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(54) **Title:** WNT PEPTIDE PHARMACOPHORE COMPOSITIONS AND METHODS OF USE THEREOF

(57) **Abstract:** Wnt compositions and methods for their use are provided. Compositions of the invention comprise fragments of wnt polypeptides having a desired biological activity, which fragments are referred to herein as "wnt peptide pharmacophores". These compositions and methods find particular use in determining binding to Wnt receptors; inhibiting Wnt signaling in a cell that expresses a Wnt receptor; in delivering a functional moiety to a cell that expresses a Wnt receptor; and as an immunogen for producing Wnt-specific antibodies.



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WNT PEPTIDE PHARMACOPHORE COMPOSITIONS AND METHODS OF USE THEREOF

GOVERNMENT RIGHTS

[0001] This invention was made with government support under Grant Number GM097015 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0002] Wnts (*Wingless* and *Int-1*) are central mediators of vertebrate and invertebrate development, due to their influences on cell proliferation, differentiation, and migration. Wnts act through activation of cell surface receptors on responder cells which activate at least three different signaling pathways including the “canonical” β -catenin pathway, and the “non-canonical” planar cell polarity (PCP) and Ca^{2+} pathways. 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) to serve as co-receptors. Wnt signaling is also regulated by several alternative receptors, such as Ryk and Ror2, and by secreted antagonists that directly interact with Wnts, such as Wnt-interacting factor (WIF-1), or engage Wnt receptors, such as Dickkopf (Dkk) and Kremen (Krm). 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.

[0003] Wnts are ~350-residue secreted, cysteine-rich glycoproteins for which there exists no structural information: the primary sequences of Wnts are not clearly related to any known protein families or folds. Wnts are hydrophobic molecules due to the post-translational addition of palmitate and/or palmitoleic acid to one or two residues (Cys77 and/or Ser209 in Wnt3a). Acylation is necessary for both Wnt intracellular trafficking and its full activity when secreted, but its precise role in Wnt action is not known. Genetic evidence suggests that Wnt-secreting cells require the action of acyltransferases (*porcupine* in *Drosophila*) for Wnt palmitoylation. 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. Wnt palmitoylation has been a major hindrance to research in Wnt signaling, as it has greatly complicated expression and purification of recombinant material since Wnts require purification and handling in either detergents or liposomes. As a result of the technical difficulties of Wnt expression, and their sensitivity to mutation, relatively few detailed structure-function studies of Wnts have been carried out that shed light on how they engage Fz. Both

N-terminal and C-terminal regions of Wnt have been implicated as being important for Fz binding.

[0004] Current structural knowledge of Frizzled receptors is limited to the unliganded Fz8-CRD and the secreted CRD antagonist sFRP, which are ~160 residue, primarily α -helical proteins. Mutational mapping studies of *Xenopus* Wnt8 (XWnt8) interactions with Fz8, Fz4, and dFz2 identified several potential patches on the CRD important for binding, but an unambiguous binding site has not been determined. 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. With respect to Fz activation, the molecular mechanisms are unknown. While Fz share several features reminiscent of G-protein coupled receptors (GPCR), they lack hallmark characteristics that would clearly place them as prototypical GPCR. Nevertheless, some principles of transmembrane signaling by GPCR may be relevant.

[0005] One of the puzzling questions about the Wnt/Fz system is the basis for functional specification in the face of the apparent ligand-receptor cross-reactivity. Unambiguous receptor-ligand matching between the 19 mammalian Wnts and 10 Fz is complicated by the fact that each Wnt can engage multiple Fz, and each Fz can respond to multiple Wnts. This pleiotropy confounds interpretation of *in vivo* functional experiments in that it is unclear if mono-specific Wnt/Fz pairs are responsible for certain biological effects and diseases, or if polyspecificity is key to the functional properties of the Wnt/Fz system. Although structural information regarding Wnt/Fz interactions has been lacking that would provide insight into this issue, one hypothesis is that the Wnt lipid group(s) may be directly involved in binding to the Fz-CRD, and this may be emblematic of a biological paradigm for CRD-containing proteins (e.g. NPC1 and Smo) to bind lipid-modified ligands (e.g. Hedgehog). A recent structure of WIF sequestering bound lipids is suggestive that the lipid group of Wnts could be used for ligand or receptor binding. 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.

[0006] The development of pharmaceutically active wnt compositions that are water soluble is therefore of great interest.

Publications

[0007] The biological activity of soluble wingless protein is described in van Leeuwen et al. (1994) Nature 24:368(6469):342-4. Biochemical characterization of Wnt-frizzled interactions using a soluble, biologically active vertebrate Wnt protein is described by Hsieh et al. (1999) Proc Natl Acad Sci U S A 96(7):3546-51. Bradley et al. (1995) Mol Cell Biol 15(8):4616-22 describe a soluble form of wnt protein with mitogenic activity. Hoppler et al. (1996) Genes

and Dev. 10:2805-2817 describe dominant negative Wnt polypeptides that are truncated Wnts (truncated Xwnt-8 and truncated mouse Wnt-1). Couso and Martinez-Arias (1994) Cell 79(2):259-72 describes a mutant allele of Dvnt-1 that encodes a secreted protein with a substantial carboxy-terminal deletion that has antimorphic effects. Other dominant negative Wnt polypeptides and methods for their use are described in WO2010/078458.

[0008] Willert et al. (2003) Nature 423:448-452 describes methods of purifying Wnt proteins with the aid of detergents. Morrell et al. (2008) PLoS One 3(8):e2930 describes the formulation of Wnt proteins into liposomes, and how Wnts packaged in liposomes retain biological activity in vivo. Patent publications include U.S. Patent nos. 7,335,643; and 7,153,832; and published U.S. Patent Application 20080226707.

SUMMARY OF THE INVENTION

[0009] Wnt compositions and methods for their use are provided. Compositions of the invention comprise fragments, particularly cyclic fragments, of wnt polypeptides having a desired biological activity, which fragments are referred to herein as "wnt peptide pharmacophores". Wnt peptide pharmacophores of interest have a high affinity to a Wnt receptor, for example a frizzled protein, but are of a small size, usually less than about 20 amino acids in length, less than about 19, 18, 17, 16, 15, 14, 13, 12, 11 amino acids in length. Such peptides, including without limitation those sequences set forth in SEQ ID NO:1-47 or fragments and conserved variants thereof, have conserved cysteine residues that allow for a cyclic structure. Included are fragments of native wnt proteins and derivatives thereof, e.g. analogs comprising one or more amino acid changes relative to the native sequence that enhance a property of interest, such as solubility, affinity or specificity for a targeted receptor. The pharmacophores are water soluble.

[0010] Compositions of interest include, without limitation, an effective dose of a wnt peptide pharmacophore in a pharmaceutically acceptable excipient. Compositions may comprise additional agents, e.g. adjuvants and the like. Wnt peptide pharmacophores may be produced synthetically; by various suitable recombinant methods, and the like, as known in the art.

[0011] These compositions and methods find particular use in inhibiting Wnt signaling in a cell that expresses a Wnt receptor, e.g. to inhibit aberrant cell proliferation; in delivering a functional moiety, e.g. a therapeutic or an imaging moiety, to a cell that expresses a Wnt receptor; and as an immunogen for producing Wnt-specific antibodies.

[0012] A wnt peptide pharmacophore of interest is derived from a conserved region of the C terminus of a wnt protein. The delineation of the carboxy terminal domain is exemplified herein with multiple human Wnt proteins, e.g. as shown in Table 1. Wnt carboxy terminal domains may also be empirically identified by alignment with sequences provided herein. In

one example, a wnt peptide pharmacophore amino acid sequence aligns by conserved residues with positions 292-304 of *Xenopus* Wnt8.

[0013] In some embodiments, the wnt peptide pharmacophore polypeptide is fused or conjugated to a functional moiety, which may be a therapeutic moiety, *e.g.* a cytotoxic moiety, or a moiety that targets a cell for ADCC- or CDC-directed cell death. Therapeutic moieties of interest include those that alters the cell's activity. In some embodiments, the functional moiety is an imaging moiety, *e.g.* a fluorophore, luminophore, radioisotope, *etc.* In some embodiments the functional moiety is an oligomerizing moiety that induces oligomerization of the wnt peptide pharmacophore into homo-dimers, -trimers, -tetramers or more, or hetero-dimers, -trimers, -tetramers or more by, *e.g.* using zippers or Fc polypeptides, or other avidity-enhancing chemical or protein agents. In some embodiments the functional moiety is a protein or chemical moiety that extends the half-life and/or increases the size of the wnt peptide pharmacophore polypeptide.

[0014] In some aspects of the invention, a method is provided for delivering a functional moiety to a cell, comprising contacting a cell of interest expressing a Wnt receptor with a wnt peptide pharmacophore comprising a functional moiety of interest. In other embodiments, methods are provided for inhibiting Wnt signaling in a cell. In such methods, a cell expressing a Wnt receptor is contacted with a concentration of a wnt peptide pharmacophore that is effective to inhibit signaling, *e.g.* to reduce signaling by 25%, 50%, 75%, 90%, 95%, or more, relative to the signaling in the absence of the wnt peptide pharmacophore. Such signaling inhibition may inhibit proliferation of the targeted cell, or may otherwise interfere with Wnt-signaling pathways active in the targeted cell. In some methods, the receptor-expressing cell is contacted *in vitro*. In other embodiments, the receptor-expressing cell is contacted *in vivo*. Cells of interest include a wide variety of Wnt-receptor expressing cells, as are known in the art.

[0015] A benefit of the compositions and methods of the invention is the specificity of targeting and the small size of the pharmacophore, where the wnt peptide pharmacophore targets the same cells as the native wnt protein from which it is derived. For example, wnt peptide pharmacophores selectively inhibit Wnt signaling in those cells that are responsive to the Wnt parent protein. In addition, a benefit of the water soluble forms of wnt peptide pharmacophores is the lack of a requirement for formulation additives that might limit their therapeutic utility.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to-scale. On the contrary, the

dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures.

[0017] 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.

[0018] 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 yellow 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. S2 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.

[0019] 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 2). 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 9 and 10). 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.

[0020] 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 0.8σ) 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 our Fz8-CRD construct, residues 150 and 151 are truncated to Ala, but are Asn and Tyr in the wild type Fz8-CRD sequence.

[0021] Figure 5. "Wnt peptide pharmacophore" binds to site 2. (A) The C-terminal 90-amino acids of XWnt8 were displayed on the surface of yeast and shown to bind to Fz4-, Fz5-, and Fz8-CRD by FACS analysis using fluorescent Fz-CRD-Streptavidin-PE tetramers. The cartoon of wnt peptide pharmacophore displayed on yeast depicts the C-terminal 90-amino acids seen in the structure. The population of wnt peptide pharmacophore 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 Fz8-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. Both equilibrium and kinetically-derived K_D 's are shown.

[0022] Figure 6. 'Pseudo site 3' mediates formation of asymmetric dimers in the crystal lattice. (A) Ribbon diagrams of two XWnt8/Fz8-CRD complexes associated through pseudo site 3 to form an asymmetric dimer. The PAM moiety is shown as red sticks. (B) Closeup view of pseudo-site 3 interface in surface representation showing complete burial of the lipid group between the two complexes. (C) Four XWnt8/Fz8-CRD complexes are shown as they pack in the crystal lattice to generate a repeating oligomer centered around the 4-fold screw axis of the crystal.

[0023] Figure 7. Representative electron density in the refined structure. (A) Region of XWnt8 N-terminal domain showing Arg-Trp stacking interactions. (B) N-linked glycan attached to Asparagine-263. Both panels show SigmaA-weighted 2Fo-Fc maps at 3.25Å resolution (0.87 sigma contour) calculated using the final refined structure.

[0024] Figure 8. Mapping Wnt sequence conservation on to the molecular surface of XWnt8. Potential co-receptor binding site is circled with dashed line. Sequences used for the analysis were XWnt8 and the 19 mammalian Wnts shown in Figure 10.

[0025] Figure 9. Sequence alignment of mouse Wnts and XWnt8. Invariant and chemically conserved residues are colored in red and yellow, respectively. Interacting residues are marked with numbers above the alignment, representing the respective binding site.

Secondary structure elements are depicted above the alignment. Alignments performed by ClustalW.

[0026] Figure 10. Sequence alignment of mouse Frizzled CRDs. Invariant and chemically conserved residues are colored in red and yellow, respectively. Interacting residues are marked with numbers above the alignment, representing the respective binding site. Secondary structure elements are depicted above the alignment. Alignments performed by ClustalW.

[0027] Figure 11. Binding of mni-Xwnt8 to Fz5-CRD can be recapitulated by a 13 amino acid cyclic peptide corresponding to the structural finger of the wnt protein.

[0028] Figure 12. Mini-Xwnt8 and cyclic peptide bind to Fz5-CRD and Fz8-CRD with micromolar affinity.

[0029] Figure 13. Summary of selection of Fz8-CRD binding peptides using yeast cell surface display

[0030] Figure 14. Avidity enhanced Fz8-CRD binding cyclic peptides.

[0031] Figure 15. Binding of selected cyclic peptides.

DETAILED DESCRIPTION OF THE INVENTION

[0032] Before the present methods and compositions are described, it is to be understood that this invention is not limited to particular method or composition described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0033] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0034] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated

herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

[0035] It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the peptide" includes reference to one or more peptides and equivalents thereof, e.g. polypeptides, known to those skilled in the art, and so forth.

[0036] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DEFINITIONS

[0037] Wnt peptide pharmacophore compositions and methods for their use are provided. These compositions and methods find particular use in inhibiting Wnt signaling in a cell that expresses a Wnt receptor, e.g. to inhibit aberrant cell proliferation; in delivering a therapeutic moiety to a cell that expresses a Wnt receptor; in delivering an imaging moiety to a cell that expresses a Wnt receptor; and in producing Wnt-specific antibodies. These and other objects, advantages, and features of the invention will become apparent to those persons skilled in the art upon reading the details of the compositions and methods as more fully described below.

[0038] A "Wnt protein" is a member of the family of highly conserved secreted signaling molecules that play key roles in both embryogenesis and mature tissues. The terms "Wnts" or "Wnt gene product" or "Wnt polypeptide" when used herein encompass native sequence Wnt polypeptides, Wnt polypeptide variants, Wnt polypeptide fragments and chimeric Wnt polypeptides. A "wnt peptide pharmacophore polypeptide" is a polypeptide that is a fragment of a full-length Wnt protein that retains the ability of the full-length Wnt protein from which it was derived to specifically bind to one or more Wnt receptors. The binding of the wnt peptide pharmacophore to a Wnt receptor may have a dominant negative effect, in that signaling from the Wnt receptor is inhibited.

[0039] The term "native sequence Wnt polypeptide" refers to Wnt polypeptides comprising sequence as they are found in nature. For example, human native sequence Wnt proteins of interest in the present application include the following: Wnt-1 (GenBank Accession No. NM_005430); Wnt-2 (GenBank Accession No. NM_003391); Wnt-2B (Wnt-13) (GenBank Accession No. NM_004185 (isoform 1), NM_024494.2 (isoform 2)), Wnt-3 (RefSeq:

NM_030753), Wnt3a (GenBank Accession No. NM_033131), Wnt-4 (GenBank Accession No. NM_030761), Wnt-5A (GenBank Accession No. NM_003392), Wnt-5B (GenBank Accession No. NM_032642), Wnt-6 (GenBank Accession No. NM_006522), Wnt-7A (GenBank Accession No. NM_004625), Wnt-7B (GenBank Accession No. NM_058238), Wnt-8A (GenBank Accession No. NM_058244), Wnt-8B (GenBank Accession No. NM_003393), Wnt-9A (Wnt-14) (GenBank Accession No. NM_003395), Wnt-9B (Wnt-15) (GenBank Accession No. NM_003396), Wnt-10A (GenBank Accession No. NM_025216), Wnt-10B (GenBank Accession No. NM_003394), Wnt-11 (GenBank Accession No. NM_004626), Wnt-16 (GenBank Accession No. NM_016087)). Although each member has varying degrees of sequence identity with the family, all encode small (i.e., 39-46 kD), acylated, palmitoylated, secreted glycoproteins that contain 23-24 conserved cysteine residues whose spacing is highly conserved (McMahon, A P et al., Trends Genet. 1992; 8: 236-242; Miller, J R. Genome Biol. 2002; 3(1): 3001.1-3001.15). Other native sequence Wnt polypeptides of interest in the present invention include orthologs of the above from any mammal, including domestic and farm animals, and zoo, laboratory or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, rats, mice, frogs, zebra fish, fruit fly, worm, etc.

[0040] A "native sequence" wnt peptide pharmacophore polypeptide is a fragment of the amino acid sequence of a sequence Wnt polypeptide. Such native sequence polypeptides can be isolated from cells producing endogenous Wnt protein or can be produced by recombinant or synthetic means. Thus, a native sequence wnt peptide pharmacophore polypeptide can have the amino acid sequence comprised by, e.g. naturally occurring human Wnt polypeptide, murine Wnt polypeptide, or polypeptide from any other mammalian species, or from non-mammalian species, e.g. *Drosophila*, *C. elegans*, and the like.

[0041] A "variant" wnt peptide pharmacophore polypeptide means a biologically active polypeptide as defined below having less than 100% sequence identity with a native Wnt sequence over the length of the fragment. Such variants include polypeptides wherein one or more amino acid residues are added at the N- or C-terminus of, or within, the native sequence; from about one to forty amino acid residues are deleted, and optionally substituted by one or more amino acid residues; and derivatives of the above polypeptides, wherein an amino acid residue has been covalently modified so that the resulting product has a non-naturally occurring amino acid. Ordinarily, biologically active variants will have an amino acid sequence having at least about 75% sequence identity, about 80% sequence identity, about 85% amino acid sequence identity, about 90% amino acid sequence identity with a native sequence polypeptide, preferably at least about 95%, more preferably at least about 99% sequence identity. For example, a peptide set forth in Table 1 can comprise up to one amino acid substitution, up to 2 amino acid substitutions, up to 3 amino acid substitutions. Various methods known in the art may be utilized in developing such variant polypeptides.

[0042] A "chimeric" wnt peptide pharmacophore polypeptide is a polypeptide comprising a wnt peptide pharmacophore polypeptide fused or conjugated to a heterologous polypeptide. The chimeric wnt peptide pharmacophore polypeptide will generally share at least one biological property with the initial wnt peptide pharmacophore polypeptide. Examples of chimeric polypeptides include wnt peptide pharmacophore polypeptides fused to one or more functional moieties such as a therapeutic moiety, an imaging moiety, an epitope tag. Typically, when the functional moiety is a polypeptide moiety, the moiety has sufficient residues to provide a function, e.g. promoting multimerization, promoting cell death, altering cell function, fluorescence signal, an epitopic sequence, *etc.*, yet is short enough such that it does not interfere with biological activity of the wnt peptide pharmacophore polypeptide. Suitable tag polypeptides for use as an epitope generally have at least six amino acid residues and usually between about 6-250 amino acid residues.

[0043] By "water soluble" it is meant a composition that is soluble in aqueous buffers in the absence of detergent, usually soluble at a concentration that provides a biologically effective dose of the polypeptide. Compositions that are water soluble form a substantially homogenous composition that has a specific activity that is at least about 5% that of the starting material from which it was purified, usually at least about 10%, 20%, or 30% that of the starting material, more usually about 40%, 50%, or 60% that of the starting material, and may be about 50%, about 90% or greater. Wnt peptide pharmacophore compositions of the present invention typically form a substantially homogeneous solution at concentrations of at least 25 μM and higher, e.g. at least 25 μM , 40 μM , or 50 μM , usually at least 60 μM , 70 μM , 80 μM , or 90 μM , sometimes as much as 100 μM , 120 μM , or 150 μM . In other words, wnt peptide pharmacophore compositions of the present invention typically form a substantially homogeneous solution at concentrations of about 0.1 mg/ml, about 0.5 mg/ml, of about 1 mg/ml or more.

[0044] "Wnt protein signaling" or "Wnt signaling" is used herein to refer to the mechanism by which a biologically active Wnt exerts its effects upon a cell to modulate a cell's activity. Wnt proteins modulate cell activity by binding to Wnt receptors, including proteins from the Frizzled (Fz) family of proteins, proteins from the ROR family of proteins, the proteins LRP5, LRP6 from the LRP family of proteins, the protein FRL1/crypto, and the protein Derailed/Ryk. Once activated by Wnt binding, the Wnt receptor(s) will activate one or more intracellular signaling cascades. These include the canonical Wnt signaling pathway; the Wnt/planar cell polarity (Wnt/PCP) pathway; the Wnt-calcium (Wnt/ Ca^{2+}) pathway (Giles, RH et al. (2003) *Biochim Biophys Acta* 1653, 1-24; Peifer, M. et al. (1994) *Development* 120: 369-380; Papkoff, J. et al (1996) *Mol. Cell Biol.* 16: 2128-2134; Veeman, M. T. et al. (2003) *Dev. Cell* 5: 367-377); and other Wnt signaling pathways as is well known in the art. For example, activation of the canonical Wnt signaling pathway results in the inhibition of phosphorylation of the intracellular

protein β -catenin, leading to an accumulation of β -catenin in the cytosol and its subsequent translocation to the nucleus where it interacts with transcription factors, e.g. TCF/LEF, to activate target genes. Activation of the Wnt/PCP pathway activates RhoA, c-Jun N-terminal kinase (JNK), and nemo-like kinase (NLK) signaling cascades to control such biological processes as tissue polarity and cell movement. Activation of the Wnt/ Ca^{2+} by, for example, binding of Wnt-4, Wnt-5A or Wnt-11, elicits an intracellular release of calcium ions, which activates calcium sensitive enzymes like protein kinase C (PKC), calcium-calmodulin dependent kinase II (CamKII) or calcineurin (CaCN). By assaying for activity of the above signaling pathways, the biological activity of a Wnt composition can be readily determined. A "biologically active wnt peptide pharmacophore" is a wnt peptide pharmacophore composition that is able to specifically bind to Wnt receptor and modulate Wnt signaling when provided to a cell *in vitro* or *in vivo*, that is, when administered to an animal, e.g. a mammal. Frequently wnt peptide pharmacophore polypeptides are dominant negative, or competitive, inhibitors of wnt signaling.

[0045] The term "specific binding" refers to that binding which occurs between such paired species as enzyme/substrate, receptor/ligand, antibody/antigen, and lectin/carbohydrate which may be mediated by covalent or non-covalent interactions or a combination of covalent and non-covalent interactions. When the interaction of the two species produces a non-covalently bound complex, the binding which occurs is typically electrostatic, hydrogen-bonding, or the result of lipophilic interactions. Accordingly, "specific binding" occurs between a paired species where there is interaction between the two which produces a bound complex having the characteristics of an antibody/antigen or ligand/receptor interaction. One may determine the biological activity of a wnt peptide pharmacophore protein in a composition by determining the level of activity in a functional assay after *in vivo* administration, e.g. decelerating bone regeneration, downregulation of stem cell proliferation, etc., quantitating the amount of wnt peptide pharmacophore protein present in a non-functional assay, e.g. immunostaining, ELISA, quantitation on coomassie or silver stained gel, etc., and determining the ratio of *in vivo* biologically active wnt peptide pharmacophore to total wnt peptide pharmacophore. A wnt peptide pharmacophore composition that is biologically active is one that modulates the activity of a Wnt protein by at least about 40%, about 60%, more usually by about 70%, 75%, or 80%, often by about 85%, 90%, or 95%, sometimes by as much as 100%, i.e. complete abrogation of Wnt signaling.

[0046] The terms "Wnt antagonist", "Wnt inhibitor", and "inhibitor of Wnt signaling" are used interchangeably herein to mean an agent that antagonizes, inhibits, or negatively regulates Wnt modulation of a cell's activity. Likewise, the phrases "antagonizing Wnt signaling" and "inhibiting Wnt signaling" are used interchangeably herein to mean antagonizing, inhibiting, or otherwise negatively regulating Wnt modulation of a cell's activity.

[0047] “Fz”, “Fz proteins” and “Fz receptors” is used herein to refer to proteins of the Frizzled receptor family. These proteins are seven-pass transmembrane proteins (Ingham, P. W. (1996) Trends Genet. 12: 382-384; Yang-Snyder, J. et al. (1996) Curr. Biol. 6: 1302-1306; Bhanot, P. et al. (1996) Nature 382: 225-230) that comprise a CRD domain. There are ten known members of the Fz family (Fz1 through Fz10), any of which may serve as receptors of Wnts. Fz receptors mediate a number of Wnt biological activities, including but not limited to the modulation of synapse formation,

[0048] By “comprising” it is meant that the recited elements are required in the composition/method/kit, but other elements may be included to form the composition/method/kit etc. within the scope of the claim. For example, a composition comprising a wnt peptide pharmacophore polypeptide is a composition that may comprise other elements in addition to wnt peptide pharmacophore polypeptide(s), e.g. functional moieties such as polypeptides, small molecules, or nucleic acids bound, e.g. covalently bound, to the wnt peptide pharmacophore polypeptide; agents that promote the stability of the wnt peptide pharmacophore composition, agents that promote the solubility of the wnt peptide pharmacophore composition, adjuvants, etc. as will be readily understood in the art, with the exception of elements that are encompassed by any negative provisos.

[0049] By “consisting essentially of”, it is meant a limitation of the scope of composition or method described to the specified materials or steps that do not materially affect the basic and novel characteristic(s) of the subject invention. For example, a wnt peptide pharmacophore polypeptide “consisting essentially of” a disclosed sequence has the amino acid sequence of the disclosed sequence plus or minus about 5 amino acid residues at the boundaries of the sequence based upon the full length parent Wnt sequence from which it was derived, e.g. about 5 residues, 4 residues, 3 residues, 2 residues or about 1 residue less than the recited bounding amino acid residue, or about 1 residue, 2 residues, 3 residues, 4 residues, or 5 residues more than the recited bounding amino acid residue.

[0050] By “consisting of”, it is meant the exclusion from the composition, method, or kit of any element, step, or ingredient not specified in the claim. For example, a wnt peptide pharmacophore polypeptide “consisting of” a disclosed sequence consists only of the disclosed amino acid sequence.

[0051] By “functional moiety” or “FM” it is meant a polypeptide, small molecule or nucleic acid composition that confers a functional activity upon a composition. Examples of functional moieties include, without limitation, therapeutic moieties, binding moieties, and imaging moieties.

[0052] By “therapeutic moiety”, or “TM”, it is meant a polypeptide, small molecule or nucleic acid composition that confers a therapeutic activity upon a composition. Examples of therapeutic moieties include cytotoxins, e.g. small molecule compounds, protein toxins, and

radiosensitizing moieties, i.e. radionuclides etc. that are intrinsically detrimental to a cell; agents that alter the activity of a cell, e.g. small molecules, peptide mimetics, cytokines, chemokines; and moieties that target a cell for ADCC or CDC-dependent death, e.g. the Fc component of immunoglobulin.

[0053] By an "imaging moiety", or "IM", it is meant a non-cytotoxic agent that can be used to locate and, optionally, visualize cells, e.g. cells that have been targeted by compositions of the subject application.

[0054] An oligomerizing moiety is a polypeptide, small molecule or nucleic acid composition that induces oligomerization of the wnt peptide pharmacophore into homo-dimers, -trimers, -tetramers or more, or hetero-dimers, -trimers, -tetramers or more by, e.g. using zippers, or Fc polypeptides, biotin and avidin/streptavidin, or other avidity-enhancing chemical or protein agents as known in the art.

[0055] The phrases "Wnt-mediated condition" and "Wnt-mediated disorder" are used interchangeably herein to describe a condition, disorder, or disease state characterized by aberrant or undesirable Wnt signaling. In a specific aspect, the aberrant Wnt signaling is a level of Wnt signaling in a cell or tissue suspected of being diseased that exceeds the level of Wnt signaling in a similar non-diseased cell or tissue. Examples of Wnt-mediated disorders include those associated with aberrant angiogenesis, e.g. retinopathies, and those associated with aberrant proliferation, e.g. cancer.

[0056] The terms "treatment", "treating" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, i.e., arresting its development; or (c) relieving the disease, i.e., causing regression of the disease. The therapeutic agent may be administered before, during or after the onset of disease or injury. The treatment of ongoing disease, where the treatment stabilizes or reduces the undesirable clinical symptoms of the patient, is of particular interest. Such treatment is desirably performed prior to complete loss of function in the affected tissues. The subject therapy will desirably be administered during the symptomatic stage of the disease, and in some cases after the symptomatic stage of the disease.

[0057] The terms "individual," "subject," "host," and "patient," are used interchangeably herein and refer to any mammalian subject for whom diagnosis, treatment, or therapy is desired, particularly humans.

[0058] General methods in molecular and cellular biochemistry can be found in such standard textbooks as Molecular Cloning: A Laboratory Manual, 3rd Ed. (Sambrook et al., CSH Laboratory Press 2001); Short Protocols in Molecular Biology, 4th Ed. (Ausubel et al. eds., John Wiley & Sons 1999); Protein Methods (Bollag et al., John Wiley & Sons 1996); Nonviral Vectors for Gene Therapy (Wagner et al. eds., Academic Press 1999); Viral Vectors (Kaplift & Loewy eds., Academic Press 1995); Immunology Methods Manual (I. Lefkovits ed., Academic Press 1997); and Cell and Tissue Culture: Laboratory Procedures in Biotechnology (Doyle & Griffiths, John Wiley & Sons 1998), the disclosures of which are incorporated herein by reference. Reagents, cloning vectors, and kits for genetic manipulation referred to in this disclosure are available from commercial vendors such as BioRad, Stratagene, Invitrogen, Sigma-Aldrich, and ClonTech.

Compositions

[0059] Wnt peptide pharmacophore compositions and methods for their use are provided. Wnt peptide pharmacophore compositions are compositions that comprise a wnt peptide pharmacophore polypeptide. As discussed above, a wnt peptide pharmacophore polypeptide is a polypeptide that is a fragment of a full-length Wnt protein that retains the ability of the full-length Wnt protein from which it was derived to specifically bind to at least one Wnt receptor. Unlike a full-length Wnt protein, which may simultaneously bind to two distinct co-receptors, a wnt peptide pharmacophore typically binds to only one of the co-receptors. A wnt peptide pharmacophore polypeptide is usually less than about 20 amino acids in length, less than about 19, 18, 17, 16, 15, 14, 13, 12, 11 amino acids in length, and of a length sufficient to provide for micromolar binding affinity to a Wnt receptor, e.g. a frizzled protein, as shown in Figure 12. In some embodiments the peptide is at least about 11 amino acids in length, and comprising at least one cysteine pair that create a bond and form a cyclic peptide. For example, without limitation the invention includes a peptide as shown in Table 1 or fragment thereof comprising at least the sequence from residue 2 to residue 12 and the conserved cysteine residues contained therein, and optionally including the contiguous amino acid residues at positions 1 and 13. The peptide in some embodiments is from about 11 to about 20 amino acids in length, comprising or consisting of a contiguous sequence of a wnt polypeptide and two pairs of cysteines forming disulfide bonds.

Table 1

XWnt8	SEQ ID NO:1	NCKFHWCCTVKCE
mWnt1	SEQ ID NO:2	NCTFHWCCHVSCR
mWnt2	SEQ ID NO:3	ECKFHWCCAVRCQ
mWnt2B	SEQ ID NO:4	ECKFHWCCAVRCK
mWnt3	SEQ ID NO:5	HCVFHWCCYVSCQ
mWnt3A	SEQ ID NO:6	HCVFHWCCYVSCQ
mWnt4	SEQ ID NO:7	GCRFHWCCFVKCR
mWnt5A	SEQ ID NO:8	HCKFHWCCYVKCK

mWnt5B	SEQ ID NO:9	HCRFHWCCFVRCK
mWnt6	SEQ ID NO:10	LCRFHWCCVVQCH
mWnt7A	SEQ ID NO:11	NCKFHWCCYVKCN
mWnt7B	SEQ ID NO:12	NCKFHWCCFVKCN
mWnt8A	SEQ ID NO:13	DCNFQWCCTVKCG
mWnt8B	SEQ ID NO:14	NCKFHWCCAVRCE
mWnt9A	SEQ ID NO:15	QCQVRWCCYVECR
mWnt9B	SEQ ID NO:16	HCQVQWCCYVECQ
mWnt10A	SEQ ID NO:17	HCRFHWCCFVUCE
mWnt10B	SEQ ID NO:18	HCRFHWCCYVLCD
mWnt11	SEQ ID NO:19	HCKYHWCCYVTCR
mWnt16	SEQ ID NO:20	ECKFIWCCYVRER
hWnt1	SEQ ID NO:21	NCTFWCCCHVSCR
hWnt2	SEQ ID NO:22	GCKFHWCCAVRCQ
hWnt2B	SEQ ID NO:23	ECKFHWCCAVRCK
hWnt3	SEQ ID NO:24	HCIFHWCCYVSCQ
hWnt3A	SEQ ID NO:25	RCVFWCCYVSCQ
hWnt4	SEQ ID NO:26	SCKFHWCCFVKCR
hWnt5A	SEQ ID NO:27	HCKFHWCCYVKCK
hWnt5B	SEQ ID NO:28	HCKFHWCCFVRCK
hWnt6	SEQ ID NO:29	LCRFHWCCVVQCH
hWnt7A	SEQ ID NO:30	NCKFHWCCYVKCN
hWnt8A	SEQ ID NO:31	NCKFQWCCTVKCD
hWnt8B	SEQ ID NO:32	NCKFHWCCAVRCE
hWnt10A	SEQ ID NO:33	HCRFHWCCFVUCE
hWnt10B	SEQ ID NO:34	HCRFHWCCYVLCD
hWnt11	SEQ ID NO:35	HCKYHWCCYVTCR
hWnt13	SEQ ID NO:36	ECKFHWCCAVRCK
hWnt16	SEQ ID NO:37	ECKFIWCCYVRER
human	SEQ ID NO:38	GCKFHWCCAVRCQ

[0060] Affinity matured wnt peptide pharmacophores include, without limitation, those peptides mutated at selected positions and having an avidity enhanced K_D of at least about 1×10^{-7} M for Frizzled; at least about 1×10^{-8} M; at least about 5×10^{-9} M, or more. Examples of affinity matured wnt peptide pharmacophores include, without limitation:

4A-8	SEQ ID NO:39	N C N R L I C C P L R C
4B-1	SEQ ID NO:40	N C N R L V C C P L R C
4B-3	SEQ ID NO:41	Y C K R V V C C P L R C
4B-4	SEQ ID NO:42	N C R R L I C C P L R C
4B-5	SEQ ID NO:43	N C A R V V C C P L R C
4B-6	SEQ ID NO:44	N C K R V V C C P I R C
4B-8	SEQ ID NO:45	N C A R I V C C P L R C
XX-FZ8-P7-2	SEQ ID NO:46	C K L H R Y F T V K C
XX-FZ8-P [^] -6	SEQ ID NO:47	C K L H R F F T I K C

[0061] A wnt peptide pharmacophore comprises or consists of a fragment the carboxy terminal domain of a wnt polypeptide, the fragment corresponding to the structural "index finger" region described in the Examples, and does not include the amino acid residues of the amino terminal domain. Wnt peptide pharmacophores may also be empirically identified by

alignment with sequences provided herein. In one example, a wnt peptide pharmacophore amino acid sequence aligns by conserved residues with positions 292-304 of XWnt8. In some embodiments a wnt peptide pharmacophore is a variant or analog as defined above. In some embodiments, the variation, or mutation, alters the affinity for its cognate receptor; solubility; and/or specificity for its cognate receptor.

[0062] Wnt peptide pharmacophores correspond to Wnt proteins from any species of organism, e.g. mouse, rat, cat, chicken, fruit fly, frog, zebra fish, dog, worm, *etc.*, and find use in the subject compositions. The polypeptide sequence for such wnt peptide pharmacophores may readily be determined by performing an alignment of the homolog or ortholog of interest with the provided Wnt sequences of Table 1, using alignment software such as NCBI BLAST, ClustalW, or other software as is well known in the art. Compositions comprising a wnt peptide pharmacophores may comprise various elements, with the exception of those elements that are specifically set forth herein as excluded from the recited wnt peptide pharmacophores, for example the wnt peptide pharmacophore may be fused to an exogenous polypeptide. The site at which the fusion is made may be selected in order to optimize the biological activity, secretion or binding characteristics of the polypeptide. The optimal site will be determined by routine experimentation.

[0063] For example, compositions comprising a wnt peptide pharmacophore optionally include polypeptides fused to the wnt peptide pharmacophore to further increase their solubility. The domain may be linked to the polypeptide through a defined protease cleavage site, e.g. a TEV sequence, which is cleaved by TEV protease. The linker may also include one or more flexible sequences, e.g. from 1 to 10 glycine residues. In some embodiments, the cleavage of the fusion protein is performed in a buffer that maintains solubility of the product, e.g. in the presence of from 0.5 to 2 M urea, in the presence of polypeptides and/or polynucleotides that increase solubility, and the like. Domains of interest include endosomolytic domains, e.g. influenza HA domain; and other polypeptides that aid in production, e.g. IF2 domain, GST domain, GRPE domain, and the like.

[0064] As another example, compositions comprising a wnt peptide pharmacophore may optionally include modifications to the wnt peptide pharmacophore to improve stability. For example, the peptide may be PEGylated, where the polyethyleneoxy group provides for enhanced lifetime in the blood stream. The polypeptide may be fused to another polypeptide to increase the *in vivo* stability. Generally such fusion partners are a stable plasma protein, which may, for example, extend the *in vivo* plasma half-life of the polypeptide when present as a fusion, in particular wherein such a stable plasma protein is an immunoglobulin constant domain. In most cases where the stable plasma protein is normally found in a multimeric form, e.g., immunoglobulins or lipoproteins, in which the same or different polypeptide chains are normally disulfide and/or noncovalently bound to form an assembled multichain polypeptide,

the fusions herein containing the polypeptide also will be produced and employed as a multimer having substantially the same structure as the stable plasma protein precursor. These multimers will be homogeneous with respect to the polypeptide agent they comprise, or they may contain more than one polypeptide agent.

[0065] Stable plasma proteins are proteins which typically exhibit in their native environment an extended half-life in the circulation, i.e. greater than about 20 hours. Examples of suitable stable plasma proteins are immunoglobulins, albumin, lipoproteins, apolipoproteins and transferrin. The polypeptide agent typically is fused to the plasma protein, e.g. IgG at the N-terminus of the plasma protein or fragment thereof which is capable of conferring an extended half-life upon the polypeptide. Increases of greater than about 100% on the plasma half-life of the polypeptide are satisfactory. Ordinarily, the polypeptide is fused C-terminally to the N-terminus of the constant region of immunoglobulins in place of the variable region(s) thereof, however N-terminal fusions may also find use. Typically, such fusions retain at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain, which heavy chains may include IgG1, IgG2a, IgG2b, IgG3, IgG4, IgA, IgM, IgE, and IgD, usually one or a combination of proteins in the IgG class. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain. This ordinarily is accomplished by constructing the appropriate DNA sequence and expressing it in recombinant cell culture. Alternatively, the polypeptides may be synthesized according to known methods.

[0066] In some embodiments, the wnt peptide pharmacophore is modified without altering its sequence. Modifications of interest that do not alter primary sequence include chemical derivatization of polypeptides, e.g., acylation, acetylation, carboxylation, amidation, etc. Also included are modifications of glycosylation, e.g. those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps; e.g. by exposing the polypeptide to enzymes which affect glycosylation, such as mammalian glycosylating or deglycosylating enzymes. Also embraced are sequences that have phosphorylated amino acid residues, e.g. phosphotyrosine, phosphoserine, or phosphothreonine.

[0067] Wnt peptide pharmacophores for use in the subject compositions and methods may be modified using ordinary molecular biological techniques and synthetic chemistry so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a therapeutic agent. Analogs of such polypeptides include those containing residues other than naturally occurring L-amino acids, e.g. D-amino acids or non-naturally occurring synthetic amino acids. D-amino acids may be substituted for some or all of the amino acid residues.

[0068] The wnt peptide pharmacophores may be prepared by *in vitro* synthesis, using conventional methods as known in the art. Various commercial synthetic apparatuses are available, for example, automated synthesizers by Applied Biosystems, Inc., Beckman, etc. By using synthesizers, naturally occurring amino acids may be substituted with unnatural amino acids. The particular sequence and the manner of preparation will be determined by convenience, economics, purity required, and the like. If desired, various groups may be introduced into the peptide during synthesis or during expression, which allow for linking to other molecules or to a surface. Thus cysteines can be used to make thioethers, histidines for linking to a metal ion complex, carboxyl groups for forming amides or esters, amino groups for forming amides, and the like.

[0069] Alternatively, the wnt peptide pharmacophore may be prepared by recombinant DNA technology in a cellular or cell-free polypeptide synthesis system, using any one of the many systems known in the art. Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required. For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform host cells, and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced.

[0070] Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells, including without limitation plant, mammal, insect, etc. cells. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *Enterobacteriaceae* such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis*, *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. These examples are illustrative rather than limiting.

[0071] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable expression hosts. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as *K. lactis*, *K. fragilis*, etc.; *Pichia pastoris*; *Candida*; *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as *Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulan*, and *A. niger*.

[0072] Suitable host cells may also be derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher

eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

[0073] Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*. During such incubation of the plant cell culture, the DNA coding sequence is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences.

[0074] The wnt peptide pharmacophores prepared by recombinant synthesis are typically isolated and purified in accordance with conventional methods of recombinant synthesis. A lysate may be prepared of the expression host and the lysate purified using HPLC, exclusion chromatography, gel electrophoresis, affinity chromatography, or other purification technique. For the most part, the compositions which are used will comprise at least 20% by weight of the desired peptide, more usually at least about 75% by weight, preferably at least about 95% by weight, and for therapeutic purposes, usually at least about 99.5% by weight, in relation to contaminants related to the method of preparation of the product and its purification. Usually, the percentages will be based upon total protein.

[0075] Wnt peptide pharmacophores of the present invention are usually biologically active in binding to a cognate Wnt receptor. In such instances, the wnt peptide pharmacophore specifically binds to a Wnt receptor and inhibits Wnt signaling when contacting a cell expressing the receptor, usually binding to one but not both or a Wnt co-receptor pair. One may determine the biological activity of a wnt peptide pharmacophore in a composition by determining the amount of Wnt activity that is inhibited by the wnt peptide pharmacophore in a functional assay, e.g. destabilization of β -catenin, inhibition of growth of stem cells, etc., quantitating the amount of wnt peptide pharmacophore present by a non-functional assay, e.g. immunostaining, ELISA, quantitation on coomassie or silver stained gel, etc., and determining the ratio of biologically active wnt peptide pharmacophore to total wnt peptide pharmacophore. An exemplary assay for wnt peptide polypeptide specific activity involves contacting cells with a Wnt-comprising composition, e.g. culture media from cells expressing

Wnt protein, for a period of time sufficient to stabilize β -catenin, usually at least about 1 hour. A wnt peptide pharmacophore composition is then provided to the cells, and the cells are incubated, usually at least about an hour, to allow the wnt peptide pharmacophore polypeptide to competitively replace the Wnt protein. The cells are then lysed, and the cell lysate is resolved by SDS PAGE, then transferred to nitrocellulose and probed with antibodies specific for β -catenin. Other assays include C57MG transformation and induction of target genes in *Xenopus* animal cap assays. An effective dose or concentration of a wnt peptide pharmacophore polypeptide is that which will reduce signaling, for example as evidenced by the presence of nuclear β -catenin, by 25%, 50%, 75%, 90%, 95%, or more, relative to the signaling in the absence of the wnt peptide pharmacophore.

[0076] An aspect of the binding activity of wnt peptide pharmacophores is the ability to determine the receptor specificity of the full-length Wnt protein from which a wnt peptide pharmacophore is derived. Typically a wnt peptide pharmacophore used in such methods will have substantial sequence identity with the corresponding full-length Wnt protein, where the wnt peptide pharmacophore is usually identical to the corresponding full-length Wnt protein over at least a stretch of from about 10 to 20 contiguous amino acids.

[0077] In methods for determining the cognate receptor for a Wnt protein, a candidate Wnt receptor or fragment thereof is contacted with a wnt peptide pharmacophore that corresponds to a full-length Wnt protein of interest, which may be a native Wnt protein; and determining the binding of the wnt peptide pharmacophore to the candidate receptor, wherein the presence of specific binding is indicative that the full-length Wnt protein is a ligand for the candidate receptor. Receptors of interest include Fz protein, a ROR protein, or an Ryk protein, which may be contacted with a Cterm wnt peptide pharmacophore; and LRP5, LRP6, or FRL1/crypto, which may be contacted with an Nterm wnt peptide pharmacophore. Various binding assays find use, for example utilizing cells expressing the candidate receptor, but of particular interest are assays that can be performed in solution, *e.g.* utilizing a soluble fragment of the receptor for binding, including without limitation interactions between Fz-CRD polypeptides and wnt peptide pharmacophores.

[0078] In some embodiments, wnt peptide pharmacophores of the present invention are conjugated to various functional moieties such as polypeptides, drugs, radionucleotides, or toxins. In other words, the subject composition comprises a functional moiety conjugated to the wnt peptide pharmacophore polypeptide. See, *e.g.*, PCT publications WO 92/08495; WO 91/14438; WO 89/12624; U.S. Pat. No. 5,314,995; and EP 396,387.

[0079] One example of a functional moiety that may be conjugated to a wnt peptide pharmacophore is a therapeutic moiety. Therapeutic moieties include, without limitation, moieties that promote cell death, and moieties that alter cellular activity.

[0080] Examples of moieties that promote cell death include cytotoxic agents, i.e. a cytotoxin, e.g., a cytostatic or cytotoxic small molecule, a polypeptide agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, teniposide, vincristine, vinblastine, colchicine, doxorubicin, daunorubicin, dihydroxy anthracenedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologues thereof. Cytotoxic agents also include proteins, peptides, or polypeptides possessing a cytotoxic biological activity, e.g., toxins such as abrin, ricin A, pseudomonas exotoxin, cholera toxin, and diphtheria toxin. Cytotoxic agents also include radioactive metal ions, i.e. radionuclides, such as alpha-emitters, e.g. Bismuth-213, Radium-226, Lead-212, Actinium-225, and Astatine-211, and β -emitters, e.g. Iodide-131, Yttrium-90, Rhenium-188, Lutetium-177, Copper-67 and Copper-64, and macrocyclic chelators useful for conjugating radiometal ions, e.g. ^{131}In , ^{131}L , ^{131}Y , ^{131}Ho , ^{131}Sm , to polypeptides or any of those listed supra. Macrocyclic chelators can be attached to the antibody via a linker molecule, e.g. as described in Denardo et al., 1998, Clin Cancer Res. 4(10):2483-90; Peterson et al., 1999, Bioconjug. Chem. 10(4):553-7; and Zimmerman et al., 1999, Nucl. Med. Biol. 26(8):943-50, each incorporated by reference in their entireties.

[0081] Moieties that promote cell death also include moieties that target a cell for antibody-dependent cell-mediated cytotoxicity (ADCC), antibody dependent cell-mediated phagocytosis (ADCP), or complement dependent cytotoxicity (CDC, also known as complement-mediated cytolysis, or CMC), e.g. the Fc component of immunoglobulin. See, for example, Raghavan et al., 1996, Annu Rev Cell Dev Biol 12:181-220; Ghetie et al., 2000, Annu Rev Immunol 18:739-766; Ravetch et al., 2001, Annu Rev Immunol 19:275-290). To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, e.g., in a animal model such as that disclosed in Clynes et al. PNAS (USA) 95:652-656 (1998). All Fc γ Rs bind the same region on Fc, at the N-terminal end of the C γ 2 domain and the preceding hinge, which region may be utilized as a functional moiety for the purposes of the invention. An overlapping but separate site on Fc serves as the interface for the complement protein C1q. In the same way that Fc/Fc γ R binding mediates ADCC and ADCP, Fc/C1q binding mediates complement dependent cytotoxicity (CDC). A site on Fc between the C γ 2 and C γ 3 domains mediates interaction with the neonatal receptor FcRn, the binding of which recycles endocytosed antibody from the endosome back to the bloodstream.

[0082] As used herein, an Fc fusion is synonymous with the terms "immunoadhesin", "Ig fusion", "Ig chimera", and "receptor globulin" as used in the art (Chamow et al., 1996, Trends

Biotechnol 14:52-60; Ashkenazi et al., 1997, Curr Opin Immunol 9:195-200). An Fc fusion combines the Fc region of an immunoglobulin with the Cterm or Nterm wnt peptide pharmacophore, for example. See for example U.S. Pat. Nos. 5,766,883 and 5,876,969, both of which are expressly incorporated by reference.

[0083] Therapeutic moieties other than those that promote cell death would include agents that alter the activity of a cell. Such therapeutic agents include, but are not limited to, cytokines, chemokines, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thiotepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

[0084] Other functional moieties suitable for conjugation to subject wnt peptide pharmacophores of the present application include imaging moieties. As discussed above, an imaging moiety is a non-cytotoxic agent that can be used to locate and, optionally, visualize cells, e.g. cells that have been targeted by compositions of the subject application. For example, fluorescent dyes may be used as an imaging moiety. In another example, radioactive agents that are non-cytotoxic may also be an imaging moiety. An imaging moiety may require the addition of a substrate for detection, e.g. horseradish peroxidase (HRP), β -galactosidase, luciferase, and the like. Alternatively, an imaging moiety may provide a detectable signal that does not require the addition of a substrate for detection, e.g. a fluorophore or chromophore dye, e.g. Alexa Fluor 488® or Alexa Fluor 647®, or a protein that comprises a fluorophore or chromophore, e.g. GFP, RFP, dsRED, phiYFP, etc. and mutants thereof.

[0085] Functional moieties that induce multimers of two or more min-wnts are also of interest, e.g. including binding pairs having a high affinity, such as biotin and avidin/streptavidin, peptide sequences such as zipper domains, and the like, as known in the art.

[0086] Techniques for conjugating functional moieties to polypeptides are well known in the art, see, e.g., Amon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin et

al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev. 62:119-58 (1982).

[0087] Functional moieties are typically bound to the wnt peptide pharmacophore of the subject compositions by covalent interactions. In some embodiments, a linker may be used, where the linker may be any moiety that can be used to link the wnt peptide pharmacophore to the functional moiety. In some embodiments, the linker is a cleavable linker. The use of a cleavable linker enables the moiety linked to the wnt peptide pharmacophore to be released from the wnt peptide pharmacophore once absorbed by the cell, and transported to the cell body. The cleavable linker may be cleavable by a chemical agent, by an enzyme, due to a pH change, or by being exposed to energy. Examples of forms of energy that may be used include light, microwave, ultrasound, and radiofrequency.

[0088] In certain applications, it may be desirable to release the functional moiety, particularly where the moiety is a therapeutic moiety, once the compound has entered the cell, resulting in a release of the moiety. Accordingly, in one variation, the linker L is a cleavable linker. This enables the moiety M to be released from the compound once in a cell. This may be desirable when, for example, the functional moiety is a therapeutic moiety which has a greater therapeutic effect when separated from the wnt peptide pharmacophore polypeptide. For example, the therapeutic moiety may have a better ability to be absorbed by an intracellular component of the cell when separated from the wnt peptide pharmacophore polypeptide. Accordingly, it may be necessary or desirable to separate the therapeutic moiety from the wnt peptide pharmacophore so that the therapeutic moiety can enter the intracellular compartment.

Methods

[0089] In methods of the present invention, an effective amount of a composition comprising a wnt peptide pharmacophore is provided to cells, *e.g.* by contacting the cell with an effective amount of that composition to achieve a desired effect, *e.g.* to inhibit Wnt signaling, inhibit proliferation or aberrant angiogenesis, deliver a therapeutic or imaging moiety, generate an antibody, *etc.*

[0090] In some methods of the invention, an effective amount of the subject composition is provided to inhibit Wnt signaling in a cell. Biochemically speaking, an effective amount or effective dose of a Wnt inhibitor is an amount of inhibitor to decrease or attenuate Wnt signaling in a cell by at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or by 100% relative to the signaling in the absence of the wnt peptide pharmacophore. In other words, the responsiveness to Wnt signaling of a cell that has been contacted with an effective amount or effective dose of a wnt peptide pharmacophore composition will be about 70% or less, about 60% or less, about 50% or less,

about 40% or less, about 30% or less, about 20% or less, about 10% or less, about 5% or less, or will be about 0%, i.e. negligible, relative to the strength of the response to Wnt signaling that is observed of a cell that has not been contacted with an effective amount/dose of a wnt peptide pharmacophore composition. The amount of modulation of a cell's activity by Wnt, that is, the responsiveness of a cell to Wnt signaling, can be determined by a number of ways known to one of ordinary skill in the art of Wnt biology. For example, the amount of phosphorylated β -catenin in a cell may be measured; the amount of cytosolic β -catenin in a cell may be measured; or the amount of activity of the transcription factors that are normally activated by Wnt signaling, e.g. TCF/LEF, may be measured, for example by measuring the RNA or protein levels of genes that are the transcriptional targets of TCF/LEF, or by transfecting/infecting the cell with a nucleic acid vector comprising a TCF binding site (TOP) operably linked to a reporter protein such as luciferase (TOPFlash), EGFP (TOP-EGFP), etc. and qualitatively or quantitatively measuring the amount of reporter protein that is produced. In this way, the antagonistic effect of the agent may be confirmed.

[0091] In a clinical sense, an effective dose of a wnt peptide pharmacophore composition is the dose that, when administered for a suitable period of time, usually at least about one week, and maybe about two weeks, or more, up to a period of about 4 weeks, 8 weeks, or longer will evidence an alteration in the symptoms associated with undesired Wnt signaling. For example, an effective dose is the dose that when administered for a suitable period of time, usually at least about one week, and may be about two weeks, or more, up to a period of about 4 weeks, 8 weeks, or longer will slow or even halt tumor growth in a patient suffering from cancer or neovascularization in the eye of a patient suffering from diabetic retinopathy. In some embodiments, an effective dose may not only slow or halt the progression of the disease condition but may also induce the reversal of the condition. It will be understood by those of skill in the art that an initial dose may be administered for such periods of time, followed by maintenance doses, which, in some cases, will be at a reduced dosage.

[0092] In some embodiments, an effective amount of the subject composition is provided to deliver a functional moiety to a cell, e.g. a therapeutic moiety or an imaging moiety. In such embodiments, an effective amount will be the amount required to achieve therapeutic or imaging efficacy by the functional moiety.

[0093] For example, in some embodiments, the functional moiety is a therapeutic moiety that is cytotoxic. An effective amount of a composition comprising a cytotoxic moiety will be the amount sufficient to promote cell death selectively in the cells targeted by the wnt peptide pharmacophore composition to which the cytotoxic moiety is fused. In some instances, the effect amount of functional moiety is well known; e.g. radionuclides are typically delivered in the range of 10-30 cGy/h, the regimen depending on the half-life of the radioisotope. In other instances, the effective amount can be readily determined by one of ordinary skill in the art

using any convenient method known in the art for assaying for cell death, e.g. TUNEL staining, Annexin staining, propidium iodide uptake, etc. It will be understood by those of skill in the art that an initial dose may be administered for such periods of time, followed by maintenance doses, which, in some cases, will be at a reduced dosage.

[0094] As another example, in some embodiments, the functional moiety is a therapeutic moiety that targets a cell for ADCC or CDC. An effective amount of a composition comprising a moiety that targets a cell for ADCC or CDC will be the amount sufficient to promote ADCC or CDC selectively in the cells targeted by the wnt peptide pharmacophore composition to which the cytotoxic moiety is fused. The effective amount can be readily determined by one of ordinary skill in the art using any convenient method known in the art for assaying ADCC and CDC.

[0095] As another example, in some embodiments, the functional moiety is an imaging moiety. The effective amount of a subject composition comprising an imaging moiety is the amount sufficient to selectively label the cells targeted by the wnt peptide pharmacophore composition to which the cytotoxic moiety is fused. The effective amount can be readily determined by one of ordinary skill in the art using any convenient method known in the art for visualizing imaging moieties, e.g. microscopy, e.g. epifluorescence or light microscopy.

[0096] The calculation of the effective amount or effective dose of wnt peptide pharmacophore composition to be administered is within the skill of one of ordinary skill in the art, and will be routine to those persons skilled in the art. Needless to say, the final amount to be administered will be dependent upon the route of administration and upon the nature of the disorder or condition that is to be treated.

[0097] Cells suitable for use in the subject methods are cells that comprise one or more Wnt receptors. As discussed above, Wnt receptors include Fz proteins, ROR proteins, Ryk, LRP5, LRP6 and EGF-CFC proteins. In some embodiments, the cell is a cell expressing a Wnt receptor comprising a CRD domain or a WIF domain. In such embodiments, the composition used in the method comprises a wnt peptide pharmacophore polypeptide that is a Cterm wnt peptide pharmacophore. Examples of Wnt receptors that comprise a CRD domain include Fz proteins and ROR transmembrane kinases. Examples of Wnt receptors that comprise a WIF domain include Derailed/Ryk. In some embodiments the cell is a cell expressing a Wnt receptor that is LRP5, LRP6, or cryptot. In such embodiments, the composition used in the method comprises a wnt peptide pharmacophore polypeptide that is an Nterm wnt peptide pharmacophore.

[0098] The cells to be contacted may be *in vitro*, that is, in culture, or they may be *in vivo*, that is, in a subject. Cells may be from/in any organism, but are preferably from a mammal, including humans, domestic and farm animals, and zoo, laboratory or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, rats, mice, frogs, zebrafish, fruit fly,

worm, etc. Preferably, the mammal is human. Cells may be from any tissue. Cells may be frozen, or they may be fresh. They may be primary cells, or they may be cell lines. More usually, they are primary cells *in vivo*.

[0099] Cells of particular interest are those that are responsive to Wnt signaling and are associated with undesirable or otherwise aberrant cell proliferation, .e.g tumorigenesis, angiogenesis, *etc.* particularly as they may relate to Wnt-mediated disease conditions described below. As an example, cells of interest include endothelial cells, which are the cells that line the interior surface of blood vessels, and which, when aberrantly active, may be associated with aberrant angiogenesis. As another example, cells of interest include cancer cells, e.g. tumor cells, e.g. a cancer stem cell, which is a type of cancer cell that possesses characteristics associated with normal stem cells, namely the ability to give rise to all cell types found in a particular cancer sample, and which is associated with aberrant cell proliferation.

[00100] Cells *in vitro* may be contacted with a composition comprising a wnt peptide pharmacophore by any of a number of well-known methods in the art. For example, the composition may be provided to the cells in the media in which the subject cells are being cultured. Nucleic acids encoding the wnt peptide pharmacophore may be provided to the subject cells or to cells cocultured with the subject cells on vectors under conditions that are well known in the art for promoting their uptake, for example electroporation, calcium chloride transfection, and lipofection. Alternatively, nucleic acids encoding the Wnt inhibitor may be provided to the subject cells or to cells cocultured with the subject cells via a virus, i.e. the cells are contacted with viral particles comprising nucleic acids encoding the wnt peptide pharmacophore polypeptide. Retroviruses, for example, lentiviruses, are particularly suitable to the method of the invention, as they can be used to transfect non-dividing cells (see, for example, Uchida *et al.* (1998) P.N.A.S. 95(20):11939-44). Commonly used retroviral vectors are "defective", i.e. unable to produce viral proteins required for productive infection. Rather, replication of the vector requires growth in a packaging cell line.

[00101] Likewise, cells *in vivo* may be contacted with the subject wnt peptide pharmacophore compositions by any of a number of well-known methods in the art for the administration of peptides or nucleic acids to a subject. The wnt peptide pharmacophore composition can be incorporated into a variety of formulations, which in some embodiments and particularly for Cterm wnt peptide pharmacophores will be formulated in the absence of detergents, liposomes, *etc.*, as have been described for the formulation of full-length Wnt proteins. More particularly, the compounds of the present invention can be formulated into pharmaceutical compositions by combination with appropriate pharmaceutically acceptable carriers or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections,

inhalants, gels, microspheres, and aerosols. As such, administration of the wnt peptide pharmacophore composition can be achieved in various ways, including oral, buccal, rectal, parenteral, intraperitoneal, intradermal, transdermal, intracheal, etc., administration. The active agent may be systemic after administration or may be localized by the use of regional administration, intramural administration, or use of an implant that acts to retain the active dose at the site of implantation. The active agent may be formulated for immediate activity or it may be formulated for sustained release.

[00102] For some conditions, particularly central nervous system conditions, it may be necessary to formulate agents to cross the blood brain barrier (BBB). One strategy for drug delivery through the blood brain barrier (BBB) entails disruption of the BBB, either by osmotic means such as mannitol or leukotrienes, or biochemically by the use of vasoactive substances such as bradykinin. The potential for using BBB opening to target specific agents to brain tumors is also an option. A BBB disrupting agent can be co-administered with the therapeutic compositions of the invention when the compositions are administered by intravascular injection. Other strategies to go through the BBB may entail the use of endogenous transport systems, including caveol-1 mediated transcytosis, carrier-mediated transporters such as glucose and amino acid carriers, receptor-mediated transcytosis for insulin or transferrin, and active efflux transporters such as p-glycoprotein. Active transport moieties may also be conjugated to the therapeutic compounds for use in the invention to facilitate transport across the endothelial wall of the blood vessel. Alternatively, drug delivery of therapeutics agents behind the BBB may be by local delivery, for example by intrathecal delivery, e.g. through an Ommaya reservoir (see e.g. US Patent Nos. 5,222,982 and 5,385,582, incorporated herein by reference); by bolus injection, e.g. by a syringe, e.g. intravitreally or intracranially; by continuous infusion, e.g. by cannulation, e.g. with convection (see e.g. US Application No. 20070254842, incorporated here by reference); or by implanting a device upon which the agent has been reversibly affixed (see e.g. US Application Nos. 20080081064 and 20090196903, incorporated herein by reference).

[00103] Therapeutic uses. As alluded to above, wnt peptide pharmacophore compositions of the present invention find use in inhibiting Wnt signaling in a cell that is responsive to Wnt signaling. The responsiveness of a cell to a Wnt may be readily determined by one of ordinary skill in the art by methods known in the art and set forth herein. Biologically active wnt peptide pharmacophore compositions will inhibit, i.e. antagonize or suppress, Wnt signaling in a cell. Put another way, biologically active wnt peptide pharmacophore compositions are dominant negative regulators of Wnt signaling.

[00104] Dominant negative regulators of Wnt signaling such as the wnt peptide pharmacophore compositions of the present invention find use in the treatment of mammals,

such as human patients, suffering from Wnt-mediated disease conditions such as disorders associated with aberrant cell proliferation or aberrant angiogenesis, including various cancers associated with Wnt. Patients suffering from diseases characterized by such conditions will benefit greatly by a treatment protocol of the pending claimed invention.

[00105] The term "cancer" refers to the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Examples of cancer include, but are not limited to: carcinoma, lymphoma, blastoma, and leukemia. More particular examples of cancers include, but are not limited to: colorectal cancer, chronic lymphocytic leukemia (CLL), lung, including non small cell (NSCLC), breast, ovarian, cervical, endometrial, prostate, colorectal, intestinal carcinoid, bladder, gastric, pancreatic, hepatic (hepatocellular), hepatoblastoma, esophageal, pulmonary adenocarcinoma, mesothelioma, synovial sarcoma, osteosarcoma, head and neck squamous cell carcinoma, juvenile nasopharyngeal angiofibromas, liposarcoma, thyroid, melanoma, basal cell carcinoma (BCC), medulloblastoma and desmoid. Cancers of interest for treatment by the subject methods include gliomas, medulloblastomas, colon cancer, colorectal cancer, melanoma, breast cancer, lung cancer, liver cancer, and gastric cancer.

[00106] A composition comprising a wnt peptide pharmacophore that inhibits the growth of a tumor is one which results in measurable reduction in the rate of proliferation of cancer cells *in vitro* or growth inhibition of a tumor *in vivo*. For example, preferred growth inhibitory Wnt antagonists will inhibit growth of tumor by at least about 5%, at least about 10%, at least about 20%, preferably from about 20% to about 50%, and even more preferably, by greater than 50% (e.g., from about 50% to about 100%) as compared to the appropriate control, the control typically being cancer cells not treated with the Wnt antagonist molecule being tested. The Wnt antagonist is growth inhibitory *in vivo* if administration of the Wnt antagonist at about 1 µg/kg to about 100 mg/kg body weight results in reduction in tumor size or cell proliferation within about 5 days to 3 months from the first administration, preferably within about 5 to 30 days.

[00107] As another example, compositions and methods of the present invention find use in inhibiting aberrant angiogenesis in a CNS cell. The term "angiogenesis" is used to describe the biological process by which new blood vessels grow or sprout from pre-existing vessels. Angiogenesis plays a critical role in the elaboration of vasculature both during embryogenesis and in the mature organism, for example, in wound healing. However, there are many disease states that are driven by persistent unregulated or improperly regulated angiogenesis. In such disease states, this aberrant angiogenesis may either cause a particular disease or exacerbate an existing pathological condition. For example, choroidal neovascularization (CNV) in the eye and the subsequent retinopathy has been implicated as the most common cause of blindness and underlies the pathology of a number of ocular diseases, most notably

diabetic retinopathy and age related macular degeneration (AMD, ARMD), particularly wet/exudative age related macular degeneration. Wnt signaling has been implicated in promoting angiogenesis in the CNS and retina during development. Accordingly, Wnt inhibitors find use in treating--i.e. arresting the development or progression of--disease conditions of the CNS wherein aberrant angiogenesis is a contributing factor.

[00108] A wnt peptide pharmacophore composition that inhibits aberrant angiogenesis in the CNS or that inhibits neovascularization in the CNS is one which results in measurable inhibition of the development of new vasculature, for example tube formation by endothelial cells in culture or blood vessel formation in a subject. Preferred Wnt antagonists inhibit the rate of development of new vasculature by at least about 10%, at least about 20%, preferably from about 20% to about 50%, and even more preferably, by greater than 50% (e.g., from about 50% to about 100%) as compared to the appropriate control, the control typically being cells not treated with the Wnt antagonist molecule being tested. The Wnt antagonist is inhibitory *in vivo* if administration of the Wnt antagonist at about 1 µg/kg to about 100 mg/kg body weight results in a slowing or cessation of the development of neovasculature within about 5 days to 6 months from the first administration of the Wnt inhibitor, preferably within about 5 days to about 2 months. Neovasculature development may be observed by a number of ways that are well-known in the art and that will be obvious to the ordinary skilled artisan. For example, the inhibition of choroidal neovascularization may be readily observed directly by fundus photography, or indirectly by assaying for improved scoring on visual acuity tests.

[00109] Furthermore, other disorders are associated with aberrant Wnt signaling, including but not limited to osteoporosis, osteoarthritis, polycystic kidney disease, diabetes, schizophrenia, vascular disease, cardiac disease, non-oncogenic proliferative diseases, and neurodegenerative diseases such as Alzheimer's disease.

[00110] As an alternative to or in addition to inhibiting Wnt signaling, wnt peptide pharmacophore compositions may be used to deliver therapeutic moieties as discussed previously for the treatment of any of the aforementioned diseases. For example, wnt peptide pharmacophore compositions may be used to deliver cytotoxic moieties to tumorigenic cells, or to tag tumorigenic cells with Fc moieties that will target the cell for ADCC or CDC-mediated cell death. As another example, wnt peptide pharmacophore compositions may be used to delivery cytokines that promote synapse formation to neurons in neurodegenerative states, or axon outgrowth at sites of CNS injury. These and other therapeutic applications for the subject wnt peptide pharmacophore compositions will be readily apparent to the ordinarily skilled artisan.

[00111] For inclusion in a medicament, wnt peptide pharmacophores may be obtained using generally accepted manufacturing methods. As a general proposition, the total

pharmaceutically effective amount of the Wnt inhibitor compound administered parenterally per dose will be in a range that can be measured by a dose response curve.

[00112] Pharmaceutical compositions can include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, buffered water, physiological saline, PBS, Ringer's solution, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation can include other carriers, adjuvants, or non-toxic, nontherapeutic, nonimmunogenic stabilizers, excipients and the like. The compositions can also include additional substances to approximate physiological conditions, such as pH adjusting and buffering agents, toxicity adjusting agents, wetting agents and detergents.

[00113] The composition can also include any of a variety of stabilizing agents, such as an antioxidant for example. When the pharmaceutical composition includes a polypeptide, the polypeptide can be complexed with various well-known compounds that enhance the *in vivo* stability of the polypeptide, or otherwise enhance its pharmacological properties (e.g., increase the half-life of the polypeptide, reduce its toxicity, enhance solubility or uptake). Examples of such modifications or complexing agents include sulfate, gluconate, citrate and phosphate. The polypeptides of a composition can also be complexed with molecules that enhance their *in vivo* attributes. Such molecules include, for example, carbohydrates, polyamines, amino acids, other peptides, ions (e.g., sodium, potassium, calcium, magnesium, manganese), and lipids.

[00114] Further guidance regarding formulations that are suitable for various types of administration can be found in Remington's Pharmaceutical Sciences, Mace Publishing Company, Philadelphia, Pa., 17th ed. (1985). For a brief review of methods for drug delivery, see, Langer, Science 249:1527-1533 (1990).

[00115] The pharmaceutical compositions can be administered for prophylactic and/or therapeutic treatments. Toxicity and therapeutic efficacy of the active ingredient can be determined according to standard pharmaceutical procedures in cell cultures and/or experimental animals, including, for example, determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds that exhibit large therapeutic indices are preferred.

[00116] The data obtained from cell culture and/or animal studies can be used in formulating a range of dosages for humans. The dosage of the active ingredient typically lines within a range of circulating concentrations that include the ED₅₀ with low toxicity. The dosage can

vary within this range depending upon the dosage form employed and the route of administration utilized.

[00117] The components used to formulate the pharmaceutical compositions are preferably of high purity and are substantially free of potentially harmful contaminants (e.g., at least National Food (NF) grade, generally at least analytical grade, and more typically at least pharmaceutical grade). Moreover, compositions intended for *in vivo* use are usually sterile. To the extent that a given compound must be synthesized prior to use, the resulting product is typically substantially free of any potentially toxic agents, particularly any endotoxins, which may be present during the synthesis or purification process. Compositions for parental administration are also sterile, substantially isotonic and made under GMP conditions.

[00118] The methods of the present invention also find use in combined therapies. For example, a number of agents may be useful in the treatment of aberrant angiogenesis, e.g. angiostatin, endostatin, VEGF inhibitors, *etc.* Likewise, a number of agents may be useful in the treatment of cancer, e.g. chemotherapeutic agents, radiotherapy, *etc.* The combined use of wnt peptide pharmacophores of the present invention and these other agents may have the advantages that the required dosages for the individual drugs is lower, and the effect of the different drugs complementary.

[00119] Delivery of imaging moieties. As alluded to above, wnt peptide pharmacophore compositions of the present invention also find use in delivering imaging moieties to cells expressing Wnt receptors. For example, wnt peptide pharmacophore compositions conjugated to fluorescent moieties may be used to label and track cells *in vitro* and *in vivo*, e.g. for research purposes.

[00120] Antibody Generation. Wnt peptide pharmacophore compositions of the present invention may also be used to generate antibodies. Antibodies may be generated by any suitable method known in the art. The antibodies of the present invention may be polyclonal or monoclonal antibodies. They may be monovalent, bivalent, or multivalent. They may be fragments, e.g. F(ab) fragments, chimeric, humanized, human, *etc.* as known in the art. Methods of preparing antibodies are known to the skilled artisan (Harlow, et al., *Antibodies: a Laboratory Manual*, (Cold spring Harbor Laboratory Press, 2nd ed. (1988), which is hereby incorporated herein by reference in its entirety).

[00121] In generating antibodies of the present invention, the subject composition comprising a wnt peptide pharmacophore is formulated for injection, e.g. with an adjuvant, and the resulting immunogen is used to immunize animals. The immunogen composition may, when beneficial, be produced as a fusion protein in which the wnt peptide pharmacophore is attached to a fusion segment. The fusion segment often aids in protein purification, e.g., by permitting the fusion protein to be isolated and purified by affinity chromatography. Fusion proteins can be produced by culturing a recombinant cell transformed with a fusion nucleic acid sequence that

encodes a protein including the fusion segment attached to either the carboxyl and/or amino terminal end of the protein. Fusion segments may include, but are not limited to, immunoglobulin Fc regions, glutathione-S-transferase, β -galactosidase, a poly-histidine segment capable of binding to a divalent metal ion, and maltose binding protein.

[00122] Candidate wnt peptide pharmacophore antibodies may be tested by enzyme linked immunosorbent assay (ELISA), Western immunoblotting, or other immunochemical techniques to confirm their affinity and specificity for the target Wnt. Assays performed to characterize the individual antibodies include, but are not limited to (1) inhibition of Wnt-autocrine proliferation of cancer stem cells; and (2) inhibition of Wnt-induced TCF-LEF-induced gene expression.

[00123] Antibodies of the present invention may also be described or specified in terms of their cross-reactivity. Antibodies that bind wnt peptide pharmacophore polypeptides, which have at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, and at least 50% identity (as calculated using methods known in the art and described herein) to human Wnts are also included in the present invention. Thus, antibodies of the present invention bind to the Wnt domain of the native parent Wnt protein from which the wnt peptide pharmacophore was derived and inhibit the activation of the receptor complexes that are normally bound by that Wnt.

[00124] Preferred binding affinities include those with an equilibrium dissociation constant or K_D from 10^{-8} to 10^{-15} M. The invention also provides antibodies that competitively inhibit binding of an antibody to an epitope of the invention as determined by any method known in the art for determining competitive binding, for example, the immunoassays described herein. In preferred embodiments, the antibody competitively inhibits binding to the epitope by at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50%.

EXAMPLES

[00125] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1

Structural basis of Wnt recognition by Frizzled

[00126] 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, that extends two 'fingers' 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 knob-in-holes amino acid contacts with a depression on the opposite side of the Fz8-CRD. The structural distribution of variable versus conserved amino acids in both interfaces appears to facilitate ligand-receptor cross-reactivity, which has important implications for understanding Wnt/Fz signaling specificity, and the development of drugs for cancer and regenerative medicine.

Results:

[00127] We screened a variety of vertebrate and invertebrate Wnts for expression, biochemical behavior, and binding to different Frizzled CRD in order to identify a crystallizable complex. These studies converged on *Xenopus* Wnt8, which expressed at high levels and could be purified as a complex with several Fz-CRD, including Fz4, Fz5 and Fz8. Focusing the structural studies on XWnt8 also offered the advantage that it has served as a model system to study Wnt/Fz interactions since it binds to and activates mammalian Fz. Initial attempts to purify XWnt8 in detergents, followed by incubation with Fz8 or Fz5-CRD did not result in efficient complex formation. 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, from conditioned media of transfected S2 cells using protein-A (Figs. 1A, B). The lack of detergent requirement of the Wnt/Fz complex compared to Wnt alone suggested that binding to the Fz-CRD was apparently shielding the lipid group(s) on Wnt from solvent. The XWnt8/Fz-CRD complex eluted from gel filtration as several inter-converting peaks with MW ranging from ~50kDa to ~200kDa (Fig. 1B).

[00128] We crystallized the fully-glycosylated XWnt8/Fz8-CRD complex in detergent-free buffers and obtained a native x-ray data set to a resolution of 3.25Å (Table 2). We determined experimental phases for the structure using isomorphous and anomalous scattering difference methodologies with crystals derived from material expressed in S2 cells supplemented with Selenomethionine (Table 2). 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. 7). 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. 7) and the lipid group.

[00129] 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 can be separated into 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 of which forms a distinct site of interaction with the Fz8-CRD (Figs. 2A-B). 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. While clearly distinct structural sub-domains that appear to associate only through a small interaction patch between the AB-loop of the NTD and a small helix (helix F) in the CTD, the NTD and CTD are intimately associated, as they remain non-covalently associated after protease cleavage of the inter-domain linker XWnt8. We were curious if conservation analysis of Wnt sequences, now in the context of the XWnt8 structure, might give clues as to the locations of potential co-receptor binding sites (e.g. Lrp5/6, Ryk). We analyzed XWnt8 sequence alignments with all 19 mammalian Wnts, which revealed a large patch of conserved residues that mapped to the top surface of the XWnt8 NTD, potentially representing an interaction site (Figs. 8, 9) (see Discussion). 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, 7). Asn263 forms extensive intramolecular interactions with surrounding amino acids on the Wnt surface, possibly serving a stabilizing role in the structure, consistent with the fact that mutation of Asn263 resulted in loss of XWnt8 expression and we could not produce a deglycosylated XWnt8.

[00130] The functional role for lipid modification of Wnts is unknown, but it is necessary for full biological activity. The structure presented here clearly implicates XWnt8 acylation directly in Fz8-CRD binding (Fig. 2A, Fig. 3). In binding site 1, a $15\text{\AA}$ long tube of continuous electron density is connected to the hydroxyl group of Ser-187 (Fig. 3A), which resides at the tip of the XWnt8 thumb that is formed by a long interhelical EF-loop 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 $600\text{\AA}^2$ of total surface area ($330\text{\AA}^2$ from the lipid, $265\text{\AA}^2$ 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 are not showing ordered electron density. Wnt acylation has been reported to be either an unsaturated palmitic acid, or a monounsaturated palmitoleic acid. We could not unambiguously determine the chemical identity of the lipid on XWnt8 using mass spectrometry, but the shape of the electron density (Fig. 3A) for the lipid is not acutely bent, implying a minor degree of unsaturation. More directly, 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. 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, 9), and so cannot serve as a lipid addition site. Therefore, the conserved Ser at this position in all Wnts (corresponding to Ser187 in XWnt8) appears to be the consensus acylation site.

[00131] The site 1 interaction is mainly comprised of the lipid on Ser187 penetrating deeply into a hydrophobic groove between helix A, helix D and the DE-loop of the Fz8CRD (Fig. 3B, Table 3). This CRD groove is lined with hydrophobic amino acids including Phe72 and Leu75 on helix A, and Met122, Tyr125 and Phe127 on helix D and the DE-loop that form extensive van der Waals interactions with the lipid (Fig. 3B, Table 3). 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, 9). Perhaps 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 to the CRD would appear to be the hydrophobic effect combined with the shape complementarity of the lipid-CRD interface, where the lipid and the 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 (discussed below).

[00132] 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 $\sim 585\text{\AA}^2$ 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 significantly 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 present at this corresponding position in all Wnts, and Glu or Asp are present at the corresponding position as Glu64 in 8 of 10 mammalian Fz-CRD, suggesting this interaction is mostly conserved (Table 3, Figs. 8 & 9). 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. Despite the buried surface between the 'non-lipid' protein components of site 1, the shape complementarity of the thumb loop with the Fz8-CRD is poor ($Sc = 0.54$). 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.

[00133] The structural disposition of the lipid attachment site, which resides at the apex of the extended Wnt thumb rather than the core of the protein (Fig. 2C), has several important implications. First, from the structure it suggests the possibility 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, and recruiting back the weakened site 1 binding for Fz 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 or shielding from aqueous solvent during expression and transport, such as with carrier proteins. In keeping with 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. The XWnt8 structure supports this idea in that the lipid is presented in a highly accessible manner (Fig. 2C), in an ideal position for anchoring Wnt to the plasma membrane.

[00134] The site 2 interaction is on the opposite side of the Fz8-CRD as 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. 3A, B, Table S2). The site 2 interface buries a total of $\sim 900\text{\AA}^2$ ($\sim 440\text{\AA}^2$ CRD, $\sim 450\text{\AA}^2$ XWnt8) and despite the "knob-in-hole" binding mode (Fig. 3A), exhibits poor overall shape complementarity ($Sc = 0.58$). 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. 8). In the Fz8-CRD Tyr48, Phe86, and Cys148, are conserved Fz-CRD residues that form side-chain specific van der Waals interactions with XWnt8 (Fig. 9). As for site 1, the picture that emerges is that both Wnt and Fz contact residues are highly chemically conserved as apolar amino acids, implying this will be an interaction mode common to all Wnt/Fz-CRD complexes (Figs. 3C, 8). Importantly, several Fz8-CRD contacts are substituted in other Fz-CRD that might contribute to Wnt sub-type preferences, such as Ile95, Leu97, Tyr100, and Met149. Met149 resides at the center of site 2 and is conserved in 5 of 10 mammalian Fz-CRD, but is substituted to Val or Asp in Fz1, 2, 3, 6 and 7. These substitutions might contribute to Wnt/Fz subtype specificity.

[00135] Given the two-site Wnt/Fz binding mode seen in the complex, a distinction of the relative binding strengths and specificities of site 1 versus 2 is important for clarifying Wnt/Fz specificity, and also for Wnt engineering and drug design. Experimentally measuring these parameters is complicated by several factors. First, site 1 is dominated by the acylation of Ser187, and numerous studies have shown that acylation is necessary for Wnt secretion and activity. We were unable to express sufficient quantities of “wild-type” versions of XWnt8 with Ser187 mutated to Ala so as to prevent acylation, complicating a fine structure-function analysis of the energetic landscape of site 1. Second, there are few published structure-function studies of Wnts due to their general intolerance to mutation. As a result, the vast majority of such studies have, instead, focused on mutation of the Fz-CRD. Nevertheless, our experimental efforts to use *in vitro* evolution to create water-soluble, non-lipidated Wnts resulted in structure-function information that is highly informative for understanding the relative importance of site 1 versus site 2 in Fz-CRD binding (Fig. 5).

[00136] We attempted to display XWnt8 on yeast and, through the creation of error-prone libraries, to discover mutations that would compensate for the loss of lipid modification and express a folded, water-soluble Wnt molecule that retained Fz-CRD binding. In so doing, we discovered a small truncation fragment, comprising the C-terminal 90 amino acids of XWnt8, which we termed “wnt peptide pharmacophore,” that retained Fz8-CRD binding activity and was water-soluble since it did not contain the Ser-187 acylation site (Fig. 5A). Yeast-displayed wnt peptide pharmacophore was stained by FACS using 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, suggesting some degree of ligand-receptor discrimination mediated by site 2. We produced wnt peptide pharmacophore in a recombinant form expressed from insect cells, and measured a K_D of ~2.4 μ M by SPR for Fz8-CRD, and K_D of ~3.5 μ M for Fz5-CRD using surface plasmon resonance (Fig. 5B). From our structure, we see that mini-XWnt8 comprises most of the site 2 binding index finger, and

so engages the Fz-CRD through single point attachment (Fig. 5A). From these studies we can conclude that site 2 alone represents a low affinity interaction site and exhibits cross-reactivity in that it binds to three different Fz, but also has clear differences in binding affinity for different Fz, which demonstrates that site 2 is not entirely degenerate. We surmise that, collectively, site 1 and site 2 combine to manifest as high affinity for Wnts through two point attachment, but that either site alone may be insufficient for an efficient interaction. It is noteworthy that in the XWnt8 structure, the distal tips of the site 1 and site 2 binding loops are near one another (Fig. 2C), providing an entropic advantage for two-site binding, and ensuring that both fingers can simultaneously 'grasp' the Fz8-CRD. Even though the loops are extended, they are highly constrained by disulfide bonds, so we predict that they would not require large conformational changes to engage the CRD.

[00137] The potential combinatorial complexity of 19 mammalian Wnts engaging 10 Fz raises the important question of whether a unique Wnt/Fz ligand-receptor matching code exists. It is currently unclear to what degree cross-reactive, or specific Wnt/Fz pairs contribute to distinct biological, and disease-related functions. The XWnt8/Fz8-CRD complex structure is informative with respect to this issue. From inspection of the chemistry and sequence conservation of the site 1 and site 2 interfaces, several conclusions emerge (Figs. 3C, 4C, 8, 9). First, the majority of Wnt/Fz-CRD contacts in both site 1 and site 2, including the lipid interactions, involve either strictly conserved, or chemically conserved residues across other Wnts and Fz. Thus, we conclude from the structures that the site1/site 2 Wnt/Fz interaction chemistry is incompatible with mono-specificity. Clearly, however, some studies, including ours here with wnt peptide pharmacophore (Fig. 5), have shown that Wnts and Fz are not completely degenerate. Rather, we propose that most Wnts would have the capacity to engage multiple, but not all, Fz-CRD through poly-specificity. Receptor and ligand-specific amino acid substitutions in the site 1 and site 2 interfaces may impart functional preferences, through higher and lower affinity, for some Wnt/Fz interactions over others such that each Wnt can engage a group of Fz preferentially. For example, since site 1 is primarily mediated through interaction of the monomorphic lipid with the CRD, it would appear that there is limited capacity for Wnt/Fz specificity to be focused on the lipid contacts. But a possible means of specificity tuning in site 1 may be through substitutions of residues on the Fz-CRD that contact the conserved Lysine (Lys182) on XWnt8. However, this would also appear limited given that 8 of 10 Fz have negatively charged residues (Asp or Glu) in the position that contacts this Lysine. Therefore, for site 1, we predict that the Wnt lipid will have the capacity to engage most, if not all, Fz-CRD. Site 2 is most likely where Wnt/Fz specificity, if any, could be manifested given that this interface is mediated by amino acids, not a chemically-invariant post-translational modification. Nevertheless, the majority of the contacts in site 2 are strictly or chemically conserved, with a minority of instances showing highly divergent Fz-specific

side chain substitutions. Given the apparent low affinity of site 2 we measured by surface plasmon resonance (Fig. 5), we speculate that this interface could play a role in qualitative discrimination of 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.

[00138] Why, then, are there 19 different forms of mammalian Wnts and 10 different Fz-CRD if there is not a unique ligand-receptor matching code? 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. These 'group' preferences may collectively serve to 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, and whose relative concentrations vary within the extracellular milieu. Instead of a specific Wnt engaging a specific Fz in a binary fashion to elicit a unique biological response, perhaps the collective balance of the cross-reactive signaling events 'tunes' the biological effects. With the structure of a Wnt/Fz-CRD pair now in hand, it is feasible to attempt to engineer Wnts that are mono-specific Fz binders in order to directly probe the role of Wnt/Fz specificity in function.

[00139] 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. There is currently no structure-function data implicating specific regions of Wnt in Lrp5/6, Ror2, or Ryk binding, but it is known that Lrp6 contains two different modules of β -propeller domains that appear to engage different subsets of Wnts. 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 7). 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. 7). This patch is comprised of 14 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. A complex structure with Wnt will be needed to definitively ascertain the binding mode.

[00140] With respect to Wnt pleiotropy versus signaling specificity, perhaps in the case of canonical signaling, variable Wnt affinities for the different Lrp6 binding modules may interplay with different Wnt affinities for Fz to generate specific biological responses. However, this

would not explain differences in non-canonical Fz signaling, that is apparently independent of Lrp5/6, although the composition of non-canonical signaling complexes remains an evolving issue. Here the activity differences may be solely reliant on small differences in Wnt/Fz affinity or recruitment of as yet undetermined co-receptors, potentially Ror2 or Ryk.

[00141] What does the structure of the XWnt8/Fz8-CRD complex tell us about Fz receptor activation? Since the Fz8-CRD represents only the Wnt-binding ectodomain, we cannot definitively assert how Wnt/Fz-CRD complex formation results in full-length Fz transmembrane receptor activation. There are no structures of full-length complexes of GPCR containing large N-terminal ligand binding domains analogous to Fz, such as some class A (LH/FSH/TSH) and class C (GABA, Glutamate receptors) GPCR, and it is still not clear if Fz operate under similar mechanistic principles as GPCR. However, it is understood that class C GPCR are obligate dimers. By analogy, mechanistic possibilities for Wnt-induced receptor activation include Fz oligomerization, and/or conformational changes in the Fz transmembrane helices initiated by the Wnt/CRD binding, with the composite module docking down on the Fz exofacial loops. To date there is no evidence that the latter interaction occurs, but the Wnt/Fz-CRD binary complex now enables investigation of this possibility through structure-function studies. Although a definitive mechanism will likely require a structure of the full-length complex, several important clues exist that allow us to make some speculations based on the Wnt/Fz-CRD structure reported here.

[00142] Given current uncertainty as to the precise compositions of Fz signaling complexes in canonical and non-canonical Wnt signaling, with regards to inclusion of co- or alternative Wnt receptors such as Lrp5/6, Ror2, and Ryk, it is difficult to speculate on oligomerization-based signaling models. However, 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, 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, which is a mechanism used by the majority of transmembrane receptors to initiate signaling. 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), 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, we speculate that Wnt may activate Fz in part through receptor dimerization, or oligomerization. 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, as also seen in some TK receptors such as erbB/EGF-R, or GDNF/RET.

[00143] 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. 6). 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. 6A, B). The physiological relevance of pseudo-site 3 is uncertain since this complex was crystallized at pH 4.5, but it is the largest interaction surface in the crystal (Fig. 6A), burying a total of $\sim 1450\text{\AA}^2$ of surface area, and it also serves to shield $185\text{\AA}^2$ of remaining lipid surface remaining exposed from site 1 (Fig. 6B). In this interface, the exposed distal tip of the lipid from one Wnt, including the 2 carbon atoms not seen in the electron density, resides within a hydrophobic depression between Wnt's A, B, and D helices of the NTD, resulting in excellent shape complementarity (Fig. 6B). Interestingly, while the residues on the Fz8-CRD contacting XWnt8 in pseudo-site 3 are mostly conserved, the residues on XWnt8 in the pseudo-site 3 interface are less conserved amongst Wnts. This raises the possibility that oligomerization mediated by pseudo-site 3 would be more ligand-specific than sites 1 or 2. The asymmetric nature of pseudo-site 3 in the crystal lattice generates repeating units of self-associating binary complexes (Fig. 6C). It is intriguing to speculate whether these asymmetrically-linked Wnt/Fz complexes represent the higher order clusters we see in gel filtration chromatography to initiate Fz signaling. The water-solubility of the recombinant XWnt8/Fz8-CRD complex, despite the exposed lipid surface at site 1, does suggest additional interactions are shielding the lipid from aqueous solvent. Nevertheless, a large complementary interface and evidence of self-association in solution does not prove functional relevance of pseudo-site 3. Unfortunately, mutational analysis of pseudo-site 3 is complicated by the fact that most of the Fz8-CRD residues important for binding to the lipid group in site 1 of the binary complex, are also engaged in the site 3 *trans* contact with a second Wnt molecule. Thus, it will be difficult to mutationally decouple Fz-CRD residues important for pseudo-site 3 *trans* contact versus lipid binding (*cis*) via site 1. A detailed analysis of pseudo-site 3 is beyond the scope of the current study, but is an important future subject for investigation.

[00144] The structure of the XWnt8/Fz8-CRD pair presented here provides the first structural access to Wnts, and can now serve as a paradigm to understand Wnt/Fz recognition and signaling, potentially facilitating the development of agonist and antagonist drugs. While many structural and mechanistic questions remain about the nature of canonical versus non-canonical signaling complexes, inclusion of co-receptors such as Lrp5/6, and how Wnt

recognition by Fz is coupled to receptor activation, these can now be addressed by future structural studies that are facilitated by the molecular and technical insights described herein.

[00145] Wnt specificity and cross-reactivity is a major issue in that it is not known which Wnt/Fz interactions are important for different diseases. From structure provided herein, Fz-specific Wnts are engineered through mutation and directed evolution of the site 1 and site 2 'fingers' for binding to specific sub-type of Fz proteins. The structure of Wnt has allowed us to identify the co-receptor (Lrp5/6 binding site on Wnt. Through conservation analysis of Wnt sequences we have mapped the Lrp binding site on to the actual Wnt structure, and this structure can now be targeted for drugs. The structure guides us in how to create single-chain Wnt-Fz proteins that could function as potential antagonists of canonical signaling or as research tools.

Material and Methods:

[00146] 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, 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 (43), 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.

[00147] To generate a stable cell line co-expressing XWnt8 and Fz8CRD-Fc, 13 μ g of XWnt8 plasmid and 7 μ g of Fz8-CRD-Fc plasmid were co-transfected with 1 μ g 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).

[00148] 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 (Fig. 1).

[00149] 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.

[00150] In order to produce recombinant Fz-CRD for yeast display selections and immobilization on Streptavidin BIAcore[™] 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 (44). After the biotinylation reaction was complete, proteins were re-purified on a Superdex 75 size-exclusion column (GE Healthcare) to remove excess of biotin.

[00151] 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 (Fig. 5B).

[00152] 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 (45). See Table 2 for data statistics.

[00153] 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. Initial selenium sites were located with SHELXD and refined within autoSHARP (Table 2). 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 E²)/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. 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 and COOT. Refinement strategies included bulk solvent correction and anisotropic scaling of the data, individual coordinated 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. 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 19.9 % and 24.4 %, respectively (Table 2). The quality of the structure was validated with MolProbity, and PROCHECK as implemented in CCP4. 86.8 % of residues are in the most favored region of the Ramachandran plot, 12.3 % in additional allowed region, 1 residue in the disallowed region. The final model contains amino acids 35-151 of Fz8-CRD and amino acids 32-220 and 234-338 of XWnt8, a palmitoleic acid at Ser187 and two N-acetylglucosamine residues on Asn49, two N-acetylglucosamine residue and two mannose residue on Asn104, and two N-acetylglucosamine residue on 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. Due to the resolution of the structure, water molecules were not placed. Interface contacts were determined using the CCP4 suite program CONTACT. Buried surface area values were calculated using Protein Interfaces, Surfaces, and Assemblies (PISA) software. Structure figures were prepared with the program PyMOL, and sequence conservation analysis was performed using ClustalW.

[00154] Yeast display of Xwnt8. We attempted to evolve a water-soluble version of XWnt8 by creating an error-prone library of the XWnt8 gene, expressing these mutants on yeast linked to the yeast Aga2 protein, and then selecting for binders to Fz-CRD. The general technical aspects of yeast display have been previously described. For our experiments, wildtype, C55A, and S187A mutants of XWnt8 DNA (residues 23 - 338) were 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 µg of error-prone PCR product ("insert DNA"). Insert DNA was combined with 15 µg linearized pCT302 and EBY100 yeast, then electroporated and rescued as previously described (59). The electroporations yielded a library of 1.2×10^8 transformants.

[00155] 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 µL 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 µL 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).

[00156] 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.

[00157] 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 "wnt peptide pharmacophore." 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 µL of human Fz4-CRD, human Fz5-CRD, or Fz8-CRD tetramers. Tetramers were formed by incubating 2 nM biotinylated Fz-CRD in PBE with 470 nM SAV-PE for 15 min on ice in a total volume of 50 µL. Finally, the labeled yeast were washed twice with 1 mL PBE and analyzed on an Accuri C6 flow cytometer (Fig. 5A).

[00158] 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 µl/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 (Fig. 5B).

[00159] The preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of the present invention is embodied by the appended claims.

THAT WHICH IS CLAIMED IS:

1. A composition comprising an isolated circular wnt peptide pharmacophore.
2. The composition of claim 1, wherein the wnt peptide pharmacophore is a polypeptide that consists of an amino acid sequence as set forth in DEQ ID NO:1-47 or a variant thereof.
3. The composition of claim 1, wherein the wnt peptide pharmacophore is from about 11 to 20 amino acids in length and comprises an amino acid sequence that aligns by conserved residues with positions 292-304 of *Xenopus* Wnt8.
4. The composition according to any one of Claim 2-3, wherein the variant comprises one or more amino acid deletions or substitutions.
5. The composition according to any one of Claims 1-4, wherein the wnt peptide pharmacophore is water soluble.
6. The composition according to any one of Claims 1-5, further comprising a fused or conjugated functional moiety.
7. The composition according to Claim 6, wherein the functional moiety is a therapeutic moiety or an imaging moiety.
8. The composition according to any one of Claims 1-7, wherein the composition is formulated with an adjuvant.
9. The composition according to any one of Claims 1-7, wherein the composition is formulated for pharmaceutical administration.
10. A method for inhibiting Wnt signaling in a cell, comprising:
contacting a cell expressing a Wnt receptor with an effective amount of a composition as set forth in any one of Claims 1-9, wherein Wnt signaling is inhibited.
11. The method according to claim 10, wherein proliferation of the cell is inhibited.
12. The method according to claim 11, wherein the cell is a cancer cell.

13. The method according to claim 12, wherein the cell is *in vitro*.
14. The method according to claim 12, wherein the cell is *in vivo*.

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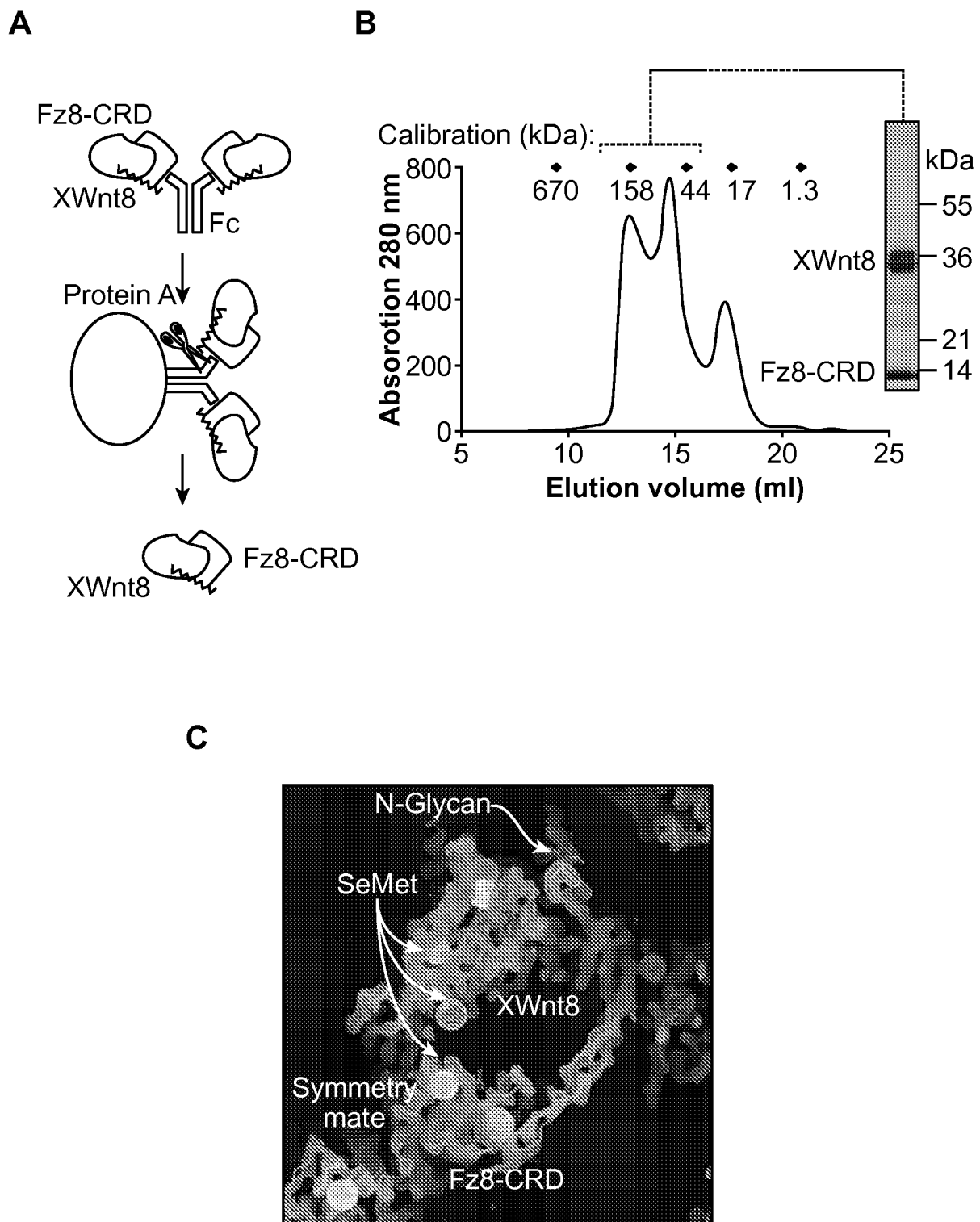
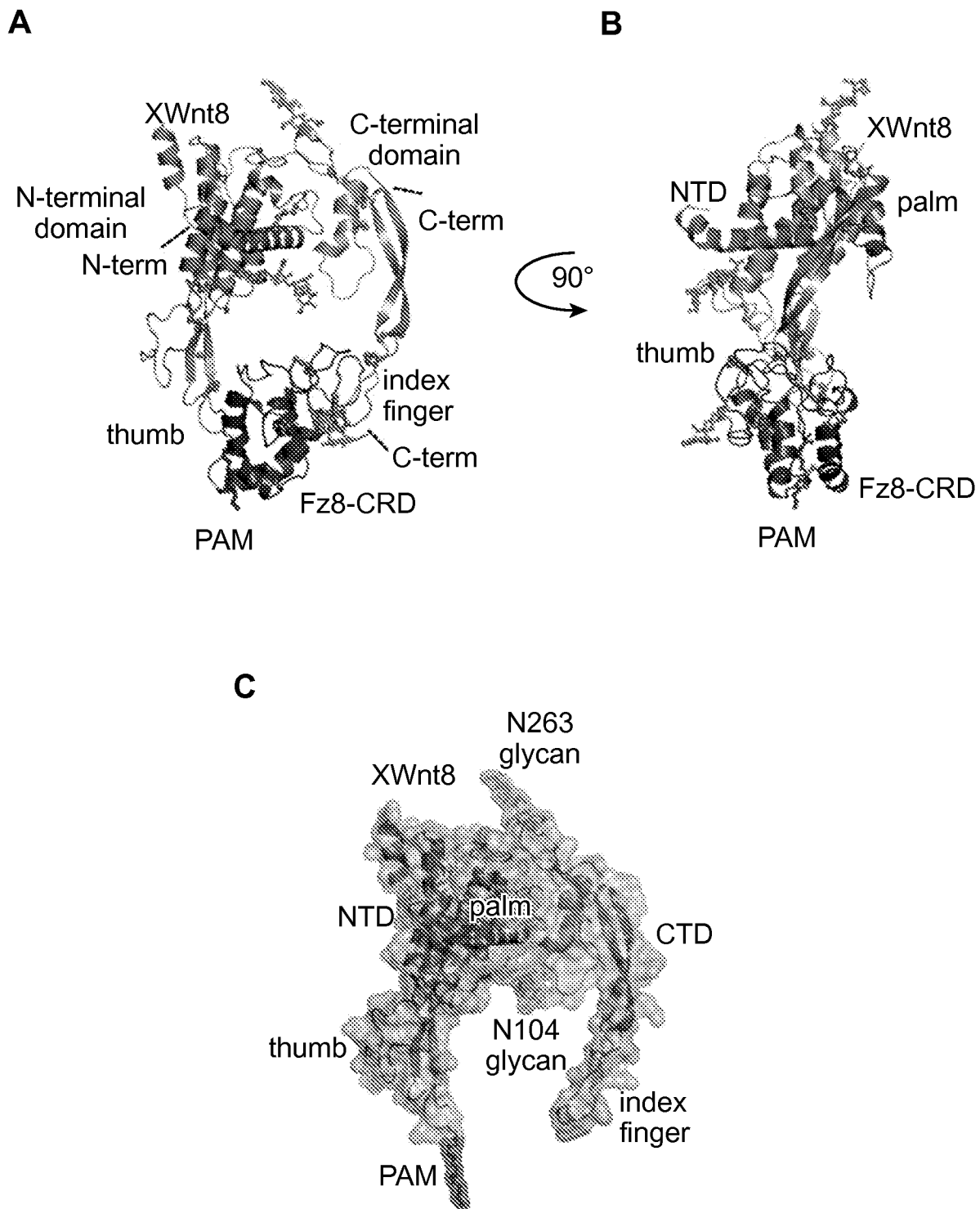


FIG. 1

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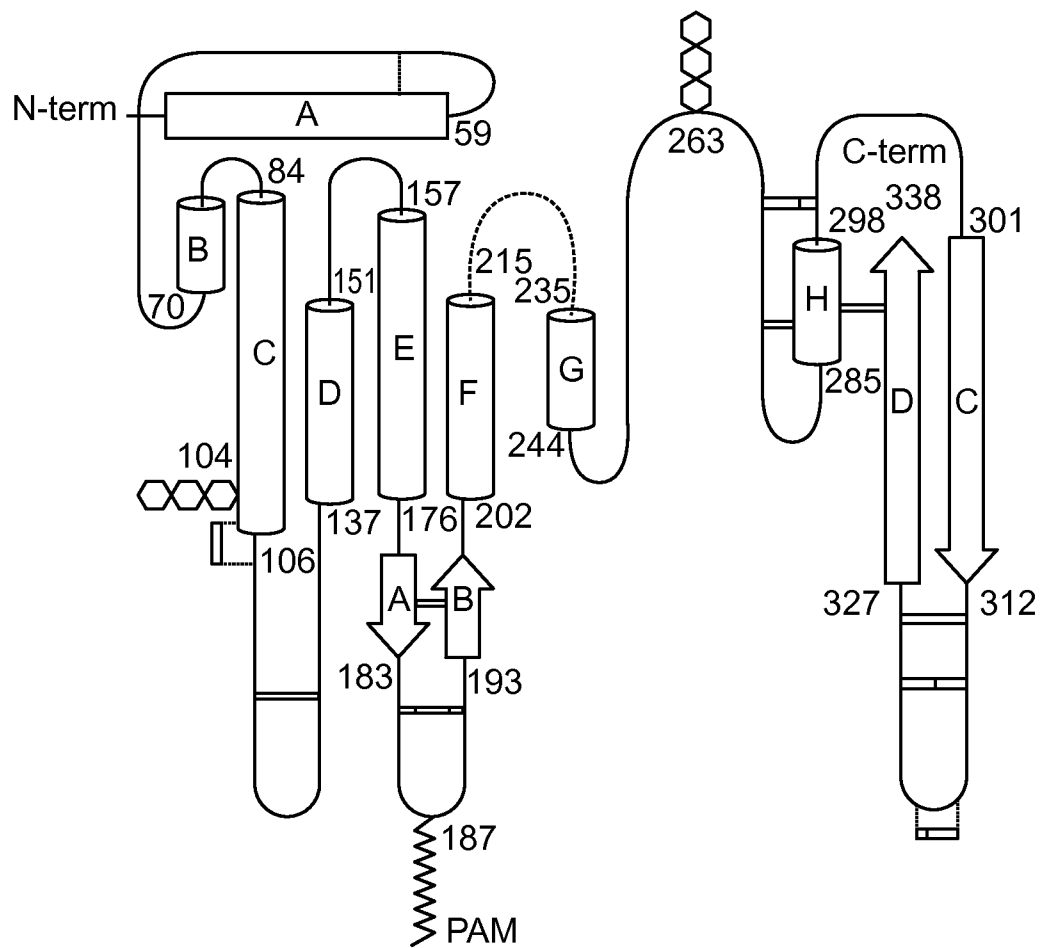
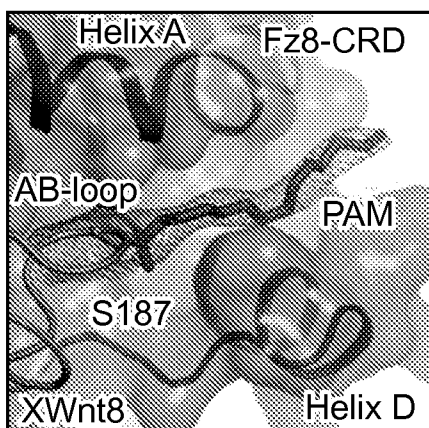
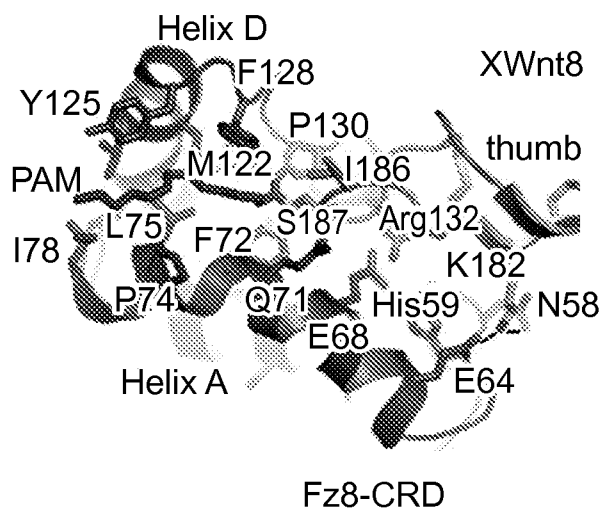
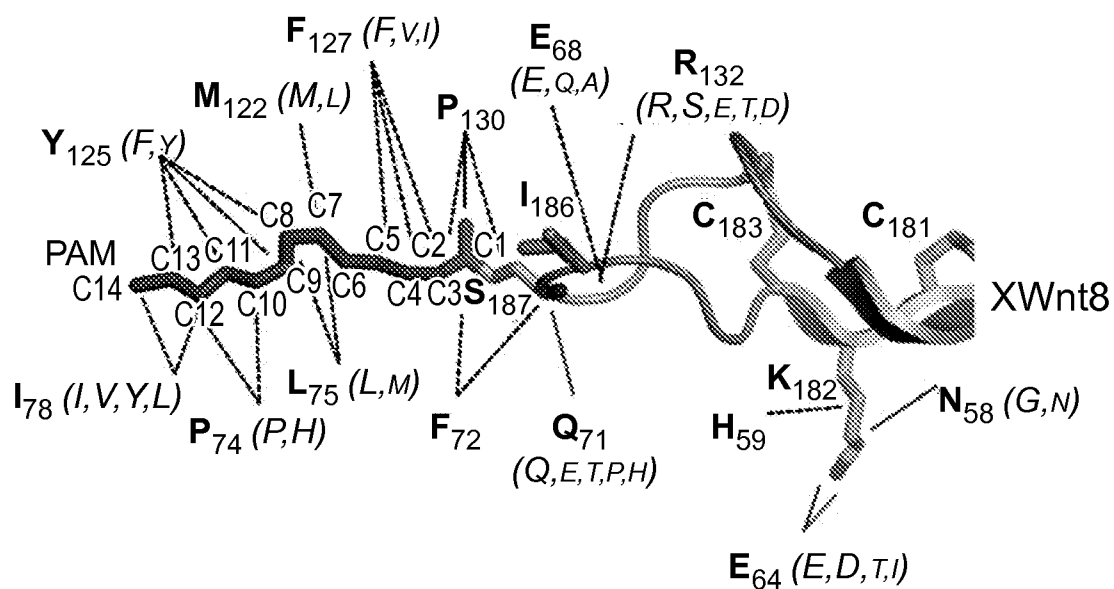
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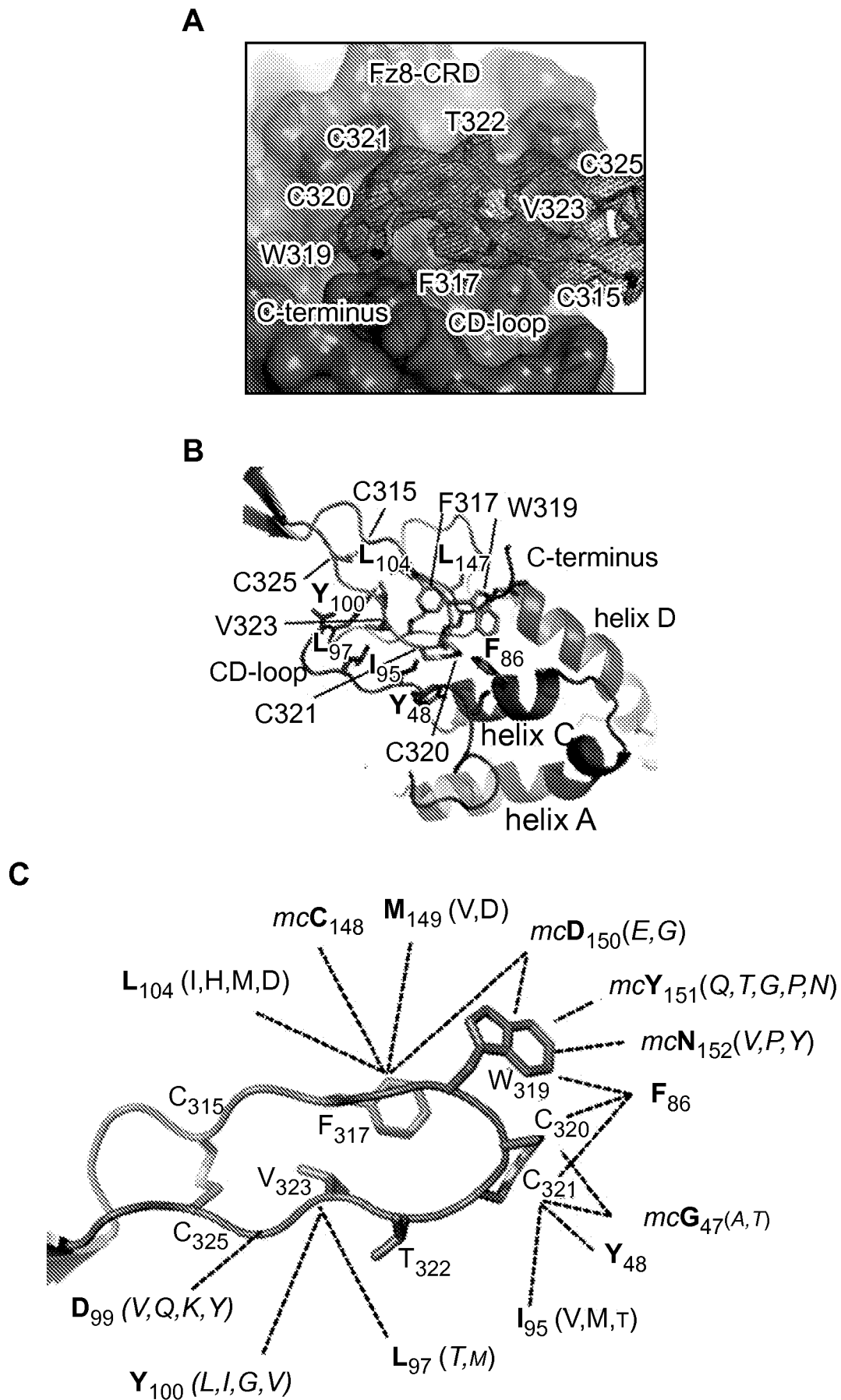
FIG. 2 (Cont.)

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A**B****C****FIG. 3**

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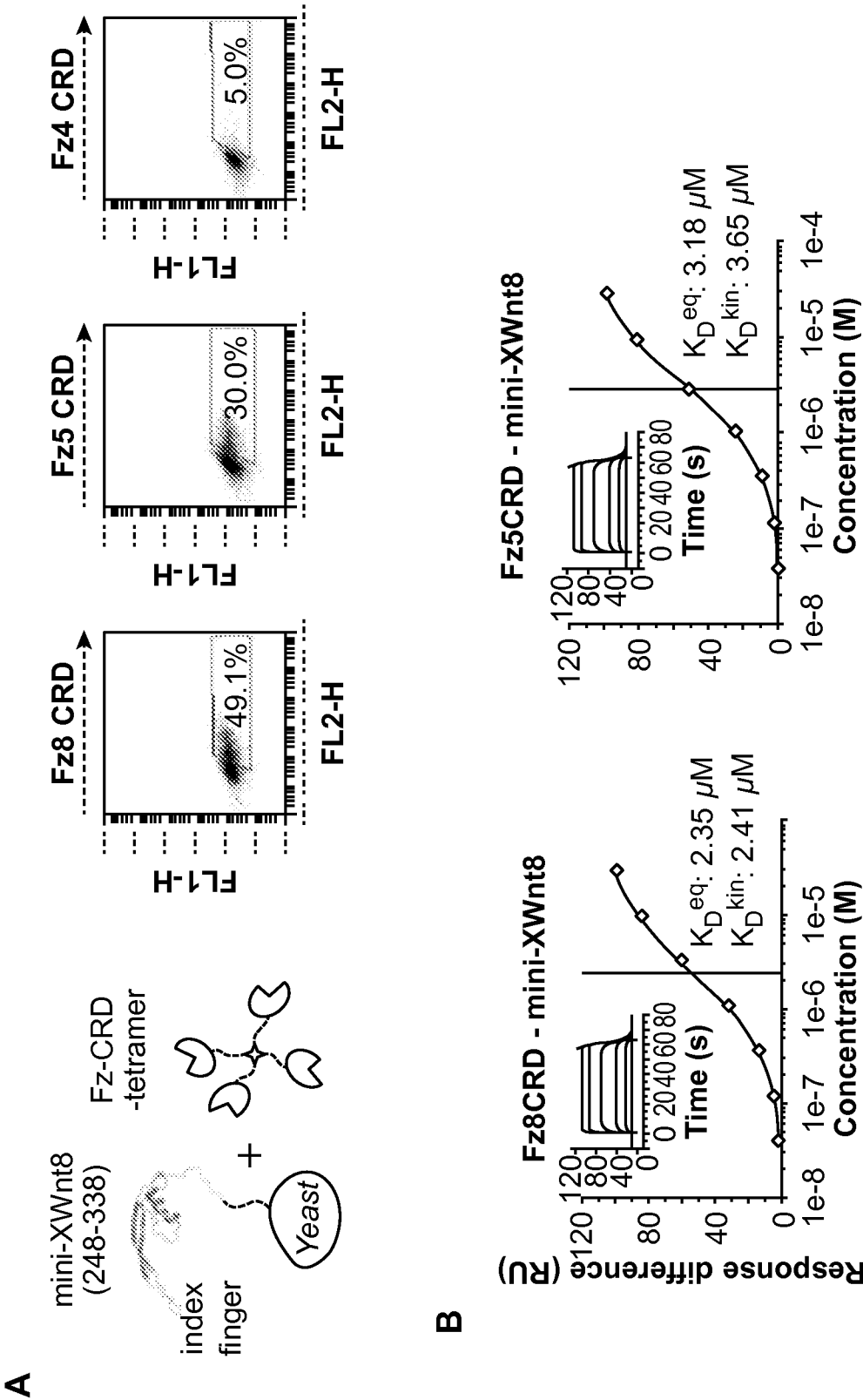


FIG. 5

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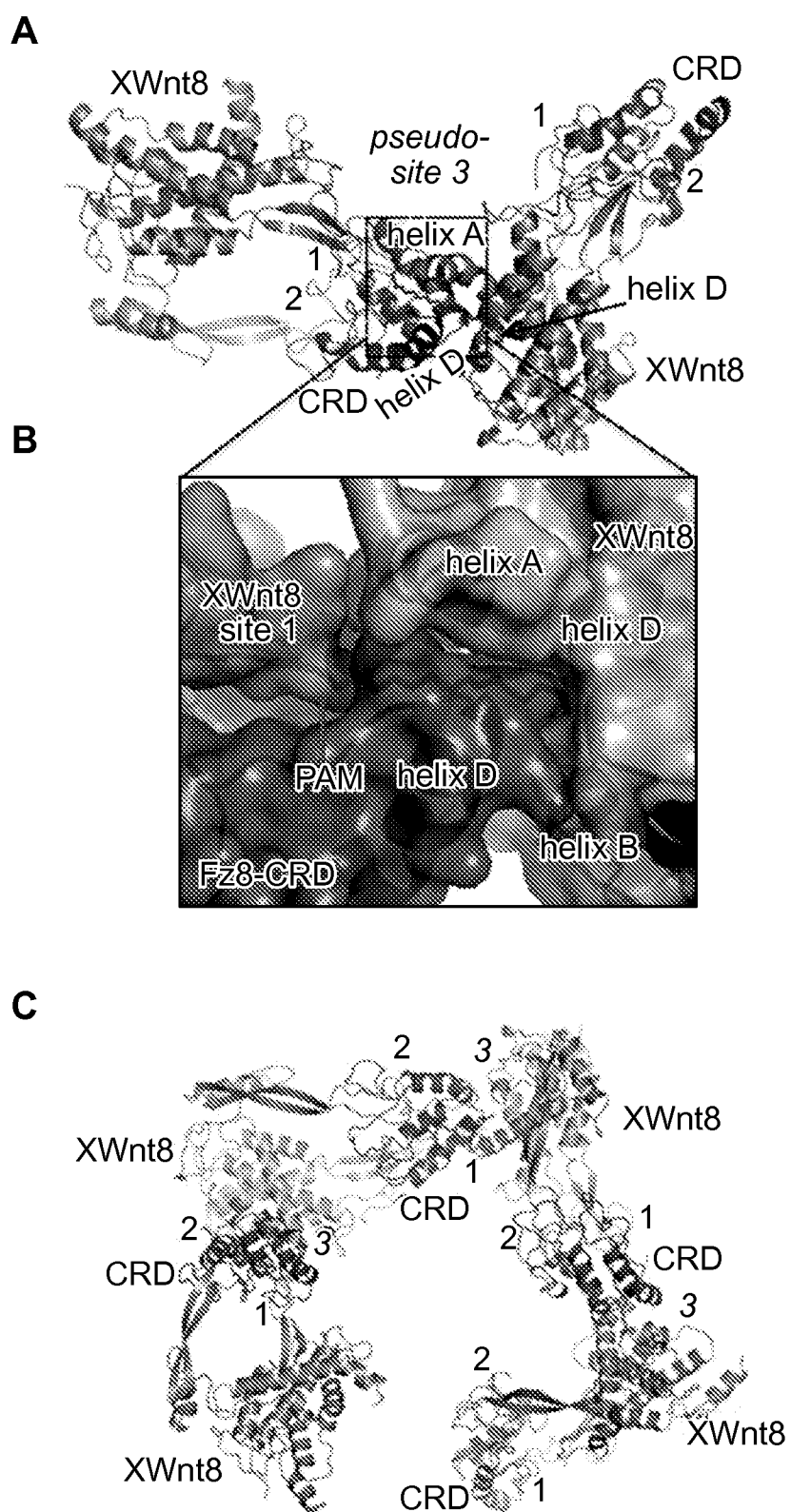
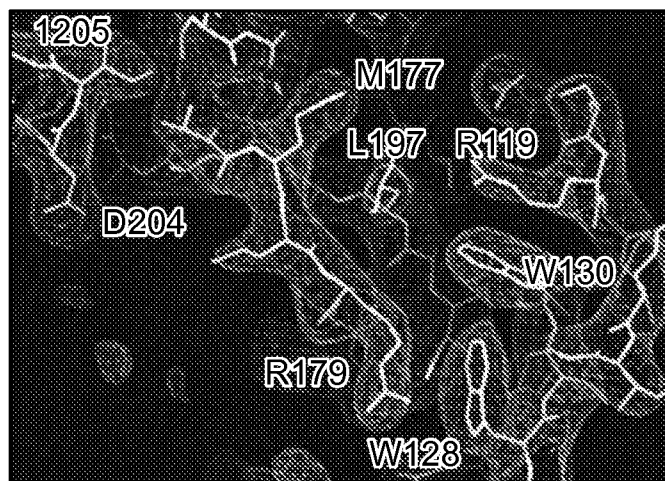


FIG. 6

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A



B

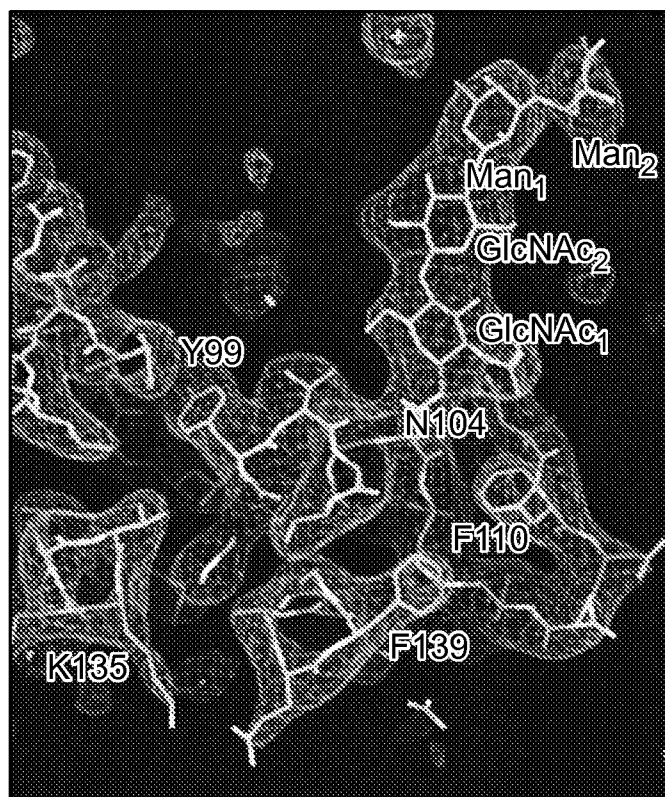


FIG. 7

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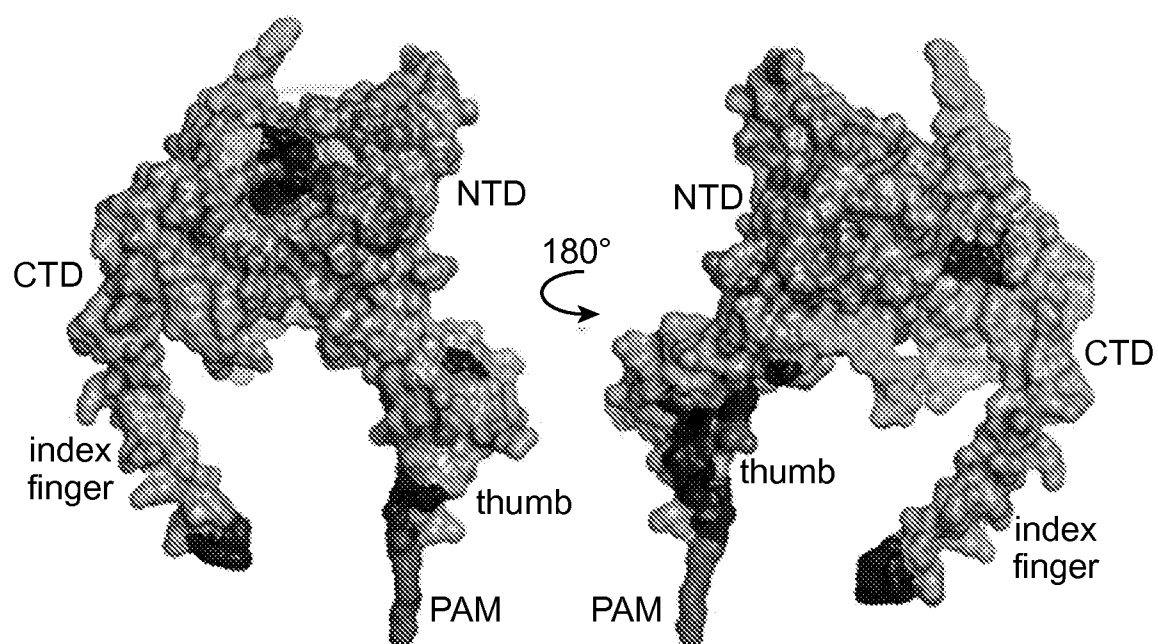


FIG. 8

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XWnt8	: : -----MQNTTILATLLIFCPFFT-----	: : 20
Wnt1	: : -----MGLWALLPSWVS-----	: : 48
Wnt2	: : -----TTLTLLALTALPAALANSSG---RWWGIVNIA--SS---TNLLT	: : 36
Wnt2B	: : -----GIWLWPLLLTWTPEVSS-----SWWYMRATG-GS-----	: : 65
Wnt3	: : MLKLQGEDEAAQLAPRRARVPVPRPTAPDVSPSSARLGLACLLLLLLTIPARVDT-----SWWYIGALG-----	: : 39
Wnt3A	: : -----MEPH-----LLGLLLGLLLSGTRVLAGYP---IWWSLALGQQYTS-----LA	: : 36
Wnt4	: : -----MAP-----LGYLL-VLCSLKQALGSYP---IWWSLAVGPQYSS-----LS	: : 37
Wnt5A	: : -----MSPRS-----CLRSRLLVFAVFSAAASN-----WLYLAKLSSVGS-----I	: : 63
Wnt5B	: : --MKKPIGILSPGVALGTAGGAMS--S-----KFFLMALATFFSFAQVVIEAN---SWWSLGMNN--PVQMSEVYII	: : 42
Wnt6	: : -----MP--S-----LLLVVVAALLSSWAQLLTDAN---SWWSLALN---PVQRPPEMFII	: : 34
Wnt7A	: : -----MLPPVPS-----RLGLLLLLCP---AHVDG---LWWAVG---SPLVM	: : 35
Wnt7B	: : -----MTRKARRCLG-----HLFSLGIVY--LRIGGFS---SVVALGAS-----	: : 35
Wnt8A	: : -----MHRNFRKWIF-----YVFLCFGVLY--VKLGALS---SVVALGAN-----	: : 19
Wnt8B	: : -----MGHLLMLWVAAGMCYPALG-----	: : 18
Wnt9A	: : -----MFLMKPVCVLLVTCVLR-----	: : 48
Wnt9B	: : -----LAAAFGLTLLLAALRPSAAFYGLTGSEPLTILP-----LTLET	: : 46
Wnt10A	: : -----MRPAPALA-----LAALC---LLVLPAAAAAAAYFGLTGREVLTFFPGL---GTAAA	: : 55
Wnt10B	: : -----MGSAPRPWLRLPQG-----PQPRPEFWALLFFLLLLAAAVPRSAPNDILGLRLPPEPV-----L	: : 42
Wnt11	: : -----MLEEPR-----SRPPPLGLAGLLFLALFSRALS-----NEILGLKLPEPP-----L	: : 39
Wnt16	: : -----MRARPQVCEALLFALALHTGVCYG-IKWLALSKTPAALA-----L	: : 42
	: : -----MDRAALLALP-----SLCALWAAVLSLLPCGTQG---NWMWLGIASFQVP-----	: : 42

FIG. 9i

	Helix A			Helix B		
	3	33	33	3	33	
XWnt8	--ASAWSVNN	-----FLMTGPKAYL	TSASVAVGAQNGIEECKYQFAWERWNCPEST	-----LQLATHNGLRSAT		83
Wnt1	DSKSLQLVLEPSLQLLSRKQRRLLIRQNPGILHSVSGGLQSAVRECKWQFRNRRWNCPTAPG			-----PHLFGKIVNRGC		121
Wnt2	--SRVMCDNVPG	-----LVSQRQLCHRHPDVMRAI	GLGVAEWTAECQHQFRQHRWNCNTLDR	-----DHSLFGRVLLRSS		105
Wnt2B	--ARVICDNIPG	-----LVSQRQLCQRYPDIMRSVGE	GAREWIRECQHQFRHHRWNCNTLDR	-----DHTVFGRAMLRSS		104
Wnt3	SQPLLCGSIPG	-----LVPKQLRFCRNYIEIMPSVAE	GVKLGIECQHQFRGRRWNCNTTIDD	-----SLAIFGPVLDKAT		109
Wnt3A	TQPILCASIPG	-----LVPKQLRFCRNYVEIMPSVAE	GVKAGIECQHQFRGRRWNCNTTVSN	-----SLAIFGPVLDKAT		106
Wnt4	SEETCEKLG	-----LIQRQVMCKRNLEVMDSVRRGAQ	LAIEECQYQFRNRRWNCSTLD	-----SLPVFGKVVTQGT		106
Wnt5A	GAQPLCSQLAG	-----LSQGQKKLCHLYQDHMQYI	GEGAKTGIECQYQFRHRRWNCSTVD	-----NTSVFGRVMQIGS		102
Wnt5B	GAQPVCSQLPG	-----LSPGQRKLCQLYQEHMSYI	GEGAKTGIRECQHQFRQRRWNCSTVD	-----NTSVFGRVMQIGS		111
Wnt6	DPTSICRKARR	-----LAGRQAEELCQAEPEVVAELAR	CARLGVRECCQYQFRFRRWNCSSHS	-----KAFGRVLQQDI		101
Wnt7A	---IICNKIPG	-----LAPRQRAICQSRPD	AIIVIGESQMGLECCQYQFRNGRWNC	-----ALGERTVFGKELKVGS		101
Wnt7B	---IICNKIPG	-----LAPRQRAICQSRPD	AIIVIGEGAQMGIDECQHQYQFRFGRWNC	-----ALGERTVFGQELRVGS		101
Wnt8A	---ASAWSVNN	-----FLITGPKAYL	TYTASVALGAQIGIEECKYQFAWERWNCPEHA	-----FQFSTHNRLRAAT		#2
Wnt8B	---SHAWSVNN	-----FLMTGPKAYL	VYSSSVAAGAQS	GIEECKYQFAWDRWNCPEHA	-----LQLSSHGGLRSAN	#1
Wnt9A	EAAAQAHYKACDR	LKLERKQRRMCRDPGVAETL	VEAVSMSALECCQYQFRFERWNC	LEGR-----YRASLLKRGF		113
Wnt9B	PAQAGAHLKQC	DLLKLSRRQKQLCRREPGLAETLRD	AAHLGLECCQYQFRQERWNC	LEG-----RTGLLQRGF		115
Wnt10A	NANTVCLTLPG	-----LSRRQMEVCVRHPDVAASA	IQGIQIAIHECCQHQYQFRDQRWNC	SLETRN-----KVPYESPIFSRGF		127
Wnt10B	LANTVCLTLSG	-----LSKRQLGLCLRSPDVTASAL	QGLHIAVHECCQHQYQFRDQRWNC	SALEGG-----RLPHHSAILKRGF		114
Wnt11	NQTQHCKQLEG	-----LVSAQVQLCRSNLELMRTI	VHAARGAMKACRRAFADMRWNC	SSIELA-----PNYLLDLERGT		108
Wnt16	-EKLGCADLP	-----LNSRQKELCKRKPYLLPSI	REGARLGIQECRSQYQFRHERWNC	MVATTTSTQLATAPLFGYELSSGT		116

FIG. 9i (Cont. 1)

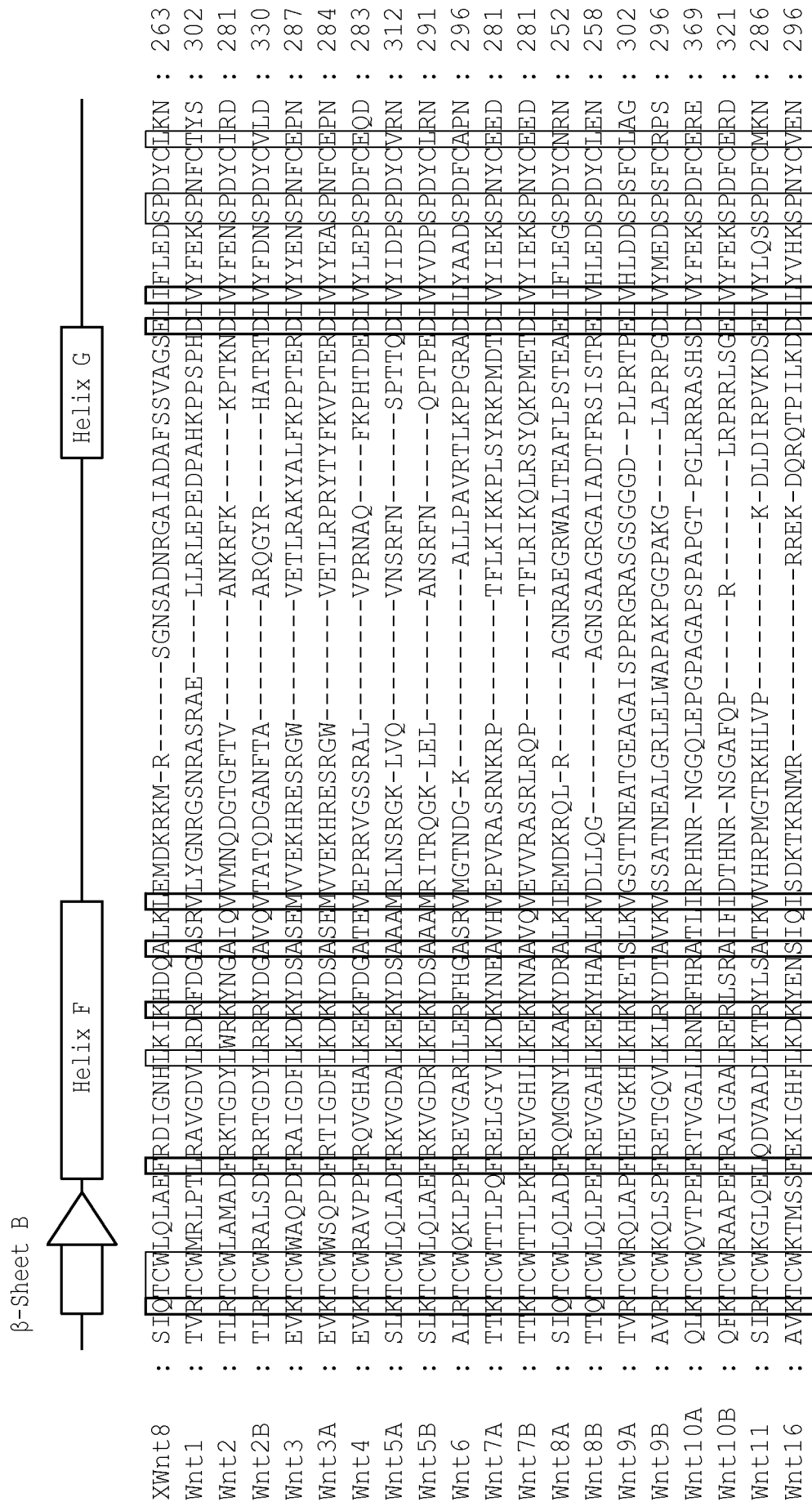


FIG. 9ii

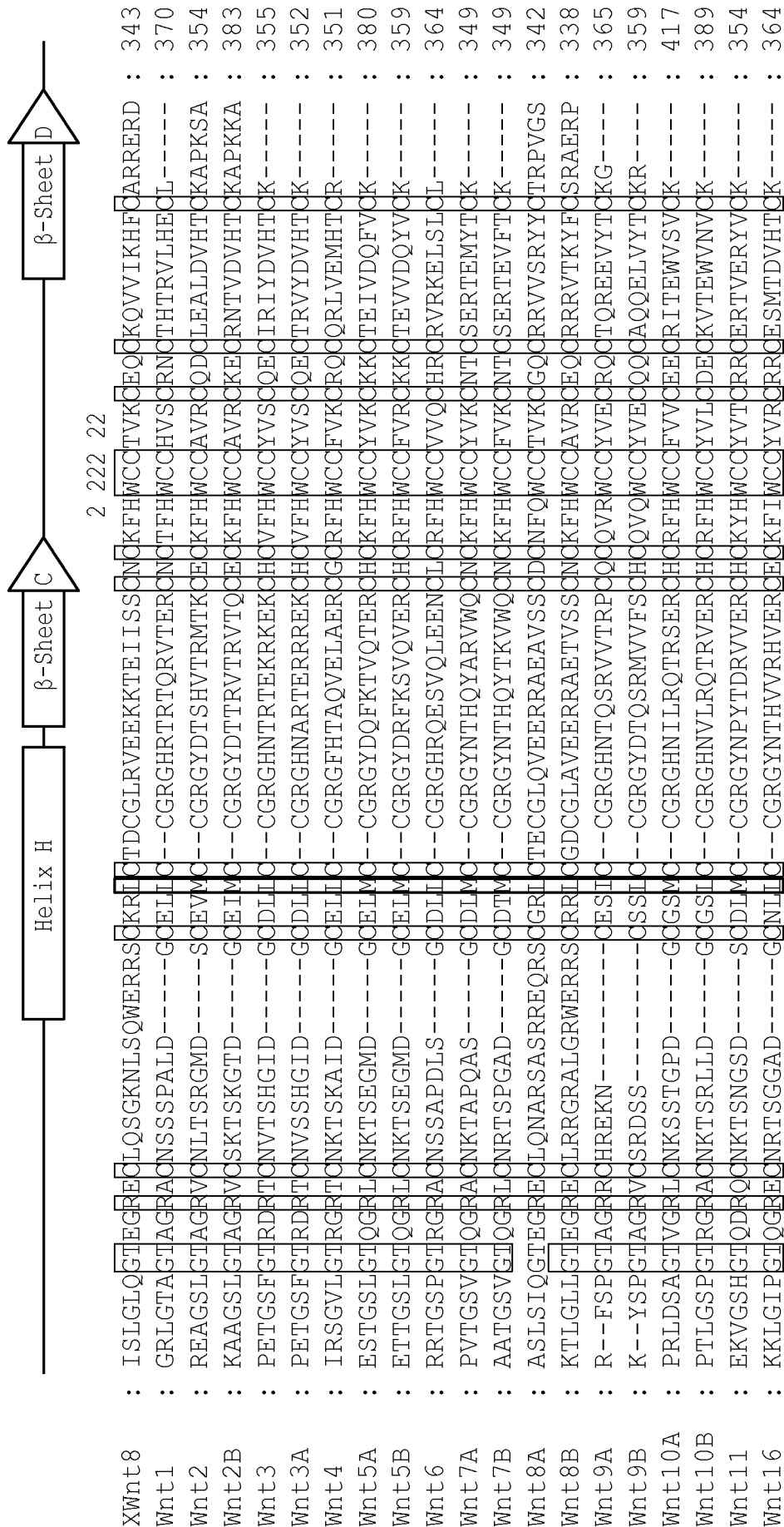


FIG. 9ii (Cont. 1)

XWnt8	: SNMLNTKRKNRGHRR	: 351
Wnt1	: -----	: -
Wnt2	: DWATPT-----	: 360
Wnt2B	: EWLDQT-----	: 389
Wnt3	: -----	: -
Wnt3A	: -----	: -
Wnt4	: -----	: -
Wnt5	: -----	: -
Wnt5B	: -----	: -
Wnt6	: -----	: -
Wnt7A	: -----	: -
Wnt7B	: -----	: -
Wnt8A	: AR--PRGRGKDSAW-	: 354
Wnt8B	: PRGAHKPGKNS---	: 350
Wnt9A	: -----	: -
Wnt9B	: -----	: -
Wnt10A	: -----	: -
Wnt10B	: -----	: -
Wnt11	: -----	: -
Wnt16	: -----	: -

FIG. 9ii (Cont. 2)

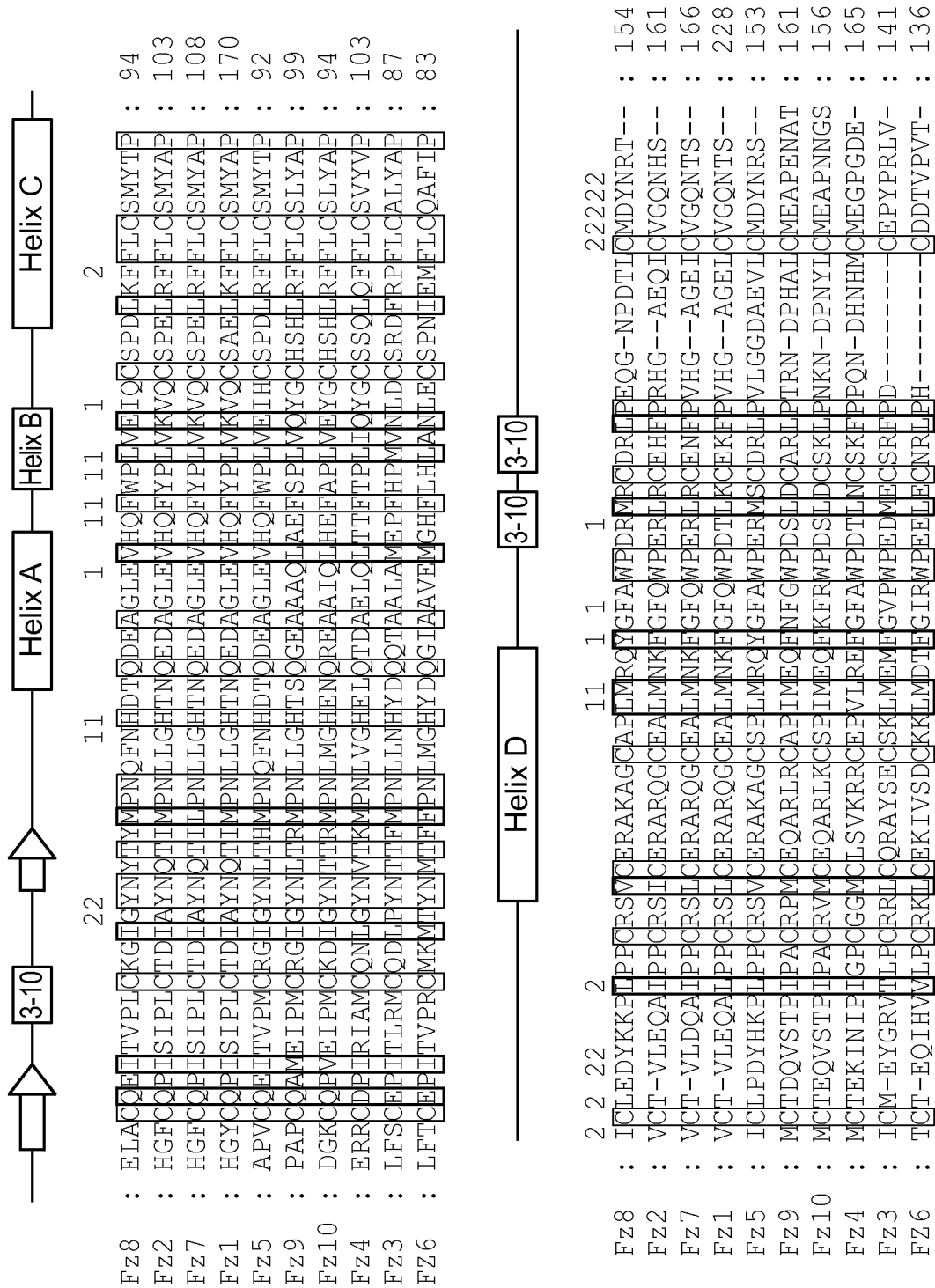


FIG. 10

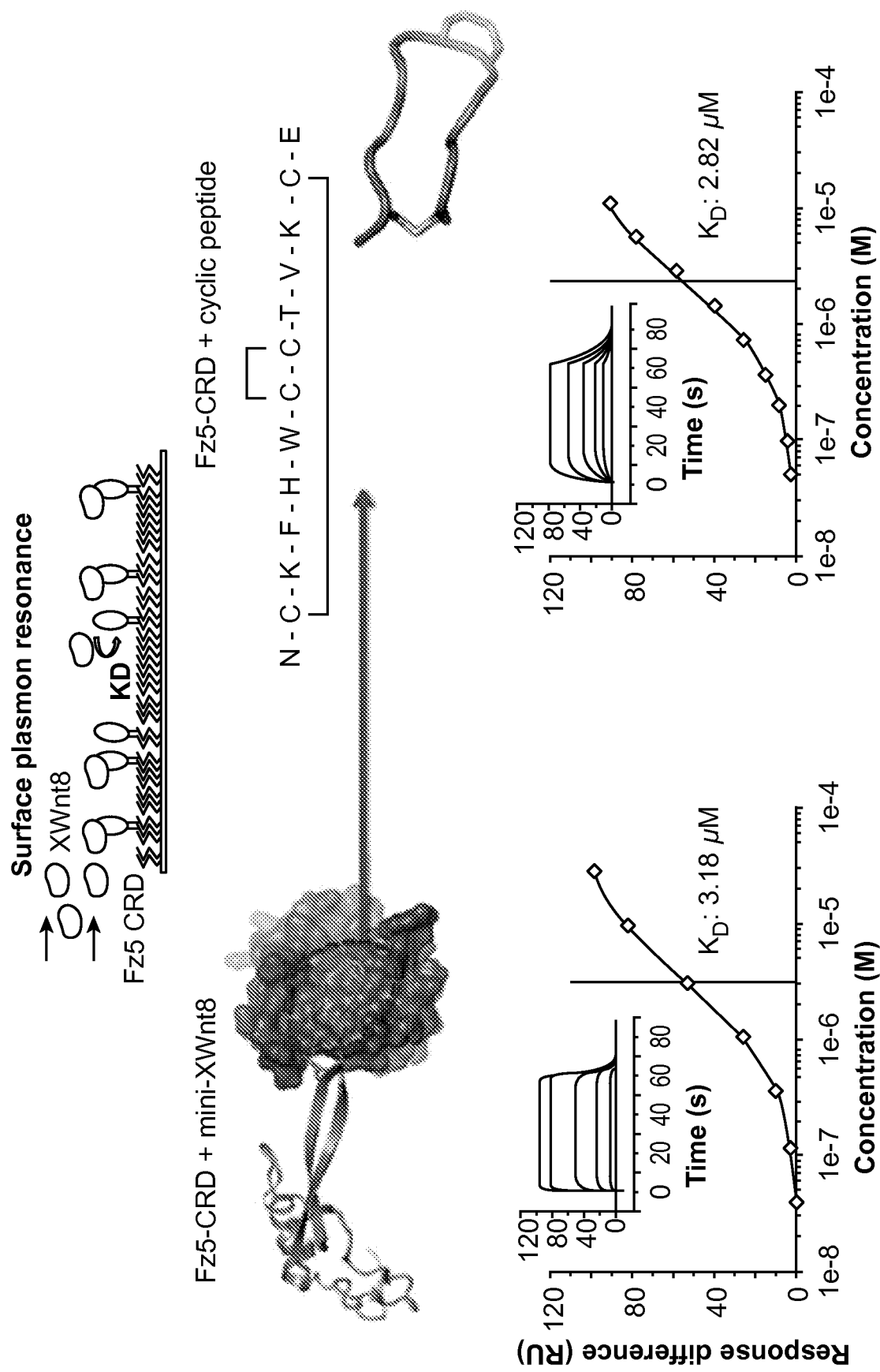


FIG. 11

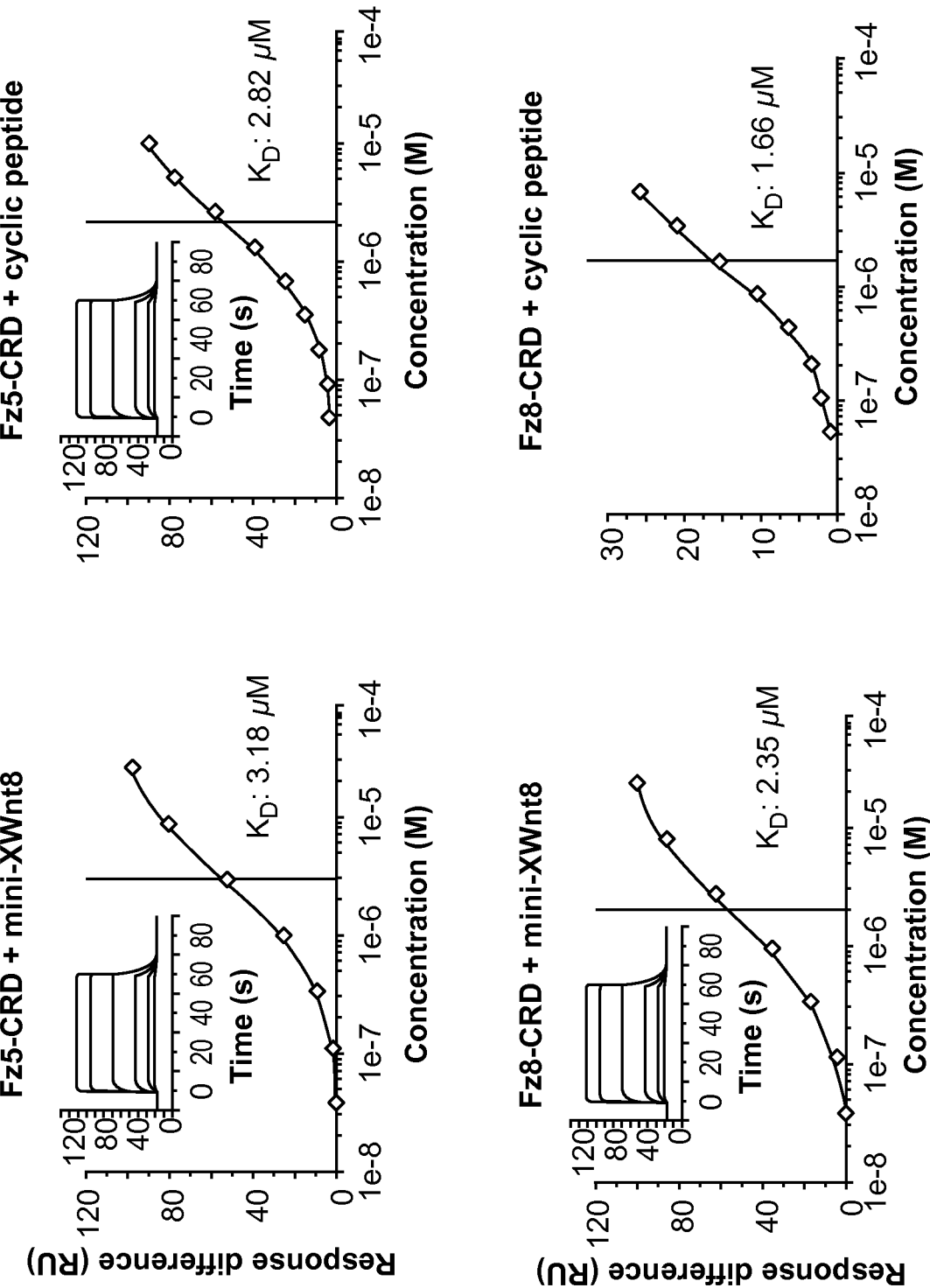


FIG. 12

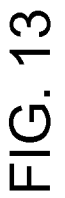


FIG. 13

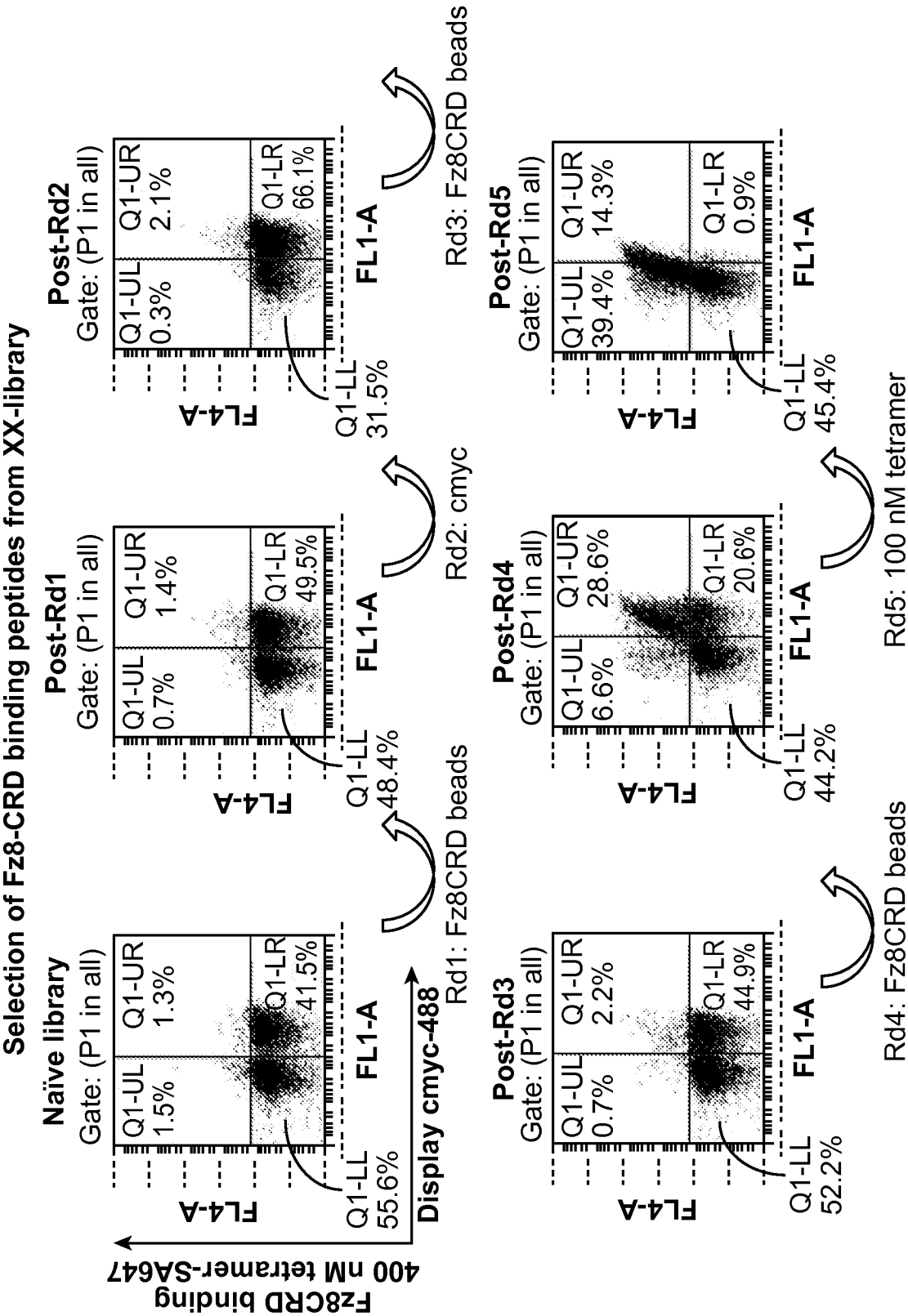
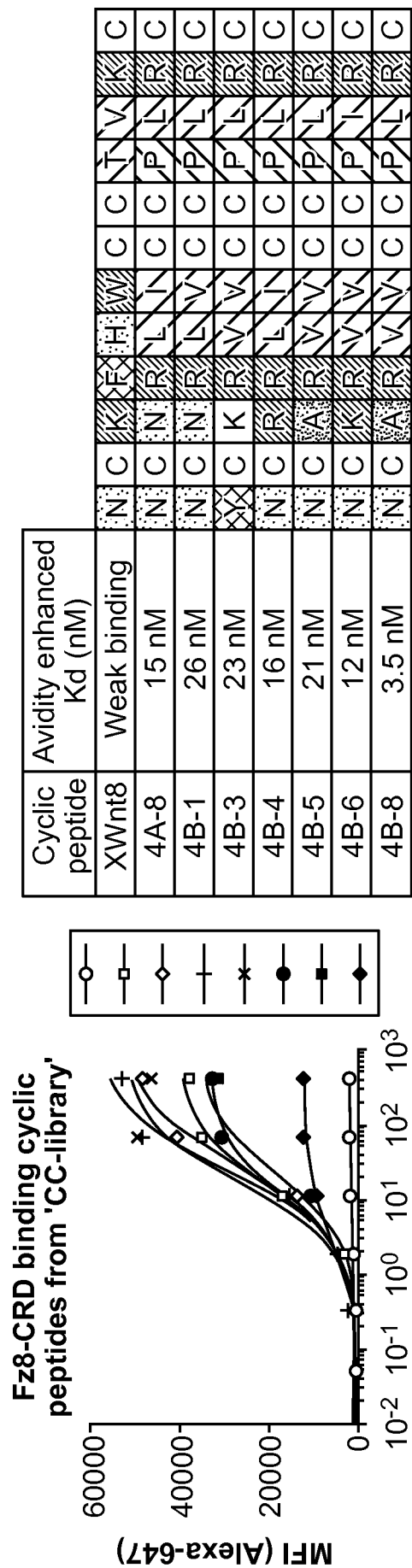


FIG. 13 (Cont.)



Fz8-CRD binding cyclic peptides from 'XX-library'

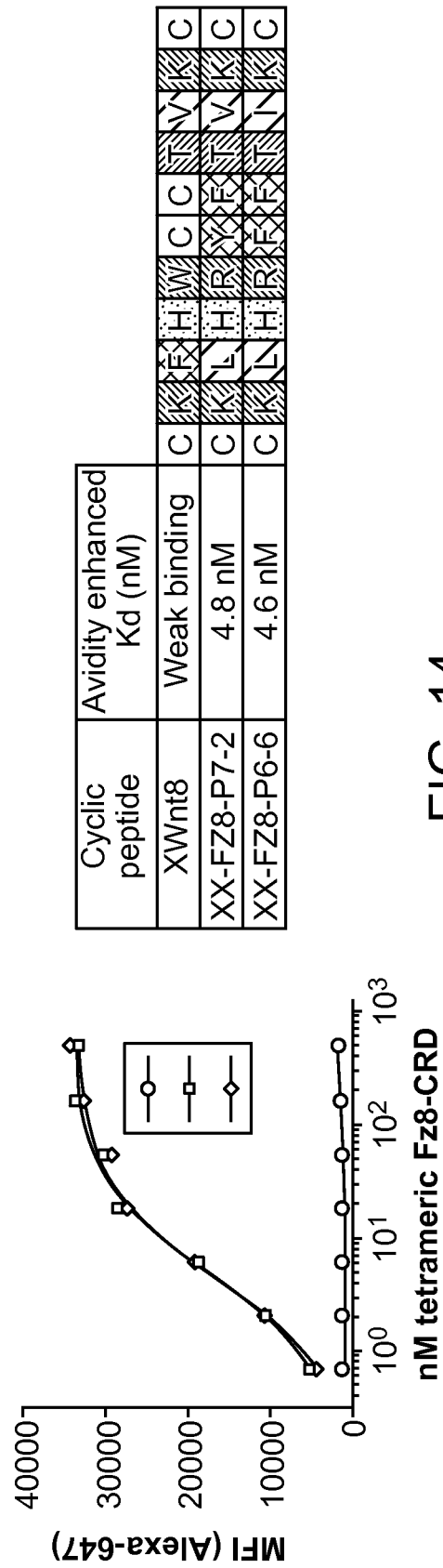


FIG. 14

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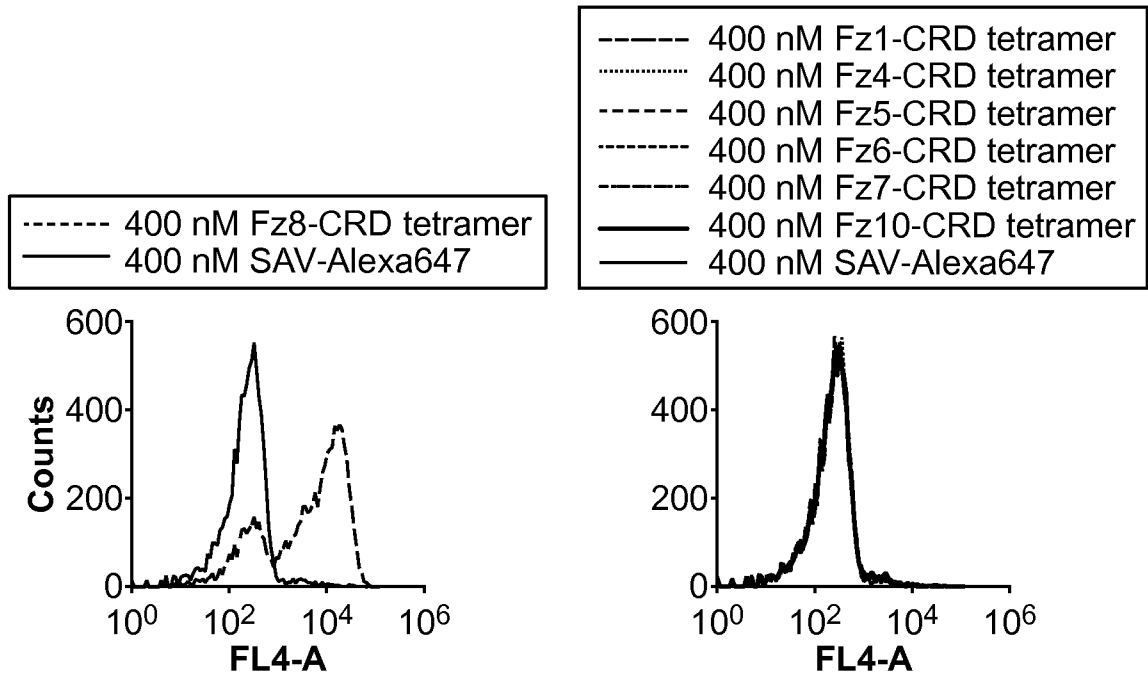
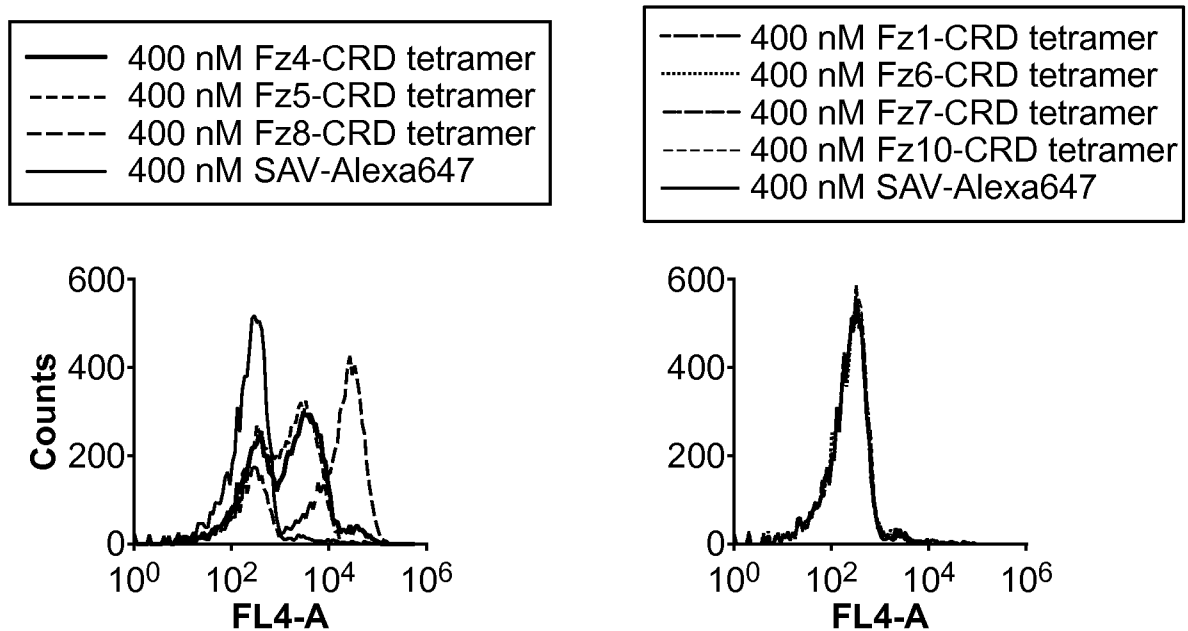
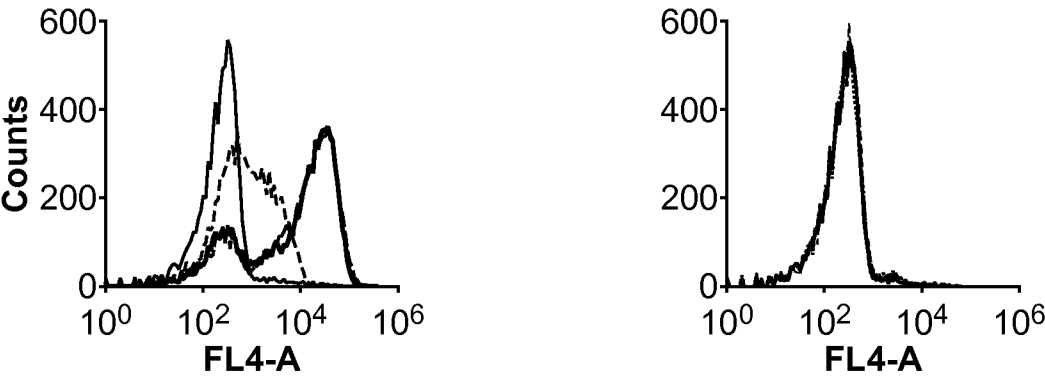
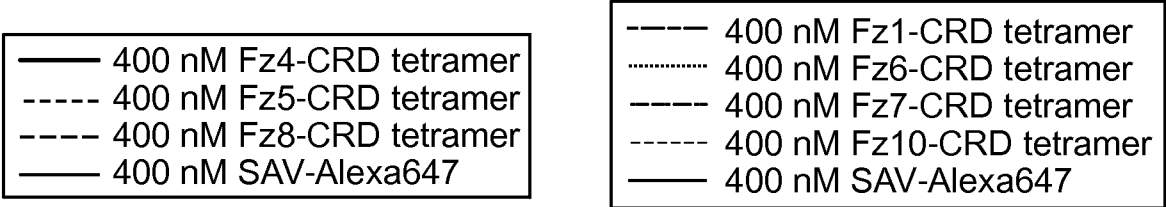
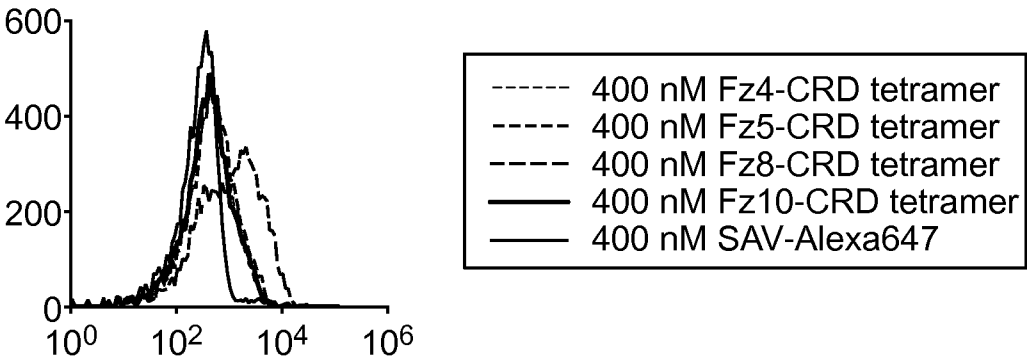
CRRVVCCPLRC**CKLHRFFTIKC**

FIG. 15

CKLHRYFTVKC



WT mini-XWnt8:



WT XWnt8	C	K	F	H	W	C	C	T	V	K	C
CC-FZ8-P73	C	R	R	V	V	C	C	P	L	R	C
XX-FZ8-P66	C	K	L	H	R	F	F	T	L	K	C
XX-FZ8-P72	C	K	L	H	R	Y	F	T	V	K	C

FIG. 15 (Cont.)