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Review

Yellow submarine of the Wnt/Frizzled signaling: Submerging from the G protein harbor to the targets☆

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ABSTRACT

The Wnt/Frizzled signaling pathway plays multiple functions in animal development and, when deregulated, in human disease. The G-protein coupled receptor (GPCR) Frizzled and its cognate heterotrimeric Gi/o proteins initiate the intracellular signaling cascades resulting in cell fate determination and polarization. In this review, we summarize the knowledge on the ligand recognition, biochemistry, modifications and interacting partners of the Frizzled proteins viewed as GPCRs. We also discuss the effectors of the heterotrimeric Go protein in Frizzled signaling. One group of these effectors is represented by small GTPases of the Rab family, which amplify the initial Wnt/Frizzled signal. Another effector is the negative regulator of Wnt signaling Axin, which becomes deactivated in response to Go action. The discovery of the GPCR properties of Frizzled receptors not only provides mechanistic understanding to their signaling pathways, but also paves new avenues for the drug discovery efforts.

Contents

1. Introduction: the sea of the Wnt/Frizzled signaling

Wnt signaling plays instructive roles in animal development, conserved from sponges to human beings, to activate the β-catenin-dependent transcriptional regulation of cell fate specification [1]. In the adult, this pathway is mostly silent. However, both improper overactivation and underactivation of this pathway can lead to diseases. Insufficient Wnt pathway activity underlies defects in tissue regeneration and the decreased proliferative potential of various stem cells [2,3]; it may also lead to certain neurodegenerative disorders [4]. On the other hand, misactivation of this signaling, e.g. through overproduction of the Wnt ligands or mutational activation of the downstream components of the pathway, promotes carcinogenesis, especially in the colon and breast [5,6]. In addition to this "canonical" β-catenin-dependent pathway, Wnt signaling also controls establishment of epithelial planar cell polarity (PCP). PCP is characterized by uniform polarization of the epithelial tissue within the plane of the epithelium, perpendicular to the typical apico-basal polarization of epithelial cells [7].

Frizzled (Fz) proteins have been identified as the receptors for the Wnt lipoglycoprotein ligands [8]. The first Fz cloned was that of

☆ The "Yellow Submarine" theme refers to the song of the same name from *The Beatles*.

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Drosophila, and the lab of Paul Adler disclosed the primary structure of this protein [9,10] as a 7-transmembrane helix (7-TM) receptor. This discovery happened 15 years after the pioneering works describing the existence of this transmembrane topology [11] and a decade since the prediction methods for this specific arrangement had emerged [12,13]. Reliability and simplicity of such methods based on the hydropathicity profile over the protein length allowed the 7-TM topology of the Fz protein to be no matter for any controversy. In contrast, the idea that 7-TM receptors may have the general feature of coupling to heterotrimeric G-proteins had been formulated only a few years earlier [14], but soon became widely accepted, and the words "7-TM receptors" and "G protein-coupled receptors (GPCR)" became essentially synonymous.

Heterotrimeric G proteins represent the immediate cytoplasmic transducers of GPCRs [15–17]. Upon binding of the agonist the receptor undergoes conformational changes, which enhance its GEF (guanine nucleotide exchange factor) activity towards the α-subunit of the heterotrimeric G protein. As a result, the α-subunit exchanges its GDP for GTP and dissociates from the βγ-heterodimer. Gα–GTP is then capable of interacting with downstream effectors until its intrinsic GTPase activity converts GTP back into GDP, leading to reassociation of the heterotrimeric complex, which brings the system to the "point zero". GPCRs interact with one or several types of G proteins, the specificity being determined by their α-subunits. Unlike more promiscuous βγ dimers [18], α-subunits are capable of signaling to specific effectors; the human genome contains 16 genes for different α-subunits constituting four families (Gαo/i, Gαq, Gαs and Gα12) [16]. Typically, any given cell expresses multiple types of heterotrimeric G-proteins [19].

Despite the growing body of evidence of GPCR coupling to heterotrimeric G-proteins, the data for the involvement of such transducers in Wnt/Fz signaling was missing for many years. Thus the G proteins were omitted from the emerging pathway architecture, being overshadowed by other major components of the cascade [8,20–22]. The first evidence which returned the missing G protein link in the chain of the Fz signal transduction was the role of pertussis toxin-sensitive G proteins obtained for the Fz-dependent non-canonical Ca^{2+}-pathway in zebrafish [23]. Later the Go-protein was found to be a transducer in both canonical and PCP pathways in Drosophila [24–26], and roles for its orthologues along with other members of the heterotrimeric GTPase superfamily in Xenopus [27,28] and mammalian models [28–31] of β-catenin stabilization as well as cell polarization [32] were established. Although these findings suggested that Fz proteins were activators of G-proteins, only recently the final biochemical proof of the ability of Fz receptors to bind heterotrimeric G-proteins and activate the nucleotide exchange on them upon engaging the Wnt ligands has been provided [33–35] – the final demonstration that these receptors are *bona fide* GPCRs [36–38]. Thus when all the i's are dotted and the t's are crossed, it is time to use the vast experience obtained in the field of GPCR functioning and pharmacology to bring us new concepts and insights into the work of the Fz pathway and ultimately to facilitate development of the drugs targeting it. This will be the focus of our review, followed by discussion of which effectors G proteins talk to upon transduction of the Wnt/Fz pathway.

2. Frizzleds as GPCRs: view from above the surface

GPCRs bear certain motifs in the extracellular and intracellular sequences, recognizable for their interactions with ligands and effectors. Fz receptors are no exception to this rule [39], and we proceed with description of the current knowledge of their features. On the extracellular side, Fz proteins carry a well-defined N-terminal cysteine-rich domain (CRD). The CRD contains ten cysteines conserved among the Fz receptor family [40]. This domain has been proposed as the main ligand-binding region of the receptor, and indeed a physical interaction of purified CRD with Wnt ligands has been demonstrated [41,42]. Additional proof for the role of CRD in Wnt binding comes from the fact that the so-called secreted Fz-related proteins (SFRPs), consisting essentially of just CRDs, serve as natural antagonists of Wnt signaling by competing with Fz for the interaction with Wnt ligands [43]. However, CRD is also present in the Smoothened receptor [44], which functions as a constitutive GPCR in the Hedgehog signaling pathway and has no influence on Wnt signaling. Additionally, experiments in Drosophila have demonstrated that a CRD-less Fz can efficiently rescue Fz loss-of-function alleles [45], suggesting that CRD is not the sole Wnt-binding region of the receptors. It is conceivable that a two-step ligand binding mechanism, known for some other GPCRs [46], also applies to Fzs, so that the Wnt-binding CRD serves to increase the local concentration of the ligand in the receptor's vicinity, while binding to the second site is required to trigger structural rearrangements in the GPCR and activate signal transduction.

Given the likely existence of several Wnt interaction regions in Fz receptors, the available information on the affinity of various Fz' CRDs for Wnt ligands [42] may not have a straightforward relevance for the physiological interactions between Wnts (19 in humans, 7 in Drosophila) and Fzs (ten in humans, four in flies). A systematic functional analysis of the Wnt–Fz interaction pairs is missing and requires an easy read-out system, measuring the most immediate events following the ligand–receptor interaction, rather than the final transcriptional response which is often affected by complicated feed-back regulations and depends on the exact composition of downstream elements of the cellular signaling machinery [47,48]. Until recently such read-outs were unavailable. Our experiments biochemically probing the GPCR activity of Fzs close this gap [33,34,49,50]. Indeed, the ability of a GPCR to catalyze guanine nucleotide substitution on the Gα-subunit is the most immediate consequence of the conformational change induced by ligand recognition, and can be easily measured. Using this assay, we have begun the systematic analysis of the Wnt–Fz coupling [33,34] and identified that human Fz1 is efficiently activated by Wnt3a and Wnt5a, Fz6 – by Wnt7a, Fz7 – by Wnt5a, and Fz10 – by Wnt3a. This set-up is also primed to be used in a high-throughput screening of small molecule antagonists of the Wnt–Fz interactions [50], the long-desired endeavor which might yield novel anti-cancer lead compounds [6].

Which factors apart from the intrinsic properties of the Fz GPCR may influence the specificity of the ligand binding? The mode of ligand interaction with a GPCR can be influenced by co-receptors – such as LRP5/6 for the canonical Fz signaling and Ror1/2 for the PCP signaling [51]. LRP5/6 are single-pass transmembrane proteins participating in a ternary complex with Wnt and Fz [52]. Recent evidence suggests that LRP5/6 may act as general GPCR accessory proteins, enabling non-Fz GPCRs to signal through the β-catenin pathway [53,54]. In this regard, LRP5/6 and Ror1/2 may be viewed as RAMPs (receptor-activity-modifying proteins): single-transmembrane accessory proteins regulating GPCRs [55].

Another potential level of complexity brought by LRP5/6 is their expected ability, as members of the lipoprotein receptor proteins, to interact with lipoprotein particles [56]. This becomes highly relevant in the context of Wnt signaling, as multiple forms of packaging of natural Wnt ligands have been proposed, including incorporation of the ligands into lipoprotein particles [57–59]. Wnts, being lipid-modified glycoproteins, easily bind to the outer cell membrane and extracellular matrix and are thus poorly diffusive as monomers [60], yet can migrate over long distances in vivo. It has been proposed that different ways to package Wnts serve to activate different groups of cells in the natural

environment, depending on the location of these cells regarding the source of production of the Wnt morphogens [61,62]. It is conceivable that different subtypes of the intracellular signaling pathways are activated by different forms of Wnts, and circumstantial evidence exists in favor of this idea [63–65]. LRP5/6, capable of interacting with lipoprotein particles, might play different roles in the recognition of the different forms of Wnt ligands and initiation of different subtypes of signaling.

Ligand recognition by Fz receptors may also be affected by Fz homo- and hetero-dimerization – a typical feature in GPCR physiology [66]. The very first evidence for Fz dimerization was provided by the structure of the mouse Fz8 CRD [67], which was crystallized as symmetrical homodimers. Subsequent investigations with full-length proteins confirmed the role of the extracellular domain in receptor dimerization and oligomerization [68]. However, human Fz4 was shown to dimerize even in the absence of the CRD through the 7-TM region of the receptor [69], similarly to some other GPCRs of the A and B class [70]. Nevertheless both studies [68,69] found di- or oligomerization essential for the proper receptor trafficking and signal transduction. Since Fz dimerization was sensitive to reducing agents [68,69], extracellular intra-molecule disulfide bridges are likely to play a role in the dimer formation, as has been proposed for some other GPCRs [71]. Heterodimerization of Fz proteins has also been proposed [69], which brings along further potential flexibility in ligand recognition and transduction. Furthermore, it is conceivable that Fz heterodimerization with other, non-Fz GPCRs may exist, which provides a possible explanation for the involvement of non-Gi/o heterotrimeric G proteins in Wnt pathways [28,30] (see below).

3. Frizzleds as GPCRs: view from the inside

On the inner side of the plasma membrane the C-terminal part and the intracellular loops of Fz receptors mediate interactions with G proteins and other regulatory components. Fzs possess rather short C-termini, with the conserved KTXXXW (X = any amino acid) motif [72] which binds the PDZ domain of the scaffolding protein Disheveled (Dvl) [73]. Dvl is one of the major transducers of Fz in both the canonical and PCP branches of Fz signaling [74,75]. Through its DIX domain, Dvl interacts with Axin – the negative regulator of the canonical Fz pathway [76,77]. This interaction contributes to the disassembly of the Axin-based β-catenin destruction complex [78,79]. In the PCP branch, Dvl accumulates together with Fz in endosomes, which are actively transported in the posterior direction and released back to the plasma membrane at the apical posterior tip of the epithelia [80,81] – phenomenon required for the amplification of the initial Fz-activating signal and establishment of the uniform planar polarization of the tissue. In addition to Dvl, other PDZ-containing proteins (e.g. Kermit/GIPC [82], PSD95 [83], GOPC [84]) have also been shown to interact with Fz. These binding partners are involved in the regulation of the Wnt pathway via modulation of the G protein activity (such as recruiting RGS proteins in case of Kermit/GIPC [85,86]) or mediating proper receptor distribution and trafficking [87].

A well-described way of GPCR regulation is their inactivation through sequential phosphorylation by GRKs (G protein-coupled receptor kinases) or other kinases, followed by interaction with β-arrestin which prevents G protein binding and triggers GPCR internalization [88]. So far, there is no evidence for β-arrestins or GRKs to directly interact with Fz proteins, although both are involved in the Wnt pathway in rather unusual ways. Specifically, it was found that the β-arrestin's role in Fz internalization was mediated by the hyperphosphorylated form of Dvl [89–91], while GRKs participated in this pathway by phosphorylating LRP6 [92] which was also important for the receptor-ligand complex internalization. Fzs themselves also have multiple putative phosphorylation sites [39], and direct phosphorylation of several Fzs by different kinases has been demonstrated [93,94]. Thus it is possible that β-arrestin or other phosphate-recognizing proteins also may directly interact with Fz.

Heterotrimeric G proteins have been shown to directly interact with Fz receptors [31,33] and to exchange their GDP for GTP upon interaction with the ligand-activated receptors [33–35]. Simple sequence alignment does not reveal G protein-activating regions in the intracellular parts of Fzs. Different GPCRs expose different G protein-activating sequences, located mainly in the intracellular loops 2 and 3, and more rarely 1 and 4, upon structural rearrangements induced by agonists [95]. Thus Fz receptors likely have non-overlapping regions interacting with the two main types of intracellular transducers: Dvl binding to the C-terminus and G proteins to the intracellular loops. It remains unknown whether simultaneous Dvl and G protein binding to Fz is possible.

Heterotrimeric G proteins are the most immediate transducers of GPCRs. About one thousand GPCRs are encoded by mammalian genomes, and roughly a dozen Gα-subunits organize G protein complexes to transduce their signals. Thus it is obvious that many GPCRs signal through the same G proteins; additionally, a certain level of promiscuity exists, such that a receptor may talk to several different G protein types [96]. Thus, the problem of how signal specificity is achieved can be raised and several ideas have been put forward to address this problem [97,98]. This issue is linked with the following question: can we reliably predict which G proteins any given GPCR is coupled to? And if yes, can we extend this prediction capacity to Fz receptors?

A significant database of experimentally established links between different types of G proteins and GPCRs has been collected in the previous 20 years. Analysis of these data shows that the specificity of GPCRs could not be deduced using conventional direct approaches to sequence comparison, as there is no single epitope which would be universally responsible for interaction with G proteins. As a result, G-protein binding sites could be reliably predicted only for small groups of closely related receptors on the basis of existing biochemical data [99]. However, computational approaches have been developed for the broader groups of GPCRs, so that algorithms can be "taught" using the known receptor – G protein combinations to predict those for primary sequences of other GPCRs [100–102].

The most efficient (94% accuracy) prediction tool to-date based on the neuronal network algorithm was developed in 2005 [101]; no Fz sequences were included in the training set of this tool. We used this tool to probe the coupling of Fz receptors to heterotrimeric G proteins of different types. We find that all 10 human Fz proteins are predicted to be Go/i-family coupled, with the confidence value ranging from 0.88 to 0.99 (Table 1). Similar results were obtained for Drosophila receptors.

Table 1
Confidence of the coupling of 10 human and 2 Drosophila Frizzled receptors to Gi/o- and Gq-type heterotrimeric G proteins.

	Gi/o	Gq
Fz1	0.98	<0.5
Fz2	0.98	<0.5
Fz3	0.94	<0.5
Fz4	0.99	0.73
Fz5	0.88	0.71
Fz6	0.98	0.74
Fz7	0.99	<0.5
Fz8	0.99	<0.5
Fz9	0.99	<0.5
Fz10	0.99	<0.5
dFz1	0.99	0.54
dFz2	0.99	<0.5

Interestingly, no significant probability of signaling through the Gs- and G12/13-type of G proteins was determined, while coupling to Gq was predicted to occur for Fz4, 5 and 6. In comparison to the results obtained with the earlier algorithms [39], our data display Fz as largely the Gi/o interacting family of receptors, in accordance with biochemical observations [23,30,32–35,103,104]. This brings us to the following question: What are the targets of these heterotrimeric G proteins in Fz signaling? As will be seen in the next section, relatively few effectors of the Gi/o proteins are known in general. However, novel transducers have recently emerged.

4. The heterotrimeric Go protein: captain and his crew

The heterotrimeric Go protein emerges as the major transducer of Fz across species [24,31,33,34]. Gαo was among the first α-subunits discovered, and was found to be the major Gα-subunit of the nervous system across the animal kingdom [105,106], controlling both development and adult physiology of the brain [107,108]. Go is also required for the proper development and functioning of the heart [109,110]. In addition to the crucial developmental and physiological functions of this G protein [111,112], it also has important pathological implications, especially in cancer [113]. Constitutive activation of Gαo induced transformation in NIH-3T3 fibroblasts and tumors in mouse xenografts [114,115]. Recently, a mutation rendering Gαo overly active [116] was described in human breast cancer samples and was shown to promote anchorage-independent growth of human mammary epithelial cells [117]. The oncogenic ability of Gαo was proposed to involve activation of the Stat3 pathway [115], but is likely also to involve overactivation of the Wnt/Fz pathway [37]. The ability of Gαo inhibitors to block the invasiveness of Wnt2-transformed human colon cancer cells has been demonstrated [118].

Despite the evolutionary conserved function, abundance, and medical importance of the heterotrimeric Go protein, the list of its known molecular targets is remarkably short [119]. One of the described targets of Gαo is RapGAPII – a GTPase-activating protein restricting the activity of the Ras-type small GTPase Rap1 [120,121]. Gαo inhibits the negative action of RapGAPII, increasing the activity of Rap1 which in turn may lead to activation of the MAPK and Stat3 pathways [115,120]. RapGAPII interacts with Gαo through the so-called GoLoco domain [121,122], present in many Gαo/i-interacting proteins [123]. Another GoLoco domain-containing protein, Pins in Drosophila, GPR-1 in *C. elegans*, and AGS3/LGN in mammals, has been shown to interact with Gαo [121,122,124]. This interaction is necessary for asymmetric cell divisions in nematodes [125,126] and in Drosophila [25,121]. Gαo has also been shown to interact with several RGS proteins [120,121]. Of those, RGS12/14 may represent not just a negative regulator of the Gαo activity, but also a target of Gαo signaling, as it contains several additional protein–protein interaction regions, such as the PDZ and Rap-binding domains [127]. The latter may represent another link between Gαo and MAPK signaling [128]; the importance of the interaction of Gαo with loco (Drosophila RGS12/14 protein) has been demonstrated in the context of septate junction integrity in cardiac development [129]. In the mammalian brain, one of the Gαo-interacting proteins is a major protein component of growth cones GAP-43 [130]. GAP-43 can activate guanine nucleotide exchange on Gαo in a GPCR-independent manner, which may be important for neurite extension [108,130]. Another brain protein capable of binding to and activating Gαo is the amyloid precursor protein (APP) [131]. Although this interaction may have implications for the etiology of the Alzheimer's disease, the GEF activity of APP (and GAP-43) towards Gαo indicate that these proteins are atypical activators, rather than targets, of Go-mediated GPCR signaling.

In essence, this description summarizes the body of existing knowledge concerning characterized interaction partners of Gαo. The scarcity of the known targets has led to a proposition that the major mechanism of Go signaling is the release of Gβγ subunits from the heterotrimeric complex upon GPCR activation [132]. Indeed, in the brain Gβγ released from the Go complex is able to regulate voltage-dependent Ca^{2+} channels, as well as inward-rectifier K^+ channels [132]. However, through several broad screening approaches we have identified a massive network of Gαo-centered protein-protein interactions ([121,133] and unpublished data). Two novel Gαo target proteins will be reviewed below: Rab5 and Axin.

5. Frizzleds and Rab-family monomeric GTPases: submerging with the signaling endosomes

GPCR internalization plays important roles in the regulation of the receptor signaling [134]. It may simply function to deplete GPCRs from the plasma membranes and thus shut the signaling off. Alternatively, through receptor recycling back to the plasma membrane, it may serve to ready the cell for the new round of agonist stimulation. If recycling is preceded by directed transport of the GPCR endosomes, this combined activity can elicit accumulation of the receptors in a particular domain of the plasma membrane, which may be required for cell polarization. Finally, in certain cases internalization produces the so-called signaling endosomes, from which the ligand-receptor complexes continue to signal inside the cell, often with an enhanced strength or changed modality.

Fz receptors are also tightly regulated by endocytosis. In the canonical β-catenin-dependent signaling, internalization of Fz was proposed to enhance the initial signal emanating from the plasma membrane [65,135,136]. In the PCP branch of the pathway, Fz is endocytosed and transported along microtubules to be released at the apical posterior membrane to elicit planar polarization of the epithelia [81]. Out of the many possible endocytosis routes [137], two have been implicated in Fz regulation: clathrin-dependent and caveolin-dependent endocytosis [138,139]. Clathrin-mediated internalization of Fz relies on Dvl and β-arrestin as discussed above [89–91], as well as on the μ-subunit of the adaptor complex 2 (AP-2) linking Dvl with clathrin; these interactions are required for the proper PCP signaling [140]. Both clathrin-dependent and caveolin-dependent endocytosis have been shown necessary also for the canonical Wnt/Fz signaling [136,141]. Similarly, the more downstream endocytic components, common for both internalization routes, have been implicated in Fz signaling. Dynamin, required for pinching off of the plasma membrane invaginations to form early endocytic vesicles has been shown to be required for the high levels of the canonical Wnt signaling [135], although conflicting data exist on its role in this pathway [138]. Finally, Rab5, the small GTPase responsible for the regulation of a number of early endocytic events, such as formation of clathrin-coated vesicles, fusion of endocytic vesicles and early endosomes, and homotypic fusion between early endosomes [142], is required for both the canonical and PCP Fz signaling [65,135].

In addition to Rab5, a number of other Rab proteins regulate GPCR trafficking. Specifically, Rab4 mediates the fast recycling of endosomes back to the plasma membrane, while Rab11 is responsible for the slower recycling path. In contrast, Rab7 controls transition from the early to late endosomes, destining the receptors for lysosomal degradation [142]. Relatively little is known about whether GPCRs or their signaling components can regulate these different Rab activities and thus influence their own trafficking fates. We have provided a mechanistic description of how Fz and Gαo as its immediate binding partner regulate Rab GTPases to differentially affect signaling in the canonical and PCP branches [65].

We have found that Fz proteins in Drosophila (dFz1 and dFz2) can interact with Rab5 genetically as well as physically as recombinant proteins [65]. This direct binding of GPCRs to a Rab protein is not unprecedented: angiotensin II type 1A receptor through its C-terminus binds Rab5, Rab4 and Rab11 and thus influences its own trafficking [143,144], while tromboxan A_2 and β2-adrenergic receptors have been shown to interact with Rab11 [145,146]. Interestingly, we have additionally found that the immediate Fz transducer Gαo also binds Rab4 and Rab5, but not Rab11 [65]. This is the first ever demonstration of a direct interaction between an α-subunit of heterotrimeric G proteins and small GTPases of the Rab family. In vitro, Rab5-GDP is the preferable binding partner of Gαo hinting at the potential of Gαo to activate Rab5. And indeed we find in cellular assays that Gαo activates Rab5; Fz proteins can do the same in the Gαo-dependent manner [65]. We provide evidence that the mechanism for the Gα-induced Rab5 activation is the recruitment of the latter from the cytoplasm to the plasma membrane, where the natural Rab5 GEFs such as Rabex5 are localized [65,147]. Through this relocalization, Rab5 is activated in the vicinity of Fz receptors, inducing their internalization [65]. Since internalized Fz complexes possess the maximal signaling strength [135,136], the Fz-Gαo-Rab5 relay serves as amplifier of the initial plasma membrane-originated signal in the canonical Wnt pathway [65]. Similarly, in the PCP branch of Fz signaling, this internalization, followed by microtubule-dependent transport [81] and Rab11-mediated recycling [65], is responsible for the enhanced receptor signaling.

In addition to the discovery of an intricate mechanism of GPCR-mediated regulation of trafficking, and trafficking-mediated regulation of GPCR signaling, our studies provide insights into the details of Fz receptor signaling. The first relates to the question how the cell decides which subpathway – canonical vs PCP – is activated, if the same receptor (dFz1 in Drosophila) can activate both pathways. Several contradicting ideas have been put forward to address this question [41,148,149]. Our findings suggest that Fz is directed to one or the other subpathway through different trafficking routes. Specifically, Rab5-dependent endocytosis, opposed by Rab4- and Rab11-mediated recycling, is required for maximization of Fz signaling in the canonical pathway. In contrast, Rab5-dependent endocytosis and Rab11-dependent slow recycling is the trafficking route required for the strong PCP signaling. Thus, Gαo, Rab5 and the recycling Rabs play the role of the pointsman, subdividing the Fz signaling into canonical and PCP subpathways [65].

Another interesting finding relates to the distinct signaling abilities of Drosophila dFz1 and dFz2 receptors. Only dFz1 is active in PCP signaling, but both are competent in the Wingless (Wg, Drosophila Wnt-1) transduction [9,150,151]. However even in this canonical signaling the two receptors bear clear signaling differences. For example, overexpressed dFz2 can potently overactivate the Wg pathway and induce ectopic sensory bristles in Drosophila wings, while dFz1 is ineffective in such overactivation [41,148,149,152]. We believe that these differences originate from the unequal activities of the two receptors in the activation of Rab5 and endocytosis. We have found that dFz2 has a strong basal, ligand-independent capacity to activate endocytosis, which is only modestly stimulated by Wg. In contrast, dFz1 in the absence of the ligand is mediocre in enhancing the endocytic potential of the cell, but becomes fully active in the presence of the Wg ligand [65]. Both receptors are dependent on Gαo for this stimulation of endocytosis [65]. In the developing Drosophila wing, Wg is produced by a narrow stripe of cells along the dorso-ventral margin – the future adult wing margin. Being a poorly diffusive morphogen, Wg concentrates close to the zone of production and its concentration in the rest of the growing wing tissue is low [58,153]. Thus dFz1, overexpressed in the regions of low Wg concentration, is poorly active in inducing its own endocytosis and cannot lead to endosome-directed high levels of signaling, necessary for induction of the high-threshold target genes such as Senseless required for the bristle development. In contrast, dFz2 overexpression in the same domain leads to the general stimulation of endocytosis, dFz2 internalization, and strong endosome-directed signaling. (Of note, endogenous dFz2 expression is low in the tissue away from the source of Wg production, as opposed to dFz1 which is ubiquitously expressed across the tissue [152].) Thus, our data provide the mechanistic explanation, in terms of differences in their basal vs ligand-stimulated Rab5 activation, to the long-known differences in the activities of Fz receptors [65].

6. Axin as Neptune of the deep-water Wnt/Frizzled signaling: the many ways to appease the evil god

Since Rab5 appears as an amplifier of the Fz–Go signaling, other effector(s) of Go must exist in this pathway, directly transducing the initial Wnt signal towards the downstream signaling machinery. We have identified Axin as one of such transducers [64]. Axin is the key negative regulator of the canonical Wnt pathway, capable of interacting with many proteins and organizing the β-catenin destruction complex [154]. Binding of the Wnt ligand to its receptors leads to reorganization of this complex through several signaling inputs into the Axin protein. One is provided by LRP5/6, which can directly bind Axin upon phosphorylation by casein kinase 1 and GSK3 [155–158]. The second input is mediated by Dvl, which inactivates Axin through the heterophilic interaction of the DIX domains present in both proteins [78,79]. This action of Dvl may in turn be enhanced by the βγ-subunits of the heterotrimeric G proteins, which bring Dvl to the plasma membrane [64,159,160]. Finally, the novel mechanism contributing to the neutralization of Axin is mediated by Gαo [64]. This α-subunit, released from the heterotrimeric Go complex by the activity of Fz receptors, directly binds Axin through its RGS domain, recruits it from the cytoplasm and thus suppresses its negative activity in the Wnt signaling [64].

Although it is probable that the three mechanisms act in parallel, they may be differentially involved in different modes of the canonical Wnt/Fz signaling. This idea brings us again to the issue of different cellular compartments, from which this signaling can emanate: low-level signaling from the plasma membrane vs high-level signaling from Rab5-positive endosomes. It is conceivable that different molecular components of the Wnt/Fz transduction machinery are involved in signaling from these two compartments, with different outcomes. For example, speaking about the developing insect wing as the read-out for Wnt signaling, it can be proposed that the plasma membrane-originating low-level signaling leads to expression of the low-threshold Wnt target genes Distal-less and Vestigial. In contrast, the endosome-originating high-level signaling drives expression of the high-threshold Wnt target Senseless and subsequent development of the sensory wing bristles. It also appears that the heterotrimeric Go protein is crucial for the signaling from the plasma membrane, but not the endosome (although it is required for the proper trafficking of Fz from the plasma membrane to endosomes). Indeed, overexpression of Gαo or its constitutively active mutant form could induce low-threshold Wnt target genes Distal-less and Vestigial, but not high-threshold targets like Senseless; as a result, no ectopic margin bristles are ever produced by Gαo overexpression [24]. Similarly, different forms of Axin show different effects regarding the high- and the low-threshold Wnt target genes: while overexpression of full-length Axin induced a complete loss of both types of Wnt target genes, the AxinΔRGS construct resulted in a complete loss of Senseless expression in wings (and failure of bristle development), but did not at all affect the expression of the low-threshold target gene Distal-less [64]. As the RGS domain is

absolutely required for the interaction with Gαo, these data cumulatively suggest that the Gαo-mediated impact on Axin is required for the plasma membrane-originating low-level signaling, leading to expression of the low-threshold Wnt targets like Distal-less. In contrast, the endosome-originating high levels of Wnt signaling apparently rely on Gαo-independent inputs into Axin to result in expression of the high-threshold target genes.

The distinction between the low-level plasma membrane signaling and the high-level endosome signaling may also be temporal: it is clear that the first precedes the second, and indeed different temporal phases in the Wnt/Fz signaling can be identified [31,161]. It should also be remembered that different packaging forms of Wnt ligands exist, destined for short vs long-range diffusion through the tissue and resulting in expression of different target genes [58]. It remains to be clarified whether these different forms induce different subtypes of intracellular signaling.

The data summarized above identify Axin as an effector in Fz/Gαo signaling [64]. Other Gα-subunits (Gαq, Gαs, Gα12) have also been proposed to interact with Axin isoforms, suggesting that non-Fz GPCRs may also feed into this scaffolding protein [31,162,163]. On the other hand, Dvl may also emerge as a more general GPCR effector, directly interacting with the PDZ-binding motif of multiple GPCRs [164]. Moreover, Gβγ subunits can bind and regulate Dvl in different organisms [64,159,160]; Gα-subunits also have a potential to interact with it (our unpublished observations). It is worth adding that Dvl can multimerize, incorporating Axin and other proteins into giant signalosomes [79,165–168]. It remains to be established whether such Dvl-based signalosomes and Axin-based protein complexes may represent a novel type of effectors in many GPCR signaling pathways.

7. Conclusions

Receptors of the Fz family, recognizing the Wnt growth factors and initiating developmentally and medically important cellular signaling pathways, have been acknowledged as potential GPCRs ever since their discovery [9,10]. However, it took decades of research to prove that heterotrimeric G proteins are required for the normal Wnt/Fz signaling in various organisms (reviewed in [37,111]). And only recently the final biochemical proof for the GPCR activity of Fzs has been provided [33–35], resolving the debate of whether Fz proteins are *bona fide* GPCRs [37]. Novel effectors of G proteins in the Fz pathways are emerging, such as the small GTPase Rab5 acting as the amplifier of signaling [65], and Axin as the key negative player in transduction, scaffold for multiple regulatory inputs including the one mediated by Gαo [64]. At the same time, it is clear that Fz pathways are complicated enough to utilize other, G protein-independent, ways to transmit the signal inside the cell [48], as is also the case for other GPCRs [169]. Different cellular compartments, different temporal phases of the signaling, and finally different forms of Wnt ligands may differentially depend on various Fz transducers. The discovery of the G protein-coupled mechanisms and targets of Fz signaling brings along further mechanistic understanding of this type of intracellular signaling. Additionally, it opens the way to utilize the vast GPCR methodology towards Fz proteins in the drug discovery endeavors, with the goal of obtaining small molecule antagonists as potential anti-cancer drugs, and agonists as agents for regenerative medicine [6,50,170].

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