Molecular mechanism of Gαi activation by non-GPCR proteins with a Gα-Binding and Activating motif

Alain Ibáñez de Opakua1,*, Kshitij Parag-Sharma2,*, Vincent DiGiacomo2,*, Nekane Merino1, Anthony Leyme2, Arthur Marivin2, Maider Villate1, Lien T. Nguyen2, Miguel Angel de la Cruz-Morcillo2, Juan B. Blanco-Canosa3, Sekar Ramachandran4, George S. Baillie5, Richard A. Cerione4,6, Francisco J. Blanco1,7 & Mikel Garcia-Marcos2

Heterotrimeric G proteins are quintessential signalling switches activated by nucleotide exchange on Gα. Although activation is predominantly carried out by G-protein-coupled receptors (GPCRs), non-receptor guanine-nucleotide exchange factors (GEFs) have emerged as critical signalling molecules and therapeutic targets. Here we characterize the molecular mechanism of G-protein activation by a family of non-receptor GEFs containing a Gα-binding and -activating (GBA) motif. We combine NMR spectroscopy, computational modelling and biochemistry to map changes in Gα caused by binding of GBA proteins with residue-level resolution. We find that the GBA motif binds to the SwitchII/α3 cleft of Gα and induces changes in the G-1/P-loop and G-2 boxes (involved in phosphate binding), but not in the G-4/G-5 boxes (guanine binding). Our findings reveal that G-protein-binding and activation mechanisms are fundamentally different between GBA proteins and GPCRs, and that GEF-mediated perturbation of nucleotide phosphate binding is sufficient for Gα activation.
**Results**

**GIV binds similarly to GDP-bound and nucleotide-free Gz13.** We focused our initial efforts on GIV, the first-identified and best-characterized member of the GBA motif-containing family of non-receptor GEFs. GPCRs bind preferentially to nucleotide-free G proteins (Gz-[-]) over G-GDP and dissociate from G-GTP. We analysed the ability of GIV to bind different states of Gz13 along the activation pathway, that is, Gz13-[GDP] → Gz13-[GTP], by depleting purified Gz13 of nucleotides and reloading it (or not) with GDP or GDP-AlF 

We found that GIV binds Gz13-[GDP] but not to Gz13-[GTP] (Fig. 1b). Moreover, GIV binds equally to Gz13-[GDP] and Gz13-[GTP] (Fig. 1b). To rule out that this observation was due to inefficient GDP depletion, we used Ric-8A as a control. The binding preference of Ric-8A for different states of Gz13 mimics that of GPCRs22, that is, Gz13-[ ] > > Gz13-[GDP] > Gz13-[GTP]. Ric-8A bound much more to Gz13-[ ] than to Gz13-[GDP] (Fig. 1b), indicating that nucleotide depletion occurred efficiently. GAIP (also known as RGS19) was used as a control to rule out that the nucleotide depletion procedure compromised Gz13 integrity. GAIP is a GAP and, as expected36, it bound to Gz13-[GTP] but not to Gz13-[GDP] (Fig. 1b), demonstrating that nucleotide-depleted Gz13 can bind nucleotides and adopt an active conformation. These results indicate that GIV, contrary to GPCRs, binds to Gz13-[GDP] and Gz13-[ ] to a similar extent. An independent demonstration that GIV binds similarly to Gz13-[GDP] and Gz13-[ ] was obtained by co-immunoprecipitation experiments in mammalian cells. Full-length GIV co-immunoprecipitated with a G-protein mutant unable to bind nucleotides, thus mimicking Gz13-[ ]37, as efficiently as with the wild-type (WT) protein (Supplementary Fig. 1). These results indicate that the association of GIV with G proteins along the activation pathway differs from that of GPCRs (Fig. 1c): GIV binds with high affinity to monomeric Gz13-[GDP], remains bound to Gz13 with similar affinity on nucleotide release and eventually dissociates on GTP binding to release the active G protein.

**NMR reveals that GIV perturbs discrete regions of Gz13.** A synthetic 16-mer GEF peptide (named KB-752) has been previously crystallized in complex with Gz11-GDP38. The sequence of this peptide is not present in any human protein but has similarity to the GBA motif (Supplementary Fig. 2). However, comparison of Gz11-GDP free or bound to this peptide shows that the nucleotide-binding pocket remains essentially unchanged (Supplementary Fig. 2), providing limited insight into the mechanism by which GBA proteins activate Gz1. This might be due to loss of structural rearrangements in Gz1 under crystallization conditions. To gain further insights into how a naturally occurring GBA sequence binds to Gz1 and structural rearrangements associated with it, we investigated the molecular mechanism of Gz1 activation by GIV using solution NMR spectroscopy. Perturbation in the NMR signals of the backbone amide groups of Gz13 protein on ligand binding should inform about direct intermolecular contacts as well as indirect structural rearrangements, providing information at the residue level.

Full-length GIV is 1,870-amino acid long and is composed of multiple domains (Supplementary Fig. 2). Attempts to purify the >220 kDa full-length protein from bacteria gave extremely poor yields of low-quality protein, making its use impossible for NMR experiments. As an experimentally tractable alternative, we used a C-terminal fragment of GIV corresponding
to the last 210 amino acids (that is, GIV-CT, residues 1,660–1,870). This fragment was chosen for several reasons: (i) a thorough characterization of GIV-CT has revealed that it recapitulates the biological properties of the full-length protein in mammalian cells, including G-protein-dependent signalling27,29; (ii) it contains the GBA motif and mutation of this motif in the context of either full-length GIV or GIV-CT leads to loss of G-protein binding and G-protein-dependent signalling18,27,29,30; (iii) GIV-CT is sufficient to bind and activate G proteins in vitro35, and G-protein binding and/or activation is comparable to that obtained with larger fragments (for example, residues 1,425–1,870 translated in vitro18 or 1,623–1,870 purified from bacteria25). Thus, GIV-CT is a reliable approximation to the G-protein-binding properties of the native protein and its signalling functions in cells.

NMR spectra of Gz3-GDP were recorded in the absence or presence of GIV-CT. Backbone amide signals of Gz3-GDP, as well as the side chain indol signals of its three tryptophans (W131, W211 and W258), were assigned in the two-dimensional TROSY and three-dimensional (3D) HNCO spectra by comparison with the published assignments under the same experimental conditions39. As expected from the increase in molecular weight and the binding of a non-deuterated ligand, there was an overall decrease in signal intensity (median ~ 3-fold) when NMR measurements were carried out in the presence of GIV-CT (residues 1,660–1,870, containing the GBA motif). However, the effect was more pronounced for certain signals, with some even disappearing (for example, W211 and W258; Fig. 2a,b). This may be due to appearance of the signal of the bound form at different frequencies that we were not able to identify, or to a change in the local dynamics that broadened the signal beyond detection. Regardless of the cause, a large intensity loss reflects perturbations specifically caused by GIV binding and it was quantified for each signal by the calculation of the intensity ratio between the free and bound forms (I_free/I_bound). In addition, we measured significant chemical shift perturbations on GIV-CT binding for a fraction (~20%) of the assigned peaks (Fig. 2a,b). Assignment of the signals in the bound form was made on a nearest-neighbour basis in both the two-dimensional and 3D spectra, and was confirmed by the similar pattern of signals observed in the presence of the shorter GIV fragment 1,671–1,696 (GIVpept; Supplementary Fig. 3). Assignments of Gz3-GDP bound to GIVpept were confirmed by a titration with the peptide. The overall decrease in signal intensities on binding of GIVpept was only modest (~15%), which increases the confidence of the identified perturbations, especially for the I_free/I_bound. The results revealed a high correlation between the perturbations induced by GIV-CT and GIVpept (Supplementary Fig. 3c,d), which indicates that the GBA motif of GIV is causing most of the perturbations observed with GIV-CT. The similar behaviour observed for the short and long fragments of GIV is consistent with the disordered nature of the 210-residue-long C-terminal region of GIV (Supplementary Fig. 4). Circular dichroism shows little, if any, secondary structure, and no cooperative transition is observed in the thermal denaturation, indicating the absence of a tertiary structure. The NMR spectrum confirms that the C-terminal region of GIV is intrinsically disordered as the backbone amide signals show very little dispersion in the proton frequency dimension.
As a first approach to interpret the NMR measurements, which cannot unambiguously differentiate between direct contacts and indirect structural rearrangements, we analysed them in conjunction with molecular modelling. For this, a 3D structural model of the GBA motif of GIV (residues 1,678–1,696) bound to Gαi3 was built based on homology with the KB-752/Gαi1 crystal structure, *ab initio* extension of the GBA sequence and docking (Fig. 3). This model provides independent information because no constrains based on the NMR data were applied to build it. Although this model does not capture the possible conformational changes distant to the peptide-binding site (absent in the model template), it informs of the protein–protein interface and provides a framework to visualize what specific perturbations might be due to direct GBA binding or indirect conformational changes. The NMR perturbations mapped predominantly to the Ras-like domain (Figs 2c and 3, and Supplementary Fig. 3d).
We observed extensive perturbations in the GIV-binding site seen in the computational model, which is formed by the α3 helix, α3/β5 loop and the Switch II (SwII) region (Figs 2c and 3). There are additional perturbations in the nucleotide-binding site, which made no direct contact with GIV in our model. More specifically, all the residues of the P-loop and the Switch I (SwI) region for which we could measure NMR data displayed significant perturbations (Figs 2c and 3). The P-loop and SwI contain the G-1 and G-2 boxes, two of the five conserved nucleotide-binding elements of G proteins. The G-1/P-loop and G-2/SwI are responsible for binding the phosphate moieties of GDP, whereas G-4 and G-5 bind the region of the nucleotide base (G-3 makes direct contact with GTP but not with GDP). Interestingly, G-4 (that is, β5–αG loop) and G-5 (β6–α5 loop) remained unperturbed by GIV binding (Figs 2c and 3). The selective effect of GIV on the phosphate-binding elements indicates a divergence from the mechanism of action of GPCRs, which alter all four G-1, G-2, G-4 and G-5 on activation. We also observed moderate to strong perturbations in the interdomain interface of Gxi3, that is, the αD–αE loop (helical domain) and SwIII (Ras-like domain; Figs 2c and 3). These perturbations are compatible with a local disruption of the interdomain interface, which might be related to the domain separation that accompanies increased nucleotide exchange on the action of both GPCRs and the non-receptor GEF Ric-8A.

These results suggest an allosteric mechanism of action for GIV, whose binding to the α3/SwII cleft induces conformational changes in the phosphate-binding region of the nucleotide-binding pocket that weaken GDP binding. The most direct communication route between the GIV-binding site and the nucleotide-binding pocket is the β1 strand, which extends from the bottom of the α3/SwII cleft, where GIV binds, into the P-loop.

Consistent with this hypothesis, the β1 strand undergoes strong NMR signal perturbations on GIV binding (Figs 2c and 3, and Supplementary Fig. 3d). This strand has also been proposed to serve as an allosteric route for GPCR-mediated activation.

Characterization of Gxi3 binding to GIV by mutagenesis. To further validate the mode of action of GIV on Gxi3, we carried out a biochemical analysis by site-directed mutagenesis. We reasoned that mutation of residues in direct contact with GIV should show a larger impact on binding than mutation of residues involved in indirect structural rearrangements. The design of the mutants was based on the NMR and modelling data, but also on in silico thermodynamics analysis of the Gxi3–GIV model, which predicts the Gxi3 residues that contribute the most to the complex formation (Fig. 4a,b). A detailed rationale of the mutant design is described in Supplementary Note 1. For the characterization of these mutants, we used purified His-tagged Gxi3. The tagged protein binds the GBA motif of GIV with the same affinity as the untagged Gxi3, used in the NMR experiments (Supplementary Fig. 3a). All mutants were purified with similar yields and purity (Supplementary Fig. 5). All mutants were capable of adopting an active conformation on GTPγS binding (Supplementary Fig. 6b), indicating that none of them had major structural defects.

We found reduced GIV binding for mutants of any of the Gxi3 residues in SwII, α3 or the α3/β5 loop that were predicted by the thermodynamics analysis to contribute to GIV binding and also displaying NMR signal perturbations on GIV binding. These results are W211, F215, L249, W258 and F259 (Fig. 4c,d, and Supplementary Figs 6 and 7). On the other hand, mutation of V218 or K257, two residues in the same region but predicted to marginally destabilize GIV binding, had little effect on binding.
activities in the absence of GIV or DAPLE are shown in Supplementary Fig. 7. ND, not determined due to severely compromised basal activity.

Right, correlation plots of Log₂(GIV) interaction but not experimentally tested by mutagenesis are labelled grey. (b) Heat map (green to red scale shown on top) of Log₂(GIV/DAPLE binding) relative to WT. Left, heat map (green to red scale shown on top) of Log₂(RGS12 binding) relative to WT. The GIV sample is highly correlated with DAPLE binding but not with RGS12 binding. Left, heat map (green to red scale shown on top) of Log₂(GIV/DAPLE binding) relative to WT. The GIV sample is highly correlated with DAPLE binding but not with RGS12 binding.

Figure 4 | Characterization of the GIV-binding site on Gαi3. (a) GIV-binding site on Gαi3 and location of residues selected for mutagenesis. Left, representation of the molecular model of Gαi3 bound to GIVpept (green ribbon) coloured as in Fig. 3. Right, ribbon representation of the view of Gαi3 on the left with residues selected for mutagenesis shown in sticks. (b) Calculation of energetic contribution of individual Gαi3 residues to the interaction with GIV. The Gαi3/GIV model was analysed using FoldX. Experimentally mutated residues are indicated with black labels. Residues predicted to stabilize the interaction but not experimentally tested by mutagenesis are labelled grey. (c) GIV and DAPLE binding to Gαi3 mutants in pull-down assays. Binding of His-Gαi3 WT or mutants to immobilized GST-GIV (left) or GST-DAPLE (right) was visualized by Coomassie blue staining. One experiment representative of at least three is shown. GST protein loading and negative controls are shown in Supplementary Fig. 5. (d) Equilibrium dissociation constants (Kd) for the binding of Gαi3 mutants to the GBA motif of GIV and DAPLE or the GoLoco motif of RGS12. ΔΔG values for fluorescein-labelled peptides derived from GIV, DAPLE or RGS12 were calculated from fluorescence polarization measurements (complete binding curve data sets in Supplementary Fig. 6). Mean ± s.e.m., n = 3–7. Fold changes in the Kd larger than 2 or smaller than 0.5 are indicated in red or green, respectively. (e) The effect of Gαi3 mutations on GIV binding is highly correlated with DAPLE binding but not with RGS12 binding. Left, heat map (green to red scale shown on top) of Log₂ΔΔG (KdMUT/KdWT) values for fluorescein-labelled peptides derived from GIV, DAPLE or RGS12. (f) GIV- and DAPLE-mediated activation of Gαi3 mutants. Steady-state GTPase activity of His-Gαi3 WT or mutants in the absence (white) or presence (black) of GIV (left) or DAPLE (right). Mean ± s.e.m., n ≥ 3. ***30–50% inhibition, *** >50% inhibition of GIV/DAPLE-mediated activation compared to Gαi3 WT. Raw basal activities in the absence of GIV or DAPLE are shown in Supplementary Fig. 7. ND, not determined due to severely compromised basal activity.
Mutation of S252 and N256, two residues in the $\alpha_3$ helix displaying large NMR perturbations (Fig. 2) but not predicted to stabilize GIV binding, severely impaired GIV binding when mutated to D and E, respectively, but not when mutated to A (Fig. 4c,d, and Supplementary Figs 6 and 7). These two residues are located in the GIV-binding site, and mutation to D or E, respectively, creates a steric/electrostatic hindrance for GIV binding that does not occur when mutated to A. Mutation of I184 in SwI also diminished binding (Fig. 4c,d). This residue is not predicted to contribute directly to GIV binding (Fig. 4a,b) but makes a direct intramolecular contact with SwII based on our homology model (Fig. 4a). On the other hand, I184 undergoes strong NMR signal perturbations in the presence of GIV (Fig. 2 and Supplementary Fig. 5). This is consistent with I184 interacting with SwII on GIV binding and possibly stabilizing a conformation of SwII that favours GIV binding. These mutagenesis results indicate that the groove formed by $\alpha_3$, SwII and $\alpha_3$/SwIII loop elements is the direct binding site for GIV.

To further characterize the Gz3/GIV-binding site, we analysed a battery of GIV mutants for binding to Gz3 in a peptide array format (Supplementary Fig. 8), GBA motif peptides in which nine selected positions were replaced by every other natural amino acid one at a time were probed for Gz3 binding (Supplementary Fig. 8b). Selected mutants were tested for confirmation of binding in solution (Supplementary Fig. 8C). The results revealed that mutation of GIV residues predicted by our model to be located in the vicinity of Gz3 residues important for binding (as determined by mutagenesis) efficiently disrupted the binding. On the other hand, mutation of residues in the same region of GIV but not predicted to be important for Gz3, had marginal or no effect on binding (see Supplementary Note 2 for a full description). These results further validate our model of GIV binding to a pocket on Gz3 formed by the $\alpha_3$, SwII and $\alpha_3$/SwIII loop.

Mutation of Gz3 residues in the $\beta_1$/P-loop axis predicted by the thermodynamics analysis in Fig. 4b to contribute to GIV binding (K35 and L39) also impaired GIV binding in pull-down or fluorescence polarization assays (Fig. 4c,d, and Supplementary Figs 6 and 7). On the other hand, mutation of residues in the same region and displaying NMR perturbations, but not making direct contact with GIV (L36, L37 and G42) did not significantly affect binding (Fig. 4c,d). Because G42 is located in the middle of the P-loop, this suggests that GIV induces indirect structural rearrangements in the nucleotide-binding pocket.

We monitored GIV-mediated enhancement of Gz3 steady-state GTPase activity (which reflects its GEF activity) for the mutants described above. Not all mutants could be analysed due to compromised intrinsic (basal) activity (Supplementary Fig. 7b), fluorescence polarization (Fig. 4d,e) and G-protein activity assays (Fig. 4f and Supplementary Fig. 7b,c) were highly correlated with those of GIV. To assess specificity, we tested the same set of mutants for binding to a peptide derived from RGS12’s GoLoco motif. We chose RGS12 GoLoco motif as a stringent test for specificity because it also docks onto the $\alpha_3$/SwII cleft of Gz3-GDP33. Consistent with binding to this common site, we found that some of the mutations that impair GIV binding also impair binding of the RGS12 GoLoco motif (Fig. 4d,e). However, the overlap of the sets of Gz mutations that affect binding to GBA or GoLoco motifs is only partial, and many of the mutations that affect binding to both motifs do so to a significantly different extent. The divergence of the effect of same Gz mutants on GoLoco or GBA binding is in agreement with the comparison of the crystal structures of Gz bound to a GoLoco motif or the GBA-like peptide KB-752, which showed that the common binding site formed by the $\alpha_3$ helix and SwII region adopts a different conformation38. The correlation of the effect of the entire set of Gz3 mutants on the dissociation constant of RGS12–Gz3 versus GIV–Gz3 is markedly reduced (Pearson’s $r = 0.58$) compared to the correlation with DAPLE–Gz3 (Pearson’s $r = 0.97$; Fig. 4d,e). A notable example is mutant W211A, which completely abolishes GIV and DAPLE binding but has no effect on RGS12 binding. Thus, the overall effect of the mutants indicates that different GBA proteins share a common and specific site of binding to G proteins, which partially overlaps with the GoLoco-binding site but engages Gz3 in a different way.

**Different GBA proteins bind similarly to Gz3.** DAPLE has been recently shown to bind and activate Gz3 via a GBA motif similar to that in GIV20. We reasoned that if the G-protein binding mode of different GBA motifs is conserved, the effect of specific Gz3 mutations on DAPLE–Gz3 coupling should closely recapitulate the observations with GIV–Gz3. Indeed, we found that the effects of Gz3 mutations in pull-down (Fig. 4c), fluorescence polarization (Fig. 4d,e) and G-protein activity assays (Fig. 4f and Supplementary Fig. 7b,c) were highly correlated with those of GIV. To assess specificity, we tested the same set of mutants for binding to a peptide derived from RGS12’s GoLoco motif. We chose RGS12 GoLoco motif as a stringent test for specificity because it also docks onto the $\alpha_3$/SwII cleft of Gz3-GDP33. Consistent with binding to this common site, we found that some of the mutations that impair GIV binding also impair binding of the RGS12 GoLoco motif (Fig. 4d,e). However, the overlap of the sets of Gz mutations that affect binding to GBA or GoLoco motifs is only partial, and many of the mutations that affect binding to both motifs do so to a significantly different extent. The divergence of the effect of same Gz mutants on GoLoco or GBA binding is in agreement with the comparison of the crystal structures of Gz bound to a GoLoco motif or the GBA-like peptide KB-752, which showed that the common binding site formed by the $\alpha_3$ helix and SwII region adopts a different conformation38. The correlation of the effect of the entire set of Gz3 mutants on the dissociation constant of RGS12–Gz3 versus GIV–Gz3 is markedly reduced (Pearson’s $r = 0.58$) compared to the correlation with DAPLE–Gz3 (Pearson’s $r = 0.97$; Fig. 4d,e). A notable example is mutant W211A, which completely abolishes GIV and DAPLE binding but has no effect on RGS12 binding. Thus, the overall effect of the mutants indicates that different GBA proteins share a common and specific site of binding to G proteins, which partially overlaps with the GoLoco-binding site but engages Gz3 in a different way.

**GBA proteins and GPCRs couple differently to G proteins.** Figure 5 depicts the mapping of the NMR perturbations induced by GIV on Gz3 and the GPCR contact sites reported for a rhodopsin–G-protein model12. Previous studies using crystallographic, biophysical, biochemical and computational approaches indicate that GPCRs make contact with the C-terminal tail of Gz subunits as well as with other regions such as the boundary between the N-terminal helix ($\alpha$N) and the $\beta$1 strand, and the $\alpha$4/$\beta$6 loop9–12. One route goes through the $\alpha$5 helix (which connects the C-terminal tail to the $\beta$6/$\alpha$5 loop that binds the guanine nucleotide ring) and the other one through the $\beta$1 strand (which connects $\alpha$N to the P-loop that binds the guanine nucleotides; Fig. 5c). Our results suggest that while GIV induces structural changes along the $\beta$1/P-loop axis (Fig. 5c), it does not perturb significantly the C terminus, $\alpha$5 or guanine-binding loops (Fig. 5a).

A subset of residues with GIV-induced NMR perturbations mapped to the $\alpha$4/$\beta$6 loop (Fig. 6a), one of the GPCR-binding sites, but mutation of two of those residues (R313 and K317) did not affect Gz3 binding and activation by either GIV or DAPLE (Fig. 6b–d). These observations indicate the NMR signal perturbations observed in the $\alpha$4/$\beta$6 loop are likely due to a secondary structural rearrangement, perhaps mediated by the $\alpha$3/$\beta$5 loop that directly binds GIV (Fig. 6a). We also tested a mutant lacking the last nine residues of Gz3 ($\Delta$C9), which removes the main GPCR-binding site, and observed no effect on binding and activation by GIV or DAPLE (Fig. 6b–d).

The results described above indicate that Gz3 mutants that interfere with GPCR coupling have no effect on how GBA
proteins couple to Gz3i. Next we tested whether the converse is also true, that is, mutants that disrupt GIV and DAPLE coupling to Gz3i have no effect on GPCR-mediated G-protein activation. We used purified bovine rhodopsin and retinal Gβγ (Gβγ3) in combination with Gz3i mutants (Fig. 7a), a previously validated system to monitor GPCR-mediated activation of G proteins in vitro5–7,11,12,40. We selected the Gz3i mutants that most markedly impair GIV and DAPLE binding (K35A, W211A, F215A, L249H, S252D and N256E), as well as Gz3i ΔC9, a mutant expected not to be activated by rhodopsin. Because GPCR-mediated activation requires an intact G-protein heterotrimer, we first tested whether any of the Gz3i mutants had altered Gβγ binding. Both W211A and F215A abolished Gβγ binding (Fig. 7b) and were excluded from subsequent experiments. The other mutants that disrupt GIV and DAPLE binding (K35A, L249H, S252D and N256E) were activated efficiently by rhodopsin, whereas the ΔC9 mutation completely abolished it (Fig. 7c). These in vitro results show that GBA proteins and GPCRs use different molecular mechanisms to couple to G proteins. We also investigated the effect of different G proteins mutants in a genetically engineered yeast strain that lacks endogenous GPCRs and expresses a single Gz3 subunit (human Gz3i with the first 35 residues (αN) replaced by the corresponding residues of yeast Gα1 (Fig. 7d). In this system, the natural pheromone response pathway that leads to activation of the ERK-like kinase Fus3 can only be activated by exogenous GEFs (Fig. 7d). Saccharomyces cerevisiae does not express GoLoco proteins. We found that GIV and DAPLE activated Gz3i WT and Gz3i ΔC9 equally, while activation of Gz3i N256E (NE, see rationale for the choice of this mutant in Methods) was blunted (Fig. 7e). Conversely, the human GPCR A2BR equally activated Gz3i WT and Gz3i N256E while activation of Gz3i ΔC9 was completely blunted (Fig. 7f). These results further confirm the divergence between the mechanisms of GPCR and GBA motif coupling to G proteins.

**Discussion**

Here we provide the first detailed characterization of the molecular basis for the activation of trimeric G proteins by non-receptor GEFs containing a GBA motif. Our conclusions are supported by consistent results from three independent and complementary approaches: biophysical (NMR); computational (modelling); and biochemical (mutagenesis). The major conclusion is that the mechanism of G-protein activation by GBA motif-containing GEFs is fundamentally different from the mechanism used by GPCR GEFs. Although results from such NMR experiments cannot be directly compared to the available crystal structure of nucleotide-free G protein in complex with a GPCR4, they can be interpreted with confidence in light of a wealth of additional information on the mechanism of GPCR-mediated activation obtained from experiments using biophysics5–9, biochemistry10,11, and computational modelling12–14. The current model for GPCRs is that activation is caused by the simultaneous perturbation of structural elements that bind both the nucleotide base (β6–α5 and β5–αG loops) and the nucleotide phosphates (P-loop and Swl). According to this model, GPCRs bind to the C terminus of Gz triggering a conformational change through the α5 helix to perturb the nucleotide base-binding site6,10–13. Perturbation of the phosphate-binding elements is currently explained by two non-exclusive models: one is that it is a consequence of the rearrangements of the nucleotide base-binding site6,12 while the other holds that it is caused by GPCR binding to the N terminus of Gz and subsequent conformational rearrangements transmitted to the P-loop through the β1 strand10,12. Our results indicate that in contrast to the GPCR mechanism, GIV does not induce perturbations in the nucleotide base-binding β6–α5 and β5–αG loops, but does cause structural changes in the P-loop and Swl phosphate-binding elements (Figs 2 and 3). Moreover, GIV, as well as other GBA proteins, binds to a Gz region different from the GPCR-binding site. The GBA motif docks in a cavity delimited by the α3/SwII/α3–β5 loop in which the β1 strand lies at the bottom...
Figure 6 | The α4-β6 loop and C terminus of Gαi3 are not required for coupling to GIV or DAPLE. (a) GIV-induced perturbations in the α4-β6 loop of Gαi3. Top, diagram of Gαi3 secondary structure elements overlaid with GIV-induced NMR perturbations reproduced from Fig. 2. Bottom, illustration of the position of α4/β6 loop and C-terminal α5 helix relative to the GIV-binding region in the GIV/Gαi3 model. Colour-coded as in the top panel. Two residues selected for mutation are labelled (R313 and K317). Some residues are displayed in sticks to indicate predicted contacts (dotted lines) between the α4/β6 loop and the α3 helix/α3-β5 loop region. The distance between the centroid of the GIV-binding site and the α4-β6 loop and C-distal part of α5 is ~16 and ~26 Å, respectively. (b) GIV or DAPLE binding to Gαi3 is not affected by mutations in the α4-β6 loop or deletion of the C terminus in pull-down assays. Binding of His-Gαi3 WT or mutants to immobilized GST-GIV (left) or GST-DAPLE (right) was visualized by Coomassie blue staining. One experiment representative of at least three is shown. GST protein loading and negative controls are shown in Supplementary Fig. 5. (c) Binding affinity of the GEF motif of GIV or DAPLE for Gαi3 is not altered by mutations in the α4-β6 loop or deletion of the C terminus. Binding of fluorescein-labelled GIV (left) or DAPLE (right) peptides to His-Gαi3 WT or mutants was determined by fluorescence polarization as in Fig. 4d. Curves were fitted to a one site binding model to calculate the $K_d$. Mean ± s.e.m., $n \geq 3$. (d) GIV- or DAPLE-mediated activation of Gαi3 is not affected by mutation of the α4-β6 loop or deletion of C terminus. Steady-state GTPase activity of His-Gαi3 WT or the indicated mutants in the absence (white) or presence (black) of GIV (left) or DAPLE (right). Mean ± s.e.m., $n \geq 3$. Raw basal activities in the absence of GIV or DAPLE are shown in Supplementary Fig. 7. ND, not determined due to severely compromised basal activity.

(Fig. 4). The β1 strand directly extends into the P-loop and acts as an allosteric route to perturb the P-loop on GPCR binding$^{10,12}$. We propose that the β1 strand also serves as a conduit to transmit information from the GIV-binding site to the nucleotide-binding pocket. GIV altering only a subset of the nucleotide-binding elements (P-loop/Swl) may be the reason behind the apparent lower GEF efficiency of GIV compared to GPCRs$^{18}$. Nevertheless, this also suggests that weakening phosphate binding is sufficient for GEF-catalysed nucleotide exchange. A recent study$^{14}$ indicates that the main driving force for nucleotide release is the structural rearrangement of the Ras-like domain instead of the separation between the Ras-like and all-helical domains as previously suggested$^{13,8,10}$. The same study also provided evidence that without destabilization of phosphate binding, nucleotides are not released. However, because GPCRs induce structural rearrangements in all the different nucleotide-binding elements of Gx, it remained unclear whether weakening of phosphate binding was necessary or sufficient to accelerate nucleotide exchange. Our results with the non-receptor GEF GIV highlight the importance of phosphate binding for nucleotide exchange and suggest that altering the interaction between GDP phosphates and the P-loop/Swl is sufficient to promote nucleotide exchange. This mechanism of G-protein activation regulates signalling in cells, as demonstrated by GIV-dependent signalling via Gαi-GTP$^{26–28}$ and free Gβγ$^{18,20,27,29}$. Moreover, GIV and GPCRs activate G-protein signalling in cells to a similar extent as determined by bioluminescence resonance energy transfer-based biosensors$^{30}$. Thus, the moderate GEF activity of GIV in vitro might be either an underestimation of its GEF activity in cells or just sufficient to elicit biological responses. This is in keeping with early observations for the α2 adrenergic receptor, a bona fide Gi GEF, which showed moderate GEF activity in vitro (three to sixfold)$^{31,42}$.

The GBA motif of GIV is present in a long disordered region of the protein (Supplementary Fig. 4), and the shorter GBA peptide recapitulated the NMR perturbations caused by GIV-CT (Supplementary Fig. 3), indicating that the GBA motif is sufficient to regulate G proteins. The disordered nature of GIV-CT is intrinsic and not a consequence of the truncation of the protein at

(44x324) representative of at least three is shown. GST protein loading and negative controls are shown in Supplementary Fig. 5. (c) Binding affinity of the GEF motif of GIV or DAPLE for Gαi3 is not altered by mutations in the α4-β6 loop or deletion of the C terminus. Binding of fluorescein-labelled GIV (left) or DAPLE (right) peptides to His-Gαi3 WT or mutants was determined by fluorescence polarization as in Fig. 4d. Curves were fitted to a one site binding model to calculate the $K_d$. Mean ± s.e.m., $n \geq 3$. (d) GIV- or DAPLE-mediated activation of Gαi3 is not affected by mutation of the α4-β6 loop or deletion of C terminus. Steady-state GTPase activity of His-Gαi3 WT or the indicated mutants in the absence (white) or presence (black) of GIV (left) or DAPLE (right). Mean ± s.e.m., $n \geq 3$. Raw basal activities in the absence of GIV or DAPLE are shown in Supplementary Fig. 7. ND, not determined due to severely compromised basal activity.

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The GBA motif of GIV is present in a long disordered region of the protein (Supplementary Fig. 4), and the shorter GBA peptide recapitulated the NMR perturbations caused by GIV-CT (Supplementary Fig. 3), indicating that the GBA motif is sufficient to regulate G proteins. The disordered nature of GIV-CT is intrinsic and not a consequence of the truncation of the protein at
residue 1,660 since disorder is predicted for the C-terminal 330 residues of the GIV sequence, and larger fragments of GIV can also recapitulate G-protein binding and/or activation in a GBA motif-dependent manner. The presence in intrinsically disordered regions might be a property common to all GBA motifs identified. Thus, it is possible that the presence of GBA motifs in intrinsically disordered regions facilitates their function by making them more readily accessible to the target G protein.

The molecular mechanism of activation of Gz by GBA motif-containing proteins has more similarities with GEF-mediated activation of small G proteins of the Ras family of CALNUC only when the protein is not calcium-bound, which is known to result in high disorder of the region comprising the EF hands (and the GBA motif). Thus, it is possible that the presence of GBA motifs in intrinsically disordered regions facilitates their function by making them more readily accessible to the target G protein.

Figure 7 | Gz3 mutants that disrupt activation by GIV are activated by GPCRs. (a) System to monitor GPCR-mediated activation of Gz3. Left, human His-Gz3 (blue) was reconstituted with bovine GB1γ1 (β; yellow; γ; turquoise) and activated with light-stimulated bovine rhodopsin (red). Diagrams on the right depict the position of mutants relative to the GPCR- and GIV-biding sites. (b) GB1γ binding to Gz3 mutants. His-Gz3 WT or mutants were incubated with bovine GB1γ1 and binding determined in pull-down assays. Equal protein loading of Gz3 ΔC9 (not detected by the Gz3 antibody) was confirmed by Ponceau S staining (bottom row). One experiment representative of three is shown. (c) Gz3 mutants that disrupt activation by GIV are activated by rhodopsin. Steady-state GTPase activity of His-Gz3 WT or mutants in complex with GB1γ1 was determined in the absence (white) or presence (black) of rhodopsin. Mean ± s.e.m., n = 3. (d) Schematic diagram and validation of a yeast-based assay used to monitor G-protein signalling in cells. A genetically engineered S. cerevisiae strain was used to determine the levels of G-protein activation on expression of exogenous GEFs like GIV and DAPLE or a GPCR (human A2b receptor) by measuring Fus3 phosphorylation (phospho(ppFus3) antibodies recognize yeast ppFus3). Insert, validation experiment showing activation of human Gz3 (as determined by Fus3 phosphorylation) in yeast strains expressing GIV WT but not the GEF-deficient F1685A mutant (FA). One experiment representative of three is shown. (e) Activation of G-protein signalling by GIV or DAPLE is blocked by the N256E mutation but not by deletion of the C terminus of Gz3. Yeast strains expressing Gz3 WT or mutants were incubated with bovine GB1γ1 and binding determined in pull-down assays. Equal protein loading of Gz3 ΔC9 was determined in the absence (−) or presence (+) of GIV (left) or DAPLE (right) co-expression were lysed and immunoblotted as indicated. NE, N256E. One experiment representative of three is shown. (f) Activation of G-protein signalling by A2bR is blocked by deletion of the C terminus of Gz3 but not by the N256E mutation. Yeast strains expressing Gz3 WT or mutants co-expressing A2bR WT or a constitutively active mutant (Act., in red) were lysed and immunoblotted as indicated. NE, N256E mutant. One experiment representative of three is shown.
The combined NMR, computational and mutagenesis analyses presented here provide a detailed view of the structural features than GPCR-mediated activation of trimeric G proteins. First, GBA proteins displace Gβγ from the trimeric complex and exert their GEF activity directly on monomeric Gα (refs 18–20,25), whereas GPCRs work most efficiently as GEFs on intact Gzβγ trimers. Second, GEFs for small G proteins perturb the phosphate-binding region of the G protein while leaving the guanine base-binding region mostly unchanged. Third, as in the case of GEFs for small G proteins, GBA proteins bind to a region that overlaps with the site where effectors and other regulators (like GAPs) bind, whereas GPCRs bind to a region that does not overlap.

GBA proteins also differ from the non-receptor GEF Ric-8A. Although both Ric-8A and GBA proteins activate monomeric Gα instead of Gzβγ trimers, this indicates a different mode of G-protein binding for Ric-8A that resembles how GPCR bind. Also, Ric-8A binds preferentially to nucleotide-free Gα (ref. 22), which again differs from GBA proteins and resembles GPCRs.

The preference of GPCRs for nucleotide-free G proteins is considered an event that favours the progression of the reaction in the direction of activation. In the case of GIV, the directionality of the reaction is still ensured by the dissociation from Gzα in GDP binding, and it is unclear why a GEF would bind to Gzα-[GDP] with an affinity comparable to nucleotide-free Gzα. We speculate that such Gzα-[GDP] binding would increase the efficiency of this class of GEFs. To activate Gzα, GBA proteins must either displace Gβγ from the trimeric complex or engage transiently formed Gzα-[GDP] en route to re-association with Gβγ after a priming cycle of activation. In both scenarios, binding with higher affinity to Gzα-[GDP] would be advantageous to facilitate GBA-mediated activation. Therefore, we propose a model to integrate the GBA-mediated mechanism of activation with the classic G-protein cycle triggered by GPCRs (Fig. 8).

In vitro experiments demonstrate that GIV and DAPLE can displace Gβγ (refs 18,20), and GIV can also induce the rapid dissociation of the Gzβγ complex in cells.

A question that cannot be unambiguously answered at this point is whether the perturbations observed in the nucleotide-binding pocket in our NMR experiments are due to lower GDP occupancy or structural rearrangements of the GDP-bound form on GIV binding. This is in part because studies in Gzt (ref. 46) and Gz1 (ref. 47; which shares >90% sequence identity with Gz1) indicate that the NMR spectra of nucleotide-free Gzα do not differ markedly from those of Gzα-GDP. Nevertheless, we favour the interpretation that our NMR experiments reflect changes in GDP-bound G proteins because they were carried out in the presence of 300 μM GDP, a 3- or 10-fold molar excess over Gz1 (for GIV-CT or GIVpept, respectively). Since G proteins bind nucleotides with submicro-molar–nanomolar affinity, we think that it is unlikely that the GEF activity of GIV will be sufficient to lower it over three orders of magnitude to result in a marked decrease of nucleotide occupancy. Moreover, an overall decrease in the nucleotide occupancy would also be expected to result in perturbations in the guanine-binding elements of the nucleotide-binding pocket, which we do not observe. Thus, our results probably reflect the structural rearrangements that precede the release of nucleotide. Regardless of the exact interpretation, it is possible that the observed structural perturbations would be even more marked in the absence of GDP. This would be in agreement with previous hydrogen–deuterium exchange experiments showing that Gs in complex with the β2AR complex in the presence of excess GDP (1 mM) undergoes changes in the exact same regions as the nucleotide-free complex but of lower magnitude (Supplementary Figs 2 and 9 of ref. 10).

The combined NMR, computational and mutagenesis analyses presented here provide a detailed view of the structural features
that govern the GBA–Gz interaction. Because some GBA proteins are specifically dysregulated in disease and disruption of Gz binding blocks their adverse effects16,20,31, the structural insights gained here should provide a solid framework for the development of new pharmacological agents. On the basis of our results, such agents would not be expected to interfere with GPCR-mediated activation, and previous data indicate that GIV’s GBA motif binding can be disrupted by mutagenesis of Gz without affecting binding to other G-protein-interacting partners like GPCRs, Gβ/γ, RGS proteins or GoLoco guanine-nucleotide dissociation inhibitors25. Although effectors bind to Gz, the form to which GBA motifs bind, thereby providing room for specificity in targeting. However, a major challenge will be to identify drugs that can discriminate among different GBA proteins. Such efforts would require atomic resolution structures to exploit small differences in the binding mode among distinct GBA proteins.

Methods
Preparation of nucleotide-free Gz3 and pull-down assay. His-tagged rat Gz3 cloned in a pET28b vector was purified from BL21(DE3) bacteria (Life Technologies) after isopropyl-[β-D-thiogalactoside (IPTG) induction25. For this, the Gz3 was run through a Superdex 75 column equilibrated with 20 mM Tris-HCl (pH 7.4), 20 mM NaCl, 1 mM MgCl2, 1 mM dithiothreitol (DTT), 10 μM GDP and 5% (v/v) glycerol (6 ml) and the protein eluted at room temperature with the same buffer. Protein-containing fractions were pooled (500–600 μl) and incubated overnight at 4 °C. After concentration to ~100–200 μl, the sample was applied to a second Superdex 200 10/300 column equilibrated with 50 mM Tris-HCl (pH 8.1), 150 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 2 mM DTT, 20% (v/v) glycerol and 0.04% (w/v) C12E10, or the same buffer supplemented with 100 μM NaF to generate GDP-loaded Gz3 (Gz3-GDP) or GDP/AF6 - loaded Gz3 (Gz3-GTP). Samples were cooled down to 4 °C and centrifuged for 10 min at 14,000 g. A volume of 30 μl of the His-Gz3-containing supernatant was used for pull-down assays in which the proteins immobilized on glutathione-agarose beads were 10 μg of glutathione-X-transferase (GST), GST-GIV (residues 1,671–1,755, containing its GBA motif)20, GST-GAIP25 or GST-Ric-8A (residues 12–49, a plasmid generously provided by Stephen Sprang, University of Montana)25. Binding reactions were carried out overnight at 25 °C in a final volume of 300 μl of 50 mM Tris-HCl (pH 8.1), 150 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 2 mM DTT, 20% (v/v) glycerol and 0.04% (w/v) C12E10, or the same buffer supplemented with 100 μM GDP or 100 μM GDP plus 100 μM GTP to generate GDP-loaded Gz3 (Gz3-GDP) or GDP/AF6 - loaded Gz3 (Gz3-GTP). Samples were cooled down to 4 °C and centrifuged for 10 min at 14,000 g. A volume of 30 μl of the His-Gz3-containing supernatants were used for pull-down assays in which the proteins immobilized on glutathione-agarose beads were 10 μg of glutathione-X-transferase (GST), GST-GIV (residues 1,671–1,755, containing its GBA motif)20, GST-GAIP25 or GST-Ric-8A (residues 12–49, a plasmid generously provided by Stephen Sprang, University of Montana)25. Binding reactions were carried out overnight at 25 °C in a final volume of 300 μl of 50 mM Tris-HCl (pH 8.1), 150 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 2 mM DTT, 20% (v/v) glycerol and 0.04% (w/v) C12E10, or the same buffer supplemented with 100 μM GDP or 100 μM GDP plus 100 μM GTP. Glutathione–agarose beads were washed with the same buffers and resin-bound proteins eluted with Laemmli sample buffer and separated by SDS–PAGE. After transfer to polyvinylidene difluoride (PVDF) membranes, GST-fused proteins were visualized by Ponceau S staining and His-Gz3 detected by immunoblotting with mouse anti-polyHis-Tag primary antibodies (1:500, Sigma, H1029) and goat anti-mouse IRDye 800F(ab')2 secondary antibodies (1:10,000, Li-Cor) using an Odyssey Infrared Imaging System (Li-Cor Biosciences). Images of uncropped scans of Photoshop and Illustrator softwares (Adobe). Images of uncoated scans of immunoblots and protein gels are provided in Supplementary Fig. 9.

Co-immunoprecipitation. HEK293T cells (American Type Culture Collection) were grown at 37 °C in DMEM supplemented with 10% FBS, 100 μM l-cysteine, 100 μg ml−1 penicillin, 100 μg ml−1 streptomycin, 1% l-glutamine and 5% CO2. DNA plasmids encoding for C-terminally 3×Flag-tagged Gz3 (ref. 25) or an empty plasmid (p3XFlag-CMV-14) were transfected using the calcium phosphate method in 10 cm dishes. The DNA amount for the Gz3 N269D plasmid was threefold (12 μg) of that for Gz3 WT (4 μg) to equalize their expression levels. Cells were collected 48 h after transfection by gently scraping in PBS, centrifuged and resuspended in 1 ml of lysis buffer (20 mM HEPES (pH 7.2), 5 mM MgCl2/CoCl2), 125 mM KCl, 20 mM Tris-HCl, 0.4% (v/v) Triton X-100, 1 mM DTT, 10 mM β-glycerophosphate and 0.5 mM Na3VO4, supplemented with a protease inhibitor cocktail (Sigma; catalog number #88380) and cleared (14,000g for 10 min) before use. Lysates were supplemented with 1 μg of purified GST-Ric-8A (1–492)25 and 2 μg of mouse anti-FLAG antibody (Sigma F1804), and incubated for 4 h at 4 °C with 100 μl of BSA-blocked Protein A/G-agarose beads (Thermo Scientific) added and tubes incubated for additional 90 min at 4 °C. Beads were washed four times (4.3 mM NaHPO4, 1.4 mM KH2PO4, pH 7.4, 37 mM NaCl, 2.7 mM KCl, 0.1% (v/v) Tween 20, 10 mM MgCl2, 5 mM EDTA and 1 mM DTT) and immunoprecipitated proteins eluted by boiling in Laemmli sample buffer. Proteins were separated by SDS–PAGE, transferred to PVDF membranes and immunoblotted with rabbit anti-GIV serum (1:500)23, rabbit anti-GST (1:500, Santa Cruz Biotechnology Z-5), mouse anti-FLAG (1:1,000) and mouse α-β2-tubulin (1:2,500) primary antibodies followed by incubation with goat anti-rabbit (Sigma F0500) and mouse Alexa Fluor 680 or IRDye 800F(ab')2 secondary antibodies (1:10,000) and imaging with an Odyssey Infrared Imaging System. Images were processed using the Image J software (NIH) and assembled for presentation using Photoshop and Illustrator softwares (Adobe).

Purification of proteins and synthesis of peptides for NMR. Full-length human Gz3 (UNIPROT entry P08754) cloned in a pET24d (+) plasmid (a generous gift from Ichio Shimada, University of Tokyo) was used to purify the protein used in NMR experiments47. For this, the protein expression was seed in BL21 Rosetta (Life Technologies) cell cultures with 1 mM IPTG for 16 h at 23 °C in a modified M9 minimal medium containing 1 g l−1 15N-HCl, 2 g l−1 13C-glucose and 1 g l−1 of 13C-3-N2, providing room for specificity in targeting. However, a major challenge will be to identify drugs that can discriminate among different GBA proteins. Such efforts would require atomic resolution structures to exploit small differences in the binding mode among distinct GBA proteins.

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silapentane-5-sulfonate (DSS, 0.00 p.p.m.), and 13C and 15N chemical shifts were indirectly referenced to DSS.83 G3i3 spectra were recorded at 30°C, 2H2O, 13C, 15N-NMR with a threefold molar excess of GDP in 10 mM HEPES (pH 7.0) with 10 mM MgCl2, 5 mM DTT, 0.01% NaN3 and 5% 2H2O. The samples were prepared by mixing different amounts of G3i3 and GIV–CT from stocks in the corresponding gel filtration buffers followed by three cycles of fourfold dilution in the NMR buffer and concentration by ultrafiltration using 10kdA cut-off membranes. Protein solubility limited the G3i3 concentration of the samples to 100 μM (free and bound to GIV-CT) or 32 μM (bound to the 1.671–1.696 GIV peptide). H–15N TROSY and HNCA spectra of G3i3 allowed transferring all of the assignments of the protein backbone resonances deposited in the BiomagResBase database entry 19015.35. We observed, however, a systematic offset of 0.09 and −1.1 p.p.m. in our spectra with respect to the published 1H and 15N chemical shifts, respectively. The assignment of G3i3 resonances in the presence of a twofold molar excess of GIV–CT was done based on 1H–15N TROSY, HNCO and HNcoCA spectra (the latter one, however, lacking many signals because of its lower sensitivity). The assignment of G3i3 resonances in the presence of a fivefold molar excess of GIV–CT was achieved from the joint analysis of HNCO and 1H–15N TROSY spectra recorded along a titration (at molar ratios 1:0, 1:2, 1:0.5, 1:1, 1:2, and 1:5). The stepwise addition of the fragment from a 5 mM stock in NMR buffer with 50% 2H2O dimethylsulfoxide (DMSO) was necessary to solubilize the peptide. Finally, a result in 3% DMSO concentration was achieved from the receptor and re-docked as above to confirm that an energetically favourable complex was generated. To compare the intensity of G3i3 mutants for biochemical studies. Human G3i3 was expressed from the same plasmid as described for the NMR studies and purified as an uncleaved His-tagged protein. Pelleted bacteria from 1 l of culture were resuspended in 25 ml of buffer (50 mM NaH2PO4, pH 7.4, 300 mM NaCl, 10 mM imidazole, 25 μM GDP and 1% (v/v) Triton X-100 supplemented with protease inhibitor cocktail (leupeptin 1 μM, pepstatin 2.5 μM, aprotinin 0.2 μM and phenylmethylsulfonyl fluoride 1 mM)). After sonication (four cycles, with pulses lasting 20 s and with 1 min interval between pulses to prevent heating), lysates were centrifuged at 12,000g for 20 min at 4°C. Solubilized proteins were affinity-purified on HisPur Cobalt Resin (Pierce) and eluted with lysis buffer supplemented with 500 mM imidazole. The buffer was exchanged for 20 mM NaCl, 1 mM MgCl2, 1 mM DTT, 10% 2H2O and 5% (v/v) glycerol using a Hitrap Desalting column (GE Healthcare). Protein samples were stored at −80°C.

Fluorescence polarization-based peptide-binding assays. Fluorescently labelled peptides derived from human G(i)3 (residues 1,671–1,701, KTGSPGSEYTVLQ QFELEENKTSVQIKSSS, DAPLE (residues 1,662–1,695, SAPSPEMVTLEEL ELENRSSPHTDTPSCRDL) or RGS12 (residues 1,185–1,221, DEAEFFELISQ AKNRANDDQRRLHIKEDVLFELR) were synthesized following a protocol as described above in ‘Purification of proteins and synthesis of peptides for NMR’ with minor modifications. Briefly, following chain elongation 5,6-carboxyfluorescein was activated with HATU and coupled to the resin-bound peptide at 65°C for 1 h to yield the fluorescence-labelled peptides. The remaining steps were then performed as described above. Fluorescence polarization measurements were carried out in 384-well plates (Black OptiPlate-384F, Perkin Elmer). G protein (0.8 μM) and 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% (v/v) NP-40, 10 mM MgCl2, 5 mM EDTA, 2 mM DTT and 30 μM GDP) and incubated 4 h at 4°C with constant rotation in the presence of tagged G3i3 WT or mutants (1 μM final concentration). 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% (v/v) Tween-20, 10 mM MgCl2, 5 mM EDTA, 1 mM DTT and 30 μM GDP) and resin-bound proteins eluted with Laemmli sample buffer by incubation at 37°C for 10 min. Proteins were separated by SDS–PAGE and stained with Coomassie blue.

In vitro protein-binding assays with GST-fused proteins. Binding of G3i3 mutants to GST-fused GIV or DAPLE was determined by a pull-down assay.57 A solution of 10 μg of GST, GST-GIV (1,671–1,755) or GST-DAPLE (1,650–1,745), created by cloning from Cazone’s clone h14721 (KIAA1509) into pGEX-4T-1) were immobilized on glutathione agarose beads for 90 min at room temperature in PBS (1.25 μM final concentration in the binding reactions). Beads were washed twice with PBS, resuspended in 250 μl of binding buffer (50 mM Tris-HCl, pH 7.4) and incubated 4 h at 4°C. For mutants not reaching binding saturation, the maximal binding of G3i3 mutants for biochemical studies. Human G3i3 was expressed from the same plasmid as described for the NMR studies and purified as an uncleaved His-tagged protein. Pelleted bacteria from 1 l of culture were resuspended in 25 ml of buffer (50 mM NaH2PO4, pH 7.4, 300 mM NaCl, 10 mM imidazole, 25 μM GDP and 1% (v/v) Triton X-100 supplemented with protease inhibitor cocktail (leupeptin 1 μM, pepstatin 2.5 μM, aprotinin 0.2 μM and phenylmethylsulfonyl fluoride 1 mM)). After sonication (four cycles, with pulses lasting 20 s and with 1 min interval between pulses to prevent heating), lysates were centrifuged at 12,000g for 20 min at 4°C. Solubilized proteins were affinity-purified on HisPur Cobalt Resin (Pierce) and eluted with lysis buffer supplemented with 500 mM imidazole. The buffer was exchanged for 20 mM NaCl, 1 mM MgCl2, 1 mM DTT, 10% 2H2O and 5% (v/v) glycerol using a HiTrap Desalting column (GE Healthcare). Protein samples were stored at −80°C.

G3i3-limited proteolysis assay. Human G3i3 (0.25 mg ml−1) was incubated for 150 min at 30°C in buffer (20 mM Na-HEPES, pH 8, 100 mM NaCl, 1 mM EDTA, 10 mM MgCl2, 1 mM DTT and 0.05% (w/v) C12E10) supplemented with G3i3 (1 μM) or GDP (300 μM) and incubated for 3 min at room temperature. Samples were rapidly transferred to ice and reactions stopped by the addition of Laemmli sample buffer and boiling for 5 min. Proteins were separated by SDS–PAGE and stained with Coomassie blue.

Fluorescence polarization-based peptide-binding assays. Fluorescently labelled peptides derived from human G(i)3 (residues 1,671–1,701, KTGSPGSEYTVLQ QFELEENKTSVQIKSSS, DAPLE (residues 1,662–1,695, SAPSPEMVTLEEL ELENRSSPHTDTPSCRDL) or RGS12 (residues 1,185–1,221, DEAEFFELISQ AKNRANDDQRRLHIKEDVLFELR) were synthesized following a protocol as described above in ‘Purification of proteins and synthesis of peptides for NMR’ with minor modifications. Briefly, following chain elongation 5,6-carboxyfluorescein was activated with HATU and coupled to the resin-bound peptide at 65°C for 1 h to yield the fluorescence-labelled peptides. The remaining steps were then performed as described above. Fluorescence polarization measurements were carried out in 384-well plates (Black OptiPlate-384F, Perkin Elmer). G protein (0.8 μM) and 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% (v/v) NP-40, 10 mM MgCl2, 5 mM EDTA, 2 mM DTT and 30 μM GDP) and incubated 4 h at 4°C with constant rotation in the presence of tagged G3i3 WT or mutants (1 μM final concentration). 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% (v/v) Tween-20, 10 mM MgCl2, 5 mM EDTA, 1 mM DTT and 30 μM GDP) and resin-bound proteins eluted with Laemmli sample buffer by incubation at 37°C for 10 min. Proteins were separated by SDS–PAGE and stained with Coomassie blue.

GTase and GTP/βS-binding assays with GIV and DAPLE. Steady-state GTase activity was measured by release of radioactive phosphate and GTP/βS binding by
Synthesis of peptide libraries and Gz3i overlay. Libraries of immobilized peptides were produced by automatic SPOT synthesis on continuous cellulose membranes supported on 0.45 μm filter membranes (Millipore, 4.5–5.5 cm final concentration) using Gz3i (fluoren-9-ylmethoxycarbonyl) chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments AG). Individual peptide–cellulose complexes were solubilized and re-spotted on Cellspot slides for subsequent probing. Slides were primed in binding buffer (4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 5 mM MgCl2, 1 mM DTT, 30 μM GDP and 1% (v:v) TX100) pre-blocked with 30 μM GDP) for 4 h at 4°C. A volume of 25 μl of BSA-blocked HisPur cobalt resin (Pierce) were added to each tube and the incubation continued for 90 min. Beads were washed four times with 1 ml of binding buffer and resin-bound proteins eluted by adding Laemmli sample buffer supplemented with 100 μM EDTA and boiling for 5 min. Proteins were separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted with rabbit anti-Gz3i (1:500, Santa Cruz Biotechnology C-10) and anti-panGz (1:250, Santa Cruz Biotechnology M-14) primary antibodies followed by incubation with goat anti-rabbit Alexa Fluor 680 (1:10,000, Life Technologies) secondary antibodies and imaging with an Odyssey Infrared Imaging System (Li-Cor Biosciences).

Steady-state GTase assays with rhodopsin. ROS membranes washed with urea were prepared as described earlier and used as the source of rhodopsin. Human His-Gz3i WT or mutants (20–25 μM) were incubated overnight at 4°C with twofold excess Gz3i; γi of 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM MgCl2, 1 mM DTT, 10 μM GDP and 5% (v/v) glycerol. Bovine rhodopsin was freshly solubilized from urea-washed ROS membranes in assay buffer (20 mM HEPEs, pH 7.5, 100 mM NaCl, 1 mM MgCl2, 1 mM DTT, 0.01% (v:v) dodecylmaltoside) for each experiment. Gz3i/Gz3iγi (200:100 nm mol) and rhodopsin (20 nmol μM) were incubated in ice for 30 min under ambient light in assay buffer. Reactions were initiated at 30°C by adding an equal volume of assay buffer containing 1 μM [γ-32P]GTP (50 μCi, 50 μM γ-32P-GTP, 100 μCi/μmol) and [32P]Pi released. Background [32P]Pi detected at 15 min in the absence of G protein was subtracted from each reaction (<5% of the counts detected in the presence of G proteins). Results were expressed as per cent of the activity of Gi3 WT in the absence of rhodopsin.

G-protein activation assay in yeast. The previously described S. cerevisiae strain CY1316 (ref. 68; MATa gpa1f1 arf3 fusi1p-HIS3 ste11p-KLY5 ste3a1 lys2a1 trpl3 his3a1) kindly provided by James Broach, Penn State University) was used for experiments with GIV and DAPLE and strain MMY11 (ref. 69; MATa gpa1f1 arf3 fusi1p-HIS3 ste2a2 fus1a1-2 lac ura3 trpl3 his3a1) kindly provided by Simon Dowell, Glaxo-Smith-Kline) for the experiments with A2b receptor. The native G-protein-dependent pheromone response pathway is similarly modified in both strains: the pheromone-activated GPCR (Ste2 or Ste3), endogenous Gz-subunit (Gpa1) and the cell cycle arrest-inducing protein far1 are deleted. In MMY11, the G-protein inhibitor Ste2 is also deleted. Both strains were transformed with a centromeric plasmid (CEN TRP) encoding a chimeric Gpa1 (1–41)/human Gz3i (36–354) protein under the control of the endogenous Gpa1 promoter68 (courtesy of Mary Cisowski, Nationwide Children’s Hospital) using the lithium acetate method69. In these strains, the pheromone response pathway can be regulated by the ectopic expression of activators of human Gz3i and does not result in the cell cycle arrest that occurs in the native pheromone response68. CY1316-derived strains were transformed with pYES2 plasmids (2 μm, URA3) encoding for GIV (residues 1660–1870, between BglII/EcoRI) and assembled for presentation using Photoshop and Illustrator softwares (Adobe).

Gz3i mutants tested in the in vitro G-protein activation experiments with rhodopsin (K35A, L249H, S252D, N256E and AC9), only N256E and AC9 were tested for different reasons. K35A was excluded because this residue is right at the boundary between the Gz3i and Gpa1 sequences of the chimera and for this reason it may yield results that are difficult to interpret. L249H and S252D were excluded because they displayed enhanced spontaneous activation in yeast.
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Author contributions

A.I.d.O. performed and analysed NMR experiments; N.M. and M.V. purified proteins for NMR studies and performed CD experiments; K.P., S.J., A.L., A.M., L.T.N. and M.G.-M. performed protein-binding experiments, yeast-based assays, G-protein activity experiments and trypsin protection assays; V.D. conducted the computational modelling and analysed data; M.A.d.L.-C. M. performed the co-immunoprecipitation experiments; J.B.B.-C. synthesized peptides in solution and G.S.B. provided the peptide arrays; S.R. and R.A.C. isolated ROS membranes and purified retinal G{alpha}; M.G.-M. conceived the study; M.G.-M and F.J.B. co-supervised the study, designed experiments and analysed data; M.G.-M, V.D. and F.J.B. wrote the manuscript with input from all the authors.

Additional information

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