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Trimeric G protein-dependent signaling by Frizzled receptors in animal development

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1. ABSTRACT

Receptors of the Frizzled family transduce important signals during animal development and are conserved from sponges to humans. Frizzled receptors belong to the superfamily of G protein-coupled receptors (GPCRs), but until recently were considered G protein-independent in their signaling. In the present article we review the extensive knowledge demonstrating the functions of trimeric G proteins in Frizzled signal transduction in vertebrates and lower animals. Other structural and functional similarities of Frizzled receptors and the GPCRs are also discussed.

2. A BRIEF HISTORY OF FRIZZLED RECEPTORS

The first Frizzled was described in *Drosophila* as the gene encoded by the cuticle polarity *fz* locus (1, 2). Subsequent cloning and biochemical characterization revealed that Fz was an integral membrane protein with seven transmembrane helixes, extracellular N-terminus, and intracellular C-terminus (3, 4), thus belonging to the superfamily of G protein-coupled receptors (GPCRs) (5). Through a series of genetic interaction experiments, Fz was placed on the top of the signal transduction cascade regulating planar cell polarity (PCP, see below) as the cell-surface receptor for the extracellular polarizing information (6). However, the nature of the Fz ligand (s) in *Drosophila* PCP remains unclear (7).

In contrast, it was soon found that another *Drosophila* Fz receptor, DFz2, bound Wingless (Wg), the founding member of the Wnt family of secreted glycoproteins (8), which initiated the Fz- beta-catenin pathway required for many steps of organism development (see below). Subsequent experiments in *Drosophila* showed that both Fz and DFz2 served as Wg receptors, and simultaneous removal of both was required to phenocopy *wg* mutations (9-12).

First non-*Drosophila* Fz was identified in rat (13), followed by cloning of multiple Fz homologs from mammals, chicken, zebrafish, sea urchin and nematode (14, 15). Today we know that Fz receptors and their Wnt ligands are omnipresent in multicellular animals and appeared in animal evolution already in sponges (16-19). The Fz family of receptors includes 10 members in humans, 4 in *Drosophila*, and 3 in *C.elegans* (20). A conserved protein Smoothened (Smo) is related to the Fz family and is involved in a separate pathway, transducing the Hedgehog signal during animal development (21, 22). Interestingly, Fz/Smo-like receptors have been identified in the social amoeba *Dictyostelium discoideum*, despite the absence of Wnt or Hedgehog genes in its genome (17, 23, 24), providing interesting implications for the possible evolution of the Fz signaling cascades.

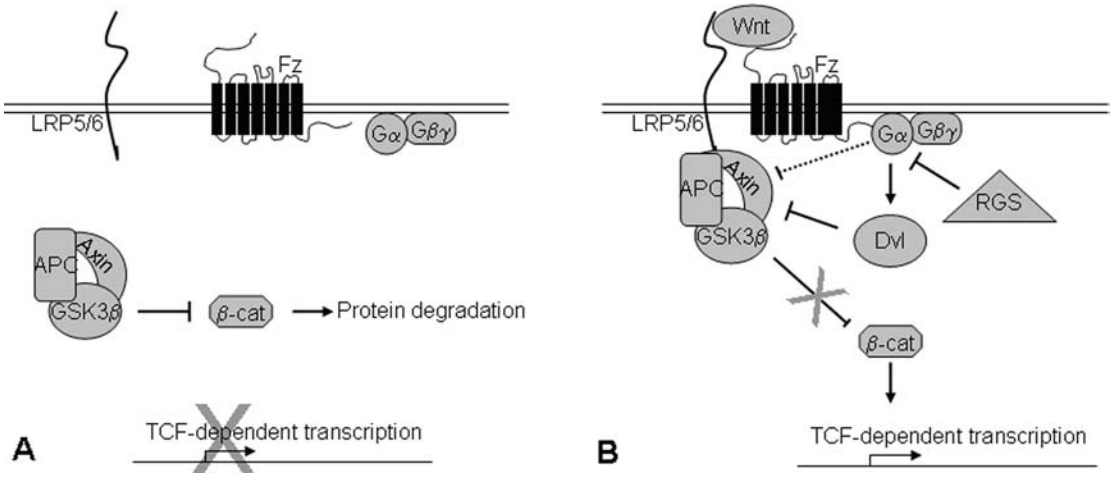

Figure 1. The beta-catenin pathway. A) In cells receiving no Wnt signal, the destruction complex containing Axin, APC and GSK3beta phosphorylates beta-catenin, which leads to its proteasomal degradation. B) The binding of Wnt to Fz and LRP5/6 activates the trimeric G protein and Dvl, and induces Axin binding to LRP5/6. This leads to inactivation of the destruction complex and to stabilization of beta-catenin. beta-catenin is then translocated to the nucleus where it acts as a transcription activator. The trimeric G proteins are also modulated by the RGS proteins.

3. THE FRIZZLED- BETA-CATENIN SIGNALING IN DEVELOPMENT

The Fz- beta-catenin pathway is also referred to as the canonical Wnt-Fz signaling. It is induced by the Wnt family of secreted glycoproteins, which are often secreted by a localized group of cells (the organizer) and spread through the tissue, creating a concentration gradient inducing different patterning responses. Thus, Wnt ligands are typical morphogens (25, 26). As Wnts are strongly hydrophobic due to lipid modifications (27, 28), their spreading through the tissue becomes a sophisticated and highly regulated process. In *Drosophila*, different ways of packing of this morphogen for the short-range *vs.* the long-range spreading have been predicted (29-31).

On the surface of the receiving cells, Wnt ligands bind to Fz and its single-pass transmembrane co-receptor LRP5/6 (known as Arrow in flies) (32). This binding, through the help of the scaffolding protein Dishevelled (Dvl) (33, 34), leads to re-organization of the Axin-APC-GSK3beta destruction complex (Figure 1). The function of this complex is to induce phosphorylation, followed by proteasomal degradation of the cytoplasmic pool of beta-catenin (35). Re-organization of the destruction complex induced by receptor activation makes it incapable of binding and phosphorylating beta-catenin. As a result, the levels of beta-catenin rise and it can enter the nucleus (Figure 1). There, beta-catenin binds to multiple proteins including the TCF transcription factor (36), inducing expression of the Wnt target genes which initiate various developmental programs. From *Drosophila* to vertebrates, the Fz- beta-catenin pathway controls numerous developmental events that include embryonic patterning, organ and limb development, and CNS formation (37).

The target genes activated by Wnt signaling are context- and tissue-specific. It is however noteworthy that such growth-promoting genes as *c-myc* and *cyclin D1* have been identified among Wnt targets (38, 39). It is thus not surprising that aberrant activation of the Wnt-Fz signaling pathway leads to tumorigenesis in many tissues (40, 41). The biomedical importance of the canonical Wnt-Fz signaling is also highlighted by findings that many types of stem cells require activation of this pathway to stimulate self-renewal and prevent differentiation (41). Growing understanding of the roles of the Fz- beta-catenin pathway in regeneration (42) and ageing (43) further adds to the importance of deciphering this type of signal transduction.

4. THE FRIZZLED-PLANAR CELL POLARITY PATHWAY IN DEVELOPMENT

Planar cell polarity (PCP) is characterized by uniform polarization of the epithelial tissue within the plane of the epithelium, perpendicular to the typical apico-basal polarization of the epithelial cells (44, 45). PCP has been most extensively studied in *Drosophila*, where it is manifest as the uniform orientation of epithelial protrusions (called trichomes or hairs) on the adult's body, or as the uniform chiral shape and orientation of the fly compound eye's ommatidia. In vertebrates, PCP can be seen in the body hair orientation (46) or mediolateral orientation of stereociliary bundles of the sensory hair cells in the inner ear (47), which is important for the proper perception of the sound. Further, the process of convergent extension happening during gastrulation, neurulation, and organogenesis is analogous to PCP and controlled by the same set of genes (48, 49). In convergent extension, a tissue narrows along one axis and elongates in a perpendicular axis through cell intercalation.

Fz is the receptor initiating PCP signaling (6). However, it is still unclear what kind of molecule(s) serves as the ligand for Fz in PCP. In vertebrates, certain members

of the Wnt family (such as Wnt5a and Wnt11) are involved in the regulation of convergent extension (50, 51) (see below). However, genetic evidence suggests that these Wnts may play a permissive rather that instructive role in this process (49, 50). Furthermore, Wnts are not involved in PCP signaling in flies (7). Protocadherins Fat and Dachsous participate in tissue polarization in flies (52, 53) and possibly vertebrates (54), and their graded expression or activity was proposed to serve as the polarizing information decoded by Fz in PCP (52, 53, 55). However, recent experiments have revealed that Fat and Dachsous represent a separate, Fz-independent pathway of cell polarization, and the two pathways are semi-redundant in ensuring correct and precise establishment of the PCP (56, 57).

Fz signal transduction in PCP is not straightforward and apparently involves feed-forward (self-amplification) loops. Two additional transmembrane proteins are involved in the Fz-PCP signaling: Van Gogh (also known as Strabismus) and Flamingo (also known as Starry Night). During the course of PCP signaling, these proteins undergo mutually-dependent re-localizations. For example, in the pupal wing epithelia, Fz re-localizes to the distal apical membrane (58), Van Gogh to the proximal apical membrane (59), and Flamingo is enriched both distally and proximally, but depleted from the lateral membranes (60). At the cytoplasmic level, Dvl is again a critical transducer of Fz and re-localizes together with Fz to the distal membranes (61-63). Interestingly, different domains of this scaffolding protein are involved in the Fz-beta-catenin *vs.* the Fz-PCP signaling (64). Additional proteins participating in the Fz-PCP signaling are Prickle and Diego (45). It is however unclear whether all these proteins actually help transmit the signal from Fz inside the cell, or are just necessary for the Fz-induced Fz re-localization and thus local amplification of the Fz signal. In case of Dvl, our experiments in $fz^{-/-}$ *Drosophila* eyes have shown that this protein is clearly involved in the signaling *per se*, and not just Fz re-localization (65). Additional experiments are required to determine whether the same is true for other PCP proteins.

In the wing, the manifestation of PCP is the coordinated growth of the actin-rich epithelial protrusions. It is thus not surprising that proteins involved in the control of the actin cytoskeleton such as RhoA, Rho-kinase, and cofilin are necessary for the correct PCP establishment (66-68). In vertebrate convergent extension, cell intercalation is also an actin-dependent process, controlled by RhoA and its effectors (69, 70). However, in other tissues such as *Drosophila* eye, PCP is less dependent on the RhoA signaling (71). This difference illustrates that there are possibly several Fz-PCP signaling pathways.

5. STRUCTURAL PROPERTIES OF FRIZZLED RECEPTORS AS GPCRS

As members of the GPCR superfamily, Fz receptors have an extracellular N-terminus, seven transmembrane helixes, an intracellular C-terminus, and three extracellular and intracellular loops. Further, a pair of cysteines creating a disulfide bond between extracellular loops 1 and 2 and stabilizing the structure of rhodopsin and other GPCRs (5, 72) is conserved in Fz receptors (73). Ligand- and effector-binding structural elements are distributed in the extracellular and intracellular portions, respectively. The extracellular N-terminus of Fz contains a conserved cysteine-rich domain (CRD), which can bind Wnt ligands *in vitro* and *in vivo* (74, 75). The function of the CRD in Wnt binding is further supported by the finding that several non-Fz Wnt receptors also contain a CRD (76, 77), as well as by the existence of secreted vertebrate CRD-containing proteins serving as Wnt antagonists (78). However, the CRD is also conserved in the Smo receptor (although one of the ten conserved cysteines is missing in Smo), as well as in several *Dictyostelium* Fz/Smo-related receptors which do not transduce any Wnt signal (21, 24). Furthermore, experiments in *Drosophila* showed that the CRD of Fz or DFz2 receptors was largely dispensable for the ability of these receptors to transduce the Wnt signal (79, 80). Thus, the function of the CRD might be to increase the local concentration of Wnt at the Fz receptor, but Wnt binding to another site in the extracellular loops of Fz might be necessary to activate signal transduction; such a two-step binding mechanism is known to activate certain GPCRs (81). Another interesting possibility for the function of the CRD comes from the fact that Fz receptors can dimerize which may be necessary (82) or even sufficient (83) for receptor activation; the CRD is required for Fz dimerization (83) and can be crystallized as a dimer (84). Homo- or hetero-dimerization of GPCRs is a frequent feature of this superfamily of receptors (85).

The C-terminus of Fz receptors contains a highly conserved KTXXXW motif directly after the last transmembrane domain (73); this motif can bind the PDZ domain of Dvl (86) and is essential for the activation of the beta-catenin pathway (87). However, as this motif is also present in Fz receptors which do not activate the canonical Fz- beta-catenin pathway, such as rat Fz2 (73), and in some Fz/Smo-like receptors of *Dictyostelium* which lacks Dvl homologs (17, 23, 24), its function can not be restricted to the beta-catenin pathway nor to Dvl binding. A more typical PDZ-binding motif S/T-X-V is present on the extreme C-terminus of some but not all Fz receptors (73). Multiple PDZ-containing proteins might thus bind Fz receptors (88, 89) and contribute to the specificity of signal transduction. Some of these PDZ-containing proteins, like Kermit, bind many other GPCRs (90) in addition to Fz (88).

Activation of trimeric G proteins is a general functional feature of GPCRs. Trimeric G proteins consist of three subunits: the GDP- or GTP-bound alpha-subunit and the tightly associated beta- and gamma-subunits. Active GPCRs serve as guanine nucleotide exchange factors (GEF) catalyzing the substitution of GDP for GTP on the Galpha subunit, which further leads to dissociation of $Galpha^{GTP}$ from Gbetagamma; both components can engage downstream signaling effectors (91). With time, GTP is hydrolyzed by the intrinsic GTPase activity of Galpha, which is further enhanced by the action of specific *R*egulators of *G*-protein *S*ignaling (RGS) proteins (92). It is

generally assumed that the resulting $Galpha^{GDP}$ is inactive and immediately re-associates with Gbetagamma, terminating the signal transduction. However, our recent modeling has demonstrated that depending on the cellular conditions, trimeric G proteins may create several kinetic modes of signaling (93). Production of significant pools of $Galpha^{GTP}$ and Gbetagamma is only one of these kinetic modes; other modes represent formation of free Gbetagamma and $Galpha^{GDP}$, without any significant amounts of $Galpha^{GTP}$, or a transient appearance of significant amounts of $Galpha^{GTP}$ with a stable release of free Gbetagamma (93). Such a multitude of signaling regimes likely contributes to the specificity in GPCR signaling. Our modeling also shows that the RGS proteins, normally believed to be terminators of GPCR signaling, can in some instances serve to enhance and even enable the signal transduction (93), confirming several experimental observations (94-96).

Four subfamilies of Galpha subunits exist: $Galpha_s$, $Galpha_{i/o}$, $Galpha_{q/11}$ and $Galpha_{12/13}$. It is believed that structural re-arrangements induced by ligand binding expose the G protein-activatory GEF sequences located in the intracellular portions of the GPCRs (97). Indeed, peptides corresponding to the intracellular regions of certain GPCRs have the capacity to activate trimeric G proteins *in vitro* (98), as does the wasp venom receptor-mimetic peptide mastoparan (99). The GEF sequences can be located on each of the three intracellular loops or the C-terminus of the GPCRs (98). Bioinformatic tools can be used to predict the G protein coupling selectivity of a given GPCR based on the sequence of its intracellular regions. Application of these techniques to human Fz receptors has predicted that Fz2, Fz4, Fz6, Fz9, and Fz10 are coupled to the $G_{i/o}$ subclass of trimeric G proteins; Fz5 and Fz8 to the $G_{q/11}$ subclass; Fz1 and Fz7 to both subclasses; and Fz3 to the three $G_{i/o}$, $G_{q/11}$ and G_s subclasses (73); these predictions are fairly well supported experimentally (see below). Physical interaction of mouse Wnt3a-responsive Fz and $Galpha_o$ was demonstrated (100), as was the binding of $Galpha_o$ to *Drosophila* Fz and DFz2 (101) and human Fz1 (102).

The intracellular regions of GPCRs have more binding partners in addition to the PDZ-containing- and G proteins. Beta-arrestin, a crucial regulator of GPCR internalization (103), can bind phosphorylated GPCRs. Most notable among the kinases phosphorylating GPCRs are the *G* protein-coupled *R*eceptor *K*inases (GRKs) (104), additional kinases which can fulfill this function are protein kinases A and C (PKA and PKC) (105); the sites of GPCR phosphorylation are usually located in the C-terminus (105). Beta-arrestin plays a significant role in the vertebrate Fz internalization and Fz- beta-catenin signaling (106, 107), as well as in *Xenopus* convergent extension movements (108). Its function in *Drosophila* PCP signaling awaits elucidation; however, Fz endocytosis is important for PCP establishment in both flies and vertebrates (109, 110). Two alternative ways of rendering Fz receptors recognizable by beta-arrestin have been reported. First, Fz can be directly phosphorylated by various kinases (73, 111, 112), creating beta-arrestin recognition sites. Second, membrane-bound Dvl can be hyperphosphorylated (34) and can serve as an intermediate between human Fz4 and beta-arrestin2 to mediate Fz4 internalization and signaling in 293 cells (106). This strategy is a curious modification of the GPCR- beta-arrestin interaction theme and compensates for the usually short length of Fz C-termini (73).

Similarly to the Fz receptor signaling, the involvement of trimeric G proteins (113), GRK (114, 115) and beta-arrestin (114, 116) in signaling by the Fz-related Smo receptor in vertebrates has also been reported.

The role of trimeric G proteins in Fz signaling pathways was questionable for a long time, but is widely accepted nowadays. However, it is probable that both G protein-dependent and -independent signaling can occur downstream from Fz receptors. The following chapters will recapitulate the findings of different laboratories concerning the interactions between Fz and trimeric G proteins in animal development.

6. TRIMERIC G PROTEINS IN THE BETA-CATENIN SIGNALING PATHWAY

6.1. In vertebrates

The involvement of trimeric G proteins in the Fz-beta-catenin pathway was first postulated by Malbon and colleagues working on mouse F9 teratocarcinoma stem cells (117). In these cells the activation of the beta-catenin pathway can be monitored by their differentiation into the primitive endoderm (PE). Stimulation of F9 cells expressing rat Fz1 (Rfz1) with *Xenopus* Wnt5a or Wnt8 resulted in PE formation. Pertussis toxin (Ptx), which selectively uncouples G proteins of the $G_{i/o}$ family (G_i, G_o, G_t) from receptor activation (118), blocked this stimulation. Antisense oligodeoxynucleotides were used to further narrow down which Galpha subunit was involved. Depletion of either $Galpha_q$ or $Galpha_o$ but not other Galpha subunits inhibited the induction of PE by *Xenopus* Wnt8 in cells expression Rfz1 (117). Additional experiments were performed using a chimeric receptor consisting of the extracellular and transmembrane portions of the beta-adrenergic receptor $beta_2AR$ and intracellular loops and the C-terminus of Rfz1 (119). Such a chimera permitted usage of conventional beta-adrenergic agonists to activate the Fz-beta-catenin pathway and the formation of PE (119). The beta2AR-Rfz1 receptor displayed a decreased affinity for the beta-adrenergic agonists in the presence of a non-hydrolyzable GTP analog - a typical feature of the receptors operating through trimeric G proteins (120). Ptx, as well as depletion of either $Galpha_q$ or $Galpha_o$ resulted in the suppression of the beta2AR-Rfz1-induced beta-catenin specific gene transcription (119). Taken together these data demonstrated that activation of Rfz-1 with Wnt5a or Wnt8 was transduced through $Galpha_q$ or $Galpha_o$ to activate the beta-catenin signaling pathway.

More evidence of a trimeric G protein involvement in the beta-catenin pathway came from experiments with RGS proteins. Injection of rat RGS4 RNA into *Xenopus* embryos showed nearly the same phenotype as injection of a dominant negative Wnt8, which

suggested that the RGS proteins could repress the *Xenopus* Wnt8 signaling to beta-catenin by restricting the activity of trimeric G proteins (121).

Comparable experiments were also made in the mouse F9 cells. A screen was performed to identify RGS proteins involved in the beta-catenin pathway. A panel of different RGS proteins was overexpressed in F9 cells and their ability to suppress the Wnt3a-induced beta-catenin-specific gene transcription was tested. Only expression of RGS19 (also known as GAIP) could downregulate the beta-catenin pathway in F9 cells (96). Next the authors sought to identify the trimeric G protein(s) affected by RGS19. To do so they expressed constitutively active mutant forms of different Galpha subunits in F9 cells already transfected with RGS19. These point mutations eliminated the GTPase activity of the Galpha subunits rendering them insensitive to the action of the RGS. Only the constitutively active mutant of $Galpha_o$ was able to rescue the Wnt3a-stimulated gene transcription in cells expression RGS19 (96). As RGS19 also prevented Dvl phosphorylation, the authors concluded that RGS19 inhibited $Galpha_o$ acting in the pathway downstream from Fz and upstream of Dvl (96). Curiously, the authors found that not only overexpression, but also the knockdown of RGS19 suppressed the Wnt3a-induced Fz- beta-catenin pathway in F9 cells (96), demonstrating a complex role of the RGS activity in this pathway. This finding agrees with our mathematical modelling predicting that RGS proteins may play both negative and positive roles in GPCR signaling (93). Interestingly, RGS19 can also interact with the scaffolding protein Kermit (also known as GIPC) (90) which in turn can bind several GPCRs including Fz (88). Thus, formation of a quaternary complex involving Fz, Kermit, RGS and $Galpha_o$ might be necessary for efficient Fz- beta-catenin signaling; formation of similar complexes has been described for other GPCRs (122).

A biochemical approach to fit the trimeric G proteins into the beta-catenin pathway was used by Kimmel and co-workers (100). The authors could show that upon the Wnt3a stimulation of the mouse L929 and 3T3-L1 cells the GSK3beta/Axin and GSK3beta/Axin2 complexes rapidly (within minutes) dissociated. If these cells had been pre-treated with small interfering RNA to either $Galpha_q$ or $Galpha_o$, or with Ptx, the Wnt-induced stabilization of beta-catenin and the dissociation of GSK3beta/Axin complexes was inhibited. Interestingly, $Galpha_q$ was responsible for dissociation of the GSK3beta/Axin, and $Galpha_o$ for dissociation of the GSK3beta/Axin2 complexes (100). The authors could further demonstrate the ability of a nonhydrolyzable analogue of GTP to mimic the effects of Wnt3a on the beta-catenin stabilization and the GSK3beta/Axin dissociation; the authors also detected a transient interaction of $Galpha_o$ with Fz and Dvl, rapidly dissociated upon Wnt3a addition (100). Existence of the distinct rapid and more time-consuming responses to Fz stimulation has been demonstrated in several investigations (123-125); it can be proposed that the later responses result from amplification of the rapid initial input. The data from Kimmel and co-workers (100) suggest that the trimeric G proteins G_q and G_o represent the immediate transducers of Fz in the rapid form of the Fz-beta-catenin pathway, targeting the GSK3beta/Axin complexes and thus contributing to the beta-catenin stabilization.

6.2. In *Drosophila*

The involvement of trimeric G proteins in the Fz signaling in *Drosophila* was for a long time doubted, because no mutations in trimeric G proteins were found in genetic screens aiming to identify components of the Fz pathways. However, as only six genes for the Galpha subunits exist in the *Drosophila* genome, each of the trimeric G protein mutations should likely have pleiotropic effects preventing isolation in direct genetic screens. We utilized a different approach expressing Ptx or cholera toxin to target specific G-proteins in the fly (126). Expression of Ptx (but not cholera toxin) under the control of an eye-specific promoter suppressed the Fz-overexpression eye phenotype, suggesting involvement of a Ptx-sensitive G-protein downstream of Fz (126, 127); the only Galpha subunit affected by Ptx in *Drosophila* is $Galpha_o$. To further clarify the role of $Galpha_o$ in the beta-catenin signaling pathway somatic clones lacking $Galpha_o$ function were analysed. These clones showed a clear reduction in Wg signal transduction monitored by the target gene expression. With the help of genetic epistasis experiments $Galpha_o$ was placed upstream of Armadillo (fly beta-catenin), Shaggy (fly GSK3beta), and Dvl, suggesting that $Galpha_o$ was an immediate transducer of Fz signaling. Over-activation of the Fz- beta-catenin pathway was observed upon overexpression of $Galpha_o$ or its constitutively active form ($Galpha_o$-GTP). Importantly, when same overexpression was performed in the $fz^{-/-}; fz2^{-/-}$ double mutant background, only the $Galpha_o$-GTP form (but not the wild-type form), independent of the receptor GEF activity, could rescue loss of Wg target gene expression, providing a genetic evidence for the GEF activity of Fz receptors in *Drosophila*. Together with the ability of *Drosophila* Fz receptors to physically interact with $Galpha_o$ (101), these data clearly demonstrate that $Galpha_o$ is indeed an immediate transducer of Fz in the beta-catenin signaling pathway (126).

7. TRIMERIC G PROTEINS IN *DROSOPHILA* PLANAR CELL POLARITY SIGNALING

As Ptx could suppress the dominant PCP phenotypes induced by Fz overexpression in *Drosophila* eye, a function of $Galpha_o$ in the Fz-PCP signalling was expected (126, 127). Somatic clones lacking the $Galpha_o$ function, as well clones overexpressing $Galpha_o$ or $Galpha_o$-GTP were analysed. Loss-of-function somatic clones in the wing showed defects in hair orientation and a strong multiple wing hair (mwh) phenotype, with up to five hairs produced per wing cell (instead of one in wild-type wings). Non-autonomous polarity defects on the proximal side of the clones were also seen. Such non-autonomy can also be induced by *fz* mutant clones, but on the opposite, distal side of the clone. The overexpression of either $Galpha_o$ or $Galpha_o$-GTP in wings also produced an mwh phenotype. Similar effects of loss- and gain-of-function of genes involved in PCP signalling are usual, showing that

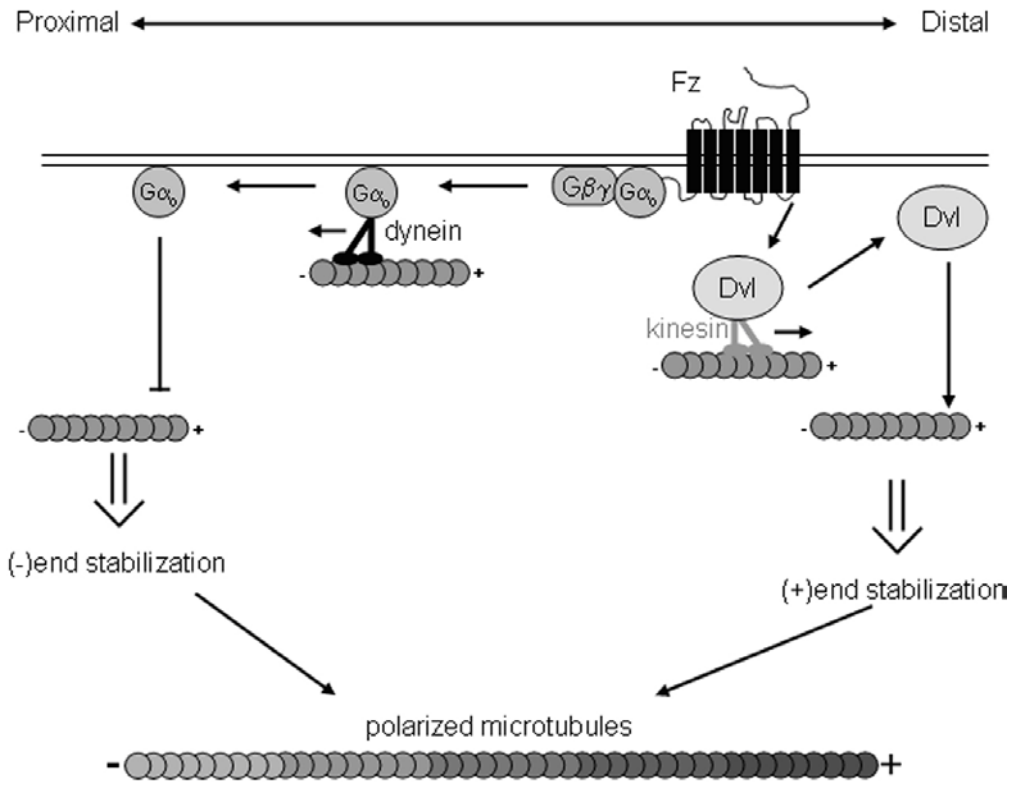

Figure 2. Proposed mechanism of action of the trimeric G protein G_o in *Drosophila* PCP. Fz activates G_o (releasing $Galpha_o$-GTP) and Dvl in parallel. $Galpha_o$ accumulates (possibly through the (-)end-directed dynein transport) at the proximal side of the cell, where it destabilizes the (+)ends of microtubules, which in consequence leads to further enrichment of the (-)ends on this side of the cell. Dvl (possibly through the (+)end-directed kinesin transport) accumulates at the distal side of the cell, where it stabilizes the microtubule (+)ends. These antagonistic effects of $Galpha_o$ and Dvl on the microtubule cytoskeleton, coupled with their opposing localizations might be the mechanism ensuring cell polarization in PCP.

the right balance of protein levels is important for the correct polarity establishment. We could further demonstrate that the effects of $Galpha_o$ overexpression were Fz-dependent, in contrast to the $Galpha_o$-GTP overexpression phenotypes which were Fz-independent, again providing a genetic evidence for a GEF activity of Fz towards G_o (126).

Additional insights into the Fz-$Galpha_o$ coupling were obtained investigating the asymmetric division of sensory organ precursor cells (SOPs). An SOP divides in the plane of the epithelium to generate the pIIa and pIIb daughter cells, unequal in content and size; these daughters again divide asymmetrically, building up a sensory bristle consisting of four to five different cells. The asymmetric SOP division depends on polarized accumulation of cell fate determinants such as Numb, followed by their exclusive segregation into one of the two daughter cells (128). Loss of Fz or its PCP transducers does not perturb the asymmetric nature of the SOP division, but leads to randomization of the plane of this division (129). Thus Fz is important for the correct positioning of the SOP division axis in the epithelial plane, but not for the intrinsic polarity of the SOP. In contrast, we found that $Galpha_o$ was important for both the intrinsic cellular polarization, as well as for the sensing of the Fz-mediated extracellular polarity signal, as mild *Galpha$_o$* mutations phenocopied *fz* loss-of-function while more severe *Galpha$_o$* mutations led to complete loss of the asymmetry of the SOP division, resulting in formation of aberrant sensory bristles (127). Thus, $Galpha_o$ likely plays an integrator role in the SOP, linking the extracellular Fz-mediated polarizing information with the intrinsic cell polarization machinery (127). Additionally we showed that $Galpha_o$ could bind to and genetically interact with Pins (127), which is an important regulator of asymmetric Numb localization (130, 131). The function of trimeric G proteins downstream from Fz receptors in regulating the asymmetric cell divisions may also be conserved in *C.elegans* (132).

What could be the mechanism of action of the trimeric G protein G_o in *Drosophila* PCP signaling? Unlike $Galpha_o$'s action in the canonical beta-catenin pathway, in the PCP pathway it does not signal through Dvl. In contrast, we found an antagonism between $Galpha_o$ and Dvl activities in Drosophila PCP (65). Together with our previous findings that $Galpha_o$ re-localized to the proximal side of the wing epithelia at the time when Fz and Dvl re-localized distally, as well that *Galpha$_o$* mutant clones induced non-autonomous PCP defects on the side opposite to those induced by *fz* clones (126), these observations suggest that the PCP signal downstream from Fz bifurcates

into the Dvl-mediated "positive" and the Galpha$_o$-mediated "negative" branches. Both branches are required for the proper cell polarization by acting at the opposite sides of the cell; their coordinated action may be mediated by their opposing regulation of the microtubule cytoskeleton (65, 127) (see below; Figure 2).

8. THE MULTITUDE OF TRIMERIC G PROTEIN-DEPENDENT NON-CANONICAL FRIZZLED PATHWAYS IN VERTEBRATES

The 'non-canonical' Fz pathways collectively refer to the pathways not relying on the beta-catenin-dependent gene transcription. While the distinction between the canonical and the PCP pathways in *Drosophila* development is clear-cut, in vertebrates several non-canonical pathways have been described in cell culture, and zebrafish, *Xenopus*, or mammalian development. These pathways partially overlap and have been described as convergent extension, Wnt-Ca^{2+}, Wnt-cGMP pathways, as well as pathways regulating cell migration and beta-catenin-independent transcription.

To illustrate the complexity and partial overlap of the non-canonical Fz pathways signaling through trimeric G proteins, we first discuss the events occurring during *Xenopus* gastrulation. Proper gastrulation requires convergent extension (through mediolateral intercalation) of the axial mesoderm, leading to lengthening of the tissue (49). This process is under the control of Wnt11 (133) and Fz7; loss or overexpression of Fz7 leads to gastrulation arrest (134-137). However, this arrest is not only due to convergent extension defects (134-136), but also due to the failure of proper cell sorting in the mesoderm and lack of tissue separation between the anterior mesoderm and the ectoderm (137). These two functions of Fz7 are transduced differently: the signaling to convergent extension is Dvl-dependent (138), and the signaling to cell-sorting is Dvl-independent (137). Iioka et al have recently demonstrated a function of Galpha$_{11}$ (a member of the Galpha$_{q/11}$ subclass of trimeric G proteins) in Wnt11- and Fz7-dependent convergent extension events and regulation of Dvl phosphorylation and plasma membrane translocation (139). Downstream from Dvl, regulation the actin cytoskeleton through the small GTPase RhoA (69), and regulation of stability of the focal adhesion-associated protein Paxillin (139) have been shown necessary for the convergent extension. Wang and Malbon have predicted bioinformatically that human Fz7 should couple to both the $G_{i/o}$ and $G_{q/11}$ subclasses of trimeric G proteins (73). In agreement with that, a Ptx-sensitive trimeric G protein of the $G_{i/o}$ subclass controls regulation of the cell-sorting behaviour downstream from Fz7; the signal is further transmitted from the trimeric G protein to PKCalpha (137). The Wnt11-Fz7-$G_{i/o}$-PKC branch of signaling further activates the small GTPase Cdc42 (140, 141), which is not necessary for the Fz7-dependent cell sorting (137), but contributes to the actin cytoskeleton regulation during convergent extension (141). It could also be demonstrated that activation of Cdc42 downstream from Fz7 is mediated by the Gbetagamma subunits of the trimeric $G_{i/o}$ protein (141).

An additional complication to this picture has been provided by the observation that PKA negatively regulates the Dvl-RhoA branch of Wnt11-Fz7 signaling (142). Curiously, this regulation is mediated by $G_{i/o}$ apparently acting upstream from Dvl. Thus, the trimeric protein $G_{i/o}$ can act both independently from Dvl (137) and upstream from Dvl (142) in Fz7 signaling; an additional G protein acting upstream form Dvl is G_{11} (139). The complexity and intertwining of several G protein-dependent pathways regulating Xenopus gastrulation movements downstream from Fz7 receptor is graphically summarized on Figure 3.

Another non-canonical Fz pathway has been described in mouse bone formation. Wnt7b *in vivo*, and Wnt3a in the culture of murine ST2 cells regulate transcriptional induction of osteoblastogenesis through a G_q- and PKCdelta-dependent signaling (143). The authors propose the action of a phospholipase C (PLCbeta) between G_q and PKCdelta; they also show that this pathway is Dvl-dependent, although their experiments do not address the issue of hierarchy between G_q and Dvl. There is a possibility that Dvl acts downstream of PKCdelta in this signaling, as has been shown in *Xenopus* convergent extension (144).

These examples of developmental Fz-G protein-PKC signaling cascades relate to the Fz-G protein-Ca^{2+} pathways described in zebrafish and cell culture. The Ca^{2+} signaling pathway is characterized by the elevation of the Ca^{2+} levels in the cytoplasm through an influx of calcium ions from the endoplasmatic reticulum (ER) or cell exterior. A typical trigger of Ca^{2+} release from the ER is inositol-1,4,5-trisphosphate ($InsP_3$), which binds to the $InsP_3$ receptor on the ER membrane, opening the calcium channel (145). $InsP_3$ is generated by the phospholipase C (PLC) activity which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2); another product of this hydrolysis reaction is diacylglycerol (DAG), an important second messenger activating several isoforms of PKC including PKCalpha and PKCdelta. PKCalpha can also be activated by calcium (146). Several subtypes of PLC exist; and among those the PLCbeta isozymes are responsive to GPCRs and can be activated by the Gbetagamma subunits of trimeric G proteins, and by the GTP-loaded forms of Galpha$_{q/11}$ (147). All this shows that there exist many possibilities of inducing the Ca^{2+} influx and activating PKC isoforms downstream of Fz receptors through trimeric G proteins.

It has been shown that *Xenopus* Wnt5a and Wnt11 can double the frequency of Ca^{2+} oscillations in zebrafish embryos (148, 149). This could be mimicked with expression of rat Fz2 (Rfz2), but not rat Fz1; co-expression of Wnt5a and Rfz2 produced synergistic effects. It could further be shown that Ptx inhibited Ca^{2+} mobilization induced by Rfz2, suggesting that a trimeric G protein of the $G_{i/o}$ subclass was involved in this pathway. Additional experiments revealed an important function of the Gbetagamma subunits and the enzyme inositol monophosphatase (IMPase) in the Wnt5a-Rfz2 pathway to calcium mobilization (150). Subsequent experiments in

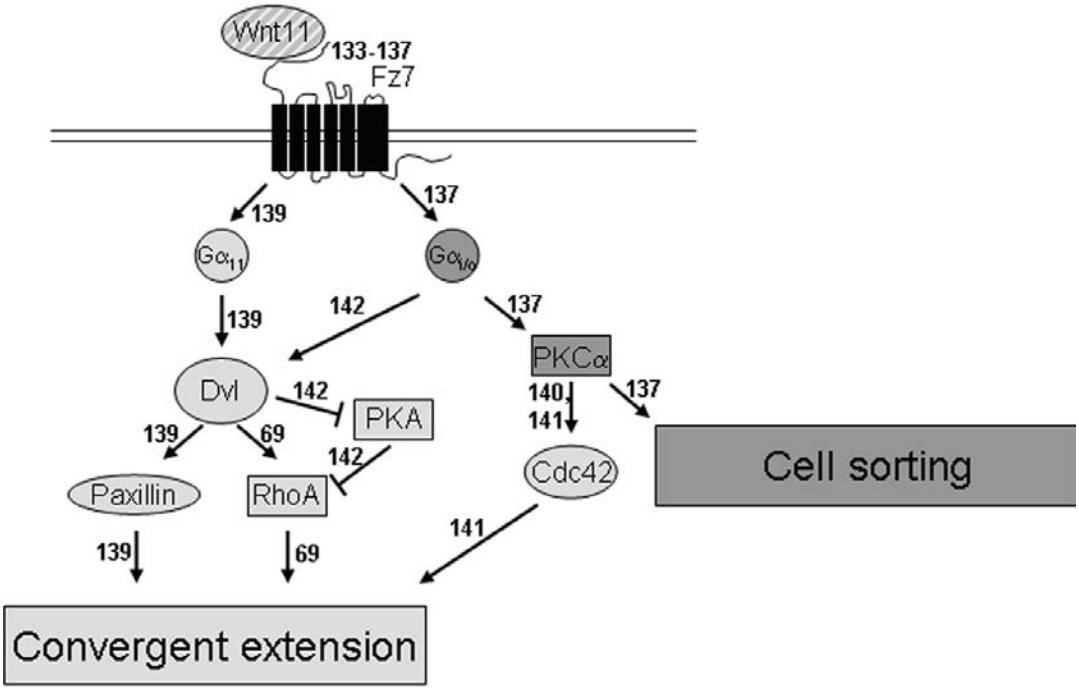

Figure 3. Non-canonical Wnt-Fz pathways in *Xenopus* gastrulation. Wnt11 and its receptor Fz7 control two pathways in parallel to enable proper gastrulation, namely the convergent extension (proteins highlighted in light grey) and the cell sorting (proteins highlighted in dark grey). For more details please refer to the text. The bold numbers near the arrows are the references to respective experimental publications.

Xenopus embryos revealed that this Wnt-Rfz2-G protein pathway also led to plasma membrane translocation and activation of PKC (151), as well as activation of the calcium/calmodulin-dependent protein kinase II (CamKII) (152). CamKII might be involved in the inhibition of the canonical beta-catenin pathway induced by Wnt11 and Wnt5a in *Xenopus* embryo (148, 152). Inhibition of the beta-catenin responses by the Fz-Ca^{2+} pathway in *Xenopus* embryos was also shown to be transduced through the Ca^{2+}-activated phosphatase calcineurin and NF-AT-dependent transcription (153). A possible function of Dvl in the Fz-Ca^{2+} pathway was also demonstrated: Dvl injection into *Xenopus* and Zebrafish embryos induced the Ca^{2+} flux and activation of PKC and CamKII in a Ptx-insensitive manner, suggesting that Dvl acted downstream or in parallel to $G_{i/o}$ in this pathway (154) (Figure 4).

Activation of a separate Wnt5a-inducible signaling pathway leading to inhibition of production of cyclic guanidine monophosphate (cGMP) by Rfz2 was found in the mouse F9 teratocarcinoma cells and Chinese hamster ovary (CHO) cells (155). $Galpha_{t2}$, a member of the $Galpha_{i/o}$-protein family, was expressed in F9 and CHO cells and showed a crucial role in this signaling (155). The signal was transduced through the enzyme phosphodiesterase (PDE) which decreased the concentration of intracellular cGMP (156). A decrease in cGMP concentration led to a decrease in protein kinase G activity, which was necessary for the Ca^{2+} mobilization (155, 156). Thus, integration of the Fz-Ca^{2+} and the Fz-cGMP pathways occurs (Figure 4). The biological role of the Wnt-cGMP pathway is not yet fully understood, but it has been shown that PDE inhibitors block the convergent extension movements in zebrafish gastrulation (155).

Additional G protein-dependent non-canonical Fz pathways regulate mammary cell adhesion and cancer cell migration. In HB2 mammary cells, Wnt5a-dependent and Ptx-sensitive signaling was necessary for cell adhesion to collagen (157). Human colon cancer cells HCT8/S11 can become invasive in collagen gels upon expression of Wnt2; this process involves signaling by the Ptx-sensitive $G_{i/o}$ trimeric G protein, its betagamma subunits, PI3 kinase and GSK3beta, which culminates at transcription under the control of AP-1 (158, 159), a major pro-invasive transcription factor (160). These results highlight the medical importance of G protein-dependent non-canonical Fz pathways.

AP-1 is a dimeric transcription factor consisting of members of the Fos and Jun gene families of nuclear phosphoproteins (160) and regulated by several members of the MAPkinase family, including JNK (Jun N-terminal kinase) (161). JNK has been implicated in the *Xenopus* convergent extension (70, 162) and *Drosophila* PCP signaling (64, 163), although only very weak PCP phenotypes are elicited by mutations in the *Drosophila* components of the JNK cascade (71). Fz signaling can also activate JNK in cell cultures (64, 162, 164). Very recently, Wnt3a-induced activation of JNK in mouse F9 teratocarcinoma cells has been shown to be mediated by the Fz1-$Galpha_o$ pathway acting upstream from Dvl and RhoA family members (164).

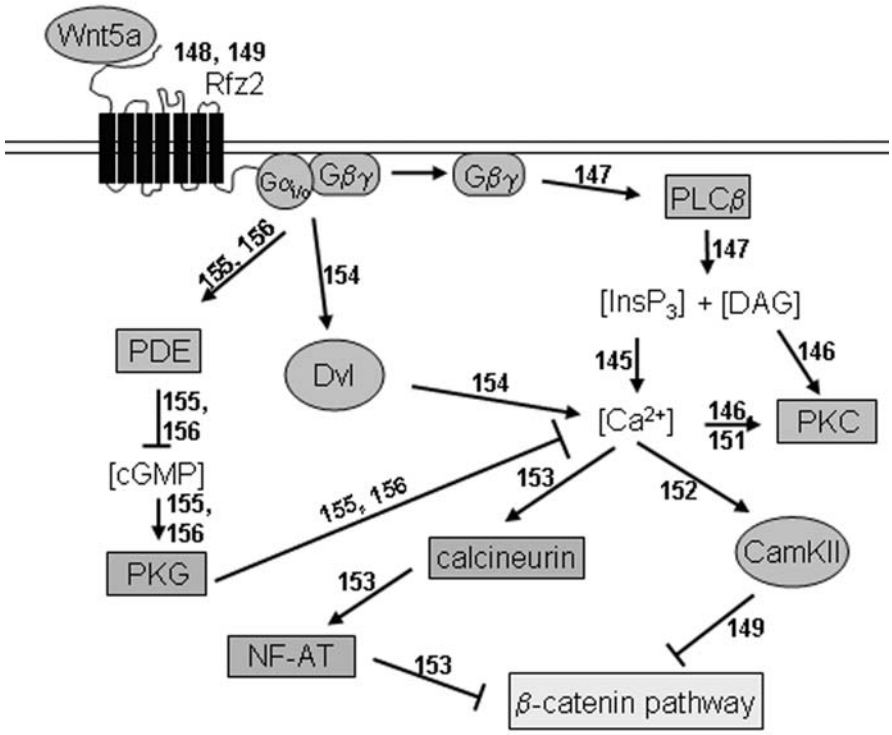

Figure 4. The Fz-Ca^{2+} and Fz-cGMP pathways in vertebrates. Upon Wnt5a binding to Rfz2, the associated trimeric G protein of the $G_{i/o}$ class is dissociated into the Gα and Gbetagamma subunits. Gbetagamma activates PLCbeta to induce an increase in the intracellular (Ca^{2+}). Additionally the Wnt5a-Rfz2-G protein link negatively controls the intracellular levels of cGMP through the activity of a phosphodiesterase; the Ca^{2+} and cGMP pathways are interconnected. See the text for more details. The bold numbers near the arrows are the references to respective experimental publications.

Another example of a non-canonical Wnt-Fz pathway leading to gene transcription was described in vertebrate muscle development. Myogenesis can be induced in cultured muscle precursors upon expression of Wnt1 or Wnt7A (165); this pathway was shown to be dependent on the adenylyl cyclase signalling via PKA and its target transcription factor CREB. Importantly, the authors could show a function of the trimeric G protein G_s upstream from the adenylyl cyclase in this pathway (165), representing the first example of a participation of G_s downstream from Fz receptors.

9. POSSIBLE MECHANISMS OF FRIZZLED SIGNALING THROUGH G PROTEINS

There are apparently multiple mechanisms of signal transduction by trimeric G proteins downstream from Fz receptors. In case of the Wnt-Ca^{2+} pathway described above, Galpha subunits of $G_{q/11}$ or the Gbetagamma subunits can act directly on PLCbeta. However, in many instances the trimeric G protein acts upstream form the scaffolding protein Dvl, which upon activation of Fz receptors is known to re-localize from the cytoplasm to the plasma membrane and become hyperphosphorylated (34). In a search for the Dvl-associated proteins, Moon and co-workers have performed a tandem-affinity purification and mass spectrometry and identified among other proteins the betagamma subunits of trimeric G proteins as the Dvl-binding partners (166). It is tempting to propose that Gbetagamma, which remains associated with the plasma membrane after dissociation form Galpha (167), can serve to recruit Dvl form the cytoplasm and thus mediate its activation. A similar mechanism of activation of PI3-kinase gamma in response to GPCR activation has been proposed (168, 169).

What could be target(s) of the activated forms of the Galpha-subunits involved in Fz signaling pathways? In the beta-catenin pathway, an interesting candidate is Axin, the organizer of the beta-catenin destruction complex. Among other domains, Axin possesses an RGS domain, known in other proteins to bind to the activated Galpha and catalyze GTP hydrolysis on Galpha (see above). Despite the fact that the Axin RGS domain does not contain many residues that directly contact Galpha and lacks the enzymatic RGS activity on tested Galpha subunits (170, 171), it can physically bind to $Galpha_s$ (172) and $Galpha_{12}$ (171); this binding has been shown to mediate the ability of non-Fz GPCRs such as the Prostaglandin E_2 receptor to activate the beta-catenin signaling (172). Since in cell culture dissociation of the Axin-GSK3beta complex by $Galpha_q$, and of the Axin2-GSK3beta complex by $Galpha_o$ has been shown (100), it seems probable that these G proteins might act on Axin in the Fz- beta-catenin pathway as well.

In the Fz-dependent control of the asymmetric SOP divisions, Pins is a likely Galpha$_o$ transducer. Indeed, Pins can physically bind Galpha$_o$; a strong genetic interaction between the two proteins exists in the SOP divisions; finally, Galpha$_o$ can re-localize Pins in dividing SOPs (127). However, Pins has no function in Drosophila PCP, as neither *pins* loss-of-function, nor overexpression produces any PCP defects (our unpublished observations).

In the PCP pathway, direct action of Galpha$_o$ on the tubulin cytoskeleton is possible. Galpha subunits of different organisms can physically bind tubulin and microtubules (173, 174). Direct binding of Drosophila Galpha$_o$ to tubulins has also been shown (127); furthermore, Galpha can destabilize the microtubule (+)ends (175). We have previously proposed a model whereby the (+)end-destabilizing activity of Galpha$_o$ coupled with (-)end-directed transport of Galpha$_o$ would lead to microtubule polarization similar to that seen in *Drosophila* wing cells (127). In the yeast two-hybrid assay of human genome proteins, binding of Galpha$_o$ to dynamitin was reported (176). Dynamitin is a component of the dynactin complex which binds to the (-)end-directed microtubule motor dynein and may provide a link between the motor and its cargo (177). Thus, a function of Galpha$_o$ in regulation of the microtubule motor-cargo interactions might be possible. Since Fz, Dvl and possibly other PCP proteins become re-localized to distinct plasma membrane poles during PCP in a microtubule-dependent manner, such a function of Galpha$_o$ might be crucial in the establishment of PCP in *Drosophila.*

10. PERSPECTIVES

The field of Fz signal transduction is rapidly developing. If originally Fz receptors were largely considered trimeric G protein-independent, the growing body of experimental evidence summarized in this review demonstrates that they are faithful members of the G protein-coupled receptor superfamily. Structural features of Fz receptors, the role of GRKs and beta-arrestin in their internalization and signaling, and the numerous examples of their signaling through trimeric G proteins align them with other GPCRs. Future research will close the gaps in our understanding of the Fz-G protein signaling in vertebrates and lower organisms, for example addressing the issue of the possible involvement of a beta-arrestin in *Drosophila* Fz pathways. Broad systems-like approaches are in need to isolate the interaction partners of the Galpha and Gbetagamma subunits in Fz signal transduction, and to identify their connections with other established Fz signaling proteins.

Trimeric G protein-independent connections between the Fz receptors and the cytoplasmic signaling components (such as Dvl) clearly exist. In this regard it is interesting to track the possible evolution of the Fz signaling cascades. The genome of the social amoeba *Dictyostelium discoideum* encodes several Fz/Smo-like receptors which contain such characteristic signatures of this group as the N-terminal CRD domain and the non-conventional PDZ-binding site in the C-terminus. Trimeric G proteins exist in *Dictyostelium*, while no Wnt, Hedgehog, Dvl or Axin genes are present in its genome (17, 23, 24). Thus, one might imagine that the trimeric G protein coupling is an ancient feature of the Fz receptors. With the development of multicellularity the Fz receptor family might have been 'charged' with the function of a master controller of development. With additional ligand and signal transduction proteins hooked up to it to facilitate this function, the reliance on the G proteins might have been reduced. However, the enduring dependence on trimeric G proteins might serve to keep 'life' in the Fz signaling cascades by the virtue of tight temporal and spatial control of Fz membrane association and integration with the downstream signaling nets.

11. ACKNOWLEDGEMENTS

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