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# *Drosophila* GoLoco-Protein Pins Is a Target of $G\alpha_o$-mediated G Protein–coupled Receptor Signaling

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**G protein–coupled receptors (GPCRs) transduce their signals through trimeric G proteins, inducing guanine nucleotide exchange on their G$\alpha$-subunits; the resulting G$\alpha$-GTP transmits the signal further inside the cell. GoLoco domains present in many proteins play important roles in multiple trimeric G protein–dependent activities, physically binding G$\alpha$-subunits of the G$\alpha_{i/o}$ class. In most cases GoLoco binds exclusively to the GDP-loaded form of the G$\alpha$-subunits. Here we demonstrate that the poly-GoLoco–containing protein Pins of *Drosophila* can bind to both GDP- and GTP-forms of *Drosophila* G$\alpha_o$. We identify Pins GoLoco domain 1 as necessary and sufficient for this unusual interaction with G$\alpha_o$-GTP. We further pinpoint a lysine residue located centrally in this domain as necessary for the interaction. Our studies thus identify *Drosophila* Pins as a target of G$\alpha_o$-mediated GPCR receptor signaling, e.g., in the context of the nervous system development, where G$\alpha_o$ acts downstream from Frizzled and redundantly with G$\alpha_i$ to control the asymmetry of cell divisions.**

## INTRODUCTION

Trimeric G proteins transduce the signals from G protein–coupled receptors (GPCRs), the largest receptor family in the animal kingdom (Pierce *et al.,* 2002). Signal specificity is mainly represented by the $\alpha$-subunits of the trimeric G proteins; 16 genes for the $\alpha$-subunits are present in the human genome, and six in *Drosophila* (Malbon, 2005). Both in flies and mammals, $G\alpha_o$ is the predominant G$\alpha$-subunit in the nervous system (Sternweis and Robishaw, 1984; Wolfgang *et al.,* 1990); up to 10% of the whole plasma membrane proteins of the neuronal growth cones is represented by the trimeric $G_o$ protein (Strittmatter *et al.,* 1990). $G\alpha_o$ is required for the proper brain functioning and development (Jiang *et al.,* 1998; Ferris *et al.,* 2006), e.g., controlling neurite outgrowth (Bromberg *et al.,* 2008). Among the brain GPCRs activating $G\alpha_o$ are the dopamine, serotonin, adenosine, cannabinoid, glutamate, and other receptors (Offermanns, 2003; Bromberg *et al.,* 2008). Additional developmental functions of $G\alpha_o$ are the transduction of the evolutionary conserved Frizzled receptors (Egger-Adam and Katanaev, 2008) and the regulation of the heart development and physiology (Valenzuela *et al.,* 1997; Fremion *et al.,* 1999).

In the resting state the trimeric G proteins exist as complexes of the GDP-bound $\alpha$-subunit and the $\beta$- and $\gamma$-subunits. On ligand activation, GPCRs serve as guanine nucleotide exchange factors, catalyzing the substitution of GDP for GTP on the G$\alpha$-subunit. This leads to dissociation of the complex into the GTP-loaded G$\alpha$ and the $\beta\gamma$-heterodimer. Both components of the initial complex can interact with downstream effectors (Gilman, 1987).

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GoLoco domains (Willard *et al.,* 2004) present in many different proteins across the animal kingdom can specifically bind $\alpha$-subunits of the $G_{i/o}$ class of trimeric G protein ($G\alpha_i$, $G\alpha_o$, $G\alpha_t$, and $G\alpha_z$) and thus might serve as a hallmark of a subclass of $G\alpha_{i/o}$ target proteins. For example, interaction of $G\alpha_{i/o}$ with the GoLoco-containing protein Rap1Gap (a negative regulator of a small G protein Rap1) has been proposed as a mechanism of GPCR-induced neurite outgrowth (Jordan *et al.,* 1999; Jordan *et al.,* 2005). However, in the majority of cases GoLoco domains bind to the GDP-, and not the GTP-loaded forms of free G$\alpha$-subunits (Willard *et al.,* 2004); furthermore, some GoLoco motifs are able to dissociate the trimeric G protein complexes without nucleotide exchange (Takesono *et al.,* 1999; Ghosh *et al.,* 2003). These observations have led to proposition that GoLoco-containing proteins may serve not as targets, but instead as activators of trimeric G proteins (Cismowski *et al.,* 2001; Hampoelz and Knoblich, 2004; Blumer *et al.,* 2005; Siderovski and Willard, 2005).

In our search for the possible targets of the activated form of the *Drosophila* $G\alpha_o$ protein, we performed a saturating yeast two-hybrid screen using $G\alpha_o$ and its constitutively GTP-loaded mutant form as the baits. Among the multiple $G\alpha_o$- and $G\alpha_o$[GTP]-binding partners, we identified all *Drosophila* GoLoco-containing proteins. Among those, Pins was unusual in its interaction not only with the wild type (mostly GDP-loaded), but also with the GTP-charged form of $G\alpha_o$. This result confirmed our previous observations of the interaction of the activated form of $G\alpha_o$ with Pins in the regulation of the asymmetric cell division during *Drosophila* sensory organ formation (Katanaev and Tomlinson, 2006a). We narrowed down the $G\alpha_o$[GTP]-interacting region to the GoLoco domain 1 of Pins, and showed that a positively charged residue, unique among *Drosophila* GoLoco domains, is necessary for the interaction with the GTP-bound form of $G\alpha_o$. Our experiments thus uncover Pins as a possible target of $G\alpha_o$-mediated GPCR signaling.

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## MATERIALS AND METHODS

### Yeast Two-Hybrid Screen

The wild-type and the Q205L mutant forms of the *Drosophila melanogaster* class II $G\alpha_o$ open reading frame (GenBank accession number gi: 45551069) were amplified from the pBluescript plasmids containing the respective wild-type or mutated cDNA (Fremion *et al.*, 1999) using the oligos: forward: TAAACTAGTGATGGGCTGCACCACATCCG and reverse: ATCTGCAGCT-TAGTACAGTCCACAGCCG. The PCR product was digested with BcuI/PstI and cloned in-frame C-terminally to the lexA sequence of the pB27 vector (Hybrigenics, Paris, France) and sequence-verified. The screen was performed by Hybrigenics using as the prey the random-primed *Drosophila* adult head cDNA library constructed into pP6 plasmid. 54 and 95 million clones (5- and 9.5-fold the complexity of the library) were screened for $G\alpha_o$ and for $G\alpha_o$[Q205L], respectively, using a mating approach with Y187 (mat$\alpha$) and L40$\Delta$Gal4 (mat**a**) yeast strains as previously described (Fromont-Racine *et al.*, 1997). His+ colonies were selected on a medium lacking tryptophan, leucine, and histidine for the wild type and the mutant (n = 225 and 171), respectively. The prey fragments of the positive clones 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 as previously described (Formstecher *et al.*, 2005). All interactions reported in this manuscript have the highest confidence score (A), *E*-value < 1e-10.

### Cloning

***Cloning $G\alpha_o$ and $G\alpha_o$[Q205L] for the Nontagged Expression.*** $G\alpha_o$ open reading frames were amplified from the pBluescript-$G\alpha_o$ and pBluescript-$G\alpha_o$[Q205L] using the oligos: forward: AACCAACCACCATGGGCTGCAC-CAC and reverse: GGTGCAGCCCATGGTGGTTGGTTTTGG and subcloned into pQE60 (Qiagen, Hilden, Germany) by NcoI/BamHI.

***Cloning $G\alpha_o$ and $G\alpha_o$[Q205L] for the $(His)_6$-tagged Expression.*** $G\alpha_o$ open reading frame from pGEX-$G\alpha_o$ (Katanaev and Tomlinson, 2006a) was subcloned into the pQE32 (Qiagen) by BamHI. $G\alpha_o$[Q205L] open reading frame was amplified from the pBluescript-$G\alpha_o$[Q205L] using the oligos: forward: AAGGATCCATGGGCTGCACCACATCCGC and reverse: ATCTGCAGCT-TAGTACAGTCCACAGCCG and subcloned into pQE30 (Qiagen) by BamHI/PstI.

***Generation of Pins Fragments.*** pMAL-Pins (Schaefer *et al.*, 2000) encoding the full-length Pins was used to generate constructs expressing individual Pins fragments using PCR amplification with the following oligos: For the tetratricopeptide repeats (TPRs; aa 49-373), forward: ATGAATTCGTCGAGGGT-GAACGCCTCTGC and reverse: ATAAGCTTCTAGTCGTGCAGCTCCTTG-GCC; for the whole-GoLoco domain region (aa 374-658), forward: ATGAATTCCCGGTGGGTGAAAGCACAGCC and reverse: ATAAGCT-TCTAGTCGTGCAGCTCCTTGGCC; for the GoLoco1 domain (aa 374-543), forward: ATGAATTCCCGGTGGGTGAAAGCACAGCC and reverse: ATA-AGCTTCTAGCTCCTGGCGAGCGGCCC; for the TPRs + GoLoco1 domain (aa 49-543), forward: ATGAATTCGTCGAGGGTGAACGCCTCTGC and reverse: ATAAGCTTCTAGCTCCTGGCGAGCGGCCC; for the GoLoco3 domain (aa 600-658), forward: ATGAATTCCCACGCGGACAGACCGGACGTGG and reverse: ATAAGCTTCTACTTTCCAGCTCCGCCGGC. PCR products were digested by EcoRI/HindIII and cloned into the pMAL-c2 plasmid (New England Biolabs, Frankfurt am Main, Germany). All plasmids were sequence-verified.

Site-directed mutagenesis of GoLoco domain 1 was performed through high-fidelity amplification (with Pfu DNA-polymerase) of the pMAL-GoLoco1 plasmid with oligos containing the point mutation (highlighted by quotation marks): forward: CGGTCGCAGTCG"G"AGCGCATGGATGAC and reverse: GTCATCCATGCGCT"C"CGACTGCGACCG. The PCR product was treated with the restriction enzyme DpnI for removal of the methylated template, purified by the gel-extraction kit (Peqlab, Erlangen, Germany), and used for bacterial transformation. The resulting plasmids were sequence-verified.

### Protein Expression

***Expression of Nontagged $G\alpha_o$ and $G\alpha_o$[Q205L].*** *Escherichia coli* strain BL21-CodonPlus RIL (Stratagene, La Jolla, CA) was transformed with pQE60-$G\alpha_o$ and pQE60-$G\alpha_o$[Q205L] and grown at 37°C to an OD(600) = 0.5 before induction with 1 mM isopropyl-1-thio-D-galactopyranoside (IPTG) and additional growth for 4 h at 37°C, followed by harvesting by centrifugation and storage at −20°C. Cell pellets were defrosted, resuspended in 1× PBS, 1 mM EGTA, 5 mM $\beta$-mercaptoethanol, and 1 mM PMSF, and disrupted by sonication on ice. Debris was removed by centrifugation at 18,000 × *g*/30 min/4°C. The resulting supernatant was diluted to 5 mg protein/ml with 50 mM HEPES-KOH, pH 8.0, 100 mM KCl, 1 mM DTT, 1× protease inhibitor cocktail (Roche, Grenzach-Wyhlen, Germany) before addition of 0.5% Nonidet P-40, 0.1% Tween20, and $MgCl_2$ to 5 mM and additional centrifugation 18,000 × *g*/10 min/4°C; the supernatant was directly used in the binding experiments. Equal amounts of $G\alpha_o$ and $G\alpha_o$[Q205L] in the bacterial extracts was verified by Western blots with rabbit anti-$G\alpha_o$ antibodies (1:1000, Calbiochem, La-Jolla, CA; cat. no. 371726).

***Expression of $(His)_6$-$G\alpha_o$ and $(His)_6$-$G\alpha_o$[Q205L].*** *E. coli* strain M15 (pREP4, Qiagen) was transformed with pQE32-$G\alpha_o$ and pQE30-$G\alpha_o$[Q205L], grown at 37°C to an OD(600) = 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 1× PBS, 1 mM EGTA, 5 mM $\beta$-mercaptoethanol, and 1 mM PMSF and disrupted by sonication. Debris was removed by centrifugation at 18,000 × *g*/30 min/4°C. The supernatant was applied to the $Ni^{2+}$ resin (Qiagen), preequilibrated in same buffer. Column purification of the $(His)_6$-$G\alpha_o$ proteins was performed using the ÄKTAprime plus protein purification system (Amersham Biosciences, Piscataway, NJ).The $Ni^{2+}$ resin was washed three times with 10 resin volumes of 1× PBS, 1 mM EGTA, 5 mM $\beta$-mercaptoethanol, 0.1 mM PMSF, and 30 mM imidazole. Proteins were eluted with 200 mM imidazole in the washing buffer. Resulting proteins were dialyzed with 50 mM HEPES-KOH, pH 7.5, 150 mM KCl, 10 mM NaCl, and 1 mM DTT in vivaspin-6 (Sartorius, Edgewood, NJ) and stored at 4°C.

For phenyl-Sepharose purification, $(His)_6$-$G\alpha_o$ was brought into 50 mM HEPES-KOH, pH 7.5, 1.2 M $(NH_4)_2SO_4$, and 50 $\mu$M GDP and applied to a phenyl-Sepharose column (bed volume 30 ml). After washing with two bed volumes of the column buffer (50 mM HEPES-KOH, pH 7.5, 1.2 M $(NH_4)_2SO_4$, 50 $\mu$M GDP, and 5 mM $\beta$-mercaptoethanol), $G\alpha_o$ was eluted with a linear gradient (10 bed volumes) of 1.2M $(NH_4)_2SO_4$ to 0 M in the column buffer. The peak fractions containing $G\alpha_o$ were pooled and ultrafiltrated against 50 mM HEPES-KOH, pH 7.5, 150 mM KCl, 10 mM NaCl, and 1 mM DTT and stored at 4°C. The purity of the single- and double-step–purified $(His)_6$-$G\alpha_o$ proteins is shown in Supplementary Figure S1A. Proteins preserved their activity for months, as measured by the BODIPY-GTP$\gamma$S assay (below).

***Expression MBP-Pins, Its Fragments, and MBP Alone.*** *E. coli* strain Top10 (Invitrogen, Carlsbad, CA) was transformed with respective plasmids, grown at 37°C to an OD(600) = 0.5 before induction with 1 mM IPTG and additional growth for 4 h at 37°C, followed by harvesting by centrifugation and storage at −20°C. Subsequent procedures were performed as above, except for application of the postsonication supernatant to the amylose resin (New England Biolabs), which was washed four times with 10 resin volumes of 1× PBS, 1 mM EDTA, 5 mM $\beta$-mercaptoethanol, and 0.1 mM PMSF before elution with 10 mM maltose in the washing buffer. The resulting purity of the MBP-Pins proteins, as determined by SDS-PAGE and Coomassie staining, was 50–90%. The major source of impurity was the MBP derived from proteolytic cleavage of the fusion proteins. All fusion proteins were used in the equal molar concentrations adjusted for the Pins content. In several pulldown experiments, the amylose resin with noneluted MBP-fusion proteins was used. In this case, amylose-binding conditions for each MBP-fusion protein were varied in order to select the conditions granting identical molar concentrations for all resin-immobilized proteins.

GST-$G\alpha_o$, GST-AGS3 (GoLoco domains 1–4), and GST-LGN (GoLoco domains 3–4) were purified as described (Bernard *et al.*, 2001; Natochin *et al.*, 2001; Katanaev and Tomlinson, 2006a). $(His)_6$-$G\alpha_i$ (Nipper *et al.*, 2007) and rat $(His)_6$-$G\alpha_o$ (Natochin *et al.*, 2001) were single-step purified, prepared as described above for *Drosophila* $(His)_6$-$G\alpha_o$.

### GTP Binding and Hydrolysis Assays

$(His)_6$-tagged $G\alpha_o$ or $G\alpha_i$ subunits were diluted to 1 $\mu$M into the buffer containing 50 mM HEPES-KOH, pH 8.0, 100 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$, 0.1% Tween20, and 1 $\mu$M BODIPY-GTP$\gamma$S or BODIPY-GTP (Molecular Probes, Eugene, OR). Fluorescence measurements were performed with a Perkin Elmer VICTOR3 multiwell reader (Norwalk, CT) with excitation at 485 nm and emission at 530 nm at 20°C for 60 min. The 36-mer GoLoco1 domain peptide (see Figure 5A for the sequence) and its Lys15Gly mutated version were synthesized by Pepscan Presto BV (Lelystad, Netherlands) and freshly dissolved in water before each experiment. Mastoparan was from Sigma (St. Louis, MO). The peptides were preincubated with $G\alpha_o$ into the binding mixture for 10 min at 20°C before addition of BODIPY-GTP$\gamma$S. The rate of GTP$\gamma$S incorporation was measured as the increase of BODIPY-GTP$\gamma$S fluorescence upon addition of the G protein over the buffer during the first 300 s (for $G\alpha_o$) or 3000 s ($G\alpha_i$) of incubation with the nucleotide.

The specific activity of $G\alpha_o$ protein after bacterial expression is measured as the percent of $G\alpha_o$ molecules that bind GTP nucleotides out of total $G\alpha_o$ present in the measurement. The GTP analogs BODIPY-GTP$\gamma$S and [$^{35}$S]GTP$\gamma$S were used in activity measurements as described below and produced similar results.

For the BODIPY-GTP$\gamma$S specific activity measurements, increasing amounts of GST-$G\alpha_o$ or $(His)_6$-$G\alpha_o$ were added to 1 $\mu$M BODIPY-GTP$\gamma$S as described above. Plateau fluorescence was measured after 75 min of incubation. The resulting curves depicting dependence of the fluorescence of BODIPY-GTP$\gamma$S on the concentration of $G\alpha_o$ were plotted and fitted to the Michaelis-Menton-like equation: [$y = a + \{bx/(c + x)\}$], using KaleidaGraph 4.0 (Synergy Software, Reading, PA) to obtain the concentration of $G\alpha_o$

providing the half-maximal fluorescence enhancement ($EC_{50}$) of 1 $\mu$M BODIPY-GTP$\gamma$S. Given the nanomolar $K_d$ value for BODIPY-GTP$\gamma$S for G$\alpha_o$ (McEwen *et al.*, 2001), the obtained $EC_{50}$ gives directly the percentage of active G$\alpha_o$. Multiple isolations of $(His)_6$-G$\alpha_o$ result in 30–50% active protein, whereas GST-G$\alpha_o$ is only 8–10% active.

The [$^{35}$S]GTP$\gamma$S-binding assay was modified from Sternweis and Robishaw (1984). $(His)_6$-tagged G$\alpha_o$ was diluted to 1 $\mu$M into the buffer containing 50 mM HEPES-KOH, pH 8.0, 100 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$, and 0.1% Tween20. GTP$\gamma$S was added to 1 $\mu$M and [$^{35}$S]GTP$\gamma$S (Hartmann Analytical, Braunschweig, Germany) to 150 cpm/pmol GTP$\gamma$S, together with GoLoco1 peptide/recombinant domain at indicated concentrations to the final volume of 100 $\mu$l. After 1-h incubation at 20°C, the samples were diluted by 900 $\mu$l of ice-cold 20 mM Tris.HCl, pH 7.5, 100 mM NaCl, 25 mM $MgCl_2$, and filtered through 0.45-$\mu$m nitrocellulose filters (Millipore, Bedford, MA; cat. HAWP02500). After washing with 10 ml of same buffer, the filters were measured in Beckman LS6500 counter (Fullerton, CA) with the Econo-Safe scintillation cocktail (RPI, St. Laurent, Canada). To measure G protein–specific activity, 100 nM $(His)_6$-G$\alpha_o$ was identically probed with 1 $\mu$M [$^{35}$S]GTP$\gamma$S (150 cpm/pmol GTP$\gamma$S). Specific activity was calculated from the ratio of radioactivity bound by the G protein to that expected to be bound, as the average of four experiments.

### Preparation of Protein Extracts from Fly Heads

Adult flies were anesthetized by $CO_2$ and stored at −20°C in a 50-ml Falcon tube. Heads were separated from the bodies using the Mini-Sieve set (Bel-Art Products, Pequannock, NJ) after freezing the flies in liquid nitrogen and vortexing and were smashed on ice in a glass-rod homogenizer (Sartorius) in a hypotonic buffer (10 mM HEPES-KOH, pH 7.5, 2 mM EGTA, and 2× protease inhibitor cocktail). After adjusting the ionic strength by adding KCl to 100 mM and HEPES to 50 mM, the debris was removed by short (15 s) centrifugation at 200 × *g* at 4°C. The supernatant was recentrifuged at 20,000 × *g*/60 min at 4°C. The pellet was resuspended in 10 mM HEPES, pH 7.5, 100 mM KCl, 5% glycerol, 2 mM EGTA, and 1× protease inhibitor cocktail, frozen in liquid nitrogen, and stored in aliquots at −80°C. On usage, the defrosted extract was resuspended with 50 mM HEPES, pH 7.5, 150 mM KCl, 10 mM NaCl, 2 mM EGTA, and 1× protease inhibitor cocktail to 1.5 mg protein/ml and solubilized with 0.5% Nonidet P-40, and 0.1% Tween20 for 4 h at 4°C on a rotator, followed by centrifugation 20,000 × *g*/30 min/4°C. The resulting supernatant was immediately used in pulldown experiments.

### Pulldown Assays

***Pins on Resin.*** Amylose resin, 100 $\mu$l, with immobilized MBP-fusion protein (50% slurry; equal molar concentrations for all resin-immobilized MBP-fusion proteins were ensured) was used per 1.5 ml of the bacterial extract containing nontagged G$\alpha_o$ or G$\alpha_o$[Q205L] (the two forms G$\alpha_o$ were present in equal amounts) or per 2 ml of the *Drosophila* head extract. When necessary, the G$\alpha_o$-containing bacterial extract (in 50 mM HEPES-KOH, pH 8.0, 100 mM KCl, 1 mM DTT, 1× protease inhibitor cocktail, 0.5% Nonidet P-40, 0.1% Tween20, and 5 mM $MgCl_2$) was preloaded with 1 mM GDP or GTP$\gamma$S for 30 min at 25°C before the experiment. Purified $(His)_6$-G$\alpha_o$ was preloaded similarly and applied to the matrix providing a 1:1 molar ratio between G$\alpha_o$ and the immobilized MBP-fusion protein. The extracts with the amylose slurry were rotated at 4°C overnight, followed by centrifugation (200 × *g*/1 min/4°C) and removal of the supernatant. The matrix was washed four times with 10 bed volumes of the binding buffer without $MgCl_2$ at 4°C. Bound proteins were eluted by 8 M urea or 1× SDS sample buffer, separated by SDS-PAGE, and probed with anti-G$\alpha_o$ antibodies.

***G$\alpha_o$ on Resin.*** $(His)_6$-tagged G$\alpha_o$ or G$\alpha_o$[Q205L] were coupled to the CNBr (cyanogen bromide)-activated Sepharose 4 Fast Flow (GE Healthcare, Waukesha, WI) according to the manufacturer. Such coupling did not decrease the guanine nucleotide-binding properties of G$\alpha_o$, as measured by the BODIPY-GTP$\gamma$S assay. MBP was similarly coupled for control experiments. 100 $\mu$M GDP or GTP$\gamma$S in 50 mM HEPES-KOH, pH 8.0, 100 mM KCl, 25 mM $MgCl_2$, and 1 mM DTT were used to preload the immobilized G$\alpha_o$ (50% slurry) for 30 min at 25°C. A 20-fold volume excess of the *Drosophila* head extract was added to the slurry for a 4 h/4°C rotation, followed by centrifugation (200 × *g*/1 min/4°C) and removal of the supernatant. The matrix was 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, 1× protease inhibitor cocktail, 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 probed with guinea pig anti-Pins antibodies (1:1000; Bellaiche *et al.*, 2001).

***Binding in Solution.*** 1 $\mu$M $(His)_6$-G$\alpha_o$ or $(His)_6$-G$\alpha_i$ was preloaded with 1 mM GDP or GTP$\gamma$S for 30 min in 1.5 ml of buffer (50 mM HEPES-KOH, pH 8.0, 100 mM KCl, 1 mM DTT, 0.5% Nonidet P-40, 0.1% Tween20, and 5 mM $MgCl_2$) before addition of the equimolar amount MBP-Pins, GST-AGS3, or GST-LGN fusion proteins. The solution was incubated 4°C/4 h before addition of 50 $\mu$l amylose or glutathione Sepharose (50% slurry) and overnight incubation at 4°C. The matrix was washed four times with 10 bed volumes of the binding buffer without $MgCl_2$ (but with 50 $\mu$M GDP or GTP$\gamma$S) at 4°C. Bound proteins were eluted by boiling in 1× SDS sample buffer, separated by SDS-PAGE, and probed with rabbit anti-G$\alpha_o$ (see above) or anti-G$\alpha_i$ antibodies (1:1000, Calbiochem; cat. no. PC61).

### Analysis of the Trimeric Complex (G$\alpha_o$-Pins-G$\alpha_i$)

CNBr-immobilized G$\alpha_o$ (50% slurry, 100 $\mu$l) was preloaded with 100 $\mu$M GDP or GTP$\gamma$S in 50 mM HEPES-KOH, pH 8.0, 100 mM KCl, 25 mM $MgCl_2$, and 1 mM DTT for 30 min at 25°C. A 10-fold molar excess of MBP-Pins was added in 1.5 ml binding buffer (50 mM HEPES-KOH, pH 8.0, 100 mM KCl, 1 mM DTT, 0.5% Nonidet P-40, and 0.1% Tween20), together with or without $(His)_6$-G$\alpha_i$ (equimolar to Pins), and incubated at 4°C for 4 h, followed by centrifugation (200 × *g*/1 min/4°C) and removal of the supernatant. The matrix was washed four times with 10 bed volumes of the binding buffer at 4°C. Bound proteins were eluted by boiling in 1× SDS sample buffer, separated by SDS-PAGE, and probed with rabbit anti-G$\alpha_i$ (see above) and anti-MBP antibodies (1:10,000, New England Biolabs, E8030S).

### Drosophila Genetics

Standard methods of *Drosophila* genetics were applied (see Roberts, 1986). The *MS1096-Gal4* (Brand and Perrimon, 1993) line driving expression in the whole wing was crossed at 25°C to the *UAS-Ptx* (Katanaev *et al.*, 2005) or the UAS-RNAi lines targeting Pins, G$\alpha_i$, or Fz (Dietzl *et al.*, 2007). Wing mounting and quantification of stout bristle defects was done as in Katanaev and Tomlinson (2006a).

## RESULTS

### A Saturating Yeast Two-Hybrid Screen Identifies all Drosophila GoLoco Domain-Containing Proteins as Potential G$\alpha_o$-Interaction Partners

To identify potential binding partners of *Drosophila* G$\alpha_o$, we performed a saturating yeast two-hybrid screen using the wild-type G$\alpha_o$ and the GTPase-deficient Q205L mutant form of G$\alpha_o$ as the baits (Katanaev *et al.*, 2005). The first mostly resides in the GDP-bound state, and the second is predominantly GTP-bound (Graziano and Gilman, 1989; Kroll *et al.*, 1992; Supplementary Figure S1C) and will be referred to as G$\alpha_o$[GDP] and G$\alpha_o$[GTP] in the rest of the text, as opposed to G$\alpha_o$-GDP and G$\alpha_o$-GTP$\gamma$S, which will refer to G$\alpha_o$ directly loaded with GDP or GTP$\gamma$S in the subsequent biochemical experiments.

*Drosophila* head cDNA library was used as the prey, and 54.40 and 95.26 million interactions were analyzed with G$\alpha_o$[GDP] and with G$\alpha_o$[GTP], respectively. Multiple G$\alpha_o$-interaction partners were identified, some binding exclusively to G$\alpha_o$[GDP] or G$\alpha_o$[GTP] and others not differentiating between the two nucleotide forms of G$\alpha_o$ (to be published elsewhere). Among the identified partners, all three *Drosophila* GoLoco domain-containing proteins were found: Pins (containing three GoLoco domains), Loco, and Rapgap1 (Table 1). No other genes encode GoLoco domains in the *Drosophila* genome (see http://www.ebi.ac.uk/interpro/DisplayIproEntry?ac=IPR003109). Although Rapgap1 was found to interact only with G$\alpha_o$[GDP], Pins and Loco bound both G$\alpha_o$[GDP] and G$\alpha_o$[GTP] in the yeast two-hybrid assay (Table 1).

### Domains of Rapgap1, Loco, and Pins, Responsible for the Interaction with G$\alpha_o$ in the Yeast Two-Hybrid System

Because of the saturating manner of our yeast two-hybrid analysis (see *Materials and Methods*), each of the GoLoco domain-containing proteins was identified in many independent hits (see Table 1). As these hits often contained only fragments of the respective cDNAs encoding partial open reading frames, simple bioinformatics analysis (Formstecher *et al.*, 2005) of the binding fragments allowed us to narrow down the regions responsible for the interaction with G$\alpha_o$ in this yeast two-hybrid system. Of the 11 G$\alpha_o$[GDP]-interacting Rapgap1 sequences, all were found to contain amino acids 95-166 (Figure 1). This minimal G$\alpha_o$[GDP]-interacting

**Table 1.** *Drosophila* GoLoco domain-containing proteins

| GoLoco protein | No. of GoLoco domains | Other domains/motifs present | No. of interactions with $G\alpha_o$ in the yeast two-hybrid screen | No. of interactions with $G\alpha_o$[Q205L] in the yeast two-hybrid screen |
|---|---|---|---|---|
| Rapgap1 | 1 | RapGAP | 11 | None |
| Loco | 1 | RGS, Ras-binding | 11 | 11 |
| Pins (Rapsinoid) | 3 | TPR repeats | 46 | 26 |

site of Rapgap1 includes the GoLoco domain (amino acids 120-142, Figure 1). Thus, the interaction between *Drosophila* $G\alpha_o$[GDP] and Rapgap1 is likely mediated by the GoLoco domain, as has been reported for the $G\alpha_{i/o}$/Rapgap interactions in other organisms (Mochizuki *et al.*, 1999; Cuppen *et al.*, 2003; Willard *et al.*, 2007).

However, similar analysis of the $G\alpha_o$-interacting regions of Loco (11 hits for each, $G\alpha_o$[GDP] and $G\alpha_o$[GTP]) points to the RGS domain (amino acids 115-231) and not the GoLoco domain of Loco as responsible for the interaction with both forms of $G\alpha_o$ in our yeast two-hybrid system. Indeed, the minimal site of binding of $G\alpha_o$[GDP] is between amino acids 72-281, and the minimal site of binding of $G\alpha_o$[GTP] between amino acids 72-238, whereas the GoLoco domain encompasses amino acids 642-664 (Figure 2). RGS (Regulator of G protein Signaling) domains are responsible for the activation of the GTPase activity of Gα-subunits of trimeric G proteins and typically interact with the GTP hydrolyzing transition-state of Gα-subunits (Ross and Wilkie, 2000). Our finding of the interaction of both $G\alpha_o$[GDP] and $G\alpha_o$[GTP] with the RGS domain of Loco in the yeast two-hybrid system is somewhat unexpected and requires additional biochemical confirmations. *Drosophila* Loco has been shown to bind $G\alpha_i$, a related Gα-subunit, both through the RGS and the GoLoco domains (Yu *et al.*, 2005). Our findings suggest that, at least in our yeast two-hybrid system, $G\alpha_o$ binds Loco only through the RGS region of the latter. Our data also identify Loco as the first RGS protein interacting with *Drosophila* $G\alpha_o$.

Lastly, the similar analysis was performed with Pins (Figure 3). The bioinformatics analysis suggests that the 46 fragments of Pins interacting with $G\alpha_o$[GDP] fall into two groups with two separate $G\alpha_o$[GDP]-interaction regions: one encompassing amino acids 420-528, and the other amino acids 614-658 (till the end of the protein, Figure 3A). Pins contains three GoLoco domains, and GoLoco1 domain is part of the first $G\alpha_o$[GDP]-interaction region, whereas GoLoco3 domain falls into the second interaction region. Out of 46 fragments interacting with $G\alpha_o$[GDP], 10 fragments contain GoLoco1 domain but not GoLoco3 domain, 18 contain GoLoco3 but not GoLoco1, and 18 contain the whole GoLoco domain region (Figure 3A).

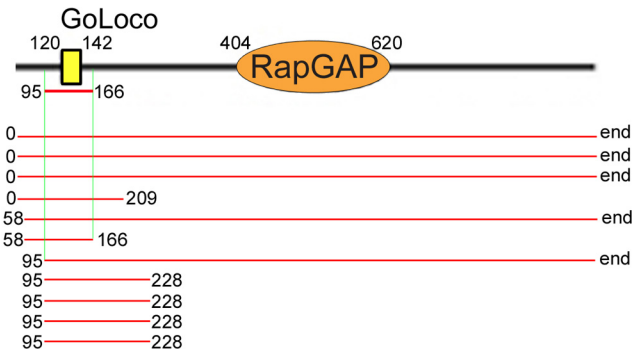


**Figure 1.** Identification of the $G\alpha_o$-binding site on Rapgap1 through the saturating yeast two-hybrid screen. The schematic domain structure of *Drosophila* Rapgap1 is shown, including the GoLoco domain (amino acids 120-142) and the RapGAP catalytic domain (amino acids 404-620). Eleven hits containing complete or partial Rapgap1 coding sequences found to interact with $G\alpha_o$ are shown in red with their starting and ending amino acids. All fragments contain amino acids 95-166, which restricts the minimal $G\alpha_o$-interacting part of Rapgap1 to the GoLoco domain-containing region.

In contrast, a single region interacting with $G\alpha_o$[GTP] can be identified from the 26 Pins fragments (Figure 3B). This region includes amino acids 470-517 and covers GoLoco1 domain. Fifteen fragments of Pins binding $G\alpha_o$[GTP] contain GoLoco domain 1 but not 3, with the remaining 11 encompassing the whole GoLoco half of Pins (Figure 3B). Because the same cDNA library was used to screen for the $G\alpha_o$[GDP] and $G\alpha_o$[GTP] binding partners, the above analysis may suggest the comparable binding of the two forms of $G\alpha_o$ to the GoLoco1 domain-containing fragments of Pins in the yeast two-hybrid screen, whereas $G\alpha_o$[GDP] additionally interacts with the GoLoco3 domain.

Among the five GoLoco domains of the *Drosophila* GoLoco proteome two did not reveal binding to any form of $G\alpha_o$ in our yeast two-hybrid screen (the Loco GoLoco domain, and Pins GoLoco2 domain), whereas two GoLoco domains suggest the "canonical" binding to the $G\alpha_o$[GDP] but not the activated form of $G\alpha_o$ (these are the Rapgap1 GoLoco domain and the GoLoco3 domain of Pins). The "noncanonical" binding to both nucleotide forms of $G\alpha_o$ in the yeast two-hybrid assay is revealed only by the Pins GoLoco1 domain. As the yeast two-hybrid assays sometimes record unnatural interactions or provide false-negative results, these preliminary conclusions require independent confirmations using biochemical assays. Such confirmations for the $G\alpha_o$-Pins interactions are detailed below.

### *Confirmation of Pins Binding to the GTP-Forms of $G\alpha_o$ In Vitro*

We previously demonstrated the ability of recombinant GST-tagged $G\alpha_o$-GTPγS to pull down Pins from *Drosophila* extracts (Katanaev and Tomlinson, 2006a). However, the same form of $G\alpha_o$ failed to efficiently bind purified recombinant Pins (Katanaev and Tomlinson, 2006a). Given the convincing yeast two-hybrid data described above that $G\alpha_o$[GTP] interacted with Pins, we hypothesized that the presence of the GST tag on $G\alpha_o$ could somehow impede the interaction with purified Pins. Thus we prepared recombinant, nontagged or $(His)_6$-tagged forms of $G\alpha_o$. We compared the enzymatic activities of the GST-tagged $G\alpha_o$ and the $(His)_6$-tagged $G\alpha_o$ using the BODIPY-GTPγS assay (McEwen *et al.*, 2001) and found that the big GST tag negatively affected the G protein activity: only 8–10% of the

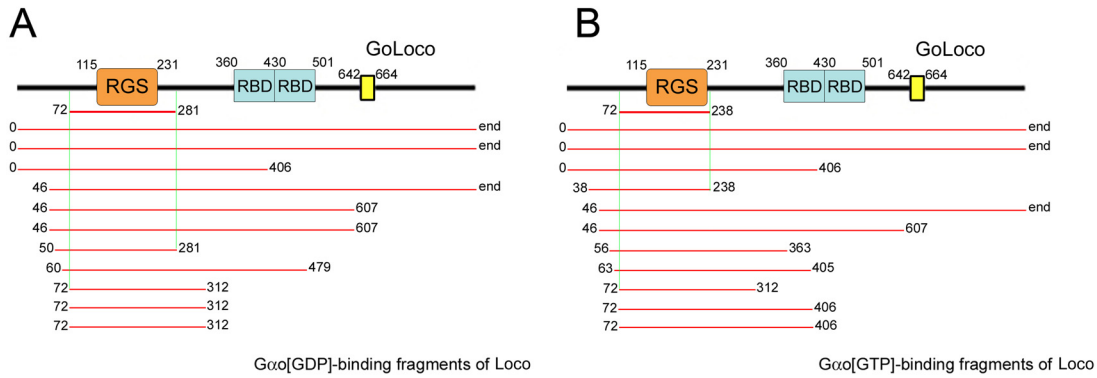


**Figure 2.** Identification of the $G\alpha_o$[GDP] -binding sites (A) and $G\alpha_o$[GTP]-binding sites (B) on Loco through the saturating yeast two-hybrid screen. The schematic domain structure of *Drosophila* Loco is shown, including the catalytic RGS domain (amino acids 115-231), the double Ras-binding domain (amino acids 360-501), and the GoLoco domain (amino acids 642-664). Eleven hits containing complete or partial Loco coding sequences found to interact with $G\alpha_o$[GDP] and $G\alpha_o$[GTP] are shown in red with their starting and ending amino acids. All $G\alpha_o$[GDP]-interacting fragments contain amino acids 72-281; all $G\alpha_o$[GTP]-interacting fragments contain amino acids 72-238. The minimal $G\alpha_o$[GDP]- and $G\alpha_o$[GTP]-interacting part of Loco is thus restricted to the RGS domain-containing region.

purified GST-$G\alpha_o$ was active, compared with 25–50% specific activity of the purified $(His)_6$-$G\alpha_o$ (Supplementary Figure S1B). An additional purification of $(His)_6$-$G\alpha_o$ on phenyl-Sepharose (see *Materials and Methods*) resulted in 80% active protein, as measured both by the BODIPY-GTP$\gamma$S and [$^{35}$S]GTP$\gamma$S-binding assays (Supplementary Figure S1B). We also hypothesized that $G\alpha_o$-GTP$\gamma$S might have not fully reflected the $G\alpha_o$ activation state represented by the activating Q205L mutation and thus also prepared nontagged or $(His)_6$-tagged forms of $G\alpha_o$[Q205L] ($G\alpha_o$[GTP]). The ability of the $(His)_6$-$G\alpha_o$[GTP] to bind but not hydrolyze GTP was confirmed using the BODIPY-GTP assay (Willard *et al.*, 2005; Supplementary Figure S1C). As a control demonstrating the completeness of the GTP charging in our $G\alpha_o$-preparations, we found that the GTP-forms of $G\alpha_o$ could not bind the $\beta\gamma$ subunits of trimeric G proteins from head extracts, whereas the GDP-forms of $G\alpha_o$ efficiently interacted with $\beta\gamma$ (Katanayeva, Kopein, Portmann, Hess, and Katanaev, unpublished data).

We next confirmed that the non-GST-tagged $G\alpha_o$ could bind Pins from *Drosophila* head extracts. Figure 4A shows that both the GDP- and the GTP-forms of $G\alpha_o$ efficiently precipitated the full-length Pins from *Drosophila* heads, confirming our previous observations (Katanaev and Tomlinson, 2006a). In addition, the GDP-forms precipitated a slower-migrating form of Pins that we had previously identified as phosphorylated Pins (Katanaev and Tomlinson, 2006a). The GTP-forms of $G\alpha_o$ ($G\alpha_o$[GTP] and $G\alpha_o$-GTP$\gamma$S) were unable to bind phosphorylated Pins (Figure 4A). These data may also serve as a control of completeness of the GTP charging in our $G\alpha_o$-GTP-forms.

In the reciprocal experiments, recombinant Pins immobilized on a resin, or the GoLoco-containing C-terminal half,

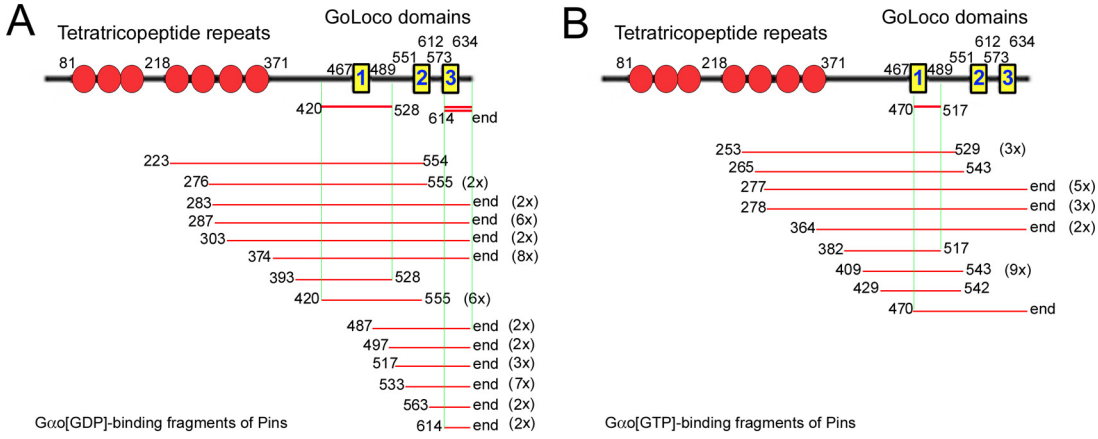


**Figure 3.** Identification of the $G\alpha_o$[GDP]-binding sites (A) and $G\alpha_o$[GTP]-binding sites (B) on Pins through the saturating yeast two-hybrid screen. The schematic domain structure of *Drosophila* Pins is shown, including seven tetratricopeptide repeats (amino acids 81-371), and the three GoLoco domain (first, amino acids 467-489; second, amino acids 551-573; third, amino acids 612-634). Forty-six hits containing partial Pins coding sequences found to interact with $G\alpha_o$[GDP] (A) and 26 hits interacting with $G\alpha_o$[GTP] (B) are shown in red with their starting and ending amino acids. Identical sequences independently identified are shown once with the description of how many times these sequences were found. (A) The $G\alpha_o$[GDP]-interacting fragments fall into two groups; all fragments of the first group contain amino acids 420-528 (underlined with a single red line); the fragments of the second group contain amino acids 614 up to the end of the C-terminus of Pins (underlined with a double red line). Thus the two distinct $G\alpha_o$-interacting parts of Pins contain the GoLoco1 domain and the GoLoco3 domain. (B) In contrast, the $G\alpha_o$[GTP]-interacting fragments of Pins fall into the single minimal $G\alpha_o$[GTP]-binding site, overlapping with the GoLoco1 domain.

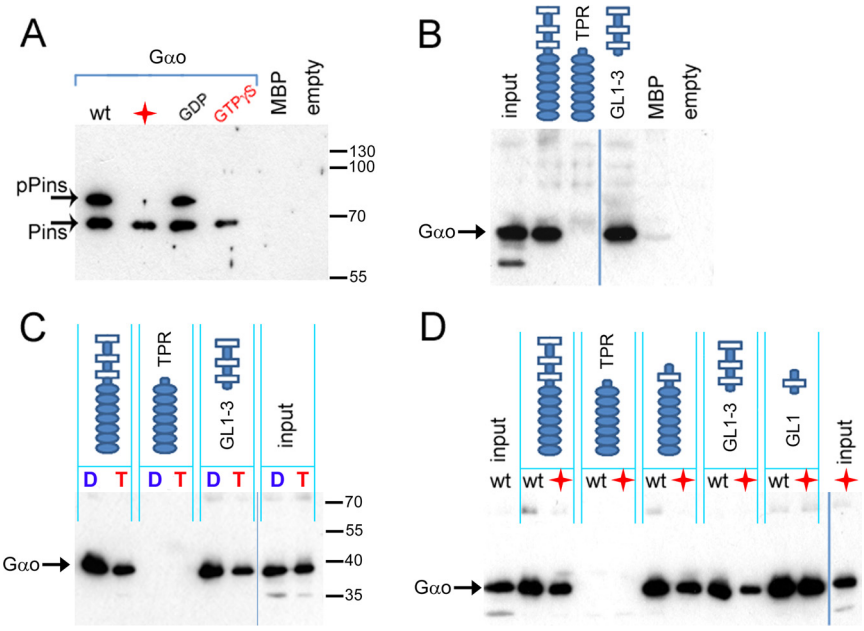


**Figure 4.** Biochemical interactions between different forms of $G\alpha_o$ and Pins. (A) Non-preloaded $(His)_6$-$G\alpha_o$ (wt) and $(His)_6$-$G\alpha_o$[Q205L] (⋆), or the GDP- and GTPγS-preloaded forms of the wild-type $(His)_6$-$G\alpha_o$ immobilized on the CNBr-Sepharose efficiently bind endogenous Pins from *Drosophila* head extracts. The GDP-forms of $G\alpha_o$ also bind the phosphorylated Pins (pPins). Control resins (empty or containing MBP) do not bind any form of Pins. (B) Full-length Pins and its C-terminal GoLoco-containing half, immobilized on the amylose resin, efficiently bind endogenous $G\alpha_o$ from *Drosophila* head extracts. Control resins (empty or containing MBP) do not bind $G\alpha_o$. The N-terminal TPR-containing half of Pins does not bind $G\alpha_o$ and is used as an internal negative control in the other panels of this and subsequent figures. (C) Full-length recombinant Pins and its C-terminal GoLoco-containing half, but not the N-terminal TPR-containing half, immobilized on the amylose resin, interact with recombinant nontagged $G\alpha_o$ preloaded with GDP or GTPγS (D or T on the Figure). (D) Full-length Pins or its fragments containing GoLoco1 domain, immobilized on the amylose resin, interact with recombinant nontagged $G\alpha_o$[GDP] (wt) or $G\alpha_o$[GTP] ($G\alpha_o$[Q205L], ⋆). Pins constructs immobilized on the resin are schematically shown with seven tetratricopeptide repeats (ovals) and three GoLoco domains (open boxes). Each image is a representative result of at least three independent experiments. Molecular weight markers are shown for the anti-Pins Western blot (A) and once for the anti-G protein Western blot (C) but is not shown for other anti-G protein Western blots of this and subsequent figures for space minimization.

but not the TPR-containing N-terminal half of the protein, could efficiently precipitate endogenous *Drosophila* $G\alpha_o$ from the head extracts (Figure 4B).

Subsequent coprecipitation experiments with recombinant proteins confirmed our yeast two-hybrid data and proved that the GTP-forms of $G\alpha_o$ could interact with Pins with a high efficiency comparable to that of the GDP-forms. These observations were made in multiple experimental setups:

1. Nontagged $G\alpha_o$, provided as bacterial lysates preloaded with GTPγS or GDP, bound efficiently to MBP-Pins immobilized on the amylose resin (Figure 4C).
2. Nontagged $G\alpha_o$[GTP] and $G\alpha_o$[GDP], provided as bacterial lysates, bound similarly to Pins (Figure 4D).
3. Purified $(His)_6$-$G\alpha_o$, loaded with GTPγS or GDP, bound equally to Pins immobilized on the resin (Supplementary Figure S2A).
4. Purified $(His)_6$-$G\alpha_o$[Q205L], directly loaded with GTPγS or GDP, bound equally to Pins (Supplementary Figure S2B). The ability of $G\alpha_o$[Q205L] to exchange its GTP for other nucleotides in our nucleotide-binding conditions (see *Materials and Methods*) is demonstrated on Supplementary Figure S2C. These experiments show that the [Q205L] form of $G\alpha_o$ is active and can bind Pins in various nucleotide-bound states.
5. Purified $(His)_6$-$G\alpha_o$, immobilized on CNBr-Sepharose and loaded with GTPγS or GDP, efficiently precipitated soluble MBP-Pins (Figure 5D, bottom panel).
6. $(His)_6$-$G\alpha_o$, double-purified on the nickel and phenyl columns (and thus 80% active; see Supplementary Figure S1B), preincubated with Pins in solution before application to the MBP-Pins–precipitating amylose resin, revealed equal binding to Pins in the GDP- and GTPγS-states (Figure 5B).

Altogether, these experiments prove that the GTP-bound forms of $G\alpha_o$ can physically bind to Pins with the efficiency comparable to that of the GDP-forms. However, we note that these pull down experiments are qualitative or at best semiquantitative in nature. Although a similar capacity of the GTP- and the GDP-bound forms of $G\alpha_o$ to interact with Pins is clearly demonstrated by these experiments, direct measurements of the affinity of these interactions are lacking and will be subject of future research.

### *The GTP-Forms of $G\alpha_o$ Bind Pins through Its GoLoco1 Domain In Vitro*

Our yeast two-hybrid data results suggest that the region of interaction of Pins with $G\alpha_o$[GTP] is restricted to the GoLoco1 domain of Pins, whereas $G\alpha_o$[GDP] binds to both GoLoco1 and GoLoco3 domains of Pins (see Figure 3). We confirmed these observations using recombinant $G\alpha_o$ and Pins proteins.

First, we found that in all experimental setups described above, $G\alpha_o$—in either GDP or GTP nucleotide state—could not interact with the TPR-containing N-terminal half of Pins, but interacted efficiently with its GoLoco1-3 domain–containing C-terminal half. This was observed when Pins fragments were used to coprecipitate endogenous $G\alpha_o$ from *Drosophila* heads (Figure 4B), recombinant nontagged $G\alpha_o$ from bacterial extracts (Figure 4, C and D), or purified $(His)_6$-tagged $G\alpha_o$[GDP] or $G\alpha_o$[GTP] (Supplementary Figure S2, A and B). The same phenomenon was also observed when two-step purified $G\alpha_o$ was allowed to interact with Pins fragments in solution before Pins precipitation (Figure 5B) or when $G\alpha_o$ was preimmobilized on a resin (Figure 5D, bottom panel).

Second, we found that GoLoco1 domain, present alone or together with the TPR part, was similarly efficient to interact with the GTP- and GDP-forms of $G\alpha_o$ in multiple setups (Figures 4D and 5B; Supplementary Figures S2, A and B, and S3, A and B).

And third, we confirmed in several types of experiments that the GoLoco3 domain could interact efficiently with the

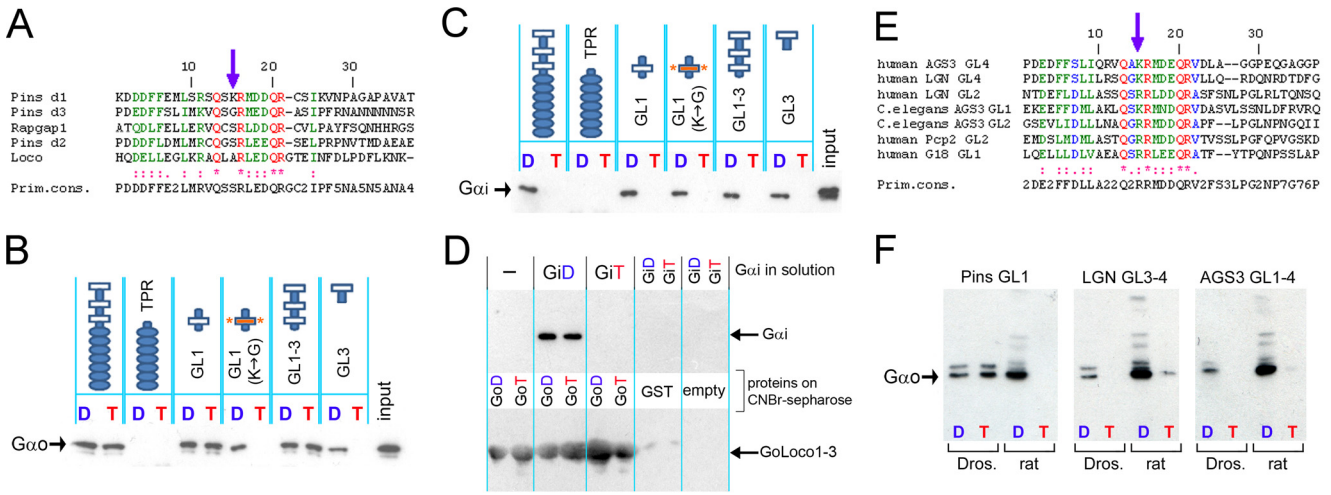


**Figure 5.** $G\alpha_o$, $G\alpha_i$, Pins, and its mammalian homologues. (A) Alignment of the extended GoLoco sequences of the five GoLoco domains of the *Drosophila* proteome. Pins GoLoco1 domain contains Lys15 (↓) preceding the conserved Asp/Glu-Gln-Arg triad. Lys15 is absent in other *Drosophila* GoLoco sequences. (B) Lys15 is crucial for the interaction of $G\alpha_o$-GTPγS with Pins. Full-length Pins, its GoLoco-containing half, or the isolated GoLoco1 domain efficiently interact with both $G\alpha_o$-GDP and $G\alpha_o$-GTPγS in solution before precipitation of Pins fragments on amylose resin. In contrast, the isolated GoLoco3 domain or the Lys15Gly mutated version of GoLoco1 domain interact only with $G\alpha_o$-GDP. TPR-containing half of Pins does not bind either form of $G\alpha_o$. (C) In a similar experimental setup, $G\alpha_i$ binds Pins constructs exclusively in the GDP state. (D) $G\alpha_o$-GDP or $G\alpha_o$-GTPγS, preimmobilized on a matrix, efficiently interact with the GoLoco1-3-containing portion of Pins. Addition of soluble $G\alpha_i$ in the GDP-loaded, but not GTPγS-loaded form, resulted in coprecipitation of $G\alpha_i$ indicating formation of a trimeric $G\alpha_o$-Pins-$G\alpha_i$ complex. (E) Sequence alignment of several non-*Drosophila* GoLoco domains shows conservation of Lys15 or Arg15 in GoLoco sequences of other organisms, including mammalian AGS3 and LGN. (F) Despite this conservation, *Drosophila* $G\alpha_o$-GTPγS fails to interact with the GoLoco-containing fragments of human LGN or rat AGS3; $G\alpha_o$-GDP robustly binds these proteins. Furthermore, rat $G\alpha_o$ also interacts with Pins, LGN, or AGS3 exclusively in the GDP-bound state. Note that the anti-$G\alpha_o$ antibodies recognize the rat $G\alpha_o$ more strongly than the *Drosophila* $G\alpha_o$ despite their equal molar amounts used in these experiments. Each Western blot is a representative result of at least three independent experiments.

GDP-, but not GTP-loaded forms of $G\alpha_o$ (Figure 5B, Supplementary Figure S3B). Thus, we conclude that the GTP-loaded $G\alpha_o$ binds Pins through the GoLoco1 domain, whereas GoLoco3 domain can sustain interaction only with the GDP-loaded $G\alpha_o$.

### *Lysine15 in GoLoco1 Domain Is Necessary for Binding to the GTP-Loaded $G\alpha_o$*

GTP-forms of $G\alpha_o$ bind Pins through its GoLoco1 domain. This distinguishes it from the other GoLoco domains in *Drosophila*. What might be so special in the GoLoco1 domain of Pins that allows it to bind GTP-loaded $G\alpha_o$? Alignment of the *Drosophila* GoLoco domain sequences reveals that a Lys residue is present in GoLoco1 domain of Pins (Figure 5A) at position 15 (position 510 of the full-length Pins), preceding the Asp/Glu-Gln-Arg triad crucial for the interaction with the Gα-subunits in the vicinity of the guanine nucleotide-binding pocket (Kimple *et al.*, 2002). We hypothesized that the positively charged side chain of this Lys might interact with the negative charge of the γ-phosphate of GTP and so stabilize the interaction between Pins and the GTP-loaded $G\alpha_o$. To test this hypothesis, we substituted this Lys with Gly found in the identical position of GoLoco3 domain of Pins (see Figure 5A), which binds exclusively the GDP-forms of $G\alpha_o$ (see above). The abilities of the intact and the (Lys→Gly)-mutated GoLoco1 domains to bind different forms of $G\alpha_o$ were compared in pulldown experiments.

Figure 5B shows that the two-step purified $(His)_6$-$G\alpha_o$, preloaded with GDP or GTPγS, interacted similarly with Pins GoLoco1 domain in solution. In contrast, binding of $G\alpha_o$-GTPγS was abolished upon introduction of the Lys→Gly mutation in GoLoco1 (Figure 5B). In essence, this single mutation converted GoLoco1 into the GoLoco3 domain in terms of their interactions with different nucleotide forms of $G\alpha_o$ (Figure 5B). Similar observations were obtained in other experimental setups, for example, using single-purified $(His)_6$-$G\alpha_o$ and Pins constructs preimmobilized on a matrix (Supplementary Figure S2A), nontagged $G\alpha_o$ provided as bacterial lysates preloaded with different nucleotides (Supplementary Figure S3B), or nontagged $G\alpha_o$[GDP] and $G\alpha_o$[GTP] provided in bacterial lysates (Supplementary Figure S3A). These experiments confirm our hypothesis that the Lys15 of GoLoco1 domain of Pins is necessary for its ability to interact with the GTP-loaded $G\alpha_o$.

### *$G\alpha_i$ Binds Pins in the GDP State But Can Participate in a Trimeric Complex with Pins and GTP-Loaded $G\alpha_o$*

Interactions of $G\alpha_i$ with Pins and its homologues have been extensively studied for both *Drosophila* and mammalian proteins (De Vries *et al.*, 2000; Bernard *et al.*, 2001; Natochin *et al.*, 2001; Adhikari and Sprang, 2003; McCudden *et al.*, 2005; Nipper *et al.*, 2007). In pulldown experiments performed in parallel to the above-described studies on $G\alpha_o$-Pins interactions, we confirmed the previous reports that $G\alpha_i$ bound to Pins exclusively in the GDP-state (Figure 5C and Supplementary Figure S3C). We also found that both GoLoco1 and GoLoco3 domains of Pins interacted efficiently with $G\alpha_i$-GDP, and that the Lys→Gly mutation on GoLoco1 domain did not affect this interaction (Figure 5C and Supplementary Figure S3C). Thus, the binding of Pins and the GTP-loaded G protein appears specific of the $\alpha_o$ but not $\alpha_i$ member of the $G\alpha_{i/o}$ subfamily of trimeric G proteins.

Because of the multiple GoLoco domains present in Pins proteins (three in *Drosophila* Pins and four in its mammalian homologues LGN and AGS3), a single Pins molecule or its GoLoco domains-containing region was found to interact

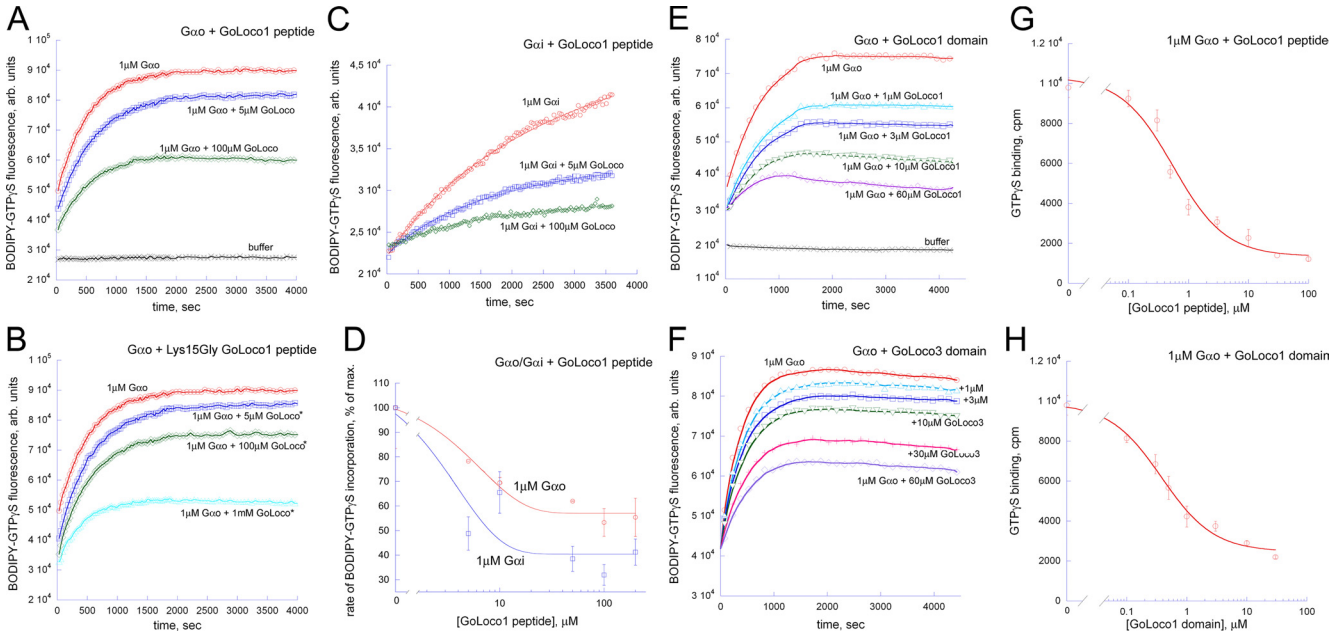


**Figure 6.** Pins GoLoco domains exerts the GDI activity toward Gα-subunits. $G\alpha_o$ (A, B, and E–H) or $G\alpha_i$ (C) at 1 μM was incubated with BODIPY-GTPγS (A–F) or [$^{35}$S]GTPγS (G and H) in the absence or presence of Pins GoLoco1 synthetic peptide (A, C, D, and G), Lys15Gly synthetic GoLoco1 peptide (B), or recombinant GoLoco1 (E and H) or GoLoco3 (F) domains. Individual BODIPY-GTPγS incorporation curves are shown with selected concentrations of GoLoco1 and represent three independent experiments (A, B and E, F). Quantifications of the effects of increasing concentrations of GoLoco1 on the rate of BODIPY-GTPγS incorporation into $G\alpha_o$ and $G\alpha_i$ (D) or on the plateau levels of [$^{35}$S]GTPγS incorporation (G and H) are presented as mean ± SEM, n = 3–5.

with several $G\alpha_i$ molecules at once (Bernard *et al.*, 2001; Nipper *et al.*, 2007), although the physiological significance of such interactions are not clear. We hypothesized that *Drosophila* Pins could also interact simultaneously with $G\alpha_i$ and $G\alpha_o$, the latter present either in the GDP- or GTP-bound form. To investigate this possibility, we incubated $G\alpha_i$, preloaded with GDP or GTPγS, with Pins GoLoco1-3 domains and Sepharose-immobilized GDP/GTPγS-loaded $G\alpha_o$ or control matrixes. Figure 5D demonstrates an efficient binding of $G\alpha_i$ to the $G\alpha_o$-containing but not control matrixes in the presence of GoLoco1-3 domains, suggesting formation of a trimeric complex between $G\alpha_o$, Pins, and $G\alpha_i$, where the two G proteins bind to different GoLoco domains of Pins. As expected, only GDP-loaded $G\alpha_i$ could be seen in the trimeric complex, whereas $G\alpha_o$ could exist either in the GDP- or the GTPγS-bound state in this complex (Figure 5D). In the latter scenario, given all the information presented above, the complex must be organized by $G\alpha_o$-GTPγS bound to GoLoco1 domain of Pins and by $G\alpha_i$-GDP bound to GoLoco3 domain (or GoLoco2 domain; see Nipper *et al.*, 2007). This trimeric complex forming in vitro may be artificial and not representing a physiologically relevant cellular event. Alternatively, such a trimeric $G\alpha_o^{GTP}$-Pins-$G\alpha_i^{GDP}$ complex might have a functional importance for the activity of these proteins in the context of the asymmetric cell divisions (see below).

### *The GDI Activity of Pins toward $G\alpha_o$*

Pins and its mammalian homologues AGS3 and LGN not only exclusively bind to the GDP-form of $G\alpha_i$, they also possess the GDI (guanine nucleotide dissociation inhibitor) activity toward $G\alpha_i$, inhibiting the loading of $G\alpha_i$ with GTP (De Vries *et al.*, 2000; Natochin *et al.*, 2001). In contrast, AGS3′ or LGN's GoLoco domains did not reveal such an activity toward $G\alpha_o$ (De Vries *et al.*, 2000; Natochin *et al.*, 2001). To test whether GoLoco1 domain of Pins modulated the GTP-binding by $G\alpha_o$, we performed the BODIPY-GTPγS-binding analysis (McEwen *et al.*, 2001) on $G\alpha_o$ with increasing concentrations of GoLoco1 synthetic peptide (see Figure 5A for the peptide sequence). Surprisingly, we found a robust GDI activity of the GoLoco1 peptide toward $G\alpha_o$ (Figure 6A). $G\alpha_i$ (Nipper *et al.*, 2007) revealed a comparable sensitivity to the GoLoco1 peptide (Figure 6, C and D), despite a generally slower rate of GTP incorporation into $G\alpha_i$ as compared with $G\alpha_o$ (Figure 6, A and C). These experiments were performed in the presence of mild detergents as were the pulldown experiments (see *Materials and Methods*). Interestingly, when detergents were omitted, the GoLoco1 peptide lost its GDI activity toward $G\alpha_o$ (Supplementary Figure S4A), but not $G\alpha_i$ (data not shown). Although we are not certain about the importance of this observation, we note that the GPCR peptide mimetic mastoparan (Higashijima *et al.*, 1990) could stimulate $G\alpha_o$ charging with BODIPY-GTPγS also only in the presence of detergents but not in their absence (data not shown).

Similarly to the synthetic GoLoco1 peptide, the recombinant GoLoco1 domain also displayed the GDI activity against $G\alpha_o$ (Figure 6E). To exclude the possibility that the GDI activity of GoLoco1 toward $G\alpha_o$ was an artifact of the experimental setup, we repeated the experiments using two-step purified (80% active) $G\alpha_o$ and the [$^{35}$S]GTPγS-binding assay. Both the peptide and the recombinant GoLoco1 domain revealed a robust GDI activity toward $G\alpha_o$ in these new conditions (Figure 6, G and H).

GoLoco3 domain of Pins fails to interact with the GTP-forms of $G\alpha_o$ but interacts robustly with $G\alpha_o$-GDP, albeit to a somewhat lesser extent than the GoLoco1 domain does (Figure 5B; Supplementary Figure S3B). Similarly, the Lys15Gly mutation of GoLoco1 domain abrogates the binding to the GTP-forms of $G\alpha_o$ but only moderately affects the interaction with $G\alpha_o$-GDP (Figure 5B; Supplementary Fig-

ures S2A, and S3, A and B). We decided to test whether the ability of Pins GoLoco sequences to act as GDIs toward $G\alpha_o$ depended on their abilities to bind GTP-loaded $G\alpha_o$. We found that recombinant GoLoco3 domain demonstrated a clear GDI activity (Figure 5F), which was however reduced compared with that of GoLoco1 domain (Figure 5E). Similarly, a synthetic GoLoco1 peptide harboring the Lys15Gly mutation possessed a clear GDI activity that was lowered compared with that of the intact synthetic peptide (Figure 6, A and B). We thus conclude that the GDI activity of GoLoco peptides did not correlate with their ability to bind GTP-forms of $G\alpha_o$, but only with their ability to bind $G\alpha_o$-GDP.

As we found that the GoLoco1 domain could efficiently bind the GTP-loaded forms of $G\alpha_o$, we decided to investigate whether this domain could in any respect influence the GTP hydrolysis reaction of $G\alpha_o$. However, neither stimulation nor inhibition of the GTPase reaction was found in the BODIPY-GTP assay (Willard *et al.*, 2005) performed either with (Supplementary Figure S4B) or without (not shown) added detergents. In contrast, the *Drosophila* homolog of RGS19 was found to efficiently stimulate GTP hydrolysis by $G\alpha_o$ in the same assay (Lin and Katanaev, unpublished). Thus, GoLoco1 domain strongly affects the GTP binding, but not hydrolysis, reaction of $G\alpha_o$ in vitro. However, the physiological importance of the in vitro GDI activity of GoLoco domains toward Gα-subunits is unclear.

### *The Interaction of Pins with GTP-Loaded $G\alpha_o$ Is Not Maintained in their Mammalian Homologues*

Although Lys15 preceding the Asp/Glu-Gln-Arg triad is unique among *Drosophila* GoLoco domains (Figure 5A), it is present in several GoLoco domains of other organisms, such as GoLoco4 domains of vertebrate AGS3 and LGN, as well as nematode AGS3 GoLoco1 domain (Figure 5E). Several GoLoco domains (GoLoco1 of mammalian G18, GoLoco2 of nematode AGS3, and GoLoco2 of mammalian Pcp-2) contain another positively charged amino acid (Arg) in this position (Figure 5E; Willard *et al.*, 2004). These data suggest that the interaction between the GTP-loaded $G\alpha_o$ and GoLoco-containing proteins might be conserved in evolution.

To investigate this possibility, we bacterially expressed the GoLoco-domain–containing fragments of mammalian LGN: GoLoco domains 3 and 4 (Natochin *et al.*, 2001) and of AGS3: GoLoco domains 1–4 (Bernard *et al.*, 2001). We also prepared $(His)_6$-tagged rat $G\alpha_o$ in parallel to *Drosophila* $G\alpha_o$ and tested whether these G proteins could interact, in their GDP- or GTPγS-loaded forms, with the GoLoco domains of Pins, LGN, and AGS3 (Figure 5F). Surprisingly, although both nucleotide forms of *Drosophila* $G\alpha_o$ bound efficiently to Pins (Figure 5F, left panel; also see above), only $G\alpha_o$-GDP bound LGN and AGS3, but $G\alpha_o$-GTPγS completely failed to interact with these two mammalian Pins homologues (Figure 5F, middle and right panels). Moreover, we find that rat $G\alpha_o$-GDP could efficiently interact with all three GoLoco domain proteins, but rat $G\alpha_o$-GTPγS was unable to interact with Pins, LGN, or AGS3 (Figure 5F).

Thus, the ability of $G\alpha_o$ in both nucleotide states to bind Pins appears specific for *Drosophila* proteins: mammalian $G\alpha_o$ fails to bind Pins, AGS3, or LGN in the GTPγS-form, and *Drosophila* $G\alpha_o$-GTPγS does not bind non-*Drosophila* Pins homologues.

### *Investigation of the In Vivo Function of the $G\alpha_o$-Pins Interactions Is Sensory Organ Development*

We previously showed the involvement of $G\alpha_o$ in the process of the asymmetric cell divisions in *Drosophila* adult sensory bristle formation (this process is schematically shown on Figure 7A); overactivation of $G\alpha_o$ resulted in defective bristle formation, while $G\alpha_o$ loss-of-function produced less frequent defects (Katanaev and Tomlinson, 2006a). We found a genetic interaction between $G\alpha_o$ and Pins, as well as a possible redundancy between $G\alpha_o$ and $G\alpha_i$ in this process (Katanaev and Tomlinson, 2006a). To extend these observations, we now used RNAi constructs to target Pins and $G\alpha_i$ (Dietzl *et al.*, 2007), and pertussis toxin to uncouple $G\alpha_o$ from interactions with GPCRs such as Frizzled. $G\alpha_o$ is the only target of pertussis toxin in *Drosophila* (Katanaev *et al.*, 2005; Katanaev and Tomlinson, 2006b). Expression of the RNAi against Pins produced visible asymmetric cell division defects resulting in formation of aberrant sensory bristles (Figure 7B; $3.3 \pm 0.4$ aberrant stout bristles per wing; mean ± SEM; $n = 16$; aberrant stout bristles per wing). In contrast, RNAi against $G\alpha_i$ was incapable to produce similar defects (similarly to genetic removal of $G\alpha_i$; Katanaev and Tomlinson, 2006a), nor did the treatment of $G\alpha_o$ with pertussis toxin (Figure 7, C and D; $0.0 \pm 0.0$ aberrant bristles per wing; $n = 16$ for both genotypes). However, a combination of $G\alpha_i$ loss-of-function and $G\alpha_o$-receptor uncoupling phenocopied Pins loss-of-function (Figure 7E; $2.5 \pm 0.3$ aberrant bristles per wing; $n = 16$).

Similar to uncoupling of $G\alpha_o$ from Frizzled with pertussis toxin, down-regulation of Frizzled receptors either by genetic loss-of-function (Gho and Schweisguth, 1998) or by expression of the Frizzled-targeted RNAi (Figure 7F) did not produce visible asymmetric cell division defects ($0.0 \pm 0.0$ aberrant bristles per wing; $n = 11$). However, a combination of Frizzled and $G\alpha_i$ down-regulation again recapitulated such defects (Figure 7G; $1.7 \pm 0.3$ aberrant bristles per wing; $n = 21$).

These results suggest that in vivo, GTP-loaded $G\alpha_o$ released from the trimeric $G_o$ complexes after activation with Frizzled receptors serves to activate Pins as one of its target proteins. A second input into Pins is provided by $G\alpha_i$ in its GDP-bound form (Schaefer *et al.*, 2001; Nipper *et al.*, 2007), Figure 7H. This redundancy between $G\alpha_o$ and $G\alpha_i$ in the regulation of the GoLoco domain function during asymmetric cell divisions is conserved in 600 million years of evolution between nematodes and insects (Gotta and Ahringer, 2001; Vavouri *et al.*, 2008).

## DISCUSSION

In the present work we demonstrate using the yeast two-hybrid and pulldown experiments that *Drosophila* Pins can interact with both GDP- and GTP-bound forms of the $G\alpha_o$ G-protein. We characterize the GoLoco1 domain of Pins as necessary and sufficient for the interaction with $G\alpha_o$-GTP, and pinpoint the Lys15 of GoLoco1 as necessary for the stabilization of the GoLoco/$G\alpha_o$ complex in the presence of GTP. Our experiments thus identify Pins as a likely target of $G_o$-mediated GPCR signaling.

These observations expand our previous report (Katanaev and Tomlinson, 2006a) that Pins could interact with $G\alpha_o$ in the context of the asymmetric cell divisions during formation of *Drosophila* adult sensory bristles. In that previous work, we had demonstrated a genetic interaction, as well as an ability of both GDP- and GTPγS-loaded forms of recombinant $G\alpha_o$ to pulldown endogenous Pins from *Drosophila* extracts (Katanaev and Tomlinson, 2006a). However, when the interaction between purified recombinant $G\alpha_o$ and Pins proteins was tested, only the GDP-loaded $G\alpha_o$ revealed the binding to Pins. We interpreted this discrepancy by proposing that certain *Drosophila* proteins could enhance the interaction between the GTP-loaded $G\alpha_o$ and Pins, while the

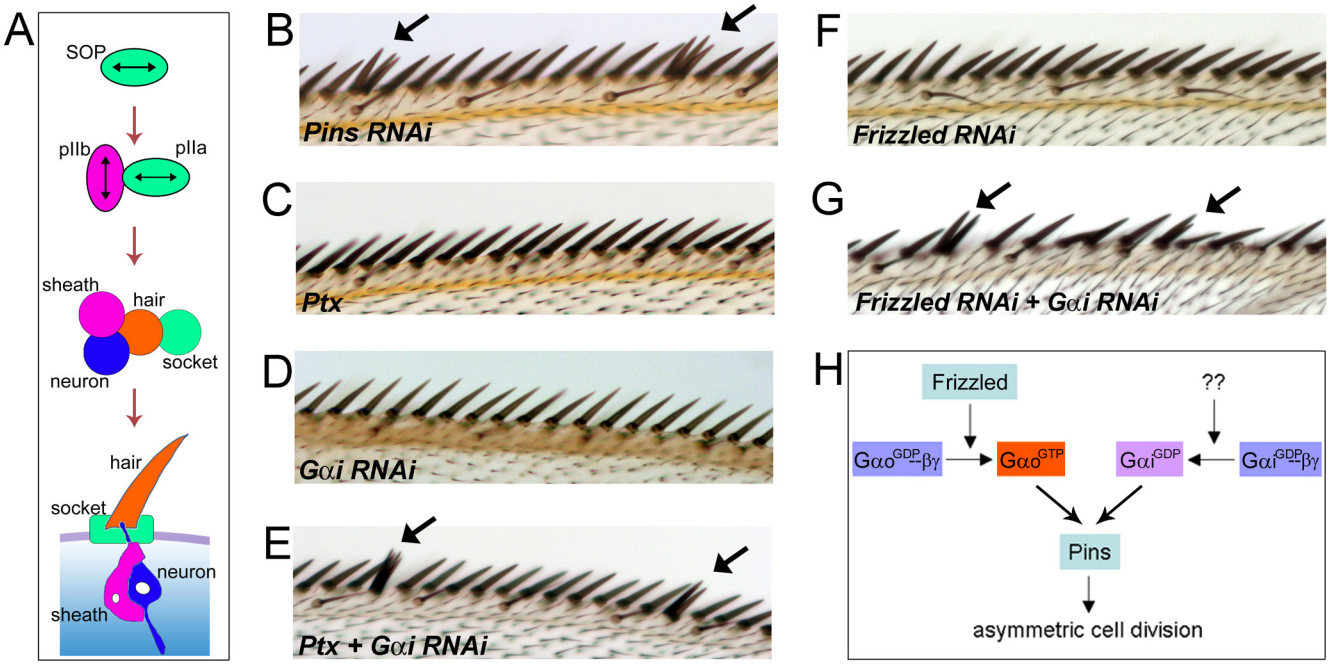


**Figure 7.** In vivo function of Pins, $G\alpha_o$, and the redundancy with $G\alpha_i$. (A) A general scheme of the set of asymmetric cell divisions generating a sensory bristle in *Drosophila*. The sensory organ precursor cell (SOP) divides asymmetrically in the anterior-posterior axis generating pIIa and pIIb cells, which are precursors of the bristle external cells (hair and socket) and internal cells (neuron and sheath), respectively. Both cells again divide asymmetrically, pIIa parallel, and pIIb perpendicular to the SOP division axis, producing the final four cells of the adult sensory bristle. A failure of the asymmetry of division at each of these stages produces aberrant sensory organs with certain cell types duplicated at the expense of the other cell types. (B) Expression of the RNAi against Pins in developing *Drosophila* wings induces defects in the asymmetric cell divisions during formation of the sensory bristles of the wing margin. Defective bristles have excessive external cells (hairs or sockets, marked by arrows) at the expense of the internal cells. (C) Expression of the pertussis toxin (Ptx) specifically uncoupling $G\alpha_o$ from GPCRs does not induce visible asymmetric cell division defects. (D) Expression of the RNAi against $G\alpha_i$ does not induce visible asymmetric cell division defects. (E) Combined expression of Ptx and RNAi against $G\alpha_i$ produces asymmetric cell division defects (arrows) identical to those induced by down-regulation of Pins. (F and G) Expression of the RNAi against Frizzled can induce asymmetric cell division defects only if coexpressed with the RNAi against $G\alpha_i$. (H) A model on the redundant activity of $G\alpha_o$ and $G\alpha_i$ upstream from Pins in the regulation of asymmetric cell divisions. Frizzled receptors activate the release of $G\alpha_o$ from the trimeric $G_o$ complexes producing $G\alpha_o$-GTP, which can activate Pins. Alternatively, Pins can be activated by $G\alpha_i$-GDP, but it is unclear how it is released from the trimeric $G_i$ complex.

interaction between the purified proteins was "canonical" and only happened in the presence of GDP (Katanaev and Tomlinson, 2006a).

Although the existence of helper proteins enhancing the in vivo interactions between GTP-loaded $G\alpha_o$ and Pins is still a possibility (Du and Macara, 2004; Nipper *et al.*, 2007), we find in our current work that the nontagged or $(His)_6$-tagged $G\alpha_o$-GTPγS efficiently binds purified Pins in multiple experimental setups, while $G\alpha_o$ used in our previous experiments was GST-tagged (Katanaev and Tomlinson, 2006a). We also find that the point Q205L mutation on $G\alpha_o$, rendering it unable to hydrolyze GTP and thus constitutively GTP-bound (Graziano and Gilman, 1989; Kroll *et al.*, 1992), allows highly efficient Pins binding comparable to that of the $G\alpha_o$[GDP]. Although we cannot fully explain why the GST-tagged $G\alpha_o$-GTPγS is unable to bind purified Pins, we note that the bulky GST tag reduces the GTP-binding activity of $G\alpha_o$ 3–5 times (Supplementary Figure S1B). Thus, we conclude that the active, GTP-loaded $G\alpha_o$ binds Pins both in vivo and in vitro.

This unusual interaction of the GTP-loaded $G\alpha_o$ and Pins is confined to the GoLoco1 domain of Pins. Lys15 of the GoLoco1 domain is necessary for the efficient binding to GTP-loaded $G\alpha_o$. Substitution of Lys15 of GoLoco1 domain with Gly located in the identical position of GoLoco3 domain uncouples the interaction with GTP-loaded $G\alpha_o$ but only moderately affects the binding to GDP-loaded $G\alpha_o$, and thus recapitulates the GoLoco3 domain-binding pattern (Figure 5B). We thus propose that Lys15 of the GoLoco1 domain might stabilize the γ-phosphate of GTP during interaction with GTP-loaded $G\alpha_o$.

Our work provides the second clear demonstration of the interaction of a GoLoco domain-containing protein with the GTP-loaded form of a Gα-subunit. The only other clearly confirmed case of a similar interaction is the binding of the activated rat $G\alpha_z$ to Rap1GAP (Meng *et al.*, 1999). It is interesting to note that Lys15 of the GoLoco1 domain of Pins is absent from the equivalent position of the Rap1GAP' GoLoco domain (Willard *et al.*, 2004). It thus might be proposed that multiple mechanisms stabilizing the GoLoco domain interaction with GTP-loaded Gα may exist (Willard *et al.*, 2007). Additional evidence is provided by our experiments with homologues of $G\alpha_o$ and Pins. $G\alpha_i$, being 69% identical to $G\alpha_o$, binds Pins or its domains exclusively in the GDP-conformation (Nipper *et al.*, 2007; Figure 5C). This biochemical result is paralleled with in vivo experiments where only $G\alpha_i$[GDP] but not $G\alpha_i$[GTP] could affect asymmetric divisions in *Drosophila* (Katanaev and Tomlinson, 2006a). Furthermore, rat $G\alpha_o$, 81% identical to *Drosophila* $G\alpha_o$, shows no ability to interact with *Drosophila* Pins in the GTPγS-loaded form, but interacts efficiently in the GDP-form (Figure 5F). Additionally, both *Drosophila* and rat $G\alpha_o$-GTPγS fail to bind the GoLoco region of mammalian Pins homologues AGS3 and LGN (Figure 5F), despite the pres-

ence of Lys15 in the GoLoco4 domain of AGS3 and LGN (see Figure 5E). It is still possible that other $G\alpha_o$/Pins homologues may reveal an interaction in the GTP state. For example, efficient binding of *Caenorhabditis elegans* AGS3 (which has Lys15 in GoLoco1 domain and Arg15 in GoLoco2 domain, see Figure 5E) to GAO-1[GDP] and GAO-1[GTP] was demonstrated in the yeast two-hybrid assay (Cuppen *et al.*, 2003), but the biochemical confirmation of this interaction is missing. The detailed information we provide on the specificity of GoLoco binding to the GTP-loaded $G\alpha_o$ ($G\alpha_o$, but not $G\alpha_i$; *Drosophila*, but not rat $G\alpha_o$; *Drosophila* Pins, but not its mammalian homologues; GoLoco1 domain of Pins, but not other *Drosophila* GoLoco domains) will help elucidate the structural mechanism of this interaction.

Pins and its homologues have the conserved activity in the regulation of the asymmetric cell divisions (Hampoelz and Knoblich, 2004; Willard *et al.*, 2004; Sanada and Tsai, 2005; Siderovski and Willard, 2005). In *Drosophila* sensory organ formation, the process of the asymmetric cell divisions appears under the redundant control of $G\alpha_o$ and $G\alpha_i$ (Katanaev and Tomlinson, 2006a). Down-regulation of $G\alpha_i$ alone, either by genetic ablation (Katanaev and Tomlinson, 2006a) or by targeted RNAi expression (this work), does not result in any defects in the structure of the adult sensory bristles (see Figure 7D), unlike same manipulations of Pins (Katanaev and Tomlinson, 2006a; and Figure 7B). In contrast, loss-of-function or overactivation of $G\alpha_o$ result in aberrations in the process of asymmetric cell divisions and visible defects in the adult bristle structure (Katanaev and Tomlinson, 2006a). However, we now show that no apparent defects are induced by targeted expression of pertussis toxin, which uncouples $G\alpha_o$ (and not any other G$\alpha$-protein in *Drosophila*) from its cognate GPCRs such as Frizzled (Figure 7C). This observation is not unexpected, as loss of Frizzled itself leads only to the randomization of the axis of the asymmetric cell divisions, but not to the loss of asymmetry or defects in the adult bristle structure (Gho and Schweisguth, 1998), Figure 7F. However, the redundancy between $G\alpha_o$ and $G\alpha_i$ is revealed by a concomitant expression of the $G\alpha_i$-RNAi and pertussis toxin, as this now phenocopies Pins loss-of-function (Figure 7, B and E). The same phenotype is produced by the concomitant down-regulation of Frizzled (acting upstream from $G\alpha_o$) and $G\alpha_i$ (Figure 7G). These data suggest that $G\alpha_o$ and $G\alpha_i$ act coordinately in the process of the asymmetric cell division of the sensory precursor cells (Figure 7H), perhaps similarly to what has been demonstrated for the asymmetric division of the *C. elegans* zygote (Gotta and Ahringer, 2001). The three individual GoLoco domains of Pins bind $G\alpha_i$ identically; furthermore, multiple $G\alpha_i$ molecules can simultaneously bind a single Pins scaffold (Bernard *et al.*, 2001; Nipper *et al.*, 2007). Similarly, we show that $G\alpha_o$ and $G\alpha_i$ can simultaneously bind Pins most likely occupying different GoLoco domains (Figure 5D). We also show that this trimeric complex exists when the two G proteins are bound to different nucleotides: $G\alpha_o$ to GTP and $G\alpha_i$ to GDP. Such a multiprotein complex might allow a more effective regulation of the process of the asymmetric cell division.

Our results on the in vivo function of Frizzled, $G\alpha_o$, $G\alpha_i$, and Pins in the *Drosophila* sensory organ lineage further support the idea that Pins acts as a target and not as an activator of G protein signaling in this physiological process. Indeed, similarity of the Frizzled-RNAi + $G\alpha_i$-RNAi phenotypes on one hand, and the pertussis toxin + $G\alpha_i$-RNAi phenotypes on the other hand clearly shows the redundancy of the Frizzled→$G\alpha_o$ module with the $G\alpha_i$ function for the process of asymmetric cell divisions (Figure 7H). This redundancy implies that both $G\alpha_o$ and $G\alpha_i$ act upstream from Pins. While generation of active $G\alpha_o$ from the trimeric $G_o$ complexes can be achieved by Frizzled receptors (Katanaev *et al.*, 2005; Katanaev and Tomlinson, 2006a; Katanaev and Buestorf, 2009 and this work), it is not clear how $G\alpha_i$ is released from the trimeric $G_i$ complexes. Ric-8 (a non-GPCR guanine nucleotide exchange factor) might be implicated in activation of $G\alpha_i$ (David *et al.*, 2005; Hampoelz *et al.*, 2005; Wang *et al.*, 2005). Downstream from Pins, a known regulator of the asymmetry of cell divisions is NuMA (known as Mud in flies) that anchors the mitotic spindle at the correct location within the plasma membrane (Du and Macara, 2004; Bowman *et al.*, 2006; Izumi *et al.*, 2006; Siller *et al.*, 2006).

While Pins and its homologues have the conserved activity in the regulation of the asymmetric cell divisions, additional functions of these proteins exist. The Pins homologues AGS3 and LGN are strongly expressed in the brain (Blumer *et al.*, 2002) as $G\alpha_o$ is (Sternweis and Robishaw, 1984), where AGS3 is involved e.g., in drug sensitization and seeking behavior (Bowers *et al.*, 2004; Yao *et al.*, 2005). At the molecular level Pins homologues regulate plasma membrane localization and activity of several transmembrane receptors and channels (Sans *et al.*, 2005; Wiser *et al.*, 2006; Groves *et al.*, 2007). *Drosophila* Pins is also expressed in the larval (Caussinus and Gonzalez, 2005) and adult brain (Figure 4A). Additionally, Pins affects motor axon guidance and synaptogenesis in *Drosophila* (Kraut *et al.*, 2001). Thus a variety of GPCRs are likely to engage Pins and potentially other GoLoco domain-containing proteins through liberation of $G\alpha_o$-subunits from the trimeric $G_o$ protein complexes. In addition, some non-GPCR guanine nucleotide exchange factors such as Ric-8 (David *et al.*, 2005; Hampoelz *et al.*, 2005; Wang *et al.*, 2005) might be involved in the generation of the Pins-interacting $G\alpha_o$-GTP. Although clear data demonstrate that Pins and its homologues can modulate activities of $G\alpha_i$ (Willard *et al.*, 2004; Blumer *et al.*, 2005; Siderovski and Willard, 2005) and $G\alpha_o$ (Figure 6), the capacity of the activated $G\alpha_o$ to bind Pins demonstrated in this study highlights the possible important function of Pins as a general transducer of GPCR signaling. Yeast two-hybrid screens have identified multiple interaction partners of Pins (Blumer *et al.*, 2003; Giot *et al.*, 2003; Marty *et al.*, 2003). The multidomain structure of Pins may suggest that this protein serves as a scaffold to organize signal transduction downstream from various GPCRs.

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