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Frizzled receptors signal through G proteins

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Abstract

Frizzled receptors have long been thought to couple to G proteins but biochemical evidence supporting such an interaction has been lacking. Here we expressed mammalian Wnt-Frizzled fusion proteins in *Saccharomyces cerevisiae* and tested the receptors' ability to activate the yeast mitogen-activated protein kinase (MAPK) pathway via heterotrimeric G proteins. Our results show that Frizzled receptors can interact with $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$ proteins, thus confirming that Frizzled functions as a G protein coupled receptor (GPCR). However, the activity level of Frizzled-mediated G protein signaling was much lower than that of a typical GPCR and, surprisingly, was highest when coupled to $G\alpha_s$. The Frizzled/ $G\alpha_s$ interaction was further established *in vivo* as *Drosophila* expressing a loss-of-function $G\alpha_s$ allele rescued the photoreceptor differentiation phenotype of Frizzled mutant flies. Together, these data point to an important role for Frizzled as a nontraditional GPCR that preferentially couples to $G\alpha_s$ heterotrimeric G proteins.

Keywords

Frizzled; G protein; GPCR; Wnt signaling; planar cell polarity; GDI

1. Introduction

Frizzleds are seven transmembrane-spanning receptors for large secreted ligands called Wnts [1-3]. Wnt signaling is essential for many developmental processes, including neural crest induction, cell fate specification and tissue morphogenesis [4-8]. In the canonical Wnt-signaling pathway, Wnt binds to Frizzled and a lipoprotein receptor-related protein (LRP) co-receptor. This results in the stabilization and translocation of β -catenin to the nucleus, where it acts on the Tcf/Lef family of transcription factors [9-12]. Characterized by the features common to all G protein-coupled receptors (GPCRs) – seven transmembrane segments, an extracellular N-terminal domain, and a cytoplasmic C-terminal tail – Frizzleds have long been considered likely GPCRs [2,13]. Until recently, however, studies aimed at showing a direct interaction between Frizzled and G proteins have proven inconclusive.

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Typically, ligand binding to a GPCR induces a conformational change that catalyzes guanosine diphosphate (GDP) release and guanosine triphosphate (GTP) capture by the α -subunits of heterotrimeric G proteins. $G\alpha$ then dissociates from the $G\beta\gamma$ portion of the heterotrimer and both components activate effector proteins. Initial studies of potential G protein signaling interactions demonstrated that rat Frizzled 2 and XWnt5a RNA injection in zebrafish embryos caused calcium mobilization [14]. More recently, studies of mouse embryonic cells stably expressing Frizzled 2 also showed Wnt5a-stimulated calcium mobilization [15]. In both cases, Wnt-stimulated activity could be blocked by pertussis toxin, which ADP-ribosylates both $G\alpha_i$ and $G\alpha_o$ subunits and turns off G protein signaling. Additional studies have demonstrated that depletion of $G\alpha_o$ or $G\alpha_q$ in cultured mammalian cells inhibits Wnt-stimulated stabilization of β -catenin, and, conversely, that direct activation of G proteins by GTP γ S leads to β -catenin stabilization in the absence of Wnt [16]. Genetic evidence for possible Frizzled-G protein signaling places *Drosophila* $G\alpha_o$ downstream of Frizzled and upstream of cytoplasmic proteins in the pathway, such as Dishevelled and glycogen synthase kinase 3- β (GSK3 β) [17]. Studies have also shown that Regulators of G Protein Signaling (RGS) proteins can modulate Wnt signaling and that effector molecules downstream of $G\alpha_s$ are involved in Wnt-regulated gene expression [6,18].

Recent studies have provided the first direct evidence that Frizzleds interact with heterotrimeric G proteins [19,20]. Isolated membranes from N13 cells showed G protein activation by GTP γ S binding in response to Wnt5a stimulation; the response was abrogated in the presence of pertussis toxin [20]. Similar work by Koval and Katanaev suggests that GTP binding activity is pertussis toxin sensitive in cells expressing human Frz1, thus also implicating $G\alpha_{o/i}$ proteins [19]. However, since the experiments were performed using intact mouse fibroblasts it is unlikely that the GTP binding activity assayed can be exclusively attributed to heterotrimeric G proteins as small G proteins are present throughout the cell.

Frizzled pharmacology studies are generally complicated by the insolubility of Wnts [21] and the potential interplay between the canonical β -catenin and G protein signaling pathways. Using *Saccharomyces cerevisiae* as a model system to study Frizzled receptor function has several advantages. Mammalian GPCR function has been studied extensively in yeast strains engineered to express chimeric mammalian $G\alpha$ subunits [22-25]. Also, yeast do not encode proteins related to the canonical Wnt signaling pathway, thus providing a null environment in which to study mammalian receptors.

In this study, we asked whether Frizzled proteins, when expressed with chimeric G proteins, could activate transcription of yeast reporter genes downstream of the yeast MAPK pathway. We found that chimeric Frizzled receptors induced G protein signaling and that chimeric G proteins containing the last five amino acids of mammalian $G\alpha_s$ were preferentially activated over chimeric G proteins containing the last five amino acids of mammalian $G\alpha_i$ or $G\alpha_q$.

2. Materials and methods

2.1 Yeast strains

Yeast strains BY1173 ($G\alpha_i$), BY1172 ($G\alpha_q$), and BY1404 ($G\alpha_s$) have previously been described [23]. BY1173 has the genotype MATa ura3 leu2 trp1 his3 can1 gpa1 Δ ::ade2 Δ ::3XHA far1 Δ ::ura3 Δ fus1 Δ ::P_{FUS1}-HIS3 LEU2::P_{FUS1}-lacZ sst2 Δ ::ura3 Δ ste2::G418R trp1::GPA1/ $G\alpha_{i3}$. BY1172 has the genotype MATa ura3 leu2 trp1 his3 can1 gpa1 Δ ::ade2 Δ ::3XHA far1 Δ ::ura3 Δ fus1 Δ ::P_{FUS1}-HIS3 LEU2::P_{FUS1}-lacZ sst2 Δ ::ura3 Δ ste2::G418R trp1::GPA1/ $G\alpha_q$. BY1404 has the genotype MATa ura3 leu2 trp1 his3 can1

gpa1Δ::ade2Δ::3XHA far1Δ::ura3Δ fus1Δ::P_{FUS1}-HIS3 LEU2::P_{FUS1}-lacZ sst2Δ::ura3Δ ste2ΔG418R lys2Δ Δtrp::GPA1/Gα_s. BY1173, BY1172, and BY1404 express a chimeric Gα subunit that is made up of amino acids 1-467 of the yeast Gα protein, Gpa1, followed by the last five amino acids of human Gα_i, Gα_q, and Gα_s, respectively. In all three strains, presence of an activated GPCR leads to signaling through the yeast mitogen-activated protein kinase (MAPK) cascade and expression of the *P_{FUS1}-lacZ* reporter gene.

2.2 Construction of Plasmids

All constructs were verified by sequencing at the Washington University Protein and Nucleic Acid Chemistry Laboratory.

2.2.1 XWnt8-rFrz1-3xHA (pBN 2245) and XWnt8-rFrz2-3xHA (pBN 2247)—

Plasmids encoding *Xenopus* Wnt8 (XWnt8, GenBank Accession NM_001088168), rat Frizzled 1 (Frz1, NM_021266) and rat Frizzled 2 (Frz2, NM_172035) were generously provided by Randall Moon. ΔN-terminal Frz1 and ΔN-terminal Frz2 were amplified with oligos containing homology to a yeast ADE2 plasmid encoding XWnt8. PCR products were recombined with the linearized yeast vector in *S. cerevisiae*, generating plasmids that encode XWnt8-Δ222 Frz1 (pBN 2060) and XWnt8-Δ156 Frz2 (pBN 2062) fusion proteins. The 3xHA-URA3-3xHA sequence was amplified from pBN 687 with homology to the 3' end of Frz1 or Frz2 and the 3' UTR. Recombination of the PCR product and linearized pBN 2060 or pBN 2062 generated yeast plasmids encoding two selectable marker genes: ADE2 and URA3. In the presence of 5-Fluoroorotic acid (5-FOA), the URA3 gene product becomes toxic to yeast (Green and Moehle, 2001). Thus, yeast subjected to homologous recombination were screened for resistance to 5-FOA, indicating loss of the URA3 gene by recombination and generation of Wnt8-Frz-3xHA fusions.

2.2.2 XWnt8-mFrz6-3xHA (pBN 2353) and XWnt8-mFrz7-3xHA (pBN 2352)—

Plasmids encoding mouse Frizzled 6 (Frz6, GenBank Accession NM_008056) and mouse Frizzled 7 (Frz7, NM_008057) were generously provided by Dr. Fanxin Long. Oligos were designed to amplify the Frizzled coding sequence excluding all but approximately 100 bp of the predicted extracellular N-terminal domain. Homologous recombination in *S. cerevisiae* was used to recombine the purified PCR product and linearized pBN 2245, thus generating plasmids encoding XWnt8-Δ113 mFrz6-3xHA (pBN 2353) and XWnt8-Δ161 mFrz7-3xHA (pBN 2352).

2.2.3 A2B-3xHA in ADE2 (pBN 2348)—The human Adenosine 2B (A2B) receptor (GenBank Accession NM_000676) was subcloned into a yeast ADE2 vector (pBN 741) by homologous recombination. Oligos TJB 2425 and 115 were used to amplify the 3xHA tag from pBN 2245. Homologous recombination was utilized to insert the 3xHA in place of the A2B stop codon in linearized pBN 2320. The generated plasmid, pBN 2348, encodes the human A2B-3xHA fusion protein.

2.2.4 LRP6 in URA3 (pBN 2361)—A cDNA clone of mouse LRP6 (GenBank Accession BC060704) was obtained from ATCC. Full length mLRP6 was subcloned into a yeast URA3 vector (pBN 2362) with EcoRI (5') and NotI (3').

2.2.5 XWnt8-Frz1 in URA3 (pBN 2369)—In order to co-transform Frz1 and Frz2, the ADE2 selectable marker gene of pBN 2245 was replaced with URA3 by homologous recombination. Briefly, oligos were designed to amplify the URA3 gene from pBN 2265, generating a PCR product with homology to both the 5' and 3' UTRs of ADE2. Recombination of the purified PCR product and linearized pBN 2245 generated a plasmid encoding XWnt8-Frz1 and the selectable marker URA3 (pBN 2369).

2.3 Yeast transformation and determination of receptor activity

Yeast were transformed with either an ADE2 plasmid or both ADE2 and URA3 plasmids using standard lithium acetate protocols. Transformed yeast were grown in suspension at 30°C under appropriate selective conditions. Following overnight growth, the culture density was measured using a Spectronic-20 spectrophotometer. The target OD₆₀₀ for assay initiation was 0.15. If necessary, cultures were diluted with selective media (pH 6.8). OD₆₀₀ of cultures at assay initiation ranged from 0.10 to 0.25. 90 μ L of yeast was seeded into 96-well plates; 10 μ L of 10 μ M of the A2B receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) was added to positive control wells. Cells were incubated at 30°C for 4 hours then lysed and monitored for β -galactosidase activity in the presence of the chromogenic substrate, chlorophenolred- β -D-galactopyranoside (CPRG, Roche) [26]. 20 μ L of substrate/lysis solution consisting of a 50/50 mixture of 5% (w/v) Triton X-100 (Bio-Rad) in 250 mM PIPES, pH 6.8, and 4.86 mg/mL CPRG in 25 mM PIPES, pH 6.8 was added to each well [25]. Assays were incubated at 37°C for 12-18 hours; reactions were terminated by adding 20 μ L of 1 M Na₂CO₃ to each well. Absorbance of the supernatant was measured at 570 nm using a BioTek plate reader. β -galactosidase activity was calculated as A₅₇₀/min/OD units (OD₆₀₀ = 1 is 1 OD unit). Statistical analysis was performed using GraphPad Prism version 4.0 (GraphPad Software, Inc.).

2.4 Western blots

Cultures of single yeast colonies were grown to log-phase (OD₆₀₀ ~ 1.0) in appropriate selective media. 3.0 OD of each yeast culture was harvested by centrifugation; cells were lysed by vortexing with 0.5-mm glass beads for 5 minutes in 1X SDS sample buffer with protease inhibitors. Lysates were treated with 200 mM dithiothreitol (DTT) and heated at 50°C for 5 minutes, then separated on a 10% SDS-PAGE gel and assayed by Western blot. An antibody specific for the hemagglutinin (HA) epitope was used at a 1:5000 dilution (0.6 μ g/mL, mouse monoclonal antibody clone 12CA5) to assess expression of HA-tagged proteins. A rabbit antibody against LRP6 was used at a 1:1000 dilution to detect LRP6 expression (C47E12, Cell Signaling). Western blots were performed using the same yeast cultures assayed for receptor activity. Quantification of receptor expression was performed using AlphaEaseFC software and normalized to a nonspecific band (~50 kD) to correct for protein content (Fig. 3).

2.5 Sectioning of *Drosophila* eyes

Fz^{KD4a}/TM3 originated from [27]; Fz^{R52}/TM6c originated from [28]. G α_s ^{R60}/Cyo strain was from Bloomington Stock Center. Sectioning was performed on adult fly eyes as described in [29], except resin was baked at 55°C instead of 70°C. For statistical analysis, 10 eyes of each genotype were scored, excluding one outlier from the +/+; Fz^{R52}/Fz^{KD4a} group which contained 35.4% R3-R3 ommatidia (out of 147). Inclusion of this data would have increased the difference between the two phenotypes. Pictures were taken using phase-contrast optics. Strains were prepared using standard techniques.

3. Results

3.1 Wnt-Frz chimeras can be expressed in yeast

Frizzled proteins have large amino-termini (200-300 amino acids) characterized by a cysteine-rich (CRD) domain containing 10 highly conserved cysteines. Full-length, wild-type Frz1 and Frz2 did not express in yeast, as assessed by fluorescence microscopy of receptors tagged at the C-terminus with YFP (data not shown). Previous studies have established that expression of a Wnt ligand-frizzled receptor transgene confers constitutive receptor signaling via canonical β -catenin both in cell culture and *in vivo* in *Drosophila* [30].

Therefore, we constructed chimeric Frizzled receptor genes that encoded full-length XWnt-8 (358 amino acids) in place of the Frizzled N-terminus and tagged the receptors with the hemagglutinin (HA) epitope at their C-termini. Wnt8 was selected for the chimera because it has previously been used as a ligand for Frizzled receptors linked to G protein-regulated signaling [6,31-33]. The chimeric Frizzled proteins were successfully expressed in yeast, as shown by Western blot (Fig. 3). The receptors ran at their expected molecular weights (Fig. 3, see open arrows) with some minor species that migrated further and likely represent proteolytic fragments of the Frizzled receptors. A non-specific yeast protein that runs at ~ 50 kDa (Fig. 3, see asterisks) was used as a loading control. Wnt8-Frz1, Wnt8-Frz2 and Wnt8-Frz7 (all ~ 93 kDa) were expressed at higher levels than Adenosine 2B receptor (A2B) (Figure 3A), whereas Wnt8-Frz6 was expressed at a level comparable to A2B. Receptor expression was nearly identical in yeast strains expressing Gpa1/Gα_i and Gpa1/Gα_q whereas overall protein expression was lower in the Gpa1/Gα_s yeast strain (Fig. 3A-C). Relative expression of each Frizzled chimera compared to A2B was the same in all yeast strains.

3.2 Wnt-Frz chimeras signal in yeast

We next asked whether Frizzled chimeras can signal directly through G proteins. In order to answer this question, we exploited the ability of mammalian GPCRs to activate the endogenous GPCR signaling cascade in genetically engineered *S. cerevisiae*. Activation of the MAPK pathway by a GPCR in yeast strains BY1172, 1173 and 1404 induces expression of the LacZ reporter gene, generating β-galactosidase that catalyzes a colorimetric reaction in the presence of substrate. Each engineered yeast strain lacks the endogenous pheromone receptor, thus allowing mammalian receptors to be introduced and studied in an otherwise GPCR-null background. In addition, the yeast express a chimeric G protein that is primarily the yeast Gα, Gpa1 (residues 1-467), followed by the last five amino acids of the human Gα_q (strain BY1172), Gα_i (BY1173), or Gα_s (BY1404); the endogenous Gβ (Ste4) and Gγ (Ste18) subunits remain unchanged (Fig. 2A). The original goal was to determine whether the Frizzled chimeras could cause constitutive activation of Gpa1/Gα_i. BY1173 yeast were transformed with Wnt8-Frz1, Wnt8-Frz2, or empty vector and then assessed for G protein signaling by performing β-galactosidase assays. Both Frizzled receptors activated signaling in the Gα_i strain although the level of signaling was less than 3% of that seen by a maximally stimulated professional GPCR (Fig. 4A, B). Predicting that other highly abundant mammalian Frizzled receptors interact with G proteins in a similar fashion, we generated and tested two additional Frizzled chimeras: Wnt8-Frz6 and Wnt8-Frz7 [34]. Surprisingly, Wnt8-Frz6 stimulated signaling was lower than basal G protein signaling in BY1173 (Fig. 4C) whereas signaling in the presence of Wnt8-Frz7 was the same as basal (Fig. 4D). These data suggest that activated Frizzleds can signal via G proteins albeit with an overall lower rate of Gβγ release than a typical activated GPCR. In addition, the results provide further evidence that mammalian Frizzled receptors are not merely redundant but likely interact differently with effector molecules.

3.3 Wnt-Frz chimeras signal more through Gα_s than Gα_i or Gα_q

Previous reports have implicated heteromeric Gα_{i/o} proteins in Frizzled signaling [6,14-17,19,20,32,35-38]. In order to test whether Frizzled proteins can interact with Gα_s and Gα_q subunits, we expressed the receptors in yeast strains that have Gpa1/Gα_s (BY1404) or Gpa1/Gα_q (BY1172) chimeras and assayed β-galactosidase activity. BY1172 transformed with Wnt8-Frz1, Wnt8-Frz2, Wnt8-Frz6 and Wnt8-Frz7 showed a low level of G protein activation (Fig. 4A-D) similar to Wnt8-Frz1 and Wnt8-Frz2 stimulated activity in BY1173. Interestingly, BY1404 yeast expressing Frizzled chimeras showed a considerably higher level of G protein activation compared to the Gα_i and Gα_q strains. Despite the increased level of activity, the signaling of each Frizzled chimera in the Gα_s strain was only

10-15% of that seen by a maximally stimulated GPCR (Fig. 4A-D). In order to determine whether signaling via G proteins is greater in the presence of Frizzled heterodimers, we co-transformed BY1404 with Wnt8-Frz1/Wnt8-Frz2, Wnt8-Frz1/Wnt8-Frz6, and Wnt8-Frz1/Wnt8-Frz7. There was no significant difference in the G protein signaling levels of yeast expressing one Frizzled receptor compared to yeast expressing two Frizzleds (Fig. 5).

3.4 LRP6 does not enhance G protein-dependent Frizzled signaling

In light of recent work showing that the Frizzled co-receptor LRP6 binds $G\alpha_s$ and regulates the localization of the $G\alpha_s\beta\gamma$ heterotrimer to the plasma membrane, we investigated whether co-expression of LRP6 would promote Frizzled-activated G protein signaling in yeast [39]. BY1172, BY1173 and BY1404 strains were each co-transformed with LRP6 and a Frizzled chimera: Wnt8-Frz1, Wnt8-Frz2, Wnt8-Frz7, or Wnt8-Frz6. G protein signaling was assessed in each strain by β -galactosidase assays. No change in Frizzled signaling was observed in the $G\alpha_i$ and $G\alpha_q$ strains with LRP6 expression (Figure 6A-D). In the $G\alpha_s$ strain, signaling of Wnt8-Frz2 and Wnt8-Frz7 was inhibited slightly by the presence of LRP6; signaling via Wnt8-Frz1 and Wnt8-Frz6 was unchanged. Concurrent expression of the two proteins was confirmed by Western blot (Figure 6E). Together, these studies suggest that the GPCR activity of Frizzled is not enhanced by LRP5/6 expression.

3.5 $G\alpha_s$ antagonizes Frizzled signaling in the regulation of photoreceptor differentiation in the *Drosophila* eye

The finding that Frizzleds in yeast can signal through $G\alpha_s$ prompted us to examine Frizzled/ $G\alpha_s$ interactions in a physiologic system. During development of the *Drosophila* compound eye, eight distinct photoreceptors (designated R1 through R8) develop in each of the ~750 ommatidial units. By light microscopy, each photoreceptor can be identified by position of its pigmented rhabdomere: the R3 rhabdomere, for instance, is positioned at the apex of the trapezoid formed by the seven photoreceptor rhabdomeres visible in a given cross-section of the eye while the R4 rhabdomere is positioned next to the R3 in a more equatorial location (closer to the base of the trapezoid, Fig. 7C inset). Differentiation of the R3/R4 photoreceptor pair is directly regulated by the level of Frizzled signaling; the cell that achieves the higher level of Frizzled signaling becomes the R3 photoreceptor, while the other becomes the R4 photoreceptor [40,41]. Loss of Frizzled signaling in these cells causes pleiotropic defects, including inappropriate specification of two R3 photoreceptors (R3-R3 phenotype), and disruption of the typical trapezoidal shape formed by the rhabdomeres (Fig. 7A inset).

In *Frizzled* loss-of-function mutants (Fz^{KD4a}/Fz^{R52}), 8.1 percent of ommatidia displayed the R3-R3 phenotype ($n=1466$ ommatidia, Table 1 and Fig. 7A, B). In this *Frizzled* mutant background, reduction of $G\alpha_s$ activity by the presence of a single copy of the $G\alpha_s^{R60}$ loss-of-function allele suppressed the phenotype: 3.7% of ommatidia showed the R3-R3 phenotype ($n=1011$, $p < 0.0001$ by χ^2 analysis, Table 1 and Fig. 7C, D). Furthermore, in the *Frizzled* mutant background 10.2% of ommatidia failed to rotate (resulting in orientation perpendicular to other ommatidia, but only 4.6% of ommatidia displayed this phenotype in flies that were also heterozygous for $G\alpha_s^{R60}$ ($p < 0.00001$ by χ^2 analysis). Eyes were also scored for defects in dorsal/ventral and anterior/posterior positioning and for aberrant photoreceptor number, but these phenotypes were not significantly modified by the $G\alpha_s^{R60}$ allele (Table 1).

4. Discussion

The results of this study provide strong evidence supporting a direct interaction between Frizzleds and G proteins. We have shown that Frizzled receptors co-expressed with chimeric

G proteins can activate the yeast MAPK pathway, thus confirming Frizzled as a GPCR. These results confirm those obtained by another group that expressed human Frizzled receptors 1 and 2 and observed activation of the yeast MAPK pathway through the yeast G proteins [42]. Our study extends this work by showing that there is specificity in the interaction between Frizzleds and chimeric G proteins, with Frz1, Frz2, Frz6 and Frz7 preferentially interacting with Gpa1/Gα_s over Gpa1/Gα_i or Gpa1/Gα_q. We have also observed in yeast that full-length Wnts can signal through Frizzled proteins that lack the native N-termini. Finally, we have identified a genetic interaction between Gα_s and Dfz-1 in *Drosophila*.

Our findings leave the catalytic activity of Frizzleds on G proteins unresolved. In the absence of receptor, the engineered Gα subunits exist in equilibrium between heterotrimeric G protein and free Gβγ. Two models can explain the increased signaling observed when Frizzled receptors are expressed in yeast strains containing chimeric Gpa1/Gα_s or Gpa1/Gα_q. In the first model, Frizzleds act as typical GPCRs that exhibit both constitutive and ligand-dependent guanine nucleotide exchange factor (GEF) activity (Fig. 8A). In the second model, Frizzled proteins function as ligand-gated guanine nucleotide dissociation inhibitors (GDIs) to sequester inactive Gα-GDP (Fig. 8B). In the yeast system, sequestration of inactive Gα-GDP results in release of Gβγ and activation of the MAPK pathway.

If Frizzled acts as a GEF, then a reduction in the level of the corresponding G protein would be predicted to enhance the Frizzled loss-of-function phenotype. In contrast, if Frizzled acts as a GDI, a reduction in G protein level would be predicted to suppress the Frizzled phenotype. Our finding that reduction of Gα_s activity in the fly eye suppresses the Frizzled loss-of-function phenotype is consistent with Frizzled acting as a GDI for Gα_s. Frizzled has been linked to heterotrimeric G protein signaling in the development of the fly pupal wing [17]. Overexpression of Gα_o in the wing induced upregulation of target of canonical and non-canonical Frizzled signaling, and this phenotype was abolished if Frizzled pathway components were removed. However, Gα_o did not colocalize with Frizzled. Gα_o or Frizzled null clones caused polarity disruption of neighboring wing cells on the opposite sides of the respective clones. Additional studies have shown that overexpression of Gα_o in *Drosophila* leads to defects in the orientation of wing margin bristles and that the phenotype can be rescued by removal of Frizzled [38]. Interpretation of the genetic interactions with Gα_s is complicated by the equilibrium that exists between Gα_s and Gα_i (and Gα_o) as activators and inhibitors of adenylyl cyclase, respectively. Therefore, an alternative interpretation of our data is that Frizzleds activate Gα_i in the fly eye to regulate R3 differentiation by inhibiting adenylyl cyclase, while an opposing signal activates adenylyl cyclase through Gα_s.

Wnt-Frz chimeras activate G protein signaling through Gα_i, Gα_s, and Gα_q, though activation by Frizzled chimeras is considerably lower than that observed for a typical GPCR (Fig. 4). Previous research has implicated Gα_o and Gα_q in Frizzled signaling through the β-catenin pathway [37] and in pertussis toxin-sensitive phosphatidylinositol signaling that may be linked to Gα_o [14,35]. Another study linked Wnt-directed myogenic gene expression to adenylyl cyclase and signaling through CREB [18]. This result implicated Gα_s as a downstream effector of Wnt signaling but did not show that direct activation of G proteins by Frizzled led to myogenesis. The work that we report here supports direct interactions between Frizzleds and multiple G protein subunits with specificity for Gα_s. This finding is particularly compelling since the yeast strains differ by only five amino acids in the chimeric Gα subunits. In addition, the identification of a genetic interaction between Frizzled and Gα_s supports a physiological role for this specific Frizzled-G protein interaction. However, determination of coupling specificity in the yeast system is limited, since the chimeric G proteins expressed in the engineered yeast strains might not fully replicate the activities of

mammalian heterotrimeric G proteins. Furthermore, yeast lack accessory proteins such as Disheveled and Axin which could modulate G protein activation or specificity in higher organisms; Disheveled has been shown to stimulate Ca^{2+} release downstream of G proteins in activation in zebrafish embryos albeit in a manner independent of G protein signaling [43].

Recent work reported by Kilander, et al. provided the first biochemical evidence of an interaction between Frizzled and G proteins [20]. Isolated membranes from N13 cells showed a dose-dependent increase in Wnt5a-stimulated [$\gamma^{35}\text{S}$]GTP binding that was sensitive to pertussis toxin, suggesting that Frizzled can activate heterotrimeric $\text{G}\alpha_{i/o}$ proteins. Another recent study showed Wnt3a-stimulated GTP binding in mouse fibroblasts overexpressing Frizzled that was also pertussis toxin sensitive [19]. Fibroblasts transfected with human Frz1 and various $\text{G}\alpha$ subunits showed Wnt3a-stimulated G protein activation for $\text{G}\alpha_o$ and $\text{G}\alpha_i$. Of note, the results supported the presence of an endogenous Frizzled receptor in L-cells that preferentially coupled to $\text{G}\alpha_s$ since overexpressing $\text{G}\alpha_s$ alone resulted in increased Wnt ligand-induced GTP binding.

5. Conclusions

The characteristic seven transmembrane structure of Frizzled receptors, combined with genetic data linking G proteins to Wnt signaling, has long supported the idea that Frizzled interacts with G proteins. However, conventional readouts for G protein signaling have not demonstrated G protein activation by Frizzled. More recent data has shown that Wnt stimulation of Frizzled receptors led to $\text{G}\alpha$ exchange of GTP for GDP, but the question of how Frizzled interacts with G proteins in a physiologically relevant context remains wide open. One possibility is that Frizzled receptors interact with G proteins in a manner identical to traditional GPCRs but with altered kinetics such that the rate of exchange of guanine nucleotides on associated G proteins is slower than with typical GPCRs. Results of our studies support both models wherein Frizzled may be acting as a partial GEF or a functional GDI. These models are also consistent with the lack of conventional downstream G protein activation assay data in response to Frizzled activation.

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Abbreviations

Frz	Frizzled
GPCR	G protein-coupled receptor
MAPK	mitogen-activated protein kinase
GEF	guanine nucleotide exchange factor
GDI	guanine nucleotide dissociation inhibitor

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Highlights

- Frizzled receptors directly interact with $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$ proteins.
- $G\alpha_s$ -mediated Frizzled signaling regulates *Drosophila* eye development.
- Frizzled receptors might act as partial GEFs or functional GDIs.

Figure 1. Schematic outlining the MAPK signaling pathway in BY1404

The single endogenous GPCR, Ste2 is not expressed in the modified *S. cerevisiae* strain. A chimeric yeast Gpa1-mammalian $G\alpha_s$ transplant is expressed in place of endogenous Gpa1. Signaling through the endogenous MAPK pathway initiates transcriptional activation of the Fus1-lacZ reporter gene.

Figure 2. Yeast strains and Frizzled proteins assayed for receptor activity

A) Schematics of the Gα subunits in yeast strains BY1173, BY1172, and BY1404 are shown from the amino terminus to the carboxyl terminus (left to right). Yeast Gpa1 residues are represented by an open bar and human Gα_i, Gα_q, and Gα_s residues are shaded black. An alignment of the last five residues of the chimeras is shown. B) Cartoon representations of the four chimeric Frizzled proteins assayed for receptor activity in yeast: Wnt8-Frz1, Wnt8-Frz2, Wnt8-Frz6, Wnt8-Frz7.

Figure 3. Expression of chimeric Frizzled proteins in yeast

Lysates of transformed yeast were treated with DTT and subjected to SDS-PAGE and immunoblotting. Receptor expression was detected by anti-HA antibody. Positions of molecular weight markers are shown at left in each panel. Expected proteins sizes are as follows: Wnt8-Frz1, 93 kDa; Wnt8-Frz2, 93 kDa; Wnt8-Frz7, 93 kDa; Wnt8-Frz6, 113 kDa; A2B, 43 kDa. A) BY1172 express Wnt8-Frz1, Wnt8-Frz2, Wnt8-Frz7 and Wnt8-Frz6 (open arrows). Five times more lysate is required to observe a similar level of expression of A2B receptor (closed arrow at ~ 37 kDa). A non-specific band corresponding to an approximately 50 kDa yeast protein was used for normalization (asterisks). Expression of Frizzled chimeras was also detected in BY1404 (B) and BY1173 (C).

Figure 4. Signaling activity of Frizzled receptors in $G\alpha_s$ -, $G\alpha_i$ -, and $G\alpha_q$ -expressing yeast Receptor activity is normalized to the signaling of stimulated A2B – a well-studied GPCR that acts as a guanine-nucleotide exchange factor (GEF) – in each yeast strain. The mean of n replicates is plotted; error bars represent standard error of the mean. A) Activity of yeast expressing Wnt8-Frz1 compared to no receptor control in yeast encoding Gpa1/ $G\alpha_s$, $G\alpha_i$, or $G\alpha_q$ chimeras, $n = 24-42$; B) activity of yeast expressing Wnt8-Frz2 compared to no receptor control in yeast encoding Gpa1/ $G\alpha_s$, $G\alpha_i$, or $G\alpha_q$ chimeras, $n = 21-53$; C) activity of yeast expressing Wnt8-Frz6 compared to no receptor control in yeast encoding Gpa1/ $G\alpha_s$, $G\alpha_i$, or $G\alpha_q$ chimeras, $n = 7-13$; D) activity of yeast expressing Wnt8-Frz1 compared to no receptor control in yeast encoding Gpa1/ $G\alpha_s$, $G\alpha_i$, or $G\alpha_q$ chimeras, $n = 11-24$. ***, $p < 0.0001$; **, $p < 0.005$; *, $p < 0.05$; ns, not significantly different ($p > 0.05$).

Figure 5. Signaling activity of co-expressed Frizzled receptors in $G\alpha_s$ -expressing yeast
 Receptor activity is normalized to the signaling of stimulated Adenosine 2B receptor. The mean of n replicates is plotted; error bars represent standard error of the mean. Activity of $G\alpha_1/G\alpha_s$ -encoding yeast co-expressing A) Wnt8-Frz1 and Wnt8-Frz2, $n = 7$; B) Wnt8-Frz1 and Wnt8-Frz7, $n = 4$; and C) Wnt8-Frz1 and Wnt8-Frz6, $n = 4$ compared to no receptor control. ***, $p < 0.0001$. One-way ANOVA analysis was performed to compare strength of signaling of the co-expressed chimeras Wnt8-Frz1/Wnt8-Frz2 with Wnt8-Frz1 and Wnt8-Frz2 alone. There was no statistical difference in the signaling of yeast expressing two receptors versus one.

Figure 6. LRP6 does not enhance G protein-dependent Frizzled signaling in yeast
 Receptor activity is normalized to the signaling of stimulated Adenosine 2B receptor in each yeast strain. The mean of n replicates is plotted; error bars represent standard error of the mean. Activity of A) Wnt8-Frz1, $n = 5-8$; B) Wnt8-Frz2, $n = 6-8$; C) Wnt8-Frz7, $n = 6-8$; and D) Wnt8-Frz6, $n = 4-8$, expressed with and without LRP6 is compared in yeast encoding $G\alpha_1/G\alpha_s$, $G\alpha_i$, or $G\alpha_q$ chimeras. E) LRP6 expression was confirmed in each yeast strain by Western blot. Closed arrows identify the molecular weight of the expected LRP6 band (~200 kDa). **, $p < 0.005$; *, $p < 0.05$; ns, not significantly different ($p > 0.05$).

Figure 7. $G\alpha_s$ antagonizes *Drosophila* Frizzled signaling in R3/R4 photoreceptor differentiation
 Cross-sections of adult fly eyes; all eyes are in Fz^{KD4a}/Fz^{R52} background. A) Frizzled loss-of-function alleles cause multiple defects in ommatidial development, including adaptation of the R3-R3 phenotype of ommatidia. A sample field of view is shown. Inset: magnification of an R3-R3 ommatidium. B) A cartoon representation of the field shown in panel A. Each ommatidium is color-coded by phenotype. Black, wild-type; yellow, R3-R3; green, failure to rotate; blue, anterior-posterior (A/P) flip; red, dorsal-ventral (D/V) flip; orange, both A/P and D/V flip; gray, two R4 photoreceptors. C) In the Frizzled loss-of-function background, flies heterozygous for the $G\alpha_s^{R60}$ allele show a suppression of the R3-R3 phenotype. Inset: magnification of a wild-type ommatidium. D) Cartoon representation of the field shown in panel C, color-coded as in (B).

Figure 8. Potential mechanisms of direct Frizzled-G protein interactions

A) Frizzled may act as a classic guanine nucleotide exchange factor (GEF) to catalyze the exchange of GDP for GTP on the $G\alpha_s$ subunit, resulting in the release of GTP-bound $G\alpha$ and $G\beta\gamma$. B) Frizzled may act like a guanine dissociation inhibitor (GDI) by sequestering $G\alpha$ in the GDP-bound form and releasing $G\beta\gamma$ without catalyzing guanine nucleotide exchange.

Summary of eye phenotypes. Types of errors in planar cell polarity and cell fate include: dorsal-ventral (D/V) flip, both A/P and D/V flip, anterior-posterior (A/P) flip, two R3 photoreceptors (R3-R3), two R4 photoreceptors, extra photoreceptors, failure to rotate.

Table 1

Genotype:	+/+; FzKD4A/FzR52		R60/+; FzKD4A/FzR52 ("rescue")						
type of error	% ommatidia	(n=1466)	sd	sem	% ommatidia	(n=1011)	sd	sem	χ^2 p value
D/V only	17.0		4.3	1.4	15.4		4.9	1.6	0.30
A/P only	11.6		2.8	0.9	14.9		6.2	1.9	0.015
D/V and A/P	3.9		2.1	0.7	2.9		1.4	0.4	0.173
R3/R3	8.1		4.6	1.5	3.7		1.8	0.6	<0.0001
R4/R4	5.2		3.7	1.2	2.8		1.3	0.4	0.003
Missing	0.3		0.4	0.1	0.0		0.0	0.0	0.06
Extra	0.5		0.6	0.2	0.0		0.0	0.0	0.03
Fail to rotate	10.2		6.4	2.0	4.6		2.4	0.8	<0.00001
total incorrect	56.8		7.1	2.3	44.3		4.4	1.4	<0.0000001