Functional dissection of the N-terminal extracellular domains of Frizzled 6 reveals their roles for receptor localization and Dishevelled recruitment

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The Frizzled (FZD) proteins belong to class F of G protein–coupled receptors (GPCRs) and are essential for various pathways involving the secreted lipoglycoproteins of the wingless/int-1 (WNT) family. A WNT-binding cysteine-rich domain (CRD) in FZDs is N-terminally located and connected to the seven transmembrane domain–spanning receptor core by a linker domain that has a variable length in different FZD homologs. However, the function and importance of this linker domain are poorly understood. Here we used systematic mutagenesis of FZD6 to define the minimal N-terminal domain that has a variable length in different FZD homologs. In this region, we identified a triad of evolutionarily conserved cysteines in the FZD linker domain that is crucial for receptor membrane expression and recruitment of DVL. Our results are in agreement with the concept that the conserved cysteines in the linker domain of FZDs assist with the formation of a common secondary structure in this region. We propose that this structure could be involved in agonist binding and receptor activation mechanisms that are similar to the binding and activation mechanisms known for other GPCRs.

Class F of G protein–coupled receptors (GPCRs) consists of 10 Frizzled homologs (FZD1–10) and Smoothened (SMO) (1).

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2 The abbreviations used are: GPCR, G protein–coupled receptor; SMO, Smoothened; FZD, Frizzled; CRD, cysteine-rich domain; 7TM domain, seven transmembrane–spanning domain; DVL, Dishevelled; DEP, Dishevelled, Egl-10 and pleckstrin domain; ECL, extracellular loop; aa, amino acids; FL, full-length; HEK, human embryonic kidney; HA, hemagglutinin; FEVR, familial exudative vitreoretinopathy; C59, 2-[4-(2-methylpyridin-4-yl)phenyl]-N-[4-(pyridin-3-yl)phenyl]acetamide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole; ANOVA, analysis of variance.

FZDs, the focus of this study, bind WNT proteins with their extracellular cysteine-rich domain (CRD) to initiate a complex network of WNT signaling pathways (2). The CRD is seen as the orthosteric binding site for WNTs and is functionally implicated in receptor dimerization and signal initiation (3–6). Structurally, the CRD is connected to the seven transmembrane (7TM) core of the receptor through a linker domain of variable length and low degree of conservation among the representatives of class F (5). A central intracellular interaction partner of FZDs is the scaffold protein Dishevelled (DVL), of which three isoforms exist in mammals (7, 8). DVL, which does not interact with SMO, is involved in several branches of WNT signaling, including β-catenin–dependent and –independent pathways. The mechanisms of interaction between FZDs and DVL are complex and multimodal. Originally, it was shown that DVL binds an unconventional PDZ ligand domain in helix 8 of FZDs with the consensus motif KTXXWX (9). In addition, three amino acids C-terminal of the KTXXWX sequence, the flanking regions of intracellular loop 3, and electrochemical interaction with the phospholipids of the membrane were shown to be important for DVL membrane recruitment (10–12). The current model, however, includes rather prominent DVL–DEP (Dishevelled, Egl-10, pleckstrin) domain interactions with FZDs, and it was recently shown that the DVL–PDZ and the DVL–DIX (derived from dishevelled and axin) domains are dispensable for FZD–DVL recruitment (13–15). Mutagenesis has further revealed sites in other regions of FZDs to regulate FZD–DVL contact (16, 17). Even though some of these results might not necessarily pinpoint direct interaction sites, such as Tyr-250 at the lower end of TM 2 of FZD4, which is most likely involved in defining an overall conformation enabling FZD to interact with DVL (13, 16).

So far, the dynamics and the regulation of the FZD–DVL interaction remain unclear. Recently, it has been proposed that FZD–DVL interactions appear to be WNT-induced when feeding into the WNT/β-catenin pathway, whereas FZD–DVL binding could be more static when signaling is pushed toward β-catenin–independent, planar cell polarity–like signaling pathways (15). WNT-induced DEP domain swapping in DVL leads to reduced DVL–FZD interaction, which appears to be an underlying mechanism of low-density lipoprotein receptor–related protein 5/6 (LRP5/6)–based signalosome formation (13, 18).
The human FZD\textsubscript{6} constructs used in this study contain an the XWNT-8/mFZD\textsubscript{8}-CRD structure (19). Instead, FZD\textsubscript{6} is known to activate β-catenin-independent signaling; for example, through planar cell polarity–like signaling or activation of heterotrimeric G proteins of the G\textsubscript{q/11} families (20–25). To define the role of the extracellular CRD and the linker region for receptor surface expression and functionality toward DVL, we use systematic mutagenesis. Furthermore, we apply pharmacological inhibition of porcupine, which is required for WNT acylation and thereby inhibits secretion of WNTs from cells (26). Mutagenesis clearly defines the linker domain and especially a triad of conserved cysteines as crucial for receptor surface expression. Neither removal of the CRD nor pretreatment with a porcupine inhibitor affect the ability of FZD\textsubscript{6} to recruit DVL to the membrane and to induce a phosphorylation-dependent shift in the electrophoretic mobility of DVL. On the other hand, Cys-to-Ala mutation of any of the three linker cysteines completely disrupts FZD\textsubscript{6} cell surface expression and the receptor’s functionality toward DVL. Thus, our findings emphasize the functional importance of the linker domain for surface expression of FZDs and receptor function.

**Results**

**Design of N-terminal deletion mutants of FZD\textsubscript{6}**

The N terminus of FZD\textsubscript{6} consists of a short signaling sequence, the CRD, and a linker region that connects the CRD to the 7TM domain (1). The receptor model (Fig. 1A) presents an overlay of the crystal structure of full-length SMO (27) and the XWNT-8/mFZD\textsubscript{8}-CRD structure (3), visualizing the areas of putative WNT-CRD binding sites in relation to the full-length class F receptor and pinpointing the position of the linker, including an antiparallel β-sheet in the linker domain. The human FZD\textsubscript{6} constructs used in this study contain an N-terminal signal sequence, a FLAG epitope tag, and a C-terminal His tag to allow careful validation of full-length receptor expression. To assess the role of the N-terminal part of the receptor that contains the WNT binding site, we created deletion mutants lacking the CRD or the CRD and the linker region, shown schematically in Fig. 1, B and C. For the construct lacking the CRD, we aimed to delete the whole CRD. To preserve the disulfide bond in the linker region, the deletion was made after Arg-155. For removal of the linker domain, the intention was to maintain the last cysteine of the linker (Cys-185), which, according to the SMO model, creates a disulfide bond with a cysteine in extracellular loop (ECL) 1 (Cys-260). Therefore, the receptor was truncated after Cys-181 (Fig. 1C).

**Expression of N-terminal deletion mutants of FZD\textsubscript{6}**

The full-length (FL) receptor (1–708 aa), FZD\textsubscript{6} lacking the CRD (ΔCRD-FZD\textsubscript{6}, 156–708 aa), and the construct lacking both the CRD and linker region (ΔLinker-FZD\textsubscript{6}, 182–708 aa) were expressed in HEK293T cells, as shown by SDS-PAGE/immunoblotting using an anti-FZD\textsubscript{6} antibody with a C-terminal epitope or an anti-FLAG or anti-His antibody (Fig. 2A). Immunoblot detection of the FZD\textsubscript{6} constructs by an anti-FZD\textsubscript{6} antibody indicates that FZD\textsubscript{6} and ΔCRD-FZD\textsubscript{6} are more extensively posttranslationally modified compared with ΔLinker-FZD\textsubscript{6} (Fig. 2A), potentially pointing to reduced maturation and glycosylation in the latter construct. Indeed, glycosylation site prediction using the NetOGlyc 4.0 and NetNGlyc 1.0 applications suggests that the linker domain of human FZD\textsubscript{6} could be O-glycosylated at Ser-168 (28, 29). Indirect immunocytochemistry using conditions with and without membrane permeabilization prior antibody staining revealed that all of the FZD\textsubscript{6} constructs are expressed (Fig. 2B), although only full-length and ΔCRD-FZD\textsubscript{6}, but not ΔLinker-FZD\textsubscript{6}, were localized at the cell surface (Fig. 2, C and D), corroborating what was surmised from the electrophoretic mobility of the constructs. Thus, removal of both the CRD and the linker domain corrupted proper membrane embedding.

![Figure 1. Design of N-terminal deletion mutants of FZD\textsubscript{6}](image-url)
DVL2 signaling and recruitment induced by FZD₆ deletion mutants

To functionally validate the receptor constructs, we analyzed the DVL shift induced by full-length and ΔCRD-FZD₆. The electrophoretic mobility shift is a validated consequence of DVL activation (30, 31). HEK293T cells, similar to many other cell types, constitutively secrete WNTs, resulting in an autocrine signaling loop. To address the influence of autocrine WNT signaling through the overexpressed FZD₆ constructs on FZD–DVL signaling, we pretreated the cells with the porcupine inhibitor C59 (26). The effectiveness of the C59 treatment was verified by increasing doses of C59, resulting in reduced formation of phosphorylated and shifted, endogenously expressed DVL2 (Fig. S1A, open triangle) in a dose-dependent manner. Upon overexpression in HEK293T cells, full-length and ΔCRD-FZD₆ induced an electrophoretic mobility shift of DVL₁, visible as a minor (Fig. 3A, open triangle) and major retardation (Fig. 3A, filled triangle) on the PAGE, similar to what was previously shown for FZD₆ (31). We refer to this as shifted and hypershifted DVL, respectively. ΔLinker-FZD₆ was not capable of altering the electrophoretic mobility of DVL. Thus, we decided to analyze the ability of full-length and ΔCRD-FZD₆ to shift DVL in more detail (Fig. 3, B and C). There was no difference in FZD-induced DVL shift/hypershift between full-length and ΔCRD-FZD₆ (Fig. 3, B and C). We performed the experiments in parallel under conditions where endogenous secretion of WNTs was blocked by C59 (Fig. 3, B and C, and Fig. S1B). As expected, the basal shift of DVL was reduced, resulting in a stronger band of the unshifted form of DVL. Despite the presence of C59, full-length and ΔCRD-FZD₆ exhibited the ability to shift hypershift DVL in the presence and absence of C59. These findings indicate that the induction of DVL shift and hypershift by FZD₆ is CRD-independent and can be achieved in a ligand-independent manner. Furthermore, we analyzed the ability of full-length, ΔCRD-FZD₆, and ΔLinker-FZD₆ to recruit DVL to the membrane, similar to previous studies (10, 13, 22, 32). In agreement with previous results, overexpression of an HA-tagged DVL2 (DVL2-HA) alone resulted in the typical cytosolic aggregates (33, 34) (Fig. 4A). Cotransfection of the different FZD₆ constructs together with DVL2-HA showed that receptor constructs that are able to reach the plasma membrane also recruit DVL2-HA, irrespective of the presence of the CRD (Fig. 4A). ΔLinker-FZD₆ was neither recruiting DVL2-HA to the membrane nor colocalizing with DVL2-HA intracellularly. C59 treatment did not affect FZD–DVL recruitment mediated by full-length or ΔCRD-FZD₆ (Fig. 4B). Furthermore, the findings with recruitment of full-length DVL2-HA can be recapitulated with the isolated GFP-tagged DEP domain derived from DVL2. Although DVL2-DEP-GFP is evenly distributed in the cell and the nucleus in the absence of overexpressed FZD, coexpression of FZD₆ as well as ΔCRD-FZD₆ resulted in distinct membrane recruitment of DVL2-DEP-GFP (Fig. 4C). ΔLinker-FZD₆, however, did not affect DEP-GFP localization. In agreement with the findings using full-length DVL2, the DEP-GFP construct was also recruited to co-expressed full-length or ΔCRD-FZD₆ in the presence of C59 (Fig. 4D). Thus, FZD₆ can recruit the DEP-GFP construct irrespective of the presence or absence of the CRD in a ligand-independent manner.

Identification of a well-conserved triad of cysteines in the linker domain

Based on our findings regarding linker domain deletion, the following question arose: what linker domain–intrinsic features are important for the structural integrity of the receptor and its ability to be embedded in the plasma membrane? The crystal structure of the full-length SMO (PDB code 5V57 (27)) indicates that the linker domain adopts an antiparallel β-sheet that is stabilized by a disulfide bond that is stabilized by a disulfide bond (Fig. 5A, red). Although Cys-193 and Cys-213 in SMO (corresponding to FZD₆ Cys-161 and Cys-181) form a linker-internal disulfide bond, Cys-217 (Cys-185 in FZD₆) is linking to ECL1 by forming a disulfide bond with Cys-295 (Cys-260 in FZD₆). Detailed inspection of all 11 published SMO crystal structures and the only Frizzled (FZD₆) structure of both full-length and ΔCRD constructs indi-
cated that all protein structures contain an antiparallel β-sheet in the linker domain (Fig. 5B) (27, 35–39). Furthermore, family-wide sequence alignment of the extracellular domains and ECL1 of all class F receptors showed that this triad of cysteines (Cys-161, Cys-181, and Cys-185 in human FZD6; Cys-161, Cys-181, and Cys-185 in human FZD6) is conserved.

Cys-161 defines the minimal length of a functional FZD6 construct

We have introduced more subtle truncations varying in the N terminus of the linker region by either including Cys-161 together with Trp-160 (FZD6 (Δ1–159)), exposing Cys-161 as a terminal amino acid (FZD6 (Δ1–160)), or removing Cys-161 (FZD6 (Δ1–161)) (Fig. 6A). All three constructs are expressed at similar levels as the full-length receptor when overexpressed in HEK293 cells. However, in accordance to what we observed (Fig. 2A) for the ΔCRD and the ΔLinker constructs of FZD6ΔCRD, the full-length FZD6ΔCRD constructs (FL) and ΔCRD-FZD6ΔCRD and ΔLinker-FZD6ΔCRD, respectively, showed in the linker domain failed to reach the plasma membrane, as assessed by indirect immunocytochemistry in nonpermeabilized cells and by cell ELISA for quantitative assessment (Fig. 7, B and C). Furthermore, the electrophoretic mobility shift (Fig. 7D) that is inherently connected with FZD-induced DVL2 plasma membrane recruitment was not observed in cells expressing any of the cysteine mutations in the full-length or ΔCRD-FZD6ΔCRD constructs (Fig. 7E).

Discussion

FZDs need to be exposed at the cell surface of the cell receiving the WNT signal to be accessible for WNT stimulation either in an autocrine or a paracrine manner. Intracellularly,
DVL is a central mediator of WNT/FZD signaling, acting at the crossroads of β-catenin–dependent and –independent signaling pathways, and the mechanisms of DVL recruitment to FZDs define a field of intense research (5, 10, 11, 13, 14, 16, 18, 22, 40, 41). The activation of DVL can be assessed as a phosphorylation-dependent electrophoretic mobility shift of DVL, which is most prominently seen upon overexpression of casein kinase 1 (15, 30, 31, 42, 43). In addition, overexpression of FZDs also results in phosphorylation and shift of DVL, which, however, appears to be independent of CK1 and is qualitatively different from the CK1-induced shift (31). Thus, the current model of FZD–DVL interaction combines direct interaction of the DEP domain with FZD and subsequent DVL phosphorylation (13, 15); both events appear intrinsically interconnected and are, as of now, not experimentally dissociable. However, it remains unclear to which degree agonist stimulation of FZDs is required for FZD–DVL interaction and which exact mechanisms lead from receptor activation to DVL phosphorylation.
In fact, the ligand dependence of the FZD–DVL complex remains a mystery even though circumstantial evidence indicates that WNT stimulation could lead to dissociation of DVL from the receptor (13). On the other hand, recent results employing a DVL recruitment bioluminescence resonance energy transfer (BRET) assay in cells overexpressing FZD4, LRP6, and TSPAN12 and stimulated with recombinant Norrin (a FZD4-selective, WNT-unrelated agonist) suggest that FZD
Figure 6. Cys-161 defines the minimal length of a functional FZD₆ construct. A, schematic presentation of FZD₆ ∆CRD constructs with differences in the N-terminal amino acid. The cysteines in the linker domain are highlighted in red. B, HEK293T cells were transfected with empty vector (pcDNA), full-length FZD₆ (FL), and N-terminal deletion mutants. The electrophoretic mobility shift of endogenous DVL2 was assessed by immunoblotting using an anti-DVL2 antibody. The filled triangle marks the band of hypershifted DVL2 induced by FZD overexpression. Protein expression was verified with an anti-FZD₆ antibody; anti-GAPDH was used as a loading control. C, HEK293T cells were transfected with various N-terminal deletion mutants. Confocal photomicrographs of HEK293T cells transiently expressing truncated FZD₆ with indirect immunofluorescence using fluorophore-labeled secondary antibody against the anti-FLAG antibody on nonpermeabilized cells (FL FZD₆ and mutant receptor surface expression). The scatter dot plot represents means with S.D. of three independent experiments performed in triplicates. Statistical analysis was done by one-way ANOVA. Significance levels denoting differences between FL FZD₆ and mutant receptor surface expression are given as follows: **, p < 0.01. E, confocal photomicrographs of DVL2 recruitment in HEK293T cells transfected with FZD₆ N-terminal deletion mutants and DVL2-HA and stained with anti-FLAG (red, FZD₆) and anti-HA (green, DVL2) antibodies. Scale bars = 10 μm.

stimulation actually promotes FZD–DVL recruitment (44). From this study, we conclude that no agonist input is required for the basal interaction between overexpressed FZD and DVL because the ∆CRD-FZD₆, a construct devoid of the orthosteric binding site for WNTs, is capable of recruiting DVL, even in the absence of WNTs. We interpret these findings in two ways: agonist input is not required to stabilize an FZD conformation that provides a high-affinity binding site for DVL, and the CRD is not required for the formation of a functional FZD–DVL complex. Furthermore, we speculate that DVL could prefer the ligand-free state of FZD over WNT-bound FZD, which would be supported by the report of WNT-induced DVL dissociation mediated modest transcriptional activity of the reporter assay when cotransfected with wingless/WNT (49).

Here we present a detailed characterization of the function of the linker domain connecting the N-terminal CRD with the 7TM core of the receptor. In our hands and with the assays employed, we could not detect more enhanced constitutive activity of FZD₆ upon removal of the CRD. In the process of defining the minimal extracellular domain of a functional FZD, we also identified a triad of conserved cysteine residues, which turned out to be crucial for receptor surface expression. Our mutagenesis experiments support the idea that the cysteine-based formation of disulfide bonds and subsequent stabilization of the linker β-sheets observed in SMO and FZD₄ could be a common, well-conserved feature of class F receptors. Overall, the formation of a secondary structure in the linker domain could render this region less flexible than surmised previously (6). Interestingly, several proline residues, which introduce turns in the peptide chain, are frequent in the linker domain and partially conserved, such as Pro-162, Pro-184, and Pro-186 in FZD₄ (Fig. 5C). These prolines could contribute to stabilization of a secondary structure in this region.

As pointed out above, linker domain glycosylation could play a role in receptor surface expression. So far, we have not defined whether either mutation of the cysteines in the linker domain and subsequent destruction of the linker domain’s secondary
structure causes loss of surface expression or whether the mutation-induced loss of glycosylation and maturation is the main cause of reduced membrane exposure (Fig. 7, B and D). However, the O-glycosylation site Ser-168 in the linker domain is not conserved among class F receptors, not even in the closely related FZD3. This suggests that the structural impact of destroying disulfide bonds rather than loss of glycosylation causes the trafficking phenotype in the FZD6 cysteine mutants.
As presented in Fig. 5B, all reported SMO crystal structures, irrespective of the presence or absence of the CRD, contain the antiparallel β-sheet in the linker domain (27, 35–38), suggesting that this secondary structure is not a packing or crystallization artifact and that it is maintained under different environmental conditions not requiring the CRD for stabilization. In the process of revision of this manuscript, the first crystal structure of a class F receptor, FZD₆, was published (39). The presented data further corroborate our conclusions regarding the importance of a structured linker domain. As shown in Fig. 5B and Fig. S2, the FZD₆ structure contains a short antiparallel β-sheet in the linker domain based on the conserved Cys triad and the Cys in ECL1. This information is of importance to understand how the linker domain could contribute to agonist binding to FZDs and the agonist-induced dynamics of FZDs leading to receptor activation. The question of whether the CRD is indeed required for WNT-induced FZD activation has been raised before (49, 50) and remains a matter of debate, especially because the current state of knowledge places the orthosteric WNT binding site on the CRD (3). The debate around the importance of the CRD for WNT-dependent signaling originated from experiments where the mutant phenotype of D. melanogaster embryos caused by depletion of FZDs was at least partially rescued by expression of ΔCRD-FZD constructs (50–52). In addition, ΔCRD-FZD constructs mediated WNT/β-catenin signaling, as assessed by the transcriptional TOPFlash reporter assay, albeit to a lesser degree than the full-length receptor (49). In the case of FZD₆, it was suggested previously that removal of the CRD abolished the receptor’s ability to counteract WNT/β-catenin (19). However, in the latter experiments, no controls for protein expression, surface embedding, or structural integrity of the deletion mutants were provided, making it difficult to interpret the loss of activity in the ΔCRD-FZD₆ versus loss of surface expression.

Even though much more work is required to shed light on the function and dynamics of the CRD/linker in relation to the receptor core, our findings emphasize a central function of the linker domain for cell surface expression of the receptors. In the context of our data, however, it is experimentally challenging to distinguish whether receptor dysfunction is due to reduced surface expression of the mutant or due to a linker-intrinsic mechanism affecting ligand–receptor interaction. It is attractive to speculate that the linker domain does not only maintain receptor surface expression but that it also has a role in the agonist-induced dynamics and a potential two-step agonist binding mode, as described for class B receptors (53, 54).

Very recently it was shown for FZD₄ that the linker domain indeed has a role in defining functional high-affinity binding of the WNT-unrelated, FZD₄-selective ligand Norrin (44). The study supports our speculation of a CRD- and linker-mediated two-step binding mode also in the case of FZD-WNT interaction. Unfortunately, it remains technically challenging to assess WNT-FZD binding using the full-length receptors in a lipid environment because of the lipophilic nature and instability of WNT proteins, preventing us from directly testing this concept (55, 56).

The importance of the linker cysteines for receptor function is further underlined by previous reports identifying FZD₄ familial exudative vitreoretinopathy (FEVR) mutations in Cys-181 and Cys-204 of human FZD₄ (57–59). These C181R, C204R, C204Y mutations (corresponding to Cys-161 and Cys-185 in human FZD₆, respectively) manifested with a trafficking phenotype and poor receptor surface expression (57, 58). Also, it was shown that the C204R FZDᵣ mutant failed to mediate Norrin-stimulated β-catenin signaling (58). In agreement with FEVR as a loss-of-function phenotype of the FZD₄ signaling system, Cys-181 and Cys-204 are causally associated with the disease (57–59). On the other hand, mutations in the three cysteines in the linker domain and the cysteine in ECL1, all predicted to result in loss of signaling, are only rarely associated with cancers. In fact, among the almost 64,000 samples included in the cBioPortal for Cancer Genomics to date (60), only two FZD₄ mutations were found to be associated with cutaneous melanoma (C192G, TCGA-EB-A42Y-01) and with papillary renal cell carcinoma (C222R, TCGA-GL-6846-01) and two SMO mutations in lung adenocarcinoma (C213F, P-0002215-T01-IM3) and in malignant peripheral nerve sheath tumors (C295S, TCGA-QQ-A8V8-01). None of the other class F members presented with similar cysteine mutations in the cancer material.

In summary, our findings highlight the importance of the hitherto poorly defined function of the linker domain connecting the CRD with the 7TM core of class F receptors. Although we show, with the example of FZD₆, that this linker region, and especially several highly conserved cysteines, are essential for receptor surface expression and recruitment of DVL, we speculate that maintenance of the secondary structure of this region has importance for receptor function. The physiological and family-wide relevance of our findings is underlined by the disease-causing mutations in the cysteines of the linker domain of FZD₄ associated with FEVR.

**Experimental procedures**

**Cloning of FZD constructs and used plasmids**

The full-length version of FZD₆ with an N-terminal cleavable hemagglutinin signal sequence (MKTIALSYIFCLVF), a FLAG epitope (DYKDDDD), and a C-terminal decahistidine His tag was subcloned using a β₂-adrenergic receptor construct in pCDNA3.1(+) with primers 5′-AAA AGC TTG CCA CCA TGA AGA CGA TCA TCG CCC TCG CCA-3′ (forward) and 5′-TTT GAA TTC GTC ATC ATC GTC CTT GTA GTC GGC GAA CAC C-3′ (reverse) and HindIII and EcoRI restriction sites (the original signal sequence consisting of 18 amino acids was removed). Additionally, the FZD₆ sequence was cloned into this construct with primers 5′-AAA AGC TTG CCA CCA TGA AGA CGA TCA TCG CCC TCG CCA-3′ (forward) and 5′-TTT GAA TTC GTC ATC ATC GTC CTT GTA GTC GGC GAA CAC C-3′ (reverse) and HindIII and EcoRI restriction sites (the original signal sequence consisting of 18 amino acids was removed). Additionally, the FZD₆ sequence was cloned into this construct with primers 5′-AAA AGC TTG CCA CCA TGA AGA CGA TCA TCG CCC TCG CCA-3′ (forward) and 5′-TTT GAA TTC GTC ATC ATC GTC CTT GTA GTC GGC GAA CAC C-3′ (reverse) and EcoRI and XbaI restriction sites from the FZD₆-GFP plasmid (23). The His₆ tag was added to the C terminus via site-directed mutagenesis using primers 5′-GGG AGG TGG TTG TCA TTC AGA TAC TCA TCA CCA CCA TCA TCA CTA ATC TAG AGG GCC CGT TTA AAC CAC C-3′ (forward) and 5′-GGG TTT AAA CCG GCC CTC TAG ATC AGT GAT GGT GAT GGT GAT GAT GAG TAT CTG AAT GAC AAC CAC CTC CC-3′ (reverse). The truncated versions were cloned from the full-length construct with restriction sites EcoRI and Xbal.
using primers 5'-AAA GAA TTC GAC ATT GGA TGG TGT CCA AGG C-3' (forward) for ΔCRD and 5'-AAA GAA TTC GCC CCT CCA TGC CC-3' (forward) for the ΔLinker together with 5'-TTT TCT AGA TCA GTG ATG ATG GTG ATG-3' (reverse) and inserted into the construct with the signal sequence and FLAG tag. The truncation mutants FZD6 Δ1-159, FZD6 Δ1-160, and FZD6 Δ1-161 were made in a similar way with primers 5'-AAA GAA TTC TGG TGT CCA AGG CAT CTT AAG AC-3', 5'-AAA GAA TTC TGT CCA AGG CAT CTT AAG ACT TCT G-3', and 5'-AAA GAA TTC CCA AGG CAT CTT AAG ACT TTG GCC C-3', respectively. The Cys-to-Ala mutant C161A was made from the full-length using primers 5'-AAA GAA TTC TGG TGT CCA AGG CAT CTT AAG ACT TTG GCC C-3' and ΔCRD, Δ1-160, and FZD6 Δ1-161 were made in a similar way with primers 5'-AAA GAA TTC GCC CCT CCA TGC CC-3' and C161A was made from the full length using primers 5'-CAA AGA ATT GGA TTT TGG GCT CCA AGG CAT CTT AAG ACT TCT G-3'.

Bienz (DVL2-HA and DVL2-DEP-GFP were kind gifts from Mariann Biel and 5'-AAAA AAT CCA ATG TCT GTG ATG-3').

Membranes were incubated in 5% low-fat milk in TBST and then washed three times in TBST. The membranes were incubated in 5% low-fat milk in TBST (25 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6), incubated with the suitable secondary antibody conjugated to horseradish peroxidase (Pierce), washed, and developed using Clarity™ Western ECL Substrate (Bio-Rad) according to the manufacturer's instructions. Primary antibodies were as follows: FZD6 (Novus Biologicals, NBP1-89702, rabbit, dilution 1:1000), His (Abcam, ab18184, mouse, 1:1000), FLAG (Sigma-Aldrich, F7425, rabbit, 1:1000), GAPDH (Cell Signaling Technology, 2118, rabbit, 1:4000), α-tubulin (Sigma-Aldrich, T6199, mouse, 1:2000), and DVL2 (Cell Signaling Technology, 3216, rabbit, 1:1000). The FZD-induced electrophoretic mobility shift of DVL2 was quantified by densitometry of the three predominant DVL2 bands in ImageLab (Bio-Rad). After background subtraction, the ratios of the values of top/bottom and middle/bottom bands were calculated. Those ratios were normalized by the average of the ratio values from each corresponding dataset (with/without treatment, separately for each experiment).

Indirect immunofluorescence and confocal microscopy

One day prior to transfection, 100,000 cells/well were seeded on coverslips coated with poly-d-lysine (Sigma-Aldrich, P0899) in 24-well plates. Cells were fixed with 4% paraformaldehyde in PBS, washed three times with PBS, and blocked with PBTA (3% BSA, 0.25% Triton X-100, and 0.01% NaN₃ in PBS) for 1 h. Then they were incubated with the primary antibodies anti-HA (Covance, MMS-101P, mouse, 1:1000) and anti-FLAG (Sigma-Aldrich, F7425, rabbit, 1:1000) in PBTA overnight at 4 °C. The next day, the cells were washed three times with PBS, blocked in PBTA for 30 min, incubated with fluorescein isothiocyanate-conjugated secondary antibody (FITC, 1:500) diluted in PBTA for 2 h at room temperature in the dark, washed three times with PBS, incubated with DAPI for 5 min, washed with PBS, and mounted using glycerol/gelatin (Sigma). For the surface staining, the secondary antibody (FITC, 1:500) was incubated at the medium at a concentration of 1:500 and incubated for 7 min at room temperature. Cells were washed in PBS, fixed with 4% PFA, washed thrice with PBS, blocked for 30 min with PBTA, and detected as described above. Staining was visualized using a Zeiss LSM710 confocal microscope.

Cell ELISA

For quantification of cell surface receptor expression, 5 × 10⁴ HEK293T cells were plated in 96-well plates coated with 0.1 mg/ml poly-d-lysine. Cells were transfected with 0.1 μg of the indicated constructs and maintained for an additional 24 h. Cells were then incubated with an anti-FLAG antibody (Sigma-Aldrich, F7425, rabbit, 1:1000) in 1% BSA/PBS for 1 h at 4 °C. Following incubation, cells were washed five times with 0.5% BSA in PBS and probed with a horseradish peroxidase-conjugated goat anti-FLAG antibody at 1:1000 dilution in 1% BSA/PBS for 1 h at 4 °C. The cells were washed five times with 0.5% BSA/PBS, and 100 μl of the peroxidase substrate 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich, T8665) was added (30 min at room temperature). After acidification with 100 μl of 2 M HCl, the yellow color was read at 450 nm using a POLARstar Omega plate reader (BMG Labtech). The basal fluorescence detected in pcDNA-transfected HEK293T cells was subtracted from all data, and mean values were normalized to FL FZD6.
MAFFT alignment

The multiple alignment of the extracellular domains and the ECL1 of the human class F receptors was done in MAFFT version 7, which is available online (https://mafft.cbrc.jp/alignment/server/). Default settings were used.

Statistical analysis

For graphical and statistical analysis, GraphPad Prism 5 and 6 software was used. For cell ELISA, values from technical triplicates were averaged, and pcDNA (background) was subtracted from the experimental conditions. Values were normalized to surface expression of full-length FZD6, which was set to 100.

Statistical significance was determined by one-way ANOVA with Tukey’s multiple comparisons test. Significance levels are given as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001. For the DVL2 shift (Fig. 3, B and C, and Fig. S1B), statistical significance was determined from three independent experiments by one-way ANOVA with Tukey’s multiple comparisons test. Significance levels are given as follows: *p < 0.05; **, p < 0.01; ***, p < 0.001.

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