Systematic Mapping of WNT-FZD Protein Interactions Reveals Functional Selectivity by Distinct WNT-FZD Pairs*

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Background: WNT-FZD specificity and signaling outcome have not been systematically mapped so far.

Results: WNTs show selective binding to FZDs, and respective WNT-FZD pairs exert functional selectivity in different downstream signaling pathways.

Conclusion: The WNT-FZD signaling system provides ligand-receptor selectivity.

Significance: Understanding WNT-FZD selectivity is crucial for development of WNT pathway inhibitors and further understanding of molecular interactions within the WNT receptor signaling complex.

The seven-transmembrane-spanning receptors of the FZD1–10 class are bound and activated by the WNT family of lipoglycoproteins, thereby inducing a complex network of signaling pathways. However, the specificity of the interaction between mammalian WNT and FZD proteins and the subsequent signaling cascade downstream of the different WNT-FZD pairs have not been systematically addressed. In this study, we determined the binding affinities of various WNTs for different members of the FZD family by using bio-layer interferometry and characterized their functional selectivity in a cell system. Using purified WNTs, we showed that different FZD cysteine-rich domains prefer to bind to distinct WNTs with fast on-rates and slow off-rates. In a 32D cell-based system engineered to overexpress FZD2, FZD4, or FZD5, we found that WNT-3A (but not WNT-4, -5A, or -9B) activated the WNT-β-catenin pathway through FZD2/4/5 as measured by phosphorylation of LRPI-6 and β-catenin stabilization. Surprisingly, different WNT-FZD pairs showed differential effects on phosphorylation of DVL2 and DVL3, revealing a previously unappreciated DVL isoform selectivity by different WNT-FZD pairs in 32D cells. In summary, we present extensive mapping of WNT-FZD cysteine-rich domain interactions complemented by analysis of WNT-FZD pair functionality in a unique cell system expressing individual FZD isoforms. Differential WNT-FZD binding and selective functional readouts suggest that endogenous WNT ligands evolved with an intrinsic natural bias toward different downstream signaling pathways, a phenomenon that could be of great importance in the design of FZD-targeting drugs.

The WNT-FZD (Wingless/Int1-Frizzled) signaling system has a prevalent role in physiology during adulthood. It also regulates a plethora of processes during embryonic development, such as stem cell proliferation, migration, and differentiation. Dysfunction of WNT-FZD signaling leads to impairment of cell regulatory mechanisms, thereby leading to disease, such as cancer and neurological and bone disorders (1–3). Despite the physiological relevance of this pathway, little is known about the specificity of the interactions between the 19 members of the WNT family of lipoglycoproteins and their respective FZD cell-surface receptors.

FZDs have the typical G protein-coupled receptor architecture of seven-transmembrane segments and also show functional features of G protein-coupled receptors (4–6). Therefore, the FZDs together with the Hedgehog signal transducer Smoothened were classified as an independent class within the superfamily of G protein-coupled receptors by the International Union of Basic and Clinical Pharmacology (7), yet, unlike Smoothened, little is known about the pharmacological features of the 10 FZD isoforms (3). FZDs can act together with a range of single-membrane-spanning co-receptors, such as LRPS (LDL-receptor-related protein 5), LRPS6, ROR1 (receptor tyrosine kinase-like orphan receptor 1), ROR2, RYK (related to receptor tyrosine kinase), and proteoglycans, which serve as binding partners for WNTs and can thereby contribute to signal specification (8–10).
WNTs interact with FZDs through their cysteine-rich domain (CRD)\(^5\) at the N terminus of the receptors (11, 12), leading to initiation of distinct downstream signaling pathways. The WNT-\(\beta\)-catenin pathway is initiated by formation of a complex between WNT, FZD, and LRP5/6, resulting in subsequent stabilization of the transcriptional regulator \(\beta\)-catenin. On the other hand, the mammalian \(\beta\)-catenin-independent pathways are transduced by WNT-FZD complexes and potentially other co-receptors, leading to complex signaling networks, such as the WNT-\(\beta\)Catenin (WNT-RAC, WNT-RHO, and planar cell polarity-like pathways (3, 13, 14). In all these different scenarios, the architecture of the WNT-receptor signaling complex may direct downstream signaling output, with the most proximal and central key players downstream of the WNT-receptor complex being the FZD-binding phosphoproteins DVL1 (Dishevelled 1), DVL2, and DVL3 (4, 15).

Although different signaling pathways initiated by WNTs have been characterized, the specificity of WNT-receptor interactions is still unclear. Using purified proteins, we showed previously that different WNT isoforms bind tightly to distinct regions on LRP6 (12), suggesting new possibilities for the molecular arrangement of WNT signaling complexes at the cell surface. However, the specificity of the biochemical interactions between WNTs and FZDs remains elusive, and consequently, the underlying mechanisms for selective activation of specific pathways by different isoforms of WNT-FZD complexes remain obscure. *Drosophila* WNTs were shown to exhibit distinct FZD isoform specificity as shown by alkaline phosphatase-based interaction assays, but the information for mammalian WNTs is very limited (16). A previous study that utilized mouse, human, and *Drosophila* FZD CRDs showed that a *Xenopus laevis* WNT-8 alkaline phosphatase fusion protein bound to the mouse FZD\(_4\) CRD with 8 nM affinity (17). Also, previous data indicate that stimulation with different recombinant WNTs in N13 microglia-like cells expressing mRNA from several FZD isoforms resulted in distinct signaling profiles depending on WNT-FZD pairings, suggesting signaling selectivity (18). Along the same lines, using fluorescence recovery after photobleaching experiments for quantification of the mobile fraction of FZD\(_4\)GFP expressed in HEK293 cells, it was shown that different recombinant WNTs differentially affect the lateral mobility of FZD\(_4\)GFP (19), again suggesting ligand-receptor functional selectivity. To date, it has not been possible to measure WNT interaction with full-length FZDs by classical quantitative receptor binding assays, such as those utilizing radioactively labeled WNT, because (i) the labeling interferes with WNT biological activity, and (ii) there are no selective small-molecule antagonists that enable distinction between specific versus nonspecific binding at the receptor’s orthosteric site.

Here, we determined the binding affinities of a subset of purified WNTs for different isoforms of soluble FZD CRDs in a cell-free system. Furthermore, employing a murine bone marrow-derived 32D cell line, which expresses little or no FZD mRNA, combined with heterologous expression of specific FZD isoforms, we characterized the cellular effects of four different WNTs (WNT-3A/4/5A/9B) in combination with FZD\(_4\), FZD\(_5\), or FZD\(_7\). The observed functional selectivity between different WNT-FZD pairs suggests a model in which downstream WNT signaling output is determined by the identity of both WNTs and FZDs present at the cell surface.

### EXPERIMENTAL PROCEDURES

#### Materials—Purified and carrier-free mouse and human WNT proteins were obtained from R&D Systems and used in the binding assays. Different isoforms of soluble mouse FZD CRDs fused to Fc were also commercially obtained (R&D Systems).

**WNT-FZD CRD-Fc Binding Assays—**Binding kinetics were measured by bio-layer interferometry on an Octet RED96 system (Pall ForteBio Corp.) as described previously (12). Briefly, biosensors (anti-human IgG-Fc capture) were loaded with different recombinant FZD CRD-Fc proteins in 50 mM Tris (pH 7.2), 300 mM NaCl, 5% (v/v) glycerol, and 0.05% (w/v) Triton X-100. The loaded biosensors were washed in the same buffer before association and dissociation measurements were performed with different purified WNTs for the indicated times. Kinetic parameters (\(k_{on}\) and \(k_{off}\)) and affinities (\(K_D\)) were determined from a nonlinear fit of the data, and the plots were generated using the Octet software. Each reported value represents the average of at least three experiments at different concentrations, with a fitted experimental curve for which the correlation coefficient (\(R^2\)) is above 0.98 and the maximal response signal for the association curve is above 0.07 nm. In the case of WNT-4, a weak response signal to FZD CRD-Fc proteins was observed, and the values derived from fitted experimental curves with maximal response above 0.03 nm and \(R^2 > 0.96\) were still considered in the calculation of kinetic parameters. The \(K_D\) represents the ratio between the \(k_{on}\) and \(k_{off}\) at equilibrium, when the rate of complex formation by WNT and FZD CRD-Fc is equal to the rate of dissociation of the two components (20):

\[
K_D (\text{nm}) = \frac{k_{off}}{k_{on}} = \frac{[\text{WNT}][\text{FZD CRD-Fc}]}{[\text{WNT-bound FZD CRD-Fc}]}.
\]

Higher affinity binding pairs have a smaller \(K_D\) because less protein is required to establish equilibrium.

#### Cell Culture and Stable Transfection—**The IL-3-dependent mouse cell line 32D was maintained in RPMI 1640 medium supplemented with 15% FBS, 50 units/ml penicillin, 50 \(\mu\)g/ml streptomycin, 2 mM L-glutamine, and 5% mouse myelomonocytic WEHI3B cell-conditioned medium and kept in a humidified atmosphere at 37 °C and 5% CO\(_2\). WEHI3B cell-conditioned medium containing IL-3 was collected from confluent monolayers, filtered, and stored at −20 °C. To obtain 32D cells expressing FZD\(_4\) or FZD\(_5\), \(1 \times 10^6\) cells were transfected with 2 \(\mu\)g of DNA from the HA-tagged FZD\(_4\) or FZD\(_5\) construct, respectively, using a Nucleofector 2b device (Lonza) according to the manufacturer’s protocol (Solution V, Program E32). FZD\(_4\)-expressing 32D cells were created using a Neon transfection system (Invitrogen) according to the manufacturer’s protocol. Briefly, 2 \(\times 10^5\) cells were mixed with 1 \(\mu\)g of DNA from the HA-FZD\(_4\) construct and electroporated in a 10-\(\mu\)l tip with two pulses having a width range of 30 ms and 1100 V. Stable clones were selected with 3 mg/ml hygromycin B (Cal-

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\(^5\) The abbreviations used are: CRD, cysteine-rich domain; qPCR, quantitative PCR.
biochem) and maintained in growth medium supplemented with 1 mg/ml hygromycin B.

RNA Isolation, Quantitative Real-Time PCR, and RT-PCR—RNA was isolated from 32D cells (1 × 10⁶ cells per isolation) using an RNeasy mini kit (Qiagen, Hilden, Germany) and transcribed using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Quantitative PCR (qPCR) was performed in triplicates with a TaqMan gene expression assay (Applied Biosystems) according to the manufacturer’s instructions. Measurements were done with an ABI PRISM 7000 sequence detector. The primer pairs were used as described previously (22, 23), and PCR products were quantified with ImageJ software. The molecular masses of FZDs were determined by Western blotting using the related Image Lab software (Bio-Rad). All data were analyzed with unpaired Student’s t test. Immunoblot data are shown as mean ± S.E., and binding data are presented as mean ± S.D. All experiments were repeated at least three independent times.

RESULTS

WNT-FZD CRD Binding Studies—To investigate the interaction between WNT and FZD, we developed a binding assay based on bio-layer interferometry. Different isoforms of the FZD (FZD₁/2/4/5/7/8) CRD fused to Fc were loaded onto anti-human IgG biosensor tips. The sensor tip was washed and then transferred to different solutions containing a concentration range of purified recombinant WNTs (WNT-3A, -4, -5A, and -5B) (Table 1 and Fig. 1, A and B) that have been functionally validated in different assays in previous studies by us and others (6, 12, 18, 19, 21, 22, 24). We performed kinetic binding assays and determined the WNT association rate (k₈), the WNT dissociation rate (k₉), and the equilibrium dissociation constant (K₈) between different WNTs and FZD CRDs.

Representative binding curves of WNT-3A and WNT-5A binding to various FZD CRDs are shown in Fig. 1A, along with the relative ranking of KD values (Fig. 1B). The k₈, k₉, and KD values for WNT and FZD CRD binding are shown in Table 1.

WNT-3A showed strong binding (+ + + +, K₈ < 10 nM) to the FZD₁/2/4/5/7/8 CRDs (Table 1 and Fig. 1, A and B), suggestive of a tight complex between WNT-3A and these FZD isoforms on the cell surface. However, WNT-3A displayed intermediate

| Binding to FZD₃ CRD | K₈ | k₉ | KD |
|--------------------|----|----|----|
| mWNT-3A            | NQ | NQ | NQ |
| mWNT-4             | 15.2 ± 3.9 | 2.0 ± 0.4 | 3.0 ± 0.4 |
| h/mWNT-5A          | 26.6 ± 6.8 | 3.1 ± 1.6 | 7.4 ± 1.3 |
| mWNT-5B            | 90.4 ± 40.4 | 1.0 ± 0.5 | 8.0 ± 1.0 |

| Binding to FZD₄ CRD | K₈ | k₉ | KD |
|--------------------|----|----|----|
| mWNT-3A            | 15.7 ± 4.7 | 2.7 ± 1.1 | 3.8 ± 0.9 |
| mWNT-4             | 103.4 ± 18.4 | 1.0 ± 0.3 | 10.0 ± 2.7 |
| h/mWNT-5A          | 24.5 ± 16.8 | 2.6 ± 1.7 | 4.4 ± 0.8 |
| mWNT-5B            | 91.4 ± 34.0 | 0.7 ± 0.4 | 5.1 ± 1.9 |

| Binding to FZD₅ CRD | K₈ | k₉ | KD |
|--------------------|----|----|----|
| mWNT-3A            | 5.4 ± 1.8 | 2.9 ± 0.9 | 1.4 ± 0.2 |
| mWNT-4             | 43.7 ± 13.4 | 1.4 ± 0.4 | 5.9 ± 1.4 |
| h/mWNT-5A          | 22.6 ± 10.1 | 2.3 ± 1.4 | 4.1 ± 0.3 |
| mWNT-5B            | 50.1 ± 18.9 | 0.6 ± 0.3 | 2.4 ± 0.9 |

| Binding to FZD₆ CRD | K₈ | k₉ | KD |
|--------------------|----|----|----|
| mWNT-3A            | 3.1 ± 1.0 | 2.5 ± 0.6 | 0.7 ± 0.2 |
| mWNT-4             | 14.6 ± 6.6 | 0.9 ± 0.3 | 1.2 ± 0.6 |
| h/mWNT-5A          | 5.1 ± 1.6 | 2.9 ± 1.6 | 1.3 ± 0.2 |
| mWNT-5B            | 22.2 ± 11.3 | 1.3 ± 0.6 | 2.5 ± 0.4 |

| Binding to FZD₇ CRD | K₈ | k₉ | KD |
|--------------------|----|----|----|
| mWNT-3A            | 5.3 ± 1.2 | 2.3 ± 0.8 | 1.2 ± 0.5 |
| mWNT-4             | 94.5 ± 47.7 | 1.0 ± 0.2 | 9.2 ± 3.8 |
| h/mWNT-5A          | 42.9 ± 28.0 | 2.1 ± 2.5 | 4.4 ± 0.8 |
| mWNT-5B            | 83.5 ± 35.7 | 0.7 ± 0.4 | 5.0 ± 0.2 |

| Binding to FZD₈ CRD | K₈ | k₉ | KD |
|--------------------|----|----|----|
| mWNT-3A            | 1.5 ± 0.9 | 2.0 ± 0.6 | 0.3 ± 0.3 |
| mWNT-4             | 9.4 ± 5.2 | 1.2 ± 1.1 | 0.8 ± 0.3 |
| h/mWNT-5A          | 4.0 ± 2.8 | 1.9 ± 1.3 | 0.5 ± 0.3 |
| mWNT-5B            | 8.0 ± 2.5 | 1.3 ± 0.9 | 0.8 ± 0.2 |

Table 1: Binding affinities and kinetic constants for various WNTs binding to various FZD CRDs as measured by bio-layer interferometry

NQ indicates weak binding, not quantifiable due to a narrow response signal window in the binding curves. m, mouse; h, human.
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A

| Ligand   | FZD₁ CRD | FZD₂ CRD | FZD₄ CRD | FZD₅ CRD | FZD₇ CRD | FZD₈ CRD |
|----------|----------|----------|----------|----------|----------|----------|
| mWNT-3A  | +++      | +++      | ++++     | ++++     | ++++     | ++++     |
| mWNT-4   | NQ       | +        | ++       | +++      | ++       | +++      |
| h/mWNT-5A| +++      | +++      | +++      | ++++     | ++       | +++      |
| mWNT-5B  | ++       | ++       | ++       | +++      | ++       | +++      |

B

The table above represents the relative binding affinities of different WNT ligands (mWNT-3A, mWNT-4, h/mWNT-5A, and mWNT-5B) to FZD receptors (FZD₁, FZD₂, FZD₄, FZD₅, FZD₇, and FZD₈). The affinities are indicated by the number of plus signs, with more signs denoting higher affinity.
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binding (++, \( K_D \sim 15 \text{ nM} \)) to the FZD1/2 CRDs (Table 1 and Fig. 1, A and B). On the other hand, WNT-4 showed strong binding to the FZD6 CRD and very weak to intermediate binding to the FZD2/4/5/7 CRDs (Table 1 and Fig. 1B). Although both WNT-5A and WNT-5B displayed strong binding (++, \( K_D = 4-8 \text{ nM} \)) to the FZD6 CRD (Table 1 and Fig. 1B) and intermediate to strong binding (++) to ++++, \( K_D = 5-22 \text{ nM} \)) to the FZD5 CRD (Table 1 and Fig. 1, A and B), WNT-5A exhibited intermediate binding (++, \( K_D = 22-26 \text{ nM} \)) to the FZD1/2/4 CRDs (Table 1 and Fig. 1, A and B) and weak binding to the FZD3 CRD, whereas WNT-5B showed weak binding (\( K_D > 40 \text{ nM} \)) to these FZD CRDs (Table 1 and Fig. 1B). Finally, WNT-7A/9B/10B/11 and FZD10 did not show detectable binding to any of the chosen FZD CRDs or WNTs in this study, respectively (data not shown).

Characterization of 32D Cells—To assess the functional selectivity of WNT-FZD combinations, we chose the myeloid progenitor cell line 32D as a cell model because it expresses little or no mRNA for FZDs as determined by qPCR (Fig. 2). Used as a control, mouse primary microglia cells express endogenous FZDs (Fig. 2A) that are responsive to different WNTs (19, 21, 22, 24, 25). The lack of expression of endogenous FZDs in 32D cells (compare \( \Delta C_T \) values of FZD expression in 32D cells and microglia cells) (Fig. 2A) enables dissection of the function of a particular FZD via generating a stable 32D/FZDx-expressing cell line. To our knowledge, a cell system that expresses little or no endogenous FZDs has not been described previously. Moreover, we mapped the expression of other WNT co-receptors by RT-PCR (Fig. 2B). LRP5/6 (but neither ROR1/2 nor RYK) was expressed in 32D cells, thus providing the opportunity to activate the LRP5/6-dependent WNT-\( \beta \)-catenin pathway without involvement of other pathways that are dependent on ROR1/2 or RYK signaling. In general, 32D cells appear to lack endogenous WNT signaling components as shown in the PCR profiling provided in Fig. 2C, even though LRP5/6 and DVL1/2/3 are expressed and the \( \beta \)-catenin destruction complex machinery is functional.

We generated 32D cell lines that stably expressed FZD\(_3\), FZD\(_4\), or FZD\(_5\). The FZD constructs contained two HA tags that were engineered at the N terminus and downstream of the signal sequence to enable proper membrane targeting of the expressed protein and to allow assessment of FZD expression levels by immunoblotting (Fig. 2C). The HA-FZD\(_{2/4/5}\) signals were detected at 53.1, 53, and 63.3 kDa, respectively. These FZD constructs represent three of the four clusters of evolutionarily conserved class FZD receptors (3). Because only a transient low
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level of FZD2 and FZD5 expression (representing the fourth cluster) was detected upon transfection of these constructs in 32D cells, we excluded these cell lines from our study.

β-Catenin Stabilization—The parental 32D cells were incubated with increasing concentrations of purified soluble WNT-3A/4/5A/9B for 2 h (Fig. 3, A and B). The lack of observed β-catenin stabilization confirmed that the parental 32D cells were not responsive to WNTs in the absence of endogenous FZDs. However, expression of FZD2, FZD4, or FZD5 in 32D cells resulted in a concentration-dependent increase in β-catenin protein levels in response to increasing concentrations of WNT-3A (Fig. 3, A and B). On the other hand, the maximal concentration of WNT-5A induced a slight stabilization of β-catenin in 32D/FZD5 (but not in 32D/FZD2 or 32D/FZD4) cells, and neither WNT-4 nor WNT-9B induced β-catenin stabilization in any of the cell lines tested.

WNT-3A (but Not WNT-4, -5A, or -9B) Induces Phosphorylation of LRP6—LRP6 serves as a co-receptor for FZDs and is central for the initiation and specification of the WNT–β-catenin pathway. The formation of a ternary WNT-FZD-LRP5/6 complex induces distinct LRP6 phosphorylation and recruitment to DVL-dependent signalosomes (26–30). Subsequently, this results in inhibition of glycogen synthase kinase 3-dependent β-catenin phosphorylation and degradation, thereby leading to β-catenin stabilization. In 32D/FZD2/4/5 cells, only WNT-3A induced phosphorylation of LRP6 (Fig. 4, A and B), consistent with the stabilization of β-catenin observed earlier (Fig. 3, A and B) and indicating that WNT-3A induces the β-catenin pathway via the aforementioned FZDs.

Differential Phosphorylation of DVL2 and DVL3—We used the same experimental setup as described above for β-catenin and phospho-LRP6 to investigate WNT-induced phosphorylation of DVL2 and DVL3 (as measured by electrophoretic mobility shift of phosphorylated and shifted DVL, known as PS-DVL). DVL is a key player in FZD signaling and mediates both β-catenin-dependent and β-catenin-independent pathways (24, 25, 31–35). Interestingly, parental 32D cells did not show a basal shift in DVL upon stimulation with WNT-3A, -4, -5A, or -9B (Fig. 5, A and B, and Fig. 6, A and B), indicating lack of PS-DVL formation under these treatment conditions. However, expression of FZD2, FZD4, or FZD5 induced PS-DVL formation even in the absence of WNT stimulation. Moreover, stimulation of 32D/FZD2/4/5 cells with increasing concentrations of the four WNTs revealed differential effects on PS-DVL2 and PS-DVL3 in a manner that was dependent on the WNT-FZD pair present. PS-DVL2 formation was not observed in response to any of the WNTs in 32D/FZD5 cells, whereas PS-DVL3 formation occurred in FZD4-expressing cells upon stimulation with either WNT-4 or WNT-5A (Fig. 5, A and B, and Fig. 6, A and B). Additionally, WNT-3A and WNT-5A induced formation of PS-DVL3 (but not PS-DVL2) in 32D/FZD5 cells. In contrast, WNT-4 induced both PS-DVL2 and PS-DVL3 in 32D/FZD5 cells. Finally, in 32D/FZD2 cells, WNT-3A, -4, and -5A caused the electrophoretic mobility shift of both DVL2 and DVL3.
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**DISCUSSION**

In this study, we investigated the direct binding of different WNTs to various FZD isoforms CRDs and assessed the functional selectivity of the WNT-FZD pairs in initiating downstream signaling pathways. The general understanding of selectivity in WNT-FZD signaling has remained elusive, primarily due to the poor availability of purified active WNTs, which are challenging to obtain due to their hydrophobic lipid modification (34, 36–38) and to a lack of suitable quantitative WNT-FZD binding assays. However, recent advances in protein expression and purification techniques have led to the development of a series of recombinant soluble WNTs (31, 39, 40), which have been validated by us and others for activity in different biological systems and functional assays (12, 18, 19, 41). However, WNT-FZD binding has not been assessed systematically in a quantitative manner.

Our data from kinetic binding assays revealed new information about the behavior of WNTs and their binding to FZDs. Some WNTs seem promiscuous, such as WNT-3A, which showed intermediate to strong binding to most of the FZD CRDs examined in this study, whereas other WNT family members, such as WNT-4, -5A, and -5B, seem more selective of different members of the FZD family. In the case of WNT-7A, -9B, -10B, and -11, no binding to the FZD CRD domains was detected by bio-layer interferometry assays. This result might be explained in light of a number of reasons: (a) these WNTs bind to other FZD CRDs that were not among the panel of proteins examined in this study; (b) these WNTs require the full-length FZD receptor to achieve high-affinity binding; or (c) additional accessory proteins or yet poorly defined plasma membrane interactions could be required for establishment of functional high-affinity states. Nonetheless, for the other WNTs that show binding, the WNT-FZD CRD interaction seems to be the primary binding mode defining initial ligand-receptor interaction, but additional contacts that span the extracellular surface loops and the FZD protein core might be involved, similar to what has been observed for other G protein-coupled receptors that bind peptide or protein ligands (42).

To assess the functional selectivity of WNTs toward individual FZDs, we chose 32D myeloid progenitor cells, which exhibit...
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little or no endogenous FZD expression, and generated versions of these cells that stably overexpressed individual representatives of class FZD receptors. Of note is that these cells also expressed endogenous LRP5/6 proteins to levels sufficient to allow activation of WNT-FZD signaling. This unique cell model enables the assessment of FZD pharmacology and can be potentially of use in drug-screening campaigns. Unlike common mammalian cell lines, this cell system offers for the first time the opportunity to assess the functionality of single FZD isoforms in a mammalian cell system. The parental 32D cells had low basal levels of β-catenin that did not change upon treatment with the different WNTs, emphasizing the notion that endogenous LRP5/6 expression in the absence of FZDs cannot initiate WNT-dependent β-catenin signaling. After heterologous expression of individual FZDs, the cells became responsive to WNTs, as shown by WNT-3A-induced stabilization of β-catenin. Moreover, the parental 32D cells lacked a basal shift in DVL, i.e. the slower migrating band on DVL immunoblots was completely absent, and treatment of these cells with any of the chosen WNTs did not result in formation of PS-DVL, consistent with the lack of FZD expression in these cells. In addition, the lack of WNT-induced signaling in the parental 32D cells functionally supports our co-receptor expression profiling, arguing that even very low levels of ROR1/2 or RYK do not initiate WNT-mediated changes as measured in this study. In contrast, most mammalian cell lines express at least one or two FZDs at the mRNA level and show basal levels of PS-DVL (6, 21, 22, 31, 43), highlighting the basal signaling input in the presence of the receptors. In 32D cells, heterologous expression of FZD2, FZD4, or FZD5 resulted in the formation of PS-DVL even in the absence of exogenous WNT stimulation. These findings indicate that expression of FZD is sufficient to induce phosphorylation of endogenous DVLs, consistent with previous observations for FZD (44). Finally, WNT-3A, -4, and -5A demonstrated functional activity in the 32D/FZD cell system as shown by β-catenin stabilization and phospho-LRP6 (WNT-3A) and PS-DVL2/3 (WNT-3A, -4, and -5A).

WNT-3A belongs to the group of WNTs that generally induce WNT-β-catenin signaling and is an established ligand for FZD1–8 as shown by immunoprecipitation, TOPflash, and binding experiments (4, 12, 40). Our data suggest that WNT-3A forms a ternary complex with overexpressed FZD14/5 in the presence of endogenously expressed LRP5/6 to induce WNT-β-catenin signaling and phospho-LRP6 irrespective of the FZD isoform present in the cells. On the other hand, WNT-5A treatment led to a slight β-catenin increase in 32D/FZD cells only at a very high concentration (1000 ng/ml), consistent with a previous report (45), but not in 32D/FZD or 32D/FZD cells. Furthermore, neither WNT-4 nor WNT-9B induced β-catenin stabilization in 32D cells stably expressing FZD2, FZD4, or FZD5. Interestingly, WNT-9B has been shown previously to signal through FZD2 to induce luciferase activity in a TOPflash assay in HEK293 cells (38). One explanation for this discrepancy is that 32D cells are nonresponsive to WNT-9B. We have previously shown that WNT-9B binds directly to LRP6 and that it induces WNT-β-catenin signaling in HEK293 and Hs578T cell lines (12, 46). On the other hand, WNT-9B induces β-catenin-independent PS-DVL formation, heterotrimeric G protein activation, and cell proliferation in N13 cells (18). These data indicate that WNT-9B, which does not show (a) binding to the FZD CRDs tested here or (b) activity in the 32D-FZD cells used in this study, can act on different pathways in cells with different receptor profiles. At this point, it is possible that WNT-9B can activate other FZDs that are not expressed in 32D cells or that have not been tested in our binding assays.

WNT-4 is known to mediate β-catenin-independent signaling (47). However, when coexpressed with LRP6, WNT-4 has been shown to signal through FZD2 and FZD5 to induce TOPflash luciferase activity (45). The lack of WNT-4-induced β-catenin stabilization in FZD-overexpressing 32D cells might be due to differences in experimental conditions. In our studies, β-catenin stabilization was measured after 2 h of WNT stimulation and is a more robust readout for WNT stimulation compared with TOPflash measurements, which are usually conducted after overnight treatment with WNTs. Long incubation times increase the possibility of cross-talk with other pathways and indirect signaling loops.

A summary of the WNT-FZD binding and signaling results presented in this study is provided in Table 2. The observed binding of WNTs to FZD CRDs seems to translate to functional activity in the 32D/FZD cell system. However, the affinity values for WNT-FZD binding do not seem to correlate with the extent of downstream signaling through activation of LRP6, DVL2/3, and β-catenin. For example, WNT-4 and WNT-5A displayed low-affinity interaction with FZD2 and FZD5, respectively, yet they both showed detectable effects on PS-DVL formation, which are similar to the effects observed with other tighter binding WNTs. At this point, we cannot rule out that other factors might be essential for the formation of a functionally high-affinity ligand-receptor complex in living cells, such as co-receptors, soluble co-factors, and the requirement of full-length FZD. Strikingly, the data summarized in Table 2 also imply a functional selectivity of WNT-FZD combinations with regard to differential phosphorylation of DVL isoforms. So far, only a few clear functional differences in DVL isoforms have been discovered and systematically described. For example, the DVL knock-out phenotypes suggest distinct functional differences but also reveal a certain degree of redundancy (48). In our studies, WNT-3A-FZD2 (but not WNT-3A-FZD4) induced formation of PS-DVL2/3, even though both WNT-FZD combina-

| WNT   | FZD | WNT-3A | WNT-4 | WNT-5A | WNT-9B |
|-------|-----|--------|--------|--------|--------|
|       | FZD3|        |        |        |        |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |
|       | ND  | ++ binding | ++ binding | ++ binding | ND    |

Table 2: Overview of the data presented in this study, including WNT-FZD CRD binding data and the functional response upon WNT-FZD binding. ND, no activity or binding detectable with the methodology used.
tions induced β-catenin stabilization and phosphorylation of LRP6 (Figs. 5 and 6 and Table 2). A similar trend was observed previously in SN4741 neuronal precursor cells, in which WNT-3A induced LRP6-dependent and PS-DVL-independent stabilization of β-catenin (24). Similarly, we showed recently that despite complete inhibition of β-catenin stabilization by DKK1 (Dickkopf-related protein 1), the WNT-3A-induced formation of PS-DVL is not blocked (33), thus uncoupling functional LRP6 signaling from the formation of PS-DVL. Even though WNT-3A did not induce a DVL2/3 shift in 32D/FZD4 cells, expression of FZD4 on its own induced basal PS-DVL compared with parental 32D cells. In contrast, WNT-4 and WNT-5A induced formation of PS-DVL without stimulating β-catenin stabilization and independently of the FZD type present (Figs. 5 and 6 and Table 2).

For the WNT-FZD system, proper quantification of the functional selectivity of WNT-FZD combinations in the form of a bias factor (49) presents a challenge due to technical shortcomings, such as unknown specific WNT activities and the lack of binding assays for full-length receptors. Despite these pitfalls, the 32D/FZD cell system described here, combined with biophysical WNT-FZD CRD binding assays, serves as a good model system to pinpoint functional selectivity, identifying WNTs as ligands with natural bias at their receptors. In summary, we systematically mapped WNT-FZD CRD binding of a set of soluble WNT and FZD CRD combinations. Furthermore, we have provided a link between direct binding and downstream signaling by systematic analysis of β-catenin stabilization and DVL activation for a selected subset of WNT-FZD combinations in a novel cell system comprising FZD-expressing 32D cells. The lack of endogenously expressed FZDs in these cells enabled for the first time the investigation of individual functional WNT-FZD pairs and their subsequent downstream signaling specificity. Our data showing preferential binding and signaling profiles of distinct WNT-FZD combinations suggest functional selectivity of WNTs. We conclude that WNT-3A, -4, and -5A are all functional binding partners for FZD2/4/5 with appreciable affinity and putative natural bias for WNT-3A, -4, and -5A are all functional binding partners for WNT-3A, -4, and -5A are all functional binding partners for WNT binding and signaling profiles of distinct WNT-FZD combinations. Our data showing preferential binding of a set of soluble WNT and FZD CRD combinations. Furthermore, we have provided a link between direct binding and downstream signaling by systematic analysis of β-catenin stabilization and DVL activation for a selected subset of WNT-FZD combinations in a novel cell system comprising FZD-expressing 32D cells. The lack of endogenously expressed FZDs in these cells enabled for the first time the investigation of individual functional WNT-FZD pairs and their subsequent downstream signaling specificity. Our data showing preferential binding and signaling profiles of distinct WNT-FZD combinations suggest functional selectivity of WNTs. We conclude that WNT-3A, -4, and -5A are all functional binding partners for FZD2/4/5 with appreciable affinity and putative natural bias for individual downstream signaling pathways. Further binding studies utilizing other soluble WNTs and full-length FZDs are still needed to fully understand the selectivity of ligand-receptor interactions in the WNT system and its functional implications. Finally, other signaling readouts, such as activity-based assays for heterotrimeric G proteins or second messenger measurements (50), could be included in the assessment of downstream mechanisms that specify WNT-FZD signaling to further investigate the pluridimensionality of WNT ligands and their FZD receptors.

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