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The Class F of G Protein-Coupled Receptors: International Union of Basic and Clinical Pharmacology CXV

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Abstract
There are ten Frizzleds (FZD1–10) and one Smoothened (SMO) G protein-coupled receptor (GPCR) in class F. Hedgehog (Hh) family morphogens acting on the transmembrane protein Patched indirectly activate SMO, while secreted lipoglycoproteins of the Wingless/Int-1 (WNT) family bind and activate FZDs. An update is warranted due to the progress made in our knowledge of FZDs and SMO as molecular machines and dynamic transmembrane receptors in the fourteen years since the first-class F GPCR IUPHAR nomenclature report. Recent developments in molecular pharmacology and structural biology have shed light on the mechanisms of ligand identification, receptor activation, signal initiation, and signal specification, among other areas. In addition, class F GPCRs are still being worked on as potential therapeutic targets, and new methods and technologies including CRISP/Cas9 edited cell systems and genetically encoded biosensors have helped to improve the functional analysis of these receptors. Cryogenic electron microscopy and crystal structure analysis have advanced to the point that our understanding of structure-function correlations is rapidly expanding, which is fantastic news for the pharmaceutical industry. The intricacy of the WNT/FZD and Hh/SMO signaling pathways is still not completely understood, despite the advances.
A great deal of structural and functional information regarding the activation processes of Frizzleds and Smoothened has been uncovered in the last several years of research. The discovery expands our knowledge of the molecular mechanisms involved in ligand recognition, receptor activation, signal specification, and initiation, and it also opens up new avenues for the use of biologics and small molecule drugs in regenerative medicine and therapy by targeting class F GPCRs.

Keywords: G protein-coupled receptor, Frizzleds, Smoothened, ligand recognition, IUPHAR, and lipoglycoproteins

1. Class F of G Protein-Coupled Receptors

1.1 Introduction

There are eleven receptors on the surface of cells that are part of the class F or Frizzled family of G protein-coupled receptors (GPCRs): the Frizzled (FZD) 1–10 and the Smoothened (SMO) (Alexander et al., 2023; Arthofer et al., 2023). It appears that FZDs within one homology cluster can behave more similarly than across clusters. The ten mammalian FZDs can be categorized into clusters based on sequence homology: FZD1, 2, 7, FZD3, 6, FZD5, 8, and FZD4, 9, 10. Lipoglycoproteins from the Wingless/Int1 (WNT) family bind and activate FZDs, whereas Hedgehog (Hh) proteins indirectly control SMO. In addition to their roles in adult mammalian tissue homeostasis and embryonic development, WNT and Hh signaling are critical communication channels in stem cell control. The purpose of this study and IUPHAR nomenclature report is to update a comparable summary from 2010 (Schulte 2010) and to examine new insights into the molecular pharmacology and molecular machinery of class F GPCRs (Fig. 1). Class F GPCRs, which include FZDs and SMO, have a more established nomenclature now than they did prior to the 2010 nomenclature report.

1.2 Recent History

We would prefer to focus on more contemporary ideas that have emerged in the past ten years or so rather than rehash the discovery of WNT and Hh signaling, the molecular basis of FZDs as WNT receptors, and SMO as an indirect mediator of Hh signaling. A significant milestone in the field of understanding SMO activation was undoubtedly the deorphanization of the orphan receptor SMO, which involved elucidating different binding sites for cholesterol and oxysterols (Siebold and Rohatgi, 2023). A fascinating dogma shift is taking place concerning FZDs and their function in ligand identification, signal initiation, and route specification. It was previously believed that the diversity of the WNT proteins (there are 19 in mammals) and the involvement of diverse coreceptors, which reduced the functionality and

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diversity of the individual FZD paralogs and their transducers, was responsible for the complexity of the WNT signaling system. As a receptor pharmacologist, I must confess that I am biased. However, I believe it is not ideal to study WNT proteins in a physiological setting without first comprehending the diversity, presence, significance, and function of WNT receptors (primarily FZDs but also others). These molecular entities selectively interpret various ligands depending on concentration and cell type and pathway. The 10 FZD paralogues contribute to the overall complexity of the WNT signaling system in terms of ligand selectivity, signal kinetics, pathway specification, and signal initiation; our understanding of their functions and selectivity has improved in recent years, but it is still incomplete. The courageous work of Benoit Vanhollebeke's lab, which produced GE HEK293 cells devoid of endogenous FZD expression (Eubelen et al., 2018), was important in enabling and undoubtedly catalyzing this advancement. This FZD-null cellular background in HEK293 cells has allowed the broad community to ask more distinct questions concerning the functional idiosyncrasies of individual FZD paralogues, even though there were other cell systems available earlier that did not have endogenously expressed FZDs (Dijksterhuis et al., 2015). Understanding the several pathways that lead to the desensitization and termination of WNT signals is still largely a frontier in our understanding of FZD function. The expanding understanding of the unique characteristics of each FZD, as revealed through functional evaluation and enhanced structural resolution, will be covered in part by this review. In order to decipher the 19 mammalian WNTs in various physiological settings, ten FZD paralogues emerged. To gain a molecular understanding of complexity and to potentially target this ligand receptor system in a mechanism-based and structure-guided manner for the therapy of human disease, it is necessary to integrate dynamic protein expression patterns during development and adulthood, in physiology and disease, with the molecular pharmacology of WNTs, FZDs, coreceptor involvement, and intracellular transducers.

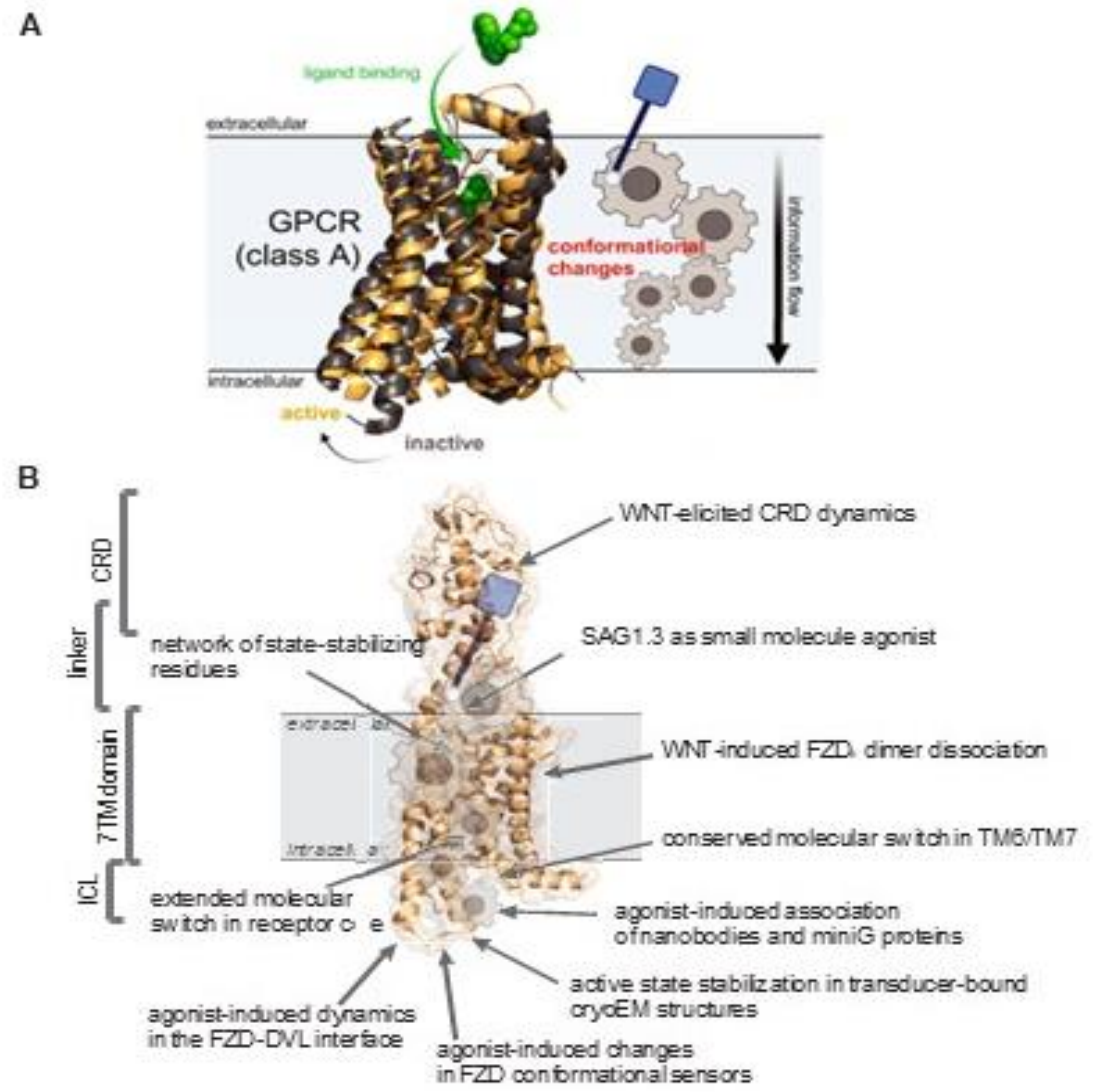

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Fig. 1. Class F receptors being like gear boxes or dynamic pharmacological entities. (A) A class A GPCR is shown in both its inactive and active states schematically. It draws attention to the fact that when an agonist binds to and activates a GPCR, it sets in motion a series of conformational rearrangements, which can be likened to a gear box in a machine that transfers mechanical changes from the outside to the inside of the cell. Based on energy landscapes of several receptor conformations and intrinsic protein dynamics, this picture of a GPCR molecular machine is presented. (B) Class F GPCRs, which are related to class A GPCRs, are likewise dynamic molecular machineries. This cartoon depicts a class F GPCR (PDB 6O3C) with a see-through surface. The receptor domains shown by the bars on the left side are as follows: CRD, which stands for cysteine-rich domain, linker, which stands for 7 transmembrane spanning helical domain, receptor core; ICL, which stands for intracellular loops 1–3, helix 8, and the far C terminus (not included); and finally, 7TM, which stands for 7 transmembrane spanning helical domain. While class F GPCR dynamics have been the subject of much discussion, this review attempts to outline the evidence that points to a connection between class F GPCR dynamics and the activation of receptors and the beginning of signals. The conformational rearrangements that occur at the intracellular transducer interface, from the CRD through the transmembrane domain, have been the focus of numerous technological investigations into the dynamics of class F receptors. The experimental detection of receptor dynamics as a measure of agonist-induced rearrangements, biosensor dynamics, or receptor activation has been documented in various studies (Bowin et al., 2023; Gratz et al., 2023a; Kowalski-Jahn et al., 2023). Arrows indicate experimental detection of receptor dynamics. When taken as a whole, our results highlight the significance of class F receptor kinetics in activating and initiating signals.

1.3 Nomenclature

In order to facilitate literature searches and prevent misconceptions, it is the responsibility of IUPHAR to standardize and simplify receptor nomenclature. Class F GPCR research has its roots in developmental biology, which has long made use of an assortment of model organisms like Caenorhabditis elegans, Xenopus laevis, and Drosophila melanogaster. The nomenclature rules used by these creatures' genes and proteins might lead to confusion when applied to mammals and humans. Now that we know FZDs and SMO are G protein-coupled receptors (GPCRs) and heptahelical transmembrane spanning receptors, we need to adopt pharmacological conventions for naming them that are consistent with how we name other ligands and receptors. As an example, the parathyroid hormone, a peptide secreted by the parathyroid glands, binds to a family of GPCRs that includes parathyroid hormone receptors 1 and 2, and hence regulates blood calcium levels. The secreted ligand in the WNT/FZD signaling pathway is known as WNT (plus a number). Although WNTs bind and activate FZDs, they are more accurately described as FZD ligands than WNT ligands. The converse is also true: FZDs are not FZD receptors but WNT receptors, and the latter does not make sense in a pharmaceutical setting because FZD is not a ligand. Since they do not follow the conventional receptor nomenclature pattern, I propose avoiding the names "WNT ligand" and "FZD receptor" to avoid misunderstanding. The preferred way to refer to the protein nomenclature of mammalian FZD paralogues, which includes subscript numbers, was already established in 2005 as FZD1–10 (Foord et al., 2005). Concerning SMO, it is clear that the logic of nomenclature departs from us. Considering that there is only one paralogue in this case that is involved in Hh signaling, it is evident that the term proposed by the fly geneticists should be used. It is important to note that SMO does not bind Hh but rather appears as a cholesterol or oxysterol receptor, so the term "Hh receptor" should obviously be avoided.

2. WNT/Frizzleds and Hedgehog/Smoothened Signaling

The purpose of this section is to provide an overview of the WNT/FZD and Hh/SMO signaling systems by outlining their primary components and providing some basic signaling principles. To stress that diverse ligands, receptors, and signal transducers provide the groundwork for intricate signaling networks, where the final result is defined by the accumulation of signaling events that may occur along potentially separate but communicating signaling pathways, I will not use the word "pathway" here. Furthermore, considering the abundance of review articles that delve into the intricate workings of class F receptor signaling in development, physiology, and disease (de Man and van Amerongen, 2021), I feel compelled to limit repetition.

2.1 The WNT/b-Catenin Pathway

Signal specification occurs through the single transmembrane spanning receptors low density lipoprotein receptor related protein 5 or 6, which are required for activation of the WNT/b-catenin pathway by many but not all WNT/FZD combinations (Davidson, 2021). According to several studies (Colozza and Koo, 2021), a signalosome is formed when WNT binds to both the FZD cysteine-rich domain (CRD) and the extracellular WNT binding site on LRP5/6. This recruitment of LRP5/6 to FZD occurs simultaneously. Axin and the phosphoprotein Dishevelled (DVL) are involved in a sequence of scaffolding processes that are initiated by the newly created and "active" receptor complex. Axin stops a cytosolic destruction complex from mediating b-catenin phosphorylation, ubiquitinylation, and proteasome-mediated degradation when it binds to the plasma membrane (de Man and van Amerongen, 2021). Therefore, b-catenin levels in the cytosol are low when WNTs are not present, but they are stabilized and translocated to the nucleus when WNTs cause the destruction complex to disassemble. For instance, b-catenin controls the transcription of "WNT genes" that lead to increased proliferation by regulating TCF/LEF-mediated gene transcription in the nucleus (Ramakrishnan and Cadigan,

2017). The role of heterotrimeric G proteins in the DVL-dependent WNT/b-catenin pathway has been the subject of long-standing controversy due to contradictory results (Schulte and Wright, 2018). Woowin et al. (2019) demonstrated that WNT-3A effectively elicits WNT/b-catenin signaling in HEK293 cells totally lacking functional heterotrimeric G protein signaling, contradicting previous publications that argued for a functional role of heterotrimeric G proteins in WNT/b-catenin signaling (Schulte and Wright, 2018). More specifics on the selectivity of transducer coupling will be covered later in the review.

Although LRP5/6 are necessary for WNT/b-catenin signaling to be specified, the majority of WNTs can activate this route via most FZDs; however, FZD3 and FZD6 form a distinct homology cluster and typically do not mediate WNT/b-catenin signaling. An increasingly complicated receptor complex has developed to specify ligand-pathway selectivity in the context of WNT7A/7B signaling in the maturing brain vasculature (America et al., 2022). A membrane-anchored glycoprotein known as Reversion-inducing-cysteine-rich protein with kazal motifs (RECK) and an adhesion GPCR (GPR124) work along with FZDs and LRP5/6 to determine the signaling result during WNT7A/B reception. It will be fascinating to observe whether additional WNTs and FZDs work with diverse receptors to accomplish cell type specificity and ligand-receptor decoding, even though this intricate WNT-decoding module is selective for WNT-7A/B recognition.

2.2 b-Catenin-Independent WNT Signaling

The functional features and network architecture of b-catenin independent pathways remain mostly unclear, despite extensive study over the past few decades. Not only is this complex network of signaling branches poorly understood in general, but there are also differences between signaling in non-vertebrates and mammals (Grainger and Willert, 2018). According to Micka and Bryja (2021), DVL is an essential component of both b-catenin-dependent and DVL-dependent but b-catenin-independent pathways, but the recruitment LRP5/6 is responsible for defining and specifying b-catenin-dependent WNT signaling. Planar cell polarity (PCP) signaling system, also known as WNT/PCP signaling, is the primary b-catenin-independent signaling idea that encapsulates the intricacy of this signaling network. This word characterizes the intricate mechanisms that control the positioning of cells in two-dimensional tissues, as well as the convergent extension motions that occur during gastrulation and neurulation. Serious diseases can arise from problems with WNT/PCP signaling; they include, but are not limited to, spina bifida, hearing loss, and limb extension abnormalities (Shi, 2022). The asymmetric arrangement of core PCP components is crucial for WNT/PCP-like signaling. These components include FZDs, the adhesion GPCR CELSR, the four transmembrane domain spanning proteins Vangl and soluble intracellular proteins Prickle and DVL. Asymmetric signaling complexes that recruit additional membrane and cytosolic proteins for functional signaling output are Celsr/Vangl/Prickle and Celsr/FZD/DVL. Another type of WNT receptor, which is not a GPCR, is engaged in signaling that is dependent on PCP and b-catenin, in addition to the FZDs. Fradkin et al. (2010) identified these as mostly ROR 1 and 2, RYK, and PTK7, which are receptor tyrosine kinases. There is new evidence that these receptors can function independently of FZDs, even though they were originally identified to work with FZDs as WNT coreceptors and define downstream signaling (Li et al., 2008; Nishita et al., 2010). In contrast to FZD CRDs, which offer a lipid-interaction site with a lipophilic groove, the WNT binding sites on these non-FZD WNT receptors—whether CRDs or WNT inhibitory factor domains—accommodate WNTs in an unusual way (Shi et al., 2021).

In order to regulate transcription and cytoskeleton dynamics, PCP-relevant signaling was described as activation of small GTPases of the RAC and RHO families, the c-jun N terminal kinase pathway, and DAAM1 signaling. Regarding biological polarity, the intriguing topic is: "what factor determines the asymmetric distribution of protein within a cell?" Several new features relating to FZD have surfaced, even if this question is poorly resolved and analogous to the one between the chicken and the hen. It is evident that FZD6 is a key WNT/PCP-mediating FZD, which is especially significant considering its extensive expression in epithelial tissue (Nabhan et al., 2023). As an example, ciliated epithelial cells in the human fallopian tube epithelium express FZD6 on their plasma membranes consistently. Casein kinase 1e and G protein-coupled receptor kinases (GRK) 2 and 5 readily phosphorylate phospho-Ser648, which can be recognized by a phospho-specific antibody (Strakova et al., 2018). This allowed us to discover an asymmetric distribution of the phosphorylated FZD6. These results point to the possibility that FZD posttranslational alterations contribute to the maintenance of PCP-relevant asymmetry. Another way that proteins like FZD6 might be unevenly distributed in cells is through differential sorting in the trans-Golgi network (Ma et al., 2018). In a two-dimensional structure like the epithelium, asymmetric protein distribution is obviously important for preserving epithelial integrity. Disruption of epithelial-mesenchymal transition may occur if asymmetry is lost.

Several important concerns about PCP signaling in mammals are still unsolved, and WNT/PCP-like signaling is not well understood. When considering signal transduction in two-dimensional tissues, planar cell polarity, or WNT/FZD signaling mediated by DVL rather than b-catenin, what type of signal transduction is this word exclusive to? I don't have the answers you're looking for, however there are additional signaling pathways that overlap and don't overlap with what is commonly thought of as WNT/PCP signaling. For instance, the Hippo pathway is closely interdependent with WNT/FZD signaling (Azzolin et al., 2014; Piccolo et al., 2014). Moreover, multiple studies have identified alternative signaling pathways that go from FZDs to RHO signaling and YAP/TAZ signaling via heterotrimeric G12/13 proteins. This connection may be

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relevant to various types of cancer (Hot et al., 2017). Furthermore, I would want to bring attention to the various methods in which WNT and FZD can interact with intracellular calcium pools. Although heterotrimeric G proteins have long been linked to this process, there are alternative pathways that can cause intracellular calcium to be mobilized through WNT and FZD, respectively. Early research indicated that cells expressing endogenous levels of signaling components were more likely to utilize the classic pathway, which involves activating phospholipase C and producing diacyglycerol and inositol phosphates (Wright et al., 2018). Also, it was found that phosphodiesterase 6 is involved in the cyclic GMP-dependent process of WNT-induced calcium mobilization. Understanding the regulation of intracellular calcium and how tissue-specific selectivity is achieved through communication with the multitude of involved assessor proteins in the membrane and cytosol, as well as the involvement of the various WNTs, FZD paralogues, and downstream signaling events, will undoubtedly require future efforts.

2.3 Hedgehog/Smoothened Signaling

Sonic, Indian, and Desert Hh are the three mammalian members of the hedgehog protein family. At their N and C termini, respectively, Hh proteins have palmitate and cholesterol, making them dually lipidated. Secretion and diffusion are made possible through a complex interaction mechanism that involves transferring lipidated Hh to carrier proteins. Next, the Patched protein—which has two paralogs, Patched (PTCH) 1 and 2—finds the secreted Hh. PTCH, a 12-transmembrane-spanning protein, is a constitutively active cholesterol transporter that decreases the accessibility of cholesterol in the plasma membrane, namely the primary cilium membrane (Siebold and Rohatgi, 2023). Because cholesterol is less accessible when Hh is not present, PTCH suppresses SMO. The action of PTCH as a cholesterol transporter is diminished when Hh is stimulated and bound to it. As a result, cholesterol becomes available for SMO, an endogenous agonist, and SMO is activated by cholesterol. The activation-induced translocation of SMO to the main cilium is a crucial event. Based on structural and functional analyses, various cholesterol/oxysterol binding sites in SMO have been identified. SMO is activated by cholesterol and various cilia-associated oxysterols (Turku et al., 2021). The SMO/zinc finger protein GLI1 (GLI; also known as glioma-associated oncogene) pathway is activated when SMO is activated in the primary cilium. GLI is a transcription factor and zinc finger protein that is controlled in a cAMP-dependent protein kinase (PKA)-dependent manner. Substantial functional evidence suggests that SMO functions as a GPCR (Qi et al., 2019). For instance, activation of inhibitory heterotrimeric Gi/o proteins could lower cAMP levels and, consequently, activate PKA, which is needed for signal transmission to GLI. Arveseth et al. (2021) described a new signaling method that augments these existing mechanisms by directly interacting between the SMO C tail and the catalytic subunit of PKA. An inhibitory peptide for proton pumping and calcium channel activation (cPKA) is located in SMO's C tail; this peptide reduces PKA activity and activates (dephosphorylated) GLI (Happ et al., 2022). Previous research has identified cilial G protein receptor kinases, like GRK2/3, as crucial signaling components of the pathway (Happ et al., 2022; M.F. Walker, preprint, DOI: 10.1101/ 2023.05.10.540226). It is evident that these involved in the process. Because GRK2/3 are controlled by bg subunits, their role in SMO recruitment is consistent with the activation of heterotrimeric G proteins and the presence of free bg subunits (Penela et al., 2019). The recently found method of inhibiting PKA by directly sequestering cPKA is an intriguing concept in and of itself, but it also gives us a transducer that interacts with SMOs and seems to rely on receptor core conformational dynamics for interaction (Turku et al., 2021).

3. Frizzleds and Smoothened Structure and Conformational Dynamics

3.1 The N Terminal Cysteine Rich Domain

The extracellular cysteine-rich domain, a globular region of approximately 120 amino acids that is positioned on top of the transmembrane receptor core and a linker domain, is one of the structural signatures of class F GPCRs (MacDonald and He, 2012). The CRD of FZDs acts as a cholesterol binding site for SMO, an orthosteric ligand binding domain for WNTs and Norrin (in the case of FZD4), and other functions (Kinnebrew et al., 2022). Removing or altering the CRD from FZDs renders them incapable of binding WNTs or Norrin. Nevertheless, there is evidence suggesting that the surviving core of the receptor protein influences, controls, or plays a role in the interaction between FZDs and WNTs or Norrin (Kozielewicz et al., 2021b). Janda et al. (2012), Chang et al. (2015), and Hirai et al. (2019) used structural analysis to determine the specifics of how WNTs and Norrin interact with the CRD. Besides FZDs and SMO, the CRD is found in several unrelated WNT receptors that bind WNTs; for instance, it is a part of receptor tyrosine kinase 7 and the ROR family, which span multiple membranes (Shi et al., 2021). All class F GPCRs have a lipophilic groove that can accommodate lipophilic moieties like the lipidations of WNT proteins or cholesterol/oxysterols that act as SMO ligands. The globular domain is stabilized by five disulphide bonds in FZDs and by four in SMOs. The muscle-specific receptor tyrosine kinase 7 and ROR use separate receptor tyrosine kinase interfaces and WNT-receptor interaction pathways (Shi et al., 2021). Due to the inherent dimerization ability of FZD CRDs, this phenomenon has been extensively studied, particularly in comparison to SMO dimerization (Kowalski-Jahn et al., 2021). It is intriguing that no overarching idea or functional function for FZD dimerization has been discovered, despite significant efforts. Although FZD6 activation requires agonist-induced dimer dissociation (Kowalski-Jahn et al., 2021), signal initiation also involves dimerization of other paralogues, such as FZD7 (Nile et al., 2017). To grasp its practical significance, the dimerization mode is crucial. Transmembrane domain dimerization between inactive FZD6 monomers, potentially independent of CRD interaction, opens different conceptual possibilities for designing small molecule drugs that block dimerization. This is in contrast to

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drugs that target CRD-CRD dimerization, like the peptides developed at Genentech (Nile et al., 2018).

Structural analysis revealed that SMO differs from FZDs in that it lacks the intracellular C terminus and instead contains the CRD and receptor core. According to Table 1, these structures (Vo et al., 2023) offered a more complete view of the CRD's location in relation to the receptor core and the extended TM6. Two distinct conformations or locations of the SMO CRD were captured, which is quite interesting. But it is still unclear what these various CRD orientations mean functionally (Huang et al., 2018). The inherent flexibility of the purified protein makes it structurally exceedingly difficult to re-solve the CRD of class F GPCRs in the context of the full-length protein. Resolving the CRD in the full-length FZD has been hindered by the intrinsic flexibility of the CRD relative to the receptor core, even though the full-length receptor was isolated and examined by cryogenic electron microscopy (CryoEM) (Xu et al., 2021). According to Kinsolving et al. (2024), not even the inclusion of a massive CRD-interacting bacterial toxin could aid in the resolution of a full-length FZD7. As a result of the purified proteins' adaptability, a model has been proposed—one that I find implausible—and call the "spaghetti mode." In this model, the agonist-binding CRD and the transmembrane receptor core are linked by a loose, completely unstructured linker, which does not indicate any structural connection between the two. Given this "spaghetti mode" (Tsutsumi et al., 2020), it follows that WNT binding to FZD and LRP5/6 mostly brings them close together, which is enough to initiate the pathway. True, that assumption has been the foundation for the design and development of numerous generations of bitopic WNT surrogates (Nabhan et al., 2023). Nevertheless, there are multiple lines of experimental evidence that cast doubt on this idea, and I will devote a considerable amount of space in this review to prove that, contrary to what the "spaghetti mode" suggests, there is in fact close allosteric contact between the CRD and the receptor core. Xu et al. (2021) found the most compelling evidence supporting the idea of an allosteric link while using cryo-electron microscopy to examine an active FZD7-Gs complex. Although the Cryo-EM analysis was unable to clarify the overall receptor structure including the CRD, it seemed that the receptor-G protein complex was stable when the CRD was present, suggesting that efficient purification of the complex was hindered when the CRD was removed from FZD7. Further functional data from live cell assays demonstrated that FZD7's constitutive activity toward heterotrimeric Gs was abolished upon CRD removal, demonstrating that the extracellular CRD communicates with an intracellular transducer, the heterotrimeric G protein, through allosteric contacts with the transmembrane domain.

TABLE 1 Class F GPCR structures

Receptor (Fusion ICL3)	Ligand	Transducer Bound	CRD Present/Resolved	CryoEM/Crystal Structure	PDB ID	Reference
SMO-BRIL	LY2940680	No	No/no	Crystal structure	4JKV	Wang et al., 2013
SMO-BRIL	LY2940680	No	No/no	Crystal structure	4JKV	
SMO-BRIL	SANT-1	No	No/no	Crystal structure	4N4W	Wang et al., 2014
SMO-BRIL	SAG1.5	No	No/no	Crystal structure	4QIN	
SMO-BRIL	ANTA_XV	No	No/no	Crystal structure	4QIM	
SMO-BRIL	Cyclopamine	No	No/no	Crystal structure	4O9R	Weierstall et al., 2014
SMO-BRIL	Cholesterol	No	Yes/yes	Crystal structure	5L7D	Byrne et al., 2016
SMO-BRIL	Vismodegib	No	Yes/yes	Crystal structure	5L7I	
SMO-Flavodoxin	TC114	No	Yes/yes	Crystal structure	5V57	Zhang et al., 2017b
SMO-Flavodoxin	TC114	No	Yes/yes	Crystal structure	5V56	
SMO-BRIL	Cyclopamine	No	Yes/yes	Crystal structure	6D32	Huang et al., 2018
SMO-BRIL	Cholesterol	No	Yes/yes	Crystal structure	6D35	
SMO	SAG21k, cholesterol	NbSmo	Yes/yes	Crystal structure	6O3C	Deshpande et al., 2019
Human SMO-Gi complex	SAG	Heterotrimeric Gi	Yes/no	CryoEM	60TO	Qi et al., 2019
SMO-G111C/I496C	24(S),25-epoxycholesterol	Heterotrimeric Gi	Yes/(yes)	CryoEM	6XBK	Qi et al., 2020
SMO	SAG	Heterotrimeric	Yes/(yes)	CryoEM	6XBL	

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Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: August 2024 Revised: September 2024 Accepted: October 2024

		Gi				
SMO	24,25-epoxycholesterol	Heterotrimeric Gi	Yes/(yes)	CryoEM	6XBM	
SMO-D384R	24(S),25-epoxycholesterol	Heterotrimeric Gi	Yes/(yes)	CryoEM	6XBJ	
SMO-BRIL V329F	Cholesterol, SAG1.3	Nb8	Yes/yes	Crystal structure	7ZI0	Kinnebrew et al., 2022
FZD_4-rubredoxin	None	no	no/no	Crystal structure	6BD4	Yang et al., 2018
FZD_5-BRIL	None	no	Yes/no	CryoEM	6WW2	Tsutsumi et al., 2020
FZD_7	None	mGs	Yes/no	CryoEM	7EVW	Xu et al., 2021
FZD_1	None	mGq	Yes/no	CryoEM	8J9N	Zhang et al., 2024
FZD_1-BRIL	None	No	Yes/no	CryoEM	8J9O	
FZD_3	None	mGs	Yes/no	CryoEM	8JHI	
FZD_3-BRIL	None	No	Yes/no	CryoEM	8JHC	
FZD_6	None	mGs	Yes/no	CryoEM	8JHB	
FZD_6-BRIL	None	No	Yes/no	CryoEM	8JH7	
FZD_7	None	No	Yes/no	CryoEM	9EPO	

The table lists class F GPCR structures comprising the receptor core with and without the CRD.

3.2 The Extracellular Linker Domain

The receptor core's transmembrane helix 1 and the CRD are sandwiched together by the extracellular linker domain. Table 1 shows that a number of structurally resolved SMO structures (Vo et al., 2023) partition this stretch of amino acids into several domains, such as hinge and linker domains. At least when the receptor is isolated, the longer and unresolvable linker in FZDs between the CRD and the receptor core is seen as more "unstructured" in comparison to SMO, according to Tsutsumi et al. 2023 have already shown that this area is frequently drawn as "spaghetti" in plans and drawings. It seems highly improbable that there is no structured connection between the CRD and the receptor core, even though this highlights our lack of understanding regarding the structure and functional relevance of the linker domain when the receptor is embedded in its physiological environment. If the CRD weren't in allosteric contact with the seven transmembrane domain (7TM) domain, how would WNTs squeezing the CRD with their thumb and index finger elicit long-range conformational rearrangements in the FZD receptor core? (Hirai et al., 2019). Class F GPCRs may be similar to class B GPCRs in that they, too, may use push and pull processes to facilitate allosteric communication between their transmembrane and extracellular domains (Duan et al., 2021). A recent study using FZD5 and FZD6 detailed the use of bioluminescence resonance energy transfer (BRET) measurements in designed FZD biosensors for the detection of WNT-induced dynamics of the FZD CRD. Biorthogonal labeling of an unnatural amino acid using amber codon suppression technology introduced the fluorescent BRET acceptor into the extracellular loop (ECL) 3 or the linker domain of the receptor, and the BRET donor Nluc was attached to the far N terminus of the receptor (Kowalski-Jahn et al., 2021). Even without the coreceptors LRP5/6, WNT-3A and WNT-5A were able to produce these dynamics. However, dynamics of the CRD induced by agonists imply that the receptor's top topology undergoes a conformational rearrangement that aids in signal initiation at the transducer interface. This, in turn, suggests that the receptor's linker region and hinge domain are crucial to this signal transduction process. According to Ko et al. (2022), the receptor surface expression and function are dependent on the conserved cysteines in the linker region and the ECL1. This highlights the significance of the structured linker domain for receptor integrity and signaling. Notably, a transmembrane cooperativity between the G protein and the CRD was proposed in recent molecular dynamics simulations of SMO/heterotrimeric G protein complexes linked to cholesterol/agonist (Vo et al., 2023). It is evident that additional research is needed to fully understand the role of the linker domain in signal transmission and how the variation in linker length across FZDs results in unique signaling characteristics.

3.3 The Seven Transmembrane Domain Receptor Core

Class F GPCRs display functionally significant and conserved residues throughout their family in their seven-transmembrane helix bundle contained in lipid bilayers (Hauser et al., 2021). With the exception of FZD4, the 7TM core is characterized by an abnormally long TM6 that extends much beyond the cell membrane (Yang et al., 2018; Fig. 2). A key component of receptor activation, TM6 works in tandem with ECL3 to facilitate information transfer from the CRD to the receptor-transducer interface, much like a lever. Class F receptors include molecular switches that define class F GPCRs as molecular machines or gear boxes, similar to class A GPCR motifs like the NPxxY (toggle switch), the D(E)RY, and the CWxP transmission switch motifs (Schulte et al., 2024). An oncogenic driver mechanism in SMO-dependent medulloblastoma and basal cell carcinoma is the opening or release of a central switch mechanism that relies on the polar interaction of a conserved basic residue Arg/Lys6.32 in the lower part of TM6 with the helix backbone at Trp7.55. This mechanism enhances the WNT-induced association of mini G proteins with class F GPCRs, increases the constitutive activity of FZDs, and has been described in various studies (Wright et al., 2019). Because it allows FZDs to

differentiate between coupling to heterotrimeric G proteins or the phosphoprotein and scaffold DVL, this entirely conserved molecular switch is of great relevance for comprehending the mechanisms of FZD route selection (Wright et al., 2019). A transcriptional reporter gene assay that mimics the stabilization of the transcriptional regulator b-catenin upon WNT stimulation, the TOPFlash signal, is rendered inactive in FZDs that can mediate WNT/b-catenin signaling due to mutations in the molecular switch (Gratz et al., 2023a). With their molecular switches intact, TM6 and TM7 remain in an inactive receptor shape that is closed. Surprisingly, this molecular switch isn't in a vacuum; rather, it's part of a larger network of interactions that travels along the receptor structure and plays a unique function in the change from an active to an inactive conformation (Turku et al., 2021). For instance, in FZD6, this expanded network comprises Tyr6.40, Trp3.43, Phe6.36, and Trp7.55, but in SMO, the network is less wide and comprises Phe3.43, Phe6.36, and Trp7.55. Additionally, the fundamental molecular mechanism of the switch was reinterpreted as a hinge limiter that restricts the swing out of TM6 in a recent high resolution cryo-electron microscopy structure of inactive FZD7 (G. Schulte et al., preprint, DOI: https://doi.org/10.21203/rs.3.rs-4322601/v1). Identifying the specific receptor activation distinctions between FZDs and SMO is also important in relation to the extended molecular switch. Salivary lipid deposition (SLD) and oxysterols are the endogenous ligands that SMO uses instead of big proteinaceous ligands bound at its CRD (Siebold and Rohatgi, 2023). We shall go into more depth about these features of cholesterol binding and SMO activation later on in the review. In contrast to other GPCRs, FZDs have a conserved Pro6.43 in the TM6 that causes the 0helix to bend when the molecular switch at the bottom of the TM6 is released. SMO, on the other hand, shows a different conformational shift because Phe6.43 is used instead of Pro6.43 (Turku et al., 2021). This aligns with the structural understanding of SMO activation, which involves either the binding of SMO agonist (SAG) 21k and the nanobody NbSmo8 complex or the binding of cholesterol/oxysterol and coupling to heterotrimeric Gi. The stabilized active SMO thus displays a straight helix that opens up at the intracellular transducer interface to accommodate the NbSmo8 or the G protein (Qi et al., 2019, 2020). Deliberate mutagenesis studies determined that the cholesterol binding site in the core of SMO would be blocked by the TM6 kink in SMO, necessitating a structurally different way to activate the receptor (Deshpande et al., 2019; Turku et al., 2021). Xu et al. (2021) reported a cryo-electron microscopy structure that resolved the features of a constitutively connected FZD7-mini Gs complex, which in turn validated the general functional predictions on FZD activation processes (Turku et al., 2021).

Macroscopic studies on the conformational dynamics of agonist-induced receptors have been carried out by genetically encoded GPCR biosensors using either conformation-sensitive fluorescent proteins (CSF), BRET (Börster resonance energy transfer), or Förster resonance (Patriarchi et al., 2018, 2019). As a basic idea of GPCR activation, the most typical design of these biosensors involves a label in intracellular loop 3 (ICL3) of the receptor, which primarily reports conformational receptor activation dynamics. Biosensors that detect agonist or mutation-induced conformational rearrangements reflecting FZD activation have been developed for FZDs, since class F receptors can also interact with heterotrimeric G proteins (Gratz et al., 2023a). Using these FZD conformational biosensors, the authors were able to decouple the WNT-induced FZD core conformational shift from the need to recruit LRP5/6 in order to specify the WNT/b-catenin pathway (Schihada et al., 2021a). The presence of DKK1 eradicates the WNT-3A-induced WNT/b-catenin signaling, which is evaluated as a TOPFlash signal, demonstrating that FZD core dynamics happen independently of LRP5/6, and WNT-3A induces full conformational dynamics even when DKK1 is present.

In addition, Gratz et al. (2023a) reported that state-stabilizing residues in the FZD core could be identified using a structure-based mutagenesis technique that compared active and inactive class F receptor structures. Through a comprehensive set of conformational dynamics evaluations, molecular dynamics simulations, signaling readouts, and genetically encoded BRET-based biosensors, groups of conserved amino acids were located that play a role in receptor activation and pathway selectivity. In particular, the study found that FZDs typically favor coupling to DVL over heterotrimeric G proteins, and that distinct molecular switches control G protein vs. DVL coupling. Class F receptors seem to use conformational dynamics for route selection, which is in line with new ideas in GPCR pharmacology such as functional selectivity and conformation-dependent transducer coupling.

Fig. 3 shows that kinetic analysis of the events in living cells, although in overexpression systems, supports the idea of a mechanistically sound process involving the chain of events from WNT binding to FZDs, agonist-induced CRD dynamics, 7TM domain dynamics, and transducer interface rearrangements. From the cell surface into the cytosol, the rate constants for the individual WNT-3A or WNT-5A-induced activities are defined in a logical order, going from fast to slower. Here, it should be mentioned that the studies that yielded these rate constants weren't actually intended for such a direct comparison (Kowalski- Jahn et al., 2021; Schihada et al., 2021a; Bowin et al., 2023). Complete kinetic study of these WNT-induced activation pathways should be provided in future work.

3.3. What Does Receptor Activation Really Mean?

Recent reviews (Schulte and Wright, 2018; Kozielewicz et al., 2020c) and an ongoing discussion (Malbon, 2011) have thoroughly examined the question of whether FZDs and SMO are GPCRs. The evidence that FZDs and also SMO functionally couple to heterotrimeric G proteins is overwhelming, and the role of FZD-G protein signaling in both overexpression cell systems and physiologically relevant cell models at endogenous receptor and transducer expression levels clearly points to a physiologically relevant liaison (Turku et al., 2021). The capacity of FZDs and SMO to couple to heterotrimeric G proteins has also been highlighted by the latest architectures of receptor-G protein complexes (Qi et

al., 2019; Xu et al., 2021). Although there is some evidence that SMO can link to heterotrimeric Gi/o proteins, the evidence for coupling to heterotrimeric G12/13 proteins is more contentious. Recent biosensor-based research argued for selective interaction but unproductive coupling between SMO and G12, evaluated as nucleotide sensitivity of SMO-G12 complex formation (Okashah et al., 2020), in contrast to studies that propose productive SMO-G12/13 coupling (Riobo et al., 2006; Guo et al., 2018).

When it comes to FZDs, it's becoming more and more clear that distinct FZD paralogs couple to different sub-families of heterotrimeric G proteins. This allows for a receptor-G protein coupling profile that defines the intracellular response to WNTs, which can be diverse and complex. As an illustration, it has been found that FZD4 interacts with G12/13 proteins (Arthofer et al., 2016; Strakova et al., 2017), that FZD5 exhibits characteristics of a Gq-coupled GPCR (Wright et al., 2018), that FZD6 couples to Gi/q proteins, that FZD7 couples to Gs proteins (von Maltzahn et al., 2012; Xu et al., 2021), that FZD9 couples to Go (Ram'ırez et al., 2016), and that FZD10 couples to G13 (Hot et al., 2017). These results show that there is functional diversity in pathways other than DVL-mediated signaling, even though the FZD-G protein coupling profile is still incomplete. An instance of this is the regulation of cAMP by either Gs or Gi, as well as the activation of mitogen-activated protein kinases through Gi/q coupling (Halleskog et al., 2012; Wright et al., 2018), calcium signaling, and the activation of RHO GTPases or the YAP/TAZ pathway through G12/13 proteins (Arthofer et al., 2016; Hot et al., 2017).

Class F GPCRs can act via heterotrimeric G proteins, but I'd rather talk about the conceptual, dogma-changing results about transducer coupling to FZDs and the conformational selection that underlies signal specification than spend too much time on it. A new idea can be formed from a deeper comprehension of FZD-transducer interaction, which unites multiple seemingly unconnected results: To start with, WNT-3A may activate the DVL-dependent WNT/b-catenin pathway—at least in HEK293 cells—even in the absence of functional heterotrimeric G proteins, as shown in G protein knockout cells (Bowin et al., 2019). Furthermore, changes to FZD4's ICL1 impair DVL-FZD interaction but have no effect on FZD4's ability to couple to heterotrimeric G proteins (Strakova et al., 2017). On the other hand, changes to FZD6's C terminus affect FZD-G protein coupling but have no effect on DVL association (Kilander et al., 2014b). In addition, mutations that have an obvious impact on the conformational landscape of FZDs, like cancer mutations in this position or the molecular switch mutation in R/K6.32 to Ala, greatly reduce the receptor's capacity to interact with full-length DVL via its DEP domain. On the other hand, when the receptor is stimulated by an agonist, it is able to recruit mini G proteins that act as sensors for the FZD conformation and are active toward heterotrimeric G protein coupling (Wright et al., 2019). Another finding is that FZDs' ability to couple to heterotrimeric G proteins is diminished when DVL is overexpressed (Kilander et al., 2014a; Hot et al., 2017). Grätz et al. (2023a) compiled these previously dispersed results into a mutagenesis strategy with family-wide implications: class F GPCRs have distinct state-stabilizing residues that determine whether FZD conformations couple to DVL or het-erotrimeric G proteins. The main takeaway is that coupling to FZDs by DVLs and heterotrimeric G proteins are incompatible. Put simply, the specific FZD conformation that is appropriate for binding heterotrimeric G proteins is incompatible with FZD-DVL interaction, and conversely, this holds true as well. Transducer coupling and, by extension, route selection, are defined by the conformational dynamics of FZDs.

Critically, DVL and heterotrimeric G proteins have been identified as FZD-binding transducers; consequently, FZD-induced signaling pathways rely on either DVL or heterotrimeric G proteins. As a result, the pharmacological rationale for the currently accepted categorization of the WNT-induced and FZD-mediated signaling pathways as either b-catenin-dependent or b-catenin-independent is ill-founded. Rather, it would be more accurate to classify WNT signaling as either DVL-mediated or DVL-independent. Since DVL-independent WNT signaling incorporates heterotrimeric G proteins and other non-DVL transducers, the possibility of discovering further transducers remains open (Fig. 4). The WNT/b-catenin route is one example of a DVL-dependent system; others include signaling via RHO GTPases and c-jun-N terminal kinases, which are similar to WNT/PCP. It seems that DVL recruits the receptor-independent guanine nucleotide factor DAPLE to FZDs to mediate Gi/o activation (Ishida-Takagishi et al., 2012; Aznar et al., 2015), which is an exception to the mutually exclusive DVL versus G protein selectivity. Despite the pharmacological relevance of this concept in relation to GPCR transducers like heterotrimeric G protein, arrestin, and GPCR kinases, I am cognizant that the suggested renaming of pathways to reflect actual receptor-transducer coupling (FZD)-DVL or G protein) will not be published anytime soon (Schulte and Wright, 2018).

According to Schulte et al. (2010), Chen et al. (2018b), and Wess et al. (2023), the scaffold protein family of arrestins is involved in GPCR desensitization, internalization, and various aspects of GPCR signaling. However, their role in FZD regulation remains poorly understood. G protein-dependent pathways may be adversely controlled by b-arrestins, as they do in class A and B GPCRs, but DVL-dependent pathways seem to depend on the functional presence of b-arrestin (Bryja et al., 2007, 2008). Although little is known about the exact processes that allow b-arrestin-dependent DVL-mediated pathways to work, it is known that b-arrestins engage in direct kinase/phosphorylation-dependent interactions with DVLs, which are crucial for both signaling pathways that rely on and do not rely on b-catenin and for FZD4 internalization (Kim et al., 2008). Unsurprisingly, b-arrestin are also linked to SMO signaling since they facilitate the internalization and cilial localization of SMO (Kovacs et al., 2008).

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The significance of class F GPCR activation is a topic that we will revisit (Kozielewicz et al., 2020c). The structural differences between receptors activated toward heterotrimeric G proteins and DVL must be understood in order to grasp the fundamental mechanisms of constitutive and agonist-induced signal initiation through FZDs. In the case of SMO, a comparable differentiation may be relevant; one form of SMO binds a transducer that mediates signaling that is not dependent on G proteins (Deshpande et al., 2019; Qi et al., 2019), while another form, one that was structurally resolved in complex with a heterotrimeric Gi, feeds into Gi/o protein coupling. However, like DVL in FZDs, no transducer has been found for mammalian SMO. This mysterious factor X may function similarly to the nanobodies in the NbSmo series, stabilizing a unique active SMO conformation (Deshpande et al., 2019). Other potential proteins include the cilial motor protein KIF7, which is related to the Drosophila melanogaster Costal2 in mammals (Jia et al., 2003; Liem et al., 2009), and the recently found SMO interaction with the catalytic subunit of PKA, which may reveal a conformation-sensitive transducer (Arveseth et al., 2021). According to Maharana et al. (2022), the SMO-cPKA interaction is similar to GPCR-arrestin interactions in that it relies on phosphorylation and involves GRKs. In GPCR-arrestin interactions, the transducer is typically recruited by interacting with the phosphorylated C terminus of the receptor, which stabilizes a different conformation of the receptor core than in GPCR-G protein coupling.

Weis and Kobilka (2018) state that the big swing out of the TM6 is the defining feature of GPCR activation, which allows the receptor core to extend into the cytoplasm and accommodate the C5 helix of the heterotrimeric G protein. Class F GPCRs can accommodate heterotrimeric G proteins through similar structural rearrangements, with the exception that FZD TM6 can kink like class A GPCRs, while SMO TM6 stays straight (Xu et al., 2021). Additionally, it has been noted in previous studies (Qi et al., 2019; Kozielewicz et al., 2020c; Xu et al., 2021) that the swing out of TM6 associated with G protein coupling is not as strong in class F GPCRs as it is in class A or class B GPCRs (Fig. 5). Curiously, a molecular explanation for the hinge limiter mechanisms that restrict receptor opening in FZDs has been provided by a recent structure of inactive FZD7 at 1.9 A˚ (G. Schulte et al., preprint, DOI: https://doi.org/10.21203/ rs.3.rs-4322601/v1). Gratz et al. (2023a), Kozielewicz et al. (2020b, 2021a), and Wright et al. (2018) all use conformational FZD sensors based on fluorescence, FRET, or BRET to detect receptor activation dynamics through an ICL3 probe. This further emphasizes that WNT-induced FZD activation manifests as conformational changes, just like class A and B GPCRs. Where does the active receptor conformation that couples to heterotrimeric G proteins diverge from the FZD-DVL active complex, and what does it look like?

In order to go further into this subject, one must be familiar with the study and operation of FZD-DVL recruitment and interaction. The original hypothesis for how FZD-DVL binding occurs was a fully conserved KTxxxW sequence in helix 8 of FZDs. The KTxxxW stretch was previously believed to have an intrinsic PDZ ligand that interacts with the PDZ domain of DVL. But PDZ domains favor linear, unstructured peptides, and the KTxxxW sequence appears as an amphipathic helix (Gayen et al., 2013; Yang et al., 2018; Xu et al., 2021), thus it's not likely to interact with them. Our present knowledge points to the DVL DEP domain as the primary interacting domain that mediates the FZD-DVL interaction (Gammons et al., 2016a,b). A discontinuous motif on ICL3 was found to mediate DEP contact when the FZD-DVL DEP interface was zoomed in on (Tauriello et al., 2012). Although the DEP domain alone cannot promote WNT signaling, it can bind to FZDs in live cells with a high affinity (Bowin et al., 2023). Thinking about DEP (instead of DVL) as a FZD-binding transducer that investigates a receptor protein's DVL-selective active conformation brings to mind the classic ternary complex model of GPCR-G protein interaction that developed in the late 1970s. Based on the work of Schulte and Wright (2018) this model explains how a high-affinity ternary agonist-receptor-transducer complex is formed, allowing the extracellular agonist and the nucleotide-free heterotrimeric G protein to undergo bidirectional transmembrane allostery. Could this also be said of the WNT-FZD-DVL complex? The simple response is "no." Actually, similar to the agonist-GPCR-nucleotide-free heterotrimeric G protein complex, there is scant evidence in the literature that the WNT-FZD-DVL combination forms a high-affinity complex. Both the presence of the CRD and WNTs do not affect DVL recruitment to FZDs in overexpression models (Tauriello et al., 2012; Valnohova et al., 2018). Recent attempts to reassemble a WNT-FZD-DEP ternary complex in vitro failed to produce an increase in agonist affinity from low to high (Mahoney et al., 2022). This research led some to conclude that "FZD does not get bent out of shape by WNTs" (Angers, 2022), in accordance with the traditional ternary complex model of GPCRs. Bowin et al. (2023) found that FZD-DVL (or DEP) interface dynamics in response to WNT stimulation in living cells, which is consistent with an allosteric coupling between the agonist binding site and the FZD-DVL interface. This was in contrast to BRET-based studies that used different assessments of FZD-DVL and FZD-DEP interaction in response to WNT-3A and WNT-5A. To differentiate FZD-DVL recruitment from structural rearrangements in the FZD-DVL (DEP) interface, a new unimolecular FZD-BRET sensor was developed. This sensor consists of a full-length FZD5, which is fused to the BRET donor at its C terminus, an acceptor that is separated from it by a flexible linker, and a minimal DEP domain at its far C terminus (Bowin et al., 2023). The design of the sensor was influenced by SPASM sensors, which are developed by Malik et al. (2013). These sensors rely on a unimolecular fusion of GPCR, linker, and Ga subunit. As shown by the WNT-induced changes in the FZD5-DEP Clamp sensor BRET, structural rearrangements at the intracellular transducer interface are induced by agonist stimulation of the extracellular portion of the receptor, even though an agonist affinity shift has not been detected thus far. The fact that the observed, WNT-induced transducer dynamics were independent of DVL DIX domain oligomerization, the coreceptors LRP5/6, and casein kinase 1-dependent phosphorylation is intriguing, and these

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data give further strong evidence against the "spaghetti mode" concept. Initiation of the WNT/b-catenin pathway signals the recruitment of LRP5/6-FZD, DVL oligomerization, and casein kinase-mediated processes, all of which are characteristic of signalosome formation (Colozza and Koo, 2021).

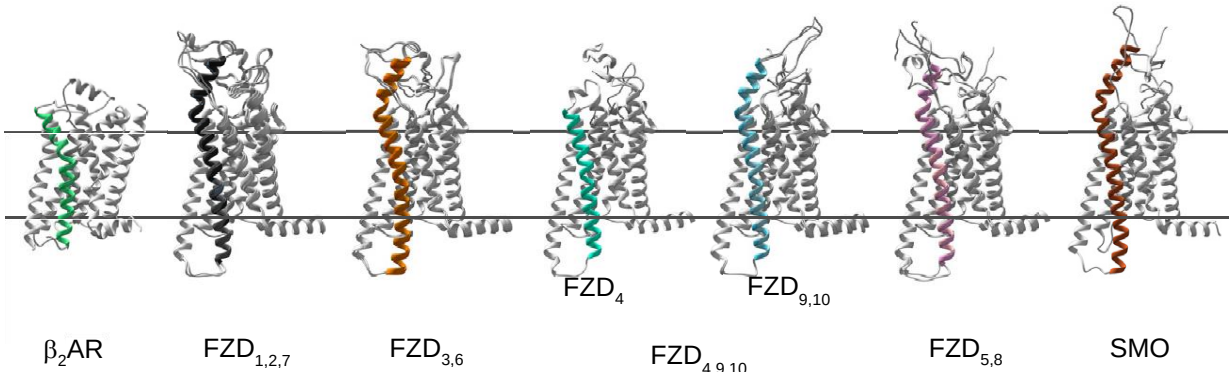

Fig. 2. Class F GPCRs' 7TM core and TM6's peculiar structure... Coloured TM6 represents the FZD1-10 and SMO alphafold models. Keep in mind that the extracellular RRD is absent from the models. In this order of homology clusters, the class F GPCRs are presented: FZD1,2,7; FZD3,6; FZD4,9,10; FZD5,8, and SMO. The b2 adrenoceptor structure (PDB ID 2RH1) is utilized for the sake of comparison.

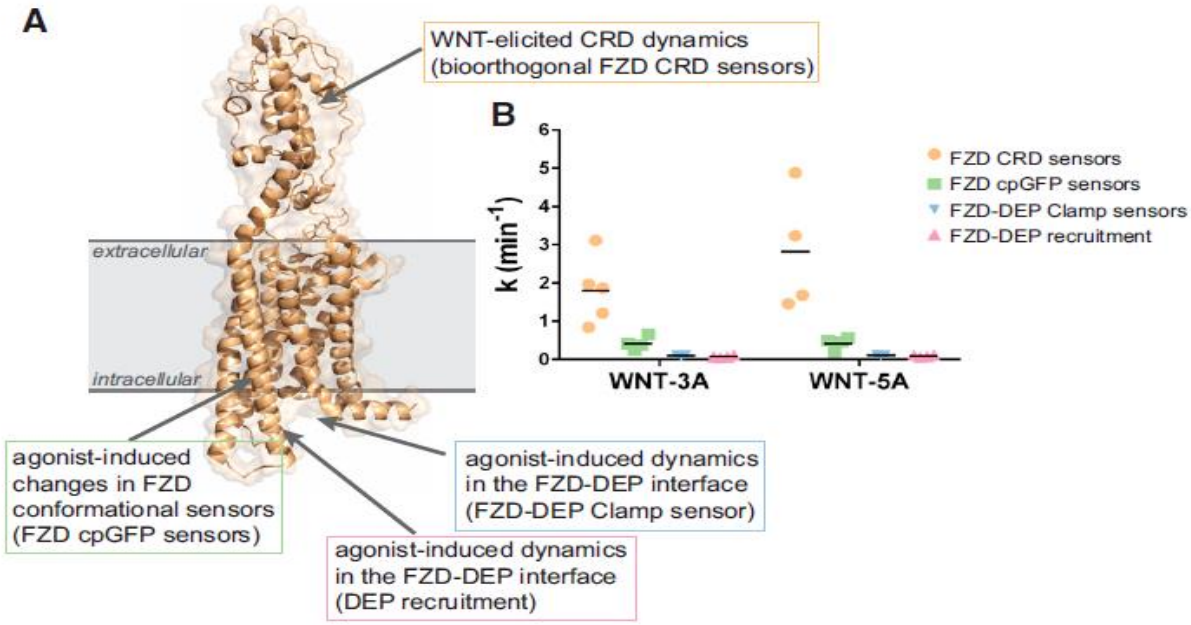

Fig. 3. The rate of dynamic rearrangements in FZDs caused by WNTs. Part A To illustrate important conformational changes in the receptor molecule, a model of a class F receptor is used. According to a study by Kowalski-Jahn et al. (2023), FZD CRD biosensors can detect ligand occupancy-proximal WNT-induced dynamic conformational changes. Schihada et al. (2021a) found that FZD cpGFP conformational sensors evaluate WNT-induced core conformational rearrangements, which represent ongoing information flow in the receptor molecule. The dynamics of the FZD-DVL interface can be measured using either a direct BRET assay or the monomolecular FZD5 DEP Clamp sensors (Bowin et al., 2023). We can get the rate constants k from the published studies' data. It is important to mention that these rate constants were derived from published data and experiments that were conducted separately and using various sensor technologies, specifically BRET and fluorescence. Because of this, we cannot rely on the rate constants as absolute values.

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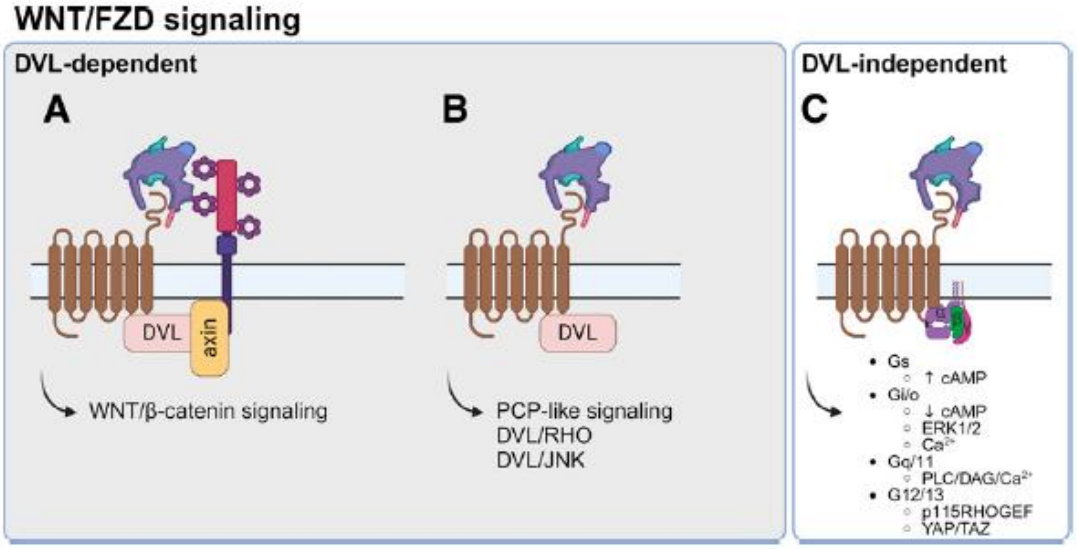

Fig. 4. Signaling pathways; WNT/FZD. Here, WNT signaling is separated into two types: one that relies on intracellular transducers like the scaffold protein DVL and another that does not. The two main types of signaling, WNT/b-catenin signaling (A) and WNT/PCP-like signaling (B), are defined by DVL-dependent signaling. The current scheme does not include single-transmembrane spanning receptors (such as ROR1/2 or receptor tyrosine kinase) in FZD-dependent WNT/PCP-like signaling, although their involvement as WNT coreceptors in b-catenin signaling is well-established (Riquelme et al., 2023). Heterotrimeric G protein signaling is considered a distinct subset of transducer-defined signaling since it is not dependent on DVL. Pointed out by Grätz et al. (2023a), new mutagenesis data reveals that FZDs typically favor DVL coupling over heterotrimeric G protein coupling. The model was made using the BioRender.com platform.

4. Class F G Protein-Coupled Receptors Ligands and Ligand Binding

4.1 WNTs Activate Frizzleds

Willert and Nusse (2012) and Grainger and Willert (2018) found that the 19 members of the secreted lipoglycoprotein family known as WNT exhibit diverse tissue expression profiles with respect to both space and time. It is conceptually difficult to grasp how mammalian WNTs function as receptor ligands or, at the very least, how they reach the orthosteric binding site of their receptors because they are lipidated (Willert et al., 2003). Wntless (WLS), also known as GPR177 or Evi, is a transmembrane protein that transports acylated WNT proteins from the endoplasmatic reticulum to the cell surface. Recent cryo-electron microscopy (Cryo-EM) structure-finding of a WLS-WNT complex has shown that, in contrast to the WNT-FZD interaction (Janda et al., 2012; Hirai et al., 2019), the WNT lipid moiety is located deep within the trans-membrane bundle of WLS helixes (Nygaard et al., 2021). The structural evidence acquired with isolated FZD CRDs and in complex with recombinant WNTs from Xenopus laevis and humans, respectively, is the molecular basis for the molecular understanding of the WNT-FZD interaction (Janda et al., 2012; Hirai et al., 2019; Tsutsumi et al., 2023). One way to think about the WNT structure is as a hand. On one side, the lipidated thumb interacts with a lipophilic groove on the CRD. On the other side, the WNT index finger loop pinches the CRD. While the WNT protein's core is distant from the FZD CRD, it is almost certainly involved in coreceptor interaction and does not appear to contribute to FZD binding (Janda and Garcia, 2015). The FZD CRD and the LRP5/6 b-propellers, which make up the LRP5/6 ligand binding domains, can both find binding sites in WNTs (Tsutsumi et al., 2023).

The process of WNT release is still not fully known, despite the identification and functional understanding of essential components (Mittermeier and Virshup, 2022). From a mechanistic and cellular biology perspective, various theories have been advanced to explain how WNTs are released from cells. These theories include vesicular transport, lipoprotein particles, heparan sulphate proteoglycans, carrier proteins, cytonemes, de Almeida Magalhaes et al., 2024, Zhang et al., 2023a, and many more. In order for a WNT molecule to be transported, the lipid component must be protected from the surrounding water, either by a protein or a membrane. Since the fatty acid is crucial for FZD CRD recognition and binding (Janda et al., 2012; Hirai et al., 2019), a significant thermodynamic barrier exists when the lipophilic group moves from the carrier's lipophilic environment to the CRD's lipophilic groove via the watery milieu. The problem of how to facilitate receptor binding by transferring the WNT protein appears to have been solved by evolution. There is still a great deal of ground to cover before we can understand these systems in depth. Interestingly, WNTs that have been synthesized or purified, whether in a serum-stabilized or "carrier free" form, can access the binding site of their target receptor, are biologically active, and can be used for pharmacological evaluation of ligand-receptor interactions (Kozielewicz et al., 2021b). Wesslowski et al. (2020) reported that a well-characterized enhanced GFP (eGFP)-tagged WNT-3A may be used

to assess WNT-receptor binding and examine binding kinetics, even if the cellular biology and underlying processes of WNT-FZD binding remain a mystery. Not only has this technological achievement proven that the CRD is crucial for WNT binding to FZDs, but it also suggests that the receptor core can play a role in the interaction between WNT and FZDs (Kozielewicz et al., 2021b). Looking at the cases of FZD4 and FZD8, comparing the binding of eGFP-WNT-3A to the full-length receptor and the membrane-anchored CRD (fused to CD86) revealed that the receptor core has the ability to either increase WNT binding in FZD4 or inhibit it in FZD8. However, coming up with a common concept has been extremely challenging thus far due to these diametral diverse consequences. Remarkably, the receptor's membrane dynamics are significantly affected by the extremely sluggish binding kinetics of eGFP-WNT-3A, which is particularly noticeable when the receptor is overexpressed to a high degree. The binding kinetics and affinity determinations in cells with high overexpression of BRET-compatible receptors differ from those in cells with endogenous promotor-driven BRET-compatible receptors that have been tagged using the CRISPR/Cas9-based technique. This finding is important to note. In addition, the addition of recombinant DKK1 did not influence WNT-FZD affinity when the WNT-LRP5/6 interaction was simultaneously blocked (Gratz et al., 2023b). The only binding tests conducted thus far have used WNT-3A that has been fluorescently tagged. With rigorous validation of the tagged proteins' biological activity, it should be technically possible to employ analogous constructions for the other 18 mammalian WNTs as well. As mentioned earlier, when WNTs bind to the CRD, a cascade of dynamic, FZD-intrinsic events such CRD dynamics, changes in core conformation, and dynamics at the receptor-transducer interface begin. These events seem to happen before or independently of coreceptors like LRP5/6.

4.2 Norrin Activates Frizzled

Along with WNTs, Norrin is an important factor in both healthy and diseased retinal vascular development; it is a FZD4-selective agonist (Ye et al., 2010). Familial exudative vitreoretinopathy is linked to malfunctioning Norrin, FZD4, and LRP5 signaling. According to Ke et al. (2013) and Chang et al. (2015), the WNT/b-catenin signaling pathway is initiated when the dimeric cysteine knot protein Norrin binds with the FZD CRD and recruits LRP5/6. Norrin and WNTs bind to FZDs in fundamentally different ways, which is an intriguing finding. The fact that dimeric Norrin can bind to FZD4 and LRP5/6 independently explains why WNTs and Norrin can both induce the WNT/b-catenin signaling pathway (Chang et al., 2015). According to Bang et al. (2018), the Norrin-FZD affinity is influenced by the Norrin-CRD interaction and the linker domain of FZD4. Evidence from structural biology and size exclusion chromatography provide credence to the idea that Norrin engages FZD4 dimers, as suggested by the arrangement of dimeric Norrin (Bang et al., 2018).

4.3 Clostridioides Difficile Toxin Binds Frizzled

One of the most highly expressed FZDs in the gastrointestinal tract is FZD7, which is involved in WNT signaling-driven intestinal epithelium renewal. The pathogenic bacterium Clostridioides difficile uses multiple toxins; one of these, Clostridioides difficile toxin B (TcdB), binds FZDs. Specifically, FZD1, 2, and 7 all share a homology cluster with other class F GPCRs (Tao et al., 2016; Chen et al., 2018a). Interestingly, from a functional standpoint, there is a density in the structure of both the Delivery/Receptor binding domain of TcdB and the CRD of FZD2. This density was thought to be a fatty acid in the lipophilic groove of the CRD, which also accommodates the lipid moiety of WNTs (Chen et al., 2018a). Thus, TcdB interacts with the FZD-CRD. When lipophilic ligands bind to CRDs without WNTs, it becomes unclear if the fatty acid is transported by TcdB or is constitutively bound in the FZD-CRD. In the second scenario, one would wonder if it's necessary to outcompete in order to enable WNT-CRD engagement. But, it seems that TcdB-FZD binding also mediates toxic effects on sensory neurons eliciting neurogenic inflammation (Manion et al., 2023) and that TcdB-FZD binding to intestinal FZDs blocks WNT/b-catenin signaling, which compromises the maintenance of the intact epithelium (Tao et al., 2016). According to Chen et al. (2018a) and Kinsolving et al. (2024), the structural resolution of the interaction between FZD2 and FZD7 was achieved by mapping the FZD-binding site on TcdB to the DRBD. It is even more astounding that the FZD-CRD's TcdB binding site is located across from the WNT binding site, enabling the binding of both TcdB and WNT to the CRD at the same time. This suggests that the FZD core is sterically collided with during the interaction between TcdB and the FZD-CRD, which could affect the receptor's structure and function.

4.4 Cholesterol and Oxysterols as Smoothened Ligands

A number of studies have shown that Hh proteins limit cholesterol availability to SMO by binding to PTCH and inhibiting the PTCH-mediated constitutive cholesterol transport (Siebold and Rohatgi, 2023; Kowatsch et al., 2019). In studies conducted by Nachtergaele et al. (2012), Nedelcu et al. (2013), Huang et al. (2016), Sever et al. (2016), Qi et al. (2019), and Raleigh et al. (2018), it was found that cholesterol and possibly oxidized cholesterol, also known as oxysterols, act as agonists at SMO. According to several studies (Ko- watsch et al., 2019; Kinnebrew et al., 2021, 2022; Sie-bold and Rohatgi, 2023), SMO was deorphanized as a sterol receptor and then discovered to be a sensor of membrane-accessible cholesterol. The two primary locations for cholesterol binding to SMO were located in the lipophilic groove on the CRD and deep in the core of the 7TM domain, respectively, as determined by structural and functional analyses of cholesterol binding to SMO and sterol-mediated regulation of SMO. In addition, the inverse agonist cyclopamine's binding site is situated in the upper portion of the 7TM domain, topographically between the CRD site and the deeper cholesterol binding site in the 7TMD. This compound is a teratogen generated from plants. So far, three allosterically communicating sterol

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binding sites have been found (Kozielewicz et al., 2020c); a receptor is primed for complete activation by occupancy of the CRD cholesterol binding site, which occurs after the 7TM domain site for cholesterol (Kinnebrew et al., 2022). This begs the question: how do cholesterol molecules reach their binding locations? According to Huang et al. (2018) and Deshpande et al. (2019), the receptor protein has a lipophilic tunnel that allows cholesterol from the membrane to enter the protein laterally, specifically at the lower 7TM core binding site. To facilitate the transport of cholesterol within the receptor molecule to the CRD, the intermediate binding site may offer a transitory cholesterol interaction site that is accessible also for outer leaflet cholesterol.
Class A GPCRs, like adrenoceptors, bind adrenaline, an endogenous agonist, singly. The main or orthosteric binding site is the name given to the agonist binding site. SMO currently has two or three sterol binding sites, which can bind either cholesterol or oxysterols, which are endogenous agonists. Are there multiple orthosteric sites, or is there just one binding site? The two primary locations may subsequently be the orthosteric sites. It is crucial to name these compounds according to current pharmacological ideas, considering the rich pharmacology of small molecules targeting SMO (Schwartz and Holst, 2007). It is still not clear whether the negative efficacy of cyclopamine impacts the efficacy or affinity of the cholesterols bound to the two primary sites, so the idea that the smoothened agonist SAG can be an allosteric enhancer or activator requires additional investigation and explanation. SANT1 is a reversible competitive antagonist.

4.5 Constitutively Active Class F Receptors

It was not until the cloning of GPCR genes enabled their overexpression in cell systems that did not express the target receptors endogenously that constitutive or ligand-independent activation of GPCRs was revealed. Signaling downstream was detected even when the receptor's ligand was not present, suggesting an agonist-independent basal activation, due to overexpression alone. The physiological significance of ligand-independent signaling can vary across receptor systems. This occurrence had pharmaco-logical consequences, including the creation of the idea of inverse agonism, the identification of inverse agonists, and the finding that some medications previously believed to be neutral antagonists were, in fact, inverse agonists, meaning they had a negative effect. The capacity of class F GPCRs to assemble an apo receptor-G protein complex even when an agonist that stabilizes the ternary complex is not present is the most striking example of their constitutive activity. Similar to human FZD7 and heterotrimeric Gs, FZDs exhibit constitutive activity functionally, which can be evaluated by measuring G protein activation with FZD overexpression (Schihada et al., 2021b; Xu et al., 2021). In addition, prior research by Bernat'ık et al. (2014) and Valnohova et al. (2018) suggests that overexpressing FZD causes a significant change in DVL's electrophoretic mobility that is dependent on phosphorylation. This change is independent of ligands and CRDs, suggesting that it may also be seen as constitutive action toward DVL. In contrast to SMOs, such as cyclopamine, which have inverse agonists, FZDs do not have any pharmacological tools that may be used to study ligand-independent receptor activation (Masdeu et al., 2006). The molecular aspects of FZD activation in cellular systems devoid of autocrine WNT exposure were better understood with the development of small molecule porcupine inhibitors like C59, which inhibit WNT acylation and prevent WNT secretion (Proffitt et al., 2013; Arthofer et al., 2016; Petersen et al., 2017). For instance, in order to differentiate between receptor-induced signaling to extracellular signal-regulated kinases 1/2 that is WNT-dependent and WNT-independent, porcupine inhibition was the only method that allowed for the detection of increased constitutive activity of FZD6 dimer interface mutants (Petersen et al., 2017). The molecular switch SMO M2 mutant (W7.55L) makes SMO more constitutively active, feeding into the glioma associated onco-gene (GLI) pathway. This finding establishes the clinically relevant concept of SMO inverse agonists, as noted in studies by Arensdorf et al. (2016).

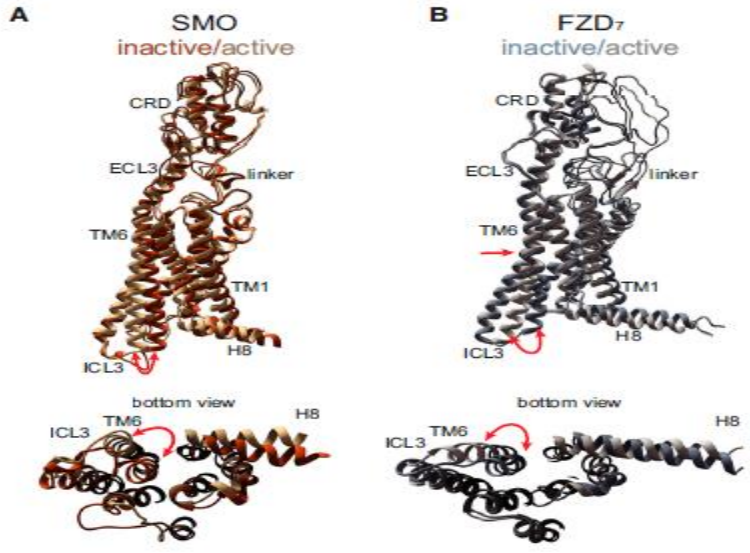

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Fig. 5. Characteristics of the structural activation of class F GPCR cells. Recent developments in the study of class F receptor structure-function relationships have provided new light on the kinetics of receptor activation. The plan showcases SMO inactive and active models that are grounded on structures that have been resolved experimentally [PDB ID 5V57 (Zhang et al., 2017b) and 6O3C (Deshpande et al., 2019)] as well as FZD7 models that are based on alphafold predictions. According to Pa'ndy-Szekeres et al. (2023), the GPCRdb has the latter. According to Turku et al. (2021), the kink (Pro6.32) in TM6 is accentuated during receptor activation induced by G protein coupling, just like the characteristics of class A GPCR activation, as seen by the straight red line. In both SMO and FZDs, the double-headed red arrow indicates the TM6 swing out. It should be noted that the SMO swing out is achieved not by a kink but by a parallel outward shift over TM6.

5. Class F Receptor Mutations in Disease

Class F GPCR molecular pharmacology is the primary emphasis of this review; nonetheless, the connection between dysregulated class F GPCRs and human disease must be addressed. The tumor-driving molecular switch mutant of SMO (SMO M2; W7.55L) that causes and therapeutically bases the treatment of SMO-dependent basal cell carcinoma and medulloblastoma is a prime example of how a deeper understanding of receptor activation mechanisms provides deeper insight into disease mechanisms. Given the same molecular switch mechanism throughout families, this insight into SMO disease process has far-reaching implications for the function of FZD molecular switch mutations, which are uncommon in various tumor types. A causal relationship between disease and molecular switch mutations in FZDs has not been established thus far, though (Wright et al., 2019).

5.1 Activating and Inactivating Mutations

Regarding FZDs mediating DVL-dependent and DVL-independent but G protein-dependent signaling pathways, it becomes more difficult to distinguish between activating and inactivating receptor alterations. Class F GPCRs (R/K6.52 and W7.55) contain a molecular switch that, when mutated, completely blocks WNT/b-catenin signaling and improves FZD coupling to heterotrimeric G proteins (Wright et al., 2019; Tsutsumi et al., 2020; Gratz et al., 2023a). As a result, the mutant cannot participate in DVL-mediated pathways and instead causes G protein signaling to gain-of-function, which may lead to harmful outcomes (Wu et al., 2019). A failure of mutant FZDs to recruit and activate DVL will impede pathway activation and hence not necessarily drive the oncogenic pathway, given that the DVL-dependent WNT/b-catenin is oncogenic. Tumor tissues from patients rarely contain molecular switch mutations in FZDs. However, there is still a lack of research on the possibility that these mutants cause developmental abnormalities. Aspects such as the improved coupling to hetero-trimeric G proteins may also contribute to many disease pathways, such as cancer, however this needs additional research. This line of reasoning does not hold water when it comes to SMO. The human SMO M2 mutant (W7.55L; in mouse SMO A1), which is the oncogenic driver mutant of the molecular switch, leads to an overstimulation of GLI signaling, making it an activating mutant that also increases the agonist-induced SMO-Gi/o coupling (Wright et al., 2019).

In Norrie disease and familial exudative vitreoretinopathy, two other types of retinal vascularization defects, a distinct set of mutations is responsible for blocking the activation of FZD4 in response to agonists, such as mutations that disrupt the binding of WNT to FZD (Le et al., 2023). In the CRD of FZD4, there are multiple disease mutations that directly cut off agonist binding. The C6.46 Ter is one such mutation that leads to an early termination codon, which entirely limits the development of a functioning receptor. The disease is linked to mutations in FZD4 and other pathway proteins like Norrin (Norrie disease protein), LRP5, and TSPAN12 Le et al., 2023.

Not only that, individuals suffering from autosomal recessive nail dysplasia were shown to have FZD6 loss-of-function mutations (Naz et al., 2012; Cui et al., 2013; Raza et al., 2013; Wilson et al., 2013). These mutations can be either nonsense or missense. One example is the missense mutation FZD6 R511C, which is located in the receptor's C terminus. Due to its strong mislocalization phenotype and functional selectivity of FZD6 of DVL over G protein coupling, this mutation has helped to shed light on FZD6 function (Kilander et al., 2014b; Petersen et al., 2017). Although the myriads of cancer mutations impacting FZD signal transduction are outside the focus of this publication, it is worth noting that certain malignancies exhibit either overexpression or downregulation of FZDs and their ligands. Public databases, like www.cbioportal.org, make these data readily available. The complex and crucial subject of transcriptional control of the WNT/FZD system in disease will hopefully be further illuminated by future investigation. On the other hand, while discussing FZD regulation, it's important to highlight a group of cancer mutations that affect oncogenic input through directly influencing FZD surface expression. In order to maintain consistently low levels of surface-expressed FZDs, such as in LGR5-positive stem cells in the intestinal niche, it is crucial to inhibit the E3 ubiquitin ligases ring finger protein 43 (RNF43)/zinc ring finger 3, which are known as the R-Spondins. This is done by acting on the WNT/b-catenin signaling enhancers (Hao et al., 2012; Koo et al., 2012). Reduced constitutive internalization and decreased FZD ubiquitinoylation result from RNF43 mutations that render the enzyme functionally impaired; this, in turn, increases cell surface expression and the responsiveness to ligand stimulation (Jiang et al., 2015; Zhong et al., 2021).

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According to Jiang et al. (2013), Planas-Paz et al. (2016), and Steinhart et al. (2017), RNF43 mutations are extremely important for understanding the control of FZD in physiology and disease/cancer, and they also present a chance for therapy that targets FZD.

6.0 Targeting Class F Receptors with Biologics

A wide variety of biologics that target FZD have emerged in recent years, including peptides, antibodies, antibody fragments, and what are essentially WNT surrogates, which function as competitive antagonists to WNTs or WNT mimetics, respectively.

An intense pursuit of peptide designs derived from WNTs resulted in the creation of FOXY5, BOX5, and UM206 (UM207, UM208), with FOXY5 making it to clinical trials (Safholm et al., 2006; Jenei et al., 2009; Laeremans et al., 2011; Yadav et al., 2021). Although the UM206 peptides showed promise in promoting wound healing during myocardial infarction, this could not be validated (Laeremans et al., 2011; Daskalopoulos et al., 2019). Peptides generated from WNTs do not selectively target any FZD paralogue from a mechanistic standpoint. Yet, peptides that target FZD7 were subsequently created; these peptides show promise as a therapy for gastrointestinal malignancies (Nile et al., 2018). In spite of early selective examples targeting, among others, FZD7 and FZD10, the lack of paralog selectivity initially eclipsed efforts to target the FZD CRD by antibodies (Fukukawa et al., 2008; Pode Shakked et al., 2011; Do et al., 2022). The most well-known FZD-targeting antibody, OMP-18R5/vantictumab, was initially designed to target FZD7 but has now shown less selective binding to five out of ten FZDs (FZD1, 2, 5, 7, 8). (Martin et al., 2022; Gurney et al., 2012). The phase I clinical trials of vantictumab were unsuccessful because of side effects that were indeed on target (Davis et al., 2020; Diamond et al., 2020). In spite of these clinical challenges, research into FZD paralog selectivity has continued to advance, with ideas like mini WNTs made of the index finger of WNTs and the idea of blocking the WNT-FZD interaction through antibodies and designer biologics (Janda et al., 2012; Steinhart et al., 2017; Pavlovic et al., 2018; Raman et al., 2019).

A new approach that is well-suited to regenerative medicine has focused on designing WNT surrogates that activate the WNT/b-catenin pathway by clustering FZD and LRP5/6, rather than interfering with the WNT-FZD interaction utilizing FZD-CRD-targeting antibodies or antibody fragments. For this, researchers have used recombinant DKK as an LRP5/6 binder in conjunction with an antibody or anti-body fragment that targets FZD CRD (Janda et al., 2017; Miao et al., 2020; Nguyen et al., 2022; Ding et al., 2023). Tetravalent trispecific diabodies targeting FZD and LRP6 are a new class of WNT surrogates that have been designed to aid in alveolar regeneration. It was surprising to find that these WNT surrogates could bind FZD6, allowing it to mediate WNT/b-catenin signaling on par with FZD5, something it cannot do when stimulated with WNTs. The restoration of the blood-retina-barrier is made possible by FZD4-LRP5 selective WNT surrogates, which can imitate Norrin-like transcriptional responses (Zhang et al., 2023b). This has great therapeutic promise for retinopathies and neurologic diseases like macular edema and stroke, which are marked by dysfunction of the barrier.

7. Targeting Class F Receptors with Small Molecule Drugs

7.1 Smoothened-Targeting Small Molecule Compounds

Numerous small compounds have been identified as targeting SMOs; a number of these have found clinical application in the treatment of medulloblastoma and basal cell carcinoma, among other malignancies (Stanton and Peng, 2010; Teglund and Toftgard, 2010). Dual targeting of SMO and another oncogene, like extracellular signal regulated kinase 1/2 or MET, may be able to mitigate treatment resistance associated with frequently occurring SMO variants (Atwood et al., 2015; Morgillo et al., 2017; Zhang et al., 2022). The center of the receptor contains several small molecule binding sites, and it is here where small molecule SMO ligands primarily exert their effects. Efficacious ligands like purmorphamine and the Smoothened agonist series SAG (SAG1.3, SAG1.5, SAG21k, etc.) primarily bind to the upper pocket, while antagonists or inverse agonists like cyclopamine or SANT-1 bind to the lower pocket, which is also a functional cholesterol binding site (Sinha and Chen, 2006; Rominger et al., 2009; Deshpande et al., 2019; Kozielewicz et al., 2020c). The fact that SMO has three separate small molecule/sterol binding sites makes the debate over which pockets are allosteric and which are orthosteric all the more pertinent. Nomenclature needs to be revised as our understanding of class F ligand-receptor interaction grows; this is due to the difficulty in accurately depicting small molecules as orthosteric ligands (agonists/antagonists and inverse agonists) and allosteric ligands (allosteric enhancers, NAM/positive allosteric modulators, full allosteric agonists, etc.).

7.2 Frizzled-Targeting Small Molecule Compounds

The pharmacological space covered by small molecule drugs that target FZD is rather restricted in comparison to the rich pharmacology relevant to SMO (Table 2; Fig. 6). As shown by recent accomplishments, the growing number of FZDs having high-resolution crystal or cryoEM structures offers a more solid foundation for large-scale in silico docking operations (Zhang et al., 2017a). Natural compound screening also provides new avenues for finding potential pharmaceuticals (Katanaev et al., 2021). A proof-of-principle demonstrating that FZDs can be targeted by small molecules and that both positive and negative regulation by small molecules is achievable was provided by the recent identification

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Vol 25, No. 2 (2024)
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Article Received: August 2024 Revised: September 2024 Accepted: October 2024

of tiny ligands with both positive efficacy (allosteric activator) and without efficacy (NAM). Since SMO is more closely related to FZD6 than any other member of the FZD family, the repurposing of SAG1.3 as an SMO agonist to a FZD ligand led to the discovery of the first small molecule allosteric activator, SAG1.3 (Kozielewicz et al., 2020b). Nevertheless, the only motivation for doing SAG1.3 experiments on FZD6 was the strong correlation between SMO and FZD6. Additional research confirmed that SAG1.3 lacked significant paralog selectivity. Purmorphamine, a structurally distinct SMO agonist, functions similarly to SAG1.3 in that it allosterically activates FZDs (Kozielewicz et al., 2020b). However, the compounds are not selective for FZD paralogs, and their positive efficacy and potency are poor as well. We were able to demonstrate that cyclopamine and its fluorescent derivative BODIPY-cyclopamine bind FZDs, although not selectively, in conjunction with the repurposing of SAG1.3 and purmorphamine from SMO to FZD6. Significantly, the BRET-based BODIPY-cyclopamine assay, originally developed to evaluate SMO binding, may be used to measure BODIPY-cyclopamine binding to FZDs, allowing for the potential to conduct competitive binding tests in a screening environment (Kozielewicz et al., 2020a,b).

The components that have been showcased thus far aim to bind to SMOs in FZDs. While FzM1 was found to be a negative allosteric modulator of FZD4, other allosteric binding sites may be targetable as well. At the FZD4-G protein contact, FzM1 binds to the intracellular domain of FZD4. The conversion of FzM1 to FzM1.8, an allosteric activator, was achieved by derivatization of FzM1. This small molecule, which targeted FZD, activated the WNT/b-catenin pathway via a G protein/PI3K-mediated pathway (Riccio et al., 2018). In addition, carbamazepine, an antiepileptic medication, appears to have no affinity for FZD5 or FZD7, although it does bind to a pocket on the CRD of FZD8 (low micromolar affinity; Kd 5 17 lM). According to Zhao et al. (2020), the CRD could potentially be a target for small molecule drugs because binding carbamazepine to it decreases the WNT-induced WNT/b-catenin pathway.

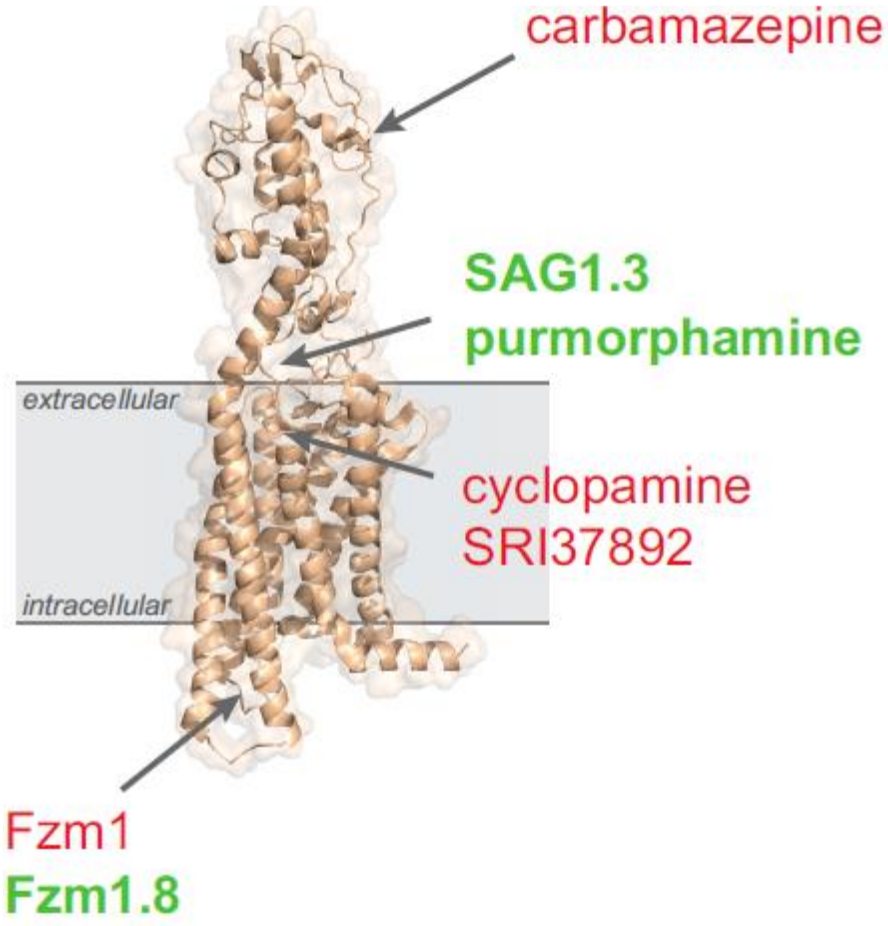

Fig. 6. FZD-targeting small molecule ligands. The following small molecule scaffolds have been found to act on FZDs: agonists (green) and negative allosteric modulators (red) with no efficacy. Citations: Generoso et al. (2015), Zhang et al. (2017a, 2020), Riccio et al. (2018), Kozielewicz et al. (2020b), FzM1, FzM1.8, SRI37892 (and related compounds), SAG1.3, purmorphamine, cyclopamine (and the BODIPY-labeled fluorescent derivative), F7H (and derivatives), and carbamazepine. No names or references for these compounds or their derivatives.

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TABLE 2 FZD-targeting small molecule compounds

Name	Proposed FZD Target	Chemical name	PubChem CID	Reference
FzM1	FZD_4	1-(3-Hydroxy-5-(thiophen-2-yl)phenyl)-3-(naphthalen-2-yl)urea	90478321	Generoso et al., 2015
FzM1.8	FZD_4	3-Hydroxy-5-(naphthalen-2-ylcarbamoylamino)benzoic acid	137553171	Riccio et al., 2018
SRI37892	FZD_7	4-(benzimidazol-1-yl)-N-[4-(2-oxo-3,4-dihydro-1H-quinolin-6-yl)-1,3-thiazol-2-yl]benzamide	41550022	Zhang et al., 2017a
SAG1.3	FZD_6 (nonselective)	3-chloro-N-(4-(methylamino)cyclohexyl)-N-((3-(pyridin-4-yl)phenyl)methyl)-1-benzothiophene-2-	5284330	Kozielewicz et al., 2020b
		carboxamide		
Purmorphamine	FZD_6 (nonselective)	2-(1-Naphthoxy)-6-(4-morpholinoanilino)-9-cyclohexylpurine	5284329	Kozielewicz et al., 2020b
Cyclopamine	FZD (nonselective)	11-Deoxojervine	442972	Kozielewicz et al., 2020b
Carbamezepine	FZD_8-CRD	5H-Dibenzo[b, f]azepine-5-carboxamide	2554	Zhao et al., 2020

8. Novel Tools to Study Class F Receptors

Class A, B, and C GPCR activation mechanisms and kinetics have been illuminated by fluorescence-based biosensors for GPCR activation, receptor-receptor interaction, transducer recruitment, second messenger detection, and enzyme activation (Lohse et al., 2012; Tenner et al., 2016; Wright and Bouvier, 2021). This comprehensive toolbox has evolved from the necessity of visualizing individual receptor paralogues in a setting where endogenous expression of both single and multiple class F receptors is present. It is being used more and more to gain a better understanding of class F GPCRs. Genetically encoded sensors are engineered proteins that can be transfected onto a plasmid. These sensors can then be used to observe molecular events like ligand binding, protein association or dissociation, second messenger production, or enzyme (kinase) activation. Using these biosensors greatly expands the range of possibilities beyond luciferase-based reporter gene tests like TOPFlash or GLI. Together, new biosensors and sensor readouts/detection enzymes (such as BRET/FRET pairs, conformation-sensitive fluorescent proteins, luciferases, and various fluorescence-or bioluminescence complementation assays) are constantly being developed.

8.1 Fluorescently Tagged Ligands for Bioluminescence Resonance Energy Transfer-Based Binding Assays

The lipophilicity of class F GPCR ligands contributes to the infamous difficulty of ligand-receptor binding experiments, which make discriminating between selective and nonspecific ligand binding a real challenge. One major cause of misunderstanding ligand behavior is the substantial amount of nonspecific binding to total ligand binding. It took a lot of washing steps to get an estimate of specific ligand binding in fluorescence-based ligand binding assays using fluorescently tagged cyclopamine for SMO. In spite of this, the binding data cannot produce a pharmacologically sound evaluation of receptor engagement due to the nonsaturability of the binding caused by the substantial contribution of nonspecific binding (Huang et al., 2018). The development of BRET-based ligand binding, however, allowed for the pharmacologically sound quantification of BODIPY-cyclopamine binding to SMO and competition binding using unlabeled small molecule SMO ligands (Kozielewicz et al., 2020a), since it effectively filters out the large contribution of nonspecific fluorescent ligand outside the effective BRET radius.

There is currently no stable, functional protein available for any of the 19 mammalian WNTs, which makes WNT binding experiments problematic. It is highly unlikely that any of these proteins exist in chemically altered forms that may be used in ligand binding tests, such as radioligand binding, since they are not commercially accessible in functional native protein forms. The functional validation of fluorescently tagged WNT-3A was accomplished relatively recently (Mii and Taira, 2009; Wesslowski et al., 2020), enabling the use of several BRET assay paradigms for ligand binding studies to WNT receptors. In order to achieve direct BRET between the Nluc-FZD and the eGFP-tagged WNT-3A in live cells, FZD constructs with a nanoluciferase at the N terminus were utilized. There are three main benefits to these experiments: First, the complete FZD, including the WNT-binding CRD, is used in the experiments. Second, the receptor core enables us to draw inferences about the core's function in ligand binding. Third, the amount of unspecific binding caused by the lipid modification of WNTs is severely reduced. The ratio of specific to unspecific binding is greatly enhanced since the BRET readout relies on the WNT receptor's closeness to the Nluc. Consequently, the assay only takes into account WNTs that are bound close to the target. Actually, nonspecific ligand binding has minimal effect on the eGFP-WNT-3A BRET binding experiment (Wesslowski et al., 2020; Kozielewicz et al., 2021b).

An improved version of this direct BRET assay was developed using the NanoBiT technology. In this setup, the WNT receptor has a tiny HiBiT tag attached to its N terminus. After that, the recombinant, cell-impermeable LgBiT protein is added to HiBiT to supplement it with LgBiT, and the result is the functioning nanoluciferase. Since the basal luciferase counts in this setup only come from receptors expressed on the cell surface and not from Nluc-tagged FZDs that are already inside the cell, the assay window is much improved (Kozie-lewicz et al., 2021b). It is worth noting that the BRET-

readout does indeed permit paralogue specificity, since this FZD-paralogue specific binding experiment yields identical results when conducted in cells with endogenously expressed FZDs or in engineered FZD-free cells. In addition, this allows us to compare several knockout cell lines that lack essential components of the WNT signaling pathway and evaluate paralogue-selective WNT-FZD binding. Recording the kinetics of ligand-receptor binding or measuring saturation binding are two ways to evaluate ligand affinity. On the other hand, eGFP-WNT-3A binding is extremely slow in cells overexpressing HiBiT-tagged FZDs, reaching a plateau after hours of ligand exposure, according to the kinetic analysis. Further limiting the experimentation range is the concentration of eGFP-WNT-3A that can be achieved during the enrichment technique (Wesslowski et al., 2020). The one apparent drawback to this WNT binding assay based on NanoBRET/Nano-BiT is the requirement to overexpress the target receptors, despite all of the other benefits. To overcome this drawback, we tagged endogenously expressed FZD7 with HiBiT in a SW480 colorectal cancer cell line using CRISPR/Cas9 (Gratz et al., 2023b). By utilizing the same eGFP-WNT-3A preparation, we were able to evaluate WNT binding to FZD7, which is expressed from its natural promoter. This finding (2) suggests that ligand binding kinetics reach saturation faster, leading to more precise affinity values for the WNT-FZD interaction in the lower nanomolar range, and 1) that cells express relatively few cell surface FZDs, with thousands of receptors per cell. It would be ideal to have multiple fluorescently labeled WNTs that are functionally well-characterized so that we can profile all WNTs in the FZD family, not just one WNT across all FZD paralogs. We should ideally use HiBiT-tagged FZDs that are expressed under the control of an endogenous and physiologically relevant promoter.

8.2 Genetically Encoded Biosensors

Using class F receptor-specific sensors and well-established GPCR sensors, we examined ligand-induced and constitutive activation of receptors and receptor-induced pathways, as well as downstream signaling, transducer association, and receptor-intrinsic conformational changes. The original purpose of receptor sensors was to track the cellular localization of receptors over time using simple receptor-fluorescent protein fusion proteins. However, new experimental paradigms have expanded their use to analyze changes in subcellular localization under various conditions, leading to more practical conclusions. Although there was a caveat of very low throughput and low kinetic resolution, we were able to draw conclusions on FZD-G protein coupling, FZD-DVL inter-action, FZD-dimerization, and the effects of mutants on receptor behavior using fluorescence recovery after photobleaching (Kilander et al., 2014a,b; Arthofer et al., 2016; Hot et al., 2017; Petersen et al., 2017; Wright et al., 2018). Optical tools, new conformation-sensitive fluorescent proteins, and flexible resonance energy transfer assays are all being developed, which is greatly influencing the chances of developing genetically encoded biosensors to understand class F GPCR function in detail (Fig. 7). By tracking their movement from the cell's periphery to its interior, several biosensors have helped shed light on the receptor kinetics, conformational rearrangements, and transducer coupling caused by WNTs. In order to study the dynamics of WNT-induced CRDs—that is, CRDs that were either rearranged in relation to the receptor core or had their range of motion restricted—intramolecular BRET sensors were made using biorthogonal labeling. In the extracellular areas of FZD5 and FZD6, the acceptor label was added using click chemistry-based coupling of Cy3 as a fluorophore to unnatural amino acids. Stimulating WNTs with an N-terminally localized Nluc and the linker domain caused a shift in BRET between the CRD and ECL3. By integrating this experimental design with pharmaceutical markers, such as the LRP5/6-binding protein DKK1, we were able to isolate the effects of WNT on BRET from those caused by LRP5/6.

Following conformity with the notion of class F receptors as molecular machines, CRD rearrangements at the receptor's surface move into the receptor's inside. Similar receptor sensors with different optical readouts have been used to evaluate the dynamics of the receptor core. When activated, each of these sensors inserts a tag into ICL3, a part of the receptor protein that goes through significant conformational changes when TM6 swings out. The probes reflect the agonist-induced activation rather than the transducer stabilizing an active conformation of the receptor core (Wright et al., 2018), because these ICL3-tagged sensors cannot couple to intracellular transducers like DVL or heterotrimeric G proteins. To measure conformational changes induced by WNTs and small molecules, researchers have used FRET sensors, which measure resonance energy transfer between blue fluorescent proteins and fluoresceine arsenic hairpin binder tags. These sensors were developed by Carsten Hoffmann and Roger Tsien (Wright et al., 2018; Kozielewicz et al., 2020b). Drawing from prior research on class A GPCRs, the sensor concept was refined by incorporating circular permutated GFP (cpGFP) and a Halo-tag into ICL3 at the same location Ravotto et al., 2020). According to Schihada et al. (2021a), the cpGFP-FZD sensors enabled the concentration-dependent characterization of WNT-induced effects on receptor core dynamics utilizing FZD4, 5, 6, and 7, which are typical receptors of the FZD family homology clusters. Although DKK1 entirely abolished WNT-induced b-catenin signaling as shown by the TOPFlash test, WNT-induced receptor core dynamics, like WNT-induced CRD dynamics, were insensitive to LRP5/6 inhibition. According to Grätz et al. (2023a), the halo-tagged FZDs were helpful in methodically evaluating how receptor mutations affected the core conformation as a whole, with basal BRET and mutation-induced alterations serving as readouts. Ollsen et al. (2020), Schihada et al. (2021b), Wright et al. (2021), Janicot et al. (2024), and Wright et al. (2024) are just a few of the many studies that have utilized the several heterotrimeric G protein activation/dissociation sensors that are available for the investigation of transducer coupling.

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http://www.veterinaria.org
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Furthermore, the FZD-DEP-Clamp sensors that were previously discussed provide valuable resources for evaluating the dynamics generated by agonists at the FZD-DVL interface (Bowin et al. 2023).

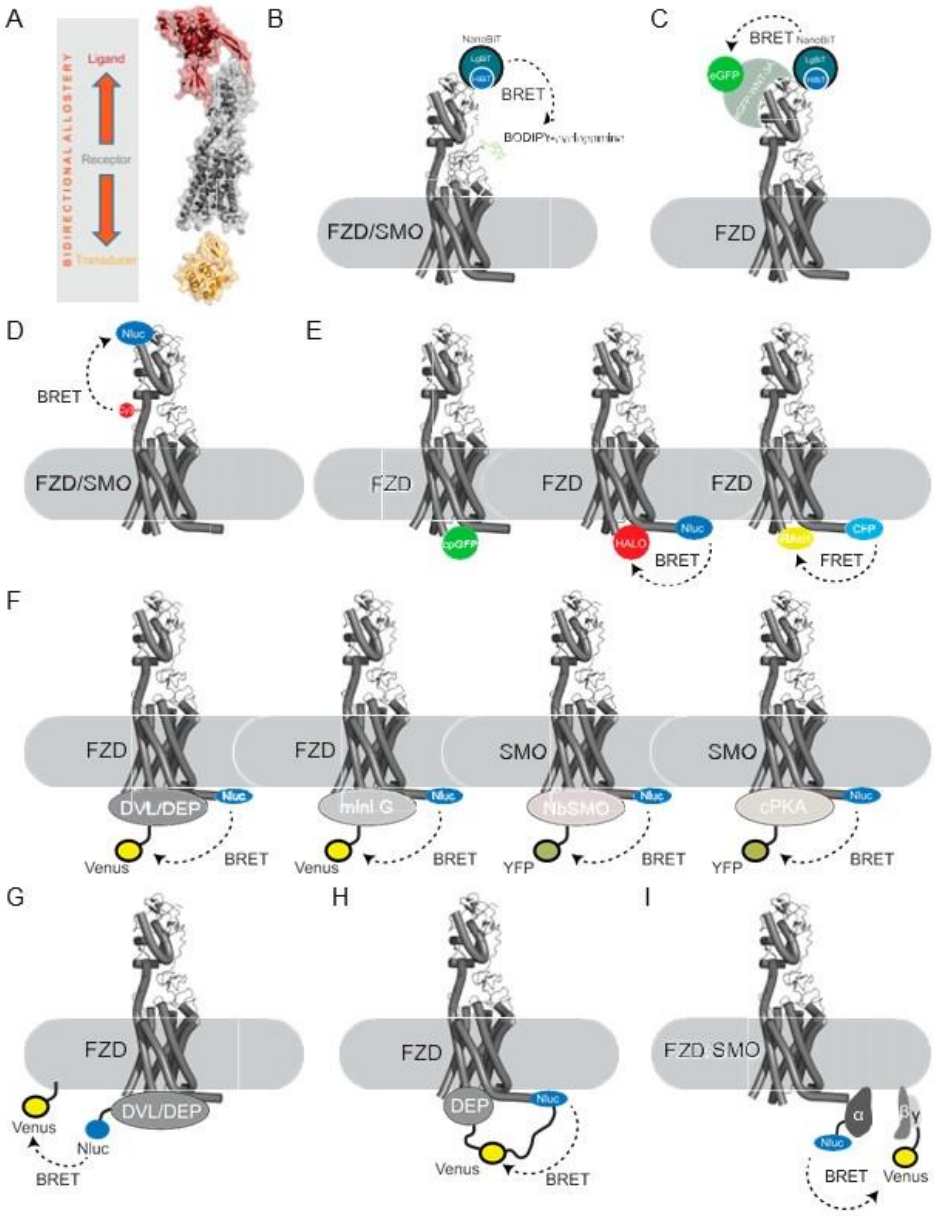

Fig. 7. Biosensors for the detection of class F receptor kinetics and activation.

(A) The ternary complex model highlights the allosteric cooperation between the intracellular transducer interaction site and the external agonist binding site, which is a two-way allostery defined by the receptor molecule.

(B) In a BRET system that combines Nluc- or HiBiT-tagged FZDs/SMO with the fluorescent BODIPY-cyclopamine, the ligand binding, including competition binding with unlabeled ligand, may be evaluated (Kozielewicz et al., 2020a,b; Kozielewicz and Schulte, 2022).

(C) Wesslowski et al. (2020) and Kozielewicz et al. (2021b) show that this technique may be modified to detect eGFP-tagged WNTs, similar to the NanoBiT/NanoBRET-based small molecule ligand binding experiment.
(D) The evaluation of WNT-induced changes in the CRD's relative position to the receptor core was made possible by biorthogonal labeling with fluorescent Cy3 in the linker domain and the ECL3 together with an N-terminal Nluc tag (Kowalski-Jahn et al., 2023).

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(E) FZD conformational biosensors equipped with a variety of fluorescent, BRET, or FRET-based techniques allowed for the evaluation of core conformational changes in response to small molecule or WNT activation in live cells.
(F) Assays based on BRET have been widely used to measure

8.3 Engineered Cell Lines

Thanks to the CRISPR/Cas9 method, genetic engineering has advanced to a point where we can evaluate the role of individual actors in a biological context (Milligan and Inoue, 2018). As indicated earlier in this paper, the FZD1-10 deletion HEK293 cell line has greatly expanded the technological options for evaluating the function of certain FZD paralogs against a comparable cellular backdrop. On the other hand, there is a catch: this cellular background doesn't really have much practical use when it comes to things like human illness or embryonic development. Another thing to keep in mind is that even when targeting the same genes, CRISPR/Cas9 cell engineering might lead to adaptive alterations that might not be the same in two cell lines that have been created in the same way. It may be challenging to inter- pret data from diverse, non-comparable cell lines due to differential rewiring caused by the absence of critical signaling components. It is important to thoroughly analyze these limitations in order to reach appropriate conclusions and provide adequate reasons for any inconsistencies (O'Hayre et al., 2017; Luttrell et al., 2018). The intriguing new prospects for understanding cellular signaling downstream of class F GPCRs are made possible by the potential to eliminate critical signaling components from the cells under examination. I understand that this list will be updated often, but for the time being I can only provide a few of cell systems that have been really beneficial in the field (Table 3). As an example, researchers are increasingly engineering tagged proteins to be expressed from their endogenous promoters, which opens up exciting possibilities beyond gene knockout in cell lines. This allows them to study ligand binding at physiological receptor levels, for example (Eck-ert et al., 2020; Gratz et al., 2023b).

TABLE 3 Relevant cellular systems engineered by CRISPR/CAS9 to study WNT/FZD or HH/SMO signaling

Protein Target	Cellular Background	Comment	Reference (Tool Generation)	Reference (Tool Use; Examples)
FZD_4 $FZD_{1,2,4,5,7,8}$	HEK293 HEK293	Deletion Deletion	Lai et al., 2017 Voloshanenko et al., 2017	Tsutsumi et al., 2020
FZD1-10	HEK293	Deletion	Eubelen et al., 2018	Bowin et al., 2023
SMO	HEK293	Deletion	Kozielewicz et al., 2020a	Kozielewicz et al., 2020b
LRP5/6	HEK293	Deletion	Lai et al., 2017; Eubelen et al., 2018	
DVL1, 2, 3	HEK293	Deletion	Cervenka et al., 2016; Gammons et al., 2016b	Bowin et al., 2023
DAAM1, 2	HEK293	Deletion	Colozza et al., 2023	
Heterotrimeric G proteins	HEK293	Deletion	Hisano et al., 2019	Bowin et al., 2019
HiBiT-FZD_7 LRP6-tdTomato	SW480 NCI-H1703	Tagged endogenous protein Tagged endogenous protein	Gratz et al., 2023b Eckert et al., 2020	

9.0 Future Prospects

The molecular pharmacology of class F GPCRs has been extensively studied since the latest report on class F GPCR nomenclature from IUPHAR (2010). New avenues for human therapy based on class F receptors have opened up thanks to structural biology advancements, a better understanding of cholesterol as the body's SMO agonist, biosensors that are relevant to class F GPCRs, small molecule compounds that target FZDs, and various biologics that target FZDs. This could lead to the development of drugs that are sensitive to FZD paralogs, which could mitigate or eliminate the severe side effects of targeting FZDs.

9.1 Structure of Signaling Complexes

A future milestone that will push the field of class F GPCR pharmacology to a new era will be a more de- tailed structural understanding of relevant smaller and larger receptor complexes. Hereby, I think about the smaller complexes being composed of ligand, full- length receptors, and transducers, such as G protein, DVL or cPKA, or others. It is relatively likely that the next update of this article can report on a series of these complexes providing insights into WNT-FZD selectivity, mechanisms of functional selectivity, and contribution of membrane lipids and cholesterol for receptor activation. With that structural information, which larger or more minute conformational differences are required in class F receptors to distinguish between transducers to achieve functional selectivity will also be resolved. Furthermore, I have larger complexes in mind, which could be resolved by state-of-the- art electron microscopy approaches including electron cryotomography. These technologies could resolve relevant signaling complexes of, for example, active SMO in the primary cilium or WNT/FZD/LRP5/6 or WNT/PCP signalosomes including additional transmembrane proteins, intracellular scaffolds, and signaling components

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Vol 25, No. 2 (2024)
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9.2 Integration of Two Opposing Concepts

Having a better grasp of the structures of important smaller and bigger receptor complexes is a potential milestone that will propel class F GPCR pharmacology into a new age. Here, I imagine that the smaller complexes include ligand, full-length receptors, and transducers like G protein, DVL, cPKA, or any number of others. It is quite probable that a number of these complexes will be reported in the next version of this article, which will provide light on the selectivity of WNT-FZD, the processes by which selectivity is functional, and the role that cholesterol and membrane lipids play in receptor activation. This structural data will also help in determining whether class F receptors need greater or smaller conformational changes to differentiate between transducers in order to attain functional selectivity. In addition, I am thinking about bigger complexes that could be solved using cutting-edge electron microscopy techniques, such as electron cryotomography. Examples of signaling complexes that could be resolved by these methods include active SMO in the primary cilium, WNT/FZD/LRP5/6 or WNT/PCP signalosomes, which contain intracellular scaffolding, signaling components, and other transmembrane proteins.

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