Golgi-Resident Gαo Promotes Protrusive Membrane Dynamics

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Graphical Abstract

Highlights

- Across animals and cell types, $G_\alpha_o$ localizes dually to plasma membrane and Golgi.
- The two pools of $G_\alpha_o$ coordinate initiation and elongation of cell protrusions.
- KDELR, an atypical Golgi GPCR, activates monomeric $G_\beta_\gamma$-free $G_\alpha_o$.
- $G_\alpha_o$, master regulator of trafficking, activates Rab1/3 at Golgi promoting secretion.

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In Brief

$G_\alpha_o$ functions in vesicle trafficking independent of canonical G protein signaling.
Golgi-Resident Ga\(\alpha\) Promotes Protrusive Membrane Dynamics

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SUMMARY

To form protrusions like neurites, cells must coordinate their induction and growth. The first requires cytoskeletal rearrangements at the plasma membrane (PM), the second requires directed material delivery from cell’s insides. We find that the Ga\(\alpha\)-subunit of heterotrimeric G proteins localizes dually to PM and Golgi across phyla and cell types. The PM pool of Ga\(\alpha\) induces, and the Golgi pool feeds, the growing protrusions by stimulated trafficking. Golgi-residing KDEL binds and activates monomeric Ga\(\alpha\), atypically for G protein-coupled receptors that normally act on heterotrimeric G proteins. Through multidimensional screenings identifying > 250 Ga\(\alpha\) interactors, we pinpoint several basic cellular activities, including vesicular trafficking, as being regulated by Ga\(\alpha\). We further find small Golgi-residing GT\(\alpha\)ases Rab1 and Rab3 as direct effectors of Ga\(\alpha\). This KDEL → Ga\(\alpha\) → Rab1/3 signaling axis is conserved from insects to mammals and controls material delivery from Golgi to PM in various cells and tissues.

INTRODUCTION

G protein-coupled receptors (GPCRs) form the biggest receptor family in animals. Main intracellular GPCR effectors are heterotrimeric G proteins composed of \(\alpha\), \(\beta\), and \(\gamma\) subunits, of which the \(\alpha\)-subunit binds guanine nucleotides. Four main subgroups of G\(\alpha\)-subunits exist: G\(\alpha\)\(_S\), G\(\alpha\)\(_\text{AR} \), G\(\alpha\)\(_{\text{i/o}}\), and G\(\alpha\)\(_{12/13}\) (Milligan and Kostenis, 2006). When bound to guanosine diphosphate (GDP), heterotrimeric G protein is competent to interact with the cognate GPCR. The activated receptor acts as a guanine nucleotide exchange factor (GEF), catalyzing exchange of GDP for GTP on the G\(\alpha\). This triggers dissociation of the G protein into G\(\alpha\)-GTP and the \(\beta\)\(\gamma\)-heterodimer, which can bind and activate downstream transducer proteins. When GTP on G\(\alpha\) is hydrolyzed, the inactive G\(\beta\)\(\gamma\)-heterotrimer re-associates for a new cycle of activation. Alternatively, the G\(\alpha\)-subunit can be reloaded with GTP and continue its signaling activity (Lin et al., 2014).

As G\(\alpha\)-subunits provide the main specificity in GPCR-initiated signaling cascades (Milligan and Kostenis, 2006), identification of the G\(\alpha\) targets is crucial to understand this type of signaling. Ga\(\alpha\) was among the first \(\alpha\)-subunits discovered and is the major G\(\alpha\)-subunit of the nervous system across the animal kingdom (Sternweis and Robishaw, 1984; Wolfgang et al., 1990), controlling both development and adult physiology of the brain (Bromberg et al., 2008). Ga\(\alpha\) is also expressed in other tissues and is a transducer of the developmentally and medically important Wnt signaling pathway (Egger-Adam and Katanaev, 2008; Koval et al., 2011).

Despite this importance, the list of known molecular targets of Ga\(\alpha\) has been remarkably short. To uncover Ga\(\alpha\) interactors, we performed several whole genome/proteome screenings resulting in > 250 candidate targets, most of which are not previously known to be regulated by G\(\alpha\) proteins. These Ga\(\alpha\) targets can be clustered into functional modules, identifying several basic cellular activities, conserved from insects to humans, as being under regulation of Ga\(\alpha\)-mediated GPCR signaling. We focus on vesicular trafficking as one of these functional modules and show that Ga\(\alpha\) controls multiple steps within it. From Drosophila epithelia to mammalian neuronal cells, Ga\(\alpha\) controls outgrowth formation through coordinated activities from the plasma membrane (PM) and the Golgi apparatus, the latter involving the KDEL receptor and small GT\(\alpha\)ases Rab1 and Rab3.

RESULTS

Massive Screenings Identify Numerous Ga\(\alpha\) Partners

With only a few Ga\(\alpha\) effectors previously known, we performed several screenings to massively identify Ga\(\alpha\) partners. Our primary screenings were: yeast two-hybrid (1), proteomic (2) and genetic suppressor-enhancer screens in Drosophila using Ga\(\alpha\) overexpression (3), and RNAi-mediated downregulation (4). In a complementary manner, we expected to cover the complete Ga\(\alpha\) interactome with these screenings. We then complemented these screens with an extensive scrutiny of the literature data (5) and bioinformatics analysis of the resulting network and translation of this network into proteins orthologous between Drosophila and humans. This type of interactome identification has not been performed for any G\(\alpha\) protein and produced an impressive list of 254 proteins being candidate Ga\(\alpha\) partners (Table S1; STAR Methods).
A

Nucleotide biosynthesis
Macromolecular complex assembly
Post-embryonic morphogenesis
Cell fate regulation
Cuticle development
Reproductive cellular process
Respiratory system development
Cell division
Cell cycle

B

Syntactic vesicle blooming
Establishment of protein localization
Membrane organization
Secretory and Recycling Pathways

C

Vesicle Trafficking and Membrane Fusion

D

dGso-GFP
F-actin
α-tubulin
GFP F-actin

30 min

120 min

E

F

G

Cells with phagosomes (%) (transfected)
Number of phagosomes per cell
Longest phagosome (μm)

Cell
dGso

ns
Next, we aimed at functional clustering of the Gαo targets, performing the gene ontology enrichment analysis of the Gαo interactome. This analysis identifies several functional modules, such as cytoskeleton organization, cell division, cell adhesion, etc., within the Gαo interaction network (Figures 1A and S1A; Table S2). These modules may represent key cellular activities being directly controlled by Gαo-mediated GPCR signaling. As opposed to analysis of isolated individual targets, we decided to select a functional module from this network and to holistically investigate the role of Gαo in the regulation of this module. For this purpose, we selected the vesicular trafficking group of Gαo targets.

**Gαo Induces Outgrowth Formation in Different Cellular Systems**

Previously, we showed that *Drosophila* Gαo (dGαo) directly interacts with Rab5 to regulate the Wnt/FRIZZLED (Fz) signaling (Purvanov et al., 2010). The screenings now identified components of the vesicular trafficking machinery as partners of Gαo (Figures 1B and 1C; Table S2), suggesting that Gαo may function as a master regulator in vesicular trafficking.

To experimentally validate our hypothesis, we looked for a Gαo-mediated cellular program that may require vesicle-mediated transport. We chose the process of neurite formation, and more broadly, outgrowth formation, for this purpose. Indeed, Gαo expression in neuronal cells coincides with, and Gαo activity is required for, neurotogenesis (Frémion et al., 1999; Lee et al., 2006; Strittmatter et al., 1994; Wolfgang et al., 1990). The need for vesicle-mediated delivery of material to growing neurites is well-known, although Gαo has not previously been implicated in this vesicle delivery. Gαo is also required for the patterning and formation of *Drosophila* wing hairs—stable actin-rich outgrowths of wing epithelial cells (Katanay et al., 2005).

Mouse neuroblastoma N2a cells rarely produce spontaneous neurites, which correlate with their low endogenous levels of Gαo (Figure S1B). As previously shown (Bromberg et al., 2008; Lüchtenborg et al., 2014), expression of Gαo in these cells induces a massive neurite outgrowth (Figures S1C and S1D) providing us with a necessary readout system.

We additionally used *Drosophila* S2 cells, which also have low endogenous Gαo levels (http://flybase.org/reports/FBgn0001122.html). Remarkably, we observed that dGαo expression induced long protrusions, not typical for these cells (Figures 1D–1G). These protrusions are initially F-actin-positive, but with time become wider and additionally filled with microtubules (Figure 1D). Live imaging reveals the dynamic nature of these structures (Movie S1). Gαo-induced formation of protrusions in S2 cells served as another, evolutionary distant, readout for the role of Gαo in trafficking.

**Gαo Shows Dual Plasma Membrane and Golgi Localization**

Before going further, we checked the subcellular localization of Gαo in our systems. In N2a cells transfected with human Gαo, immunostainings showed expected PM localization and a strong perinuclear accumulation co-staining with the trans-Golgi marker GaIT-GFP (Figure S1E). A functional C-terminal GFP-fusion of Gαo (Gαo-GFP) (Figures S1C and S1D) also stained PM and Golgi, the latter recognized by the cis-Golgi marker GM130 and a broader Golgi marker MannII-blue fluorescent protein (BFP) (Figure 2A). Gαo localization of Gαo was not affected by cycloheximide (up to 6 hr, not shown), and live imaging of Gαo-GFP revealed a rather static Golgi localization (Movie S2) as opposed to the more dynamic GaIT-GFP marker (Movie S3), suggesting that Gαo does not merely stain Golgi on its way to PM after synthesis. Endogenous Gαo was also found dually at PM and Golgi in human neuroblastoma BE(2)C cells (Figure 2B) and in primary mouse cortical neurons (Figure S1F).

A Golgi localization of mammalian Gαo, with unclear biological relevance, has been seen previously (Akgoz et al., 2004), while such localization has not been described for dGαo or any insect Gs-subunit. In *Drosophila*, Golgi stacks spread all over cytoplasm (Kondylis and Rabouille, 2009), and dGαo-GFP in S2 cells localized at PM and at cis- and trans-Golgi stacks, identified, respectively, with the GMAP-210 and GaIT-mRFP (Figure 2C). Upon heterologous expression in N2a cells, dGαo-GFP also localized at PM and Golgi (Figure S1G).

As a *Drosophila* tissue with endogenous dGαo, pupal wings expressing dArf79F-GFP as a cis-Golgi marker (Shao et al., 2010) revealed dGαo localized at PM at compartments overlapping with this marker (Figure 2D). Some dArf79F-negative dGαo clusters may represent medial- and trans-Golgi stacks, although we cannot exclude additional intracellular compartments. The immunostaining specificity was confirmed by RNAi knockdown (k/d) of dGαo in pupal wings (Figure S1H).

Cumulatively, our findings in different cellular and organism readouts show a dual localization of Gαo to the PM and Golgi compartments.

**Gαo Regulates Vesicular Trafficking at Golgi**

To test for the role of the Golgi-localizing Gαo, we first roughly approached the function of the Golgi apparatus in Gαo-induced neuritogenesis using brefeldin A (BFA). As expected (Nakamura et al., 1995), BFA induced diffuse GM130 staining.
in G\text{xo}-transfected N2a cells; loss of G\text{xo}-GFP from the perinuclear region was also seen leaving the PM pool intact (Figure S1I), while total G\text{xo} levels did not change (Figure S1J). We found that BFA-treated G\text{xo}-expressing cells still formed neurites, with the percentage of cells with neurites and neurite number not significantly affected (Figures 2E–2Q). However, these neurites were much shorter and thinner than in the control cells (Figures 2E and 2H).

Similarly, BFA treatment in Drosophila S2 cells had no effect on dG\text{xo} protein levels (Figure S1K), but significantly reduced the length of protrusions (Figures 2I and 2L), confirming that G\text{xo} functions are also required for protrusion elongation in S2 cells. However, BFA additionally reduced the average number of protrusions per cell (Figures 2I–2K), which may be due to instability of these structures.

These results indicate that G\text{xo} is required for membrane trafficking needed during elongation of G\text{xo}-induced protrusions. To test this directly, we quantified the PM-directed transport using GFP-fusion of the thermosensitive vesicular stomatitis virus glycoprotein (VSVG\text{GFP}-GFP). This construct is retained in endoplasmic reticulum (ER) at 42°C, being released at 32°C for its transport to PM through Golgi (Presley et al., 1997).

Analyzing kinetics of surface accumulation of this construct at 32°C, we found strong acceleration of PM delivery of VSVG\text{GFP}-GFP upon co-expression of G\text{xo} (Figures 2M and 2N).

To provide an independent meter of the effect of G\text{xo} on the Golgi-emanating trafficking, we employed the reverse dimerization (RD) system, whereby the GFP-FM4-hGH fusion protein aggregates in ER until addition of the D/D solubilizer drug, permitting then the secretory trafficking (Gordon et al., 2010). We found that expression of G\text{xo} in N2a cells strongly speeds up secretory protein trafficking of this construct (Figures 2O and 2P). Further, we show with two independent small hairpin RNAs (shRNAs) (Figures S2A and S2B) that k/d of endogenous G\text{xo} from BE(2)C cells significantly slows down the secretory trafficking effect rescued by re-expression of G\text{xo} (Figures 2Q and 2R). It can be seen from Figures 2M–2R that the speed of secretory trafficking is reduced ~2-fold upon k/d of G\text{xo} in BE(2)C cells and increased > 2-fold upon expression of G\text{xo} in N2a cells.

Collectively, these data suggest that Golgi G\text{xo} regulates the PM-directed transport. Our data also indicate that the two pools of G\text{xo} may function cooperatively in the outgrowths: the PM pool of G\text{xo} providing the initial inductor signal for the outgrowth formation, and the Golgi pool maintaining and elongating the outgrowth through stimulating material delivery. In order to separate the PM and Golgi functions of G\text{xo}, we generated a Golgi-only form, goG\text{xo} (STAR Methods). In N2a cells, goG\text{xo} shows a prominent Golgi but essentially no PM localization (Figure 3A) and robust interaction with Golgi partners of G\text{xo} (Figure S2C; see below). Fully supporting our expectations, goG\text{xo} fails to induce any neurite outgrowths (Figures S1C and S1D). At the same time, goG\text{xo} parallels wild-type (WT) G\text{xo} in the speeding up of material delivery from Golgi in the RD assay (Figures 3B and 3C).

G\text{xo} Physically and Functionally Interacts with Small GTPases at Mammalian Golgi

How does the Golgi pool of G\text{xo} activate cargo delivery to PM? We hypothesized that this is achieved through direct interaction with G\text{xo} targets of the vesicular trafficking module (Figure 1C; Table S2). The following mammalian orthologs of the dG\text{xo} targets were chosen for physical interaction analysis: small GTPases of the Rab (Rab1a, Rab3a, Rab4a, Rab5a, Rab7a, and Rab11a) and Arf (Arf1–Arf6) families, dynamin-1 and -2, and clathrin, all crucial components of the secretory and/or endocytic pathways. GFP-fusions of these proteins were expressed in N2a cells together with G\text{xo}-GST (that displayed correct dual localization) (Figure S2D) for the pull-down analysis; empty plasmid or GST-fusion of the Golgi-resident KDEL receptor (KDEL) (Figure S2D) (Townsley et al., 1993) were used as controls. Pull-downs showed robust binding of G\text{xo}-GST to Rab1a, Rab3a, Rab4a and Rab5a; Rab7a and Rab11a where only weakly co-precipitated (Figures 3D, S2E, and S2F). From the Arf family, G\text{xo}-GST strongly interacted with all Golgi-associated Arfs (Arf1–Arf5) but not with the endocytic Arf6 (Figures 3D, S2E, and S2G). In contrast, no direct interactions were detected in this system for clathrin and dynamins (Figure S2H).

We next analyzed co-localization, expressing G\text{xo}-mRFP with the GFP-fusions of the target proteins. Perinuclear G\text{xo} strongly

**Figure 2.** Evolutionary Conserved Localization of G\text{xo} at Golgi

(A–C) Golgi localization of G\text{xo}-GFP determined in mouse N2a cells (A) by co-localization with Golgi markers GM130 and Mannin-BFP. Human BE(2)C cells (B) showed endogenous G\text{xo} co-localizing with GM130 at Golgi. In Drosophila S2 cells (C), dG\text{xo}-GFP labeled Golgi stacks marked by GaIT-mRFP and GMAP-210. Color-channels are listed vertically top-to-bottom and selected areas are magnified with the channels displayed horizontally in the same order left-to-right. Scale bars, 10 μm (A and B); 5 μm (C).

(D) Immunostaining of endogenous dG\text{xo} in Drosophila pupal wings expressing the cis-Golgi marker Arf79F-GFP at 22 hr after puparium formation (APF). A selected region is magnified; dG\text{xo}/Arf79F-positive clusters indicated by arrowheads. Scale bar, 50 μm.

(E–L) Development of protrusions in N2a (E–H) and S2 cells (I–L) induced by G\text{xo} in the absence or presence of BFA. In N2a cells, BFA-treatment affected only neurite length (E–H), whereas both protrusion number and length were reduced in S2 cells (I–L). Quantification of parameters linked to neurite and protrusion formation (F–H and J–L). Scale bar, 50 μm (E); 10 μm (I).

(M and N) G\text{xo} speeds up trafficking to PM as measured with the YSVG\text{GFP}-GFP assay in N2a cells (M). Surface biotinylation determined PM levels of YSVG\text{GFP}-GFP at different time points at 32°C. Biotinylated and input samples were tested with Abs against G\text{xo} and G\text{xo}. Quantification of (N).

(O–R) Reverse dimerization assay in N2a (O and P) and BE(2)C (Q and R) cells. D/D solubilizer induces reduction of the full-length GFP-FM4-hGH and appearance and decrease of a furin-cleaved product (O and Q). G\text{xo} overexpression strongly sped up secretory trafficking in N2a cells (O and P), and its downregulation significantly slowed it down in BE(2)C cells (Q and R). G\text{xo} re-expression in knockdown BE(2)C cells rescued GFP-FM4-hGH secretion (Q and R). Cell extracts were tested with Abs against G\text{xo}, G\text{xo}, α-tubulin (α-tub) and/or GST. Vertical line indicates that the two sides of the same membrane are shown with different exposition times for better visualization (O). Arrowheads point to relevant bands (O and Q). Quantification of the effect of G\text{xo} overexpression (P) and its downregulation and re-expression (R).

Data represent mean ± SEM. ns, not significant; *p ≤ 0.01, **p ≤ 0.005, ***p ≤ 0.001. See also Figures S1 and S2, and Movies S2 and S3.
co-localized with the Golgi-associated Rab1a and Arf1-5 and to a lesser extent with Rab3a (Figures 3E and S3A). A limited co-localization was observed between perinuclear Goxo and Rab11a, clathrin and dynamin, and even less with the endocytic Rab4a, Rab5a, and Rab7a (Figures S3B and S3C). Together, these results identify the small GTPases Rab1a, Rab3a, and Arf1–Arf5 as potential players in the Goxo functions at Golgi.

Next, we analyzed cooperation of Goxo with these partners in neurite outgrowth, using WT and dominant-negative (DN) versions of Rab1a, Rab3a, and Arf1. While Rab1a, Rab3a, or Arf1 alone did not induce any neurite formation nor affected the length of the few spontaneous N2a cell neurites (Figures S3D–S3F), WT versions of Rab1a and Rab3a strongly potentiated the number and length of Goxo-induced neurites in N2a cells (Figures 3F and 3I). At the same time, the proportion of neurite-forming cells and the neurites-per-cell numbers were not influenced by WT and DN forms of Rab3a and modestly affected by the forms of Rab1a (Figures 3F–3H), confirming the interaction between Goxo and these GTPases is important for elongation but not induction of protrusions. In contrast to Rabs, Arf1 WT showed no functional interaction with Goxo, while Arf1DN reduced all Goxo-dependent responses (Figures 3F–3I) through a yet unclarified reduction of Goxo protein levels not seen in other co-transfections (Figures S3G–S3I). Arf1DN also induced a predicted BFA-like phenotype (Dascher and Balch, 1994): loss of the Golgi marker GM130 and of the perinuclear Goxo (Figures S4A and S4B). Conversely, the DN versions of Rab1a and Rab3a had no obvious effect on Goxo localization (Figure S4B).

Together, these data show that physical and functional interactions of Goxo with Rab1a and Rab3a at Golgi are required for neurite elongation in N2a cells.

Goxo Functionally Interacts with Small GTPases at Drosophila Golgi

To support the above conclusion in an independent setting, we performed a similar set of experiments in Drosophila S2 cells. Upon co-transfection of mRFP-fusion constructs of dRab1, dRab3, and dArf79F with dGoxo-GFP, all three small GTPases co-localized with dGoxo at Golgi stacks stained by anti-GMAP–210 (Figures S4C and S4D). While expression of the GTPases alone did not induce protrusions (Figures S4E and S4F), dRab1 and dRab3 potentiated the number and length of dGoxo-induced outgrowths (Figures 4A–4D) without changing the dGoxo-GFP expression levels (Figures S4G and S4H). In contrast, dRab1DN and dRab3DN suppressed dGoxo-induced protrusions (Figures 4A–4D). To continue the similarity with the mammalian system, the dArf79FDN mutant caused an ~50% drop in dGoxo protein levels (Figures S4G and S4H) resulting in reduction in all parameters related to the formation of protrusions (Figures 4A–4D); it also was unique in disassembling Golgi stacks (Figure S5A).

Using live imaging, we found that the dynamics of dGoxo-induced protrusions in S2 cells was strongly suppressed by dRab1DN and strongly enhanced by dRab1WT (Movies S1, S4, and S5). These data show that the function of Goxo as a key player in the Golgi-controlled elongation of outgrowths is conserved from insects to mammals.

Goxo Genetically Interacts with Small GTPases in Developing Drosophila Tissues

We next aimed at testing the role of Goxo in regulation of vesicular trafficking through Golgi-residing small GTPases in vivo, using two well-characterized phenotypes induced by dGoxo: planar cell polarity (PCP) and wing hair formation defects in developing insect’s wings (Katanayev et al., 2005) and neuromuscular junction (NMJ) phenotypes in larvae (Lu¨cchenborg et al., 2014). Both phenotypes are linked to aberrant signaling by Fz receptors, which are GPCRs largely dependent upon Goxo for proper signal transduction (Egger-Adam and Katanayev, 2008; Koval et al., 2011). We hypothesized that these developmental processes may require not only the PM-associated activity of Goxo as a GPCR transducer but also its Golgi function as a regulator of vesicular trafficking.

In pupal wings, each epithelial cell produces a stable outgrowth called trichome or hair. Aberrant Fz and dGoxo activity results in the multiple wing hair (mwh) phenotype, when some cells form two or more hairs instead of one (Katanayev et al., 2005). Remarkably, co-overexpression of dGoxo with dRab3 and especially dRab1 produced a marked, up to 15-fold, enhancement of the dGoxo-induced mwh phenotype, whereas co-expression of dArf79F showed no enhancement (Figures 4E and 4F). Because the sole overexpression of each dRab produced no effect (Figures 4E and 4F), this result illustrates that dGoxo synergistically interacts with dRab1 and dRab3 in this in vivo setting.

Drosophila NMJ is a glutamatergic synapse made by several distinct circular structures—the synaptic boutons—at the axon terminus. Boutons can be visualized with the postsynaptic CD8-GFP-Sh and the presynaptic anti-HRP staining. In larval NMJs, loss or RNAi-mediated k/d of dGoxo leads to strong reduction in the number of boutons and to morphological abnormalities seen as elongated structures with defective overlap of

Figure 3. Functional Interaction of Goxo with Key Small GTPases

(A) In N2a cells, a Goxo-only form of Goxo (goGoxo-GFP) is mostly absent from PM as seen by co-expression of Goxo-mRFP. Scale bar, 10 μm.

(B and C) Reverse dimerization assay in N2a cells. D/D solubilizer induces reduction of the full-length GFP-FM4–HGH and appearance of a furin-cleaved product (B). goGoxo sped up secretory trafficking as efficiently as WT Goxo did (C). Cell extracts were tested with Abs against GFP, GST and α-tubulin (α-tub). Vertical lines indicate different exposure times for each membrane part for better visualization (B).

(D) Pull-downs from N2a cells transfected with Goxo-GST and GFP-fusions of the small GTPases Rab1a, Rab3a, and Arf1.

(E) Co-localization of Goxo-mRFP with GFP-tagged Rab1a, Rab3a, and Arf1 in N2a cells. Boxed areas are magnified (right). Scale bar, 10 μm.

(F–I) Neurite formation in N2a cells co-expressing Goxo-GFP with GFP-fusions of WT or DN forms of Rab1a, Rab3a, and Arf1 (F). Quantification of neurite-associated parameters (G–I). Scale bar, 20 μm.

Data represent mean ± SEM; ns, not significant; *p < 0.01, **p < 0.005, ***p < 0.001. See also Figures S2, S3, and S4 and Table S2.
Figure 4. Gxo Interacts with Small GTPases in Drosophila

(A–D) Protrusions in S2 cells co-expressing dGxo-GFP with GFP-fusions of WT or DN forms of dRab1, dRab3, and dArf79F (A). Quantification of protrusion linked parameters (B–D). Scale bar, 5 μm.

(E and F) In Drosophila wings, the mwh phenotype induced by dGxo overexpression (red ovals) is boosted by dRab1 and dRab3 but not dArf79F (E). Quantification (F).

(G and H) The NMJ phenotypes induced by knockdown of dGxo in Drosophila larvae are suppressed by the overexpression of dRab1 and dRab3 overexpression (G). Note that the sole overexpression of dRab1 and dRab3 produced no phenotype compared to control (G). Synaptic boutons are visualized by CD8-GFP-Sh and anti-HRP staining. Boxed regions are zoomed-in (right). Quantification of synaptic bouton numbers (H). Scale bar, 50 μm.

Data represent mean ± SEM. Numbers in columns represent sample sizes. ns, not significant; *p ≤ 0.01, **p ≤ 0.005, ***p ≤ 0.001.

See also Figures S4 and S5 and Movies S1, S4, and S5.
pre- and postsynaptic markers (Figures 4G and 4H) (Lüchteneborg et al., 2014). We speculated that motoneuron-specific overexpression of dRab1 or dRab3 may revert this phenotype, provided that these Rab5 act downstream of dGso in NMJ formation. Indeed, we found that the dGso k/d phenotype (both the number of boutons and the NMJ morphology) was fully rescued by overexpression of dRab1 or dRab3, while these overexpressions on the wild-type background produced no effects (Figures 4G and 4H). As the k/d effect of the dGso-targeting RNAi cannot be down-titrated by overexpression of an unrelated protein (Lüchteneborg et al., 2014), these data demonstrate the in vivo functional interaction of dGso with dRab1 and dRab3 during NMJ development.

The findings in this section provide evidence for the genetic interactions of Gso with Rab1 and Rab3 in vivo, in two different tissues, supporting our cellular observations.

**Gso Activates Small GTPases at Golgi**

Experiments described above show that Gso physically and functionally interacts with Golgi-residing small GTPases. Next, we bacterially produced GST-tagged Ras1a, Ras3a, and Arf1, as well as previously characterized His6-tagged Gso (Lin et al., 2014) and preloaded these G proteins with GDP or GTPyS mimicking their inactive and active conformations. Subsequent pull-downs confirmed that Gso interactions with Rab1a, Rab3a, and Arf1 are direct (Figure 5A). Importantly, while the nucleotide state of the small GTPases did not affect the interaction with Gso, Gso-GTPyS bound Rab1a and Rab3a by folds more efficiently than Gso-GDP did (Figures 5A and 5B). In contrast, binding to Arf1 was not influenced by the nucleotide state of Gso (Figures 5A and 5B). These data might suggest that Rab1a and Rab3a are effectors of activated Gso at the Golgi apparatus.

Thus, we analyzed if Gso regulates Rab1a and Rab3a activities. Morphologically, co-expression of Gso-mRFP and GFP-Rab1a in N2a cells induced a striking enlargement of the perinuclear region positive for both proteins—the phenotype not seen for Gso co-expressions with other targets (Figure 3F). Similarly, in Drosophila S2 cells, co-expression of dGso-GFP with mRFP-dRab1 induced clustering and tubulation of the Golgi stacks, also not seen in other experimental conditions (Figures S4C and S4D). As Rab1 has been previously associated with Golgi enlargement (Romero et al., 2013), we hypothesized that co-expression of Gso stimulates Rab1a that in turn increases the Golgi size, in a manner conserved from insect to mammalian cells.

To address this, we measured the Golgi area marked by GM130 in N2a cells co-expressing non-tagged Gso and GFP-Rab1a. While overexpression of Gso or Rab1a alone had no effect, their co-expression significantly increased the Golgi size (Figures 5C and 5E). Proving that Rab1a activation is the cause, we found a similar enlargement of the Golgi area induced by the constitutive active Q70L mutant of Rab1a (Figures 5D and 5E), but not by other mutant forms of Rab1a or Gso (Figure S5B). Co-expression with Rab1b also enlarged Golgi (Figure S5C), suggesting that Gso activation of Rab1 is not isoform-specific.

To further assess activation of Rab1 by Gso, we used a FAPP1-PH-GFP construct, commonly served to evaluate phosphatidylinositol 4-phosphate (PI4P) levels in Golgi membranes (Balla et al., 2005). Because Rab1 activation increases PI4P production, FAPP1-PH-GFP recruitment to Golgi indirectly monitors the activity of endogenous Rab1 (Dumareogo-Doroin et al., 2010). Gso overexpression in N2a cells significantly increased Golgi accumulation of FAPP1-PH-GFP (Figures 5F and 5G) without any changes in its protein levels (Figure 5H). As FAPP1-PH can also interact with Arf1-GFP (Balla et al., 2005), we separately employed a GST fusion of the Arf1 effector GGAG (Dell’Angelica et al., 2000). Gso overexpression did not increase the amount of Arf1-GFP pulled down from N2a cell extracts by GST-GGA3 (Figures 5I and 5J). Thus, Gso can activate endogenous Rab1, but not Arf1, at Golgi. Similarly, pull-down of Rab3a-GTP by its effector Rim2 was used as a probe to monitor Rab3 activation (Fukuda, 2004), revealing that Gso overexpression increased 2-fold the amount of activated Rab3a (Figures 5I and 5J). We further found that Gso overexpression increased Golgi accumulation (Figures S5D and S5E) but not protein levels of GFP-Rim2 (Figure S5F) in N2a cells. Because Rab3a also localizes to regions other than Golgi (Figure 3F), these data suggest that Gso...
can activate endogenous Rab3 at Golgi, which results in recruitment of GFP-Rim2 to this compartment.

Overall, these results speak for Rab1 and Rab3 being direct binding partners of Gαo. Further, Gαo can activate these small GTPases in vivo, resulting in enhanced Golgi-derived vesicular transport, necessary for the stabilization and elongation of membrane protrusions.

**Gαo Interacts with the αGDI-Complexed Rab1/3 at Golgi**

We hypothesized that Gαo might act as a GDI displacement factor, “handing over” Rabs from GDI to a bona fide GEF localized to the Golgi. To test this, we co-overexpressed Rab-αGDI—efficient GDI for both Rab1 and Rab3 (Yang et al., 1994)—together with Gαo and GFP-Rab1a/3a. Surprisingly, we found αGDI to promote Gαo-Rab1a/3a interactions (Figures 5K and 5L). Gαo also pulled down αGDI without co-expression of any Rab (Figure S5G); this interaction is likely mediated by endogenous Rabs as recombinant Gαo and αGDI did not directly interact (Figure 5M). In order to test if Gαo is able to directly interact with Rab/αGDI complexes, we purified Rab1a and Rab3a using the baculovirus expression system preserving post-translational prenylation of Rabs, crucial for their interaction with GDIs (Maltese et al., 1996). As expected, prenylated Rab1a/Rab3a were also able to interact with Gαo (Figure S5H) but are not activated by Gαo in vitro (Figures S5I and S5J). Notably, we also succeeded in reconstituation of the Gαo-Rab1/3-αGDI complexes in vitro (Figure 5M). Cumulatively, these data indicate that multimeric Gαo-Rab1/3-αGDI complexes may exist at Golgi.

**Stimulation of KDEL Activation by γ-Free Gαo at Golgi**

We next investigated how Gαo activation at Golgi is organized, considering two possibilities: translocation of active Gαo from PM to Golgi post-activation by GPCR versus the independent activation of Gαo at Golgi. It was suggested that Gβγ heterodimers, but not Gβ2-subunits, could translocate from PM to Golgi after GPCR activation (Akgoz et al., 2004). Despite poor expression (Figures S6A and S6B), the GαoGly92-GFP construct used in those previous experiments revealed the dual PM and Golgi localization similar to our GFP-tagged Gαo construct (Figure S6C). We then applied 3 different means of Gαo activation at PM: co-transfection with muscarinic acetylcholine receptor 2 used in previous translocation studies (Akgoz et al., 2004) or with neuronal cannabinoid receptor type-1, followed by stimulation with acetylcholine or HU-210, respectively. Alternatively, we used the Gi/o-activating peptide mastoparan (Higashijima et al., 1988). In agreement with prior work (Akgoz et al., 2004), none of these treatments increased the perinuclear fluorescence of Gαo-GFP or GαoGly92-GFP (Figures S6D–S6I), confirming that Gαo is not translocated from PM to Golgi upon activation.

Thus, Gαo is a resident of Golgi and must be activated at this compartment. A GPCR-like activity of KDEL has been implicated in activation of the Golgi pool of Gαq and Gαs, which in turn regulate anterograde and retrograde trafficking, respectively (Cancino et al., 2014; Giannotta et al., 2012). KDEL is abundantly localized to Golgi, where it is constantly activated by the C-terminal KDEL sequence of chaperones delivering cargo from ER. A dominant-negative D193N mutant (KDELΔ193N) can bind the KDEL peptide but does not recycle to ER and cannot activate Gαq/Gαs (Cancino et al., 2014; Giannotta et al., 2012; Townsley et al., 1993).

We found that KDEL-GFP co-localized with Gαo-mRFP at Golgi (Figure 5N) and was efficiently pulled down by Gαo-GST (Figure 5O) from N2a cells. Similarly, dGαo and dkDEL co-localize at Golgi stacks in Drosophila S2 cells (Figure 6A). We generated a transgenic Drosophila line for in vivo overexpression of dkDEL (Figure S6J). Co-expression with dkDEL strongly enhanced the Gαo-induced mwh phenotype in Drosophila wings, while the single overexpression of dkDEL produced no PCP phenotype (Figures 6B and 6C), demonstrating that Gαo synergistically interacts with dkDEL in vivo. Together, these data show that Gαo and KDEL physically and functionally interact in an evolutionary conserved manner.

To test whether KDEL may possess a GEF activity toward Gαo, we employed the GTP-Eu loading assay in saponin-permeabilized HeLa cells (Koval and Katanava, 2011). We found that expression of Gαo renders them responsive to the external stimulation with a KDEL-containing synthetic peptide (Figure 6D).
Co-expression of the D/N, but not WT KDELR, blocked the KDEL-peptide-mediated activation of G\(\alpha_o\) (Figure 6D).

We next tested where KDELR activates G\(\alpha_o\), using an antibody specific for the GTP-bound form of G\(\alpha_o\) (Figures S6K and S6L). We generated a secretable GFP construct carrying a C-terminal KDEL signal (ssBFP\(_{KDEL}\)) that acts as a long-lasting KDELR ligand (Figures S6M–S6O) (Pulverenti et al., 2008). N2a cells were co-transfected with G\(\alpha_o\)-GFP and ssBFP\(_{KDEL}\) or a control (ssBFP). The anti-G\(\alpha_o\)-GTP staining and GFP-fluorescence were used to estimate the GTP-loading of G\(\alpha_o\) relative to its total protein level at PM versus Golgi. We found that ssBFP\(_{KDEL}\) significantly increased the G\(\alpha_o\)-GTP/G\(\alpha_o\)-total ratio at Golgi but not PM without changing the G\(\alpha_o\) Golgi/PM distribution (Figures 6E–6G), proving that stimulation of endogenous KDELR-induced activation of G\(\alpha_o\) at Golgi. Furthermore, KDELR overexpression (known to induce its self-stimulation) (Hsu et al., 1992) also activated the G\(\alpha_o\) (Figures S7A and S7B).

Although GPCRs act on heterotrimeric G\(\beta\gamma\) proteins, we found no Golgi localization of the ubiquitous G\(\beta_1\)-subunit (endogenous nor overexpressed) in G\(\alpha_o\)-expressing N2a cells (Figure S7C). Even the triple co-expression of G\(\alpha_o\), G\(\beta_1\), and the neuronal G\(\gamma_3\) (the complex previously reported to exist in Golgi) (Aijth Karunarathne et al., 2012) revealed essentially no localization of G\(\beta\gamma\) to the Golgi enriched in G\(\alpha_o\) in N2a cells (Figure 6H). We next performed pull-downs using GST-G\(\alpha\) or GST-G\(\beta\)-G\(\gamma\)1 from cells expressing GFP-fusions of KDELR, G\(\gamma_3\), and G\(\beta\)1 or G\(\alpha_o\). As expected, G\(\alpha_o\)-GST efficiently precipitated not only KDELR-GFP but also GFP-G\(\beta\)1 and GFP-G\(\gamma_3\) (Figure 6I). On the other hand, GST-G\(\beta\)1 effectively pulled down G\(\alpha_o\) and G\(\gamma_3\) whereas binding with KDELR was totally absent (Figure 6I). Together, these experiments suggest that uniquely for GPCRs, KDELR interacts with and activates G\(\beta\gamma\)-free G\(\alpha_o\) rather than a heterotrimeric G\(\alpha\)\(\beta\)\(\gamma\) complex.

Next, we analyzed if the KDELR \(\rightarrow\) G\(\alpha_o\) activation influences neuritogenesis. We co-transfected N2a cells with both proteins and quantified the neurite outgrowth. We additionally used KDELR\(_{D/N}\), which did not affect G\(\alpha_o\) localization (Figure 6N) and interacted as efficiently as the WT with G\(\alpha_o\)-GST (Figure 5O). The sole expression of KDELR did not induce neurite outgrowth, and co-expression of G\(\alpha_o\) with the WT or D/N mutant of KDELR did not change the percentage of cells forming neurites (Figures 6J and 6K). Yet, co-expression of WT KDELR increased 3-fold the total neurite length accompanied by mild augmentation in neurite numbers (Figures 6J–6M) without varying G\(\alpha_o\) protein levels (Figures S7D and S7E). Conversely, co-expression with KDELR\(_{D/N}\) did not affect neurite number and length (Figures 6J–6M), implying that this mutant is unable to potentiate G\(\alpha_o\) functions.

Similarly, dKDELR co-expression increased the length and number of dG\(\alpha_o\)-induced protrusions in Drosophila S2 cells (Figures 6N–6Q), whereas the sole expression of dKDELR had no effect (Figures S4E and S4F). On the other hand, dKDELR\(_{D/N}\) reduced both the length and number of protrusions induced by dG\(\alpha_o\) (Figures 6N–6Q), without impact on dG\(\alpha_o\) localization and expression (Figures 6A, S4G, and S4H). Regarding the KDELR\(_{D/N}\) mutant, we provide evidence in three different cell types, in two of which it behaves dominant negatively (HeLa and S2) (Figures 6D and 6N–6Q) and in one, just negatively (N2a) (Figures 6J–6M). We suspect that the negative activity of this form of KDELR becomes dominant depending on the cell type and/or relative expression levels.

Thus, KDELR emerges as an evolutionary conserved activator of the G\(\beta\gamma\)-free Golgi pool of G\(\alpha_o\) required for the elongation of cellular protrusions.

**KDELR Acts on a Multiprotein Complex Containing G\(\alpha_o\) and xGDI-Rab Complexes**

As G\(\alpha_o\) interacts with the xGDI-Rab1a/Rab3a pair, we wondered whether this pair might be present at the KDELR-G\(\alpha_o\) complexes instead of G\(\beta\gamma\). Indeed, we found that the pull-down of xGDI (and by inference of xGDI-Rab complexes) is increased by several folds upon KDELR overexpression (Figures 7A and 7B), although KDELR does not interact with xGDI (Figure S5G) nor Rab1 (Figure S2E) in the absence of G\(\alpha_o\). Thus, we infer formation of multi-subunit assemblies containing KDELR, G\(\alpha_o\), xGDI, and Rab at Golgi.

Next, we tested the reaction of these assemblies to KDELR activation. We showed above that it results in the nucleotide exchange on G\(\alpha_o\), as seen for other GPCRs. To continue this parallel, we find that KDELR activation with ssBFP\(_{KDEL}\) dissociates G\(\alpha_o\) from the receptor (Figures 7C and 7D). In further resemblance to the heterotrimeric G protein activation by GPCRs whereas the G\(\beta\gamma\) dissociates from G\(\alpha_o\), we find that the interaction of G\(\alpha_o\) with xGDI (Figures 7A and 7B) and Rab1a/Rab3a (Figures 7E–7H) is strongly diminished upon KDELR activation. G\(\alpha_o\) does not act as a GEF for Rab1a/Rab3a (Figures S5I and SSJ). Further, nucleotide exchange on the small G proteins is not a prerequisite for the KDELR-induced dissociation of Rab1a/Rab3a from G\(\alpha_o\), as the Rab1aDN and Rab3aDN mutant forms incapable of GTP-loading are also dissociated from G\(\alpha_o\) upon KDELR activation (Figures 7E–7H). These findings imply an involvement of a bona fide Rab GEF localized to Golgi in the final step of Rab activation upon the release of Rabs from G\(\alpha_o\). The interaction between G\(\alpha_o\) and Rabs, dissociated by KDELR activation, is a prerequisite for this final step.

To test if KDELR activation indeed results in Golgi Rab activation, we took the BE(2)C cells naturally expressing G\(\alpha_o\), stimulated endogenous KDELR by expression of ssBFP\(_{KDEL}\), and monitored Rab1 activation by the Golgi enrichment of FAPP1-PH-GFP (Figures 5F–5H). We indeed found that KDELR stimulation leads to activation of Rab1 on Golgi (Figures 7I and 7J). This phenomenon is critically G\(\alpha_o\)-dependent, as removal of G\(\alpha_o\) by shRNA completely blocks activation of Rab1 by KDELR, and re-expression of G\(\alpha_o\) rescues this block (Figures 7I and 7J).

Our work discovers a mechanism of KDELR-induced activation of G\(\alpha_o\) and Rab GTPases at Golgi needed to speed up material delivery to the growing cellular protrusions. The overall sequence of events starting from activation of KDELR by the arrival of the KDEL-containing chaperones accompanying the ER-delivered cargos and leading, in the G\(\alpha_o\)-dependent manner, to activation of Rabs, is schematized in Figure 7K and is further detailed in the Discussion.

**DISCUSSION**

Intracellular signaling pathways currently emerge more as dynamic networks of protein interactions rather than linear cascades.
of activation/inactivation reactions. In this regard, thorough elucidation of the interaction targets of heterotrimeric G proteins—the immediate transducers of GPCRs—is of crucial importance to advance the understanding of this type of signal transduction. It is especially true for Goα. Being the most abundant G protein in the nervous system and controlling multiple evolutionary conserved developmental, physiologic, and pathologic programs, it has been remarkably shy in revealing its signaling...
partners. Here, we disclose results of our multiple overlapping screens, identifying > 250 interaction partners of Gαo. Each of the screens performed has its inherent advantages and limitations (Beltrao et al., 2012), and by complementation, we expect to have reached a near complete coverage of the Gαo interactome—an endeavor rarely performed for a signaling protein. Cherry-picking of individual proteins from this network resulted in detailed descriptions of mechanisms of Gαo-controlled regulation of Wnt/Fz signaling, synapse formation, PCP, asymmetric cell divisions, endocytic regulation, etc. (Egger-Adam and Katanaev, 2010; Kopelin and Katanaev, 2009; Lin and Katanaev, 2013; Lin et al., 2014; Lüchtingborg et al., 2014; Purvanov et al., 2010), validating the interactome findings.

As opposed to characterizations of selected individual Gαo partners, we now aimed at identifying functional modules within the interactome. For this, we performed bioinformatics analysis clustering the individual components by their functions. This resulted in appearance of several major cellular activities, which now emerge to be regulated by Gαo-dependent GPCR signaling. We selected one of them, vesicular trafficking, for detailed investigation. Many important components of this cellular function, both endocytic and exocytic, are found among Gαo targets. We previously characterized interaction of Gαo and the endocytic master regulator Rab5, important for GPCR internalization and signaling (Purvanov et al., 2010). Now, we focus more on the exocytic function of Gαo. In various cell types (neuronal, epithelial, mesenchymal) of different animal groups (insect and mammalian) we now find a dual localization of Gαo to Golgi and PM, and we find the coordinated action of the two pools in exocytosis and formation of various types of cellular protrusions. We further uncover the evolutionary conserved KDEL → Gαo → Rab1/Rab3 pathway at Golgi, required for stimulated material delivery to PM and the growing protrusions.

KDEL is a Golgi-residing GPCR-like receptor, activated by the cargo delivery from ER and regulating both anterograde and retrograde trafficking from Golgi (Cancino et al., 2014; Giannotta et al., 2012; Townsley et al., 1993). Here, we show that from Drosophila to mammals, KDEL binds Gαo and activates it, potentiating Gαo-induced cellular responses. Intriguingly, we show that it is the βγ-free form of Gαo, which is the binding and activation partner of KDEL—in a sharp contrast to the action of typical PM-localized GPCRs that act on heterotrimeric Gαβγ complexes. We further find that KDEL and Gαo form a multi-subunit complex, additionally containing Rab1/Rab3 GTPases and αGDI. Activation of KDEL results in the nucleotide exchange on Gαo and its dissociation from KDEL. Although recombinant Rab5 interacts stronger with the GTP-loaded Gαo in vitro in absence of αGDI, in cells we find that activation of Gαo leads to dissociation of the Rab1/Rab3-αGDI complexes, ultimately resulting in activation of the small GTPases and stimulated anterograde material delivery, necessary for the growth and stabilization of cellular protrusions. Activation of KDEL is known to induce formation of multicomponent aggregates recruiting a number of additional proteins (Majoul et al., 2001); recruitment of Rab-GEFs to these complexes to mediate ultimate activation of Rab1/Rab3 is also conceivable but will require further investigation. Importantly, the Golgi pool of Gαo plays key roles in these processes, as the anterograde transport as well as KDEL-mediated Rab1 activation are inhibited upon depletion of Gαo.

Based on the data presented here, a model emerges whereas specific Gαo pools at PM and Golgi play different but cooperative roles during neuritogenesis and protrusion formation in general. At PM, Gαo initiates neurite formation regulating actin and microtubule cytoskeletons (Bromberg et al., 2008; Lüchtingborg et al., 2014) in response to activation by specific GPCRs. At Golgi, the atypical GPCR KDEL induces activation of βγ-free Gαo, which subsequently activates Rab1 and Rab3, and the combined action of these proteins potentiates the PM-directed trafficking required for elongation and stability of membrane protrusions (Figure 7K). Being conserved from Drosophila to mammals, this molecular mechanism is of basic importance for the understanding of G protein functions in development, physiology, and disease.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures, three tables, and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2017.07.015.
AUTHOR CONTRIBUTIONS

G.P.S., O.B., A.-M.L., C.L., and A.K. performed experiments. G.P.S. and V.L.K. designed the work and wrote the paper.

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links Wnt-Frizzled signaling with ankyrins to regulate the neuronal microtubule cytoskeleton. Development 141, 3399–3409.


# STAR★METHODS

## KEY RESOURCES TABLE

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Proteomic analysis | Proteomics Facility University of Konstanz | https://www.biologie.uni-konstanz.de/proteomics-centre/

### Experimental Models: Cell Lines

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### Experimental Models: Organisms/Strains

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<td>lines containing P-element insertion mutations</td>
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<td><em>D. melanogaster</em></td>
<td>a cohort of transgenic RNAi lines</td>
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**Oligonucleotides**

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**CONTACT FOR REAGENT AND RESOURCE SHARING**

Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Vladimir L. Katanaev (vladimir.katanaev@unil.ch).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Drosophila Stocks**

The following *Drosophila* lines were used: MS1096-Gal4, UAS-Gaq (Katanaev et al., 2005), OK371-Gal4; CD8-GFP-Sh (Lüchtenborg et al., 2014); MS1096-Gal4 (8860), pnr-Gal4 (25758), hs-hid (24638), UAS-Rab1 (24104) and UAS-Rab3 (9763) – from Bloomington Drosophila Stock Center (BDSC); UAS-Arf79F-GFP was kindly provided by Tony J. C. Harris (University of Toronto); UAS-RNAi-Gaq (110552 and 19124) as well as the other UAS-RNAi lines – from Vienna Drosophila Resource Center (VDRC); lines containing...
**P-element insertional mutations** – from the Szeged Drosophila Stock Centre. UAS-KDEL-mRFP line was generated through site-specific germ-line transformation of oX-22A line with attP-landing site on the chromosome arm 2L (24481, BDSC). All crosses were performed at 25°C on the standard cornmeal medium.

**Cell lines and culture conditions**

Male mouse neuroblastoma Neuro-2a (N2a) and female human epithelial HeLa cells were maintained in MEM (Thermo Fisher Scientific), supplemented with 10% FCS, 2 mM L-glutamine, 1 mM pyruvate, and 1% penicillin-streptomycin at 37°C and 5% CO2.

Male human neuroblastoma BE(2)C cells (ATCC CRL-2268) were generously provided by Karim Abid (University Hospital of Lausanne). Cells were grown in DMEM/F-12 (Thermo Fisher Scientific), supplemented with 10% FCS, 2.5 mM L-glutamine, 1200 mg/L sodium bicarbonate, and 1% penicillin-streptomycin at 37°C and 5% CO2.

Male *Drosophila* Schneider-2 (S2) cells were maintained in Schneider’s *Drosophila* Medium (Lonza) supplemented with 10% FCS and 1% penicillin-streptomycin at 28°C.

Female *Spodoptera frugiperda* (Sf9) cells were maintained in TNM-FH (Sigma-Aldrich) supplemented with 10% FCS and 1% penicillin-streptomycin at 28°C either as shaken suspension (60 rpm).

Poly-L-lysine-coated coverslips containing primary mouse cortical neurons obtained from E16 female and male embryos (C57BL/6J mice) grown in Neurobasal medium (Thermo Fisher Scientific) containing 2% B27 supplement, 1 mM L-glutamine and 1% penicillin-streptomycin were kindly provided by Omar Aljievic (University of Lausanne).

All vector transfections were carried out with X-tremeGENE 9 or HP (Roche). Briefly, DNA plasmids were mixed to equal mass rations and incubated for 5 min at room temperature. Then, the transfection reagent was diluted in Opti-MEM (Thermo Fisher Scientific) to a ratio of 5 μL reagent/100 μL medium, vortexed and added to a plasmid mix of 1.5 μg, vortexed and incubated for 20 min at room temperature, and finally added dropwise on cells grown in 12-well plates to 80%-90% confluence. Transfection were lineally scale up when needed.

**METHOD DETAILS**

**Screens**

**Two-hybrid screening**

A saturating yeast two-hybrid screening was performed by Hybrigenics (Hybrigenics-Services). As the prey, a random-primed *Drosophila* adult head cDNA library constructed into pP6 plasmid and 54 and 95 million clones (5- and 9.5-fold the complexity of the library) were screened for Gαo and for Gzol[Q205L], respectively, using a mating approach with Y187 (mata) and L40ΔGal4 (mata) yeast strains. His+ colonies were selected on a medium lacking tryptophan, leucine, and histidine for the Gαo WT and mutant (n = 225 and 171, respectively). Positive prey fragments were amplified by PCR and sequenced at their 5′ and 3′ junctions. The resulting sequences were used to identify the corresponding interacting proteins in the FlyBase database using a fully automated procedure. A confidence score (predicted biological score) was attributed to each interaction (Table S1). Many Gαo interaction partners were identified in multiple independent hits, and half of these interactions showed the highest confidence scores. The other half had a lower or non-computable confidence score, and was represented by only one or two hits, suggesting a lower probability of these interactions.

**Affinity purification screening**

Unlike the yeast-two hybrid screen which identifies proteins directly interacting with the bait, the affinity purification screen has the advantage of identification of multiprotein complexes binding the bait. Thus, *Escherichia coli* Rosetta-gami 2(DE3) (Novagen) was transformed with His6-Gzol, His6-Gzol[Q205L] or His6-Gzl as control (Kopein and Katanaev, 2009), grown at 37°C to an OD600 = 0.5 before induction with 1 mM IPTG and additional growth overnight at 28°C, followed by harvesting by centrifugation and storage at –20°C. All subsequent procedures were performed at 4°C. Cell pellets were resuspended in PBS supplemented with 1 mM EGTA, 5 mM β-mercaptoethanol, and 1 mM PMSF, and lysed by a cell disruptor at 0.8 psi (Constant Systems). Debris was removed by centrifugation at 18,000xg for 30 min at 4°C. The supernatant was applied to Ni-NTA agarose beads (QIAGEN) pre-equilibrated in the same buffer and purification of the His6-Gzol proteins was performed using the ÄKTAprime plus protein purification system (GE Healthcare). The Ni-NTA beads were washed three times with 10 resin volumes of washing buffer (PBS supplemented with 1 mM EGTA, 5 mM β-mercaptoethanol, 0.1 mM PMSF, and 30 mM imidazole). Proteins were eluted with 200 mM imidazole in the washing buffer. The buffer of purified proteins was exchanged to 50 mM HEPES, pH 7.5, 150 mM KCl, 10 mM NaCl, and 1 mM DTT using Amicon 10kDa Centrifugal Filter Units (Millipore), and proteins were coupled to CNBr-activated Sepharose 4 Fast Flow beads (GE Healthcare). This coupling does not decrease the guanine nucleotide-binding properties of Gzol (Kopein and Katanaev, 2009). CNBr-immobilized Gzol (50% slurry, 100 μl) was preloaded with 100 μM GDP or GTPγS in 50 mM HEPES, pH 8.0, 100 mM KCl, 25 mM MgCl2, and 1 mM DTT for 30 min at 25°C. A 20-fold volume excess of *Drosophila* head extracts was added to the slurry and incubated for 4 hr at 4°C under rotation. Matrixes were centrifuged (2000g for 1 min at 4°C), supernatant discarded, and beads were washed two times with 10 bed volumes of the binding buffer (50 mM HEPES, pH 7.5, 150 mM KCl, 10 mM NaCl, 2 mM EGTA, protease inhibitor cocktail (Roche), 0.5% Nonidet P-40, and 0.1% Tween20) at 4°C. Bound proteins were eluted by 8 M urea, separated by SDS-PAGE, and numerous bands seen to be specifically retained on the Gzol-matrixes were subjected to proteomic analysis at the Proteomics Facility University of Konstanz, Germany.
For the generation of Drosophila head extracts, adult flies were anesthetized by CO₂ and frozen using liquid nitrogen. Heads were separated from the bodies using the Mini-Sieve set (Bel-Art Products) and smashed on ice in a glass-rod homogenizer (Sartorius) in a hypotonic buffer (10 mM HEPES, pH 7.5, 2 mM EGTA, and protease inhibitor cocktail). After adjusting the ionic strength by adding KCl to 100 mM and HEPES to 50 mM, the debris was removed by a 15 s spin at 200xg and 4 °C. The supernatant was centrifuged at 20,000xg for 60 min at 4 °C. The pellet was solubilized by rotation for 4 hr at 4 °C in 50 mM HEPES, pH 7.5, 150 mM KCl, 2 mM EGTA, 0.5% NP-40, 0.1% Tween 20, and protease inhibitor cocktail at a concentration of 1.5 mg/ml. Extracts were cleared by a final centrifugation at 20,000xg for 30 min at 4 °C. The resulting supernatant was immediately used in pull-down experiments.

**Genetic screening on essential genes**

To take advantage of Drosophila genetics in order to find physiologically relevant Gαo partners, we overexpressed Gαo in developing Drosophila wing. Such overexpression leads to a defect in wing spreading due to perturbation in the late stages of wing maturation. We recombined the MS1096-Gal4 line driving expression within the wing with the UAS-Gαo transgene on the first chromosome for the ease of screening. The resulting MS1096-Gal4, UAS-Gαo line had the following phenotypes: 1) 78% of heterozygous female flies had folded wings; 2) 93% of hemizygous male flies had folded wings; 3) the hemizygous male viability was decreased; 4) the size of hemizygous male flies was severely decreased as compared to the females and normal male flies; 5) asymmetric cell divisions in the sensory organ lineage were often defective, resulting in aberrant sensory bristles of the wing margin; 6) planar cell polarity defects were seen in the wing blade, mainly as production of multiple wing hairs. In this suppressor-enhancer screen, we used the wing spreading and the male viability defects as the main readout phenotypes; in some cases, the planar cell polarity phenotypes of the resulting wings were also analyzed (Table S1). The MS1096-Gal4, UAS-Gαo line was crossed to a collection of the P-element insertional mutant lines, carrying aberrations in ca. 50% of the second chromosome essential genes which is ca. 25% of the total vital genes of the Drosophila genome (Szeged Drosophila Stock Centre). At least twenty individuals from each cross were collected and analyzed. Genes, mutations of which suppressed or enhanced Gαo gain-of-function readout phenotypes, were considered as hits. Statistical differences in the penetrance of these phenotypes from the control group (MS1096-Gal4, UAS-Gαo line) were used to evaluate the confidence scores.

**Loss-of-function genetic screening**

In contrast to Gαo mutants which are early embryonic lethal (Frémond et al., 1999) its tissue-specific knockdown by UAS-RNAi transgenes gives viable phenotype. The effectiveness of these constructs was previously confirmed (Lüchtenborg et al., 2014). Based on that, we designed genetic loss-of-function suppressor-enhancer screen using RNAi-mediated downregulation of the targeted genes. Thus, we created MS1096-Gal4 line driving expression of MS1096-Gal4, UAS-RNAi-Gαo and UAS-RNAi-Gαo lines, driving expression of the UAS-RNAi transgenes in the wing and notum tissues, respectively. Combination of the two different systems using same approach allows screening for Gαo partners implicated in different tissue developmental programs, thus potentially uncovering more genetic interactions. For the ease of screening, created lines also contained an hs-hid transgene on the Y chromosome to obtain virgin females in large numbers: 3rd instar larvae were exposed to a heat-shock at 37 °C for 1 hr to kill only males carrying this transgene. This suppressor-enhancer screen was carried out using a cohort of transgenic RNAi lines targeting ca. 10% of protein-coding genes of Drosophila genome according to the latest Flybase (FB2017_03) release. The RNAi lines were successively crossed to the lines described above. Minimum flies of both sexes (amount of the analyzed individuals varied largely depending on the viability of the progeny) from each cross were analyzed: phenotypes were identified, their strength judged in a semiquantitative manner. Since Gαo downregulation itself produced no or just very mild phenotypes in these screen-systems, we considered as hits the genes which loss-of-function phenotypes (if any) was suppressed or enhanced by co-downregulation of Gαo. The false-positive and false-negative rates for the VDRC RNAi library are estimated to be 7 and 29.4%, respectively. All results and supporting information of the screenings can be found in Table S1.

Noteworthy, each of these screening approaches has its own advantages and shortcomings, essentially targeting different subspaces of the interactome (Beltrao et al., 2012) and resulting in a limited overlap of the interaction partners identified in the different screens (Table S1). In a complementary manner, the interaction partners from the different screens build up a near-complete Gαo interactome.

**Bioinformatics analysis**

Gene Ontology (GO) terms enrichment analysis was done by DAVID (https://david.ncifcrf.gov/) using the whole Drosophila genome as background. Over-represented GO terms from the “biological process” domain were used to generate a Functional Enrichment Map. For that we applied an Enrichment Map plugin (http://www.baderlab.org/Software/EnrichmentMap) which uses Cytoscape (http://www.cytoscape.org/) as a software platform to visualize the outcome as a network. Tune parameters were: P value cut-off of ≤ 0.001, Q-value cut-off of ≤ 0.05 (equals Benjamini correction values), overlap coefficient cut-off of ≤ 0.6. Resulting network was clustered using MCL clustering algorithm and then annotated by ClusterMaker (http://www.ruby.ucsf.edu/cytoscape/cluster/clusterMaker.shtml) and WordCloud (http://baderlab.org/Software/WordCloudPlugin) plugins, respectively. Annotations of the final functional modules were manually edited. The same bioinformatics analysis was applied to generate the Enrichment Map of human orthologs. For orthology prediction, we used an in-house-made PERL scripts which extract and unify the data from the 10 major orthology databases (Bilousov et al., 2014). The programs created in-house are available at the laboratory’s web-page (https://www.unil.ch/dpt/home/menuinst/recherche/groupe-katanaev/files.html). To identify the genes involved in different aspects of vesicle-mediated transport we selected genes associated with the respective term as the backbone, and supplemented this list by adding
manually the genes having related GO annotations, and also genes whose human orthologs are known to be involved in vesicle-mediated trafficking. All results of the bioinformatics analysis can be found in Table S2.

**Permanently-transfected BE(2) cells**

Permanent Goxo depletion in BE(2)C cells was obtained by shRNA interference with annealed primers (Table S3) expressed in the pRetroSuper vector (Oligoengine) as previously described (Solis et al., 2012). Thus, annealed primers were cloned using the BamHI and HindIII sites of the pRetroSuper vector, and the empty vector was used as control. For generation of stable-transfected cell lines, shRNA vectors were transfected into BE(2)C cells, and after 48 hr cells were cultured under selection in 5 μg/ml puromycin (Invitrogen). Cell lines were grown in medium supplemented with 2 μg/ml puromycin to maintain selection pressure.

**Plasmids and molecular cloning**

Wild-type (WT) and Q205L mutant of human Goxo in pcDNA3.1+ (Thermo Fisher Scientific) were obtained from Missouri S&T cDNA Resource Center and used as template to generate C-terminal GFP, mRFP and BFP fusions. Specifically, Goxo cDNAs were PCR-amplified (primers listed in Table S3) and cloned into the EcoRI and Apal sites of pEGFP-N1 (Clontech), pmRFP-N1 (Claudia Stuermer, University of Konstanz) and pEBFP2-N1 (Addgene, 54595). For the generation of the Golgi-only construct of Goxo (goGoxo-GFP), the cDNA fragment containing the amino acids 30 to 354 of the rat Goxo (Kopein and Katanaev, 2009) was PCR-amplified and cloned in frame into the R1WTSH-GFP vector (Claudia Stuermer, University of Konstanz) in the BamHI/Agel sites lying between R1WTSH and GFP. The R1WTSH sequence encodes for the N-terminal 30 amino acids of reggie-1/flotillin-1, and showed an almost exclusive Golgi localization in N2a cells (Figure S7F) due to myristoylated and palmitoylated residues within this region. Deletion of the N-terminal 29 amino acids of Goxo (highly conserved among Gox/o members) was chosen because a corresponding Gzi1 construct showed that this region is necessary for membrane binding but dispensable for pivotal biochemical properties, such as GPCR binding and GPCR-mediated activation. To generate the Goxo-GST constructs, the GST cDNA was PCR-amplified from pGEX-4T-1 (GE Healthcare) and used to replace the Agel/NotI GFP sequence from Goxo-GFP and goGoxo-GFP. The Drosophila Goxo cDNA (Kopein and Katanaev, 2009) was PCR-amplified and cloned into pEGFP-N1 by EcoRI and BamHI to generate a C-terminal GFP-fusion. The dGoxo-GFP sequence was then digested with EcoRI and NotI, and subcloned into the pAc5.1/V5-HisA plasmid (Thermo Fisher Scientific) for its expression in Drosophila cells. The His6-tagged Goxo was previously reported (Lin et al., 2014), and the GoxoG92-GFP construct was kindly provided by Narasimhan Gautam (Agkoz et al., 2004). The BamHI/Xhol insert containing the Rab1a cDNA was cut from the pMyc-Rab1a plasmid (Dupré et al., 2006) and ligated into the BFP and Sall sites of pEGFP-C1 (Clontech) and into pGEX-4T-1 by the same restriction sites to generate the GFP-Rab1a and the bacterial GST-Rab1a constructs, respectively. The Rab3a sequence was PCR-amplified from the pRetroSuper vector (Oligoengine) as previously described (Solis et al., 2012). Thus, annealed primers were cloned using the BamHI and XhoI sites of the pGEX-4T-1 plasmid to produce the GST-Rab3a bacterial construct. For baculovirus expression, the BamHI/NotI coding inserts of Rab1a and Rab3a cut from the GST plasmids were ligated into the same sites of the pFastBac-NT vector with a N-terminal His6c-tag (Thermo Fisher Scientific). The Arf1-GFP plasmid (Chun et al., 2008) was used to create the GST-Arf1 plasmid for bacterial expression by PCR amplification, digestion with BamHI and XhoI, and insertion into the pAc5.1/V5-HisA plasmid. The S25N and Q70L mutants of Rab1a, the T36N mutant of Rab3a and the T31N mutant of Arf1 were obtained by point mutagenesis. The Rab1b cDNA was amplified from the plasmid pSV-Rab1b provided by Angelika Barnekow (University Muenster), cut with BamHI and KpnI, and inserted into pEGFP-C1 by BgIII and KpnI. The plasmids pJAF-Arf2, pJAF-Arf3 and pJAF-Arf4 obtained from Gregory J. Paozur (University of Massachusetts Medical School) were used to generate GFP-fusions of Arf2, Arf3 and Arf4, respectively, by cutting the Arf cDNAs with KpnI and ligating into the same site of pEGFP-N1. The GST-zGDI plasmid was kindly provided by Jean Grunenberg (University of Geneva) and Marino Zerial (Max Planck Institute of Molecular Cell Biology and Genetics), and the GFP-zGDI construct was generated by ligating the KpnI/Xmal fragment from the original GST-zGDI into the same sites of pEGFP-C1. The GalT-GFP, -mRFP and -BFP constructs were created by replacing the Agel/NotI mTurquoise2 sequence of the Gaft-mTurquoise2 plasmid (Goedhart et al., 2012) by the corresponding GFP, mRFP and BFP cDNAs cut from pEGFP-N1, pmRFP-N1 and pEBFP2-N1, respectively. The resulting GalT-mRFP sequence was digested using Afl and NotI, and inserted into pAc5.1/V5-HisA by EcoRV and NotI for expression in Drosophila cells. The Agel/BsrGl GFP insert from the pEGFP-C1 was used to replace the corresponding mRFP sequence from the mRFP-CI plasmid (Claudia Stuermer, University of Konstanz). The KDEL-CFP was generously provided by Angel Velasco (University of Seville) and the KDELARF and -GST constructs were created by replacing the Agel/NotI CFP sequence by the corresponding GFP or GST inserts cut from pEGFP-N1 and goGoxo-GST, respectively. The KDELARF D193N mutant was generated by point mutagenesis. The secretable GFP construct (ssGFP) containing the prion protein secretion signal upstream of GFP in the pEGFP-C1 plasmid (provided by Edward Malaqa-Trillo, Universidad Peruana Cayetano Heredia) was used to generate the ssBFP construct by exchanging the Agel/BsrGl GFP sequence with the matching BFP insert from pEBFP2-N1. A C-terminal KDEL retention sequence was introduced into the ssBFP plasmid to generate the ssBFP-GDEL plasmid by point mutagenesis. The GFPT7-Rim2-RBD plasmid obtained from Mitsunori Fukuda (Tohoku University) was used to generate the bacterial GST-Rim2 construct by cutting with the BamHI and NotI enzymes, and ligating into pGEX-4T-1 by the same sites. The GST-Rim2 vector was then digested with BamHI and EcoRI, and the insert containing the Rim2-RBD sequence was subcloned into the BgIII and EcoRI sites of pEGFP-C1 to create the GFP-Rim2 plasmid. The GFP-Gji1 construct was created by replacing the Agel/BsrGl mCerulean sequence of the mCerulean-Gji1 plasmid (Thaler et al., 2005) with the corresponding GFP cDNA cut from pEGFP-C1. For the GST-Gji1, the GST sequence was PCR-amplified...
from the pGEX-4T-1 plasmid, and used to replace the Agel/Xhol mCerulean cDNA from the original plasmid. The Gy3 cDNA was
digested from the pHA-Gy3 plasmid (Missouri S&T cDNA Resource Center) using HindIII and Xhol, and ligated into pEGFP-C3 (Clontech) by HindIII and SalI to generate a GFP-Gy3 construct. The Drosophila cDNAs for Rab1 (F10154), Rab3 (LP05860), Arf79F (LD24904) and KDEL (LD06574) were obtained from the Drosophila Genomics Resource Center (DGRC). The mRFP-dRab1 was

immunofluorescence and microscopy

For immunostaining, N2a and BE2/C were transfected for 6 hr, trypsinized and seeded on poly-L-lysine-coated coverslips for additional 18 hr before fixation. S2 cells were transfected for 24 hr, washed and resuspended in complete media, and seeded on poly-L-lysine-coated coverslips for 30 or 120 min before fixation. All cells were fixed and permeabilized for 30 min with 0.1% BSA, incubated with primary in blocking buffer for 2 hr at room temperature, washed and subsequently incubated with fluorescent secondary antibodies in blocking buffer for 2 hr cells at room temperature. All fluorescent-labeled secondary antibodies were from Jackson ImmunoResearch. Coverslips were finally mounted with Vectashield (Vector Labs) for microscopy analysis. Samples were recorded with a Plan-Neofluar 10x/0.3 objective on an Axio Imager.M1 microscope equipped with an AxioCam HRc camera, and analyzed using the AxioVision software (all from Zeiss) and ImageJ. The number of transfected cells displaying neurites, neurites per cell and total neurite length were scored from 10-20 randomly taken images (≥ 100 cells per condition), and statistical analysis were determined using one-way ANOVA or Student’s t test.

Neurite outgrowth assay

The Neurite outgrowth assay was as previously described (Lüchtenborg et al., 2014). Briefly, N2a cells were co-transfected for 18 hr with the plasmids indicated in the corresponding figures. Cells were trypsinized and seeded on poly-L-lysine-coated coverslips for additional 24 hr to allow neurite formation. For Brefeldin A (BFA; Sigma-Aldrich) treatment, transfected N2a cells were allowed to adhere on coverslips for 6 hr and incubated for additional 18 hr with 1 μg/ml of BFA or 1 μg/ml methanol as control in complete MEM. Cells were finally PFA-fixed, and stained with rhodamine-phalloidin (Molecular Probes) and DAPI (Sigma-Aldrich), and mounted for microscopy analysis. Samples were recorded with a Plan-Neofluor 10x/0.3 objective on an Axio Imager.M1 microscope equipped with an AxioCam HRc camera, and analyzed using the AxioVision software (all from Zeiss) and ImageJ. The number of transfected cells displaying neurites, neurites per cell and total neurite length were scored from 10-20 randomly taken images (≥ 100 cells per condition), and statistical analysis were determined using one-way ANOVA or Student’s t test.

Protrusion formation assay in S2 cells

S2 cells were transfected for 24 hr with the plasmids indicated in the corresponding figures. Cells were washed, resuspended in fresh media and seeded on poly-L-lysine-coated coverslips for 30 or 120 min at 28 °C to allow formation of protrusions. For BFA treatment, transfected S2 cells were resuspended in fresh media supplemented with 20 μg/ml of BFA (or methanol as control) and incubated for 30 min prior to the seeding on poly-L-lysine-coated coverslips for additional 120 min all at 28 °C. Cells were finally PFA-fixed, and directly stained with rhodamine-phalloidin or permeabilized, immunostained and mounted for microscopy analysis. Samples were recorded with a Plan-Neofluor 20x/0.50 objective on an Axio Imager.M1 microscope equipped with an AxioCam HRc camera and analyzed using AxioVision software and ImageJ. The amount of transfected cells displaying protrusions, the number of structures involved in protrusion formation and the size of the area covered by protrusions were scored from 10-20 randomly taken images (≥ 100 cells per condition), and statistical analysis were determined using one-way ANOVA or Student’s t test.
per cell and the length of the longest protrusion were scored from 15-20 randomly taken images (≥ 100 cells per condition), and one-way ANOVA or Student’s test was used for statistical analysis.

**Biochemical analyses**

N2a, BE(2)C and S2 cells were lysed with ice-cold Lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 2 mM EDTA, 1% Triton X-100 and 10% glycerin) supplemented with a protease inhibitor cocktail. Extracts were cleared by centrifugation at 15,000g and 4°C, boiled at 95°C for 5 min and finally analyzed by SDS-PAGE and western blots (WBs). Alternatively, transfected N2a and S2 cells were treated with BFA as described above (Neurite outgrowth assay and Protrusion formation assay in S2 cells), and directly lysed and prepared for SDS-PAGE and WBs. All HRP-labeled secondary antibodies were from Jackson ImmunoResearch. Quantification of blots was done using ImageJ from at least 3 independent experiments and statistical analysis was carried out using one-way ANOVA or Student’s test.

**GST-based pull-down**

N2a cells were harvested after 24 hr post-transfection with GST-lysis buffer (1% Triton X-100 and 10% glycerol in PBS) supplemented with a protease inhibitor cocktail. Cleared cell extracts were incubated with 30 μL of Glutathione Sepharose 4B beads (GE Healthcare) overnight at 4°C. Beads were repeatedly washed with GST-lysis buffer, prepared for SDS-PAGE and finally analyzed by western blot using anti-GXo, anti-GFP and anti-GST Abs. When required, Student’s test was used for statistical analysis of at least 4 independent experiments.

**Pull-down of GTP-loaded Rab3a and Arf1**

For the pull-down of active GTP-loaded Rab3a and Arf1, the GST-fusion constructs of Rim2 and GGA3 were expressed in *Escherichia coli* BL21-CodonPlus (DE3)-RIPL (Stratagene) and isolated from bacteria extracts using glutathione Sepharose 4B beads (GE Healthcare) in GST-lysis buffer. After several wash steps, loaded beads were stored in GST-lysis buffer at a 50% slurry and 4°C. N2a cells were co-transfected for 24 hr with GXo and the GFP-fusion constructs of Arf1 or Rab3a, and the empty pcDNA3.1+ plasmid was used as control. Cell lysates were obtained using the GST-lysis buffer described above (GST-based pull-down) and cleared extracts were incubated under rotation with 20 μL of the corresponding GST-loaded beads for 1 hr at 4°C. Beads were repeatedly washed, and bound proteins were eluted with 40 mM reduced glutathione (Sigma-Aldrich) in TBS buffer (20 mM Tris-HCl, pH 8.0, and 150 mM NaCl). Samples were prepared for SDS–PAGE, and WBs carried out using anti-GFP and anti-GXo Abs. Student’s test was used for statistical analysis of 4 independent experiments.

**In vitro binding assay**

Recombinant His₆-tagged GXo and the GST-fusions of Arf1, Rab1a, Rab3a and αGDI were expressed in *Escherichia coli* Rosetta-gami (Novagen) and purified with the corresponding tag-specific affinity beads Ni-NTA agarose (QIAGEN) and glutathione Sepharose 4B beads. Protein purity was assessed by SDS-PAGE and Coomassie blue staining, and GTP-binding activities were controlled as previously described (Lin et al., 2014).

For the in vitro binding assay, His₆-GXo was preloaded with 1 mM GDP or GTP-Y-S in TBS buffer supplemented with 1 mM DTT and 5 mM MgCl₂ for 2 hr at 22°C. The excess of nucleotides was removed by a 10,000x buffer exchange using Amicon 10kDa Centrifugal Filter Units (Millipore). Samples were cleared by centrifugation at 15,000g for 5 min, and protein concentrations were normalized. Simultaneously, the GST-tagged small GTPases or pure GST were incubated with 1 mM GDP or GTP-Y-S in TBS supplemented with 5 mM EDTA for 1 hr at 22°C. Proteins were subsequently bound to glutathione Sepharose 4B beads at 50 nmol per ml of resin, and washed with binding buffer (TBS containing 1% Triton X-100, 1% glycerol and 0.2 mM DTT). After the last wash, 20 μL of beads were mixed with GDP- or GTP-Y-S-loaded His₆-GXo to a final concentration of 1 μM and incubated under rotation for 1 hr at 22°C. Beads were thoroughly washed and bound proteins were eluted with binding buffer supplemented with 10 mM reduced glutathione. Eluates were prepared for SDS–PAGE and WB analysis was done using an antibody against the RGS-His epitope. A ponceau S solution (Sigma-Aldrich) was used to detect GST proteins on nitrocellulose membranes prior to the WB. Statistical analysis of 3 independent experiments was done using Student’s t test.

**Baculovirus protein expression and analysis**

Human Rab1a and Rab3a were produced in SF9 cells using Bac-to-Bac baculovirus expression system (Thermo Fisher Scientific). The baculovirus stock obtained after 5 days post initial transfection of E.coli-derived bacmid was amplified as follows: it was diluted 1:10 in the fresh SF9 culture with ca. 0.5x10⁷ cells/ml and left for 4 days on the shaker. The cell debris was removed by centrifugation for 5 min at 1,000xg to produce cleared supernatant. This cycle has been repeated until the total volume of the baculovirus supernatant was enough to inoculate preparative amounts of the culture. For the expression, 150 mL of SF9 cells at 5x10⁶ cells/ml were infected with freshly produced viral stock and shaken for 2 days at 60 rpm. The cells were harvested, washed and the membrane-bound Rabs were purified. Briefly, cells were lysed by a cell disruptor at 0.8 psi (Constant Systems), cell debris was removed by 5 min centrifugation at 1,000xg and 4°C, and cell membranes were harvested by ultracentrifugation at 100,000xg for 1 hr and 4°C. Membrane-associated (geranylated) Rabs were solubilized from the membrane pellets in TBS buffer containing 1% CHAPS and purified using Ni-NTA agarose beads (QIAGEN).
Purified His$_o$-Rabs were bound to GST-αGDI (see in vitro binding assay) by a detergent gradual dilution. Briefly, 1 mg of GST-αGDI were mixed with 2-3 mg of Rab proteins in TBS buffer containing 1% CHAPS, 1 mM GDP and 5 mM MgCl$_2$. Subsequently, the mixture was diluted on ice by adding TBS buffer in order to reduce CHAPS concentration by 0.1% every 30 min. When the concentration of CHAPS was reduced to 0.3%, the mixture was incubated overnight on ice, any precipitate was removed by 10 min centrifugation at 4,000xg and 4°C, and the complex was isolated by sequential tandem purification using first glutathione Sepharose 4B and second Ni-NTA beads. The formation and purity of the His$_o$-Rab/GST-αGDI complexes were confirmed by SDS-PAGE followed by Coomasie staining.

A His$_o$-Gαo in vitro binding assay using glutathione Sepharose 4B beads was performed essentially as described above (in vitro binding assay) with following modifications: the Gαo binding to the Rab/αGDI complexes was performed in His$_o$-tag elution buffer (TBS supplemented with 0.1% CHAPS and 300 mM imidazole, pH 7.6). Final concentration of the complex was 100 μg/ml for Rab1a and 50 μg/ml for Rab3a, and Gαo was supplemented at 500 μg/ml. Samples were finally analyzed by SDS-PAGE and WB against GST and RGS-His epitope.

Binding of Gαo to Rab proteins immobilized on the CNBr Sepharose (GE Healthcare) was analyzed essentially as described in (Lin et al., 2014). Briefly, the coupling buffer for CNBr beads was 20 mM HEPES, pH 7.4, 150 mM NaCl, 1% CHAPS. Subsequently, immobilized proteins were prepared by mixing a total of 100 μg of His$_o$-Rab1a, His$_o$-Rab3a or purified GST (as control) with 30 μl of the beads. Binding was performed in a total volume of 100 μl and His$_o$-Gαo was added to a final concentration of 1 mg/ml. Samples were repeatedly washed and beads directly boiled for SDS-PAGE and western blot analysis.

The Rab1a and Rab3a kinetic assays using fluorescent BODIPY-GTP (Molecular Probes) were performed as described previously (Lin et al., 2014) with the following modifications: Rab proteins were diluted at 3 μM in assay buffer (TBS, 0.5% CHAPS, 5 mM MgCl$_2$, 0.1% BSA) and mixed in 96-well plates with GTPγS-loaded His$_o$-Gαo or MBP as control at a final concentration of 10 μM. Following 10 min incubation, the reaction was started by addition of BODIPY-GTP to the final concentration of 0.6 μM, and recorded using a time-lapse fluorescence measurement in a VICTOR3 plate reader (Perkin Elmer). To control whether absence of Gαo on Rab proteins is not due to already maximal rate of nucleotide incorporation at given conditions, we additionally performed the same assay in presence of 10 mM EDTA, which is known to strip off Mg$^{2+}$ from Rab-GDP and drastically enhance the nucleotide uptake. For baseline, 10 μM GTPγS-loaded Gαo was measured in the same experiment in the absence of Rabs. Preloading of Gαo with GTPγS was produced by incubation of 100 μM His$_o$-Gαo with 1 mM GTPγS in TBS supplemented with 10 mM MgCl$_2$ for 2 hr at RT, and the removal of excess GTPγS by a 10,000-fold buffer exchange to TBS using Amicon 10kDa Centrifugal Filter Units (Millipore).

**VSVG transport assay**

N2a cells were co-transfected with VSVG$^{	ext{ts045}}$-GFP and Gαo or empty pcDNA3.1+ as control for 18 hr at 40°C to allow the expression and accumulation of the VSVG mutant in the ER. Then, cells were quickly cooled down at 32°C with serum-free MEM supplemented with 20 mM HEPES, pH 7.4 and 50 μM cyclohexamide to block de novo synthesis of VSVG$^{	ext{ts045}}$-GFP, and kept at 32°C for additional 30 and 60 min. Biotinylation of cell surface proteins was carried out as previously described (Solis et al., 2012). Briefly, cells were incubated on ice with 0.5 mg/ml sulfo-NHS-SS-biotin (Pierce) in PBS for 30 min and free sulfo-NHS-SS-biotin was quenched by two 5 min washes with ice-cold 50 mM NH$_4$Cl in PBS. Cell extracts were done with Lysis buffer as described above (Biochemical analyses) and biotinylated proteins were collected from cleared extracts with 20 μL of NeutrAvidin beads (Pierce) overnight under rotation at 4°C. Samples were finally analyzed by western blot using Abs against GFP and Gαo. One-way ANOVA was used for statistical analysis of 3 independent experiments.

**Reverse dimerization assay**

The reverse dimerization assays was carried out as previous described (Gordon et al., 2010). Briefly, N2a or BE(2)C cells were co-transfected with GFP-FM4-hGH and Gαo, Gαo-GST, goGαo-GST, or empty pcDNA3.1+ for 18 hr, and additionally incubated for 30 min at normal culture conditions with MEM or DMEM/F-12 supplemented with 20 mM HEPES, pH 7.4 and 50 μM cyclohexamide to block de novo synthesis of GFP-FM4-hGH. Then, the D/D solubilizer (Clontech) was added to the cells at a final concentration of 1 μM to allow GFP-FM4-hGH secretion. The assay was stopped by adding ice-cold PBS at the time points indicated in the corresponding figures, cells were washed 3 times with ice-cold PBS, lysed as described above (Biochemical analyses) and prepared for SDS-PAGE and WB using Abs against GFP, Gαo, GST and α-tubulin. Statistical analysis of at least 3 independent experiments was done using one-way ANOVA or Student’s t test.

**Quantification of Golgi area**

To determine the area covered by the Golgi apparatus in N2a cells, an immunostaining against GM130 was carried out as described above (Immunofluorescence and microscopy) using a Cy3-conjugated secondary antibody. Briefly, N2a cells were co-transfected with the constructs indicated in the corresponding figure, and the empty pEFGP-N1 and pcDNA3.1+ plasmids were used as control and/or to normalize DNA quantities for transfection. The co-expression of the non-tagged Gαo was confirmed by immunostaining using an antibody against Gαo/3 and a Cy5-coupled secondary antibody. The area covered by the GM130 staining was measured from confocal images using the AxioVision software (> 100 cells per condition), and statistical analysis was carried out using one-way ANOVA.
Golgi accumulation of FAPP1-PH-GFP and GFP-Rim2

Confocal images of N2a cells co-transfected with FAPP1-PH-GFP and Gxo or control pcDNA3.1+, or BE(2)C cells co-transfected with FAPP1-PH-GFP and BFP, ssBFPKDEL, or ssBFPKDEL plus Gxo-GST were used to determine FAPP1-PH-GFP accumulation at the Golgi. To avoid interferences due to different expression levels of the construct among single cells, GFP-fluorescence was measured at the Golgi region indicated by the GM130 immunostaining (not shown in the final images) as well as at the total cell area, and the ratio values were used to determine the relative accumulation of the construct at the Golgi apparatus. The co-expression of Gxo was confirmed by immunostaining using an antibody against Gxo/3 and a Cy5-coupled secondary antibody. Similarly, confocal images of N2a cells co-expressing GFP-Rim2 and Gxo-BFP or control GalT-BFP were analyzed as above. Student’s t test was used for statistical analysis of ≥ 50 cells per condition.

Europium-labeled GTP assay

HeLa cells seeded on 96-well plates until 60%–70% confluence were transfected with the 12 µg/ml of total plasmid DNA of the constructs indicated in the corresponding figure. After 24 hr, the medium was removed, the cell layer was quickly rinsed 2 times with 100 µl per well of Europium-labeled GTP (GTP-Eu) buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM MgCl2), and cellular membranes were permeabilized with GTP-Eu buffer containing 0.5% saponin for 10 min at RT and shaking. Then, permeabilized cells were incubated with 100 µM control (DTSEKDA) or KDEL-containing (DTSEKDEL) peptides in DMSO, the reaction was started by adding 5 µl of 50 nM GTP-Eu (Perkin Elmer), and was allowed to proceed for 15 min with shaking. Cells were then scraped and transferred to 96-well AcroWell filter plates (Sigma-Aldrich). Wells were washed once with TBS supplemented with 100 µM MgCl2 and measured in a VICTOR3 plate reader (Perkin Elmer) using a built-in protocol for Europium label (Koval and Katanaev, 2011). The activation of the Gxo was measured as a ratio (in %) between the fluorescence of wells stimulated by KDEL-containing peptide (n = 7) and basal fluorescence in control wells (n = 5) with n = 4-9 independent repeats for each condition. Statistical significance was determined by Student’s t test.

Activation of Gxo by KDEL

The ability of the antibody against active Gxo to detect GTP-loaded Gxo was determined by an immunoprecipitation assay. N2a cells expressing Gxo were harvested using the Assay-lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM MgCl2 and 1% Triton X-100) supplemented with protease inhibitors, the cleared lysate was split in two equal parts and incubated for 90 min at 30°C in the presence of 1 mM GDP or 100 µM GTPγS. Then, the antibody and protein A/G beads were added to each sample and rotated for 30 min at 4°C. Beads were washed with Assay-lysis buffer and finally prepared for SDS-PAGE and WB analysis. The specificity of the anti-active Gxo antibody was parallelly tested by immunostaining performed as described above (Immunofluorescence and microscopy) and using a Cy3-coupled secondary antibody. Stained N2a cells expressing the Gxo-GFP WT or Q205L mutant we recorded using the same confocal settings to assess fluorescence intensity.

To characterize the ssBFP and ssBFPKDEL constructs, N2a cells were separately co-transfected with Gxo-GFP and one of the constructs for 6 hr, the transfection media was exchanged by fresh complete media, and cells were incubated by additional 18 hr at normal culture conditions. Then, media were collected and cell extracts prepared using Lysis buffer as described above (Biochemical analyses). Cleared media and cell extracts were prepared for SDS-PAGE, and WB analysis was done against GFP and α-tubulin. A ponceau S solution was used as loading control for culture media. The retention at the ER of the ssBFPKDEL construct was determined by confocal microscopy in transfected N2a cells.

Then, N2a cells co-expressing Gxo-GFP and ssBFPKDEL or control ssBFP were immunostained using an antibody against GTP-loaded Gxo and a Cy3-conjugated secondary antibody to determine the levels of Gxo activation. The fluorescence mean intensities derived from the Cy3 and GFP fluorophores were measured from confocal images at the PM and perinuclear regions marked by Gxo-GFP, and their ratio values at each compartment were used to determine the GTP-loading of Gxo over total level of the protein. A similar analysis was done in N2a cells co-transfected with Gxo-GFP and KDEL-GST or control pcDNA3.1+ plasmids. At least 50 cells per condition were analyzed and Student’s t test was used for statistical analysis.

RT-PCR

Total RNA was extracted from N2a cells using the NucleoSpin RNA kit (Macherey-Nagel). The cDNA synthesis was done by the HIV Reverse Transcriptase (Thermo Fisher Scientific), and the resulting product was used as template for the PCRs with the ThermoPol DNA Taq polymerase (New England Biolabs). Briefly, 1 µg of total RNA were mixed with 2 µl of 50 µM oligo(dT) primer to a 12 µl final volume, incubated 3 min at 85°C, and then placed on ice. Then, 2 µl of the 10X Reaction buffer, 1 µl of dNTPs (10 mM each), and 1 µl of the HIV Reverse Transcriptase (freshly diluted to a 1 U/µl in 1X Reaction buffer) were added to a final volume of 20 µl. The reverse transcription reaction was achieved incubating at 43°C for 1 hr and then at 92°C for 10 min to inactivate the HIV Reverse Transcriptase. Finally, 1 µl of the resulting cDNA was used to run each PCR, adding 5 µl of 10X ThermoPol Reaction Buffer, 1 µl of dNTPs, 1 µl of Forward and Reverse primers (20 µM), 0.25 µl of the Taq DNA Polymerase to a final concentration of 50 µl. The PCR cycle was as follows: Initial denaturation at 95°C and 30 s, 30 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 1 min. Amplification of β-actin was used as control. All primers (listed in Table S3) were designed using the Primer3Plus web-interface (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).
Live imaging

N2a cells were transfected for 6 hr with the plasmids indicated in the corresponding movie, trypsinized and seeded on poly-L-lysine-coated coverslips, and further kept under normal culture conditions overnight. Then, coverslips were washed with a Hank's Balanced Salt Solution (GIBCO) supplemented with 10 mM HEPES (pH 7.4), mounted on a Chamlide CMB magnetic chamber (Live cell instrument), and kept at 37°C until recording. Cells expressing Gαo-GFP or GalT-GFP were recorded at one image per second for 3 min. Cells expressing Gαo-GFP or Gαo83992-GFP were recorded at one image per second for 2 min prior to the stimulation with 100 μM Acetylcholine (Sigma-Aldrich), 10 μM HU-210 (Cayman Chemical) or 5 μM Mastoparan-7 (Sigma-Aldrich) for additional 3 min. Recordings were done in a VisiScope Cell Explorer System (Visirion Systems) equipped with a Plan-Apochromat 63x/1.4 oil objective on an Axio Observer.A1 microscope (Zeiss), a CoolSNAP HQ2 CCD camera (Photometrics), a VisiChrome Polychromator with a Xenon-lamp 75 Watts, and the MetaFluor Fluorescence Imaging software (Molecular Devices). For analysis, movies were generated from stacks using the “Invert LUT” function of the ImageJ software, and the fluorescence mean intensity of an area at the center of the Golgi region was measured from stacks in 10 cells per condition.

Transfected S2 cells expressing dGαo-GFP alone or together with mRFP-dRab1 WT or DN were washed with complete media, mounted on poly-L-lysine-coated coverslips placed into a Chamlide CMB magnetic chamber, and allowed to spread and form protrusions for 30 min at 28°C before recording. Images were then recorded every 5 s for 15 min as described above and edition was done with ImageJ.

Analysis of Drosophila tissues

Adult wings were collected in 70% ethanol, subsequently replaced by isopropanol, and then mounted in GMM for the light microscopy analysis (Katanaev et al., 2005). Multiple wing hair (mwh) events were counted in the B1 region of the wing blade. Samples were recorded in a Plan-Neofluar 20x/0.50 objective on an Axio Imager.M1 microscope equipped with an AxioCam HRc camera and analyzed using ImageJ. Statistical analysis was determined using Student’s t test.

For immunostaining of pupal wings, white prepupae were staged at 25°C, pupae were collected at 22 or 30 hr after puparium formation (APF), cleaned from viscera, and then fixed with 4% paraformaldehyde solution in PBS for 20 min. After fixation, pupal wings were decuticized and permeabilized first with 0.5% NP-40 in PBS for 30 min, and then with 0.2% Tween-20 in PBS for 10 min. Samples were then immunostained and mounted with Vectashield for microscopy analysis.

Drosophila NMJs were obtained as in (Lüchtenborg et al., 2014). Shortly, 3rd instar larvae were collected in cold PBS, dissected and then fixed for 20 min with 4% paraformaldehyde in PBS. After permeabilization in PBS supplemented with 0.05% Triton X-100 and 5% goat serum for 1 hr, samples were immunostained and mounted with Vectashield for fluorescence microscopy. Statistical analysis was determined using Student’s t test.

Pupal wings and NMJs were recorded with a Plan-Apochromat 63x/1.4 oil and a Plan-Apochromat 20x/0.8 objectives, respectively, on a LSM780 Quasar Confocal Microscope and further processed using the ZEN blue software.

QUANTIFICATION AND STATISTICAL ANALYSIS

While mentioned, n represents the number of flies used in the experiment. Data are represented as mean ± SEM. Statistics analysis was performed using Prism v5.03. Student’s t test was used in Figures 1F, 1G, 2F–2H, 2J–2L, 2R, 3C, 4F, 4H, 5B, 5G, 5J, 5L, 6C, 6D, 6F, 6G, 6H, 7D, 7G, 7H, 7J, S1D, S4H, S5E, and S7B. ANOVA was used in Figures 1E, 2N, 2P, 3G–3I, 4B–4D, 5E, 6K–6M, 6O–6Q, S3E, S3F, S3H, S4F, and S7E. In all figures, *p < 0.01; **p < 0.005; ***p < 0.001.

DATA AND SOFTWARE AVAILABILITY

For orthology prediction (see Bioinformatics analysis), we used an in-house-made PERL scripts which extract and unify the data from the 10 major orthology databases. The programs created in-house are available at the laboratory’s web-page (https://www.unil.ch/dpt/home/menuinst/recherche/groupe-katanaev/files.html).
Figure S1. Partners, Expression, and Localization of Gαo, Related to Figures 1 and 2

(A) Enrichment Map of over-represented Gene Ontology (GO) terms clustered in functional modules built from the human orthologs of the interacting partners of Drosophila Gαo. Each node represents a set of genes associated with a particular GO annotation. The size of nodes indicates the relative number of genes.
and its color intensity represents highly over-represented terms (low p value). Thickness of edges shows the degree of overlap between the set of genes within nodes.

(B) RT-PCR analysis showed the expression of the Gα-subunits of the Gi/o subfamily in N2a cells. Amplification of actin was used as control.

(C and D) Comparison between non-tagged (Gαo), GFP-fused Gαo (Gαo-GFP, right) and its Golgi-only form (gGαo-GFP) in the induction of neurite outgrowth in N2a cells (A). A control GFP showed a very limited formation of neurites (Control). Quantification of transfected cells displaying neurites (D). Scale bar, 20 μm.

(E) N2a cells were transfected with a GFP-fusion of the trans-Golgi marker β-1,4-galactosyltransferase (GaT-GFP) and the non-tagged human Gαo. Immunostaining against Gαo revealed its co-localization with GaT-GFP at the Golgi region. Color-channels are listed vertically top-to-bottom and a selected area is magnified with the channels displayed horizontally in the same order left-to-right. Scale bars, 10 μm.

(F) Image of a mouse cortical neuron at 7 DIV immunostained against endogenous Gαo and the cis-Golgi marker GM130. Boxed area is enlarged as in (E). Scale bar, 10 μm.

(G) N2a cells were transfected with a GFP-fusion of Drosophila Gαo (dGαo-GFP) and immunostained against GM130. A selected is zoomed-in as in (E). Scale bars, 10 μm.

(H) Immunostaining of endogenous dGαo in Drosophila pupal wings co-expressing an RNAi against dGαo and the cis-Golgi marker Arf79F-GFP at 22 hr APF. The specificity of the Ab against dGαo is indicated by the almost total lack of signal by the downregulation of dGαo. Scale bar, 50 μm.

(I) N2a cells expressing a GFP-fusion of Gαo (Gαo-GFP) were treatment with 1 μg/ml Brefeldin A for 18 hr (bottom) or with methanol as control (1 μl/ml, top) and then immunostained against the cis-Golgi marker GM130. Brefeldin A induced disassemble of the Golgi apparatus and the loss of Gαo-GFP from the perinuclear region without any noticeable effect on its plasma membrane localization. Boxed areas are enlarged as in (E). Scale bar, 10 μm.

(J and K) Western blot of N2a cells expressing the non-tagged Gαo treated with 1 μg/ml Brefeldin A for 18 hr (B) and of S2 cells expressing a GFP-fusion of the Drosophila Gαo (dGαo-GFP) treated with 20 μg/ml Brefeldin A for 2.5 hr (C). Controls represent cells treated with the corresponding volumes of methanol. Samples were tested with Abs against Gαo, GFP and α-tubulin (α-tub) as loading control, and no apparent variations in Gαo protein levels were observed. Data represent mean ± SEM. ns, not significant; ***p < 0.001.
Figure S2. Gαo Pull-Downs, Related to Figure 3

(A and B) Characterization of the shRNA permanently transfected BE(2)C cell lines. Specific immunostaining (A) and western blot (B) showed the almost complete depletion of Gαo in the lines shGαo-A and shGαo-B, but not in the parental and shControl lines. Phallodin was used to label F-actin as control staining (A) and an Ab against α-tubulin (α-tub) used as loading control (B). Scale bar, 50 μm.

(C) Western blot of pull-downs from N2a cells transfected with Gαo-GST and its Golgi-only form goGαo-GST. The goGαo-GST construct was able to efficiently interact with GFP-fusions of the small GTPases Rab1a, Rab3a and Arf1 as well as KDELR. Abs against GFP, GST and Gαo were used for detection.

(D) The correct expression of the GST-fusion constructs of Gαo (Gαo-GST, top) and KDELR (KDEL-R-GST, bottom) were confirmed by immunostaining against GST in N2a cells co-expressing the Golgi marker GalT-GFP. Scale bar, 10 μm.

(E–H) Western blots of the pull-downs from N2a cells co-transfected with Gαo-GST and GFP-fusions of the partners specified in each panel. The KDELR-GST construct served as control. Abs against GFP and GST were used for detection.
Figure S3. Co-localization of Gαo with Small GTPases, Related to Figure 3

(A–C) Confocal images of N2a cells co-transfected with an mRFP-fusion of Gαo (Gαo-mRFP) and GFP-constructs from the Arf (A) and Rab (B) families as indicated in each panel as well as dynamin-1 (Dyn1-GFP; C) and –2 (Dyn2-GFP; C), and clathrin light chain (GFP-clathrin; C). Selected areas are zoomed-in to the right as in Figure S1E. Scale bars, 10 μm.

(D–F) The sole expression of GFP-fusions of Rab1a (left), Rab3a (middle) or Arf1 (right) in N2a cells did not increase neurite outgrowth nor total length of spontaneously formed neurites. Quantification of transfected cells displaying neurites (E) and neurite total length (F) compared to GFP control. Scale bar, 20 μm.

(G and H) Western blot of N2a cells co-expressing Gαo-GFP with GFP-fusions of wild-type (WT) or DN mutant constructs of Rab1a, Rab3a and Arf1 (G). Abs against GFP and α-tubulin (α-tub) were used. Note the reduced Gαo protein level detected by the co-expression of Arf1DN. Quantification of Gαo-GFP levels (H).

(I) Western blot of N2a cells co-transfected with the non-tagged Gαo and GFP-fusions of the DN mutants of Rab1a, Rab3a and Arf1. Reduced Gαo protein level were observed by the co-expression of Arf1DN. Abs against Gαo, GFP and α-tubulin (α-tub) as loading control were used.

Data represent mean ± SEM. ns, not significant; ***p ≤ 0.001.
Figure S4. Co-localization of Drosophila Gαo with Small GTPases, Related to Figures 3, 4, and 6

(A and B) Confocal images of N2a cells expressing the DN mutant of Arf1 (Arf1DN-GFP) showed disassembly of the Golgi apparatus indicated by the immunostaining against the cis-Golgi marker GM130 (A). Gαo-mRFP was co-expressed together with the mutants GFP-Rab1aDN (top), GFP-Rab3aDN (middle) and Arf1DN-GFP (bottom) in N2a cells (B). Marked regions are zoomed-in to the right as in Figure S1E. Scale bars, 10 μm.

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(C and D) Confocal images of S2 cells co-transfected with a GFP-fusion of the Drosophila Gox (dGox-GFP) and the mRFP-construct of dRab1 (mRFP-dRab1) showed their co-localization at tubulated Golgi-stacks visualized by the cis-Golgi marker GMAP-210 (top, C). The sole expression mRFP-dRab1 did not produce this phenotype (bottom, C). dGox-GFP co-localized at the Golgi with mRFP-dRab3 (top, D) and dArf79F-mRFP (bottom, D). Scale bars, 5 μm.

(E and F) S2 cells co-expressing GFP (for visualization) and mRFP-fusions of dRab1, dRab3, dArf79F and dKDELR did not form protrusions after 30 (top) or 120 min (bottom) seeding (E). Quantification of transfected cells forming protrusions after 120 min seeding (F). Scale bar, 10 μm.

(G and H) Western blot of N2a cells co-transfected with dGox-GFP and mRFP-constructs of the wild-type and mutant forms of dRab1, dRab3, dArf79F, and dKDELR (G). Quantification of dGox-GFP protein levels (H). Abs against GFP and α-tubulin (α-tub) as loading control were used. Data represent mean ± SEM. ns, not significant; *p ≤ 0.01.
Figure S5. Gαo Functional Interaction with Small GTPases at the Golgi, Related to Figures 4 and 5

(A) Confocal images of S2 cells co-expressing dGαo-GFP and the mRFP-dRab1DN (top), mRFP-dRab3DN (middle) and dArf79FDN-mRFP (bottom) were immunostained against GMAP-210. Note the almost complete disassembly of Golgi-stacks by the mutant of dArf79F. Scale bar, 5 μm.

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(B) Immunostaining against the cis-Golgi marker GM130 in N2a cells expressing the constitutive active (CA) Gαo(Q205L) mutant of Gαo (GαoCA-GFP, top) or the DN mutant of Rab1a (GFP-Rab1aDN, bottom) showed no apparent effect on Golgi morphology. Selected areas are zoomed-in to the right as in Figure S1E. Scale bar, 10 μm.

(C) An enlargement of the Golgi apparatus is observed in N2a cells co-expressing the non-tagged Gαo and a GFP-fusion of Rab1b (GFP-Rab1b). Immunostainings against Gαo (squares at right bottom corners of GFP-Rab1b panels) and GM130 confirmed co-expression and marked the Golgi, respectively. Marked regions are magnified to the right as in Figure S1E. Scale bar, 10 μm.

(D-F) The expression of the BFP-fusion of Gαo (Gαo-BFP) enhanced the accumulation of the GFP-construct of Rim2 (GFP-Rim2) at the GM130-positive Golgi region in N2a cells (D). Gαo-BFP co-expression is shown in the square at the right bottom corner of the corresponding GFP-Rim2 panel (bottom, D). Boxed areas are magnified (right, as in Figure S1E). Mean fluorescence intensity ratios of GFP-Rim2 at the Golgi versus total cell (E). Western blot for the expression of GFP-Rim2 using Abs against GFP, Gαo and α-tubulin (α-tub) as loading control (F). No evident variation in the levels of GFP-Rim2 was observed by Gαo co-expression. Data represent mean ± SEM. ***p < 0.001. Scale bar, 10 μm.

(G) Western blots of the pull-down from N2a cells co-transfected with Gαo-GST and a GFP-fusion of α-GDI. The KDELR-GST construct served as control. Abs against GFP and GST were used for detection.

(H) A western blot of an in vitro binding assay confirmed the interaction of recombinant His6-tagged Gαo and baculovirus-purified Rab1a and Rab3a. An Ab against His-tag was used for detection, and the Ponceau S staining were used to visualize Rab1a and Rab3a.

(I and J) A BODIPY-GTP uptake assay indicates that GTPγS-loaded Gαo does not possess GEF activity toward baculovirus-purified Rab1a (I) and Rab3a (J). BODIPY-GTP uptake in presence of EDTA (I and J) was used to control the maximal rate of nucleotide loading of Rab1a and Rab3a.
Figure S6. Goα Does Not Translocate From the PM to the Golgi upon Activation, Related to Figures 5 and 6

(A and B) Western blots for the expression of GFP-fusions of Goα at its C terminus (Goα-GFP) or inserted downstream of the glycine residue at position 92 (GoαGly92-GFP). Note the lower expression levels of GoαGly92-GFP (arrowheads) compared to Goα-GFP (A) and to the endogenous Goα13 in non-transfected N2a cells (B). Abs against GFP, Goα/i3 and α-tubulin (α-tub) as loading control were used.
(C) The GaO Gly92-GFP construct (right) showed a similar subcellular localization than GaO-GFP (left) at the perinuclear Golgi region and the plasma membrane. Scale bar, 10 μm.

(D–I) N2a cells were co-transfected with GaO-GFP (top) or GaO Gly92-GFP (bottom) and the muscarinic acetylcholine receptor 2 (M2R, D and E) or the cannabinoid receptor type-1 (CB1R, F and G). Cells were recorded every second for 2 min before stimulation (arrowheads) with 100 μM Acetylcholine (Ach, D and E) or 10 μM HU-210 (F and G) during 3 min. Cells expressing only the GFP-fusions of GaO were stimulated with 5 μM Mastoparan-7 (H and I) and recorded as above. Mean fluorescence intensities of selected Golgi regions were measured from 10 different cells (colored lines) and an empty region was used as background (black line). Low expression level of GaO Gly92-GFP correlates with low fluorescence intensities (E, G and I) compared to GaO-GFP signals (D, F and H).

(J) Confocal image of a Drosophila pupal wing at 30 hr after puparium formation (APF) showed the correct expression of the Drosophila KDELR transgene (dKDELR-mRFP). Scale bar, 50 μm.

(K) Western blot of the GaO immunoprecipitation (IP) using the Ab against active GaO (GaO-GTP). N2a cell extracts expressing non-tagged GaO were pre-incubated with GDP or GTPγS previous to the IP. The Ab against total GaO used for detection revealed the specificity of the GaO-GTP Ab toward GTPγS-loaded GaO (arrowhead).

(L) N2a cells expressing the wild-type (top) or the constitutive active Q205L mutant (bottom) of a GFP-fusion of GaO (GaO-GFP) were immunostained using the GaO-GTP Ab. Confocal images recorded using equal settings confirmed the specificity of the GaO-GTP Ab for the detection of GaO on its active conformation. Scale bar, 10 μm.

(M–O) N2a cells were co-transfected with GaO-GFP and a secretable BFP construct containing the KDELR retention signal (ssBFP kdel) or a control ssBFP, and cell extracts (M) and culture media (N) were analyzed by western blot. An Ab against GFP was used for detection, and the anti-α-tubulin (α-tub) Ab and Ponceau S staining were used as loading controls. The ssBFP kdel construct was detected in cell extracts (M) but not in the media (N), whereas the control ssBFP was almost completely secreted. No apparent variation in GaO-GFP expression levels were observed in extracts (M). Confocal images of N2a cells expressing cytosolic BFP (left), control ssBFP (middle) or ssBFP kdel (right) showed the retention of the latter in a compartment resembling the endoplasmic reticulum (O). A selected area is zoomed-in (right). Scale bar, 10 μm.
Figure S7. KDEL Activates Gαo at the Golgi, Related to Figures 3 and 6

(A and B) Overexpression of KDEL increased Gαo activity at the Golgi region in N2a cells (A). Active Gαo-GFP was detected by immunostaining against GTP-loaded Gαo (Gαo-GTP). Marked regions are magnified (right, as in Figure S1E). Mean fluorescence intensity ratios of active versus total Gαo calculated at the PM and Golgi (B).

(C) Overexpression of Gαo-GFP (above) or Gαo-mRFP (below) did not induce the co-accumulation of endogenous Gβ1 (above) or GFP-Gβ1 (below) at the Golgi apparatus, indicating that Golgi membranes contain mostly monomeric Gαo in N2a cells. Selected areas are magnified to the right as in Figure S1E.

(D and E) Western blot of N2a cells co-expressing Gαo-GFP with KDEL- or KDELΔH-GFP. Anti-GFP and α-tubulin (α-tub) Abs were used (D). Quantification of Gαo-GFP levels (E).

(F) Confocal image of N2a cells expressing the R1WTSH-GFP construct were immunostained against the cis-Golgi marker GM130. Note the almost exclusive Golgi localization of R1WTSH-GFP. A boxed area is magnified to the right as in Figure S1E.

Data represent mean ± SEM. ns, not significant; ***p < 0.001. Scale bars, 10 μm.