, containing both the GBA and Dishevelled binding motifs) into frog embryos caused gastrulation defects with high frequency (Fig. 5 A and B). Coexpression of GBAi wt with DAPLE almost completely blunted the gastrulation defects induced by DAPLE, while the same amount of GBAi wt alone had no effect (Fig. 5 A and B). On the other hand, coexpression of GBAi W211A with DAPLE did not diminish the frequency of gastrulation defects (Fig. 5 A and B). These results indicate that DAPLE induces gastrulation defects via its GBA motif. To further validate this using a GBAi-dependent approach, we carried out analogous experiments in frog embryos to compare the effect of DAPLE wt and a validated GEF-deficient mutant of its GBA motif [i.e., F1675A (FA) (6)] on gastrulation. We found that the FA mutation dramatically reduced the frequency of gastrulation defects induced by DAPLE (Fig. 5 A and B), validating that this phenotype is caused by the DAPLE GBA motif. The incomplete reduction of the phenotype with the FA mutant is not surprising because it has been previously shown that the FA mutation does not completely abolish GBA function in other experimental settings (6, 15, 54). However, this suggests that GBAi can be more efficient than the FA mutation in inhibiting the function of GBA motifs. Previous reports have shown that, much like the FA mutation, deletion of the Dishevelled binding motif of DAPLE also impairs its role in embryonic development in the same experimental system as described here (23, 24). Thus, it is conceivable that both G protein and Dishevelled binding by DAPLE work coordinately in this context.

We performed additional experiments with GBAi to substantiate that DAPLE causes gastrulation defects via its GBA motif by impinging on a process that relies heavily on noncanonical Wnt signaling. Gastrulation defects, such as those we observed in Fig. 5 A, frequently arise from alterations in a set of cell movements commonly referred to as convergent extension (64). Noncanonical Wnt signaling is an obligatory requirement for convergent extension movements (64). Experimentally, convergent extension can be assessed with an assay that involves the treatment of embryo explants with activin (Fig. 5C). Briefly, stimulation of animal caps excised at stage 8 with activin induces the expression of mesodermal differentiation genes, which, in turn, causes the release of noncanonical Wnt ligands that drive convergent extension cell movements and subsequent explant elongation (65). Expression of DAPLE by mRNA microinjection led to a marked attenuation of activin-induced animal cap elongation (Fig. 5 D and E). The effect of DAPLE was efficiently reverted by GBAi wt, but not by the GBA-binding deficient W211A mutant (Fig. 5 D and E), indicating that the GBA motif of DAPLE is required for the observed attenuation of animal cap elongation. We noted that the observed effects of DAPLE were due to disruption of activin-stimulated mesodermal differentiation by performing RT-PCR assays (Fig. 5F). We found that activin stimulation led to mesodermal differentiation in all experimental conditions, as determined by induction of the mesodermal markers *Xbra* and *Chordin* (Fig. 5F). Taken together, these findings indicate that the underlying cause of the gastrulation phenotypes caused by DAPLE via its GBA motif is a defect in convergent extension movements.

In summary, we have described here the rational design, validation, and implementation of a synthetic protein that can be used as a tool to investigate the functions of GBA motifs in vitro and in vivo. The development of tools to interdict specific steps of G protein signaling has proven extremely valuable in the past to advance our knowledge of this core mechanism of cell communication (26, 29). There is a parallelism between GBAi and one of the tools that has historically contributed to major advances in the field of G protein signaling (i.e., pertussis toxin). While pertussis toxin uncouples Gui subunits from their canonical GEFs (i.e., GPCRs), GBAi uncouples Gui subunits from a family of atypical GEFs that are not membrane receptors. In the same way that pertussis toxin serves as a tool to generally assess the role of GPCR-Gi coupling in a myriad of biological contexts, we hope that GBAi will serve a similar purpose for investigating the consequences of GBAi-Gi coupling.

**Materials and Methods**

*K*~S~ were determined by FP measurements, and protein–protein binding was assessed by GST pulldown or immunoprecipitation assays as previously described (6, 7, 9, 53, 66), with minor modifications. Collagen I stimulation experiments were carried out exactly as in the study by Leyme et al. (18), and BRET-based measurement was carried out as described previously (52, 56, 57, 67), with minor modifications. For *X. laevis* experiments, fertilized eggs were microinjected by in vitro-transcribed mRNA and analyzed as indicated in the main text. Statistical significance between various conditions was assessed by the Student’s *t* test or *χ*~2~ test. A full description of materials and methods used in this study is provided in Supporting Information.

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  guanine nucleotide exchange factor GoLoco is sufficient for activating heterotrimeric G proteins. J Biol Chem 291: 
  27098–27111.

15. Coleman BD, et al. (2016) Evolutionary conservation of a GPCR-independent mecha-

16. Weitz RJ, et al. (2003) GoLoco γδ binding to calnexin on Golgi membranes in living cells 
  monitored by fluorescence resonance energy transfer of green fluorescent protein 

  distribution of Galphai but not Gbeta-gamma subunits and modulates ACTH secretion in 

  activate trimeric G proteins via the nonreceptor protein GIV/Gidrin. J Cell Biol 210: 
  1165–1184.

  therapy in cancer: End of a long futile campaign striking heads of a Hydra. Aging 
  (Albany NY) 7:469–474.


22. Nakai T, et al. (2014) Girdin phosphorylation is crucial for synaptic plasticity and 
  memory: A potential role in the interaction of BDNF/Tkβ/αTtk signaling with NMDA 


  beta-catena pathway and the Wnt/INK pathway in Xenopus. Mech Dev 128: 
  1138–1153.

  an autosomal recessive non-syndromic hydrocephalus with medial diverticulum. Mol 
  Syndromol 1:99–112.

26. Smrcka AV (2013) Molecular targeting of G alpha subunit of Gi3 with a reduced affinity for 
  beta gamma dimers and altered conformation of the GDP-Pi binding and activating motif. 

27. Nakai T, et al. (2014) Girdin phosphorylation is crucial for synaptic plasticity and 
  memory: A potential role in the interaction of BDNF/Tkβ/αTtk signaling with NMDA 

  terminus of the G alpha Q subunit determines the species specificity of G protein 


33. Marvin A, et al. (2016) Dominant-negative Gs subunits are a mechanism of dysre-


  exchange factor (GEF) motif of GIV protein reveals a threshold effect in signaling. 

  GPCRs to G proteins: Role of the G alpha N-terminal region in rhodopsin-transducin 

  terminus of the G alpha Q subunit determines the species specificity of G protein 


Supporting Information

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S1 Materials and Methods

Reagents and Antibodies. Unless otherwise indicated, all chemical reagents were obtained from Sigma or Fisher Scientific. The Escherichia coli DH5α strain was purchased from New England Biolabs. Pfu Ultra DNA Polymerase was purchased from Agilent. Rat tail collagen I (catalog no. 354236) was purchased from BD Biosciences. Puromycin (catalog no. P-600-1) was purchased from Gold Biotechnology. Lipofectamine LTX with PLUS Reagent was purchased from Life Technologies (catalog no. 15338100). Human activin A (SRP3003) and mouse monoclonal antibodies against α-tubulin (T0074), FLAG tag (F1804), or hexahistidine (His) tag (H1029) were obtained from Sigma.

Rabbit sera against GIV coiled-coil domain, against DAPLE, and against GAIP (N terminus) were kindly provided by M. Farquhar, University of California, San Diego (68, 69). Rabbit polyclonal antibodies against pan-GF (M-14) and mouse monoclonal antibodies raised against total Akt (B-1) and total ERK (MK1) were purchased from Santa Cruz Biotechnology. Rabbit antibodies for pan-Goi (5090S), phosphorylated Akt (pAkt; S473) (clone D9E), phosphorylated ERK1/2 (pERK1/2; T202/Y204) (4370P), and the mouse antibody for Myc tag (clone 9B11, used for immunoblotting) were obtained from Cell Signaling. Mouse monoclonal antibody for Myc tag (clone 9E10) used for immunoprecipitation was obtained from the Developmental Studies Hybridoma Bank (University of Iowa) created by the National Institute of Child Health and Human Development. Rabbit polyclonal antibody for RFP was purchased from Abcam (ab62341), and the mouse monoclonal antibody for GFP was purchased from Clontech (JL-8, catalog no. 632380). Goat anti-rabbit Alexa Fluor 680 and goat anti-mouse or IRDye 800 secondary antibodies were from Life Technologies and LiCor, respectively.

Plasmids. Plasmids for the expression of rat Gai3 (pDNA3-Go3i), rat Gai3-FLAG (p3x FLAG-CMV14-Go3i, C-terminal 3x FLAG tag), and rat FLAG-Go3i (p3xFLAG-CMV10-Go3i, N-terminal 3x FLAG tag) in mammalian cells have been described previously (7, 10). The pcDNA3.1-adenosine 1 receptor (A1R), and pcDNA3.1-VEGFR1 (residues 1–158) (7) was generously provided by S. Goto, University of California, San Diego, and subsequent insertion into pLIC-Myc. The plasmid for in vitro mRNA transcription of myc-DAPLE was generated previously (18), as well as the plasmid encoding for Calnuc-GFP (73). A plasmid construct for the expression of His-GBAi in bacteria was generated in two steps. First, the sequence corresponding to rat Gai3 residues 26–345 (i.e., lacking the first 25 N-terminal and last nine C-terminal amino acids) was amplified by PCR and inserted into a previously described plasmid (pMCSG7, also known as pLIC-His) via a ligation independent cloning (LIC) procedure (74). Then, point mutations G203A and N149I were introduced separately. Plasmid constructs for the expression of Myc-GBAi in mammalian cells and GST-GBAi in bacteria were generated by PCR amplification of the GBAi sequence from pLIC-His-GBAi and subsequent insertion into an LIC plasmid with an N-terminal Myc tag (pLIC-Myc) derived from pcDNA3.1/Hygro(+) or an LIC plasmid with an N-terminal GST tag (pLIC-GST) (both kindly provided by J. Sondek, University of North Carolina at Chapel Hill, Chapel Hill, NC) (75). A plasmid encoding for FLAG-GBAi was generated by insertion of GBAi between the EcoRI and KpnI sites of p3xFLAG-CMV (N-terminal 3x FLAG tag). Plasmids for the bacterial expression of GST-fused GBAi motifs of human GIV (residues 1,671–1,701, GST-GIV), human DAPLE (residues 1,661–1,691, GST-DAPLE), human Calnuc (residues 303–333, GST-Calnuc), and human NUCB2 (residues 304–334, GST-NUCB2) were generated by LIC (74) of PCR-amplified fragments into a previously described plasmid (pLIC-GST; kindly provided by J. Sondek) (75). The construct encoding for Myc-NUCB2 (rat) was created by LIC cloning into the pLIC-Myc vector. To generate the construct encoding for GST–KB–1753, complementary primers corresponding to the KB–1753 sequence (SSRGyyHGWVGEegSlrS) (37) were annealed and ligated into EcoRI/HindIII sites of the pGEX-KG plasmid. The plasmid used for the expression of full-length, myc-tagged DAPLE was generated by PCR amplification from a previously described plasmid (6), a generous gift from P. Ghosh, University of California, San Diego, and subsequent insertion into pLIC-Myc. The plasmid for in vitro mRNA transcription of myc-DAPLE (residues 1,217–2,028) was used for microinjections in Xenopus laevis was cloned by PCR amplification from Kazusa’s clone fh14721 (KIAA1509) and ligation into the EcoRI/XhoI sites of a modified pcS2(+) vector incorporating an N-terminal 6x Myc tag to create pcS2(+)–myc-DAPLE. Similarly, the plasmid encoding for myc-GBAi used in frog experiments was generated by amplification of myc-tagged GBAi from pLIC-myc-GBAi and inserted into the XhoI/XbaI sites of pcS2(+) to create pcS2(+)–myc-GBAi. All point mutations were generated using specific primers following the manufacturer’s instructions (QuikChange II; Agilent), and all constructs were checked by Sanger DNA sequencing.

Protein Expression and Purification. His-Gai3 (wt or S47R mutant), His-GBAi wt, His-GBAi W211A, GST-Gai3, GST-GBAi, GST-GIV, GST-DAPLE, GST-Calnuc, GST-NECU2, GST–KB–1753, and GST-GAIP were expressed in BL21(DE3) E. coli was transformed with the corresponding plasmids by overnight induction at 23 °C with 1 mM isopropyl-β-D-thiogalactopyranoside. GST–Ric-8A induction was carried out exactly as described by Thomas et al. (44). Protein purification was carried out following previously described protocols (7, 10). Briefly, bacteria pelleted from 1 L of culture were resuspended in 25 mL of buffer [50 mM NaH2PO4 (pH 7.4), 300 mM NaCl, 10 mM imidazole, and 1% (vol/vol) Triton X-100 supplemented with protease inhibitor mixture (1 mM leupeptin, 2.5 μM pepstatin, 0.2 μM aprotinin, 1 mM PMSF)]. For Gai3, GBAi wt, and GBAi W211A, this buffer was supplemented with 25 μM GDP and 5 mM MgCl2. After sonication (four cycles, with pulses lasting 20 s per cycle, and
with a 1-min interval between cycles to prevent heating), lysates were centrifuged at 12,000 × g for 20 min at 4 °C. The soluble fraction (supernatant) of the lysate was used for affinity purification on HisPur Cobalt or Glutathione Agarose resin (Pierce) and eluted with lysis buffer supplemented with 250 mM imidazole or with 50 mM Tris-HCl (pH 8), 100 mM NaCl, and 30 mM reduced glutathione, respectively. GST-GIV, GST-DAPLE, GST-Calnuc, GST-NUCB2, and GST-Ric-8A proteins were dialyzed overnight at 4 °C against PBS. GST-Ric-8A was supplemented with 10% (vol/vol) glycerol before freezing. For His-Ga3, His-GBAi, GST-Ga3, and GST-GBa1, the buffer was exchanged for 20 mM Tris-HCl (pH 7.4), 20 mM NaCl, 1 mM MgCl2, 1 mM DTT, 10 μM GDP, and 5% (vol/vol) glycerol using a HiTrap Desalting column (GE Healthcare). All protein samples were aliquoted and stored at -80 °C.

**Differential Scanning Fluorimetry Thermal Shift Assays.** This assay was adapted from the procedures described by Sun et al. (51). Thermal shift assays were carried out in 96-well PCR plates (T-3108-I; GeneMate) using His-Ga3, His-GBAi, and His-Ga3 S47R. GDP (J61646MC; Alfa Aesar), GTPγS (10220647001; Sigma), or water control was added to recombinant proteins in buffer containing 20 mM Tris-HCl (pH 7.4), 20 mM NaCl, 11 mM MgCl2, 10 μM GDP, and 5% (vol/vol) glycerol. Following a 10-min incubation on ice, SYPRO Orange protein stain (S6650, 5,000x stock; Life Technologies) diluted in the assay buffer to a 150x concentration was added to the G protein-nucleotide mix in a final assay volume of 50 μL at room temperature. The final assay conditions contained 5 μM recombinant protein, 10 μM GDP (for the baseline conditions) or 10 μM GDP plus 250 μM added guanine nucleotide (GDP or GTPγS as indicated), and 20× SYPRO Orange. Fluorescence data for SYPRO Orange signal were collected with a ViIA 7 Real-Time PCR System (Applied Biosystems) and QuantStudio Real-Time PCR Software v1.2 using 470 nm excitation and 586 nm emission filters. The plates were held at 25 °C for 2 min to stabilize the sample temperature, after which initial fluorescence was measured. Subsequent reads were taken at each temperature interval (0.5 °C steps) after 20 s of temperature stabilization for each step. Melting curves are generated by reads spanning 25–99 °C and are normalized to the fluorescence intensity of the initial 25 °C read. The thermal denaturation temperature (Tm) was determined by plotting normalized intensity as a function of temperature and fitting the transition region to a Boltzman sigmoid. The Tm is the midpoint between the non-denatured baseline intensity (Ib) and the peak intensity plateau value (Ip, maximum denaturation): f(x) = Ip + (Ib - Ip) * e(-x/Tm)

Changes in denaturation temperature (∆Tm) were determined by subtracting the nucleotide-shifted (i.e., stabilized) Tm from the baseline Tm (no additional nucleotide added). Analysis and significance testing with the Holm–Sidak multiple comparisons t test were performed with Prism (GraphPad).

**Peptide Synthesis.** Peptides corresponding to the GBA motif of human GIV (residues 1,671–1,701, KGSPGSPVTLQFLEE-SNKLTSVQIKSS), human DAPLE (residues 1,662–1,695, SASPSVMTVEELEENRSHUSDPTSCPDDL), human Calnuc (residues 303–333, NVDTNQDLVTLVEELASTQRK-EEGDTGEGW), human NUCB2 (residues 304–334, EVDTN-KDRLVTLVEEFLKATKEKFEELPSWE), the GoLoco motif of human RGS12 (residues 1,185–1,221, DEAAEFELISKAQSN-RADDQRQGLRRKEDLVLPEFLR), or the KB-1753 sequence (SSRGYYHGIWVGEEGRLSR) were synthesized using the in situ neutralization protocol for Boc-solid phase peptide synthesis on a p-methylbenzhydrylamine resin (0.67 mmol/g, 100–200 mesh; Novabiochem). Following chain elongation, 6-carboxyfluorescein was activated with 1-[bis(dimethylamino)methylene]-1-H-I,1,2,3,4-tetrazol-5-yl)triazolo[1,5-a]pyridinium 3-oxid hexafluorophosphate (HATU) and N,N-diisopropylethylamine (DIEA) (4, 4, and 8 eq with regard to the amount of peptide-resin, respectively) and coupled to the resin-bound peptides at 65 °C for 1 h to yield the fluorescein-labeled peptides. Peptides were cleaved from the resin using a solution of hydrofluoric acid (HF) containing 5% anisole for 1 h at 0 °C. Next, the HF solution containing the peptides was removed under vacuum, and the resulting residues were crushed out with Et2O and filtered. The collected solids were redissolved in a 50% CH3CN/H2O solution containing 0.1% trifluoroacetic acid (TFA), frozen down, and lyophilized. Crude peptides were purified by reverse phase (RP)-HPLC using an XBridge BEH C18 OBD prep column (130 Å, 5 μm, 19 mm × 150 mm) at a flow rate of 20 mL/min using H2O (0.1% TFA) and CH3CN (0.1% TFA) as eluents. The identity and final purity (>97%) of the peptide were determined by analytical RP-HPLC and mass spectrometry (electrospray ionization-TOF).

**FP-Based Peptide Binding Assays.** FP measurements were carried out in 384-well plates (Black OptiPlate-384F; PerkinElmer). His-Ga3 or His-GBAi (wt or W211A) protein (0–16 μM) and fluorescein-labeled peptide (0.025 μM) were mixed at room temperature for 10 min in a final volume of 20 μL of binding buffer [50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.4% (vol/vol) Nonidet P-40, 10 mM MgCl2, 5 mM EDTA, 2 mM DTT] supplemented with 30 μM GDP or with 30 μM GDP and 30 μM AlCl3 plus 10 mM NaF (GDP-AlF3−). For some experiments, His-Ga3 or His-GBAi was preincubated with 120 μM GDP or 120 μM GTPγS for 210 min at 30 °C before mixing with an equal volume of fluorescently labeled peptide (0.05 μM) in buffer (not supplemented with nucleotides). FP (excitation of 485 ± 10 nm, emission of 528 ± 10 nm) was measured every 2 min for 30 min at room temperature in a Biotech H1 synergy plate reader to ensure that the signals were stable in time. FP was averaged at different times, normalized to maximal binding, and fitted to a one-site binding model to determine the KD using Prism (GraphPad).

**In Vitro Protein Binding Assays with GST-Fused Proteins.** GST pull-down assays were carried out as described previously (9, 70) with minor modifications. The indicated amounts of the following GST proteins were immobilized on glutathione agarose beads for 90 min at room temperature in PBS: GST (25 μg), GST-GIV (15 μg), GST-DAPLE (15 μg), GST-Calnuc (15 μg), GST-NUCB2 (15 μg), GST–KB–1753 (30 μg), GST-GAI4 (5 μg), and GST–Ric–8A (20 μg). Beads were washed twice with PBS, resuspended in 300 μL of binding buffer [50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.4% (vol/vol) Nonidet P-40, 10 mM MgCl2, 5 mM EDTA, 2 mM DTT] supplemented with 30 μM GDP or with 30 μM GDP and 30 μM AlCl3 plus 10 mM NaF (GDP-AlF3−), and incubated for 4 h at 4 °C with constant rotation in the presence of 1 μg of His-Ga3 or His-GBAi purified proteins. For some experiments, His-Ga3 or His-GBAi was preincubated with 120 μM GDP or 120 μM GTPγS for 210 min at 30 °C before addition to tubes with the immobilized GST-fused proteins in a buffer supplemented with 30 μM corresponding nucleotide. Beads were washed four times with 1 mL of wash buffer [4.3 mM NaH2PO4, 1.4 mM KH2PO4 (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 0.1% (vol/vol) Tween-20, 10 mM MgCl2, 5 mM EDTA, 1 mM DTT] supplemented with 30 μM GDP, 30 μM GDP and 30 μM AlCl3 plus 10 mM NaF (GDP-AlF3−), or 30 μM GTPγS. For experiments comparing the binding of full-length GIV, DAPLE, Calnuc, or NUCB2 to GST-Ga3 or GST-GBAi, GST-fused proteins were preincubated with 40 μM GDP, 40 μM GDP plus 40 μM AlCl3 and 12 mM NaF, or 40 μM GTPγS for 180 min at 30 °C in binding buffer [50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM DTT] supplemented with nucleotides. FP (excitation of 485 ± 10 nm, emission of 528 ± 10 nm) was measured every 2 min for 30 min at room temperature in a Biotech H1 synergy plate reader to ensure that the signals were stable in time. FP was averaged at different times, normalized to maximal binding, and fitted to a one-site binding model to determine the KD using Prism (GraphPad).

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NaCl, 0.4% (vol/vol) Nonidet P-40, 10 mM MgCl₂, 5 mM EDTA, 2 mM DTT] before immobilization in glutathione-agarose beads. Beads were washed twice and resuspended in 250 μL of the same binding buffer supplemented with GDP, GDP + AlF₄⁻, or GTPγS as described above. Tubes were transferred to ice, and 50 μL of HEK293T cell lysates (made from a 10 cm dish transfected with 10 μg of DNA of the corresponding plasmid in a final volume of 500 μL) was added before incubation for 4 h at 4 °C with constant rotation. The remaining steps were as described above. GST-Goα3 and GST-GBAi amounts were 7.5 μg per condition for GIV and DAPL and 15 μg per condition for Calnuc and NUCB2. Resin-bound proteins were eluted with Laemmli sample buffer by incubation at 37 °C for 10 min, except for the experiments with GST-Goα3/GST-GBAi, in which the temperature was 65 °C. Proteins were separated by SDS/PAGE, transferred to PVDF membranes, stained with Ponceau S, and immunoblotted with the corresponding antibody. For experiments with Calnuc and NUCB2, the Laemmli sample buffer was supplemented with 5 M urea and samples were run by SDS/PAGE in 4 M urea gels.

Cell Culture. HEK293T cells (CRL-3216; American Type Culture Collection) were grown at 37 °C with 5% CO₂ in DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1% t-glutamine. The generation of MCF-7 cell lines stably expressing full-length, myc-tagged GIV or bearing an empty plasmid (control) has been previously described (18). Briefly, MCF-7 stable cell lines were generated by transduction with lentivirus packaged from pLVX-GIV-2

For Gβγ communoprecipitation experiments, 1 million MCF-7 cells were seeded on 60-mm dishes and transfected the day after using Lipofectamine LTX with PLUS Reagent (Life Technologies) according to the manufacturer’s instructions with plasmids encoding for the following constructs (DNA amounts in parentheses): Goα3-FLAG (1.25 μg), Myc-GBAi (wt or W211A (5 μg), VC-Gβγ (0.5 μg), and VN-Gγ2 (0.5 μg). Cell media were changed 5 h after transfection. Twenty-four hours after transfection, cells were lysed on ice with lysis buffer [20 mM Hepes (pH 7.2), 5 mM Mg(CH3COO)2, 125 mM K(CH3COO), 0.4% (vol/vol) Triton X-100, 1 mM DTT, 10 mM β-glycerophosphate, and 0.5 mM Na3VO4 supplemented with a protease inhibitor mixture (SigmaFAST)] and cleared (14,000 × g, 10 min). Cleared lysates were used for communoprecipitation with antibodies immobilized on Protein G agarose beads (ThermoScientific). Thirty-five microliters of Protein G agarose beads preblocked with 5% BSA (2 h at room temperature) was incubated with 2 μg of either FLAG (F1804) or Myc (clone 9E10) antibody for 90 min at 4 °C with rotation and washed. Cell lysates were added to the beads with bead-coupled antibodies and incubated for 4 h at 4 °C with constant tumbling. Beads were washed three times in wash buffer [4.3 mM Na2HPO4, 1.4 mM KH2PO4 (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 0.1% (vol/vol) Tween-20, 10 mM MgCl2, 5 mM EDTA, 1 mM DTT], and proteins were eluted by adding Laemmli sample buffer and boiling for 5 min. Proteins were separated by SDS/PAGE and immunoblotted with the corresponding antibodies.

For the remaining communoprecipitation experiments, approximately 2 million HEK293T cells were seeded in 10-cm dishes and transfected the day after using calcium phosphate with plasmids encoding for the following constructs (DNA amounts in parentheses): FLAG-Goα3 (1.5 μg), FLAG-GBAi (4 μg), GAIP (1.5 μg), RGS12ΔN (2 μg), and Ric-8A (2 μg). Cell media were changed 5 h after transfection. Cleared lysates were incubated with 2 μg of FLAG antibody for 4 h at 4 °C and then supplemented with 35 μL of Protein G agarose beads preblocked with 5% BSA for an additional 90 min at 4 °C. The remaining steps were as described above. All buffers used in the communoprecipitation experiments with GAIP were supplemented with 30 μg/MGDP and 30 μg/MGDP AlCl3 plus 10 mM NaF (GDP-AlF₄⁻), and with 30 μg GDP for the experiments with RGS12ΔN.

MCF-7 Stimulation with Collagen I. This assay was performed exactly as previously described (18) except that cells were transiently transfected with plasmids encoding for Myc-GBAi (wt or W211A) the day before the assay. Briefly, 8 × 10⁶ MCF-7 cells stably expressing a control vector (control) or myc-tagged GIV were seeded on 60-mm dishes and transfected with 5 μg of plasmids encoding for Myc-GBAi (wt or W211A) using Lipofectamine LTX with PLUS Reagent as described above. The remaining steps were performed exactly as described previously (18). Briefly, cells were detached by incubation with PBS supplemented with 20 mM EDTA (pH 7.4), washed three times in serum-free media, and left in suspension for 1 h at room temperature in serum-free media. Approximately 600,000 cells were seeded on collagen I-coated 60-mm dishes (1.6 μg/cm² of collagen I) at 37 °C in the absence of serum. Stimulation was stopped at 30 and 60 min by placing the plates on ice, washing with cold PBS, and addition of lysis buffer [20 mM Hepes (pH 7.2), 5 mM Mg(CH3COO)2, 125 mM K(CH3COO), 0.4% (vol/vol) Triton X-100, 1 mM DTT, 10 mM β-glycerophosphate, and 0.5 mM Na3VO4 supplemented with a protease inhibitor mixture (SigmaFAST)]. Time 0 corresponds to lysisates of cells in suspension (i.e., nonstimulated by collagen I) harvested at the time of seeding. Lysates were cleared by
PVDF membranes were blocked with 5% milk test. MMR and trans-

SEM or as one at 4 °C, supernatants were collected and supplemented

with a protease inhibitor mixture (SigmaFAST, catalog no. S8830; Sigma) and ethanol. Purified mRNAs were quantified spectroscopically, followed by sequential precipitations in isopropanol and ethanol. Purified mRNAs were diluted to the desired final concentrations and served as a template for each mRNA in vitro transcription reaction with the SP6 mMessage mMachine Kit (catalog no. AM1340; Ambion). In vitro mRNA transcription reactions were treated with DNase I (catalog no. M0303; New England Biolabs) to eliminate the template, and mRNA was purified by alkaline phenol/chloroform extraction, followed by sequential precipitations in isopropanol and ethanol. Purified mRNAs were quantified spectroscopically, and their quality was checked in a 1% agarose/ formaldehyde gel.

For embryo morphology experiments, mRNAs were injected equatorially into both dorsal blastomeres at the two- to four-cell stage (stages 2–3). Embryos were subsequently incubated at 16 °C and phenotypes were assessed at stage 30. Embryos were scored as normal or dorsally bent (mild or severe) as previously described (76). Egg laying was induced by dorsal lymph injection of human chorionic gonadotrophin (500 units, catalog no. 057176; Merck). In vitro fertilization and embryo culture were carried out in 0.1× Marc’s modified Ringer’s medium (MMR) as described. Staging was according to Nieuwkoop and Faber (78).

For embryo morphology experiments, mRNAs were injected equatorially into both dorsal blastomeres at the two- to four-cell stage (stages 2–3). Embryos were subsequently incubated at 16 °C, and phenotypes were assessed at stage 30. Embryos were scored as normal or dorsally bent (mild or severe) as previously described (76) using a Leica MZ6 dissection microscope. The χ2 test was used in Prism (GraphPad) to assess statistically significant differences (P < 0.05) in the distribution of phenotypes among the different experimental groups (27–30 embryo caps per group from three independent experiments). Induction of mesodermal genes by RT-PCR was performed at stage 10.5, with animal caps prepared and stimulated with activin as described above. Total mRNA from 10 caps was extracted with TRIzol (catalog no. 15596026; Invitrogen) and subsequently precipitated with isopropanol. Nucleic acids were treated with DNase I, and 1 μg of RNA was used as a template for RT using a SuperScript first-strand kit with random hexamers (Superscript First-Strand, catalog no. 11904-018; Life Technologies). PCR assays for Xbra, Chordin, and EF1α were performed as previously described (79). At least three independent frog fertilizations were used in all of the experiments described above.

Immunoblotting. PVDF membranes were blocked with 5% milk (or 5% BSA for phosphoantibodies) for 30 min and incubated overnight at 4 °C under constant agitation with the corresponding primary antibodies at the following dilutions: pan-GO, 1:250; pan-GI, 1:250; tubulin, 1:2,500; His, 1:2,500; pERK1/2 (T202/Y204), 1:1,000; total ERK, 1:100; pAkt (S473), 1:1,000; total Akt, 1:250; GIVcc, 1:1,000; Myc, 1:1,000; DAPLE, 1:1,000; and FLAG, 1:1,000. Following the incubation in the primary antibody, membranes were washed and incubated in secondary antibodies (goat anti-rabbit Alexa Fluor 680 and goat anti-mouse or IRDye 800) at 1:10,000 dilution. Infrared imaging and quantification of Western blots were performed according to the manufacturer’s protocols using an Odyssey Infrared Imaging System (Li-Cor Biosciences). Akt activation was determined by calculating the pAkt/total Akt ratio and normalizing it to the maximum activation in each experiment (% of maximum). Images were processed using ImageJ software (NIH) and assembled for presentation using Photoshop and Illustrator software (Adobe).

Statistical Analysis. Each experiment was performed at least three times. Data shown are expressed as mean ± SEM or as one representative experiment. Statistical significance between various conditions was assessed by determining P values using the Student’s t test or χ2 test.
GBAi is stabilized by the binding of GDP or GTPγS nucleotides in thermal shift assays. (A–C) Thermal denaturation curves of Gai3, GBAi, and Gai3 S47R proteins alone (gray lines, in buffer containing 10 μM GDP) or in the presence of added GDP (250 μM) or GTPγS (250 μM). SYPRO Orange fluorescence (Fluor.) was used to track the progression of denaturation upon heating of recombinant proteins, and the signals were normalized to maximal intensity. One representative melting curve from three independent experiments is shown for each protein. The GBAi + GTPγS condition displays a lower Tm peak that corresponds to a GDP-bound population. The larger intensity of this “GDP peak” compared with that in Gai3 under the same conditions is likely due to the previously reported (35) decrease in GTPγS affinity caused by the G203A mutation present in GBAi. (D) Changes in melting temperature (ΔTm) for the GDP- or GTPγS-stabilized Gai3 and GBAi proteins and for the Gai3 S47R-negative control. TmS were first determined from the midpoint of the sigmoidal transition region, and ΔTms were calculated by subtracting baseline (no additional nucleotide) Tm values. The Tm of both Gai3 and GBAi is increased moderately upon addition of GDP and more markedly upon addition of GTPγS, suggesting GBAi can bind guanine nucleotides normally. The Gai3 S47R mutant protein, previously reported as GTP binding-deficient, is not stabilized well by addition of GTPγS. Results from three independent experiments are shown as mean ± SD.
Fig. S2. GBAi binds to the GBA motif of GIV with the same affinity as Gai3, and GBAi-GIV binding is not affected by GTPγS. Binding of a fluorescently labeled GIV peptide corresponding to its GBA motif (residues 1,671–1,701) to the indicated concentrations of purified His-tagged Gai3 (black), GBAi wt (blue), or GBAi W211A (red) was determined by FP after preincubation (120 μM nucleotide, 210 min at 30 °C) with GDP (Top) or GTPγS (Bottom). Data were normalized to maximal binding and fitted to a one-site binding model (solid lines). Results from three independent experiments are expressed as mean ± SEM.
GBAI binds to the GBA motifs of DAPLE, Calnuc, and NUCB2 with the same affinity as Gαi3, and the GBAi-GBA binding is not affected by the GTP mimetics GTPγS or GDP-AlF4⁻.

(A) Sequence alignment of the regions of human GIV, DAPLE, Calnuc, and NUCB2 containing the GBA motif. A consensus sequence of the highly conserved core of eight residues is indicated underneath the alignment (ψ = hydrophobic). Mutation of the highly conserved Phe residue (“F” in red) to Ala has been previously shown to abolish binding of each one of the GBA proteins to Gαi3. The region labeled as “GBA motif” corresponds to the sequences used in the protein–protein binding experiments presented in this study. Binding of fluorescently labeled peptides corresponding to the GBA motif of DAPLE (residues 1,662–1,695), Calnuc (residues 303–333), and NUCB2 (residues 304–334) to the indicated concentrations of purified His-tagged Gαi3.

Legend continued on following page.
(black), GBAi wt (blue), or GBAi W211A (red) was determined by FP in the presence of GDP (B, Top) or GDP-AlF₄⁻ (B, Bottom), or after preincubation (120 μM nucleotide, 210 min at 30 °C) with GDP (C, Top) or GTPγS (C, Bottom). Data were normalized to maximal binding and fitted to a one-site binding model (solid lines). Results from three independent experiments are expressed as mean ± SEM. (D) GBAi binds the same to full-length DAPLE, full-length Calnuc, or full-length NUCB2 in the presence of GDP, GDP-AlF₄⁻, or GTPγS, whereas Gαi3 binds to all of them only in the presence of GDP. Lysates of HEK293T cells expressing full-length, myc-tagged DAPLE, full-length, GFP-tagged Calnuc, or full-length, myc-tagged NUCB2 were incubated with GST, GST-Gu3, or GST-GBAi immobilized on glutathione-agarose beads in the presence of GDP, GDP-AlF₄⁻, or GTPγS (as indicated). Resin-bound proteins were eluted, separated by SDS/PAGE, and analyzed by Ponceau S-staining and immunoblotting (IB) as indicated. Input = 10% of the amount of lysate used in each pulldown. One experiment of three is shown.

**Fig. S4.** Mutation of the conserved phenylalanine in the GBA motif of GIV, DAPLE, Calnuc, and NUCB2 to alanine abolishes binding to GBAi. GST-fused GBA motifs of GIV (residues 1,671–1,701), DAPLE (residues 1,661–1,691), Calnuc (residues 303–333), and NUCB2 (residues 304–334) bearing an Ala mutation (or not) in the position of the conserved Phe were immobilized on glutathione-agarose beads and mixed with His-GBAi in the presence of GDP. Resin-bound proteins were eluted, separated by SDS/PAGE, and analyzed by immunoblotting (IB) or Ponceau S staining as indicated. One experiment representative of at least three is shown.

**Fig. S5.** GBAi does not bind to Gβγ in MCF-7 cells. MCF-7 cells were transfected with plasmids encoding for Venus(155–239)-Gβ₁ (VC-Gβ₁), Venus(1–155)-Gγ₂ (VN-Gγ₂), Gαi3-FLAG, and Myc-GBAi (wt or W211A) as indicated. One day after transfection, cell lysates were subjected to immunoprecipitation (IP) with anti-FLAG or anti-Myc antibody as indicated. Immunoprecipitates (IPs) (Top) and aliquots from the lysates (Bottom, "5% input") were immunoblotted (IB) with the indicated antibodies.
Fig. S6. GBAi does not interfere with Gαi-mediated regulation of cAMP levels in response to activation of different GPCRs. (A) HEK293T cells were transfected with plasmids encoding for Nluc-EPAC-VV (0.05 μg), GABA_BRs (0.2 μg), and Gαi3 (0.5 μg) in the presence (+GBAi) or absence (control) of GBAi wt (2 μg). BRET was measured every 4 s. Forskolin (Fsk, 1 μM) and brimonidine (Brimo., 5 μM) were added (sequentially) at the indicated times. The blue trace corresponds to unstimulated control cells and is duplicated in both panels as a visual reference of the baseline. Results from three independent experiments are expressed as mean ± SEM. (B) Experiments were carried out exactly as in Fig. 2D except that the order of addition of Fsk and GABA was inverted. The blue trace corresponds to unstimulated control cells and is duplicated in both panels as a visual reference of the baseline. Results from three independent experiments are expressed as mean ± SEM.
**Fig. S7.** GBAi does not bind to the KB-1753 peptide or GAIP. (A) Purified GST or GST–KB-1753 was immobilized on glutathione-agarose beads and incubated with purified His-β3 or His-GBAi in the presence of GDP-AlF₄⁻ or GTPγS. Resin-bound proteins were eluted, separated by SDS/PAGE, and analyzed by Ponceau S-staining and immunoblotting (IB) as indicated. GST–KB-1753 binds His-β3, whereas it does not bind to GBAi. One representative experiment of three is shown. (B) GBAi does not bind to the RGS GAP protein GAIP. Experiments were carried out exactly as for the GTPγS conditions in A except that GST-GAIP was used instead of GST–KB-1753. GST-GAIP binds His-β3, whereas it does not bind to GBAi. One representative experiment of three is shown.

**Fig. S8.** GBAi does not bind to GAIP, RGS12ΔN, or Ric-8A in HEK293T cells. HEK293T cells were transfected with plasmids encoding for GAIP (A), RFP-RGS12ΔN (residues 828–1,381, which contain its GoLoco motif but lack the RGS domain) (B), or RFP-Ric-8A (C), along with FLAG-β3i, FLAG-GBAi, or an empty plasmid (−) as indicated. One day after transfection, cell lysates were subjected to immunoprecipitation (IP) with anti-FLAG antibodies. For the experiments shown in A, buffers were supplemented with GDP + AlF₄⁻, and for the experiments shown in B, buffers were supplemented with GDP, as indicated in Materials and Methods. Immunoprecipitates (IPs) (Top) and aliquots from the lysates (Bottom, “2.5% input”) were immunoblotted (IB) with the indicated antibodies.
**Fig. S9.** Phospho-Akt levels in unstimulated MCF-7 cells are not affected by expression of GIV and/or GBAi. MCF-7 cells stably expressing a vector control (Ctrl) or full-length GIV (GIV) were transfected to transiently express GBAi wt or GBAi W211A as indicated. Cells were lifted and kept in suspension in serum-free media for 1 h before seeding them for the experiments shown in Fig. 4. The immunoblots shown here correspond to lysates prepared from the cells in suspension right at the time of seeding (time 0). No Akt activation (as measured by levels of pAkt) is detected under these conditions for any of the experimental groups. tAkt, total Akt.

**Table S1.** Binding affinity ($K_d$ in micromolar) of Gαi3 and GBAi for the GBA motif of GIV, DAPLE, Calnuc, or NUCB2 in the presence of GDP

<table>
<thead>
<tr>
<th>GBA motifs, aa</th>
<th>Gαi3 wt</th>
<th>GBAi wt</th>
<th>GBAi W211A</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIV (1,671–1,701)</td>
<td>0.69 ± 0.06</td>
<td>0.24 ± 0.03</td>
<td>ND*</td>
</tr>
<tr>
<td>DAPLE (1,662–1,695)</td>
<td>0.55 ± 0.05</td>
<td>0.27 ± 0.02</td>
<td>ND*</td>
</tr>
<tr>
<td>Calnuc (303–333)</td>
<td>1.54 ± 0.19</td>
<td>1.15 ± 0.14</td>
<td>ND*</td>
</tr>
<tr>
<td>NUCB2 (304–334)</td>
<td>2.20 ± 0.34</td>
<td>2.38 ± 0.19</td>
<td>ND*</td>
</tr>
</tbody>
</table>

Each $K_d$ value was determined from the FP experiments shown in Fig. 1D or Fig. S3, and results are expressed as mean ± SEM of $n = 3$ independent experiments.

*Not determined due to low binding. $K_d$s for these conditions are assumed to be at least two to three orders of magnitude larger than for Gαi3 or GBAi wt.