Themed Section: Molecular Pharmacology of GPCRs

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WNT/Frizzled signalling: receptor–ligand selectivity with focus on FZD-G protein signalling and its physiological relevance: IUPHAR Review 3

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The wingless/int1 (WNT)/Frizzled (FZD) signalling pathway controls numerous cellular processes such as proliferation, differentiation, cell-fate decisions, migration and plays a crucial role during embryonic development. Nineteen mammalian WNTs can bind to 10 FZDs thereby activating different downstream pathways such as WNT/ β -catenin, WNT/planar cell polarity and WNT/ Ca^{2+} . However, the mechanisms of signalling specification and the involvement of heterotrimeric G proteins are still unclear. Disturbances in the pathways can lead to various diseases ranging from cancer, inflammatory diseases to metabolic and neurological disorders. Due to the presence of seven-transmembrane segments, evidence for coupling between FZDs and G proteins and substantial structural differences in class A, B or C GPCRs, FZDs were grouped separately in the IUPHAR GPCR database as the class FZD within the superfamily of GPCRs. Recently, important progress has been made pointing to a direct activation of G proteins after WNT stimulation. WNT/FZD and G protein coupling remain to be fully explored, although the basic observation supporting the nature of FZDs as GPCRs is compelling. Because the involvement of different (i) WNTs; (ii) FZDs; and (iii) intracellular binding partners could selectively affect signalling specification, in this review we present the current understanding of receptor/ligand selectivity of FZDs and WNTs. We pinpoint what is known about signalling specification and the physiological relevance of these interactions with special emphasis on FZD–G protein interactions.

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This article, written by members of the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) subcommittee for the Frizzled Class GPCRs, confirms the existing nomenclature for these receptors and reviews our current understanding of their structure, pharmacology and functions and their likely physiological roles in health and disease. More information on this receptor family can be found in the Concise Guide to Pharmacology (<http://onlinelibrary.wiley.com/doi/10.1111/bph.12445/abstract>) and for each member of the family in the corresponding database <http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=25&familyType=GPCR>

Abbreviations

7TM, seven transmembrane; APC, adenomatous polyposis coli; CLL, chronic lymphocytic leukaemia; CRD, cysteine-rich domain; DARPP-32, dopamine- and cAMP-regulated neuronal phosphoprotein-32; DKK, Dickkopf; DVL, Dishevelled; FZD, Frizzled; GSK, glycogen synthase kinase; LEF, lymphoid enhancer-binding factor; LRP, lipoprotein receptor-related protein; PCP, planar cell polarity; PTX, pertussis toxin; ROR, receptor TK-like orphan receptor; SMO, Smoothed; TCF, T-cell factor; WNT, wingless/int1

Links to online information in IUPHAR-DB and the BPS Concise Guide to PHARMACOLOGY

Targets		Ligands	
5-HT _{2c}	GSK-3 β	β -catenin	WNT-1
Akt	iNOS	cAMP	WNT-2
Alzheimer's Disease	JNK	cGMP	WNT-2B
COX-2	mTOR	DKK1	WNT-3
ERK1	nemo-like kinase	GDP	WNT-3A
ERK2	PDE6	GTP	WNT-4
FZDs	PI3K	IFN γ	WNT-5A
FZD ₁	PKA	IL-6	WNT-5B
FZD ₂	PKC	IL-8	WNT-6
FZD ₃	PKC δ	IL-15	WNT-7A
FZD ₄	ROR1	Norrin	WNT-7B
FZD ₅	ROR2	Rac1	WNT-8B
FZD ₆	RYK	R-Spondin	WNT-9B
FZD ₇	SMO		WNT-10B
FZD ₈			
FZD ₉			
FZD ₁₀			
GPCRs			

This table lists chemical names, words and phrases that are hyperlinked to relevant entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014; PMID: 24234439) and the Concise Guide to PHARMACOLOGY 2013/14 (<http://onlinelibrary.wiley.com/doi/10.1111/bph.2013.170.issue-8/issuetoc>, Alexander *et al.*, 2013).

Introduction

Frizzleds (FZDs) are seven-transmembrane (7TM) domain-spanning cell surface receptors, very much like the classical GPCRs. They are activated by the wingless/int1 (WNT) family of lipoglycoproteins (Schulte, 2010; Willert and Nusse, 2012). Cellular communication mediated by WNTs and FZDs is crucial for proper embryonic development, stem cell differentiation, organogenesis and patterning. Also in the adult organism, stem cells are regulated by WNTs and it is also emerging that WNTs have an important role in the regulation of basic physiology. Furthermore, WNT/FZD signalling plays a crucial role in the adult in the maintenance of tissue homeostasis, regeneration, plasticity and repair (Logan and Nusse, 2004; van Amerongen and Nusse, 2009; Chien *et al.*, 2009). Even though many cellular processes are regulated by WNTs, surprisingly little is known about the underlying mechanisms involving the 10 different mammalian FZDs, their 19 WNT ligands and signal initiation at the level of the cell membrane.

From a pharmacological and mechanistic point of view, class FZD receptors as 7TM receptors are poorly understood. Hardly any quantitative information is available about ligand selectivity or efficacy and the most proximal signalling events induced by activated receptors have not been fully elucidated. The field is hampered by a complete lack of pharmacological compounds that can directly target FZDs, which originates in the long-standing difficulties to obtain recombinant pure and biologically active WNTs. Even though WNT/ β -catenin pathway activation can be studied through the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) reporter assay called TOPflash assay (Molenaar *et al.*, 1996; Van de Wetering *et al.*, 1996), there is a lack of suitable tools for studying the β -catenin-independent pathways. This methodological imbalance has resulted in a strong bias towards WNT/ β -catenin signalling.

The possibility of targeting WNT signalling therapeutically is widely regarded as attractive and molecules such as glycogen synthase kinase 3 (GSK3) inhibitors,

tankyrase inhibitors, secreted FZD-related proteins, R-spondin, Dickkopf1 (DKK1), Norrin and molecules targeting the co-receptors of the WNT/FZD pathway are being considered as putative drugs for diseases such as different types of cancer, neurodegenerative diseases and osteoporosis (Logan and Nusse, 2004; Moon *et al.*, 2004; Chien and Moon, 2007). Drug screening employing, for example, TOPflash reporter assays has identified novel and apparently promising compounds but has never identified FZD-targeting molecules. A recent screen with FZD internalization as readout has identified the antihelminthic drug niclosamide, even though the molecular mechanism and direct action through FZD remains elusive (Chen *et al.*, 2009). Thus, more appropriate screening technologies could aid the development of FZD targeting molecules, so providing useful research tools and therapeutics (Koval and Katanaev, 2012).

In this review we aim to summarize the status quo of our knowledge about WNT/FZD selectivity, signalling trafficking and the functionality of class FZDs as GPCRs *in vitro* and *in vivo*. The nomenclature used in this review conforms to the BJP's Concise Guide to PHARMACOLOGY (Alexander *et al.*, 2013).

WNT/FZD signalling pathways

So far, the underlying mechanisms and associated components that determine the outcome of WNT-induced FZD activation are poorly understood. First of all, the degree of WNT/FZD binding specificity in mammals is generally unknown and the question of whether certain WNT/FZD combinations can selectively activate certain signalling routes over others has not been systematically addressed. Secondly, coupling selectivity of activated FZDs to downstream signalling pathways remains obscure. There is emerging evidence that FZDs serve to induce complex signalling networks integrating multiple pathways rather than activating a single, linear signalling pathway. Moreover, different WNT signalling branches crosstalk as exemplified by the inhibition of WNT-3A-induced signalling by WNT-5A (Topol *et al.*, 2003; Mikels and Nusse, 2006). Further, the selective recruitment and participation of co-receptors, such as single transmembrane-spanning receptors of the low-density lipoprotein receptor-related protein family (LRP5/6) and receptor TK-like orphan receptor (ROR1/2) and receptor-like TK, can influence the signalling outcome (van Amerongen *et al.*, 2008; Hendrickx and Leyns, 2008). WNTs are also known to bind to transmembrane heparin sulphate proteoglycans such as glypican and syndecan, resulting in the accumulation of WNT molecules at the cell surface and the prevention of aggregate formation in the extracellular environment (Fuerer *et al.*, 2010). The importance of this phenomenon for the WNT/FZD interaction is unclear, and the role of these cofactors and other extracellular matrix components create an additional level of complexity for ligand/receptor binding studies.

To date, several distinct FZD signalling pathways have been identified and can be divided into β -catenin-dependent and β -catenin-independent branches (Macdonald *et al.*, 2007; Semenov *et al.*, 2007; Schulte, 2010). In the β -catenin-dependent pathway, WNT binding to FZD induces FZD to interact with the co-receptor LRP6, or the similar LRP5 (van Amerongen *et al.*, 2008; MacDonald *et al.*, 2009; MacDonald

and He, 2012). The formation of a WNT-FZD-LRP5/6 complex and the recruitment of the scaffolding protein Dishevelled (DVL) leads to the co-clustering of these proteins in signalosomes, which in turn triggers LRP6 phosphorylation (Bilic *et al.*, 2007). Subsequently, the activated co-receptor causes a redistribution of axin to the cell membrane and thus an incomplete and dysfunctional destruction complex – composed of GSK3 β , axin, adenomatous polyposis coli (APC) and other proteins – in the cytosol. This, in turn, causes β -catenin to accumulate within the cytosol and enter the nucleus, where it regulates TCF/LEF-dependent transcription. Classical WNT/ β -catenin target genes include cyclin D1, c-myc, COX-2 and inducible NOS (Ramsay *et al.*, 2003; Barker, 2008; Du *et al.*, 2009; MacDonald *et al.*, 2009). β -Catenin regulation of TCF/LEF transcription factors can be assessed with the TCF luciferase reporter/TOPflash assay (Molenaar *et al.*, 1996; Van de Wetering *et al.*, 1996), a method that is extensively used. The components of the WNT/ β -catenin pathway have been and are being mapped in detail (MacDonald *et al.*, 2009). However, the signal initiation events involving FZD-DVL-LRP5/6 complex formation, kinase activation and recruitment, phosphorylation of DVL and inhibition of the destruction complex are not understood at a molecular level. Generally, this pathway is considered to be independent of heterotrimeric G proteins, although accumulating evidence suggests the opposite (Liu *et al.*, 2001; Katanaev *et al.*, 2005; Halleskog and Schulte, 2013a). For example, $G_{\alpha q}$ and $G_{\alpha o}$ have been shown to dissociate the axin-GSK3 β and axin2-GSK3 β complex, respectively (Liu *et al.*, 2005), and $G_{\alpha 12}$ and $G_{\alpha s}$ act on axin, which is the key negative regulator of the β -catenin-dependent WNT/FZD signalling pathway (Castellone *et al.*, 2005; Stemmler *et al.*, 2006).

The β -catenin-independent pathways comprise a steadily increasing number of complex signalling routes: FZD/planar cell polarity (PCP), WNT/ Ca^{2+} , WNT/cAMP, WNT/ROR, WNT/RAP and WNT/RAC and WNT/RHO pathways (Semenov *et al.*, 2007; Schulte, 2010). In fact, these pathways are so diverse that it is a strong oversimplification to name them collectively as 'non-canonical' or ' β -catenin-independent'.

Because detailed information about the individual signalling pathways is available in recent, detailed review articles, we will focus here on what is relevant for the discussion about FZDs as unconventional GPCRs (Macdonald *et al.*, 2007; Semenov *et al.*, 2007; Chien *et al.*, 2009; Schulte, 2010).

The first indications of a G protein-dependent WNT/FZD/ Ca^{2+} pathway were obtained using injections of RNA coding for different WNTs and FZDs into *Danio rerio* and *Xenopus laevis* embryos. The induction of intracellular calcium release upon XWNT-5A and rat FZD₂ could be mimicked by 5-HT_{2C} receptor overexpression and inhibited by using agents that inhibit specific G protein subunits such as pertussis toxin (PTX) or overexpression of $G\alpha$ sequestering $\beta\gamma$ subunits (Slusarski *et al.*, 1997a,b). These studies were followed up by experiments addressing WNT-induced PKC membrane recruitment in *X. laevis* embryos, a pertussis-sensitive response to intracellular Ca^{2+} mobilization (Sheldahl *et al.*, 1999). After having named this WNT-induced and G protein-dependent pathway WNT/ Ca^{2+} pathway (Kuhl *et al.*, 2000), PTX-sensitive and rapid Ca^{2+} release upon WNT stimulation has been shown to be functional in several cellular systems (Sheldahl *et al.*, 2003; Kremenevskaja *et al.*, 2005; Dejmeek *et al.*, 2006; Ma and Wang, 2006; Halleskog *et al.*, 2012; Halleskog and Schulte, 2013a). In

addition to the WNT/ Ca^{2+} pathway, other G protein-dependent signalling routes involving classical GPCR-associated second messengers and signalling pathways emerge, such as the WNT/cAMP, WNT/ERK1/2 and inositol phosphate signalling (Saneyoshi *et al.*, 2002; Semenov *et al.*, 2007; Bikkavilli and Malbon, 2009; Hansen *et al.*, 2009; Cervenka *et al.*, 2011; Halleskog *et al.*, 2012; Halleskog and Schulte, 2013a).

In accordance with the ternary complex model known from classical GPCRs (De Lean *et al.*, 1980), which describes the allosteric interaction between heterotrimeric G proteins and agonists mediated by the GPCR, it is natural to assume that FZDs directly interact with heterotrimeric G protein to regulate distinct signalling routes, such as WNT/ Ca^{2+} and WNT/cAMP signalling. As a side note, it should be mentioned that the same could be assumed for FZD- β -arrestin interaction, which, however, appeared to be indirectly bridged by the phosphoprotein DVL to mediate agonist-induced internalization (Chen *et al.*, 2003). So far, a growing body of evidence indicates that WNT binding to FZDs induces G protein activation, clearly suggesting that FZDs can interact with heterotrimeric G-proteins. For example, WNTs induce G protein activation by inducing the guanine-nucleotide exchange on $\text{G}\alpha_{i/o}$ proteins (Katanaev and Buestorf, 2009; Kilander *et al.*, 2011a,b; Koval and Katanaev, 2011; Halleskog *et al.*, 2012). Although these studies provide a direct measure of WNT-induced activation of heterotrimeric G proteins in reconstituted systems, membrane preparations from primary cells and brain tissue, the underlying mechanisms of FZD-G protein coupling remain obscure.

Historically, the WNT/ β -catenin, PCP, WNT/RHO and WNT/RAC signalling are seen as heterotrimeric G protein-independent, whereas the WNT/ Ca^{2+} branch is recognized as the G protein-dependent WNT pathway. However, over the years several important studies have shown that heterotrimeric G proteins play a more global role in the WNT signalling network including the WNT/ β -catenin pathway (Liu *et al.*, 2001; Katanaev *et al.*, 2005; Egger-Adam and Katanaev, 2010). In a recent study from our laboratory using primary microglia cells, we have, for example, shown that PTX does not only block the WNT/ Ca^{2+} -mediated ERK1/2 phosphorylation but also the parallel WNT/ β -catenin signalling, including the WNT-3A-induced phosphorylation of LRP6, the formation of PS-DVL3 and the stabilization of β -catenin (Halleskog and Schulte, 2013a). In addition, when comparing the signalling capacities of several recombinant WNTs in the N13 microglia-like cell line, we observed that only WNT-3A induced WNT/ β -catenin signalling, whereas all the WNTs (WNT-3A, -4, -5A, -5B, -7A, -9B) led to the activation of PTX-sensitive heterotrimeric G proteins (Kilander *et al.*, 2011b). These findings show that heterotrimeric G proteins probably have a more central role in the field of WNT signalling than previously appreciated.

Intracellular binding partners for FZDs: heterotrimeric G proteins and DVL

The two most prominent and widely discussed intracellular binding partners for class FZD receptors are the scaffold protein DVL and heterotrimeric G proteins.

In humans, there are three variants of DVL, which consist of 670 (DVL1), 736 (DVL2) or 716 (DVL3) amino acids. The

protein has three main functional domains: the N-terminal end of the protein consists of a so-called DIX (Dishevelled and Axin) polymerization domain, at the C-terminal region, there is a DEP (Dishevelled, Egl-10 and Pleckstrin) domain, functionally involved in the regulation of small GTPases. In addition, the protein has a centrally located PDZ (Psd-95/Disc large/ZO-1 homologous) binding domain, which enables interactions with other proteins. DVL is a central scaffold protein localized at the crossroads of several FZD-induced pathways and plays not only an important role in the β -catenin-dependent but also in the independent signalling pathway, such as the WNT/ β -catenin, the PCP pathway, the WNT/RAC, the WNT/RHO and possibly also the WNT/ Ca^{2+} pathway (Gao and Chen, 2010). DVL exists in multiprotein complexes with punctuate appearance in the cytosol (Schwarz-Romond *et al.*, 2005). These multiprotein complexes can be dissolved, for example, by casein kinase overexpression, by WNT-5A stimulation and by expression of FZD (Cong *et al.*, 2004a; Strutt *et al.*, 2006; Bryja *et al.*, 2007a,b). Co-expression with FZDs recruits DVL to the membrane and so far, it is generally unknown if this interaction is static or dynamic, for example, upon agonist treatment (Cong *et al.*, 2004b).

In addition to DVL, the solid evidence for G protein involvement in WNT/FZD signalling shows that heterotrimeric G proteins – according to the ternary complex model valid for GPCRs – interact directly with FZDs. Agonist-bound GPCRs act as a guanine nucleotide exchange factor for the G proteins inducing the GTP-bound active state (Pfeuffer and Helmreich, 1988; Chung *et al.*, 2011). The G protein heterotrimer consists of three subunits, $\text{G}\alpha$, $\text{G}\beta$, $\text{G}\gamma$. Currently, it is known that there are 16 $\text{G}\alpha$, 5 $\text{G}\beta$ genes and 12 genes encoding $\text{G}\gamma$ (Downes and Gautam, 1999). Commonly, the G proteins can be divided according to their $\text{G}\alpha$ subunit into four groups: $\text{G}\alpha_i$, $\text{G}\alpha_s$, $\text{G}\alpha_q$ and $\text{G}\alpha_{12/13}$ (Neubig and Siderovski, 2002). See also Figure 1.

Despite the fact that there is experimental evidence indicating that both DVL and heterotrimeric G proteins bind and signal through class FZD receptors, the exact binding sites have not yet been clearly defined. Thus, it is still unclear whether these two proteins compete for FZD binding or if they actually cooperate. While it is known that DVL-FZD interaction is mainly based on three mechanisms involving (i) the KTxxxW–DVL interaction; (ii) DEP-domain interaction with a bipartite motif in the i3 of FZD; and (iii) DEP-domain-mediated electrostatic interactions with the negatively charged head groups of membrane lipids, we can only extrapolate from what is known for other GPCRs and speculate about FZD-G protein contact surfaces. Because classical GPCRs engage mainly the i3 and the C terminus for G protein interaction (Wess, 1998), it appears that the G protein and DVL contact surfaces on FZDs show substantial overlap.

Mutagenesis has revealed several residues in the intracellular loops and in the C-terminal domain of FZDs that are crucial for FZD signalling. Specifically, the mutation of the highly conserved internal KTxxxW motif after the seventh hydrophobic domain, or single amino acid exchanges in the first (R340A) or the third (L524A) intracellular loops of human FZDs, completely abolished FZD signalling (Cong *et al.*, 2004b; Schulte and Bryja, 2007). The KTxxxW motif, which is supposedly located two amino acids after the seventh transmembrane domain on the intracellular HELIX 8

G protein	α -subunit subfamily	Effect in WNT/FZD signalling
G_s	Gα_s, Gα_{olf}	- G α_s is associated with FZD ₇ in myotubes (von Maltzahn <i>et al.</i> , 2012)
		- G α_o is associated with FZD ₇ in myotubes (von Maltzahn <i>et al.</i> , 2012)
		- Formation of primitive endoderm formation out of F9 stem cells in response to WNT-8 stimulating FZD ₁ is G α_o and G α_q related (Liu <i>et al.</i> , 1999)
		- FZD ₆ activated G α_o in the presence of WNT-7A and to a lesser extent WNT-5A in membranes of <i>E. coli</i> (Katanaev <i>et al.</i> , 2009)
G_{i/o}	Gα_{1-3i}, Gα_o, Gα_z, Gα_{t1-2}, Gα_{gust}	- WNT-3A, WNT-5A, WNT-5B and WNT-7B activate G α_o through FZD ₁ in membranes of <i>E. coli</i> (Katanaev <i>et al.</i> , 2009)
		- FZD ₇ -dependent G α_o activation is induced by WNT-5A, WNT-3A and WNT-7A in membranes of <i>E. coli</i> (Katanaev <i>et al.</i> , 2009)
		- WNT-3A induced a G protein (G α_o , G α_{i1} and G α_{i2}) activation in FZD ₁ expressing L-cells (Koval <i>et al.</i> , 2011a)
		- Formation of primitive endoderm formation out of F9 stem cells in response to WNT-8 stimulating FZD ₁ is G α_o and G α_q related (Liu <i>et al.</i> , 1999)
G_q	Gα_q, Gα_{11}, Gα_{14}, Gα_{16}	
G₁₂	Gα_{12}, Gα_{13}	

Figure 1

Heterotrimeric G protein α -subunit subfamilies and their effects in WNT/FZD signalling. This figure provides an overview of all 16 heterotrimeric G protein α -subunits, which are categorized according to their G α subunit (G α_i , G α_s , G α_q and G $\alpha_{12/13}$). In addition, information about specific G α -subunits in direct relation to certain FZDs and WNT proteins is listed.

(Wang *et al.*, 2013), is essential for the activation of the WNT/ β -catenin signalling pathway (Umbhauer *et al.*, 2000). Flanking sequences of the KTxxxW motif significantly influence the affinity of peptides derived from FZD₇ for the PDZ domain of DVL (Punchihewa *et al.*, 2009) suggesting a role in FZD–DVL interaction. Mutation in the KTxxxW motif of *Drosophila melanogaster* FZD, which blocks DVL binding, results in a protein that is deficient in cell-autonomous planar polarity activity (Wu and Mlodzik, 2008; Wu *et al.*, 2008). Apart from the KTxxxW motif, the C-terminal tail is not well conserved among FZDs, suggesting differences in the pattern of downstream signalling pathways.

Structural features of FZDs and WNTs

Class FZD receptors were separated from the classical class A, B, C family GPCRs due to particular structural and functional features (Foord *et al.*, 2005). Class FZD receptors contain conserved cysteines in the extracellular loops 1 and 2, as is common for GPCRs. These cysteines could engage in stabilizing disulfide bonds. Also, a series of charged residues at the C- and N-terminal ends of intracellular loop 3, which are known to be essential for receptor–G protein coupling, are present in FZD. But class FZD receptors also lack other domains, which are important in many GPCRs. For example,

they lack the DRY motif at the C-terminal end of the third transmembrane domain known to be important for G protein coupling and specificity (Schulte, 2010). The recent X-ray structure of the related Smoothened (SMO) indicates clearly that domains common to class A GPCRs are not present in class FZD receptors (Wang *et al.*, 2013). Even though the SMO structure provides an interesting insight into possible mechanisms, more detailed structural and functional analysis is required to understand the functional features of this receptor family. Nevertheless, SMO does signal through G proteins and can couple to them, as demonstrated in biochemical and genetic models (Riobo and Manning, 2007; Philipp and Caron, 2009; Ayers and Therond, 2010). There is strong experimental evidence that the constitutive active state of SMO results in GDP/GTP exchange in heterotrimeric G proteins (Riobo *et al.*, 2006), and genetic experiments indicate that SMO recruits $G_{ai/o}$ proteins to modulate cAMP levels (Ogden *et al.*, 2008). Furthermore, in a study using micelles, an eighth helix – similar to other GPCRs including SMO – was found in the c-terminal region of FZD₁ (Gayen *et al.*, 2013).

Taken together, these findings indicate that so far very little is known about the exact FZD structure. Nevertheless, it has been possible to crystallize and analyse the mouse FZD8-cysteine-rich domain (CRD) domain together with the secreted CRD antagonist FZD-related protein 3. Thereby, it was shown that the CRDs are ~160 residue and primarily consist of α -helical proteins (Dann *et al.*, 2001; Janda *et al.*, 2012). In addition, lipid modification of WNTs complicates the expression, purification and crystallization of active WNTs (Hausmann and Basler, 2006). However, recently, Janda *et al.* were able to crystallize a *Xenopus* WNT-8 (XWNT8) in complex with mouse FZD8-CRD. This study revealed an unusual two-domain WNT structure, not obviously related to known protein folds, and suggests that binding to the FZD8-CRD shields the WNT lipid from aqueous solvent (Janda *et al.*, 2012). These results highlight the presumed selectivity of WNT/FZD.

WNT binding to FZD and physiological relevance

Very few studies provide direct evidence and quantitative measurements of the WNT/FZD interaction. The conventional idea of a ligand-binding assay between hydrophilic small molecule ligands or peptides with their receptors cannot be applied to assess the WNT/FZD interaction quantitatively due to the lipophilic characteristics of the WNT proteins (Willert *et al.*, 2003). Several studies have used isolated FZD-CRDs to measure WNT/FZD selectivity and affinity and have quantified WNT-CRD affinities in the lower nanomolar range (Kawasaki *et al.*, 2007; Carmon and Loose, 2008; Sato *et al.*, 2010). In the following paragraphs we attempt to provide a WNT/FZD binding profile based on what is known about the interactions between the ten mammalian FZD isoforms and the 19 WNTs in the context of their physiological relevance (mammalian vs. more primitive organisms). Figure 2 lists putative WNT/FZD combinations based either on direct binding experiments determined by immunoprecipitation or similar experiments that allow conclusions on protein–protein interaction, such as mass spectrometry, functional testing and signalling outcome. However, immunoprecipitation in this case is the most powerful and direct evidence for a receptor–ligand interaction, depending on the experimental conditions.

FZD₁

FZD₁ has been associated with neuroprotective effects in combination with for example WNT-3A (Chacon *et al.*, 2008). To date, WNT-1, -2, -3, -3A, -5A and -7B are known to serve as binding partners for FZD₁ (Gazit *et al.*, 1999; Wang *et al.*, 2005; Koval and Katanaev, 2011). In a study, where TOPflash reporter activity was used as an outcome for WNT activity, only WNT-1, -3, -3A and to a lesser extent WNT-2 were revealed as activators of this FZD₁-dependent pathway. In

WNT	1	2	2B	3	3A	4	5A	5B	6	7A	7B	8A	8B	9A	9B	10A	10B	11	16
FZD ₁	x	x		o	x		x	o		o	x								
FZD ₂		o		o	o		x		o	o	o		o						
FZD ₃	x				x		x												
FZD ₄	x	x		o			o				x								
FZD ₅	o			x	x		x			x					o		o		
FZD ₆					x	x	x	o		o									
FZD ₇				x	x		o			o									
FZD ₈					x										o				
FZD ₉	x																		
FZD ₁₀											x								

Figure 2

WNT/FZD interaction. This figure provides an overview of reported WNT/FZD combinations and their possible interaction. Crosses (x) indicate evidence for direct binding based on immunoprecipitation, whereas circles (o) indicate colocalization, signalling outcomes such as TCF reporter activity, TOPflash or internalization. Green indicates data already present in the IUPHAR Database (<http://www.iuphar-db.org/DATABASE/FamilyIntroductionForward?familyId=25>). All references can be found within the text or at the IUPHAR Database homepage. Today, all WNTs are available from different companies such as R&D systems, Abcam, ABMGood or Genemed. However, activity and purity of these WNTs should be carefully controlled.

addition, the authors identify WNT-5A as a binding partner for FZD₁ by co-immunoprecipitation experiments, even though this particular WNT did not enhance TOPflash reporter activity (Gazit *et al.*, 1999). WNT-7B can bind to overexpressed FZD₁ on the cell surface of HEK293 cells. Furthermore, it cooperatively activates the TOPflash reporter in the presence of LRP5 in HEK293 and PAC-1 cells (Wang *et al.*, 2005). L'Episcopo *et al.* demonstrated, by use of *in vitro* experiments in neuronal cultures, that WNT-1/FZD₁ binding, via β -catenin stabilization, has neuroprotective effects. Exogenous WNT-1 increased the survival of dopaminergic neurons after treatment with 6-hydroxydopamine and MPP⁺ (L'Episcopo *et al.*, 2011) – neurotoxic components that are known to mimic the biochemical characteristics of Parkinson's disease (Blum *et al.*, 2001). Further, FZD₁ and FZD₅ were significantly up-regulated in *Mycobacterium tuberculosis*-infected mice. The authors suggested that WNT-3A is a possible ligand for FZD₁ during this process, based on the soluble FZD₁/Fc fusion protein-mediated inhibition of WNT-3A-induced Axin2 mRNA expression levels after addition to macrophages (Neumann *et al.*, 2010). Additionally, WNT-3A stimulation initiates DVL3 mobilization to mouse F9 cell membranes through FZD₁ upon which TCF/LEF-sensitive transcription is activated. This activation depends on inositol polyphosphate multikinase (Wang and Wang, 2012). In a study with FZD₁-expressing L-cells, WNT-3A increased G protein activation substantially during which members of the G_{o/i} subtype (G_{ao}, G_{ai1} and G_{ai2}) were responsive, whereas G_{az}, G_{as}, G_{aq} and G_{ai2} were not (Koval and Katanaev, 2011).

Additionally, formation of primitive endoderm from F9 stem cells in response to WNT-8 can be blocked by treatment with PTX and by the depletion of the G proteins G α_o and G α_q (Liu *et al.*, 1999).

In membranes of *E. coli* overexpressing FZD₁, WNT-3A, WNT-5A, WNT-5B and WNT-7B specifically activated the trimeric G α_o protein. WNT-3A and WNT-5A were the most active, whereas WNT-5B and WNT-7A demonstrated a more modest stimulation (Katanaev and Buestorf, 2009).

FZD₂

FZD₂ triggers both β -catenin-dependent signalling as well as β -catenin-independent signalling, among which the Ca²⁺ pathway has been studied in more depth for this receptor (Sato *et al.*, 2010). For example, after FZD₂/WNT-5A binding, PDE6 is activated after which calcium mobilization occurs in melanoma cell lines. Pretreatment with an antibody against FZD₂ suppressed this effect as well as pretreatment with PTX, indicating the specific binding of WNT-5A to FZD₂ and the role of G proteins in this pathway (Bazhin *et al.*, 2010). Additionally, Ahumada *et al.* showed that WNT-5A/FZD₂ binding in F9 cells induced a PTX-sensitive and PDE6-dependent decrease in cGMP and increase in Ca²⁺ further confirming a role for heterotrimeric G proteins (Ahumada *et al.*, 2002). Furthermore, WNT-5A and FZD₂ binding simultaneously expressed in HeLaS3 cells co-localize in the membrane of the leading edge of the cell and the border of the cell-to-cell adhesion, revealing a function for this binding pair in cell motility (Matsumoto and Kikuchi, 2012). Further, this ligand/receptor pair seems to play a role in WNT/RAC signalling, yet another pathway in the β -catenin-independent signalling

network. Knockdown of FZD₂ in the cervical cancer cell line HeLaS3 suppressed the WNT-5A-dependent activation of RAC (Sato *et al.*, 2010). However, in FZD₂-transfected human pulmonary carcinoma H441 cells, a 20-fold increase in the TOPflash response was observed after WNT-3A stimulation. This response was even stronger after co-transfection of FZD₂ and ROR2, depicting β -catenin-dependent signalling as a possible downstream event of FZD₂ activation (Li *et al.*, 2008). These data are supported by a study from Yu *et al.*, where the authors created a heat map of TOPflash luciferase activity following transient transfection of different WNTs and FZDs in HEK293 cells. WNT-2, -3A and -3 increased β -catenin-dependent TOPflash luciferase activity through FZD₂ in over-expressing cells, similar to WNT-6, -7A, -7B and -8B, but to a lesser extent (Yu *et al.*, 2012).

The signal specification present downstream of FZD₂ is likely to be WNT isoform-dependent. For example, WNT-5A competed with WNT-3A for binding to the CRD domain of FZD₂, suggesting competition at the receptor level as a possible mechanism for the WNT-5A-mediated inhibition of WNT-3A-dependent accumulation of β -catenin and phosphorylation of the co-receptor LRP6 (Sato *et al.*, 2010).

FZD₃

FZD₃ is of particular interest for chronic lymphocytic leukaemia (CLL) because expression levels are significantly higher than in normal B cells (Lu *et al.*, 2004; Kaucka *et al.*, 2013). WNT-3A is also noted to be a 'signature gene' in CLL (Rosenwald *et al.*, 2001), suggesting that this ligand/receptor pair are significantly involved with this disease. WNT-3A activates the TOPflash reporter in HEK293 cells overexpressing WNT-3A/FZD₃/LRP6 (Lu *et al.*, 2004). However, it also mediates neurite outgrowth through FZD₃ in Ewing sarcoma cells, seemingly independent of β -catenin, but instead mediated by NK₁ receptors (Endo *et al.*, 2008).

WNT-5A has also been shown to be a binding partner for FZD₃, triggering downstream pathways independent of β -catenin. For example, it was found to activate the G α_s /cAMP/PKA pathway, resulting in the phosphorylation of Thr³⁴ of DARPP-32 (dopamine- and cAMP-regulated neuronal phosphoprotein-32), which in turn leads to anti-migratory effects in breast cancer cells. The authors observed a strong inhibition of the phosphorylation of DARPP-32 after the use of siRNA for G α_s in MCF-7 cells, even after stimulation of WNT-5A (Hansen *et al.*, 2009). Furthermore, WNT-5A can activate the PI3K/Akt signalling pathway through FZD₃ and, thereby, promotes adhesion of human dermal fibroblasts *in vitro*. This effect could be blocked with recombinant FZD₃-CRD, but not by FZD₆-CRD, suggesting that WNT-5A is a binding partner for FZD₃ but not FZD₆ (Kawasaki *et al.*, 2007). Co-immunoprecipitation studies have shown that WNT-2 can interact with FZD₃ in human cumulus cells, but it is not known which downstream signalling pathways are activated after this binding interaction (Wang *et al.*, 2009).

FZD₄

Investigations of FZD₄ have focused on the relationship between receptor mutations and familial exudative vitreoretinopathy, an impairment of retinal vascularization that leads to blindness (Robitaille *et al.*, 2002). In this context,

Norrin, structurally not related to WNTs, was found to serve as a ligand of FZD₄, capable of activating β -catenin signalling in the retina. The CRD of FZD₄ is essential in this binding interaction (Zhang *et al.*, 2011). FZD₄-deficient mice have defects in their intraretinal capillary bed (Xu *et al.*, 2004). Similar effects can be seen in LRP5 and Norrin knockout models, suggesting a ligand–receptor connection between the three (Luhmann *et al.*, 2005; Xia *et al.*, 2008).

WNT-5A induces the endocytosis of FZD₄-GFP in HEK293 cells, shown using the adaptor protein β -arrestin2 and the phosphorylation of DVL2 (Chen *et al.*, 2003). Further, WNT-5A triggers β -catenin signalling in HEK294/FZD₄ cells, a response that can be inhibited when it is co-transfected with the receptor ROR2 (Mikels and Nusse, 2006). Further, inhibition of clathrin-mediated endocytosis of FZD₄ by monodansylcadaverine inhibited the WNT-5A-dependent accumulation of β -catenin in the nucleus (Despeaux *et al.*, 2012). Additionally, WNT-5A-mediated endothelial cell proliferation could be blocked by the addition of FZD₄-CRD. However, these authors suggested that this response is independent of β -catenin signalling but rather mediated through DVL and ERK1/2 phosphorylation (Masckauchan *et al.*, 2006). Furthermore, WNT-2B (Ohta *et al.*, 2011), WNT-2 (Klein *et al.*, 2008) and WNT-7B (Wang *et al.*, 2005) have been identified as FZD₄-binding partners in immunoprecipitation experiments and WNT-3A, through increased accumulation of phosphatidylinositol 4,5-bisphosphate (Pan *et al.*, 2008).

FZD₅

There is abundant evidence that WNT-5A and FZD₅ are associated with different disease models. For example, both proteins are up-regulated in mouse models of Alzheimer's disease (AD), and WNT-5A enhances A β -evoked neurotoxicity by the induction of TNF- α and IL-1 (Li *et al.*, 2011). A β by itself in turn stimulates WNT-5A and FZD₅ expression in primary cortical cultures *in vitro*. Also in the synovium tissue rheumatoid arthritis (RA), the WNT-5A/FZD₅ pair is up-regulated compared with other human adult tissue. Also transfection of normal synovial fibroblasts with WNT-5A results in RA fibroblast-like synoviocytes, as revealed by enhanced IL-6, IL-8 and IL-15 mRNA levels (Sen *et al.*, 2000). More direct proof of the identification of FZD₅ as a receptor for WNT-5A comes from He *et al.*; they showed that co-injection of hFZD₅ and XWNT-5A RNA induced the formation of dorsal axis duplication in *X. laevis* embryos, although structural and putative functional differences between XWNT-5A and mammalian WNT-5A should not be disregarded. The axis duplication was suppressed after co-injection of RNA for human GSK-3 β , suggesting the involvement β -catenin-dependent signalling in this receptor–ligand combination (He *et al.*, 1997). Furthermore, in additional axis induction assay experiments in *Xenopus* embryos, it was shown that mouse FZD₅ specifically has a synergistic effect with mouse WNT-2, WNT-5A and WNT-10B (Ishikawa *et al.*, 2001). TOPflash assay experiments revealed WNT-9B as a binding partner for FZD₅/LRP-6 in HEK293 cells (Liu *et al.*, 2008). Finally, WNT-7A and WNT-3A bind to FZD₅/CRD, as shown by an ELISA-based protein binding assay (Carmon and Loose, 2008; 2010). Knockdown of FZD₅ or the addition of CRD-FZD₅ blocked the ability of WNT-7A to stimulate synaptogenesis in cultured hippocampal neurons (Sahores *et al.*, 2010).

FZD₆

Historically, FZD₆ was thought to be involved in the PCP pathway, where it plays an important role in the orientation of hair follicles (Wang *et al.*, 2006a), auditory sensory cells and neural tube closure (Wang *et al.*, 2006b). Recently, it was shown that FZD₆ mutations can result in defects in nail and claw formation. The mutated form of FZD₆ (R511C) alters the subcellular distribution of the receptor and this results in dysfunctional WNT/FZD signalling. The WNT-3A-induced stabilization of β -catenin and WNT-5A-dependent increase in the WNT inhibitor DKK1 shown in primary human fibroblasts was completely abolished in fibroblasts from patients with the FZD₆ mutant (Frojmark *et al.*, 2011; Naz *et al.*, 2012).

Most studies have demonstrated that FZD₆ is associated with β -catenin-independent signalling. In fact, some studies have identified FZD₆ as a negative regulator of the β -catenin-dependent signalling cascade (Golan *et al.*, 2004). Combining FZD₆, TCF/LEF luciferase reporters and WNT-1, -3, -3A, -4 or -5A in HEK293T cells, did not induce increased reporter activity with any of the chosen WNTs. On the contrary, FZD₆ inhibited the reporter activity induced by FZD₁ and WNT-3A. This inhibitory effect of FZD₆ results from the activation of different pathways, for example, Nemo-like kinase, which leads to the phosphorylation of TCF/LEF reducing its binding to target DNA (Golan *et al.*, 2004). This crosstalk could be highly relevant in colon cancer with constitutively active APC mutations (Rubinfeld *et al.*, 1996). Furthermore, WNT-3A stimulated G protein activation in L-cells expressing FZD₆ (Koval and Katanaev, 2011).

Heinonen *et al.* identified FZD₆ as a receptor for WNT-4 in immature haematopoietic progenitor cells, based on a decrease in WNT-4-mediated expansion of the cell pool in the absence of FZD₆ (Heinonen *et al.*, 2011). Furthermore, WNT-4 binds the CRD of FZD₆, but this receptor–ligand pair did not induce TOPflash activation in Madin–Darby Canine Kidney cells (Lyons *et al.*, 2004). Despite the strong link between FZD₆ and β -catenin-independent signal transduction, FZD₆ was also shown to be associated with WNT/ β -catenin signalling. A positive correlation between FZD₆ and WNT-3A was reported for β -catenin activation in human mesenchymal stem cells (Kolben *et al.*, 2012).

Lastly, WNT-7A, and to a lesser extent WNT-5A and WNT-5B but not WNT-3A, were shown to induce human FZD₆-mediated G α_o activation in an *E. coli* reconstitution system (Katanaev and Buestorf, 2009).

FZD₇

So far, FZD₇ has mostly been studied in combination with PCP signalling in vertebrates. The combination of WNT-7A-FZD₇ activates the PCP pathway by a mechanism involving Vangl2, demonstrated by the expansion of satellite stem cells resulting in the repair of mouse muscle (Le Grand *et al.*, 2009). In addition, the interaction between WNT-3 and FZD₇ leads to increased stabilization of β -catenin in human hepatocellular carcinoma cells and may therefore play a role in hepatocarcinogenesis (Kim *et al.*, 2008).

Interestingly, in myotubes it was shown that FZD₇, WNT-7A, G α_{12} and PI3K interact to induce the subsequent activation of the Akt/mammalian target of rapamycin growth pathway (von Maltzahn *et al.*, 2012). The broad signalling network induced by FZD₇ means the receptor is involved in

many pathological conditions. Up-regulation of FZD₇ is denoted in different types of cancer such as hepatocellular carcinoma, colorectal- and breast-cancer – to name but a few (King *et al.*, 2012). Also an increased expression of FZD₇ has been found to be associated with a less favourable clinical prognosis in CLL patients (Kaucka *et al.*, 2013). Furthermore, tumour growth could be reduced by the use of FZD₇shRNA in mice with xenografts of triple negative breast cancer cells (Yang *et al.*, 2011). Interestingly, calcium-dependent PKC signalling is also closely linked to FZD₇. In *X. laevis* embryo experiments, tissue separation was restored by co-expression of PKC α after blocking G protein-mediated signalling with the inhibitor PTX (Winklbauer *et al.*, 2001). In this context, *in vitro* and *in vivo* disruption of the FZD₇-DVL protein–protein interaction with the small peptide RHPD-P1 shows anticancer properties, probably through the involvement of β -catenin and PKC δ (Nambotin *et al.*, 2011). Lastly, the CRD of FZD₇ binds WNT-3A, as shown by the inhibition of WNT-3A-induced β -catenin stabilization in L-cells after addition of FZD₇-CRD (Kemp *et al.*, 2007).

In *E. coli* experiments, WNT-5A induced FZD₇-dependent G α_o activation, whereas WNT-3A and WNT-7A were less effective (Katanaev and Buestorf, 2009).

FZD₈

So far, FZD₈ is mostly thought to be a WNT receptor mediating β -catenin-independent signalling. Initially, evidence that FZD₈ is important for β -catenin-independent signalling was obtained in gastrulating *X. laevis* embryos. FZD₈ led to activation of JNK and rapid apoptotic cell death (Lisovsky *et al.*, 2002). In addition, purified, secreted forms of FZD₅, FZD₇ and FZD₈ CRDs can antagonize WNT-3A-induced β -catenin accumulation in L-cells, whereas in mouse embryonic stem cells, the same CRDs can inhibit spontaneous mesoderm formation and promote neural differentiation, indicating that FZDs interpret WNT signals during embryonic mesoderm and neural induction (Kemp *et al.*, 2007). FZD₈ together with Flamingo – a central component of WNT/PCP signalling – maintains haematopoietic stem cells in a long-term quiescent state through their cooperation in the osteoblast niche. The mechanism is based on the suppression of a Ca²⁺-NFAT-IFN γ (calcium–nuclear factor of activated T-cells–IFN γ) pathway. Furthermore β -catenin-dependent WNT signalling can also be antagonized by this receptor in this situation (Sugimura *et al.*, 2012).

Recently, the structural complex of XWNT8 and FZD₈-CRD has been clarified to a resolution of 3.25 Å. Guided by the structure, the authors were able to generate a biochemically tractable version of XWNT8, the so-called ‘mini-WNTs’, and discovered that they discriminate between binding to the CRD of FZD_{4,5,8} (Janda *et al.*, 2012).

Further, the soluble CRD of FZD₈ was used as a WNT inhibitor in many ways and studies. For example, this WNT inhibitor can block endogenous WNTs and the effects of WNT-1 and -5A on proliferation and the acquisition of a dopaminergic phenotype in precursor cultures (Castelo-Branco *et al.*, 2003). In addition, the soluble CRD of FZD₈ binds the CRDs of FZD₁ and FZD₈ receptors, reducing cell sensitivity to WNT-3A (Hendaoui *et al.*, 2012). Additionally, WNT-9B can activate TOPflash activity through FZD₈ in HEK293 cells (Liu *et al.*, 2008).

FZD₉

The FZD₉ gene was identified in 1997 and is located in the chromosomal region 7q11.23, which is the same location at which a 2 Mb deletion occurs in the developmental disorder called Williams–Beuren syndrome (Wang *et al.*, 1997). To determine whether specific symptoms of the syndrome can be explained by the deletion of FZD₉, 2 years later, murine FZD₉ was characterized. Expression analysis revealed that FZD₉ is expressed in the mouse in many tissues, including heart, brain and skeletal muscle (Wang *et al.*, 1999; Zhao and Pleasure, 2005). To analyse the physiological relevance of FZD₉, two FZD₉-deficient mouse models were generated and analysed independently (Ranheim *et al.*, 2005; Zhao *et al.*, 2005). In both studies, the mice developed normally, were fertile and showed no external abnormalities. However, in one study, differences in the hippocampus and learning defects were diagnosed in FZD₉^{−/−} mice (Zhao *et al.*, 2005), whereas in the second study, a defect in the development of B cells, as well as reduced life expectancy were observed (Ranheim *et al.*, 2005). Nevertheless, no symptoms similar to the human Williams–Beuren syndrome were found.

FZD₉ could also be involved in β -catenin stabilization and recruitment of DVL1 from the cytoplasm to the cell membrane. In addition, it was shown that WNT-2-activated FZD₉ results in TOPflash activity. Deletion mutant analysis demonstrated that there is a difference in FZD₉ C-terminal residues required for the modifications of DVL1 and those required for the inductions of β -catenin stabilization and TCF transactivation. It was also shown that most of the C-terminal tail domain of FZD₉ is not required for the induction of DVL1 hyperphosphorylation and relocalization. However, all DVL-C mutants used in this study except for one failed to induce β -catenin accumulation (Karasawa *et al.*, 2002). This highlights the role of the C terminus for the stabilization of β -catenin and suggests a separate interaction/binding site for DVL1. FZD₉ shows a rapidly increasing expression pattern during early differentiation of osteoblasts, in parallel with established osteoblast marker genes such as Runx2, bone sialoprotein and osteocalcin (Karsenty, 2008). In an Affymetrix GeneChip hybridization study, the FZD₉ gene, as the only gene out of the class FZD, was induced during osteoblast differentiation, in parallel with known osteoblast markers. In addition, the expression of FZD₉ in osteoblasts has been detected by *in situ* hybridization. Thus, it was concluded that FZD₉ has an important role in the differentiation and function of osteoblasts and, therefore, in bone formation (Albers *et al.*, 2011). This indicates that FZD₉ is important physiologically for the differentiation or function of osteoblasts.

FZD₁₀

FZD₁₀ is involved in the formation of colorectal cancer, mainly through the WNT/ β -catenin pathway (Terasaki *et al.*, 2002). In addition, FZD₁₀ can be co-immunoprecipitated together with WNT-7B. This WNT/FZD combination also activates β -catenin-dependent signalling in PAC-1 cells (Wang *et al.*, 2005). However, in synovial sarcomas, the transactivation of FZD₁₀, which is highly up-regulated in synovial sarcomas, can be linked to the β -catenin-independent DVL-Rac1-JNK pathway and plays a critical role in the development/progression of synovial sarcomas (Fukukawa *et al.*, 2009).

Furthermore, an enhanced expression of FZD₁₀ occurs during embryonic development of the CNS (Yan *et al.*, 2009).

In situ hybridization experiments have been used to visualize FZD₁₀ mRNA expression during mouse development and it was found that FZD₁₀ mRNA showed a similar expression pattern to WNT-7A in the neural tube, limb buds and Müllerian duct in mouse embryos suggesting that these two interact (Nunnally and Parr, 2004).

Also, WNT-3A is known to stimulate G protein activation in L-cells expressing FZD₁₀ (Koval and Katanaev, 2011).

Evidence for the physiological relevance of FZD-G protein signalling

As pointed out in the previous sections, evidence exists on WNT/FZD selectivity but this has obviously not been systematically investigated, partly due to the difficulties in obtaining recombinant, pure and active WNTs (Willert *et al.*, 2003; Schulte *et al.*, 2005; Willert, 2008; Willert and Nusse, 2012). With increasing knowledge about the underlying signalling mechanisms and the refinement of methodologies to quantify pathway activation, the structure–function relationship between the 19 WNTs and 10 FZDs can be investigated in more detail and questions regarding β -catenin-dependent and -independent signalling and its association with FZD-induced activation of heterotrimeric G proteins can be studied in more depth.

As early as 1999, the first proof was obtained that WNT/ β -catenin signalling is inhibited in F9 teratocarcinoma cells after inhibition of G α_i and G α_o via PTX (Liu *et al.*, 1999). In parallel, biochemical evidence emerged supporting the hypothesis that FZDs that mediate β -catenin stabilization are pre-coupled to G α_o (Liu *et al.*, 2005). As mentioned earlier, studies of β -catenin-independent WNT pathways have also provided evidence for G protein signalling linked with FZDs. Studies with zebrafish embryos showed that inhibition of G proteins blocked the changes in Ca²⁺ influx, which occur in response to WNT-5A and FZD₂ (Slusarski *et al.*, 1997a,b). Further, Katanaev *et al.* showed *in vivo* that FZDs act as guanine nucleotide exchange factors of PTX-sensitive G proteins mediating both WNT/ β -catenin and PCP signals in *Drosophila melanogaster* (Katanaev *et al.*, 2005).

For example, loss of WNT-7A resulted in a reduction in satellite cells in skeletal muscle 3 weeks after injury, demonstrating its physiological role in regulating the homeostatic level of satellite cells (Le Grand *et al.*, 2009; von Maltzahn *et al.*, 2012). In the same study, mass spectrometry and co-immunoprecipitation studies revealed an association between FZD₇ and G α_i and possibly G α_o .

Physiologically, the effect of XWNT-11/Xfz7 signalling was shown to regulate convergent extension movements. These movements affected the dorsal marginal zone of gastrulating *Xenopus* embryos. Interestingly, FZD₇ and the $\beta\gamma$ -subunits of heterotrimeric G proteins were required for this process (Penzo-Mendez *et al.*, 2003).

WNT-3A signals through the G $\alpha_{q/11}$ subunits of G proteins to activate phosphatidylinositol signalling and PKC δ in murine ST2 cells. However, direct evidence for any regulation of FZD was not obtained (Tu *et al.*, 2007). Furthermore, PKA/

CREB signalling may contribute to other WNT-regulated processes, such as stem cell renewal, cancer and embryonic patterning. The authors also suggested that G α proteins modulate adenylyl cyclase activity, which may be a mechanism by which WNT proteins, mainly WNT-1 and WNT-7A, signal possibly through class FZD receptors (Chen *et al.*, 2005). Lately, there has been an emerging interest in WNTs and their role in inflammation and immune response including neuroinflammatory diseases (Gattinoni *et al.*, 2010; Miao *et al.*, 2013). For example, it has been suggested that genetic variations within WNT signalling components contribute to the initiation and maturation of prevalent neurological disorders (De Ferrari and Moon, 2006). Studies from our lab characterized WNTs as modulators of the CNS immune response and showed that β -catenin levels are elevated in pro-inflammatory microglia of AD patients (Halleskog *et al.*, 2011). WNT-3A and WNT-5A induce a pro-inflammatory response in primary mouse microglia through an ERK1/2-dependent pathway (Halleskog *et al.*, 2012; Halleskog and Schulte, 2013a). In contrast, after LPS induced pro-inflammatory transformation of microglia, WNT-3A and WNT-5A were found to induce a dose-dependent decrease in the pro-inflammatory marker COX2.

Most interestingly, both the WNT-3A-induced ERK1/2 pathway and the β -catenin signalling require heterotrimeric G proteins (Halleskog and Schulte, 2013b).

When investigating physiological responses of WNT signalling, the possibility of heterotrimeric G protein binding to class FZD receptors is often being overlooked or simply not addressed, possibly due to lack of direct and straightforward methodological readouts.

Today, the TOPflash assay, which is a powerful tool for measuring transcriptional activity of β -catenin levels, has been preferentially used to study activation of the WNT signalling. This assay is also frequently used in the industry to find molecules that will interact with the WNT/FZD signalling pathway.

A direct and less pathway-biased readout system for class FZD receptors would be of great value. Recent data pinpoint the activation of heterotrimeric G proteins as a more general downstream event activated by WNTs (Katanaev and Buestorf, 2009; Kilander *et al.*, 2011a). Using, for example, a [³⁵S]-GTP γ S or the non-radioactive alternative europium-labelled GTP analogue (Eu-GTP) assay – frequently used for other GPCRs – as a readout system would potentially improve screening efforts for FZD-targeting drugs (Koval *et al.*, 2010).

Conclusions

The signalling network induced by WNTs acting at class FZD receptors is complex. In order to understand signalling specification, it is crucial to systematically address receptor–ligand interactions and quantify which WNT/FZD combinations activate which downstream events.

The role of FZDs in many types of cancer and other diseases is diverse, rendering class FZD receptors an obvious target for therapeutic intervention (Logan and Nusse, 2004; Janssens *et al.*, 2006; van Amerongen and Nusse, 2009; Chien *et al.*, 2009; King *et al.*, 2012). Small molecular libraries are waiting to be screened for specific interaction with FZDs.

Agonists and antagonists targeting FZD as well as positive and negative allosteric modulators would be of great value and could serve as pharmacophores for the development of new drugs (Chien and Moon, 2007; Anastas and Moon, 2013). Nevertheless, suitable readout assays are the key to successful high throughput screening (Koval and Katanaev, 2012). TOP-flash assays reporting the transcriptional activity of β -catenin have been of great importance in the understanding of the biological importance of the WNT/ β -catenin pathway and have proven partially successful in drug screening as well (Huang *et al.*, 2009). However, additional responses, such as activation of heterotrimeric G proteins or second messengers, which have been used for GPCR screening for decades, could complement the assay repertoire very well (Koval *et al.*, 2011).

The discussion about signal specification also opens up possibilities to employ pathway-selective ligands for putative therapeutic intervention. It might be possible to develop biased ligands that, for example, can block the WNT- β -catenin pathway at the same time as they maintain β -catenin-independent pathways. So far, this is purely hypothetical, but the proof-of-concept for biased drugs with class A GPCRs might be transferrable to class FZD receptors (Whalen *et al.*, 2011).

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Conflict of interest

None.

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