Protein family review

The Frizzled family: receptors for multiple signal transduction pathways

Hui-Chuan Huang* and Peter S Klein*†

Addresses: *Department of Medicine and †Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, 415 Curie Blvd., Philadelphia, PA 19104-6148, USA.

Correspondence: Peter S Klein. E-mail: pklein@mail.med.upenn.edu

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Summary

Frizzled genes encode integral membrane proteins that function in multiple signal transduction pathways. They have been identified in diverse animals, from sponges to humans. The family is defined by conserved structural features, including seven hydrophobic domains and a cysteine-rich ligand-binding domain. Frizzled proteins are receptors for secreted Wnt proteins, as well as other ligands, and also play a critical role in the regulation of cell polarity. *Frizzled* genes are essential for embryonic development, tissue and cell polarity, formation of neural synapses, and the regulation of proliferation, and many other processes in developing and adult organisms; mutations in human *frizzled-4* have been linked to familial exudative vitreoretinopathy. It is not yet clear how Frizzleds couple to downstream effectors, and this is a focus of intense study.

Gene organization and evolutionary history

The *frizzled* genes were first identified in *Drosophila* in a screen for mutations that disrupt the polarity of epidermal cells in the adult fly [1]. Subsequently, *frizzleds* have been found in diverse metazoans [2], including at least ten in vertebrates, four in *Drosophila*, and three in *Caenorhabditis elegans*. *Frizzleds* have also been identified in primitive metazoans, including the sponge *Suberites domuncula* [3] and in *Hydra vulgaris* [4], but they have not been described in protozoans. They have been shown to encode receptors for Wnt proteins [5]. The *smoothened* (*smo*) gene, which functions in the Hedgehog signaling pathway in various developmental processes, is distantly related to *frizzled* genes. Additional information on the Wnt pathway can be found on the Wnt gene homepage [6] and in various comprehensive reviews [1,7-9].

Sequence analysis suggests that the ten human *frizzled* (*FZD*) genes fall into four main clusters [10]. *FZD1*, *FZD2*, and *FZD7* share approximately 75% identity; *FZD5* and *FZD8* share 70% identity; *FZD4*, *FZD9*, and *FZD10* share 65% identity; and *FZD3* and *FZD6* share 50% amino acid identity [10]. *Frizzled* genes from different clusters share between 20% and 40% sequence similarity. A dendrogram of human and selected invertebrate *frizzled* genes is shown in Figure 1. The overall genomic organization of *frizzled* genes does not appear to be highly conserved across this broad species diversity. Several *frizzled* genes appear to lack introns, however, including vertebrate orthologs of human *FZD1, FZD2*, and *FZD7* to *FZD10* (this is also a feature of many G-protein-coupled receptor (GPCR) genes); other *frizzled* genes, such as human *FZD5* and *Drosophila frizzled2* (*Dfz2*), contain one intron but the entire open reading frame is encoded by a single exon. Interestingly, the intron-deficient *frizzled* genes appear to be derived from a common ancestor, as they cluster into a subfamily that includes *Dfz2* (Figure 1).

Characteristic structural features

Frizzled proteins range in length from about 500 to 700 amino acids (Figure 2). The amino terminus is predicted to

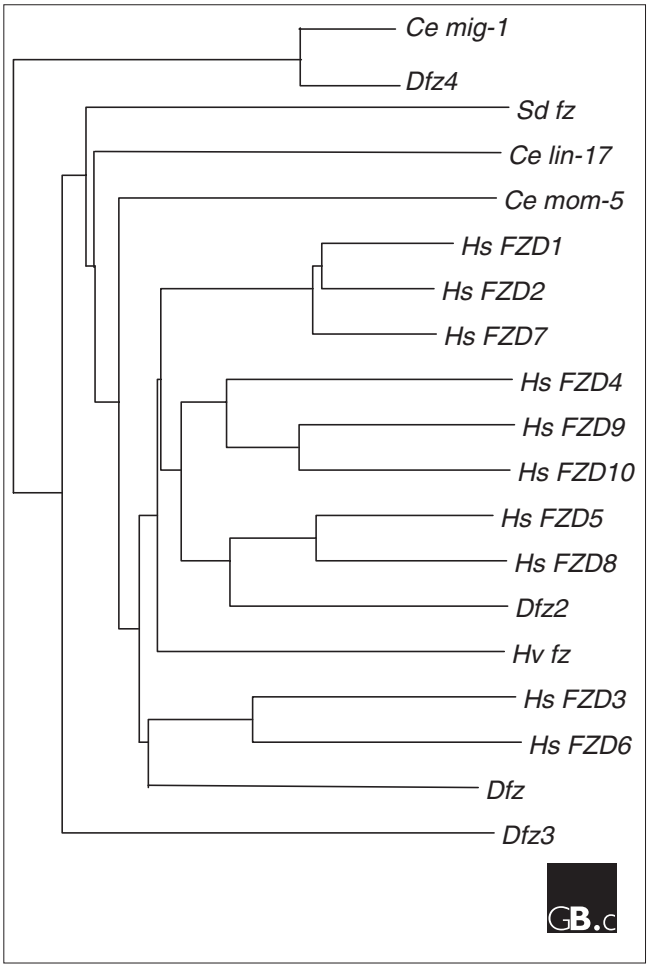

Figure 1
A phylogenetic tree of *frizzled* sequences. *Ce*, *C. elegans*; *D*, *D. melanogaster*; *Hs*, human; *Hv*, *Hydra vulgaris*; *Sd*, *Suberites domuncula*. The dendrogram was generated using the ClustalW alignment program in MacVector and is meant to show qualitative groupings of related *frizzled* genes. For more extensive and authoritative sequence analysis, see [3,4,6,10,53].

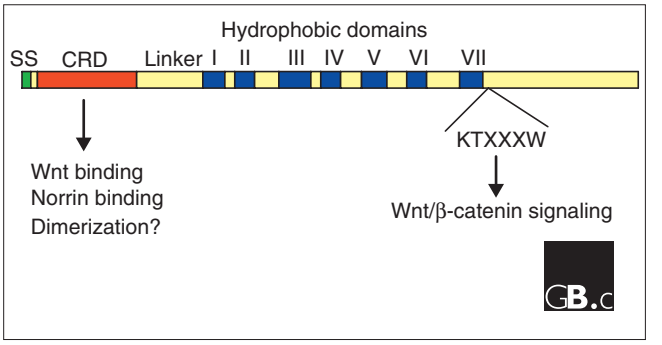

Figure 2
Motifs in Frizzled proteins. SS, signal sequence; CRD, cysteine-rich domain. The CRD is extracellular and binds ligands, including Wnts and Norrin. The carboxyl terminus is intracellular and contains a proximal KTXXXW motif (in the single-letter amino-acid code, where X is any amino acid), which is highly conserved in Frizzleds and is required for canonical signaling.

be extracellular and contains a cysteine-rich domain (CRD) followed by a hydrophilic linker region of 40-100 amino acids. The proteins also contain seven hydrophobic domains that are predicted to form transmembrane α-helices. The intracellular carboxy-terminal domain has a variable length and is not well conserved among different family members [2].

The CRD, which is necessary and sufficient for binding to Wnt molecules, consists of 120-125 residues with ten conserved cysteines, all of which form disulphide bonds [5,11]. The crystal structures of the CRDs from mouse Frizzled 8 (mFz8) and mouse secreted Frizzled-related protein 3 (sFRP-3) reveal that CRDs are predominantly α-helical and form a previously unknown protein fold [11]. A ligand-binding interface, involving a single region of the CRD surface, was predicted from analysis of the crystal structure integrated with comprehensive mutagenesis. Within the crystal, the CRDs form a conserved dimer interface, although in solution they appear to exist as monomers. Whether dimerization of the CRD has a role in ligand binding *in vivo* is not yet known [11].

The presence of seven hydrophobic domains has raised speculation that these receptors are related to the GPCR superfamily. The sequence similarity to GPCRs is low, however, and is limited to the hydrophobic domains, which might be expected to have some similarity because of the shared higher frequency of hydrophobic residues. An intriguing sequence similarity, potentially derived from evolutionary conservation, has been described between Frizzleds and members of the Taste2 subfamily of taste receptors (which are GPCRs) [10].

A motif (KTXXXW) located two amino acids after the seventh hydrophobic domain is highly conserved in Frizzleds and is essential for activation of the Wnt/β-catenin pathway [12]. Point mutations affecting any of the three conserved residues are defective in Wnt/β-catenin signaling (see below for more details on this pathway). A peptide derived from this conserved motif interacts *in vitro* with a peptide from the PDZ domain of mouse Dishevelled 1 - an intracellular signal-transduction protein - suggesting that this motif might mediate interaction between Frizzled proteins and Dishevelled proteins, although an interaction between the full-length proteins has not yet been demonstrated [13]. Apart from the KTXXXW motif, the carboxy-terminal tail is not well conserved among Frizzleds. The carboxy-terminal S/T-X-V motif found in some Frizzleds is apparently not required for Frizzled function [14]. The distantly related protein Smo also contains an amino-terminal CRD and seven hydrophobic domains, but it lacks the KTXXXW motif and does not bind Wnts [5,15].

Localization and function

Frizzled proteins are found exclusively at the plasma membrane. They are located at the surface of Wnt-responsive cells, although recent evidence has suggested that they may be internalized as part of a mechanism for regulating the extracellular level of Wnt protein and/or the cellular response to Wnts [16,17]. The tissue-specific expression of *frizzled* genes is complex, given that numerous *frizzleds* have been described in metazoans. In general, *frizzleds* are widely and dynamically expressed and, indeed, it is rare to find a cell that does not express one or more *frizzleds*. Specific expression patterns of *frizzleds* in model organisms have been described [2,6,14,18].

Frizzleds function in three distinct signaling pathways, known as the planar cell polarity (PCP) pathway, the canonical Wnt/β-catenin pathway, and the Wnt/calcium pathway. The PCP pathway is defined by the set of genes that, when mutated, result in defects in the polarity of cells in a planar tissue, as described below; the canonical Wnt/β-catenin pathway is characterized by stabilization of β-catenin protein in response to ligand binding; and the Wnt/calcium pathway is defined by the ability of overexpressed Wnts and Frizzleds to cause increases in intracellular calcium. As discussed above, the *frizzled* gene (*fz*) was first identified genetically from mutations that cause a PCP phenotype in *Drosophila* [1]. Asymmetric subcellular distribution of Frizzled has a central role in establishing cell polarity in flies, and most likely in other organisms as well. The dorsal epidermis of the adult fly shows a highly polarized pattern referred to as planar cell polarity, in which a single hair extends from the posterior end of each cell and points from anterior to posterior. The PCP pathway also regulates the organization of photoreceptor cells in the *Drosophila* eye. Frizzled and Dishevelled proteins become asymmetrically localized at the distal boundary of each pupal wing cell during the generation of polarity [7,8]. Furthermore, polarization of sensory organ precursor (pI) cells in developing bristles requires *fz*, and Frizzled protein is localized to the posterior apical cortex of the pI cell prior to mitosis. The *C. elegans frizzled* genes *lin-17* and *mom-5* are also required for asymmetric cell divisions (Table 1) [14]. A role for *frizzleds* in vertebrate gastrulation movements was first suggested by the observation that expression of a truncated form of *Xenopus fz8* that encodes just the CRD, which inhibits full-length Fz8 function, blocks convergent-extension movements in *Xenopus* gastrulae [19], in a similar way to overexpression of Wnt-5a [20] and a dominant-negative form of Dishevelled [21]. Subsequent work in zebrafish and *Xenopus* suggested this convergent-extension phenotype arises through disruption of a PCP pathway that orients cell movements during gastrulation [7].

The first evidence that Frizzled proteins can function as receptors for canonical Wnt signaling was the observations that *Drosophila frizzled-2* (*Dfz2*) can make *Drosophila* S2 cells responsive to the Wnt protein Wingless (Wg); these cells normally do not respond to Wg [5]. Although *fz* interacts genetically with *dishevelled* in the PCP pathway, a *fz* loss-of-function mutant does not disrupt canonical Wnt signaling in the fly, as *fz* and *Dfz2* are functionally redundant for canonical signaling [22]. Evidence that *frizzleds* are required for Wnt signaling therefore required removing both *fz* and *Dfz2*, which was accomplished by RNA interference against *Dfz2* in an *fz* mutant background, by analysis of chromosomal deficiencies that delete *Dfz2* (see Table 1), and by identifying mutations in *Dfz2* and crossing these mutants to *fz* flies [22]. In vertebrates, overexpression studies suggest that different Frizzleds function in either the canonical or the noncanonical pathways [23], but at least some vertebrate *frizzleds* appear to function in multiple pathways, including the PCP, Wnt/calcium, and canonical Wnt/ß-catenin pathways [12].

Description of the Wnt/calcium pathway derives originally from the observations that overexpression of *Wnt5a* or rat *frizzled2* can cause an increase in intracellular calcium in zebrafish and can activate protein kinase C and calcium/calmodulin-dependent protein kinase (CaM kinase) in *Xenopus* [7]. This pathway appears to require G proteins and Dishevelled, although a distinct Wnt/calcium pathway has also been proposed to regulate protein kinase C independently of Dishevelled in a *frizzled7* pathway that maintains the separation of mesoderm and ectoderm during gastrulation in *Xenopus* [24].

The specific functions of Frizzled proteins are as varied as the number of cell types that express them. In addition to *Drosophila* and *C. elegans*, *frizzled* mutants have also been described in mouse and humans, and interference with *frizzled* function using antisense or dominant-interfering constructs has been described in *Xenopus* and zebrafish. Some of the phenotypes associated with loss of function of Frizzleds in various organisms are listed in Table 1. Of particular note is the fact that mutations in human *FZD4* are found in familial exudative vitreoretinopathy (FEVR), an inherited form of retinal degeneration with associated progressive hearing loss [25]; investigation into the related Norrie's disease, which arises from mutations in a novel, secreted protein called Norrin, led to the exciting recent discovery that Norrin is a ligand for Fz4 that can activate canonical Wnt signaling and yet is distinct from the Wnt proteins [26].

Mechanism

Wnts bind to Frizzleds with high affinity (where tested) through the Frizzled CRD [5,15,27,28]. Furthermore, expression of the CRD alone antagonizes Wnt/β-catenin signaling [19], as does expression of secreted Frizzled-like proteins, such as Frzb-1, which have sequence similarity to the extracellular CRD domain of Frizzleds [6]. The amino-terminal extracellular region, including the CRD, has also been proposed to play a role in dimerization of the receptor and activation of

Table 1

Loss-of-function phenotypes of *frizzled* genes

Species	Genotypes*	Phenotypes	References
Drosophila	$fz^{-/-}$	Disruption of planar cell polarity in sensory bristles, dorsal epidermis, and ommatidia	[1,39]
Drosophila	$Dfz2^{-/-}$	Viable	[22] (see also [40-42])
Drosophila	$Fz^{-/-}$; $Dfz2^{-/-}$	Wg signal transduction is abolished in embryos and the wing imaginal disk	[22]
Drosophila	$fz^{-/-}$; *Dfz2* deficiency	Mimics loss of *wg* in embryonic epidermal patterning, neuroblast specification, midgut morphogenesis, and heart formation	[40-42]
Drosophila	fz^{RNAi}; $Dfz2^{RNAi}$	Defects in embryonic patterning that mimic *wg* loss of function	[43]
Drosophila	$Dfz3^{-/-}$	Suppresses a hypomorphic *wg* mutation	[44]
C. elegans	$mom\text{-}5^{-/-}$	Embryos lack endoderm and overproduce pharyngeal tissue	[45]
C. elegans	$mig\text{-}1^{-/-}$	Abnormal migration of the Q neuroblast	[46]
C. elegans	$Lin\text{-}17^{-/-}$	Disruption of a variety of asymmetric cell divisions	[47]
Mouse	$mfz3^{-/-}$	Severe defects in major axon tracts within the forebrain	[48]
Mouse	$mfz4^{-/-}$	Defects in cell survival in the cerebellum; vascular defects in retina, cochlea, and cerebellum	[26,49]
Mouse	$mfz5^{-/-}$	Embryonic lethal (at day 10.75) because of defects in yolk-sac angiogenesis	[50]
Human	$hFZD4^{+/-}$	Familial exudative vitreoretinopathy	[25]
Xenopus	$Xfz3^{MO}$	Loss of neural crest induction	[51]
Xenopus	$Xfz7^{AS}$	Depletion of maternal *Xfz7* disrupts dorsal anterior development	[52]
Xenopus	$Xfz7^{MO}$	Severe gastrulation defect arising from inability of involuted anterior mesoderm to separate from the ectoderm	[24]

*MO, morpholino oligos; AS, antisense oligos; RNAi, RNA interference. See also [6].

canonical Wnt/β-catenin signaling; Carron *et al*. [29] reported that *Xenopus* Frizzled3 (Xfz3) dimerizes to activate canonical signaling and that Xfz7, which is monomeric, can activate Wnt/β-catenin signaling if artificially forced to dimerize but not when it is a monomer. In *Drosophila*, the CRD of Fz has an approximately ten-fold lower affinity for Wg protein than does the CRD of Dfz2, and ligand affinity is one determinant in the specificity of different Frizzled proteins for different pathways downstream of Wnt signaling [27].

The mechanism by which Frizzled proteins transduce signals once ligand has bound is largely unknown for any of the Frizzled-mediated signaling pathways. Screens for *Drosophila* mutations that disrupt canonical Wnt signaling in embryonic segments and in imaginal disks identified a number of downstream components, including *dishevelled*, *shaggy/zeste-white-3* (homologous to vertebrate glycogen synthase kinase 3), and *armadillo* (homologous to β-catenin), but none of the proteins encoded by these genes has been shown to interact directly with Frizzled proteins. Dishevelled is recruited to the membrane if Frizzleds are overexpressed (reviewed in [7,9,30]), and it has been proposed to interact directly, through its PDZ domain, with the carboxyl terminus of Frizzleds, but this interaction has not yet been demonstrated with full-length proteins and the physiological significance of Dishevelled membrane recruitment is not known [13]. *Xenopus* Kermit, a PDZ domain protein of previously unknown function [31], interacts directly with the cytoplasmic domain of Frizzled proteins and is recruited to the cell surface specifically by Fz3. Kermit is required for Wnt1/Fz3-mediated induction of neural crest, but it is not yet known whether Kermit functions in other settings involving Wnt/Fz signaling, and corresponding Kermit-like molecules for Frizzleds other than Fz3 have not yet been identified. PSD-95, a mouse PDZ-domain protein, can interact with mouse Fz1, Fz2, Fz4, and Fz7 [32], and the fly PDZ-domain protein GOPC interacts with the carboxyl terminus of *Drosophila* fz [33], but the functional significance of these interactions is not yet known.

The *arrow* gene of *Drosophila*, which is required for canonical Wnt signaling, was recently found to encode a type-1 membrane receptor similar to low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6; [34]). Disruption of *LRP6* in mouse causes multiple phenotypes consistent with loss of Wnt signaling [9]. A dominant negative form of LRP6 inhibits Wnt signaling in *Xenopus*, and human LRP6 protein co-immunoprecipitates with the Fz8 CRD in a Wnt-dependent manner, suggesting that binding of Wnt to Frizzleds generates a ternary signaling complex of ligand (Wnt), receptor (Frizzled), and coreceptor (LRP) [9]. Co-immunoprecipitation of Wnts with LRPs has also been

described by others using vertebrate proteins [9], but not with *Drosophila* Frizzled, Arrow, and Wg proteins [6]. Expression of a chimeric molecule in which the carboxyl terminus of Arrow has been fused to Dfz2 robustly activates canonical Wnt signaling in the wing, supporting the hypothesis that binding of Wnts to Frizzleds somehow leads to interaction with and activation of Arrow/LRPs [35]. In addition, LRP5 and Arrow interact directly with Axin, a cytoplasmic scaffold protein that is the hub of cytoplasmic regulation of Wnt signaling, recruiting Axin to the membrane [35,36]. These observations are consistent with the idea that the Wnt signal is transduced through Frizzled proteins to Arrow/LRP, which then modulates cytoplasmic signaling through recruitment of the Axin complex. Arrow is not apparently required for PCP signaling [34].

In *Drosophila*, no ligand has been identified for activation of the PCP pathway. As discussed above, a number of components have been shown to be required for PCP signaling, and many localize at either the posterior region of the cell (Frizzled and Dishevelled) or in the anterior of the adjacent cell (for example, Strabismus, a novel transmembrane protein, and Prickle, a LIM-domain protein). Many of these components have been implicated in the regulation of convergent-extension movements in vertebrate embryos, and a role for Wnts, including Wnt11 and Wnt5a, is supported by genetic evidence in zebrafish and by the use of dominant-negative ligands in *Xenopus* [5]. The mechanism by which Frizzleds communicate with other components of the PCP pathway remains an intriguing mystery, however.

Regarding Wnt/calcium signaling, overexpression of rat *Fz2* by injection of mRNA causes an increase in intracellular calcium in zebrafish embryos, and overexpression of Frizzleds in *Xenopus* can lead to activation of protein kinase C (PKC) [23]; these effects are sensitive to pertussis toxin and other G-protein antagonists [37]. In addition, a complex chimeric molecule that incorporates the extracellular and ligand binding domains of the β-adrenergic receptor and the intracellular sequence of rat Fz2 was shown to cause intracellular calcium release within minutes after addition of adrenergic agonists [37]. Although this chimeric receptor is artificial, this was an important experiment because purified Wnts were not available until recently and the chimera provided a clever and novel approach to activate the pathway rapidly.

A similar chimeric receptor involving rat Fz1 and the β-adrenergic receptor has also been used to support a role for G proteins in canonical Wnt signaling [37]; additional support for a role of G proteins was provided by the observation that overexpressed RGS4, a G-protein antagonist, appears to block canonical Wnt signaling in *Xenopus* axis-duplication assays. These indirect assays support a potential role of G proteins in mediating the canonical and Wnt/calcium pathways, although a requirement for G proteins has not yet been established by loss-of-function experiments [7]. A new, noncanonical pathway involving Dwnt4, Frizzleds, and PKC has also recently been described in *Drosophila* in the developing ovary [38].

Frontiers

An important remaining question is how Frizzleds transduce a signal upon binding of the ligand. For the canonical Wnt pathway, as discussed above, ligand binding may initiate interaction with Arrow/LRPs, but the nature of the interaction is not known. Arrow/LRP does not appear to be involved in the PCP pathway, and other potential coreceptors have not been identified for this pathway. Whether Frizzleds are regulated by a secreted ligand in the PCP pathway also remains an open question, at least in *Drosophila*. The mechanism of signal transduction in the Wnt/calcium pathway is also an area of intense research, and the exciting possibility that Frizzleds couple directly to G proteins is still a controversial area, perhaps in part because of the lack of genetic data to support the idea of this interaction.

Information on the specificity of ligand-receptor interaction is also limited. Direct binding assays have been performed for a limited number of ligands, although this is likely to change now that a purification protocol has been established for Wnt proteins [6]. A classification of Wnt proteins has suggested that some ligands, such as Wg, Wnt1, and Wnt3a, function as ligands that activate the canonical pathway, whereas others, such as Wnt5a, Wnt11, and Dwnt4, function in noncanonical pathways. Whether this distinction applies to Frizzleds remains to be resolved. In *Drosophila*, Fz functions in both pathways but Dfz2 functions only in canonical signaling; in vertebrates, this distinction is less clear (compare [23] with [12]).

Frizzled proteins are asymmetrically distributed in tissues that exhibit planar polarity in the fly, and PCP signaling has been proposed to regulate oriented cell movements in vertebrate gastrulation; so far, however, an asymmetric subcellular distribution of vertebrate Frizzled proteins has not been demonstrated, largely because of the difficulty in generating antibodies sensitive enough to detect the endogenous protein. In addition, the biochemistry of PCP signaling is in its early stages, mainly because a biochemical readout for this pathway has not been clearly established, and it remains unclear whether PCP is regulated by a ligand-receptor interaction.

Finally, Wnt/Frizzled signaling clearly plays important roles in adult tissues as well as embryonic development. The limited number of human diseases found so far to be linked to mutations in *frizzled* genes is likely to expand in the near future.

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