International Union of Basic and Clinical Pharmacology. LXXX. The Class Frizzled Receptors^[S]

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[S] The online version of this article (available at <http://pharmrev.aspetjournals.org>) contains supplemental material.

This article is available online at <http://pharmrev.aspetjournals.org>.

doi:10.1124/pr.110.002931.

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Abstract—The receptor class Frizzled, which has recently been categorized as a separate group of G protein-coupled receptors by the International Union of Basic and Clinical Pharmacology, consists of 10 Frizzleds (FZD_{1–10}) and Smoothened (SMO). The FZDs are activated by secreted lipoglycoproteins of the Wingless/Int-1 (WNT) family, whereas SMO is indirectly activated by the Hedgehog (HH) family of proteins acting on the transmembrane protein Patched (PTCH). Recent years have seen major advances in our knowledge about these seven-transmembrane-spanning proteins, including: receptor function, molecular mechanisms of signal transduction, and the receptor's

role in embryonic patterning, physiology, cancer, and other diseases. Despite intense efforts, many question marks and challenges remain in mapping receptor-ligand interaction, signaling routes, mechanisms of specificity and how these molecular details underlie disease and also the receptor's important role in physiology. This review therefore focuses on the molecular aspects of WNT/FZD and HH/SMO signaling discussing receptor structure, mechanisms of signal transduction, accessory proteins, receptor dynamics, and the possibility of targeting these signaling pathways pharmacologically.

I. Introduction: The Class Frizzled and Recommended Nomenclature

The Frizzled family of seven-transmembrane-spanning receptors (7TMR¹) and the closely related Smoothened (SMO) have been included in the International Union of Basic and Clinical Pharmacology (IUPHAR) G

protein-coupled receptor (GPCR) list as a separate family—the class Frizzled (Foord et al., 2005). In parallel, IUPHAR recommended a unifying Frizzled receptor nomenclature for the mammalian receptors in which isoform numbers are given as subscripts and Frizzled is abbreviated FZD with capital letters, rather than previously used names, such as Fz, Fzd, Frz, or Hfz. The class FZD includes 10 FZD isoforms in mammals; these are denoted FZD_{1–10}. Smoothened, the eleventh member of the family, is abbreviated SMO. Nomenclature of FZDs in nonmammalian species, such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Xenopus laevis* is handled independently (see Table 1), although a unifying terminology would be advantageous. Nomenclature for most mammalian GPCRs is based on the endogenous ligand that binds and activates the cognate receptor. In the case of the class Frizzled receptors, however, the receptor names are derived from *D. melanogaster* phenotypes and associated gene loci coding for these transmembrane proteins. Therefore it is also worthwhile to mention the origin of the name “Frizzled”: this refers to the irregularly arranged and tightly curled hairs and bristles on thorax, wings, and feet of the *frizzled* mutant of *D. melanogaster*, described long before the discovery of the class Frizzled receptors (Bridges and Brehme, 1944). The *smoothened* locus was identified as a segment

¹ Abbreviations: 7TMR, seven-transmembrane-spanning receptor; aa, amino acid(s); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; CK1, casein kinase 1; CRD, cysteine-rich domain; CREB, cAMP response element binding protein; DKK, Dickkopf; DOCK4, dedicator of cytokinesis 4; DVL, Disheveled; FJ9, ((2-(1-hydroxypentyl)-3-(2-phenylethyl)-6-methyl)indole-5-carboxylic acid; FZD, frizzled; GEF, guanine nucleotide exchange factor; GLI, glioma-associated oncogene; GPCR, G protein-coupled receptor; GRK, GPCR kinase; GSK3, glycogen synthase kinase 3; HEK, human embryonic kidney; HH, Hedgehog; IP₃, inositol trisphosphate; IUPHAR, International Union of Basic and Clinical Pharmacology; KRM, Kremen; LEF, lymphoid enhancer factor; LRP, low-density lipoprotein receptor-related protein; PCP, planar cell polarity; PDE, cGMP-specific phosphodiesterase; PDZ, postsynaptic density 95/disc-large/zona occludens-1; PKA, cAMP-dependent protein kinase; PKC, Ca²⁺-dependent protein kinase; PS-DVL, phosphorylated and shifted DVL; PTCH, Patched; PTX, pertussis toxin; ROCK, RHO-associated kinase; ROR, receptor tyrosine kinase-like orphan receptor; RYK, receptor tyrosine kinase; SAG, smoothened agonist; SFRP, soluble Frizzled-related protein; SMO, Smoothened; TCF, T-cell factor; UM206, CNKTSEGMGCEL; WGEF, weak-similarity GEF; WIF, WNT inhibitory factor; WNT, *wingless* and *int-1*.

TABLE 1
Class FZD receptors in mammals (mouse and human), *D. melanogaster*, *X. laevis*, and *C. elegans*

For more details, see van Amerongen and Nusse (2009).

Mouse and human	<i>D. melanogaster</i>	<i>X. laevis</i>	<i>C. elegans</i>
FZD ₁₋₁₀ SMO	Fz, Dfz2, Dfz3, Dfz4 SMO	XFz1, 2, 3, 4, 5, 7, 8, 9, 10A, 10B SMO	MOM-5, LIN-17, CFZ-2, MIG-1 No expression of SMO homolog

Fz/Dfz, FZD in *D. melanogaster*; XFz, FZD in *X. laevis*; MOM-5, more of mesoderm (MS) family member-5; LIN-17, abnormal cell LiNeage family member-17; CFZ-2, *C. elegans* Frizzled homolog family member-2; MIG-1, abnormal cell MIGration family member-1.

polarity gene in *D. melanogaster* by Nüsslein-Volhard (Nüsslein-Volhard et al., 1984).

II. Brief History of Discovery and Some Phylogenetics

Before the discovery of FZDs, the mammary protooncogene *int-1* in mice was described previously (Nusse and Varmus, 1982) as the mammalian counterpart of the *wingless* gene in *D. melanogaster* (Cabrera et al., 1987; Rijsewijk et al., 1987). These findings laid the basis for the characterization of the WNT family of secreted lipoglycoproteins and provided the WNTs with their name, an acronym combining *wingless* and *int-1* (Nusse et al., 1991). They also opened up an enormous field of research providing mechanistic insights into molecular signaling in development, disease, and physiology as well as putative targets for pharmacological intervention (Klaus and Birchmeier, 2008). Shortly after, a seven-transmembrane protein with a large, extracellular N terminus was shown to be the product of the *frizzled* locus in *D. melanogaster*. This protein affected tissue polarity; i.e., the *frizzled* gene product was required for the development of a parallel orientation of the cuticular wing hairs and bristles (Vinson and Adler, 1987; Vinson et al., 1989). The *frizzled* gene product was structurally reminiscent of a GPCR.

The *D. melanogaster* model for the analysis of Wingless signaling was also essential to reach the conclusion that WNTs act on FZDs as their ligands: because the effects of Wingless—one of the seven *D. melanogaster* WNTs—were known to be mediated by the product of the *disheveled* locus (Klingensmith et al., 1994) and because mutations in *frizzled* and *disheveled* resulted in similar phenotypes (Krasnow et al., 1995), it was first surmised (Krasnow et al., 1995) and later shown (Bhanot et al., 1996; Wang et al., 1996) that *D. melanogaster* Frizzled 2 functions as *Wingless* receptor. These

and other milestones in the history of WNT/FZD signaling are illustrated in a recent review by Klaus and Birchmeier (2008).

The *smoothened* locus, originally called *smooth*, was identified as a segment polarity gene at the same time as the *hedgehog* and *patched* loci in a mutational screen in *D. melanogaster* (Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1984). Although the HH protein was cloned in the early 1990s and it soon became evident that HH was a secreted protein, it took several years to identify the role of PTCH and SMO as transmembrane-spanning proteins and signaling partners mediating HH effects (Ingham and McMahon, 2001).

Now we know that the WNT/FZD and the HH/SMO signaling systems are evolutionarily highly conserved. With evolution, an intricate and versatile WNT/FZD signaling system developed reflected by a varying number of WNTs (Prud'homme et al., 2002) and FZDs (Schiöth and Fredriksson, 2005) in different organisms (see also Table 1) (Richards and Degnan, 2009). In fact, FZDs are the most highly conserved 7TMRs throughout the animal kingdom, from worm (*C. elegans*) to fly (*D. melanogaster*), fish (*Danio rerio*, *Takifugu rubripes*), and mammals (*Homo sapiens*, *Mus musculus*) (Schiöth and Fredriksson, 2005).

The main focus of this review will be the molecular aspects of FZDs and SMO pharmacology, focusing on structure, signaling, and possibilities for pharmacological targeting of these receptors and the signaling pathways they induce. Because of space limitations, the vast literature on their role in embryonic development, physiology, and disease, as well as the receptor function in specific tissues, will be touched upon only briefly.

III. Class Frizzled Receptors

According to International Union of Basic and Clinical Pharmacology classification, the class Frizzled receptors

FIG. 1. Phylogenetic tree of the human class Frizzled receptors FZD₁₋₁₀ and SMO created with the ClustalW2 software (ver. 2.0.12; <http://www.ebi.ac.uk/Tools/clustalw2/index.html>) using multiple sequence alignment of FZD₁₋₁₀ and SMO as shown in the Supplemental material. Default settings were used. Output format: phylip. Tree type: dist UniProtKB/Swiss-Prot accession numbers used for alignment; see Table 2.

contains 10 mammalian FZDs and SMO (see Fig. 1; for class FZD protein sequence alignments, see Supplemental Fig. S1) (Foord et al., 2005).

Furthermore, homology analysis indicates that FZDs share 20 to 40% identity, which is higher within certain clusters [FZD_{1, 2, 7} (75%), FZD_{5,8} (70%), and FZD_{4,9,10} and FZD_{3,6} (50%)] (Fredriksson et al., 2003) and is also visible in the phylogenetic dendrogram (Fig. 1). The human *frizzled* and *smoothed* genes are distributed on chromosomes 2, 7, 8, 10, 11, 12, and 17 (for exact chromosomal location, see table 1).

A. Frizzled and Smoothed Structure

The first analysis of the *frizzled* locus suggested a basic structure of FZDs with seven-transmembrane-spanning domains of putatively helical character, an extracellular N terminus, and an intracellular C terminus (Fig. 2) (Vinson et al., 1989). The structural information from the primary sequence, therefore, was immediately interpreted to mean that FZD was a G protein-coupled receptor. However, even though the basic architecture is reminiscent of GPCRs, the FZDs and SMO are distinctly different from classic GPCRs. In fact, the differences are so striking that they justified classification of the FZDs into a class Frizzled, a family distinct from the conventional class A, B, or C GPCRs (Foord et al., 2005).

1. The Cysteine-Rich Domain. The extracellular region (Fig. 2; see also Table 2) of all class Frizzled receptors consists of an N-terminal signal sequence (aa 1–36

of the 537 aa² in human FZD₄) that guarantees proper membrane insertion of the protein. This short peptide stretch is followed by the Frizzled domain, the highly conserved cysteine-rich domain (CRD; aa 40–161 in human FZD₄), which may constitute the orthosteric binding site for WNTs (Xu and Nusse, 1998). The three dimensional structure of mFZD₈-CRD, solved by Dann et al. (2001), shows that the CRD consists mainly of α -helical stretches and two short sequences forming a minimal β -sheet at the N terminus representing a novel protein fold. Furthermore, distinct residues and surfaces could be identified that are important for interaction with WNTs. In addition, even though the FZD-CRD in solution exists as a monomer at approximately 100 μ M, the analysis of the crystal, resembling a FZD-CRD concentration of 50 mM, revealed the existence of CRD-CRD dimers.

Ten cysteines that form five disulfide bonds (Cys⁴⁵-S-S-Cys¹⁰⁶; Cys⁵³-S-S-Cys⁹⁹; Cys⁹⁰-S-S-Cys¹²⁸; Cys¹¹⁷-S-S-Cys¹⁵⁸; Cys¹²¹-S-S-Cys¹⁴⁵ in human FZD₄) (Dann et al., 2001; Chong et al., 2002) are highly conserved throughout the FZD isoforms over species as well as in SMO (nine conserved Cys). Given that SMO does not bind to WNTs (Wu and Nusse, 2002), the conserved sequence suggests that the CRD plays an important functional role other than ligand recognition. Moreover, similar CRDs with functional relevance for WNT signaling are present in other proteins (Xu and Nusse, 1998), such as the closely related soluble Frizzled-related pro-

² Sequence and structural information on FZDs/SMO comes from UniProtKB/Swiss-Prot database available at <http://www.uniprot.org>.

FIG. 2. Simplified scheme of Frizzled structure and extracellular and intracellular binding partners. Extracellular and intracellular interaction partners are listed in the light gray squares. For details see section III and Schulte and Bryja (2007); Wawrzak et al. (2009). GIPC, GAIP interacting protein, C terminus; GRIP-1, glutamate receptor interacting protein 1; MAGI-3, membrane-associated guanylate kinase inverted-3; PTP-BL, protein tyrosine phosphatase-basophil-like; GOPC, Golgi-associated PDZ and coiled-coil motif-containing protein; PATJ, PALS-1-associated tight junction protein; SHISA, named after Shisa, a form of sculpture common to southern Japan with a large head similar to the Egyptian sphinx.

TABLE 2
Characteristics of human Class FZD receptors

Receptor	Gene Name: Human (Rat/Mouse)	NCBI Accession No.	Chromosomal Location	UniProtKB/ SwissProt Accession No.	Protein Length aa	Molecular Mass kDa	Number of		IUPHAR Database URL
							Transcripts	Exons	
FZD ₁	<i>FZD1 (Fzd1)</i>	NP_003496	7q21	Q9UP38	647	71.158	1	1	ETTV (+) http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?familyId=25&objectId=229
FZD ₂	<i>FZD2 (Fzd2)</i>	NP_001457	17q21.1	Q14332	565	63.554	1	1	ETTV (+) http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?familyId=25&objectId=230
FZD ₃	<i>FZD3 (Fzd3)</i>	NP_059108	8p21	Q9NPG1	666	76.263	1	8	GTTSA (+) http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?familyId=231&familyId=25
FZD ₄	<i>FZD4 (Fzd4)</i>	NP_036325	11q14.2	Q9ULV1	537	59.881	1	2	ETTV (+) http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?familyId=232&familyId=25
FZD ₅	<i>FZD5 (Fzd5)</i>	NP_003459	2q33-q34	Q13467	585	64.507	1	2	LSHV (+) http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?familyId=233&familyId=25
FZD ₆	<i>FZD6 (Fzd6)</i>	NP_003497	8q22.3-q23.1	O60353	706	79.292	1	7	HSDT (-) http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?familyId=234&familyId=25
FZD ₇	<i>FZD7 (Fzd7)</i>	NP_003498	2q33	O75084	574	63.620	1	1	ETAV (+) http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?familyId=235&familyId=25
FZD ₈	<i>FZD8 (Fzd8)</i>	NP_114072	10p11.22	Q9H461	694	73.300	1	1	LSQV (+) http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?familyId=236&familyId=25
FZD ₉	<i>FZD9 (Fzd9)</i>	NP_003499	7q11.23	O00144	591	64.466	1	1	PTHL (-) http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?familyId=237&familyId=25
FZD ₁₀	<i>FZD10 (Fzd10)</i>	NP_009128	12q24.33	Q9ULW2	581	65.336	1	1	PTCV (+) http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?familyId=238&familyId=25
SMO	<i>SMO (Smo)</i>	NP_005622	7q32.3	Q99835	787	86.397	4	25	DSDF (+) http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?familyId=239&familyId=25

+ or - in the C Terminus column indicates experimental evidence for or against, respectively, the functionality of the class Frizzled receptor C terminus as PDZ ligand domain.

teins (SFRPs) (Rattner et al., 1997), the receptor tyrosine kinase-like orphan receptor (ROR) (Masiakowski and Carroll, 1992), the muscle-specific tyrosine kinase (Masiakowski and Yancopoulos, 1998), carboxipeptidase Z (Moeller et al., 2003), and an isoform of collagen XVIII (Quélard et al., 2008). The FZD-CRD also contains Asn residues predicted to be N-glycosylated. This post-translational modification could have importance for ligand recognition and thereby receptor function.

2. *Transmembrane and Intracellular Domains.* The CRD of FZDs is connected to the transmembrane core of the receptor by a linker region of variable length (aa 161–222 in human FZD₄). Furthermore, seven transmembrane regions (TM1, aa 223–243; TM2, aa 255–275; TM3, aa 303–323; TM4, aa 345–365; TM5, aa 390–410; TM6, aa 437–457; TM7, aa 478–498) give rise to three intracellular loops, three extracellular loops, and a C terminus of variable length (see also Fig. 2 and Supplemental Fig. S2). As is common for GPCRs, class FZD receptors contain conserved cysteines in the extracellular loops 1 and 2, which could engage in stabilizing disulfide bonds. In fact, disruption of the cysteine on extracellular loop 2 of SMO leads to disruption of receptor function (Lagerström and Schiöth, 2008). Other domains are lacking in class Frizzled receptors that are important in many GPCRs; for example, for G protein coupling and specificity, the DRY motif at the C-terminal end of TM3, as well as the NPxxxY motif at the end of TM7 (Wess, 1998). However, a series of charged residues at the C- and N-terminal ends of intracellular loop 3, which are also known to be essential for receptor-G protein coupling, are present in both FZDs and SMO (see also Supplemental Material).

The recent progress in structural analysis of GPCRs suggested also the presence of an α -helical stretch at the C terminus of GPCRs, the so called helix 8 (Palczewski et al., 2000; Rasmussen et al., 2007; Jaakola et al., 2008). This helix stands almost perpendicular to TM7, parallel and close to the plasma membrane. The prerequisite for helix 8 formation and presence seems to be one or more cysteine residues that anchor the C terminus by palmitoylation in the membrane, allowing stabilization of an additional loop and formation of the short helix 8. Helix 8 was implicated as a determinant of G protein coupling (Wess et al., 2008). In addition, some of the class FZD receptors can putatively form a helix 8 at their C termini, although direct experimental evidence is lacking. However, because of the lack of C-terminal cysteines, which could serve as palmitoylation site, human FZD_{1, 2, 7} cannot form helix 8, whereas the other FZDs and SMO contain C-terminal cysteines (Supplemental Fig. S2). In conclusion, the presence of some but not all common GPCR motifs underlines again the separate classification of FZD and SMO into a separate class of receptors (Foord et al., 2005).

3. *Motifs for Post-Translational Modifications.* Intracellular domains are functionally essential because

they provide a platform for various protein-protein interactions, and post-translational processing through phosphorylation, ubiquitination, nitrosylation, or hydroxylation known to regulate GPCR function (DeWire et al., 2007; Ozawa et al., 2008; Xie et al., 2009). Analysis of the intracellular regions with the MiniMotifMiner, a search motor for the identification of conserved sequence motifs (Balla et al., 2006), shows that class FZD receptors provide an intricate interaction surface for serine/threonine and also some tyrosine kinases (Supplemental Fig. S3, Table 3). This topic has so far been almost completely neglected (Yanfeng et al., 2006) in the case of FZDs. In SMO, however, mass spectroscopic studies have identified 26 Ser/Thr residues in the SMO C terminus that were phosphorylated in HH-stimulated cells; these residues partially resemble cAMP-dependent protein kinase (PKA) and casein kinase 1 (CK1) phosphorylation sites (Zhang et al., 2004). HH-mediated phosphorylation of the SMO C terminus was shown to be required for SMO activity (Jia et al., 2004) and to induce a conformational switch required for the disruption of intramolecular electrostatic interaction of an autoinhibitory domain and a phosphorylation-dependent increase in SMO surface expression (Zhao et al., 2007). In addition the Ser/Thr GPCR kinase 2 (GRK2) was shown to associate with and phosphorylate SMO in an activity-dependent manner (Chen et al., 2004b). It should be mentioned at this stage that GRK consensus motifs are not yet well characterized, and even though GRKs are

well known regulators of the function of GPCRs, including FZD and SMO, these motifs appear not to be detected by the Mini Motif Miner software.

In addition to constructive post-translational modification, proteolytic cleavage was described as a means of FZD signal transduction downstream of FZD₂ in *D. melanogaster* (Mathew et al., 2005; Ataman et al., 2006). Wingless stimulation of FZD₂ in neurons induces the endocytosis of FZD₂, its translocation to the cell body, and C-terminal cleavage. Subsequently, the C terminus locates to the nucleus for transcriptional regulation. So far, however, it is unclear whether this C-terminal cleavage of FZD₂ in *D. melanogaster* is a species- or receptor-specific feature or represents a more general means of FZD-mediated transcriptional regulation.

B. Class Frizzled Receptors as PDZ Ligands

Despite our limited knowledge of post-translational modifications on the intracellular domains, we know more about protein-protein interactions with FZDs. Most striking is the completely conserved KTxxxW domain in the C terminus of all FZDs, but not SMO, starting very close to the membrane (Fig. 2). This sequence is essential for FZD signaling and serves as an internal, atypical postsynaptic density 95/disc-large/zona occludens-1 (PDZ) ligand domain for interaction with the PDZ domain of the phosphoprotein Disheveled (DVL), an important signaling platform for many WNT/FZD signaling pathways (Umbhauer et al., 2000; Wall-

TABLE 3
Phenotypes of mice with altered class Frizzled receptor expression

Receptor	Genotype	Phenotype	References
FZD ₁	Unknown	Viable	van Amerongen and Berns, 2006
FZD ₂	Unknown	Viable	van Amerongen and Berns, 2006
FZD ₃	FZD ₃ (-/-)	Postnatally lethal, Severe defects in major axon tracts within the forebrain	Wang et al, 2002
		Anterior-posterior guidance of commissural axons	Lyuksyutova et al, 2003
FZD ₄	FZD ₃ (-/-), FZD ₆ (-/-) FZD ₄ (-/-) FZD ₄ (-/-)	Severe midbrain morphogenesis defect Cerebellar, auditory, and esophageal dysfunction Infertility and impaired corpora lutea formation and function	Stuebner et al, 2010 Wang et al, 2001 Hsieh et al, 2005
FZD ₅	Fz4 ^{CKOAP/-} ;Tie2-Cre (endothelium) FZD ₅ (-/-)	Defects in vascular growth, endothelial defects Embryonically lethal, essential for yolk sac and placental angiogenesis	Ye et al, 2009b Ishikawa et al, 2001
	Fz5LoxP/LoxP-K19Cre (intestinal epithelium) Fz5 ^{CKO-AP/lacZ} ;Sox2-Cre and Fz5 ^{CKO-AP/lacZ} , R26-CreER	Paneth cell phenotype Neuronal survival, ocular development and late onset retinal degeneration	van Es et al, 2005 Liu and Nathans, 2008; Liu et al, 2008a
FZD ₆	Fz6(-/-) nlacZ	Hair patterning, tissue polarity	Guo et al, 2004
FZD ₇	Unknown	Viable	van Amerongen and Berns, 2006
FZD ₈	Unknown	Viable	van Amerongen and Berns, 2006
FZD ₉	FZD ₉ (-/-)	Abnormal B cell development, moderately reduced lifespan, splenomegaly, and accelerated thymic atrophy	Ranheim et al, 2005
	FZD ₉ (-/-) IRES lacZ	Developmental neuroanatomical defects in hippocampus and visuospatial learning deficits (comparable to Williams syndrome)	Zhao et al, 2005
FZD ₁₀			van Amerongen and Berns, 2006
SMO	SMO(-/-)	Embryonically lethal, defects in L/R patterning	Zhang et al, 2001

ingford and Habas, 2005; Gao and Chen, 2010). However, mutation analysis with FZD-mediated DVL recruitment as the measure revealed that not only the lysine in the KTxxxW sequence but also residues in the intracellular loops 1 and 3 (R340A, L524A, and K619A in rat FZD₁) are required for signaling and DVL interaction, indicating that DVL might also engage surfaces different from the FZD C terminus in FZD-DVL binding (Cong et al., 2004a). Furthermore, electrostatic interactions between DVL and negatively charged phospholipids in the plasma membrane regulate FZD recruitment of DVL (Simons et al., 2009). Even though the KTxxxW sequence is 100% conserved among all FZDs, the affinity of FZD-derived peptides spanning the KTxxxW and amino acids adjacent to DVL differs among FZD isoforms (Punchihewa et al., 2009). This suggests that some FZDs bind DVL loosely or through structures other than the KTxxxW ligand sequence.

It is noteworthy that a very recent study shows not only that the FZD-DVL interface is conserved in FZDs but also that unrelated GPCRs can interact with DVL through their C-terminal tails (Romero et al., 2010). The parathyroid hormone receptor contains a KSxxxW sequence, which is suitable for DVL binding and stimulation of the parathyroid receptor results in dephosphorylation and stabilization of β -catenin downstream of DVL.

In contrast to the internal PDZ ligand domain, the far C-terminal stretch of some but not all FZDs serves as a conventional, terminal class I PDZ ligand (Jeleń et al., 2003; Nourry et al., 2003) (see also Table 2) for a steadily growing list of PDZ domain proteins of various and partly unknown function (Fig. 2) (Schulte and Bryja, 2007; Wawrzak et al., 2009). As indicated in Table 2, the C-terminal sequence of the class Frizzled receptors varies. The common PDZ ligand motif resembles the sequence X-S/T-X Φ , where Φ represents a hydrophobic residue. Thus, so far, PDZ interaction has been shown for FZD_{1, 2, 3, 4, 5, 7, 8} but not for FZD_{6, 9, 10} and SMO (Tan et al., 2001; Yao et al., 2001, 2004; Hering and Sheng, 2002) as could be expected by the presence of the PDZ ligand motif in those receptors.

C. Lipoglycoproteins as Receptor Ligands

FZDs were originally identified as WNT receptors in *D. melanogaster* (Bhanot et al., 1996). With the limited number of FZDs and WNTs in *D. melanogaster*, it was also possible to investigate WNT-FZD interaction profiles (Hsieh et al., 1999b; Wu and Nusse, 2002), but the specificity of interaction between WNTs and FZDs in vertebrates remains largely unmapped and obscure (Hsieh, 2004; Kikuchi et al., 2009). As detailed in section VIII, HH does not bind SMO but rather the regulatory transmembrane-spanning protein PTCH; thus, it would be misleading to discuss HH and SMO as a ligand-receptor pair.

WNTs and proteins of the HH family are morphogenes affecting embryonic patterning (Nusse, 2003). Their involvement in multiple aspects of development and devastating diseases such as cancer made them a major focus of biomedical research (Taipale and Beachy, 2001; Chien et al., 2009). The WNT family is presently known to contain 19 mammalian WNTs,³ whereas there are three members of the HH family: sonic (SHH), indian, and desert HH. Despite intensive efforts, isolation of pure and biologically active WNTs was not possible until a major breakthrough when Willert et al. (2003) discovered that WNT-3A is lipid-modified, a fact that needs to be taken into consideration during purification.

Purification of WNT-2, WNT-3A, and WNT-5A from conditioned medium was described by the Willert group and others (Willert et al., 2003; Schulte et al., 2005; Mikels and Nusse, 2006; Willert, 2008; Sousa et al., 2010). For this purpose, conditioned medium from WNT overexpressing mammalian cells is harvested and fractionated with affinity chromatography using BlueSepharose, which binds rather selectively to WNT proteins. The next step is immobilized metal affinity chromatography followed by Superdex gel filtration and, finally, heparin cation exchange (affinity) chromatography (Willert, 2008). This protocol with minor modifications has successfully been used to purify WNT-2, WNT-3A, -5A, -7A, -16, *D. melanogaster* WNTD, and Wingless (Willert, 2008; Sousa et al., 2010). Even though several WNTs could successfully be purified according to the protocol established by Karl Willert, it should be emphasized that remaining WNTs still resist purification in a biologically active form. Hopefully our increasing understanding of WNT-binding proteins that might be necessary for stabilizing (or solubilizing) WNTs will lead to increasing availability of pure and active WNTs (Lorenzowicz and Korswagen, 2009).

Heparin affinity was also used for purification of active SHH-N (Roelink et al., 1995). However, in the case of SHH, it was possible—in contrast to WNT purification—to yield active protein from overexpression in *Escherichia coli*. Furthermore, some purified and biologically active WNTs and HHs are commercially available from R&D Systems (Minneapolis, MN). The crucial factor for successful purification of WNTs was a high concentration of detergents (for example, 1% CHAPS). Detergents are required to solubilize the intact, biologically active, and lipophilic WNT. Palmitoylation and glycosylation are important for WNT secretion, activity, stability, and function (Port and Basler, 2010). The emerging picture ascribes glycosylation an important function in WNT processing and secretion as shown in the case of WNT-3A and -5A (Komekado et al., 2007; Kurayoshi et al., 2007). The role of the lipid modifications, however, seems to be more complicated and divergent. WNTs

³ For more information on WNTs, visit Roel Nusse's WNT homepage at <http://www.stanford.edu/~rnusse/wntwindow.html>.

carry two lipidations, a palmitate (at Cys77 of WNT-3A) and a palmitoleic acid (at Ser209 of WNT-3A) (Willert et al., 2003; Takada et al., 2006). Apparently, these post-translational modifications have different tasks: the palmitate renders the WNT highly lipophilic and is necessary for receptor binding, internalization, and signaling (Willert et al., 2003; Schulte et al., 2005; Komekado et al., 2007; Kurayoshi et al., 2007). On the other hand, removal of the palmitoleic acid impedes transport of WNT from the endoplasmic reticulum to the Golgi apparatus, thereby impairing WNT secretion (Neumann et al., 2009). Recent *in vivo* data even suggest that the palmitoleic acid modification is not absolutely required for secretion but has an important part in maintaining WNT signaling capacities (Franch-Marro et al., 2008). In this context, it is noteworthy that the *D. melanogaster* WNTD carries no lipid modifications and is still properly secreted and functional (Ching et al., 2008).

Post-translational modifications suggest a highly regulated processing and secretion of these proteins but also indicate that some kind of trick is required to make the proteins soluble and enable extracellular diffusion in aqueous solutions (Lorenowicz and Korswagen, 2009). Indeed the recent discovery of carrier proteins that transport WNTs extracellularly, such as high- and low-density lipoprotein, explains why a high concentration of detergent is needed to harvest WNTs from conditioned medium in an active form and the apparent conundrum that lipophilic molecules are solubilized in aqueous medium (Neumann et al., 2009).

HH proteins, such as SHH, not only undergo N-glycosylation and lipid modification but also are proteolytically cleaved to give rise to a 19-kDa N-terminal peptide and a 27-kDa C-terminal peptide of which the SHH-N is the active ligand (Bumcrot et al., 1995; Ingham and McMahon, 2001).

IV. Coreceptors

A. Extracellular Matrix Components

FZDs and PTCH are not the only membrane-associated molecules binding WNTs and HHs. Other WNT/HH binding sites have been described. Interactions with extracellular matrix components such as heparan sulfate-modified glypicans (Filmus et al., 2008) are generally important for growth factor signaling and are known to both positively and negatively modify WNT and HH signaling (Fico et al., 2007). Glypicans (Knypek in zebrafish, Dally in the fruit fly) were shown to cooperate with FZDs to mediate signaling and to assist short- and long-distance WNT and HH communication (Lin and Perrimon, 1999; Eugster et al., 2007). This is achieved by concentration of WNT molecules at the cell surface and maintenance of solubility of WNT proteins, which is strictly required for their biological activity (Fuerer et al., 2010). The complexity of glypican action on WNT signaling also became evident in a mouse model of glypi-

can loss of function, which led to the inhibition of a WNT-5A pathway and an enhancement of WNT/ β -catenin signaling (Song et al., 2005), indicating a modulator effect of glypicans on different branches of WNT signaling. How is WNT interaction with the extracellular matrix relevant for FZD pharmacology? It seems that more WNT molecules can bind a cell than are engaged in signal transduction. In other words, WNT binding to cells might not all interact with the transducing receptor. This has important implications for pharmacological *in vitro* experiments, such as the generation of dose-response relationships: because the extracellular matrix could serve as a ligand buffer, the added ligand concentration does not necessarily reflect the actual concentration of the ligand close to the receptor (M. Kilander, G. Schulte, unpublished observations: WNT-5A binding to living mammalian cells is unsaturable in reasonable range of concentrations). In addition, it is currently impossible to distinguish between FZD and non-FZD WNT binding sites because of the lack of appropriate technology. In the case of HH signaling, intriguing species differences appeared (Beckett et al., 2008), indicating that glypicans could mediate both positive and negative effects on HH-induced SMO signaling.

B. Low-Density Lipoprotein Receptor-Related Protein 5/6

A number of single transmembrane domain receptors have been identified that can act as either coreceptors with FZDs and/or autonomous WNT receptors, such as LRP5/6, ROR1/2, and RYK. The LDL receptor-related proteins 5/6 (LRP5/6) belong to a superfamily of at least 10 LRPs that have important functions in endocytosis, cellular communication, embryonic development, lipid homeostasis, and disease (Li et al., 2001; May et al., 2007). Regarding WNT/FZD communication, LRP5/6 are crucial for the transmission of β -catenin-dependent signaling through, for example, WNT-1 or WNT-3A. Arrow, the *D. melanogaster* counterpart to LRP5/6, was discovered in genetic studies in which Arrow mutants showed a phenotype similar to that of Wingless mutants (Wehrli et al., 2000). LRP5/6 collaborate with FZDs through formation of a ternary WNT/FZD/LRP5/6 complex (Tamaï et al., 2000; Wehrli et al., 2000). In contrast to FZDs, LRP5/6 do not contain any CRD-WNT binding domains; rather, their N-terminal β propeller epidermal growth factor repeats are essential for the interaction with the WNT/FZD complex (Hey et al., 1998; Liu et al., 2009; Bourhis et al., 2010). In fact, a recent mapping study identified unique binding patterns of different WNTs to the extracellular domains of LRP6, showing that several WNTs, such as WNT-3A and WNT-9B, can bind simultaneously (Bourhis et al., 2010).

C. Receptor Tyrosine Kinases as WNT Receptors

Whereas LRP5/6 act as WNT coreceptors with FZD, it remains somewhat unclear whether the receptor

tyrosine kinases ROR1/2 (ROR in *D. melanogaster*; CAM-1, CAN cell migration defective in *C. elegans*) and RYK [called Derailed (Callahan et al., 1995) in *D. melanogaster*; LIN-18 in *C. elegans*] should be seen as autonomous WNT receptors, FZD coreceptors, or possibly both. ROR is associated with genetic skeletal disorders such as dominant brachydactyly and recessive Robinow syndrome (Minami et al., 2009). ROR1/2 use their N-terminal CRD for WNT binding followed by WNT-induced receptor dimerization, subsequent kinase activation, and cross-autophosphorylation of the intracellular domains. This indicates that ROR1/2 could act independent of FZD, similar to classic receptor tyrosine kinases (Liu et al., 2008b; Minami et al., 2009) with important roles for convergent extension movements, a key tissue movement organizing mesoderm, ectoderm, and endoderm in vertebrate embryos (Unterseher et al., 2004; Schambony and Wedlich, 2007; Davidson et al., 2010). Furthermore, it has been shown that the presence of ROR2 in HEK293 cells is required for WNT-5A-mediated inhibition of FZD-dependent WNT/ β -catenin signaling (Mikels and Nusse, 2006; Witte et al., 2010), emphasizing that receptor context underlies signaling trafficking through WNT receptors (van Amerongen et al., 2008). Furthermore, WNT-3A/FZD signaling to β -catenin is modulated by ROR2 through selective cooperation with FZD₂ (Li et al., 2008). The cooperation between FZD and ROR is—at least under some circumstances—mediated by a secreted glycoprotein called CTHRC1, which aids to stabilize a ROR/FZD/WNT complex (Yamamoto et al., 2008).

RYK [receptor tyrosine kinase; Derailed (DRL) in the fly, and LIN-18 in the worm] is a single transmembrane receptor with a glycosylated N-terminal ligand-binding domain and an internal C terminus harboring an S/T domain, a protein tyrosine kinase domain, and a PDZ domain (Gao and Chen, 2010). The WNT binding domain of RYK shows no characteristics of a CRD but has homology to WNT inhibitory factor (WIF) (Patthy, 2000; Fradkin et al., 2010). Although the kinase domain of this atypical receptor tyrosine kinase is unusual in structure and is nonfunctional (Hovens et al., 1992; Katso et al., 1999; Yoshikawa et al., 2001) RYK nonetheless transduces important signals upon WNT binding, such as mediating axon guidance (Yoshikawa et al., 2003) and neurite outgrowth (Lu et al., 2004). In cooperation with FZD and DVL, RYK supports signaling induced by WNTs that mainly act through the WNT/ β -catenin pathway (Lu et al., 2004). WNTs that act in a β -catenin-independent manner, such as WNT-5A, can also recruit RYK, thereby increasing the release of intracellular Ca^{2+} (Li et al., 2009). Furthermore, RYK was identified as an important cofactor in WNT-11-induced internalization of FZD₇ in complex with DVL and β -arrestin in *X. laevis* (Kim et al., 2008).

D. Class Frizzled Receptor Dimerization

To be complete, any discussion of FZD coreceptors must include the FZDs themselves. It is well established that homo- and heterodimerization or -oligomerization is a general feature of classic GPCRs that can be required for receptor maturation and ontogeny, signaling, and modulation (Bulenger et al., 2005; Pin et al., 2007; Milligan, 2009). Not surprisingly, FZDs (FZD₃ in *X. laevis*) have been reported to dimerize through CRD-CRD interaction (Carron et al., 2003), as could be expected from the FZD-CRD structure (Dann et al., 2001). It is noteworthy that FZD-FZD interaction is sufficient to activate WNT/ β -catenin signaling. In theory, the presence of a CRD in both FZDs and SMO suggests a possible homo/heterodimerization of these receptors. In the case of SMO, receptor interaction can also be promoted by HH-induced SMO phosphorylation of an autoinhibitory domain in the C-terminal tail, which results in SMO-SMO interaction (Zhao et al., 2007). Class Frizzled receptor-receptor interaction would have a major impact on their signaling, pharmacology, and ligand binding modes and should also be taken into account, in addition to the possibility for class Frizzled receptors to establish contact to other GPCRs.

V. Patched—The Hedgehog Receptor

PTCH is a 12-membrane domain-spanning molecule that exists in two mammalian isoforms, PTCH1 and -2. PTCH is a central figure in HH/SMO signal transduction because it acts as a constitutive SMO inhibitor and is regulated by HH binding (Chen and Struhl, 1996; Stone et al., 1996). PTCH shows homology to proton-driven transmembrane molecular transporters in bacteria, and mutations in residues that are essential for transporter function affect PTCH (Taipale et al., 2002). The dissection of the molecular mechanisms underlying PTCH-mediated inhibition of SMO and the release of inhibition through HH binding to PTCH has been difficult and is still ongoing. It is noteworthy that PTCH-mediated inhibition is accomplished not by direct physical interaction with SMO but by a catalytical process based on lipids derived from lipoproteins that destabilize SMO (Taipale et al., 2002; Khaliullina et al., 2009). In addition, the PTCH-dependent inhibition and its termination by HH stimulation depend on a complicated cycle of protein dynamics as described in section X.B in the discussion of class Frizzled receptor dynamics (Fig. 5).

VI. Non-WNT Extracellular Binding Partners of Frizzleds

A. Soluble Frizzled-Related Proteins

The family of soluble Frizzled-related proteins, the SFRP1–5, is structurally related to the WNT-binding domain of the FZDs and was suggested to sequester WNTs, thereby blocking their interaction with FZDs

(Rattner et al., 1997; Kawano and Kypta, 2003). Indeed, SFRPs were shown to interact with Wingless but with a surprising ratio larger than 1:1, indicating not only WNT-CRD interaction but also higher complex formation (Rattner et al., 1997; Uren et al., 2000). This is indeed further supported by the fact that SFRP mutants lacking the CRD retain the ability to interact with Wingless (Uren et al., 2000). Similar to WNT/FZD binding, the Wingless/SFRP1 interaction could be enhanced by addition of heparin. In functional assays, such as the β -catenin stabilization assay, biphasic effects of SFRPs were reported, showing that low SFRP1 concentrations promote, whereas high SFRP1 concentrations decrease Wingless-induced β -catenin stabilization (Uren et al., 2000).

Unlike WNTs, SFRPs are not lipid-modified and are therefore more readily available in purified form (Wolf et al., 2008c). From the initial assumption that SFRPs functioned merely as scavengers of WNTs, thereby affecting WNT/FZD interaction negatively, the spectrum of known SFRP-mediated effects has developed dramatically and now includes essential functions in development and disease (Bovolenta et al., 2008). It is noteworthy that SFRPs, as CRD-containing proteins, can dimerize with other CRD proteins and thus also with FZDs (Bafico et al., 1999; Dann et al., 2001). In fact, SFRP1 was identified as a regulator of axonal growth by direct agonistic interaction with FZD₂ (Rodriguez et al., 2005). Most recent evidence indicates that WNT/SFRP interaction increases WNT diffusion and enhances long-distance signaling via WNTs (Mii and Taira, 2009).

B. *Norrin*

The Norrie disease in humans is an X chromosome-linked eye disorder resulting in postnatal blindness as a result of impaired retinal development (Berger, 1998). It is caused by disruption of the gene coding for the Norrie disease protein, also termed *norrin* (Hendrickx and Leyns, 2008). The observation that mice with mutations in FZD₄ had phenotypes strikingly similar to those of mouse models of Norrie disease (Berger et al., 1996) prompted investigation of the molecular relationship between these two proteins. To general surprise, Xu et al. (2004) discovered that *Norrin* is indeed a FZD₄-selective endogenous agonist with no structural resemblance to WNTs. Interaction of *Norrin* with FZD and the coreceptor LRP5/6 activates the WNT/ β -catenin signaling pathway. Careful analysis of the *Norrin*-FZD₄ interactions revealed that *Norrin* binds to the CRD of FZD₄ at regions overlapping those engaged by WNT-8, whereas no binding could be detected to any other FZD- or SFRP-CRD (Smallwood et al., 2007).

C. *Dickkopf*

Another group of proteins should also be discussed in this context, namely the *Dickkopf* family (DKK) (MacDonald et al., 2009). Although these proteins interact

with accessory proteins and not with FZD directly, they affect the formation of LRP5/6-FZD complexes and are therefore considered WNT blockers.

DKKs, DKK1–4 (no counterpart in *D. melanogaster*), and the DKK-like protein 1 (*soggy* in *D. melanogaster*) are secreted glycoproteins (Krupnik et al., 1999; Niehrs, 2006) and are seen as negative regulators of WNT/ β -catenin signaling (Glinka et al., 1998). DKK, especially the structurally and functionally more closely interrelated homologs 1, 2, and 4, exert their effect by direct interaction with the extracellular β propeller domains of LRP5/6, thereby preventing formation of the ternary complex WNT/FZD/LRP5/6 (Mao et al., 2001; Bourhis et al., 2010). The inhibition of WNT/ β -catenin signaling is substantiated through DKK-induced and Kremen (KRM)-mediated endocytosis of LRP5/6 (Davidson et al., 2002; Mao et al., 2002), even though the general importance of KRM for DKK function is limited (Ellwanger et al., 2008). It has become clear that KRM can inhibit or augment signaling to β -catenin, depending on whether or not DKK is present. Although DKK binding to LRP5/6 will lead to a KRM-mediated internalization and thereby a desensitization of the pathway, unbound KRM can also stabilize LRP5/6 at the plasma membrane, thereby hypersensitizing the system (Cselenyi and Lee, 2008).

D. *R-Spondin*

The *R-spondin* family consists of four homologs of secreted molecules with no representatives present in *C. elegans*, *D. melanogaster*, or *Saccharomyces cerevisiae*, indicating that they are restricted to vertebrates (Kim et al., 2006; Hendrickx and Leyns, 2008). *R-spondin* binds LRP6, induces its phosphorylation, and promotes β -catenin stabilization, similar to WNTs (Wei et al., 2007). According to the current model, *R-spondin* does not directly activate LRP6; it requires the presence of WNTs and blocks DKK-induced endocytosis of LRP6 to ensure an appropriate receptor density in the membrane for WNT signaling. In detail, *R-spondin* interferes with the DKK-dependent association of LRP6 and KRM, thereby uncoupling them from the endocytotic machinery (Binners et al., 2007).

In summary, the presence of various secreted modulators of WNT/FZD function (Fig. 3) allows distinct fine-tuning of signaling where the cellular response is determined, not only by the density of receptor expression and the ligand concentration, but also by the kind and concentration of the third party modulator. To further complicate an already complex system, even more additional factors are expressed that modify WNT action, but do not relate directly to FZDs and are therefore not discussed here in detail: WIF and connective tissue growth factor (Hsieh et al., 1999a; Mercurio et al., 2004; Semenov et al., 2005; MacDonald et al., 2009). However, another endogenous peptide that has been shown to bind FZDs, the amyloid peptide β , accumulates and forms

FIG. 3. Soluble factors affect WNT/FZD signaling through the interaction with WNT, FZD, or LRP5/6. Factors depicted in red act negatively on WNT signaling by direct interaction with WNT or interference with WNT/FZD/LRP5/6 complex formation (DKK). Factors depicted in green, on the other hand, can act as agonists on FZDs (norrin acts specifically through FZD₄ in collaboration with LRP5/6). Mechanisms of action of *R*-spondin (RSPO) are still unclear but involve interaction with FZD and LRP5/6 and possibly cooperation with WNTs. SFRP act through direct binding to FZDs by CRD-CRD interaction.

senile plaques in the brain of patients with Alzheimer's disease (Magdesian et al., 2008). Amyloid peptide β directly interacts with the FZD₄- and FZD₅-CRD and acts there as a competitive antagonist, blocking WNT-induced signaling to β -catenin. This could be important for the development of Alzheimer's disease.

VII. WNT/Frizzled Signaling Paradigms

In the past, WNT/FZD signaling (Fig. 4) was categorized according to the ability of WNT ability to transform mammary C57MG cells (Wong et al., 1994), which is correlated to the recruitment of β -catenin-dependent or -independent signaling. Because β -catenin-dependent WNT/FZD signaling was identified first, this was designated "canonical," and pathways independent of β -catenin were consequently named "noncanonical" WNT signals. However, WNT/ β -catenin signaling can by no means be seen as a default pathway, and in view of the increasing complexity of the WNT signaling networks, this review avoids this outdated nomenclature and instead refers to the pathways by the main components involved.

So far, it is unclear what defines the bias of a WNT toward a certain signaling path (Kikuchi et al., 2009). Several factors *X* have been suggested and identified. The most obvious way to achieve specificity in a signaling system composed of 10 FZDs and 19 mammalian WNTs would be a specific ligand-receptor interaction profile (Hsieh, 2004). Some degree of specificity was shown for the *D. melanogaster* FZDs and WNTs (Hsieh et al., 1999b; Wu and Nusse, 2002), whereas WNT-FZD interaction profiles and binding affinities of the mammalian proteins have not yet been completely mapped (Hsieh, 2004; Kikuchi et al., 2009). In addition, recruit-

ment of certain coreceptors is important (Mikels and Nusse, 2006; van Amerongen et al., 2008; Kikuchi et al., 2009).

A. Molecular Details: WNT/ β -Catenin Signaling

Despite intensive studies aimed at clarify aspects of WNT/ β -catenin signaling, important mechanisms in this branch remain partially obscure. It is well established that the WNT/ β -catenin pathway is initiated through a close collaboration of FZD with LRP5/6. The ternary complex of WNTs, such as WNT-1 or -3, FZD, and LRP5/6, mediates the inhibition of a constitutively active destruction complex. In the absence of WNTs, the cytosolic destruction complex keeps soluble β -catenin levels low (Klaus and Birchmeier, 2008; MacDonald et al., 2009) through constitutive phosphorylation by glycogen synthase kinase 3 (GSK3) and casein-kinase 1 (CK1). Phosphorylated β -catenin is then directed to β -transducin repeat-containing protein-dependent ubiquitinylation and subsequent proteasomal degradation. The destruction complex is a complicated, multifunctional protein assembly, with important constituents, such as β -catenin kinases, the tumor suppressor gene product adenomatous polyposis coli, and axin, a negative regulator/repressor of WNT/ β -catenin signaling.

To transduce a WNT signaling from the cell surface to the destruction complex, WNTs induce collaboration of FZDs and the coreceptor LRP5/6 through direct interaction with both transmembrane receptors. This in turn results in rapid Ser/Thr phosphorylation of LRP5/6 on five PPPPS/TP motifs in a CK1- and GSK-3-dependent manner (Zeng et al., 2005; MacDonald et al., 2008; Wolf et al., 2008b; Niehrs and Shen, 2010). These kinases, however, are not the only LRP5/6 kinases so far identified (Niehrs and Shen, 2010). In fact, a cyclin-dependent kinase L63 was recently pointed out as a cell cycle- and cyclin Y-dependent LRP5/6 kinase, opening the possibility that other proline-directed kinases regulate LRP5/6 (Davidson et al., 2009). In addition, GRK5/6 were identified as LRP6 kinases able to phosphorylate the identical PPPPS/TP motifs targeted by GSK3 as well as additional residues in the C terminus of LRP6 (Chen et al., 2009a).

WNT binding to FZDs and LRP5/6s induces a series of protein redistributions finally leading to the stabilization of β -catenin (Yokoyama et al., 2007). LRP5/6 phosphorylation and subsequent activation of signaling requires the recruitment of components of the destruction complex, such as GSK3, CK1, and axin. Reorganization of these compounds to the cell surface is a crucial event in WNT/ β -catenin signaling because it decreases activity of the destructon complex, resulting in decreased β -catenin phosphorylation and subsequent stabilization in the cytosol. LRP5/6 is activated and phosphorylated, and the consequent formation of a submembraneous complex with many different partners goes hand in hand with rapid LRP5/6 relocalization from a broad mem-

FIG. 4. Overview of WNT/FZD signaling. Schematic view of the WNT/ β -catenin, WNT/RAC and RHO, WNT/ Ca^{2+} , PCP, WNT/cAMP and WNT/ROR pathways is shown. Observe that the compiled information originates from different animal kingdoms and species. For detailed information see section XII. AC, adenylyl cyclase; ATF2, activating transcription factor 2; β -arr, β -arrestin; DARPP32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; DAG, diacylglycerol; JNK, c-Jun N-terminal kinase; c-JUN, oncogene, JUN family of transcription factors; CDK, cyclin-dependent kinase; DAAM, Dishevelled-associated activator of morphogenesis; G_s , stimulatory heterotrimeric G protein; G_i , inhibitory heterotrimeric G protein; MKK7, mitogen-activated protein kinase kinase 7; NFAT, nuclear factor of activated T cells; PAR1, Ser/Thr kinase, partitioning defective mutant in *C. elegans*; PI3K, phosphatidylinositol-3'-kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; RHO/RAC/RAP/CDC42, small RHO-like GTPases; SIPA1L1, a GTPase-activating protein of RAP small GTPases.

brane distribution to caveolin-rich areas. This redistribution of the ligand-receptor complex attached to intracellular scaffold molecules results in the formation of a WNT-FZD-LRP5/6-DVL-axin signaling platform, a so-called LRP5/6 signalosome (Bilic et al., 2007; MacDonald et al., 2009).

Stabilization of cytosolic β -catenin and its nuclear translocation ultimately lead to transcriptional regulation of many target genes in collaboration with T-cell factor (TCF)/lymphoid enhancer factor (LEF) family transcription factors (Vlad et al., 2008; MacDonald et al., 2009; Mosimann et al., 2009). The TCF/LEF binding site (i.e., the WNT responsive element) is a CCTTTG(A/T)(A/T) sequence upstream of the regulated gene (Mac-

Donald et al., 2009). Transcription through TCF/LEF in cooperation with β -catenin is modulated by a complicated network of cofactors, allowing potential cross-talk at this level of communication (Jin et al., 2008; MacDonald et al., 2009) as well as pharmaceutical interference (Klaus and Birchmeier, 2008). The first target genes to be identified were proliferative genes such as *c-myc* and *cyclinD1* (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999), indicating the importance of WNT/ β -catenin in the regulation of cell growth and differentiation and its central role in tumorigenesis. A more extensive collection and references of WNT target genes can be found on the WNT homepage: <http://wnt.stanford.edu>.

B. A Network Approach: β -Catenin-Independent WNT/ Frizzled signaling

The complexity of the FZD signaling map is steadily increasing, and attempts to describe it in terms of isolated pathways will inevitably lead to confusion, because the level of cross-talk and amount of networking among signaling branches is still unclear. Many central components, such as FZDs, DVL, β -arrestin, casein kinases, and—as discussed in section IX—heterotrimeric G proteins participate in several branches of WNT signaling, making it difficult to distinguish between exclusive, parallel, interacting, and overlapping signaling routes. So far, it seems logical to subdivide FZD-mediated and β -catenin-independent signaling into the following branches: FZD/PCP, WNT/RAC, WNT/RHO, WNT/ Ca^{2+} , WNT/cAMP, WNT/RAP, and WNT/ROR, similar to a previous division by Semenov et al. (2007).

C. Planar Cell Polarity Signaling

The phenomenon of PCP signaling (Seifert and Mlodzik, 2007; James et al., 2008; Wu and Mlodzik, 2009) offers an additional challenge to nomenclature. Tissue or planar cell polarity refers to the phenomenon of cellular orientation in a two-dimensional epithelial cell layer, such as the *D. melanogaster* wing (Wang and Nathans, 2007). The wing building blocks are hexagonal cells, each carrying a wing hair that is strictly ordered, pointing distally. A similar developmental program dictating tissue arrangement and asymmetric organization of photoreceptor rhabdomers can be observed in the ommatidia of the insect eye. Thus, PCP is intensively studied in the *D. melanogaster* wings and eyes (Fanto and McNeill, 2004). In vertebrates, the definition of PCP is less clear; however, processes similar to PCP in *D. melanogaster* are present. Developmental processes, such as convergent extension movements, neural tube and eyelid closure, hair bundle orientation in the sensory cells of the inner ear, and hair follicle orientation in the skin are important examples, in which cell polarity in an epithelial plane is affected and at least one of the genes that were identified as PCP core genes in *D. melanogaster* is involved (Wang and Nathans, 2007). Disturbances in vertebrate PCP signaling can, for example, be monitored in so-called Keller open-face explants in the developing *X. laevis* embryo, a classic model for convergent extension, which is elongation and narrowing of the embryo (Keller et al., 2003). In mammals, on the other hand, malfunctional PCP signaling becomes evident through failure of neural tube or eyelid closure in the offspring as it is evident in the FZD_{3/6} double knockout mouse (Wang et al., 2006b). Furthermore, these mice reveal also that FZD_{3/6} regulate the ordered arrangement of the auditory hairs on vestibular sensory cells, which can be employed as a mammalian readout for PCP function (Wang et al., 2006b; Wang and Nathans, 2007). Mice deficient in FZD₆ display a truly frizzled appear-

ance, with whorls dominating the macroscopic hair pattern, indicating that FZD₆ controls hair patterning in the mouse.

The molecular aspects of PCP signaling are complex and evolutionarily conserved involving different branches of FZD-dependent and -independent communication. Even though components are conserved among species throughout the animal kingdom, important and striking species differences exist (Wang and Nathans, 2007; Wu and Mlodzik, 2009). For example, although it is known that FZD/PCP signaling in *M. musculus* is regulated by a FZD ligand (Qian et al., 2007), it remains unclear whether this is also the case in *D. melanogaster* (Klein and Mlodzik, 2005). In general, the mechanism that orchestrates global planar cell polarity of tissues remains obscure (Wu and Mlodzik, 2009). PCP genes were defined in *D. melanogaster* and can be subdivided into core PCP genes/proteins, which are of general importance (such as *frizzled*/Fz, *disheveled*/Dsh, and *prickle*/Pk), and tissue-specific factors, which have less general impact. Additional core PCP genes in *D. melanogaster* are *van gogh*/Vang (or *strabismus*), *diego*/Dgo, and *flamingo*/Fmi (or *starry night*) (James et al., 2008). These are grouped together into the *frizzled-flamingo* group and are evolutionarily conserved (even though the nomenclature is not unified among species; see also James et al., 2008; Wu and Mlodzik, 2009). The apical-basal orientation of cells, as in the *X. laevis* animal cap or the *D. melanogaster* wing, which are often used as experimental models, is achieved by a selective redistribution and asymmetric arrangement of the Fz/Fmi group proteins determining cellular orientation. Before the activation of the PCP program in a tissue, the components are evenly distributed in the membrane. PCP signaling supports the asymmetrical redistribution of cellular components: Fz/Dsh/Dgo and Vang/Pk complexes repel each other resulting in distal accumulation of Fz/Dsh/Dgo and enrichment of Vang/Pk on the proximal side of *D. melanogaster* wing cells (Fig. 4). The asymmetric distribution within one cell will then translate into planar polarity by interaction between the extracellular domains of Fz/Fmi and Vang/Fmi of neighboring cells (Seifert and Mlodzik, 2007; Wu and Mlodzik, 2009). The principal and most well characterized downstream pathway propagating signaling to the cytoskeleton involves DVL and a RAC/c-Jun-N-terminal kinase and a RHO/ROCK signaling axis (Wallingford and Habas, 2005) as detailed in the next paragraph.

D. WNT/RAC and WNT/RHO

Many functions of PCP or PCP-like signaling in different species can be assigned to small monomeric G proteins. Signaling to GTPases such as the small RHO-like guanine nucleotide binding proteins is known to affect cytoskeletal organization but also subserves transcriptional regulation (Brown et al., 2006; Heasman and Ridley, 2008). WNT and FZD-mediated RHO and RAC

signaling is crucial for proper gastrulation in vertebrates, and Habas et al. (2003) identified WNT/RAC and WNT/RHO branches as separate signaling routes important for vertebrate gastrulation. Furthermore, WNT-induced activation of RAC can enhance tumor aggressiveness in the form of augmented capability of invasion and migration (Kurayoshi et al., 2006), processes that are dependent on cytoskeletal reorganization. An important initial indication that WNT/RHO and WNT/RAC pathways are separate came from time-course experiments, where RAC1 activation upon stimulation with WNT-1-conditioned medium was fast and lasted for up to 3 h, whereas the RHO activation followed a slower time course (Habas et al., 2003). Both RHO and RAC signaling require membrane-associated DVL (Park et al., 2005) but depend on different domains of this phosphoprotein. Although WNT/RHO signaling involves the PDZ domain of DVL and the Disheveled-associated activator of morphogenesis DAAM1, a formin homology protein, WNT/RAC signaling is mediated by the DVL DEP domain (Habas et al., 2001). Because small GTPases undergo a GDP-GTP exchange upon activation, their function relies on a guanine nucleotide exchange factor (GEF). The only GEF so far identified in the WNT/RHO pathway is weak-similarity GEF (WGEF), which binds both DVL and DAAM1. Overexpression of WGEF activates RHO and compensates for WNT-11-induced convergent extension defects in *X. laevis* as measured by axis- and explant elongation. Furthermore, FZD₇ overexpression promoted colocalization of DVL and WGEF at the plasma membrane (Tane-gashima et al., 2008). Another DVL-interacting GEF, xNET1, inhibits gastrulation movements in a RHO-specific manner and was suggested to serve also as an important player in the WNT/RHO pathway (Miyakoshi et al., 2004). An essential specification factor of DVL-mediated signaling to RHO/RAC is the scaffold protein β -arrestin, which was shown to be crucial for the RAC- and RHO-dependent regulation of convergent extension movements in *X. laevis* (Kim and Han, 2007) and for the WNT-5A-induced activation of RAC1 (Bryja et al., 2008). DVL, β -arrestin, and CK1 are important in defining the signaling route of WNT-5A-induced signaling: the balance between β -arrestin and CK1 dictates whether the WNT/RAC1 pathway or one of the alternative pathways is activated (Bryja et al., 2008). A recent study underlines the role of β -arrestin for WNT-5A-induced and FZD₂-mediated activation of RAC1 and provides further evidence that clathrin-mediated endocytosis and ROR1/2 are required for this pathway (Sato et al., 2010).

The differential regulation of RAC and RHO signaling by DVL obviously means that downstream signaling diverges at this point: RAC activation results in phosphorylation and activation of the stress-activated protein kinase c-Jun-N-terminal kinase, which subsequently leads to the activation of transcription factors, such as c-Jun and c-Fos, which form the activator pro-

tein 1 (Rosso et al., 2005; Bryja et al., 2008). RHO, on the other side, activates RHO-associated kinase (ROCK) (Marlow et al., 2002).

The involvement of RHO and RAC in WNT-3A signaling has been shown. WNT-3A mediates cellular migration through DVL, RHO-A, and ROCK (Kishida et al., 2004; Endo et al., 2005; Kobune et al., 2007). Furthermore, RAC and the GEF DOCK4 are required for WNT/ β -catenin signaling, more precisely for the nuclear translocation of β -catenin (Upadhyay et al., 2008; Wu et al., 2008). WNT-3, which typically activates WNT/ β -catenin signaling, was also shown to induce WNT/RHO signaling in an autocrine way in melanoma cells. In contrast to those findings on WNT/RAC signaling as a component of the WNT/ β -catenin pathway, WNT-3 was shown to activate RHO-A/ROCK signaling in an LRP5/6-independent manner (Kobune et al., 2007). In summary, this strongly indicates that WNTs, which generally activate WNT/ β -catenin signaling, are able to induce β -catenin- and LRP5/6-independent pathways in parallel.

E. WNT/ Ca^{2+} Signaling

The ability of FZDs to mediate elevation of intracellular Ca^{2+} levels was first reported in *D. rerio*, where overexpression of FZD₂ but not FZD₁ induced Ca^{2+} transients in a G protein-dependent manner (Slusarski et al., 1997). This study indicated that FZD₂ could recruit the $\text{G}_{i/o}$ family of heterotrimeric G proteins to communicate with phospholipases C and thereby provided an upstream mechanism for the previously reported communication with PKC (Cook et al., 1996) through inositol phosphates and diacylglycerol. Later an additional route from FZD to the increase of $[\text{Ca}^{2+}]_i$ was discovered that is reminiscent of phosphodiesterases (PDE)- and cGMP-dependent visual signal transduction (Ahumada et al., 2002; Wang et al., 2004; Ma and Wang, 2006, 2007). This pathway involves transducin (G_t) and the activation of cGMP-selective phosphodiesterases, which results in a drop in intracellular $[\text{cGMP}]_i$, leading to mobilization of Ca^{2+} through as-yet-unidentified mechanisms. In parallel, transducin signaling activates the stress-activated protein kinase p38, necessary for the WNT-5A-induced and cGMP-dependent rise in $[\text{Ca}^{2+}]_i$ (Ma and Wang, 2007). It is noteworthy that this pathway has convincingly been shown to function in cells depleted of DVL by siRNAs (Ma and Wang, 2007) in contradiction to studies indicating a role for DVL in the Ca^{2+} response (Sheldahl et al., 2003).

Ca^{2+} is a central regulator of cell function and its putative downstream targets are numerous. So far, Ca^{2+} /calmodulin-dependent kinase, Ca^{2+} -dependent protein kinase (PKC), (Kühl et al., 2000, 2001; Sheldahl et al., 2003) and nuclear factor of activated T cells (Saneyoshi et al., 2002; Dejmek et al., 2006) have been identified.

It is still unclear whether the two Ca^{2+} -pathways—one depending on PLC, the other on cGMP-PDE—are generally activated in parallel, whether they are exclusive or complementary, and which factors might convey specificity to the Ca^{2+} response pattern of WNTs. The WNT-induced Ca^{2+} responses have shown certain selectivity for pertussis toxin (PTX)-sensitive $G_{i/o}$ family proteins, including G_{t2} or transducin. This heterotrimeric G protein was initially shown to have specific tissue distribution with high concentrations in the retina (Raport et al., 1989) but was also shown to be present in other tissues, such as brain.

Molecular details of WNT/ Ca^{2+} signaling were mainly dissected in cells overexpressing FZD₂ (Ahumada et al., 2002; Ma and Wang, 2007). In particular, the comparison between chimeric adrenergic/FZD₂ receptors and full-length FZD₂ reveals an interesting but still puzzling difference in kinetics of signaling through Ca^{2+} (Ma and Wang, 2006). Although isoproterenol-induced and $\beta_2\text{AR}$ /FZD₂-mediated Ca^{2+} mobilization was rapid, resembling a classic GPCR response, the WNT-5A-induced and FZD₂-mediated response was much slower. A possible explanation put forth by the authors was the difference in solubility and distribution on the cell surface between a hydrophilic and lipophilic ligand. Another possibility could be differences in the signal transduction between those two receptors, because WNT-5A-induced Ca^{2+} transients in other cells endogenously expressing FZDs were shown to be fast (Dejmek et al., 2006; Jenei et al., 2009), more closely resembling a classic GPCR response (Schulte and Fredholm, 2002).

F. WNT/cAMP signaling

An early analysis of the primary sequence of FZD predicted that FZD₃ could couple to stimulatory G_s proteins (Wang et al., 2006a). So far, however, the experimental evidence for a FZD-mediated activation of G_s -adenylyl cyclase-cAMP-PKA-cAMP response element binding protein (CREB) signaling route is sparse. In vivo evidence for the involvement of FZD in this pathway—one of the archetypical pathways activated by GPCRs—comes from a mouse study indicating that WNT-1 and WNT-7A regulate myogenic determinant genes in an adenylyl cyclase-, PKA-, and CREB-dependent manner (Chen et al., 2005). More recent evidence from mammalian systems shows that WNT-5A can exert anti-apoptotic effects through PKA, cAMP, and CREB (Torii et al., 2008). Furthermore, WNT-5A stimulation of breast cancer cells induces cAMP production. Subsequent phosphorylation of a classic cAMP target, the Thr34 of dopamine and cAMP-regulated phosphoprotein of 32 kDa, through FZD₃ inhibits the formation of filopodia, necessary for cancer cell migration (Hansen et al., 2009). This suggests the existence of a FZD- G_s -cAMP-PKA-CREB signaling axis.

On the other hand, inhibition of adenylyl cyclase and PKA via PTX-sensitive G proteins was identified as a

regulatory mechanisms of the WNT/RHO pathway on the level of DVL/Dishevelled-associated activator of morphogenesis (Park et al., 2006). It is surprising, however, that negative regulation of cAMP, despite numerous reports on involvement of PTX-sensitive $G_{i/o}$ proteins, has not yet been established as a standard measure of WNT signaling in mammalian cells, because $G_{i/o}$ proteins by default should communicate with adenylyl cyclase.

G. WNT/RAP Signaling

The discussion of FZD signaling through small GTPases of the RHO family and cAMP signaling leads naturally to the small GTPase RAP. RAP is closely related to RHO-like proteins and, among other stimuli, is regulated by cAMP through a cAMP-binding GEF called exchange protein directly activated by cAMP (de Rooij et al., 1998). With the above-mentioned signaling axis through cAMP (Hansen et al., 2009) in mind, it seems likely that exchange protein directly activated by cAMP could mediate RAP activation. This connection however, is purely hypothetical and awaits experimental confirmation. WNT signaling to RAP1 was discovered through a proteomic approach characterizing CK1 binding proteins. In fact, a RAP-GTPase-activating protein called SIPA1L1/E6TP1 was found to be a CK1 target. Phosphorylation of SIPA1L1/E6TP1 leads to reduced GAP activity through protein destabilization, thereby increasing RAP1 activity (Tsai et al., 2007). Thus, WNT/RAP signaling so far represents an indirect pathway revealing an inhibitory input of a GAP on RAP, and it should be pointed out that the role of FZD has not been addressed in the experimental set-up. Furthermore, the *X. laevis* RAP2 was shown to modulate DVL localization, thereby affecting β -catenin stabilization as well as FZD-induced membrane recruitment of DVL (Choi and Han, 2005).

H. WNT/ROR Signaling

As already mentioned, it is unclear whether or under which circumstances WNT/ROR signaling involves cooperation with FZDs or if ROR1/2 acts as autonomous WNT receptors. The secreted glycoprotein CTHRC1 seems to be an important factor mediating formation of and stabilizing a WNT/ROR/FZD complex (Yamamoto et al., 2008). Restricted expression patterns in various tissues suggest, on the other hand, that CTHRC1-mediated communication between FZDs and ROR is not a ubiquitous mechanism (Durmus et al., 2006). FZD-ROR interaction does not necessarily require additional factors but can be mediated by CRD-dependent dimerization as shown in transfected HEK293 cells (Oishi et al., 2003). Furthermore, ROR expression is required for the WNT-5A-mediated inhibition of WNT/ β -catenin signaling (Mikels and Nusse, 2006), and recent data imply that direct interaction of ROR with PS-DVL is required for

this negative input on WNT/ β -catenin pathway (Witte et al., 2010).

ROR expression is also crucial for the WNT-5A-induced internalization of FZD₂ and cooperative signaling to small GTPases, such as RAC. WNT-3A signaling through FZD₂ and LRP6 to β -catenin, however, is not sensitive to ROR knock down, indicating that ROR plays an important role in signal trafficking of WNT/FZD signals (Sato et al., 2010).

It seems also that WNT-5A is able to induce distinct, FZD-independent signaling pathways through ROR, which involve PI3K, the small RHO-like GTPase CDC42, c-Jun-N-terminal kinase, and the transcription factors ATF2 and c-JUN to regulate the expression of paraxial protocadherin and thereby convergent extension movements in *X. laevis* (Oishi et al., 2003; Unterseher et al., 2004; Schambony and Wedlich, 2007).

VIII. Smoothened Signaling

A. Regulation of Smoothened by Patched

SMO-mediated HH communication (Fig. 5) is different from WNT/FZD signal transduction, basically because SMO does not function as the HH receptor (Riobo and Manning, 2007; Jiang and Hui, 2008). In addition SMO is a constitutively active receptor (Riobo et al., 2006) that is kept in an inactive state by PTCH (Alcedo et al., 1996; Chen and Struhl, 1998). On the other hand, the agonist-induced stabilization of the transcriptional regulator glioma-associated oncogene (GLI) is reminiscent of WNT signaling, especially because common kinases, such as CK1 and GSK3 are involved in these mechanisms (Teglund and Toftgård, 2010).

HH, a family of lipoglycoproteins (Nusse, 2003; Bürglin, 2008) of which there are three mammalian representatives (sonic, indian, and desert HH) bind to the 12

FIG. 5. Overview of HH/SMO signaling. The inactive state shows the HH/SMO signaling system in the absence of HH. PTCH and SMO undergo internalization and membrane-embedding cycling, keeping inactive SMO in endosomal compartments. PTCH is localized to primary cilia. In the presence of HH, the endosomal cycling is interrupted, leading to an exchange of SMO for PTCH in the primary cilia, allowing activation of SMO and SMO/GLI signaling. For detailed information, see section 8. KIF3A, kinesin-like protein.

membrane-spanning domain protein PTCH (Stone et al., 1996) independently of SMO (Chen and Struhl, 1998). PTCH acts as a constitutive repressor of SMO. It is noteworthy that the stoichiometry of PTCH/SMO repression is not 1:1, as one might expect, but 1:250, implicating catalytic rather than scaffold-based mechanisms of inhibition (Ingham et al., 2000; Taipale et al., 2002), possibly dependent on lipoprotein-derived lipids (Khaliullina et al., 2009). Even though HH does not bind to SMO, a functional SMO-CRD seems to be required for successful signal transduction. Mutations in the CRD of SMO, such as C155Y, disrupt SMO signaling, suggesting a functional role of the CRD in HH signaling (Chen and Struhl, 1998).

PTCH-mediated inhibition of SMO is accentuated by promotion of SMO internalization and degradation, which are supported by GRKs and β -arrestin (Chen et al., 2004b; Wilbanks et al., 2004). This repression of SMO is lost when HH binds to PTCH and SMO signaling is induced. The molecular basis of the inhibition/activation cycle between PTCH and SMO is a segregation of these molecules in late endosomes (Piddini and Vincent, 2003): In the absence of HH, PTCH and SMO are internalized and processed together, whereas addition of HH leads to a segregation of PTCH from SMO in late endosomes and a re-embedding of active SMO in the membrane, allowing relocation to the primary cilium and signaling (Wilson et al., 2009; Teglund and Toftgård, 2010).

B. Transcriptional Regulation via Glioma-Associated Oncogene

The most important group of SMO transducers is the GLI family of transcriptional modulators, of which there are three mammalian isoforms: GLI1, -2, and -3. The counterpart in *D. melanogaster* is called cubitus interruptus (Orenic et al., 1990). These zinc finger proteins vary in their ability to be regulated by SMO signals that transform GLI into transcriptional repressors through phosphorylation, degradation, and proteolytic cleavage (Teglund and Toftgård, 2010). PKA, CK1, and GSK3 are kinases that can phosphorylate GLI proteins, thereby promoting their interaction with β -transducin repeat-containing protein, an E3 ubiquitin ligase, and thus lead to GLI degradation, mechanisms reminiscent of β -catenin regulation in WNT signaling. Recent evidence indicates that to various degrees, GLI proteins can turn from being transcriptional activators (GLI^A) to being repressors (GLI^R) upon cleavage and that only a small fraction of the total GLI pool is turned to GLI^R (Wang et al., 2000; Litington et al., 2002; Bai et al., 2004; Pan et al., 2006). The net outcome of HH signaling is determined by a balance between transcriptional activation and repression (i.e., the levels of GLI^A and GLI^R) (Teglund and Toftgård, 2010). HH-mediated activation of SMO prevents GLI phosphorylation, seemingly through reduced levels of cyclic AMP and reduced activation of

PKA as well as reduced phosphorylation by GSK3 and CK1, thereby stabilizing cytosolic GLI (Zhang et al., 2005). Transcriptional regulation is then accomplished by nuclear translocation and interaction with GLI-responsive elements (Katoh and Katoh, 2009).

C. The Role of the Primary Cilium

A dynamic ciliary structure, the primary cilium, is crucial for the proper regulation of HH/SMO signaling (Wong and Reiter, 2008). The structure of the primary cilium as a dynamic microtubule-based protrusion has long been known, and it has become evident that the primary cilium can be regarded as a signaling center in cells (Eggenchwiler and Anderson, 2007). Defective function of the primary cilium is associated with a series of human diseases, underlining its importance (Wong and Reiter, 2008).

Furthermore, dysfunctional cilia disturb information flow through the HH/SMO pathway, and proteins important for intraflagellar transport have been shown to act downstream of PTCH/SMO and upstream of GLI (Huangfu et al., 2003; Huangfu and Anderson, 2005). The localization of PTCH and SMO upon HH stimulation is dynamically regulated in the primary cilium (Fig. 5). In the absence of HH, PTCH is located to the cilium and SMO is kept outside, whereas these proteins exchange places upon exposure to HH (Milenkovic et al., 2009). The exchange process is supported by β -arrestin, which links SMO physically to the kinesin motor protein KIF3A (Kovacs et al., 2008). Even though ciliary translocation is required for activation of the HH/SMO pathway, it is unclear whether translocation is intrinsically connected to the receptor activation process, because also antagonist-bound SMO, apparently in an inactive conformation, is forced to the cilium (Wilson et al., 2009). Information transfer downstream of SMO is then established through shuttling of GLI from the cilium to the nucleus in a microtubule-dependent manner (Kim et al., 2009).

The primary cilium is undoubtedly crucial for signal transduction through SMO, and its proper function is essential to maintaining a healthy organism (Veland et al., 2009). Indeed, the primary cilium is important not only for HH/SMO signaling but also for other very important signals, such as growth factors, MAPK signaling, and not least for WNT- β -catenin signals (Gerdes et al., 2007; Corbit et al., 2008). Inversin, a ciliary protein, seems to function as a molecular switch between WNT/FZD signaling pathways: inversin inhibits WNT/ β -catenin signaling, whereas it is required for β -catenin-independent mechanisms regulating convergent extension in the *X. laevis* embryo (Simons et al., 2005). On the other hand, there is also evidence against requirement of the primary cilium for WNT signaling (Huang and Schier, 2009; Ocbina et al., 2009).

IX. Frizzleds and Smoothed as G Protein-Coupled Receptors

A. Pros and Cons of G Protein Coupling

When FZDs and SMO were cloned, their primary amino acid sequence indicated the presence of seven transmembrane-spanning domains and, thus, a putative relationship to GPCRs, especially to secretin-like receptors (Barnes et al., 1998). Since then, strong evidence for FZD and SMO signaling requiring heterotrimeric G proteins has accumulated (Fig. 6) (Riobo and Manning, 2007; Egger-Adam and Katanaev, 2008; Philipp and Caron, 2009; Ayers and Thérond, 2010). There is no doubt, however, that FZDs and SMO are both atypical receptors and that they are not GPCRs employing heterotrimeric G proteins in general and under all circumstances. Egger-Adam and Katanaev (2008) recently presented the hypothesis that FZDs could be a kind of (evolutionarily) modern receptors that became master regulators of development, that they required other signaling paradigms in addition to coupling to heterotrimeric G proteins, and that the same could be true for SMO. Under some circumstances, for instance in certain cellular compartments, in certain cell types, or at certain stages of development—conditions that have yet to be defined—FZDs or SMO might be more biased to classic G protein communication over signaling through DVL, LRP6, or GLI. It remains unclear in which way G protein-independent FZD/SMO signaling is intertwined with the G protein-dependent mechanisms. In the case of FZD signaling, experimental evidence indicates heterotrimeric G proteins acting both upstream and downstream of DVL in both β -catenin-dependent and -independent pathways (Sheldahl et al., 2003; Liu et al., 2005; Bikkavilli et al., 2008); mechanistic details, on the other hand are still confusing (Egger-Adam and Katanaev, 2008). Experimental proof for FZD and SMO interaction with heterotrimeric G proteins and for the WNT-induced and FZD-mediated guanine nucleotide exchange at heterotrimeric G proteins is still lacking. In the case of SMO, there is strong experimental evidence that the receptor's constitutive activity results in GDP/GTP exchange in heterotrimeric G proteins (Riobo et al., 2006), and genetic experiments indicate that SMO recruits $G_{i/o}$ proteins to modulate cAMP levels (Ogden et al., 2008). Thus, SMO inflicts double impact on GLI through activation of $G_{i/o}$ proteins as well as G protein-independent signals involving the C terminus of SMO (Riobo et al., 2006). In addition, coexpression of SMO with the promiscuous heterotrimeric G protein $G_{\alpha_{15}}$ enabled HH signaling to phospholipase C, an interaction that could be purely artificial (Masdeu et al., 2006).

The initial functional indication for an involvement of heterotrimeric G proteins in class Frizzled receptor signaling comes from loss-of-function experiments (Slusarski et al., 1997; Sheldahl et al., 1999; Liu et al., 2001; Ahumada et al., 2002) based on treatment with a toxin

from *Bordetella pertussis*, PTX, which ADP-ribosylates the α subunit of $G_{\alpha_{i/o}}$ proteins (except G_{α_z}) (Birnbaumer et al., 1990). RNA interference- and antisense-oligonucleotide-based approaches allowed selective knockdown of G proteins, yielded similar results, and also made it possible to address the involvement of PTX-insensitive G proteins of the G_{α_q} , G_{α_s} , and $G_{\alpha_{12}}$ families (Liu et al., 1999, 2001).

For decades, the lack of purified and biologically active WNTs and the biochemical character of WNT as a lipoprotein with high affinity to heparin sulfates in the extracellular matrix hampered the development of WNT-FZD ligand binding assays. In fact, the guanine nucleotide-dependent affinity shift of GPCRs that led to the discovery of heterotrimeric G proteins in the first place (Lefkowitz, 1994) could also be seen as the ultimate proof of G protein coupling (Schulte and Bryja, 2007). This aspect has been studied intensively by the group of Craig Malbon (Liu et al., 1999, 2001; Ahumada et al., 2002; DeCostanzo et al., 2002; Li et al., 2004; Wang et al., 2006a), employing a bold approach: to circumvent the requirement of WNTs for receptor stimulation and WNT-FZD binding assays, chimeric receptors were designed and created that contain the extracellular and transmembrane domains of a bona fide GPCR, in this case adrenergic receptors, and the intracellular loops and C terminus of FZDs. With these chimeric constructs, which showed FZD-like behavior with regard to signaling, it was possible to use water-soluble, well characterized adrenergic ligands for receptor stimulation and radioligand binding experiments. Indeed, agonist but not antagonist affinity changed in the presence and absence of guanine nucleotides, emphasizing that the heterotrimeric G protein affects receptor-ligand affinity in a typical allosteric manner as described in the original ternary complex model of GPCRs (De Lean et al., 1980). Despite the fact that the chimeric receptors as a whole are very different from FZDs, this series of experiments provides strong evidence for FZD-G protein coupling and a role for G proteins in FZD signaling. So far, however, it has not been shown that either agonist stimulation at the adrenergic-FZD chimeric receptors or WNT stimulation of FZDs induces the GDP/GTP exchange in a heterotrimeric G protein. Exchange of GDP for GTP at the heterotrimeric G protein is also intrinsically connected to a structural rearrangement or dissociation of the G protein from the GPCR (Galés et al., 2005; Lohse et al., 2008). Indeed, WNT-3A induces the fast release of $G_{i/o}$ proteins from FZD as well as the dissociation of DVL from the complex as shown by immunoprecipitation in the presence and absence of WNT-3A (Liu et al., 2005). So far, these results present the most compelling biochemical evidence in cells endogenously expressing FZDs and G proteins for their dynamic interaction according to the ternary complex model. Limitations in the interpretation arise from the use of nonisoform selective FZD antibodies and—as also

ylglycerol and inositol trisphosphate (IP₃) and, on the other hand, a pathway reminiscent of visual rhodopsin-dependent signal transduction, the G α_t -phosphodiesterase-cGMP pathway (Ahumada et al., 2002). Furthermore, recent evidence indicates a more general role of G proteins also in WNT/ β -catenin signaling, suggesting a functional role of both PTX-sensitive G proteins as well as G α_q proteins (Liu et al., 2001, 2005; Katanaev et al., 2005; Egger-Adam and Katanaev, 2010).

The main challenge in the quest for signaling specificity regarding G protein coupling is to define the involved factors that determine under which circumstances FZDs can act as GPCRs. On the other hand, it becomes more and more obvious that the distinction between G protein-dependent and -independent pathways cannot be seen as a sharp line, because substantial overlap between the branches exists. For example, membrane-tethered G α and $\beta\gamma$ subunits recruit components of the WNT/ β -catenin pathway to accomplish signaling compartmentation (Egger-Adam and Katanaev, 2010). The balance between G protein-dependent and -independent WNT signaling might be delicate, underlining the importance of experimental studies in biologically relevant systems with physiological receptor/G protein ratios compared with recombinant systems.

B. Second Messenger Signaling

Ever since the discovery of cAMP, second messengers have been seen as central players in GPCR signaling (Sutherland and Robison, 1966). By definition, second messengers—as opposed to the first messenger (i.e., the extracellular ligand)—are small, intracellular molecules that are rapidly produced by enzymes in response to receptor stimulation and that can be rapidly degraded to ensure short-lived signaling. Classic GPCRs are able to communicate through an immense network of second messengers, and selective and dynamic coupling to heterotrimeric G proteins allows strict regulation of signaling and signaling trafficking (Dorsam and Gutkind, 2007; Woehler and Poni-maskin, 2009), which presents a challenge to systems biology (Heitzler et al., 2009).

Information on second messenger responses downstream of FZDs and SMO is currently sparse, but more and more reports indicate that classic second messengers serve as a means of signal transduction in FZD and SMO signaling. It is again noteworthy that what is canonical to classic GPCRs does not seem to be “canonical” for FZD/SMO signals, even though they are considered GPCRs. β -Catenin-dependent but also classic β -catenin-independent FZD signaling is so far thought to be mainly independent of second messengers. The mechanistic contribution of heterotrimeric G proteins and downstream second messengers is still unclear, as discussed in section IX.A. However, second messengers, such as inositol phosphates, inositol pentakisphosphates (Gao and Wang, 2007), inositol 3-phosphates (Slusarski

et al., 1997), phosphatidylinositol 4-trisphosphate (Qin et al., 2009), phosphatidylinositol 3,4,5-trisphosphate (Pan et al., 2008), calcium, or the cyclic nucleotides cGMP (Ahumada et al., 2002) and cAMP (Hansen et al., 2009), were reported to mediate WNT effects (see also Fig. 5).

cAMP and PKA were discovered early on to be components of SMO signaling in *D. melanogaster* (Ohlmeyer and Kalderon, 1997), although the evidence for the role of cAMP/PKA in vertebrates for the regulation of GLI is controversial. Although PKA phosphorylation of the SMO C terminus is important for SMO activity in *D. melanogaster*, it plays a minor role in vertebrates because PKA target sites are not conserved (Teglund and Toftgård, 2010). The G $_{i/o}$ -mediated reduction of cAMP and the consequent reduction in PKA-dependent GLI phosphorylation counteracts GLI degradation.

In addition, SMO signaling to phospholipases C was observed in HEK293 cells overexpressing SMO and G α_{15} (Masdeu et al., 2006). Activation of phospholipases C generates two second messengers (IP₃ and diacylglycerol) that lead to the mobilization of intracellular calcium and activation and membrane recruitment of Ca²⁺-dependent protein kinase (Oude Weernink et al., 2007). It remains to be seen, however, whether this forced liaison has physiological relevance.

C. Kinetics of Signaling

Not all experimental models applied for the investigation of FZD or SMO signaling provide sufficient resolution to allow temporal analysis of signal initiation after addition of agonist. In particular, techniques based on overexpression of ligands, such as WNTs and HHs, cannot provide any information on signaling kinetics and could also induce long-term adaption to ligand exposure. Thus, the temporal aspects of signaling were initially studied in conditioned medium and later using purified, recombinant agonists. Different time courses of signaling routes could be an indication that they are regulated separately as shown for WNT/RHO and WNT/RAC signaling (Habas et al., 2003). For technical and historical reasons, many studies have focused on the slower pathways downstream of WNTs and HHs, such as the formation of phosphorylated and shifted DVL (PS-DVL), transcriptional regulation of target genes monitored by luciferase reporters (TOPflash and GLI reporters), morphological changes (C56MG transformation), and cellular proliferation, to name but a few (Wong et al., 1994; Molenaar et al., 1996; Cong et al., 2004b). With recent developments, faster processes, such as protein redistribution, second messenger production, and protein phosphorylation, have increasingly come into focus. Figure 7 is a schematic compilation of different intracellular changes in response to acute WNT stimulation. This graph clearly indicates that responses to WNTs range in kinetics from fast and transient to slow and persistent. Most importantly, this figure indicates that slow DVL

FIG. 7. Schematic presentation of WNT/FZD signaling kinetics. Information was compiled from the following original articles: dephosphorylated, active β -catenin, ABC (black), WNT-3A response (Bryja et al., 2007c); P-LRP6 (blue), WNT-3A response (Sakane et al., 2010); PS-DVL formation (red), similar for WNT-3A and WNT-5A (Bryja et al., 2007d); RAC1 (orange), response to WNT-1 conditioned medium (Habas et al., 2003); GSK3/axin interaction (yellow), WNT-3A response (Liu et al., 2005); phosphorylated mitogen-activated protein kinase p -38 (P-p38, pink), WNT-5A response (Ma and Wang, 2007); WNT-5A/ Ca^{2+} (light green) (Jenei et al., 2009); WNT-5A/ Ca^{2+} (dark green) (Ma and Wang, 2007). Observe that the results extracted from the literature originate from different cell types and experimental settings.

responses, for instance PS-DVL formation, are preceded by responses that are presumed to be DVL-dependent, such as RAC1 activation (Habas et al., 2003) or β -catenin dephosphorylation (Bryja et al., 2007c). Thus, the slow PS-DVL formation is likely to be preceded by DVL-activation, which is not detectable as an electrophoretic mobility shift. Similar considerations are valid for SMO transduction, regarding fast signaling to adenylyl cyclase or IP_3/DAG compared with slower translocation and transcriptional activation of GLI (Masdeu et al., 2006; Riobo and Manning, 2007).

X. Class Frizzled Receptor Dynamics

Transmembrane receptors are dynamic molecules. This is true not only of the receptor molecules themselves, which shift between different activity conformations, but also of their intracellular locations, because there is a dynamic redistribution of receptors between different cellular compartments [here, the recently suggested possibility that WNT receptor signaling changes with the cell cycle (Davidson et al., 2009) is consciously disregarded]. In the case of GPCRs, most functions have been assigned to receptors that are localized to the plasma membrane. However, mutations or regulatory mechanisms can affect—among other aspects—receptor ontogeny and maturation, resulting in failure of membrane embedding of the receptor. FZD_4 mutations responsible for the familial exudative vitreoretinopathy, for example, trap wild-type FZD_4 in the ER and prevent proper membrane embedding (Kaykas et al., 2004). In the case of FZD, an endoplasmic protein called SHISA was identified that keeps immature receptors in the ER; it has been implicated as a means of negative regulation of the pathway (Yamamoto et al., 2005). Receptor homo- or heterodimerization has been shown to be necessary for proper presentation on the cell for at least some GPCRs (Milligan, 2009). Furthermore, receptors can lo-

calize to different membrane compartments, such as synapses, cell bodies, or dendrites/axons, thus creating different signaling compartments, for example, on specialized neuronal cells. Regarding microlocalization, some receptors are differentially distributed to membrane domains of varying lipid composition, such as lipid rafts (Patel et al., 2008). This kind of subcellular localization determines the place of action for a given GPCR and with which signaling components it is associated. Furthermore, receptors undergo ligand-specific recruitment to membrane compartments suitable for endocytosis, such as highly specialized caveolae or clathrin-coated pits. By this means, the activated—and possibly desensitized—receptors can be directed into different endosomal pathways, where the ultimate results are receptor degradation, recycling, or formation of signaling endosomes.

A. Frizzled Dynamics

Recent progress in the field of receptor dynamics has also provided some insight into the importance of endocytosis for FZD signaling (Kikuchi and Yamamoto, 2007; Gagliardi et al., 2008). In the context of WNTs as morphogens, endocytosis of WNTs plays an important role to establish the morphogen gradient in *D. melanogaster* wing discs (Marois et al., 2006). During wing development in *D. melanogaster*, WNT/Wingless gradients are maintained through two independent, endocytotic pathways: on the apical side, Wingless is internalized together with FZD and LRP5/6 (Arrow), whereas Wingless internalization is independent of both FZD and LRP5/6. However, no conclusions can be drawn from Marois et al. (2006) regarding the importance of endocytosis on WNT signaling.

Two main endocytic pathways are relevant for receptor signaling and regulation of receptor number on the cell surface: caveolae-mediated and clathrin-dependent endocytosis (Liu and Shapiro, 2003). Caveolae are small, flask-shaped invaginations in the cell membrane that are characterized by a cholesterol- and sphingolipid-rich composition, so called lipid rafts, and by caveolin, a transmembrane protein that can be used as a caveolae marker. Membrane proteins and submembraneous proteins can be selectively recruited to these membraneous microdomains. The fate of the endocytotic vesicles that originate from caveolae, so called caveosomes, remains obscure. In contrast, the fate of proteins internalized in a clathrin-dependent manner is clearer. Transmembrane proteins are actively recruited to hot spots of endocytotic activity, so called clathrin-coated pits. Clathrin assembles on the intracellular side of the membrane to form the clathrin-coated pit and to invaginate a vesicle, which, in cooperation with the GTPase dynamin, then pinches off to form an early endosome. These endosomes, which contain early endosomes antigen 1 and the small GTPase RAB5 can enter either a recycling pathway or a destructive lysosomal route.

Because turnover of transmembrane proteins requires endocytosis and protein degradation in lysosomes, it is not surprising that WNT receptors are subject to constitutive but also agonist-induced endocytosis (Kikuchi and Yamamoto, 2007). So far, FZD₁, FZD₂, FZD₄, FZD₅, and FZD₇ have been shown to be internalized through clathrin-dependent endocytosis in response to WNT-5A (Chen et al., 2003, 2009b; Kurayoshi et al., 2007; Yu et al., 2007; Sato et al., 2010) and WNT-11 (Kim et al., 2008). In addition, WNT-3A induces clathrin-mediated internalization of FZD₅ if overexpressed alone in HEK293 or HeLaS3 cells (Yamamoto et al., 2006). However, in cells overexpressing both FZD₅ and LRP6, WNT-3A-induced receptor internalization is pushed to caveolae-dependent mechanisms (Yamamoto et al., 2006). Depending on the cell type, overexpression of FZDs results in different subcellular distribution of the receptor indicating—in the case of FZD₄—constitutive internalization, autocrine receptor stimulation, and agonist-dependent endocytosis or possibly low-efficiency embedding in the plasma membrane (Chen et al., 2003; Bryja et al., 2007a). A FZD₅-selective mechanism dependent on coated vesicle-associated kinase of 104 kDa (CVAK104) guides the receptor in a stimulation-independent manner to lysosomal degradation (Terabayashi et al., 2009), whereas it is the protease calpain that regulates FZD₇ turnover (Struewing et al., 2007).

Several biochemical methods have been used to investigate endocytosis in WNT signaling. Endocytosis can be blocked by hyperosmolaric means, by suppression of clathrin or caveolin by siRNA, or by dominant-negative dynamin, a GTPase responsible for pinching off internalizing vesicles from either caveolae or clathrin-coated pits. These studies suggest that endocytosis is not simply a means of signaling turn-off and desensitization but that the localization of WNT receptors to endosomes and possibly also the recruitment of different signaling factors to those signaling endosomes could be critical for WNT signaling to β -catenin (Blitzer and Nusse, 2006; Yamamoto et al., 2006; Bryja et al., 2007a). In this context, it is important to mention that hyperosmolaric treatments, such as addition of sucrose or potassium depletion, can affect expression levels of DVL thereby complicating conclusions on the requirement of endocytosis or DVL for signaling (Bryja et al., 2007a). Thus, despite recent progress, the question of whether WNT receptor endocytosis is a positive or negative regulator of WNT signaling (Gagliardi et al., 2008) does not yet have a clear answer.

B. Smoothed Dynamics

In recent years it has become evident that PTCH and SMO undergo highly dynamic cycling between the membrane and endosomal compartments as well as between the cell surface and the primary cilium (Denef et al., 2000; Wong and Reiter, 2008). To accomplish the inhibition of constitutively active SMO PTCH drives SMO

into late endosomes, where both seem to colocalize and to be processed and recycled together (Piddini and Vincent, 2003). Upon HH stimulation of PTCH, the dynamic pathways diverge and SMO is activated and embedded into the membrane. This segregation becomes most obvious at the primary cilium: in the absence of HH, PTCH is localized to the cilium, whereas PTCH and SMO switch places when HH is added (Milenkovic et al., 2009). On a molecular level, it became evident that phosphorylation of a C-terminal SMO autoinhibitory domain is essential for HH-induced increase in surface expression by promotion of a conformational switch and dimerization of SMO C-terminal tails (Zhao et al., 2007). On the other hand, GRK2 phosphorylates SMO in a PTCH- and HH-dependent manner to promote interaction with β -arrestin and activity-dependent internalization in clathrin-coated vesicles and early endosomes (Chen et al., 2004b).

SMO located to the primary cilium is complexed with GRK2 and β -arrestin (Kovacs et al., 2008) and is able to activate GLI locally, inducing its nuclear translocation (Kim et al., 2009) (Fig. 5). Surprisingly, SMO is directed to ciliary localization by different active and inactive conformation (Wilson et al., 2009) indicating that activation and relocation are parallel rather than interdependent processes.

C. β -Arrestin

One important factor connecting class Frizzled receptor endocytosis, dynamic location to the primary cilium and intracellular communication is the scaffold protein β -arrestin (Kovacs et al., 2009). β -Arrestin was originally identified as a cofactor required for desensitization of adrenergic receptors, class A (rhodopsin-like) GPCRs (Lohse et al., 1990). β -Arrestin function is now understood in greater detail; in particular, three properties make β -arrestin highly interesting: it desensitizes G protein activation through GPCRs by sterical hindrance; it guides ligand-bound and phosphorylated receptors (and not only GPCRs or 7TMRs) to the clathrin-dependent endocytotic machinery; and it can mediate G protein-independent signaling through complexation of signaling compounds such as tyrosine or serine/threonine kinases, small GTPases, E3 ubiquitin ligases, phosphodiesterases, and more (DeWire et al., 2007). In recent years, it has emerged that β -arrestins also form part of the FZD/SMO signaling systems, both as an important factor for agonist-induced internalization and as a signaling cofactor or scaffold (Chen et al., 2001, 2003, 2004b; Wilbanks et al., 2004; Bryja et al., 2007b, 2008; Kim and Han, 2007; Yu et al., 2007; Kim et al., 2008; Kovacs et al., 2009; Schulte et al., 2009). In HH signaling, arrestin was identified as a crucial signaling component in *D. rerio*. Arrestin knockdown could be rescued by compensating for HH signaling through overexpression of downstream HH components (Wilbanks et al., 2004). These findings were supported by later stud-

ies identifying GRK2 and β -arrestin2 as SMO-interacting proteins, interactions that were inhibited by PTCH and SMO inhibitors, such as cyclopamine, and that induced clathrin-dependent SMO internalization into endosomes (Chen et al., 2004b) and PTCH-independent down-regulation (Cheng et al., 2010). β -Arrestin is also important for SMO localization to the primary cilium, which is accomplished by coupling phosphorylated and β -arrestin-bound SMO with the molecular motor protein KIF3A and microtubule-based transport (Kovacs et al., 2008).

The first evidence for an involvement of β -arrestin in FZD signaling was based on β -arrestin's ability to modify DVL-induced β -catenin signaling to TCF/LEF transcription factors (Chen et al., 2001). Furthermore, WNT-5A triggers FZD₄-GFP internalization in a clathrin-dependent manner, requiring PKC phosphorylation of DVL as adaptor between FZD₄ and β -arrestin (Chen et al., 2003). Later on, it emerged that β -arrestin interacts and colocalizes with DVL, forms a ternary complex together with DVL and axin, and that β -arrestin was required for WNT-3A/ β -catenin signaling in vitro as well as for WNT-induced axis duplication in *X. laevis* (Bryja et al., 2007b). Furthermore, β -arrestin turned out to be crucial for convergent extension movements mediated by small GTPases of the RHO/RAC family (Kim and Han, 2007; Bryja et al., 2008). In addition to β -catenin and RHO/RAC GTPases induced by WNT-3A and WNT-5A, respectively, β -arrestin-independent WNT pathways have also been identified: because WNT-5A-induced convergent extension movements in *X. laevis*, which are known to be mediated by RORs (Schambony and Wedlich, 2007), are not affected by β -arrestin knock down or overexpression, Bryja et al. (2008) concluded that WNT-5A/ROR2 signaling does not depend on β -arrestin. Furthermore, it was recently suggested that the WNT/CK1 ϵ /RAP1 signaling axis might also function independently of β -arrestin (Schulte et al., 2009).

As described above, WNT-induced internalization of a FZD/DVL/ β -arrestin complex requires DVL-phosphorylation via PKC (Chen et al., 2003). RYK was identified as an additional component required for the WNT-11-induced internalization of FZD₇ in *X. laevis* (Kim et al., 2008). RYK interacts with both β -arrestin and WNT-11 to support endocytosis of FZD₇ and DVL. However, it remains to be established whether this mechanism is generally applicable for other FZDs and in other species. Because cells exist that do not express RYK but do express an elaborate network of FZDs (e.g., mouse microglia cells; Halleskog et al., 2010), it seems likely that FZD dynamics are maintained even in cells devoid of RYK.

The link between WNT/FZD signaling and β -arrestin has a number of important implications, which so far are only partially supported by direct experiments. First, β -arrestin-mediated internalization of WNT-bound FZDs could contribute to an agonist-dependent desensi-

tization, both short and long term, which could serve as a regulator of WNT/FZD signaling. Furthermore, as shown for classic GPCRs, β -arrestin could be a tool to achieve signaling specificity and signaling trafficking, which has been indicated in the WNT/RAC pathway (Bryja et al., 2008). A highly speculative but interesting possibility is that β -arrestin could mediate WNT-bias to FZDs and certain signaling pathways, thereby determining WNT-FZD outcome, as is known to occur in other examples of β -arrestin-mediated signaling trafficking (Lefkowitz, 2007; Schulte and Levy, 2007).

However, the initial assumption (Bryja et al., 2007b) that β -arrestin could serve as a signaling platform, similar to its function downstream of conventional GPCRs, recruiting mitogen-activated protein kinases for example, has previously been questioned (Force et al., 2007) and has still not been confirmed.

XI. Mechanisms for Signaling Specificity

One of the challenges in the field of WNT/FZD signaling is the manner in which specificity in communication is achieved. SMO signaling is relatively straightforward, because only one mammalian isoform of SMO and two isoforms of PTCH exist (Teglund and Toftgård, 2010). For WNT/FZD, however, a multitude of combinations between 19 WNTs, other extracellular ligands, and the 10 FZDs are theoretically possible. Nonetheless, the cells seem to be able to make sense of this confusing variety.

Even though the selectivity between WNTs and FZD has not yet been mapped—especially in the case of mammalian FZDs—it seems from *D. melanogaster* that WNT binding profiles differ between FZDs, which surely presents the first level of selection of a putative downstream response (Nusse et al., 2000; Rulifson et al., 2000; Wu and Nusse, 2002). Differences in ligand affinity to the CRD of *D. melanogaster* Fz and Frizzled 2 determine signaling outcome of chimeric receptors, indicating that WNT affinity to FZDs is important for signaling trafficking (Rulifson et al., 2000). Furthermore, WNT-5A, a WNT that generally activates β -catenin-independent pathways (Schulte et al., 2005; Mikels and Nusse, 2006), can be forced to activate WNT/ β -catenin signaling by overexpression of the WNT-5A receptor FZD₄ (Mikels and Nusse, 2006). It is less clear so far, however, how WNT-3A and WNT-5A, engaging the same receptor, such as FZD₂, can recruit different signaling pathways (Sato et al., 2010).

This is where accessory proteins, such as the coreceptors LRP5/6, come into the picture, because formation of a ternary complex of WNT-3A/FZD and LRP5/6 promotes β -catenin signaling (Cadigan and Liu, 2006). In the case of WNT-5A, LRP5/6 is not recruited and the β -catenin response is not activated but rather inhibited (Topol et al., 2003; Mikels and Nusse, 2006; Bryja et al., 2007d; Nemeth et al., 2007), a phenomenon that re-

quires another coreceptor, ROR2 (Mikels and Nusse, 2006) and CK1-phosphorylated DVL (Witte et al., 2010). Different mechanisms for this phenomenon have been suggested, such as increase of GSK-3-independent β -catenin degradation and WNT-3A/WNT-5A competition at LRP5/6 (Bryja et al., 2009) and FZD₂ (Sato et al., 2010).

It is in this context tempting to assume that a factor X, a coreceptor for example, is required to distinguish between WNT-3A and WNT-5A responses acting at FZDs, as proposed previously (Bryja et al., 2007d). In the case of WNT-11-induced FZD₇ internalization in *X. laevis*, RYK could fulfill this function (Kim et al., 2008).

Related to the question of specificity between WNT-3A and WNT-5A signaling is the function and signaling capacity of the downstream effector DVL. Stimulation with either of the WNT ligands leads to an undistinguishable formation of PS-DVL through activation of CK1 ϵ , but the outcome is different, even opposing (Bryja et al., 2007c,d). Here again, the coreceptors LRP5/6, which recruit DVL in the case of WNT-3A signaling, could provide the distinction because they bring about a different compartmentation of the active DVL pool.

DVL, recently described as a signaling hub, plays a major role in relaying WNT signaling (Gao and Chen, 2010). We have identified the DVL kinases CK1/2 as a switch in β -catenin-independent WNT signaling, in which the balance of CK1/2 and β -arrestin seems to determine the outcome of WNT signaling (Bryja et al., 2008). If CK1/2 predominates, signaling promotes PS-DVL and as-yet-unidentified downstream pathways, whereas predominance of β -arrestin with low CK1/2 activity supports signaling to small GTPases, such as RAC1.

The emerging picture is that cells have various tools to interpret and modify the signaling outcome in response to WNTs (Kikuchi et al., 2009). The transcriptional alteration of the receptor and coreceptor repertoire is an obvious way to adjust a cell's capability to react to WNTs (van Amerongen et al., 2008). Compartmentation of signaling to caveolae/lipid rafts, endocytosis, and formation of signaling endosomes, or restriction to other subcellular signaling rooms, could also affect responsiveness (Kikuchi et al., 2009), and scaffold molecules such as β -arrestin play an important role here (Force et al., 2007; Kovacs et al., 2009; Schulte et al., 2009).

Another possibility to regulate different WNT pathways is the regulation of DVL degradation through, for example, invasin, which serves as a switch between different WNT signaling cascades (Simons et al., 2005). Furthermore, reversible C-terminal palmitoylation of GPCRs is implicated in receptor-G protein specificity. This has not yet been investigated in the case of class Frizzled receptors but could offer additional possibilities to relay signaling to different pathways (Wess, 1998; Qanbar and Bouvier, 2003).

XII. Short Overview of Class Frizzled Receptor Function in Physiology and Disease

An exhaustive review of the function of the class FZD receptors in physiology and pathophysiology is not possible here, but there have been a number of excellent review articles (Chien and Moon, 2007; Luo et al., 2007; Malaterre et al., 2007; Nusse, 2008; Chien et al., 2009; van Amerongen and Nusse, 2009; Freese et al., 2010; Inestrosa and Arenas, 2010; Teglund and Toftgård, 2010; Traiffort et al., 2010). Before the presentation of an overview of possibilities to pharmacologically target class Frizzled signaling, however, it seems important to shortly introduce the role of class Frizzled receptors in physiology and disease.

The physiological function that has been most important for our knowledge accumulated during the last 20 years is the crucial role of FZDs and SMO in embryonic development (Riobo and Manning, 2007; Jiang and Hui, 2008; van Amerongen and Nusse, 2009). Genetically modified mice are important tools for the investigation of FZD/SMO function in vivo (see Table 3).

Regulation of cell fate, proliferation and differentiation of stem and progenitor cells, and tissue patterning are of importance not only during embryonic development but also in the adult, not least in cancer and cancer stem cells (Taipale and Beachy, 2001; Nusse et al., 2008; Espada et al., 2009; Teglund and Toftgård, 2010). With regard to their involvement in proliferation and differentiation, it seems obvious that deregulation of FZD and SMO signaling leads to various forms of cancer (van de Schans et al., 2008; Peukert and Miller-Moslin, 2010; Teglund and Toftgård, 2010). The intricate regulation of cell fate through these pathways is required on the one hand to allow sufficient activity to do the job they are aimed to do and on the other hand to carefully restrict signal intensity, spreading, and duration to prevent the pathway from turning oncogenic. Direct linkage between mutations in FZDs or SMO and disease are rare. According to the Human Protein Reference database (<http://www.hprd.org>) only FZD₄ and SMO mutations link directly to disease [i.e., familial exudative vitreoretinopathy/advanced retinopathy of prematurity (Robitaille et al., 2002; MacDonald et al., 2005; Nikopoulos et al., 2010) and sporadic basal cell carcinoma (Reifenberger et al., 1998; Xie et al., 1998), respectively]. In addition, SMO mutations were found in medulloblastoma (Reifenberger et al., 1998; Lam et al., 1999). However, with continuing research efforts, the involvement of FZD and SMO signaling components is being and will be associated to an increasing number of important diseases (Chien and Moon, 2007; Luo et al., 2007; Teglund and Toftgård, 2010). Of utmost importance are recent advances showing the role of WNT/FZD and HH/SMO signaling in the nervous system, with important implications for neuronal tube patterning, axonal remodeling, neurotransmitter release, regenerative and degenera-

tive processes (related for instance to Parkinson's and Alzheimer's disease), depression, anxiety, and hypoxia/ischemia (Salinas, 2005; Malaterre et al., 2007; Inestrosa and Arenas, 2010; Traiffort et al., 2010). Our increasing understanding will lead to novel and effective therapies, such as stem cell-based replacement therapies for various diseases. Because WNT-5A is an important factor for the differentiation of dopaminergic mid-brain neurons (Castelo-Branco et al., 2003; Schulte et al., 2005), it could be very useful for engineering of dopaminergic precursor cells for a stem cell-replacement therapy for Parkinson's disease (Parish and Arenas, 2007; Parish et al., 2008). Similar approaches are conceivable in, for example, skin, corneal or β cell replacement, hemapoietic disorders, liver disease, or heart failure) (Mimeault and Batra, 2006).

Genetic association of class FZD receptors with disease possibly reveals novel targets for future therapy. For example, FZD₃ association with schizophrenia has been intensively studied in various human populations, albeit with contradictory results (Katsu et al., 2003; Yang et al., 2003; Wei and Hemmings, 2004). Similar discrepancies have been found for the proposed association between WNT-2 and autism (Wassink et al., 2001; McCoy et al., 2002).

WNT/FZD signaling is also involved in the development of the heart and the vasculature (Masckauchán and Kitajewski, 2006). Even though WNT/FZD activity is low in the mature heart, signaling can be activated under pathophysiological conditions, such as artery injury and myocardial infarction (Blankesteyn et al., 2008; van de Schans et al., 2008). Strong evidence points to a possible therapeutic strategy exploiting the WNT/FZD signaling system for the treatment of cardiac hypertrophy and myocardial infarction, not least employing GSK3 inhibitors or FZD antagonists. Obviously, the effects of WNTs on vasculature and angiogenesis also play an important role in tumor growth and cancer as well as in eye disorders linked to incomplete retinal vascularization.

In recent years, defective WNT/FZD signaling has also been linked to diseases of the endocrine system, not least to type 2 diabetes mellitus (Welters and Kulkarni, 2008; Schinner et al., 2009), where TCF7 was identified as a risk factor for the disease (Jin and Liu, 2008). Osteoblast proliferation and differentiation are regulated by WNT/ β -catenin; therefore, alterations in this pathway result in either low or high bone mass. The association of WNT/FZD signaling with bone disease such as osteoporosis and osteoarthritis points at possible clinical importance (Hoepfner et al., 2009; Lodewyckx and Lories, 2009).

As a result of the ubiquitous expression of FZDs and SMO, the putative links between these signaling systems and disease seem almost endless. Care needs to be taken to identify the role of class Frizzled signaling in disease and to define whether dysregulation presents

cause or consequence for the disorder. It is likely that many physiological processes are modulated by class Frizzled receptor signaling: tissue injury and regeneration, for example, related to nerve injury and regeneration, responses to hypoxic injury, inflammation, and possibly also infection and immune responses (Beachy et al., 2004; Stoick-Cooper et al., 2007; Sen and Ghosh, 2008).

XIII. Class Frizzled Signaling as a Pharmacological Target

A. A Pharmacologist's View on Frizzled Ligand Binding Modes

As stated in the beginning of this review, I consider FZDs unconventional GPCRs and that translates to structure, signaling, and also to ligand binding. In comparison with adrenergic receptors binding adrenalin according to class A ligand-receptor interaction (Gether and Kobilka, 1998), one needs to consider many more complicating factors when imagining WNT/FZD interaction. First, the lipoglycoproteins cannot be regarded as freely diffusible ligands. They are probably bound and transported by lipoprotein particles (Hausmann et al., 2007; Morrell et al., 2008); show high affinity to extracellular matrix components (Yan and Lin, 2009), such as the glypicans; and are anchored in the membrane (Nusse, 2003), which restricts their free diffusion. The classic concept of on- and off-rate according to Langmuir's adsorption isotherm is invalid in this case, where chemical characteristics of the ligand suppress free diffusion and accessory factors can support (glypicans) or prevent (DKK, SFRPs) ligand-receptor interaction (Neubig et al., 2003).

Furthermore, studies of ligand binding between immobilized FZD-CRDs and tagged WNTs yielded binding affinities in the lower nanomolar range (Hsieh et al., 1999b; Rulifson et al., 2000), which in fact resembles corresponding affinities of conventional peptide ligands to their receptors. However, the conundrum that the CRD domain of FZDs is conserved even in proteins that do not bind WNTs (such as SMO and others) (Xu and Nusse, 1998) and data showing that the CRD might be dispensable for WNT-induced FZD-mediated effects (Chen et al., 2004a) suggest that the main binding mode of FZDs to their ligands could involve non-CRD parts of the receptor as well (Chen et al., 2004a). The CRD possibly acts as a ligand fishing rod (Povelones and Nusse, 2005), bringing the ligand close enough to the FZD that it can engage other regions of the receptor and establish the high-affinity complex required for signal induction. Unfortunately, to clarify this issue, we must establish an assay that monitors FZD-WNT interaction; this has proven difficult. Tagging WNTs changes the ligand's properties and affects biological activity (especially tags such as huge fluorescent proteins).

The next step related to the reasoning on WNT/FZD interaction and ligand binding modes touches upon the concept of putative drugs that could target the WNT/FZD interaction site. If in fact the CRD is dispensable for WNT action of FZD—or if it at least is not involved in establishment of a high-affinity complex—the strategy of a CRD-based competitive ligand for agonist or antagonist action might be futile. Ligands that target the core of the FZD and affect the classic helix 3/6 protrusion (Gether and Kobilka, 1998) could possibly be effective drug candidates for FZDs. Such ligands would not need to involve the WNT/CRD interface and might also increase the possibilities to achieve FZD selectivity. Similar drugs have been designed, for example, for mGlu₅ receptors class C, where the small ligand Glu binds at a large N terminus. 2-Methyl-6-(phenylethynyl)-piperidine, a highly effective receptor blocker and, in fact, a negative allosteric modulator, targets the TM3 and TM7 of mGlu₅R (Kuhn et al., 2002) without affecting other mGlu receptors.

B. General Considerations on “Druggable” Mechanisms

Before going into detail with drugs targeting class Frizzled receptors, it seems reasonable to discuss the molecular basis of disease that could actually successfully be treated by targeting receptor-activating processes or initiation of signal transduction by the receptors (Kiselyov et al., 2007). This review focuses on drugs that act to prevent ligand binding, act on the receptors themselves, or act to prevent receptor-mediated intracellular communication. Thus, drugs that attack downstream signaling events such as GSK3/axin/ β -catenin [e.g., LiCl, ICG-001, XAV939 etc. (Barker and Clevers, 2006; Huang et al., 2009)] or GLI-mediated transcriptional regulation (e.g., GANT61, HPI-1, -2, -3, -4 [Hyman et al., 2009; Stanton and Peng, 2010]) are not in the scope of this chapter.

In the case of WNT/Frizzled signaling, overexpression or lack of WNTs could be compensated pharmacologically by FZD antagonists or WNT mimetics, respectively. Furthermore, WNT excess can be counteracted by WNT-sequestering molecules, of which nature has invented a whole battery, such as SFRPs and WIF. These molecules target the receptor-ligand interface. Increased expression of various WNTs was reported in different forms of cancer (Rhee et al., 2002; Weeraratna et al., 2002; Sato et al., 2003), schizophrenia (Miyaoaka et al., 1999), acute renal failure (Terada et al., 2003; Surendran et al., 2005), pulmonary disease (Königshoff et al., 2008), inflammatory bowel disease (You et al., 2008a), and rheumatoid arthritis (Sen et al., 2000), to name but a few. On the other hand, reduced expression of WNTs is associated with diverse cancers such as colon cancer (Shu et al., 2006; Ying et al., 2008), leukemia (Liang et al., 2003), neuroblastoma (Blanc et al., 2005), breast cancer (Leris et al., 2005), and other disorders such as obesity (Christodoulides et al., 2006).

Classic GPCR-targeting drugs are often designed to act in the 7TM core of the receptor resembling the orthosteric site of rhodopsin-like class A receptors as either agonists, inverse agonists, or antagonists. In addition, allosteric modulators, such as 2-methyl-6-(phenylethynyl)-piperidine (mentioned in section VIII.A) are conceivable also in the case of class Frizzled receptors. It should be mentioned clearly that no such small-molecule drugs that act on FZDs—although highly desirable—are available in clinics, in clinical trials, or as research tools.

In addition, it is possible to direct drugs to proximate events downstream of the receptor. In the case of FZD signal transduction, it is obvious to aim at DVL as a FZD binding partner. FZD-DVL interaction is considered to be highly selective and unique for WNT signaling and therefore considered a suitable target for drug development (Fujii et al., 2007; Gao and Chen, 2010). This notion, however, has to be slightly revised with regard to a recent publication showing the interaction between parathyroid hormone receptor, a class B GPCR, and DVL via a KSxxxW sequence (Romero et al., 2010).

In the case of HH/SMO signaling as a drug target, the situation is a bit different. First, SMO is a constitutively active receptor, and endogenous ligands binding to and acting through SMO are so far only postulated (Taipale et al., 2002). Thus, the concept of interference with the ligand-receptor interaction cannot be applied in this case. However, PTCH-HH interaction and HH sequestration could be possible approaches. Furthermore, natural small molecules exist, such as the alkaloid cyclopamine (Fig. 8), that target SMO as inverse agonists to reduce SMO constitutive activity (Taipale et al., 2000; Masdeu et al., 2006). Cyclopamine has served as a pharmacophore for further drug refinement; in addition, structurally unrelated compounds could be identified as SMO agonists and inverse agonists (Chen et al., 2002b; King, 2002; Kiselyov et al., 2007; Stanton et al., 2009; Tremblay et al., 2009). It is important from a pharmacological perspective to distinguish between antagonists, inverse agonists, and allosteric modulators (Neubig et al., 2003; Kenakin, 2004), especially in the case of SMO. Antagonists, which by definition have an efficacy of zero, could be designed to compete with an as-yet-unidentified endogenous agonist. Real antagonists, however, will not reduce the constitutive activity of SMO on their own. On the other hand, the development of inverse agonists with a negative efficacy or allosteric modulators could reduce SMO activity.

Many disorders are linked to dysregulation of HH, SMO, and PTCH expression, as diverse as cancer (Reifenberger et al., 1998; Kirikoshi et al., 2001; Rhee et al., 2002; Merle et al., 2004; Nagayama et al., 2005; Zhang et al., 2006; Caldwell et al., 2008; Teglund and Toftgård, 2010), cardiac hypertrophy (van Gijn et al., 2002), inflammatory bowel disease (You et al., 2008a), and schizophrenia (Xie et al., 1998; Katsu et al., 2003). Thus,

FIG. 8. Structures of compounds targeting FZD, DVL, SMO and SHH. FOXY-5, WNT-5A mimetic peptide (Säfhölm et al., 2006). BOX-5, antagonistic peptide derived from WNT-5A (Jenei et al., 2009). Niclosamide acts on FZD₁ to induce internalization, DVL down-regulation, and block of WNT/ β -catenin signaling (Chen et al., 2009b). NSC668036 interferes with the DVL PDZ domain (Shan et al., 2005). Cyclopamine acts on SMO to inhibit HH signaling (Taipale et al., 2000); IPI-926 and GDC-0449 are clinical drug candidates and analogs to cyclopamine (Tremblay et al., 2009; Dierks, 2010). Robotnikinin targets SHH directly and prevents its effects (Stanton et al., 2009; Peukert and Miller-Moslin, 2010). The small-molecule agonist SAG, however, competes with cyclopamine for direct SMO binding (Yang et al., 2009). Structures were drawn with ACD/ChemSketch Software.

the therapeutic potential of selective drugs targeting these pathways is enormous.

C. Frizzled-Targeting Drugs

The quest for FZD ligands, either agonists or antagonists, has begun (Rey and Ellies, 2010) and offers novel possibilities to combat disease (Barker and Clevers, 2006). Difficulties that high-throughput design encounters are the presence of several FZD isoforms on many mammalian cells (Uren et al., 2004) and the proper design of a global measure covering both β -catenin-dependent and -independent WNT/FZD signaling. The TOPflash assay is well suited for HTS, with the disadvantage that it covers only β -catenin-dependent pathways (Molenaar et al., 1996; McMillan and Kahn, 2005). Immunoblotting of DVL, which is involved in β -catenin-dependent and -independent FZD signaling (Gao and

Chen, 2010), and the detection of PS-DVL would be a more general measure, but it is challenging to adjust immunoblotting for HTS. We have recently related the subcellular distribution of DVL2-MYC to the protein's electrophoretic mobility (Bryja et al., 2007d), showing that CK1 ϵ or WNT-5A promotes even distribution over punctate distribution of DVL2-MYC, a change that could be used for HTS in connection with automated image analysis. Another highly specific way to monitor substances that affect FZDs would be to observe receptor internalization (Chen et al., 2009b) or the recruitment of β -arrestin (Verkaar et al., 2008) in living cells. Furthermore, monitoring second messenger production or G protein activation could be suitable and well established methods for drug screening (Koval et al., 2010). Dirnberger and Seuwen (2007) described an attractive yeast-based system, employing the yeast mating pathway,

which is dependent on heterotrimeric G proteins, as a potential method to screen for drugs that activate human FZDs. The possibilities to screen for functional WNT/FZD signaling in HTS format are obviously numerous and limited only by the specificity of the response of FZDs to the activating ligand used.

So far, academic efforts have barely succeeded to identify small molecules that bind and affect FZDs. A recent success pinpointed the antihelminthic niclosamide (Fig. 8), which induces FZD₁ endocytosis, DVL down-regulation and block of WNT-3A-induced WNT/ β -catenin signaling (Chen et al., 2009b). However, it remains unclear whether the niclosamide effects on FZD and FZD signaling are directly mediated through drug-receptor interaction.

Furthermore, short peptides have been established as WNT-5A mimetics or as FZD antagonists. A formylated hexapeptide (formyl-Met-Asp-Gly-Cys-Glu-Leu) derived from WNT-5A was first shown to mimic the effects of WNT-5A on human breast epithelial cells, suggesting a possible use for cancer therapy (Säfhölm et al., 2006). This peptide, named FOXY-5 (see Fig. 8), indeed turned out to impair the progression of estrogen receptor α -deficient breast cancer: FOXY-5 increases cell adhesion, thereby reducing migration of breast cancer cells, and induces expression of estrogen receptor α , rendering the cells sensitive to tamoxifen (Ford et al., 2009). The same research group also developed a *t*-butyloxycarbonyl-modified WNT-5A-derived hexapeptide (*t*-butyloxycarbonyl-Met-Asp-Gly-Cys-Glu-Leu), named BOX-5 (see Fig. 8), which blocks both the WNT-5A- and FOXY-5-induced effects in invasive melanoma cells (Jenei et al., 2009). Thus, BOX-5 represents an antimetastatic therapy for rapidly progressive melanoma, and for WNT-5A-stimulated invasive cancers. It is noteworthy that FOXY-5 and BOX-5 differ only in the N-terminal head group and seem to have opposing effects. Similar changes in agonist/antagonist characteristics of peptide ligands depending on formyl- or *t*-butyloxycarbonyl N termini have also been described for bacterial and mammalian *N*-formyl peptides acting at formyl peptide receptors (Ye et al., 2009a). Further studies to investigate FZD interaction sites and mode of action are required to fully understand these two ligands.

Another FZD antagonist developed at the Cardiovascular Research Institute Maastricht is currently available for licensing (Laeremans et al., 2009; Blankestijn et al., 2010). This drug, a short 13-aa peptide (CNKT-SEGMDGCEL) termed UM206 shows an IC₅₀ at FZD₁ and FZD₂ in the lower nanomolar range without affecting signaling through FZD₄ and FZD₅. It was developed as a tool to prevent excessive fibrosis as a consequence of tissue damage or infarction and, thus, to prevent malfunction of organs such as heart, lung, kidney, and liver. With a half-life of 90 min in mice and rats, this drug can be used in vivo as a FZD_{1/2} antagonist or diagnostic tool for imaging studies.

Nature has been quite inventive with the design of FZD-CRD-interacting compounds. The recent finding that amyloid peptide β can bind and competitively antagonize WNT/ β -catenin signaling could support a rational design of drugs targeting the CRD in addition to its putative relevance for Alzheimer's disease (Magdesian et al., 2008).

D. Small-Molecule Compounds Targeting Disheveled

As one of the known WNT/FZD-selective signaling compounds, DVL is of pharmacological interest, and the search for small-molecule blockers targeting DVL has met with some success. A small-molecule compound (NSC668036 from the National Cancer Institute small-molecule library; for structure, see Fig. 8) has been identified that disturbs binding and communication between FZD and DVL by binding the DVL-PDZ domain (Shan et al., 2005). In *X. laevis*-based assays, this compound was capable of inhibiting WNT-3A-induced signaling. Targeted drug development yielded ((2-(1-hydroxypentyl)-3-(2-phenylethyl)-6-methyl)indole-5-carboxylic acid (FJ9), an indole-2-carbinol-based compound that prevents the interaction between FZD₇ and the PDZ domain of DVL and thereby diminishes growth of tumor cells in a β -catenin-dependent manner (Fujii et al., 2007). A similar structural approach was then further developed to optimize indole-2-carbinol-based compounds that selectively target the DVL1-TCF pathway in cells and to implement a screening platform for the development of compounds with higher potency, efficacy, and selectivity (Mahindroo et al., 2008; You et al., 2008b). In addition, a recent study employing NMR-assisted virtual screening identified another compound targeting the DVL-PDZ domain (Grandy et al., 2009).

E. WNT and Frizzled Antibodies

Because of the lack of small-molecule inhibitors, antibodies targeting WNTs or FZDs were suggested for therapeutic strategies that aim to reduce WNT/FZD signaling. For example, Rhee et al. (2002) employed WNT-1 antibodies in a head and neck squamous cell carcinoma cell line to reduce cyclin D1 and β -catenin levels, reduce proliferation, and induce apoptosis. A WNT-2 antibody was able to induce apoptosis in malignant melanoma cells and to reduce tumor growth (You et al., 2004). Monoclonal antibodies MAb 92-13 raised against FZD₁₀ and coupled to yttrium-90 were capable to reduce tumor growth of synovial sarcoma in a Xenograft mouse model suggesting its use for radioimmunotherapy even in patients (Fukukawa et al., 2008). Antibody strategies were used not only in cancer models but were also extended to rheumatoid arthritis, where anti-FZD₅ antibodies reduced IL-6 and IL-15 expression in fibroblast-like synoviocytes (Sen et al., 2001).

F. Recombinant Frizzled-Cysteine-Rich Domains

Endogenously expressed and secreted molecules containing a CRD, such as the SFRPs, bind WNTs and—at least partially—prevent their interaction with FZDs and other WNT receptors and can thereby reduce tumor growth in vivo (Finch et al., 1997; Bovolenta et al., 2008; Hu et al., 2009). From a conceptual standpoint, the same is true for recombinant CRDs derived from the FZDs themselves. These FZD-CRDs are useful WNT-sequestering tools in the laboratory (Castelo-Branco et al., 2003); more importantly, they are about to make their way into the clinics as effective therapeutics for cancer. The FZD₇ ectodomain containing the CRD was used initially in a xenograft tumor model in which FZD₇-CRD expressing cells were able to reduce tumor size (Vincan et al., 2005). Furthermore, the recombinant FZD₈-CRD fused to the human Fc domain was used directly to treat teratocarcinomas in vivo, indicating its potential clinical application (DeAlmeida et al., 2007).

G. Drugs Targeting Hedgehog-Smoothed Signaling

Because dysfunctional SMO signaling is highly oncogenic, it has been suggested as an attractive target of pharmacological intervention (Tremblay et al., 2009; Peukert and Miller-Moslin, 2010). HH-sequestering antibodies have been studied in this context (Ericson et al., 1996). In addition, the classic SMO antagonist cyclopamine, a teratogenic *Veratrum* species alkaloid, is known to interfere with SMO activation (Cooper et al., 1998), blocking the effects of oncogenic mutations that both disturb PTCH function and activate SMO (Taipale et al., 2000) by direct interaction with SMO (Chen et al., 2002a). Furthermore, additional chemical modulators of HH signaling could be identified (King, 2002); some have been patented and partially proven to be useful even in clinical trials (see also Fig. 8) (Kiselyov et al., 2007; Lauth et al., 2007; Tremblay et al., 2009; Dierks, 2010).

Cyclopamine and drugs acting in a similar way on SMO, such as GDC-0049 and IPI-926, are basically known as antagonists (Peukert and Miller-Moslin, 2010). However, a HEK293 cell system overexpressing SMO and α_{15} revealed that several compounds, such as cyclopamine, Cur61414, and SANT-1, display inverse agonist properties, reducing phospholipase C activity induced by constitutively active SMO (Masdeu et al., 2006).

It is noteworthy that small-molecule agonists called purmorphamine and SAG (Fig. 8) have been identified to compete with cyclopamine for SMO binding and to promote SMO activity (Chen et al., 2002b; Stanton and Peng, 2010).

In addition to drugs targeting SMO, another small molecule was identified on the basis of its binding to SHH and its inhibitory action (Stanton et al., 2009). This molecule, called robotnikinin (Fig. 8), blocks SHH action

but does not inhibit signaling induced by SAG and purmorphamine.

In addition to their potential clinical use (Stecca and Ruiz i Altaba, 2002), the discovery of small molecules regulating SMO supports the recently emerging hypothesis that SMO activity might not be regulated solely by PTCH but also directly through endogenous small molecules (King, 2002). The strategies to target the HH/SMO pathway are diverse and not necessarily restricted to PTCH or SMO (Kiselyov et al., 2007; Hyman et al., 2009; Stanton and Peng, 2010). For example, Hyman et al. (2009) characterized four different compounds with regard to their point of interference with the HH/SMO system. One compound blocked GLI1/2 activity, possibly acting independently of the primary cilium. Two compounds interfered with the maturation of GLI2 into a transcriptional activator, whereas another compound disrupted ciliogenesis and thereby ciliary processes that are required for SMO signaling, more specifically for GLI function.

XIV. Future Directions

Our understanding of embryonic development, stem cell regulation, cancer, and many other diseases has increased immensely with the discovery of the class Frizzled receptors. However, even though some of these discoveries were made decades ago and our knowledge of molecular details has improved dramatically, many questions and challenges remain.

With regard to WNT/FZD signaling and FZD pharmacology, the lack of purified, active, and labeled WNTs is still a major obstacle. Since the original purification of WNT-3A, when it became possible to acutely stimulate cells with relatively well defined concentrations of agonists, kinetic analysis of WNT/FZD signaling has enabled us to characterize receptor signaling and pharmacology in greater detail. To completely understand WNT/FZD binding, receptor specificity, and possibly signaling trafficking, we will need access to all WNTs in pure form. Furthermore, the establishment of a method to monitor and quantify WNT/FZD interaction would enhance our knowledge of receptor function, interaction between ligands, and endogenous WNT inhibitors and could aid screening for small-molecule drugs targeting FZDs.

In parallel with more information on receptor function, it is necessary to map signaling routes activated by FZDs and SMO, especially the various factors that define the signaling outcome of receptor stimulation in a given context of coreceptors, scaffold molecules, and cellular state. These studies will lead to common signaling concepts but also distinct species differences. In this context, the advantage of a unifying species-independent nomenclature for both receptors and signaling components should be mentioned, which surely is a challenge for many research fields.

This review has focused mainly on the molecular pharmacology of the class FZD receptors. Further detailed knowledge on signaling paradigms will also help us to understand the biological roles of these receptors. Because the field has its origin in developmental biology, the role of HH/SMO and WNT/FZD in the healthy and diseased adult is less well understood. There is strong evidence that these signaling systems regulate many different aspects of physiology and pathophysiology, which so far are not necessarily considered to be affected by FZD or SMO. As we continue to make novel discoveries about the molecular signaling mechanisms of class FZD receptors and delineate their roles in biology, it will become increasingly relevant to focus on the quest for drugs that target these central pathways of cellular communication.

Acknowledgments. This work was supported by the Department of Physiology and Pharmacology, Karolinska Institute; the Foundations of the National Board of Health and Welfare of Sweden; the Swedish Medical Research Council [Grants K2008-68P-20810-01-4, K2008-68X-20805-01-4]; the Swedish Foundation for International Cooperation in Research and Higher Education [Grant YR2007 7014]; the Swedish Cancer Foundation [Grant CAN2008/539]; the Åhlén-Foundation [Grant mE7/09]; the Signhild Engkvist Foundation; and the Knut and Alice Wallenberg Foundation [Grant KAW2008.0149]. I am very grateful to Janet Holmén, Vitezslav Bryja, Jens Henrik Nørum, and Jon Lundberg for critical and constructive comments on the manuscript. I apologize to all authors in the field of class Frizzled receptors whose relevant and original work has not been cited in this review.

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