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Structural basis of Wnt recognition by Frizzled

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Abstract

Wnts are lipid-modified morphogens that play critical roles in development principally through engagement of Frizzled receptors. The 3.25Å structure of *Xenopus* Wnt8 (XWnt8) in complex with mouse Frizzled-8 cysteine-rich domain (CRD) reveals an unusual two-domain Wnt structure, not obviously related to known protein folds, resembling a “hand” with “thumb” and “index” fingers extended to grasp the Fz8-CRD at two distinct binding sites. One site is dominated by a palmitoleic acid lipid group projecting from Serine 187 at the tip of Wnt’s thumb into a deep groove in the Fz8-CRD. In the second binding site, the conserved tip of Wnt’s ‘index finger’ forms hydrophobic amino acid contacts with a depression on the opposite side of the Fz8-CRD. The conservation of amino acids in both interfaces appears to facilitate ligand-receptor cross-reactivity, which has important implications for understanding Wnt’s functional pleiotropy and for developing Wnt-based drugs for cancer and regenerative medicine.

Introduction

Wnts (*Wingless* and *Int-1*) are central mediators of vertebrate and invertebrate development, due to their influences on cell proliferation, differentiation, and migration (1–5). Wnts, which are ~350-residue secreted, cysteine-rich glycoproteins (5) activate cell surface receptors on responder cells to initiate at least three different signaling pathways including the “canonical” β -catenin pathway, and the “non-canonical” planar cell polarity (PCP) and Ca^{2+} pathways (1, 4–6). The seven-pass transmembrane receptor Frizzled (Fz) is critical for nearly all Wnt signaling, and the N-terminal Fz cysteine rich domain (CRD) serves as the Wnt binding domain. In addition to Fz, the Wnt/ β -catenin pathway requires the Low-density lipoprotein receptor related proteins 5 and 6 (Lrp5/6) co-receptors (7). Wnt signaling is also regulated by several alternative receptors, such as Ryk and Ror2, and by secreted antagonists (8) that directly interact with Wnts, such as Wnt-inhibitory factor (WIF-1)(9), or engage Wnt receptors, such as Dickkopf (Dkk) (10) and Kremen (Krm)(11, 12). Dysregulation of the Wnt/Fz system is associated with a variety of human hereditary diseases and modulation of Wnt signaling is actively targeted for cancer, regenerative medicine, stem cell therapy, bone growth and wound healing (13–17).

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Supporting Online Material

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Materials and Methods

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There exists no structural information for Wnts: their primary sequences are not clearly related to any known protein folds. Wnts are hydrophobic due to the post-translational addition of palmitate and/or palmitoleic acid to one or two residues (Cys77 and/or Ser209 in Wnt3a) (18, 19). Acylation is necessary for both Wnt intracellular trafficking and its full activity when secreted, but its precise role in Wnt action remain unclear. It has been speculated that the Wnt lipid group(s) may directly engage the Fz-CRD (20), and could also mediate binding to WIF (21, 22). Genetic evidence suggests that Wnt-secreting cells require the action of acyltransferases (*porcupine* in *Drosophila*) for Wnt palmitoylation (23). As Wnts are morphogens, the acylation is thought to localize Wnts to cell membranes, and circulating Wnts may be bound to carrier proteins that shield the lipid from solvent (24). Wnt palmitoylation has complicated expression and purification of recombinant material (25). As a result of these technical difficulties, relatively few detailed structure-function studies of Wnts have been carried out that shed light on how Wnts engage Fz.

Current structural knowledge of Frizzled receptors is limited to the unliganded Fz8-CRD and the secreted CRD antagonist sFRP, which are ~160 residue, primarily β -helical proteins (26). Mutational mapping studies of *Xenopus* Wnt8 (XWnt8) interactions with Fz8, Fz4, and dFz2 identified several potential patches on the CRD important for binding (26, 27). Interestingly, several potential Wnt-binding patches on the Fz4-CRD also appear to mediate binding to the Norrie disease protein Norrin, which is a cysteine-knot growth factor unrelated in sequence to Wnt, that has been shown to activate Fz4 (28). With respect to Fz activation, the molecular mechanisms are unknown. While Fz share several features of G-protein coupled receptors (GPCR), they lack hallmark characteristics that would clearly place them as prototypical GPCR (4, 29). Nevertheless, some principles of transmembrane signaling by GPCR may be relevant (30).

A confounding feature of the Wnt/Fz system has been how functional specification is achieved when each Wnt can engage multiple Fz, and each Fz can respond to multiple Wnts (5, 27, 28, 31–34). This pleiotropy confounds interpretation of *in vivo* functional experiments. Fz receptors and Wnt ligands have not been unambiguously matched, and it is unclear if mono-specific Wnt/Fz pairs are responsible for certain biological effects and diseases (31, 32). Structural information on Wnt and Wnt/Fz interactions can shed light on the critical issues of Wnt/Fz specificity, the functional role of Wnt acylation, and begin to give insight into a mechanism of receptor activation. Here we present the structure of XWnt8 in complex with the Fz8-CRD to a resolution of 3.25Å.

Results

We screened a variety of vertebrate and invertebrate Wnts for expression and found that *Xenopus* Wnt8 (XWnt8) expressed at high levels and could be purified as a complex with several Fz-CRD. XWnt8 is advantageous for structural studies as it has served as a model system to study Wnt/Fz interactions because it binds to and activates mammalian Fz (27). A key enabling finding was that co-expression of XWnt8 with an Fc-Fz8CRD fusion allowed efficient affinity-based purification of XWnt8/Fz8-CRD complexes in the absence of detergent. In contrast, purification of Wnt alone requires detergent, suggesting that binding to the Fz-CRD shields the Wnt lipid from aqueous solvent (Figs. 1A, B). The XWnt8/Fz-CRD complex eluted from gel filtration as two inter-converting oligomeric forms with MW ranging from ~50kDa to ~200kDa (Fig. 1B).

We crystallized the glycosylated XWnt8/Fz8-CRD complex in detergent-free buffers and obtained a native x-ray data set to a resolution of 3.25Å (Table S1). Experimental phases were determined using isomorphous and anomalous scattering difference methodologies with crystals derived from material expressed in S2 cells supplemented with

Selenomethionine (Table S1). The experimental phases yielded an excellent electron density map in which XWnt8 could be traced, the Fz8-CRD located (Fig. 1C), and the complex structure refined (Fig. S1). The amino acid register of XWnt8 was confirmed using the Selenium sites as guides (Fig. 1C), as well as the locations of disulfide bridges, N-linked glycans (Fig. S1) and the lipid group.

The complex structure is a striking donut shape (Fig. 2A, B) in which XWnt8 appears to grasp the Fz8-CRD at two opposing sites using extended thumb and index fingers projecting from a central “palm” domain, to contact “site 1” and “site 2,” respectively, burying a total of $\sim 2000\text{\AA}^2$ of surface area. Neither the structure of XWnt8, nor the manner of Fz binding, bears a clear resemblance to known protein folds or complexes, respectively. XWnt8 comprises an N-terminal α -helical domain (NTD) from residues ~ 1 –250 (helices A through F) that contains the lipid-modified thumb, and a C-terminal cysteine-rich region (CTD) from residues 261–338. Each domain forms a distinct interaction with the Fz8-CRD, whose conformation is essentially unchanged compared to the unliganded structure (Fig. S2A), leaving a large hole in the center of the complex (Figs. 2A–B, S2B). The XWnt8 NTD is comprised of a seven α -helical bundle palm, containing two large inter-helical loop insertions that are stabilized by 4 disulfide bonds (Figs. 2A, D). The principal feature of the CTD is a long 40 amino acid β -strand hairpin that is also stabilized by an extensive network of disulfide bonds. The distinct structural sub-domains associate through a small interaction patch between the AB-loop of the NTD and a small helix (helix F) in the CTD. There is clear electron density for high-mannose glycan additions at two of the three Asparagine-linked glycosylation sites on XWnt8, Asn104 and Asn263 (Figs. 2C, S1b).

The functional role for lipid modification of Wnts is unknown, but has been shown to be necessary for full biological activity (18). The structure shows XWnt8 lipidation directly involved in Fz8-CRD binding in binding site 1 (Fig. 2A, Fig. 3). A $15\text{\AA}$ long tube of continuous electron density is connected to the hydroxyl group of Ser-187 (Fig. 3A), which is located at the tip of the thumb projecting from the XWnt8 NTD. The length of the electron density corresponds to a 14-carbon lipid chain. The lipid dominates the contact interface, burying approximately $580\text{\AA}^2$ of total surface area ($330\text{\AA}^2$ from the lipid, $250\text{\AA}^2$ from the CRD), contacting 11 Fz8 residues, and completely traversing the cleft on the Fz8-CRD surface (Fig. 3B). The lipid electron density is consistent with a 16-carbon palmitoleic acid (or derivative thereof) modification to XWnt8 where the terminal two carbons of the acyl chain have exited the CRD groove and do not show ordered electron density. Wnt acylation has been reported to be either an unsaturated palmitic acid, or a monounsaturated palmitoleic acid (23). We could not unambiguously determine the chemical identity of the lipid on XWnt8 using mass spectrometry (Fig. 3A). However, based on identification of the lipid attached to the corresponding Ser209 of human Wnt3a as palmitoleic acid, we assigned the lipid attached to XWnt8 Ser187 as palmitoleic acid - but it is formally possible that the lipid is palmitic acid. Serine acylation in the complex structure also resolves uncertainty regarding the location of the lipid attachment sites on Wnts. Both conserved Serine (209) and Cysteine (77) residues have been reported as acylation sites on human Wnt3a, and it has been speculated that other Wnts are acylated at one or both of the corresponding positions (18, 19, 23). In XWnt8, we find that Cys55, the Cys residue analogous to Cys77 in Wnt3a, is engaged in a disulfide bond that will be conserved across all Wnts (Figs. 2D, S3), and so cannot serve as a lipid addition site. Therefore, the conserved Ser (corresponding to Ser187 in XWnt8) appears to be the consensus acylation site.

The cleft on the Fz8-CRD surface that is traversed by the lipid is made up of helix A, helix D and the DE-loop (Fig. 3B, Table S2), and is lined with hydrophobic amino acids that form extensive van der Waals interactions with the lipid (Fig. 3B, Table S2). The high degree of conservation of apolar amino acids in the region of the CRD contacting the acyl group

implies that the lipid-binding site is conserved in other Fz-CRD (Fig. 3C, S4). The conservative substitutions seen for these residues in other Fz-CRD could modulate lipid-binding affinity and impart a degree of Wnt specificity (Fig. 3C). The driving force for lipid binding appears to be the hydrophobic effect combined with shape complementarity of the lipid-CRD interface, where the lipid and apolar Fz-CRD core residues are driven to associate by solvent exclusion. Although approximately 60% of the total accessible surface area ($\sim 530\text{\AA}^2$) of the lipid is buried when bound to the Fz8-CRD, one face and the distal 2–3 carbon atoms of the lipid are still exposed to solvent. These exposed regions, $\sim 200\text{\AA}^2$ of hydrophobic surface, would be highly energetically unfavorable in aqueous solvent and may still require shielding.

While the site 1 interaction appears to a large degree mediated by the lipid on Wnt, thumb loop amino acids (residues 181–188) form protein-protein contacts with the Fz8-CRD that account for an additional $\sim 600\text{\AA}^2$ of buried surface area (Fig. 3C). At the extreme tip of the thumb loop (residues 186–188) several main chain van der Waals contacts are formed with the Fz8-CRD that would have limited capacity to contribute to ligand specificity (Fig. 3C). At the base of the thumb loop, Wnt Lys182 forms a salt bridge with the Fz8 Glu64 and a hydrogen bond with Fz Asn58. Lys or Arg are conserved at this corresponding position in all Wnts, and Glu or Asp are conserved at the Glu64 position in 8 of 10 mammalian Fz-CRD (Table S2, Figs. S3 & S4). However, the substitution of Thr and Ile in Fz3 and Fz6, respectively, raises the possibility of some degree of ligand specificity modulated through this interaction. We surmise that the principal driving force for the site 1 binding is the lipid-in-groove contact, with the residues at the base of the thumb contributing secondarily.

The highly exposed structural disposition of the lipid attachment site has several important implications. First, it suggests that lipid attachment may not be integral to the tertiary structural stability of the folded Wnt molecule. Clearly, acylation is necessary for proper secretion of Wnts, and our complex structure also reveals its centrality in Fz binding. But it should be possible to create viable Wnt protein therapeutics by genetically engineering “lipid-free” water-soluble Wnts through affinity maturation of Fz-contacting residues at the tip of the Wnt thumb. Second, the highly exposed position of the lipid suggests it would require sequestration from aqueous solvent during expression and transport, such as with carrier proteins (24). In Wnt’s role as a morphogen, it has been suggested that Wnts may use acylation to partition into the cell membrane in order to increase local concentrations and restrict availability to specific target tissue (18, 19). The XWnt8 structure supports this idea in that the lipid is accessible (Fig. 2C), ideally positioned for anchoring Wnt to the plasma membrane.

The site 2 interaction is on the opposite side of the Fz8-CRD from site 1 (Fig. 2A), and is comprised of residues between the Cys315-Cys325 disulfide at tip of the XWnt8 CTD index finger, engaging in hydrophobic contacts within a depression between inter-helical loops on the CRD (Figs. 4A, B, Table S2). The site 2 interface buries a total of $\sim 840\text{\AA}^2$ ($\sim 400\text{\AA}^2$ CRD, $\sim 440\text{\AA}^2$ XWnt8) and despite the “knob-in-hole” binding mode (Fig. 4A), exhibits poor overall shape complementarity ($Sc = 0.48$). The XWnt8 index finger presenting the site 2 residues is a long, twisted β -strand, rigidified by a ladder of disulfide bonds, and spans from Gly299 to the C-terminal Cys338 (Fig. 2C). In site 2, the underside of the finger loop positions hydrophobic residues Cys315, Phe317, Trp319, an unusual tandem Cys320-Cys321 disulfide bond, and Val323 to form the major van der Waals interactions with main chain and apolar residues on the Fz8-CRD (Figs. 3B, 3C). The XWnt8 Trp319 side chain at the tip of the finger loop occupies a pocket on the Fz8-CRD surface and engages primarily the main-chain of Fz8-CRD residues 150–152, and the side chain of conserved Phe86. The XWnt8 site 2 contact residues are invariant in all Wnts (Fig. S3). In the Fz8-CRD Tyr48 and Cys148, are conserved residues that form van der Waals interactions with XWnt8 (Fig. S4).

As for site 1, Wnt and Fz contact residues are conserved apolar amino acids (Figs. 3C, S3). Importantly, several Fz8-CRD contacts are substituted in other Fz-CRDs and thus could contribute to Wnt sub-type preferences. For example, Met149 at the center of site 2 is conserved in 5 of 10 mammalian Fz-CRD, but is substituted to Val, Glu or Asp in Fz1, 2, 3, 6 and 7.

Given the technical difficulties of expressing recombinant Wnts, there is a dearth of structure-function data, or biochemical measurements between Wnt and Fz. Here, guided by the structure of the complex, we engineered a biochemically tractable version of XWnt8 in order to determine three previously unknown interaction parameters: 1- the degree to which XWnt8 site 1 versus site 2 binding determines Fz specificity, 2- if the site 1 and 2 interactions can occur independently, or whether both sites are reliant on simultaneous engagement to achieve productive binding, and 3- measurement of an accurate binding affinity of site 2 alone. For the first experiment, we displayed a water-soluble, C-terminal 90 amino acid sub-domain of XWnt8, containing the site 2 binding index finger (which we term “mini-Wnt”) on yeast. We tested Fz1, 2, 4, 5, 7 and 8 CRD for binding and clearly observed that mini-XWnt8 was stained by FACS with several Fz-CRD (Fz4, Fz5 and Fz8) that were presented as fluorescent tetramers by forming complexes with Streptavidin-PE (Fig. 5A). Interestingly, we see stronger binding to Fz8 (~50% staining) and Fz5 (~30% staining) than Fz4 (~5% staining), as measured by FACS staining intensity. The soluble mini-Wnt binding to Fz8, Fz5, and Fz4 is in accord with full-length XWnt8 previously determined in a cell binding assay (27). The concordance of binding specificity between mini-Wnt and full-length XWnt8 demonstrates that Fz discrimination is mediated primarily, although we cannot say exclusively, by XWnt8 site 2, and also that the site 2 contact can occur independently of site 1. The lipid on the Serine in the site 1 contact by full-length Wnt, which is clearly necessary for full Wnt activity, may also be important for affinity enhancement and tissue localization. In order to determine an accurate affinity of site 2, we produced soluble mini-Wnt in a recombinant form expressed from insect cells, and measured a K_D of ~2.4 μ M for Fz8-CRD, and ~3.5 μ M for Fz5-CRD using surface plasmon resonance (Fig. 5B). From these studies we conclude that site 2 alone represents a moderate affinity interaction site that binds to three different Fz, but also has differences in binding affinity for different Fz, demonstrating that site 2 is not entirely degenerate. While the mini-Wnt affinity difference between Fz5 and Fz8 is small, it is consistent with the yeast display rank order of preference, and the relative difference may be amplified in the context of both sites in full-length Wnt to a degree that could functionally discriminate Fz receptors. We surmise that site 1 and site 2 combine to manifest as high affinity for Wnts through two-point attachment. Collectively, the biological relevance of the site 1 and site 2 interactions we see in the crystal structure are supported three lines of evidence: 1- the concentration of amino acid conservation patterns in both XWnt8 and the Fz8-CRD interfaces, 2- the direct involvement of the lipid group in binding given functional data showing the necessity of Wnt3a Serine palmitoylation for activity, and 3- both current (Fig. 5) structure-function data on XWnt8 (Fig. 5), and prior mutational data mapping the Wnt binding site on Fz-CRD (26, 27).

Discussion

The potential combinatorial complexity of 19 mammalian Wnts engaging 10 Fz invokes an important question: do cross-reactive, or specific Wnt/Fz pairs contribute to distinct biological, and disease-related functions? From the XWnt8/Fz8-CRD complex structure, we see that the majority of Wnt/Fz-CRD contacts in both site 1 and site 2, including the lipid interactions, involve either strictly, or chemically conserved residues. Thus, we conclude that the site1/site2 Wnt/Fz interaction chemistry is incompatible with mono-specificity and therefore a binary, or highly-restricted ligand-receptor matching code most likely does not

exist. Clearly, however, some studies, including those shown here with mini-Wnt (Fig. 5), have shown that Wnts and Fz are not broadly degenerate (27, 32, 33). Rather, it appears that most Wnts have the capacity to engage multiple, but not all, Fz-CRD through poly-specificity mediated by amino acid substitutions in the site 1 and site 2 interfaces. Since site 1 is primarily mediated through interaction of the monomorphic lipid with the CRD, it would seem there is limited capacity for Wnt/Fz specificity to be focused on the lipid contacts with the possible exception of substitutions of residues on the Fz-CRD that contact the conserved Lysine (Lys182) on XWnt8. Site 2 appears to qualitatively discriminate between specific Wnt/Fz pairs, and this is borne out by the weaker binding we see for mini-XWnt8 to Fz4-CRD versus Fz5 and Fz8. Collectively, then, we suggest that there exist subtle sub-type specific Wnt/Fz affinity differences within a background of broader Wnt/Fz poly-specificity, which is consistent with our binding data (Fig. 5), as well as prevailing literature (5, 27, 31–34). These ‘group’ preferences may fine-tune Wnt/Fz signaling through a combinatorial signaling output where a given Fz can respond with different signaling amplitudes to a range of different Wnts possessing different binding affinities. With the structure of a Wnt/Fz-CRD complex, it is feasible to attempt to engineer Wnts that are mono-specific for Fz in order to probe the role of Wnt/Fz specificity in function.

For canonical signaling, one also needs to factor in the necessity of Wnt interaction with Lrp5/6, as well as the possibility of additional co-receptors such as Ryk and Ror2, in non-canonical signaling (4, 35). Lrp6 contains two different modules of β -propeller domains that appear to engage different subsets of Wnts (36–39). We carried out a conservation analysis of Wnt sequences in order to identify locations on the Wnt structure that might serve as potential co-receptor (e.g. Lrp5/6, Ryk) binding sites (Figure S5). Clusters, or patches, of phylogenetically-conserved amino acids on protein surfaces often demarcate ligand or receptor binding sites. From an alignment of 20 Wnt sequences, we found that there are four main regions of concentrated amino acid conservation that map onto the Wnt structure: the tips of the thumb and finger loops, the core of the NTD helical bundle, and a large continuous patch at the “top” of XWnt8 (Fig. S5). This patch is comprised of approximately 10 residues derived from three discontinuous regions of sequence primarily on solvent exposed inter-helical loops: residues 216–219, 249–252, and 256–259. The location of the conserved patch at the opposing end of XWnt8 from the Fz-CRD binding region, and its solvent exposure, leads us to propose this region as a likely Lrp5/6 and/or Ryk binding site on Wnts that would enable bridging with Fz to form a ternary complex.

What does the structure of the XWnt8/Fz8-CRD complex tell us about Fz receptor activation? Wnt receptor clustering appears to be crucial for signalling and Dishevelled signalosome assembly (40), but given current uncertainty as to the compositions of Fz signaling complexes in canonical and non-canonical Wnt signaling, it is difficult to speculate on oligomerization-based signaling models (4, 35). Since Wnts activate Fz in the presence (canonical signaling, e.g. Wnt1, Wnt3a, Wnt8) or absence (non-canonical signaling, e.g. Wnt4, Wnt5a, Wnt11) of Lrp5/6 (4), cross-linking of Fz with Lrp5/6 does not appear to be absolutely required for all types of Fz signaling. Therefore, an activation mechanism may exist where Wnt/Fz engagement alone is sufficient for signaling in some settings, and this could involve dimerization (41). Important to this point is the fact that Wnt5a activates Ror2, which is a Tyrosine Kinase (TK) receptor containing a CRD in its extracellular region that presumably serves as the Wnt-binding domain. Since TK receptors are activated by ligand-induced homo- or hetero-dimerization (e.g. EGF receptor, etc)(42), it is plausible that Wnt5a activates Ror2 through dimerization via the CRD. By extension, if the structural mode of Wnt/CRD interaction for Ror2 is analogous to that seen here for Wnt/Fz, Wnt may activate Fz in part through receptor dimerization. Canonical Wnts appear to have evolved an additional binding site in order to recruit Lrp5/6 into this signaling complex. Recent studies implicating Wnt5a as heterodimerizing Ror2 with Fz to initiate

non-canonical signaling through a TK-independent mechanism, further suggests the possibility of Wnt-induced heterodimerization of signaling receptors (35).

Although we see evidence of higher order species of the XWnt8/Fz8-CRD complex in solution that would support an oligomerization model (Fig. 1B), we do not see evidence of a *symmetric* dimer in the crystal. We do, however, see a third site of contact in the crystal mediating an *asymmetric* Wnt/Fz dimer, that we term ‘pseudo-site 3’ (Fig. S6). The interface is formed by one Wnt molecule binding to the composite Wnt-lipid/CRD site 1 surface presented by a different Wnt/Fz binary complex (Figs. S6A, B). The physiological relevance of pseudo-site 3 is not known, but it is the largest interaction surface in the crystal, and buries ~200Å² of lipid surface left exposed to solvent in site 1 (Fig. S6B). Interestingly, while the residues on the Fz8-CRD contacting XWnt8 in pseudo-site 3 are mostly conserved (Fig. S4), the residues on XWnt8 in the pseudo-site 3 interface are less conserved amongst Wnts (Fig. S3). The asymmetric nature of pseudo-site 3 in the crystal lattice generates repeating units of self-associating binary complexes (Fig. S6C). Since Dishevelled signalosome assembly is itself based on DIX-dependent ‘head-to-tail’ polymerization (41, 43), it is intriguing to speculate whether pseudo-site 3 provides an underlying basis for both phenomena – ligand-induced receptor clustering and signalosome assembly. The structure of the XWnt8/Fz8-CRD pair presented here provides the first structural access to Wnts, and can now serve as a conceptual and technical framework to address remaining questions about the nature of canonical versus non-canonical signaling complexes, and how Wnt recognition by Fz is coupled to receptor activation.

Material and Methods

Recombinant expression

The XWnt8 construct used for crystallization contains residues from the mature N-terminus (+22) to residue 338. The length of the construct design was based on the observation that the C-termini of Wnts are very conserved, usually terminating two residues after the last Cysteine residue corresponding to Cys337 in XWnt8. XWnt8 contains an additional positively charged extension of 20 amino acids beyond this Cys337, which is not required for Frizzled binding and signaling activation (27), and hence was omitted from our constructs due to its likely flexibility. The gene encoding XWnt8 (23–338) was codon-optimized for expression in insect cells (GenScript Inc.), and cloned in frame with the artificial signal sequence of the human CD8 α chain (residues 1 – 21) into a modified *Drosophila* S2 expression vector pACTIN-SV (44), which contains the *Drosophila* actin 5c promoter driving constitutive expression. A stop codon was introduced after the 3′ end by the 3′ PCR primer. The coding sequence of mouse Fz8-CRD (residues 28–150) containing the artificial human CD8 signal sequence, and a C-terminal 3C protease cleavage site (LEVLFQ/GP), Fc-tag (constant region of human IgG), six-histidine tag, and a stop codon was cloned into the same vector. In this fashion, XWnt8 contains no affinity tags and can be affinity-purified with the Fz8-CRD-Fc.

To generate a stable cell line co-expressing XWnt8 and Fz8CRD-Fc, 13μg of XWnt8 plasmid and 7 μg of Fz8-CRD-Fc plasmid were co-transfected with 1 μg of pCoBlast (Invitrogen) into *Drosophila* S2 cells using the calcium phosphate precipitation kit (Invitrogen) according to the manufacturer’s protocol. Stably transfected S2 cells were selected for 2 weeks in the presence of 10 mg/ml Blasticidin (Invitrogen) in Schneider’s *Drosophila* medium (Lonza) supplemented with 10 % (vol/vol) heat-inactivated fetal bovine serum (FBS) and L-glutamine. Cells were maintained in Schneider’s medium containing 10 % FBS, L-glutamine and 10 mg/ml Blasticidin and gradually scaled up for protein expression in Schneider’s *Drosophila* medium containing 10 % FBS and L-glutamine or serum-free Insect Xpress medium (Lonza).

Conditioned medium for large-scale protein purification was harvested at a cell density of 10×10^6 cells/ml. Supernatant was clarified by passing through a glass fibre prefilter (Millipore). Fz8-CRD-Fc and bound XWnt8 were captured on a protein A-Sepharose Fast Flow (Sigma) column and resin was washed with 10 column volumes 1xHBS (10 mM HEPES pH 7.3, 150 mM NaCl). Fz8-CRD and bound XWnt8 were eluted from protein A resin by cleaving the linker between Fz8-CRD and the Fc-tag with 3C protease, leaving the Fc-tag bound to the resin. The proteolysis reaction was performed in one column volume 1xHBS while gently agitating over night at 4°C. Protein for crystallization was treated with carboxypeptidase A (Sigma) and carboxypeptidase B (Calbiochem) at the same time. The XWnt8-Fz8-CRD complex was further purified on a Superdex 200 size-exclusion column (GE Healthcare) equilibrated in 1xHBS. Fractions containing the XWnt8-Fz8CRD complex were concentrated to ~ 5–6 mg/ml for crystallization trials.

To prepare selenomethionine (SeMet)-labeled XWnt8-Fz8-CRD complex, XWnt8 and Fz8-CRD-Fc co-expressing S2 cells were grown in Insect Xpress medium (Lonza, Inc) to a density of 10×10^6 cells/ml. The medium was replaced with ESF 921 serum-free and methionine-free medium (Expression Systems) and cells were starved for 12–24 hrs. The medium was then replaced with fresh ESF 921 serum-free methionine-free medium, supplemented with 150 mg/L L-SeMet (Acros Organics). Another 200 mg/L L-SeMet were added after 24 hrs and expression was allowed to proceed for 4–5 days. Purification of the SeMet labeled XWnt8-Fz8CRD complex was similar to that of the native complex.

In order to produce recombinant Fz-CRD for yeast display selections and immobilization on Streptavidin BIAcoretm chip (see below), the CRDs of human Fz4 (residues 42 – 161), human Fz5 (residues 30 – 150) and mouse Fz8 (residues 32 – 151) containing a C-terminal 3C Protease site, a biotin acceptor peptide (BAP)-tag (GLNDIFEAQKIEWHE) and a six-histidine tag were cloned into the pAcGP67-A vector (BD Biosciences, San Diego, CA) for expression using the baculovirus expression system. The Fz-CRDs were secreted from High Five insect cells in Insect Xpress medium and purified using Ni-NTA affinity purification and size exclusion chromatography. Enzymatic biotinylation of the BAP-tag on the CRDs was performed at 40 μ M substrate concentration in 50 mM bicine pH 8.3, 10 mM ATP, 10 mM Mg(OAc)₂, 50 μ M d-biotin with GST-BirA ligase overnight at 4°C (45). After the biotinylation reaction was complete, proteins were re-purified on a Superdex 75 size-exclusion column (GE Healthcare) to remove excess of biotin.

In order to produce mini-XWnt8 for surface plasmon resonance measurements, the coding sequence of the C-terminal fragment of XWnt8 (residues 248 – 338), also referred to as mini-XWnt8, with a C-terminal six-histidine tag was cloned into the pAcGP67-A baculovirus expression vector and expression and purification was performed as described above.

Crystallization and data collection

XWnt8/Fz8-CRD complex crystals were grown by hanging-drop vapor diffusion at 295 K, by mixing equal volumes of protein (~5.5 mg/ml) and reservoir solution containing 4–10 % PEG 400, 100 mM NaOAc pH 4.5, 15–25 mM Zn(OAc)₂. Crystals were cryo-protected in reservoir solution supplemented with 55 % sucrose and flash frozen in liquid nitrogen. Crystals of the SeMet labeled XWnt8/Fz8-CRD complex grew under the same conditions. The native and SeMet substituted crystals both grew in space group P4₁ with one molecule in the asymmetric unit. Data were collected for native and SeMet derivative crystals at a temperature of 100K at beamline 11–1 at the Stanford Synchrotron Radiation Laboratory (SSRL). All data were indexed, integrated, and scaled with the XDS package (46). See Table S1 for data statistics.

Structure determination and refinement

The structure was determined by multiple isomorphous replacement with anomalous scattering (MIRAS) using a combination of a selenomethionine single-wavelength anomalous diffraction (SAD) data set, the peak wavelength data set of a multi-wavelength anomalous diffraction (MAD) experiment, and a native data set (47). Initial selenium sites were located with SHELXD (48) and refined within autoSHARP (49) (Table S1). Ten selenium and three zinc sites were finally found. The overall figures-of-merit for acentric and centric reflections were 0.26735 and 0.24246, respectively. Phases were improved by density modification within autoSHARP, assuming a solvent content of 73.5%. The (correlation on E^2)/contrast score before density modification was 0.4613. Density modification improved the score to 3.3788 and figure of merit to 0.876. This experimental map was exceptionally clear, and secondary structure elements were recognized, showing continuous density for a large portion of the XWnt8/Fz8-CRD complex main chains and side chains. Fz8-CRD was placed into the map with only minor adjustments, and an initial XWnt8 model was built manually in Coot (50). The register of the polypeptide in the experimental electron density was determined using the selenomethionine sites determined in autoSHARP, predicted Asparagine-linked glycosylation sites, disulfide bonds, characteristic side chain densities of aromatic amino acids, and the position of the fatty acid. This initial model was refined in Phenix (51) and COOT. Refinement strategies included bulk solvent correction and anisotropic scaling of the data, individual coordinate refinement using gradient-driven minimization applying stereo-chemical restraints, and restrained individual atomic displacement parameters (ADP) refinement. Initial rounds of refinement were aided by incorporation of experimental phase restraints (Hendrickson-Lattman coefficients) and restraints from the reference model using the high-resolution structure of mouse Fz8-CRD (PDBID: 1IJY.pdb). Geometric restraints for heteroatoms that were not in the CCP4 monomer library (eg. PAM and BMA) were included. The phased and unphased maximum-likelihood functions were used as a refinement and scale target (52). Real-space refinement was performed in Coot into a likelihood-weighted SigmaA-weighted 2mFo-DFc map calculated in Phenix. The final model was refined to 3.25 Å with R_{work} and R_{free} values of 19.7 % and 24.3 %, respectively (Table S1). The quality of the structure was validated with MolProbity (53). 93.8 % of residues are in the favored region of the Ramachandran plot, 6.2 % in additional allowed region, and no residues in the disallowed region. The final model contains amino acids 35–152 of Fz8-CRD and amino acids 32–220 and 235–338 of XWnt8, a palmitoleic acid at XWnt8 Ser187 and two N-acetylglucosamine residues on Fz8CRD Asn49, two N-acetylglucosamine residues and two mannose residues on XWnt8 Asn104, and two N-acetylglucosamine residues and one mannose residue on XWnt8 Asn263, and three Zn^{2+} ions. Residues 221–234, which correspond to a solvent exposed loop between XWnt8 NTD helix F and helix G, did not have clear enough electron density for confident tracing, and so were not included in the final model. Side chains of six lysines (Lys101 and Lys102 in Fz8-CRD and Lys281, Lys316, Lys324 and Lys329) were omitted due to poor density. Due to the resolution of the structure, water molecules were not placed. Interface contacts were determined using MolProbity. Buried surface area values were calculated using Protein Interfaces, Surfaces, and Assemblies (PISA) software (54). Structure figures were prepared with the program PyMOL (55), and sequence conservation analysis was performed using ClustalW (56).

Yeast display of Xwnt8

We engineered a water-soluble, CTD site 2 binding fragment of XWnt8 using yeast display. The general technical aspects of yeast display have been previously described (57). For our experiments, residues 23 to 338 of XWnt8 was cloned into yeast display vector pCT302 and subjected to error-prone PCR using the GeneMorphII kit (Stratagene). The two user-determined variables in the kit were the starting concentration of DNA template and the

number of cycles. We used 100 ng template and 30 cycles in an effort to maximize the number of errors. The primers used for error-prone PCR were: 5'-ACGCTCTGCAGGCTAGTG-3' for the forward primer and 5'-GTTGTTATCAGATCTCGAGCAAG-3' for the reverse primer. These two primers each bind to vector pCT302 approximately 60 bp outside of the XWnt8 gene, thereby adding flanking homologous (to pCT302) regions to the XWnt8 DNA and could be used for homologous recombination when creating the yeast display library. The PCR product was further amplified using the same primers to yield 74 μ g of error-prone PCR product ("insert DNA"). Insert DNA was combined with 15 μ g linearized pCT302 and EBY100 yeast, then electroporated and rescued as previously described (58). The electroporations yielded a library of 1.2×10^8 transformants.

XWnt8 library selections

Selections were performed using magnetic activated cell sorting (MACS, Miltenyi). First-round selection was performed with 2×10^9 yeast, more than 10-fold coverage of the number of transformants. Yeast were stained with 10 mL of 470 nM human Fz5-CRD SAV-PE tetramers in PBE (PBS, pH 7.4 + 0.5% BSA + 2 mM EDTA) for 2 h in a 15 mL conical tube with slow rotation at 4 °C. Cells were pelleted at 5000xg for 5 min, buffer aspirated, and washed with 14 mL PBE. The pellet was resuspended in 9.6 mL PBE and 400 μ L Miltenyi anti-PE microbeads, incubated for 20 min with slow rotation at 4 °C, pelleted, and washed with 14 mL PBE. The yeast were then resuspended in 5 mL PBE and magnetically separated by a Miltenyi LS column, following the manufacturer's protocols. Subsequent rounds of selection (rounds 2–7) used 1×10^8 yeast cells and 100 μ L of Miltenyi microbeads to capture labeled yeast-displayed XWnt8 variants. In rounds 2 and 4, the yeast library was selected for display of a c-myc epitope. This was performed by labeling yeast with a 0.5 mL solution of anti-cmyc-FITC antibody diluted 1:80 in PBE, incubating for 2 h with rotation at 4 °C, and washing and selecting as above (with Miltenyi anti-FITC beads).

Yeast displayed XWnt8 library screening

Ninety-six individual yeast clones from round 6 were grown in 96-well blocks containing 1 mL SDCAA for one day at 30 °C, then transferred via 1:10 dilution into a new 96-well block containing 1 mL SGCAA and allowed to induce at 20 °C for two days. The yeast clones were labeled and analyzed separately with 1:50 anti-cmyc-FITC (Miltenyi), 470 nM mouse Fz8-CRD tetramer (SAV-PE), and 470 nM human Fz5-CRD tetramer (SAV-PE) in a 96-well culture plate (Nunc) using an Accuri C6 cytometer. Yeast clones that displayed c-myc and bound to both Fz5-CRD tetramer and mouse Fz8-CRD tetramer were sequenced.

Yeast displayed mini-XWnt8 binding to human Fz4, human Fz5, and mouse Fz8

Sequencing of selected clones revealed a consensus sequence that represented 90-amino acid C-terminal fragment of XWnt8 (residues 248–338) that we refer to as "mini-Wnt." These C-terminal truncations of XWnt8 were cloned into yeast display vector pCT302 and induced to display on the yeast surface by growing in SGCAA for two days at 20 °C. 1×10^6 yeast were washed with 1 mL PBE and incubated for 2 h at room temperature with 50 μ L of human Fz4-CRD, human Fz5-CRD, or Fz8-CRD tetramers. Tetramers were formed by incubating 2 mM biotinylated Fz-CRD in PBE with 470 nM SAV-PE for 15 min on ice in a total volume of 50 μ L. Finally, the labeled yeast were washed twice with 1 mL PBE and analyzed on an Accuri C6 flow cytometer (Fig. 5A). We also tested binding of mini-XWnt8 to Fz1, Fz2, and Fz7-CRD but did not see convincing binding above background.

Affinity measurements of mini-XWnt8 interaction with Fz-CRD

Mini-XWnt8 was expressed in baculovirus infected insect cells in order to measure solution affinity for Fz-CRD. Binding measurements were performed by surface plasmon resonance on a BIAcore T100 (GE Healthcare) and all proteins were subject to gel filtration immediately prior to experiments. Biotinylated Fz5-CRD and Fz8-CRD were coupled at a low density (150 –160 RU) to streptavidin on a SA sensor chip (GE Healthcare). An unrelated biotinylated protein was captured at equivalent coupling density to the control flow cells. Measurements were performed in 1xHBS-P containing 0.01 % BSA (GE Healthcare) at 40 μ l/ml. Due to rapid dissociation, no regeneration of the surface was required between runs. All data were analyzed using the Biacore T100 evaluation software version 2.0 with a 1:1 Langmuir binding model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Formation of the XWnt8 complex with mouse Fz8-CRD for structure determination (A) Strategy for purification of the XWnt8/Fz8-CRD complex. The mouse Fz8-CRD was co-expressed as an Fc-fusion protein with XWnt8 in *Drosophila* S2 cells and the complex was captured with Protein-A. The XWnt8/Fz8-CRD complex was eluted from the resin using 3C protease, which cleaved the Fz8-CRD from the Fc. (B) The complex was then purified by gel filtration chromatography. The doublet band for XWnt8 represents glycosylation heterogeneity. (C) Initial density-modified electron density map calculated with experimental phases derived from Selenomethionine sites (shown in green spheres). N-Glycan evident in experimentally-phased map is labeled. The initial backbone trace built into this map is shown within the electron density, along with neighboring symmetry mates. See also Table S1, and Fig. S1 for electron density of the refined structure.

Figure 2. Overall structure of XWnt8 in complex with Fz8-CRD

Ribbon models of XWnt8 (violet) and Fz8-CRD (blue) as viewed ‘face on’ (A) and ‘side-on’ (B). N-linked glycans are drawn as green sticks, disulfide bonds are drawn as orange sticks. (C) Surface representation of XWnt8 after removal of the Fz8-CRD from the complex structure. The extended palmitoleic acid (PAM) group is shown in red extending from the Wnt thumb. See also Fig. S5 for mapping of potential Lrp5/6 binding site. (D) Secondary structure diagram of the XWnt8 fold. Disulfide connectivity is indicated by orange lines, visible N-glycan addition sites by green cartoon, PAM addition site by red cartoon. See also Fig. S2 for images of the bound versus unbound structure of the Fz8-CRD, and a molecular surface of the entire complex.

Figure 3. Acylation of the XWnt8 thumb loop mediates site 1 binding to Fz8-CRD

(A) Shape and chemical complementarity of the lipid-CRD interaction is evident when the electron density of the lipid modification (red lipid in grey mesh, sigmaA-weighted 2Fo-Fc map contoured at 0.8σ) at Ser-187 of XWnt8 (violet) is shown together with the molecular surface of the site 1 groove in the Fz8-CRD (blue). (B) Amino acid interactions mediating recognition of the XWnt8 thumb by the Fz8-CRD at site 1 (Table S1). Several hydrogen bonds are drawn as dashed lines. (C) Site 1 recognition occurs largely through chemically or strictly conserved Wnt and Fz amino acids (see also Figs S3 and S4). Residues of the Fz8-CRD that contact XWnt8 or the lipid are indicated with blue labels. Alternative residues at each position in other Fz-CRD are indicated by residues within parentheses. The relative font sizes of the different amino acids within the parentheses reflects an approximate percentage of the ten Fz that use that amino acid at the respective position. "mc" label indicates that the residue contacts Wnt through main chain interaction rather than side chain.

Figure 4. A conserved Wnt/Fz recognition mode in the site 2 interface

(A) Electron density of the XWnt8 finger loop (grey mesh, sigmaA-weighted 2Fo-Fc map contoured at 0.83) bound in a concave depression on the Fz8-CRD surface (blue). (B) XWnt8 index finger loop (violet) and Fz8-CRD (blue) amino acids mediating recognition at site 2 (Table S1). Disulfide bonds are drawn as yellow sticks. (C) Conservation analysis of site 2 interactions reveals that the majority of side-chain specific interactions are either strictly or chemically conserved in Fz (see also Figs S3 and S4). Alternative residues at each position in other Fz-CRD are indicated by residues within parentheses. The relative font sizes of the different amino acids within the parentheses reflects an approximate percentage of the ten Fz that use that amino acid at the respective position. “mc” label indicates that the residue contacts Wnt through main chain interaction rather than side chain. At the C-terminal end of the Fz8-CRD construct, positions 151 and 152 are linker-derived residues (Ala), but are Asn and Tyr in the wild type Fz8-CRD sequence.

Figure 5. “Mini-Wnt” discriminates between different Fz-CRD and engages site 2 independently of site 1

(**A**) The C-terminal 90-amino acids of XWnt8 were displayed on the surface of yeast and shown to bind robustly to Fz5-, and Fz8-CRD, and weakly to F4-CRD by FACS analysis using fluorescent Fz-CRD-Streptavidin-PE tetramers. The cartoon of mini-Wnt displayed on yeast depicts the C-terminal 90-amino acids seen in the structure. The population of mini-Wnt yeast stained by the Fz-CRD tetramers by FACS was ~50%, ~30%, and ~4% for Fz8, Fz5, and Fz4-CRD, respectively. (**B**) Surface plasmon resonance analysis of mini-XWnt8 binding to Fz5- and F8-CRD immobilized on a BIAcore T100 sensor chip. Dose-response titrations are plotted of the equilibrium binding experiments, the insets of each panel show the titration data and that each concentration reached steady state.