WNT ANTAGONISTS AND METHODS OF TREATMENT AND SCREENING

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. Provisional Application No. 61/294,270, filed January 12, 2010, U.S. Provisional Application No. 61/393,675, filed October 15, 2010, and U.S. Provisional Application No. 61/424,408, filed December 17, 2010, each of which is hereby incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[0002] The present invention provides novel compositions and methods for treating cancer and other Wnt-associated diseases or disorders, as well as novel screening methods for identifying additional novel therapeutic agents. In particular, the present invention provides Wnt antagonists including soluble receptor proteins useful for the treatment of solid tumors and other Wnt-associated diseases and conditions.

BACKGROUND OF THE INVENTION

[0003] Cancer is one of the leading causes of death in the developed world, with over one million people diagnosed with cancer and 500,000 deaths per year in the United States alone. Overall it is estimated that more than 1 in 3 people will develop some form of cancer during their lifetime. There are more than 200 different types of cancer, four of which - breast, lung, colorectal, and prostate - account for over half of all new cases (Jemal et al., 2009, *Cancer J. Clin.*, 58:225-249).

[0004] The Wnt signaling pathway has been identified as a potential target for cancer therapy. The Wnt signaling pathway is one of several critical regulators of embryonic pattern formation, post-embryonic tissue maintenance, and stem cell biology. More specifically, Wnt signaling plays an important role in the generation of cell polarity and cell fate specification including self-renewal by stem cell populations. Unregulated activation of the Wnt pathway is associated with numerous human cancers where it can alter the developmental fate of tumor cells to maintain them in an undifferentiated and proliferative state. Thus carcinogenesis can proceed by usurping homeostatic mechanisms controlling normal development and tissue repair by stem cells (reviewed in Reya & Clevers, 2005, *Nature,* 434:843-50; Beachy et al., 2004, *Nature,* 432:324-31).

[0005] The Wnt signaling pathway was first elucidated in the Drosophila developmental mutant wingless (wg) and from the murine proto-oncogene int-1, now Wnt1 (Nusse & Varmus, 1982, *Cell,* 31:99-109; Van Ooyen & Nusse, 1984, *Cell,* 39:233-40; Cabrera et al., 1987, *Cell,* 50:659-63; Rijsewijk et al., 1987, *Cell,* 50:649-57). Wnt genes encode secreted lipid-modified glycoproteins of which 19 have been identified in

mammals. These secreted ligands activate a receptor complex consisting of a Frizzled (FZD) receptor family member and low-density lipoprotein (LDL) receptor-related protein 5 or 6 (LRP5/6). The FZD receptors are seven transmembrane domain proteins of the G-protein coupled receptor (GPCR) superfamily and contain a large extracellular N-terminal ligand binding domain with 10 conserved cysteines, known as a cysteine-rich domain (CRD) or Fri domain. There are ten human FZD receptors, FZD1, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9 and FZD10. Different FZD CRDs have different binding affinities for specific Wnts (Wu & Nusse, 2002, *J. Biol. Chem.,* 277:41762-9), and FZD receptors have been grouped into those that activate the canonical β-catenin pathway and those that activate non-canonical pathways described below (Miller et al., 1999, *Oncogene,* 18:7860-72). To form the receptor complex that binds the FZD ligands, FZD receptors interact with LRP5/6, single pass transmembrane proteins with four extracellular EGF-like domains separated by six YWTD amino acid repeats (Johnson et al., 2004, *J. Bone Mineral Res.,* 19:1749).

[0006] The canonical Wnt signaling pathway activated upon receptor binding is mediated by the cytoplasmic protein Dishevelled (Dsh) interacting directly with the FZD receptor and results in the cytoplasmic stabilization and accumulation of β-catenin. In the absence of a Wnt signal, β-catenin is localized to a cytoplasmic destruction complex that includes the tumor suppressor proteins adenomatous polyposis coli (APC) and Axin. These proteins function as critical scaffolds to allow glycogen synthase kinase-3β (GSK3β) to bind and phosphorylate β-catenin, marking it for degradation via the ubiquitin/proteasome pathway. Activation of Dsh results in phophorylation of GSK3β and the dissociation of the destruction complex. Accumulated cytoplasmic β-catenin is then transported into the nucleus where it interacts with the DNA-binding proteins of the TCF/LEF family to activate transcription.

[0007] In addition to the canonical signaling pathway, Wnt ligands also activate β-catenin-independent pathways (Veeman et al., 2003, *Dev. Cell,* 5:367-77). Non-canonical Wnt signaling has been implicated in numerous processes but most convincingly in gastrulation movements via a mechanism similar to the Drosophila planar cell polarity (PCP) pathway. Other potential mechanisms of non-canonical Wnt signaling include calcium flux, JNK, and both small and heterotrimeric G-proteins. Antagonism is often observed between the canonical and non-canonical pathways, and some evidence indicates that non-canonical signaling can suppress cancer formation (Olson & Gibo, 1998, *Exp. Cell Res.,* 241:134; Topol et al., 2003, *J. Cell Biol.,* 162:899-908). Thus in certain contexts, FZD receptors act as negative regulators of the canonical Wnt signaling pathway. For example, FZD6 represses Wnt3a-induced canonical signaling when co-expressed with FZD1 via the TAK1-NLK pathway (Golan et al., 2004, *JBC,* 279:14879-88). Similarly, FZD2 was shown to antagonize canonical Wnt signaling in the presence of Wnt5a via the TAK1-NLK MAPK cascade (Ishitani et al., 2003, *Mol. Cell. Biol.,* 23:131-39).

[0008] The canonical Wnt signaling pathway also plays a central role in the maintenance of stem cell populations in the small intestine and colon, and the inappropriate activation of this pathway plays a

prominent role in colorectal cancers (Reya & Clevers, 2005, *Nature,* 434:843). The absorptive epithelium of the intestines is arranged into villi and crypts. Stem cells reside in the crypts and slowly divide to produce rapidly proliferating cells that give rise to all the differentiated cell populations that move out of the crypts to occupy the intestinal villi. The Wnt signaling cascade plays a dominant role in controlling cell fates along the crypt-villi axis and is essential for the maintenance of the stem cell population. Disruption of Wnt signaling either by genetic loss of TCF7/2 by homologous recombination (Korinek et al., 1998, *Nat. Genet.,* 19:379) or overexpression of Dickkopf-1 (Dkk1), a potent secreted Wnt antagonist (Pinto et al., 2003, *Genes Dev.,* 17:1709-13; Kuhnert et al., 2004, *PNAS,* 101:266-71), results in depletion of intestinal stem cell populations.

[0009] A role for Wnt signaling in cancer was first uncovered with the identification of Wnt1 (originally int1) as an oncogene in mammary tumors transformed by the nearby insertion of a murine virus (Nusse & Varmus, 1982, *Cell,* 31:99-109). Additional evidence for the role of Wnt signaling in breast cancer has since accumulated. For instance, transgenic overexpression of β-catenin in the mammary glands results in hyperplasias and adenocarcinomas (Imbert et al., 2001, *J. Cell Biol.,* 153:555-68; Michaelson & Leder, 2001, *Oncogene,* 20:5093-9) whereas loss of Wnt signaling disrupts normal mammary gland development (Tepera et al., 2003, *J. Cell Sci.,* 116:1137-49; Hatsell et al., 2003, *J. Mammary Gland Biol. Neoplasia,* 8:145-58). More recently mammary stem cells have been shown to be activated by Wnt signaling (Liu et al., 2004, *PNAS,* 101:4158-4163). In human breast cancer, β-catenin accumulation implicates activated Wnt signaling in over 50% of carcinomas, and though specific mutations have not been identified, upregulation of Frizzled receptor expression has been observed (Brennan & Brown, 2004, *J. Mammary Gland Neoplasia,* 9:119-31; Malovanovic et al., 2004, *Int. J. Oncol.,* 25:1337-42).

[0010] Colorectal cancer is most commonly initiated by activating mutations in the Wnt signaling cascade. Approximately 5-10% of all colorectal cancers are hereditary with one of the main forms being familial adenomatous polyposis (FAP), an autosomal dominant disease in which about 80% of affected individuals contain a germline mutation in the adenomatous polyposis coli (APC) gene. Mutations have also been identified in other Wnt pathway components including Axin and β-catenin. Individual adenomas are clonal outgrowths of epithelial cells containing a second inactivated allele, and the large number of FAP adenomas inevitably results in the development of adenocarcinomas through additional mutations in oncogenes and/or tumor suppressor genes. Furthermore, activation of the Wnt signaling pathway, including gain-of-function mutations in APC and β-catenin, can induce hyperplastic development and tumor growth in mouse models (Oshima et al., 1997, *Cancer Res,.* 57:1644-9; Harada et al., 1999, *EMBO J.,* 18:5931-42).

BRIEF SUMMARY OF THE INVENTION

[0011] The present disclosure provides a variety of agents that bind to one or more human Wnt proteins, including, but not limited to, soluble FZD receptors and other agents comprising a Fri domain, and novel methods of using those agents. The disclosure further provides methods of using the agents in the treatment of cancer by administering the agents to a subject in need thereof. In some examples, the methods comprise inhibiting the growth of cancer cells. In certain examples, the Wnt-binding agent is a Wnt antagonist. Novel methods of screening for such Wnt-binding agents are also provided. Polynucleotides encoding the agents, methods of making the agents, and a variety of compositions comprising the agents are likewise provided.

[0011a] In one aspect, there is provided a Wnt-binding agent comprising:

(a) a first polypeptide consisting of SEQ ID NO:39; and

(b) a second polypeptide comprising SEQ ID NO:43;

wherein the first polypeptide is directly linked to the second polypeptide.

[0011b] In another aspect, there is provided a Wnt-binding agent comprising:

(a) a first polypeptide comprising amino acids X to Y of SEQ ID NO:30; and

(b) a second polypeptide comprising SEQ ID NO:43;

wherein the first polypeptide is directly linked to the second polypeptide; and

wherein X = amino acid 25, 26, 27, or 28; and

Y = amino acid 158, 159, 160, 161, 162, 163, or 164.

[0011c] In yet another aspect, there is provided a Wnt-binding agent comprising the amino acid sequence of SEQ ID NO:53.

[0011d] In yet another aspect, there is provided a Wnt-binding agent consisting of SEQ ID NO:53.

[0011e] In yet another aspect, there is provided a polypeptide comprising the Wnt-binding agent as defined herein, and further comprising the amino acid sequence of SEQ ID NO:72.

[0011f] In yet another aspect, there is provided a polypeptide consisting of SEQ ID NO:72 and SEQ ID NO:53.

[0011g] In yet another aspect, there is provided a cell producing the Wnt-binding agent as defined herein.

[0011h] In yet another aspect, there is provided a cell comprising the polypeptide as defined herein.

[0011i] In yet another aspect, there is provided a composition comprising the Wnt-binding agent as defined herein, wherein at least about 80% of the Wnt-binding agent has an N-terminal amino acid sequence of ASA.

[0011j] In yet another aspect, there is provided a pharmaceutical composition comprising the Wnt-binding agent as defined herein, and a pharmaceutically acceptable carrier.

[0011k] In yet another aspect, there is provided a polynucleotide comprising a polynucleotide that encodes the Wnt-binding agent as defined herein.

[0011l] In yet another aspect, there is provided a polynucleotide comprising a polynucleotide that encodes the polypeptide as defined herein.

[0011m] In yet another aspect, there is provided a cell comprising the polynucleotide as defined herein.

[0011n] In yet another aspect, there is provided the use of the Wnt-binding agent as defined herein in the manufacture of a medicament for the treatment of cancer.

[0011o] In yet another aspect, there is provided the use of the Wnt-binding agent as defined herein in the manufacture of a medicament for treatment of a disease associated with Wnt signaling activation.

[0011p] In yet another aspect, there is provided a method of producing the Wnt-binding agent as defined herein, wherein at least about 80% of the Wnt-binding agent has an N-terminal amino acid sequence of ASA, the method comprising using a signal sequence of SEQ ID NO:72for production of the Wnt-binding agent.

[0011q] In yet another aspect, there is provided a polypeptide comprising:
(a) a signal sequence of SEQ ID NO:72;
(b) a Fri domain of human FZD8; and
(c) a human Fc region.

[0011r] In yet another aspect, there is provided a polynucleotide comprising a polynucleotide that encodes the polypeptide as defined herein.

[0011s] In yet another aspect, there is provided a cell comprising the polynucleotide as defined herein.

[0011t] In yet another aspect, there is provided a cell comprising the polypeptide as defined herein.

[0011u] In yet another aspect, there is provided a method of producing a soluble Wnt-binding agent which comprises a Fri domain of human FZD8 in a cell, wherein at least about 80% of the Wnt-binding agent has an N-terminal amino acid sequence of ASA, the method comprising using a signal sequence of SEQ ID NO:72 for production of the Wnt-binding agent.

[0011v] In yet another aspect, there is provided a soluble Wnt-binding agent produced by the method as defined herein.

[0011w] In yet another aspect, there is provided a composition comprising a soluble Wnt-binding agent which comprises a Fri domain of human FZD8, wherein at least about 80% of the Wnt-binding agent has an N-terminal sequence of ASA.

[0012] Also disclosed is a method of inhibiting the growth of a tumor. In certain examples, the method comprises contacting the tumor with an effective amount of an agent that binds to one or more Wnt proteins (e.g., human Wnt proteins). The method may be *in vivo* or *in vitro*. In certain examples, the tumor is in a subject, and the contacting of the tumor with the agent comprises administration of a therapeutically effective amount of the Wnt-binding agent to the subject.

[0013] Also disclosed is a method of reducing the frequency of cancer stem cells in a tumor comprising cancer stem cells. Accordingly, the disclosure also provides methods of reducing the tumorigenicity of tumors. In some examples, the methods comprise contacting the tumor with an effective amount of an agent that binds to one or more Wnt proteins (e.g., human Wnt proteins). The method may be *in vivo* or *in vitro*. For example, the contacting may comprise administration of the effective amount of the Wnt-binding agent to a human having the tumor.

[0014] Also disclosed is a method of inducing cells in a tumor to differentiate or inducing expression of differentiation markers in a tumor. In certain examples, the method comprises contacting the tumor with an effective amount of an agent that binds to one or more Wnt proteins (e.g., human Wnt proteins). The method may be *in vivo* or *in vitro*.

[0015] Also disclosed is a method of treating a cancer in a subject, comprising administering a therapeutically effective amount of the Wnt-binding agent to the subject.

[0016] Also disclosed is a method of treating a disease in a subject wherein the disease is associated with Wnt signaling activation, comprising administering a therapeutically effective amount of the Wnt-binding agent to the subject.

[0017] Also disclosed is a method of treating a disorder in a subject, wherein the disorder is characterized by an increased level of stem cells and/or progenitor cells, comprising administering a therapeutically effective amount of the Wnt-binding agent to the subject.

[0018] In certain examples of each of the aforementioned aspects, as well as other aspects provided herein, the Wnt-binding agent is a polypeptide. In certain examples, the agent is a soluble receptor.

[0019] Also disclosed herein, the agent comprises a Fri domain of a FZD receptor, or a fragment of a FZD Fri domain that binds one or more Wnt proteins. In certain examples, the FZD receptors are human FZD receptors. For example, the Fri domain may be from human FZD4 or human FZD5. In certain alternative examples, the agent may comprise a Fri domain of a human FZD8 receptor or a fragment of that Fri domain that binds one or more Wnt proteins. In certain examples, the Wnt-binding agent is a soluble FZD receptor. In alternative examples, the Wnt-binding agent does not comprise a Fri domain of a FZD receptor.

[0020] Also disclosed herein, the agent comprises a Fri domain of a soluble Frizzled-related protein (SFRP), or a fragment of a SFRP Fri domain that binds one or more Wnt proteins. In certain examples the SFRP is a human SFRP.

[0021] Also disclosed herein, the agent comprises a Fri domain of a Ror protein or a fragment of a Ror Fri domain that binds one or more Wnt proteins. In certain examples the Ror protein is a human Ror protein.

[0022] Also disclosed herein, the agent further comprises a human Fc region. In certain examples, the Wnt-binding agent is a fusion protein. In certain examples, the Wnt-binding agent comprises SEQ ID NO:1. In certain examples, the Wnt-binding agent comprises SEQ ID NO:46. In certain examples, the Wnt-binding agent comprises SEQ ID NO:48. In certain examples, the Wnt-binding agent comprises SEQ ID NO:50. In certain examples, the Wnt-binding agent comprises SEQ ID NO:53. In some examples, the Wnt-binding agent (before signal sequence cleavage) comprises SEQ ID NO:50 and a signal sequence selected from the group consisting of SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, and SEQ ID NO:74. In some examples, the Wnt-binding agent (before signal sequence cleavage) comprises SEQ ID NO:50 and a signal sequence selected from the group consisting of SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, and SEQ ID NO:74. In some examples, the Wnt-binding agent (before signal sequence cleavage) comprises SEQ ID NO:50 and SEQ ID NO:71. In some examples, the Wnt-binding agent (before signal sequence cleavage) comprises SEQ ID NO:53 and a signal sequence selected from the group consisting of SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, and SEQ ID NO:74. In some examples, the Wnt-binding agent (before signal sequence cleavage) comprises SEQ ID NO:53 and a signal sequence selected from the group consisting of SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, and SEQ ID NO:74. In some examples, the Wnt-binding agent (before signal sequence cleavage) comprises SEQ ID NO:53 and SEQ ID NO:71.

[0023] Also disclosed herein, the Wnt-binding agent binds to one or more, two or more, three or more, or four or more human Wnt proteins selected from the group consisting of Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt10a, and Wnt10b. In certain examples, the agent binds to Wnt1, Wnt2, Wnt3, Wnt3a, and Wnt7b.

[0024] Also disclosed herein, the agent is a Wnt antagonist. In certain examples, the agent inhibits Wnt signaling. In certain examples, the agent inhibits Wnt canonical Wnt signaling.

[0025] Also disclosed herein, the tumor or cancer is a tumor/cancer selected from the group consisting of colorectal tumor/cancer, pancreatic tumor/cancer, lung tumor/cancer, ovarian tumor/cancer, liver tumor/cancer, breast tumor/cancer, kidney tumor/cancer, prostate tumor/cancer, gastrointestinal tumor/cancer, melanoma, cervical tumor/cancer, bladder tumor/cancer, glioblastoma, and head and neck tumor/cancer.

[0026] Also disclosed herein, the methods further comprise contacting the tumor with a second therapeutic agent or administering a second therapeutic agent to the subject. In certain examples, the second therapeutic agent is a chemotherapeutic agent. In certain examples, the second chemotherapeutic agent is an antimetabolite (e.g., gemcitabine) or an antimitotic agent (e.g., a taxane such as paclitaxel).

[0027] Also disclosed is a polypeptide comprising a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:65 and SEQ ID NO:66, as well as cells producing and compositions comprising the polypeptide. Pharmaceutical compositions comprising the polypeptide and a pharmaceutically acceptable carrier are also provided. In addition, polynucleotides comprising a polynucleotide that encodes the polypeptides of SEQ ID NO:1, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:65 and SEQ ID NO:66 or having the sequence of SEQ ID NO:2 are also provided. Vectors and cells comprising the polynucleotides are likewise provided.

[0028] Also disclosed is a method of screening an agent for anti-tumor activity and/or activity against cancer stem cells. In certain examples, the method comprises comparing the level of one or more differentiation markers and/or one or more stemness markers in a first solid tumor (e.g., a solid tumor comprising cancer stem cells) that has been exposed to an agent to the level of the one or more differentiation markers in a second solid tumor that has not been exposed to the agent. In some examples, the method comprises: (a) exposing a first solid tumor, but not a second solid tumor, to the agent; (b) assessing the level of one or more differentiation markers and/or one or more

stemness markers in the first and second solid tumors; and (c) comparing the level of the one or more differentiation markers in the first tumor to the level of the one or more differentiation markers in the second solid tumor. In certain examples, the (a) increased levels of the one or more differentiation markers in the first solid tumor relative to the second solid tumor indicates anti-tumor or anti-cancer stem cell activity of the agent; and/or (b) decreased levels of the one or more stemness markers indicates anti-tumor or anti-cancer stem cell activity of the agent. In certain examples, the agent binds one or more Wnt proteins. In certain examples, the agent is a soluble FZD receptor. In certain methods, the agent is an antibody, such as an anti-FZD or anti-Wnt antibody. In certain alternative examples, the agent is a small molecule.

[0029] Where examples of the disclosure are described in terms of a Markush group or other grouping of alternatives, the present disclosure encompasses not only the entire group listed as a whole, but each member of the group individually and all possible subgroups of the main group, but also the main group absent one or more of the group members. The present disclosure also envisages the explicit exclusion of one or more of any of the group members in the claimed disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0030] Figure 1. Pharmacokinetics of FZD8-Fc (54F03) in the rat. Administration of a single dose (10mg/kg) of FZD8-Fc was followed by assessment of the pharmacokinetic properties of FZD8-Fc. Serum concentrations of FZD8-Fc were determined at 1, 24, 48, 72, 96, 168, 240 and 336 hours post-administration.

[0031] Figure 2. Inhibition of PN4 pancreas tumor growth by FZD8-Fc (54F03) treatment. PN4 pancreas tumor cells were injected subcutaneously into NOD/SCID mice. Mice were treated with FZD8-Fc (-▲-), gemcitabine (-■-), a combination of FZD8-Fc and gemcitabine (-Δ-), or a control antibody (-●-). Data are shown as tumor volume (mm^3), over days post treatment.

[0032] Figure 3. Reduction of $CD44^{hi}$ cell population in PN4 tumors treated with FZD8-Fc (54F03). Cell surface staining for ESA and CD44 on tumor cells treated with control antibody, FZD8-Fc, gemcitabine, or a combination of FZD8-Fc and gemcitabine was performed. For each treatment group, single cell suspensions from five tumors were pooled prior to staining.

[0033] Figure 4. *In vivo* limiting dilution assay of FZD8-Fc (54F03)-treated PN4 pancreatic tumors.

[0034] Figure 5. Increased cell differentiation of PN4 pancreatic tumors treated with FZD8-Fc (54F03). Paraffin embedded sections of PN4 tumors treated with control antibody, FZD8-Fc, gemcitabine, or a combination of FZD8-Fc and gemcitabine were stained with alcian blue to detect mucin-expressing cells.

[0035] Figure 6. Increased cell differentiation of PN8 pancreatic tumors treated with FZD8-Fc (54F03). Paraffin embedded sections of PN8 tumors treated with control antibody, FZD8-Fc, gemcitabine, or a combination of FZD8-Fc and gemcitabine were stained with alcian blue to detect mucin-expressing cells.

[0036] Figure 7. Increased cell differentiation and reduced proliferation in PN13 pancreatic tumors following treatment with FZD8-Fc (54F03). Paraffin embedded sections of PN13 tumors treated with control antibody or FZD8-Fc were stained with alcian blue to detect mucin-expressing cells. In addition, the sections were stained for Ki67, a marker of actively proliferating cells.

[0037] Figure 8. Increased Muc16 staining in PN13 pancreatic tumors following treatment with FZD8-Fc (54F03). Paraffin embedded sections of PN13 tumors treated with a control antibody or FZD8-Fc were stained for Muc16 protein.

[0038] Figure 9. Increased CK20 staining in PN13 pancreatic tumors following treatment with FZD8-Fc (54F03). Paraffin embedded sections of PN13 tumors treated with a control antibody or FZD8-Fc were stained for CK20 protein.

[0039] Figure 10. Inhibition of PE13 breast tumor growth following treatment with FZD8-Fc (54F03) in combination with paclitaxel. PE13 breast tumor cells were injected subcutaneously into NOD/SCID mice. Mice were treated with a control antibody (-●-), FZD8-Fc (□), paclitaxel (-▲-), or a combination of FZD8-Fc and paclitaxel (-○-). Data is shown as tumor volume (mm^3) over days post treatment.

[0040] Figure 11. Dose-dependent inhibition of C28 colon tumor growth with FZD8-Fc (54F03). C28 colon tumor cells were injected subcutaneously into NOD/SCID mice. Mice were treated with FZD8-Fc 1.5mg/kg twice weekly (-▲-), 5mg/kg once weekly (-▼-), 5mg/kg twice weekly (-○-),15mg/kg once weekly (-□-) or 15mg/kg twice weekly (-Δ-) or control antibody (-■-). Data is shown as tumor volume (mm^3) over days post treatment (Fig. 11A). Inhibition of colon tumor growth with FZD8-Fc in combination with irinotecan. Mice were treated with FZD8-Fc (-▲-), irinotecan (-▼-), a combination of FZD8-Fc and irinotecan (-●-), or a control antibody (-■-). Data is shown as tumor volume (mm^3) over days post treatment (Fig. 11B).

[0041] Figure 12. Increased CK20 staining in C28 tumors following treatment with FZD8-Fc (54F03). Paraffin embedded sections of C28 tumors treated with a control antibody or FZD8-Fc were stained for CK20 protein.

[0042] Figure 13. Inhibition of PN21 pancreatic tumor growth and decrease in cancer stem cell frequency by FZD8-Fc (54F03) treatment. PN21 pancreatic tumor cells were injected subcutaneously into NOD/SCID mice. Mice were treated with FZD8-Fc (-▼-), gemcitabine (-▲-), a combination of FZD8-Fc and gemcitabine (-■-), or a control antibody (-●-). Data are shown as tumor volume (mm^3), over days post treatment (Fig. 13A). *In vivo* limiting dilution assay of FZD8-Fc treated PN21 pancreatic tumors (Fig. 13B).

[0043] Figure 14. Increased cell differentiation of PN21 pancreatic tumors following treatment with FZD8-Fc (54F03). Paraffin embedded sections of PN21 tumors treated with control antibody or FZD8-Fc were stained with alcian blue to detect mucins.

[0044] Figure 15. Increased cell differentiation and reduced proliferation in PN21 pancreatic tumors following treatment with FZD8-Fc (54F03). Paraffin embedded sections of PN21 tumors treated with control antibody, FZD8-Fc, gemcitabine, or a combination of FZD8-Fc and gemcitabine were stained with alcian blue to detect mucins. In addition, the sections were stained for Ki67, a marker of actively proliferating cells.

[0045] Figure 16. Pharmacokinetics of FZD8-Fc variants in monkeys. Administration of a single dose (30mg/kg) of FZD8-Fc variants 54F15 and 54F16 was followed by assessment of the pharmacokinetic properties of the variants. Serum concentrations of 54F15 (-○-) and 54F16 (-♦-) were determined at 1, 6, 12, 24, 48, 72, 96, 168, 240 and 336 hours post-administration.

[0046] Figure 17. Inhibition of C28 colon tumor growth following treatment with FZD8-Fc variants. C28 colon tumor cells were injected subcutaneously into NOD/SCID mice. Mice were treated with a control antibody (-X-), 54F03 (-□-), 54F09 (-▲-), 54F12 (-▼-), 54F13 (-♦-), 54F15 (-○-) or 54F16 (-Δ-). Data is shown as tumor volume (mm^3) over days post treatment.

[0047] Figure 18. Inhibition of PN4 pancreatic tumor growth following treatment with FZD8-Fc variants. PN4 colon tumor cells were injected subcutaneously into NOD/SCID mice. Mice were treated with a control antibody (-●-), 54F03 (-■-), 54F09 (-▼-), 54F12 (-○-), 54F13 (-▲-), 54F15 (-□-) or 54F16 (-♦-). Data is shown as tumor volume (mm^3) over days post treatment.

[0048] Figure 19. Reduction of $CD44^{hi}$ and $CD44^{+}CD201^{+}$ cells in PN4 tumors treated with FZD8-Fc variants 54F03 and 54F16. Cell surface staining for ESA, CD44 and CD201 on tumor cells treated with control antibody, FZD8-Fc variant 54F03 or variant 54F16 was performed and analyzed by FACS.

[0049] Figure 20. Characterization of N-termini of FZD8-Fc proteins. FZD8-Fc variants were analyzed by mass spectrometry and results for 54F16 (Fig. 20A), 54F26 (Fig. 20B), 54F28 (Fig. 20C), 54F30 (Fig. 20D), and 54F32 (Fig. 20E) are shown.

[0050] Figure 21. Inhibition of C28 colon tumor growth following treatment with FZD8-Fc variants. C28 colon tumor cells were injected subcutaneously into NOD/SCID mice. Mice were treated with a control antibody (-■-), 54F03 (-Δ-), 54F23 (-▼-), or 54F26 (-○-). Data is shown as tumor volume (mm^3) over days post treatment.

DETAILED DESCRIPTION OF THE INVENTION

[0051] The present invention provides novel agents, including, but not limited to, polypeptides comprising the Fri domain of human Frizzled (FZD) receptors, human secreted Frizzled-related proteins (SFRPs), or Ror proteins that bind to one or more human Wnts. Related polypeptides and polynucleotides, compositions comprising the Wnt-binding agents, and methods of making the Wnt-binding agents are also provided. Methods of using the Wnt-binding agents, such as methods of inhibiting

tumor growth, treating cancer, inducing differentiation, and reducing tumorigenicity are further provided. Methods of screening agents to identify novel Wnt-binding agents with anti-tumor activity and/or anti-cancer stem cell activity are also provided.

[0052] A Wnt-binding agent comprising the Fri domain of human FZD8 and a Fc domain was produced and referred to herein as FZD8-Fc or FZD8-Fc (54F03) (Example 1). A number of variants of the FZD8-Fc protein were generated (Example 10). FZD8-Fc proteins were produced that were shown to be approximately 95% or greater homogeneous at the N-termini (Example 16). Pharmacokinetic studies using several of the FZD8-Fc variants were done in rats showing that the half-life of the FZD8-Fc variants was at least 100 hours (Examples 2 and 12; Figure 1 and Table 4). A pharmacokinetic study was undertaken in monkeys with FZD8-Fc variants 54F15 and 54F16, which demonstrated that these proteins had a half-life of at least 100 hours (Example 13 and Table 5). Treatment with FZD8-Fc (54F03), either alone or in combination with a chemotherapeutic agent was shown to reduce the growth of pancreatic tumors, breast tumors and colon tumors (Examples 3, 5, 6 and 8 and Figures 2, 10, 11B, and 13A). Furthermore, the treatment was shown to decrease the percentage of $CD44^{+}$ cells and to reduce the frequency of cancer stem cells in the pancreatic model (Examples 3 and 8 and Figures 3, 4, and 13B). Treatment with FZD8-Fc (54F03), either alone or in combination with a chemotherapeutic agent was shown to increase cell differentiation of pancreatic tumor cells and colon tumor cells (Examples 4, 7 and 9 and Figures 5-9, 12, 14, and 15). Treatment with FZD8-Fc variants demonstrated inhibition of tumor growth in colon and pancreatic tumors, with the extent of inhibition depending upon the variant (Examples 14, 15, and 17 and Figures 17, 18, and 21). Treatment with FZD8-Fc variant 54F03 and variant 54F16 was shown to decrease the percentage of $CD44^{hi}$ cells, as well as $CD44^{+}CD202^{+}$ cells in pancreatic tumors (Example 15 and Figure 19).

I. Definitions

[0053] The term "antagonist" is used herein to include any molecule that partially or fully blocks, inhibits, or neutralizes the expression of or the biological activity of a protein, (e.g., a cancer stem cell marker). The blocking, inhibiting, and/or neutralizing of biological activity includes, but is not limited to, inhibition of tumor growth. The term "antagonist" includes any molecule that partially or fully blocks, inhibits, or neutralizes a biological activity of the Wnt pathway. Suitable antagonist molecules include, but are not limited to, fragments and/or amino acid sequence variants of native FZD receptor proteins including soluble FZD receptors, as well as derivatives of SFRPs and derivatives of Ror proteins.

[0054] A polypeptide, antibody, polynucleotide, vector, cell, or composition which is "isolated" is a polypeptide, antibody, polynucleotide, vector, cell, or composition which is in a form not found in nature. Isolated polypeptides, antibodies, polynucleotides, vectors, cells or compositions include those which have been purified to a degree that they are no longer in a form in which they are found in nature. In

some embodiments, an antibody, polynucleotide, vector, cell, or composition which is isolated is substantially pure.

[0055] As used herein, "substantially pure" refers to material which is at least 50% pure (i.e., free from contaminants), more preferably at least 90% pure, more preferably at least 95% pure, more preferably at least 98% pure, more preferably at least 99% pure.

[0056] As used herein the term "soluble receptor" refers to an N-terminal extracellular fragment of a receptor protein preceding the first transmembrane domain of the receptor that can be secreted from a cell in soluble form. In some embodiments, the receptor protein is a FZD receptor. In some embodiments, the receptor protein is a Ror receptor.

[0057] As used herein the term "FZD soluble receptor" refers to an N-terminal extracellular fragment of a human FZD receptor protein preceding the first transmembrane domain of the receptor that can be secreted from a cell in soluble form. Both FZD soluble receptors comprising the entire N-terminal extracellular domain (ECD) (referred to herein as "FZD ECD") as well as smaller fragments are envisioned. FZD soluble receptors comprising the Fri domain (referred to herein as "FZD Fri") are also disclosed. FZD Fri soluble receptors can demonstrate altered biological activity, (e.g., increased protein half-life) compared to soluble receptors comprising the entire FZD ECD. Protein half-life can be further increased by covalent modification with polyethylene glycol (PEG) or polyethylene oxide (PEO). FZD soluble receptors include FZD ECD or Fri domains linked in-frame to other functional and structural proteins including, but not limited to, a human Fc region (e.g., human Fc derived from immunoglobulins IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, or IgM); protein tags (e.g., myc, FLAG, GST); other endogenous proteins or protein fragments; or any other useful protein sequence including any linker region between a FZD ECD or Fri domain and a linked protein. In certain embodiments, the Fri domain of a FZD receptor is directly linked to a human Fc region. In certain embodiments, the Fri domain of a FZD receptor is linked to human IgG1 Fc (referred to herein as "FZD Fri.Fc"). In some embodiments, the Fri domain of a FZD receptor is linked to a human Fc region with a peptide linker. FZD soluble receptors also include variant proteins comprising amino acid insertions, deletions, substitutions, and/or conservative substitutions.

[0058] As used herein, the term "linker" or "linker region" refers to a linker inserted between a first polypeptide (e.g., a FZD component) and a second polypeptide (e.g., a Fc region). In some embodiments, the linker is a peptide linker. Linkers should not adversely affect the expression, secretion, or bioactivity of the polypeptides. Preferably, linkers are not antigenic and do not elicit an immune response.

[0059] As used herein, the terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals in which a population of cells are characterized by unregulated cell growth. The term cancer is understood to encompass Wnt-dependent cancers. Examples of cancers include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such

cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, skin cancer, melanoma, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

[0060] The terms "proliferative disorder" and "proliferative disease" refer to disorders associated with abnormal cell proliferation such as cancer.

[0061] "Tumor" and "neoplasm" as used herein refer to any mass of tissue that results from excessive cell growth or proliferation, either benign (noncancerous) or malignant (cancerous) including pre-cancerous lesions. In certain embodiments, the tumor is an epithelial tumor. In certain embodiments, the tumor is a Wnt-dependent tumor.

[0062] As used herein, the term "subject" refers to any animal (e.g., a mammal), including, but not limited to humans, non-human primates, canines, felines, rodents, and the like, which is to be the recipient of a particular treatment. Typically, the terms "subject" and "patient" are used interchangeably herein in reference to a human subject.

[0063] The terms "cancer stem cell" and "CSC" and "tumor stem cell" and "solid tumor stem cell" are used interchangeably herein and refer to a population of cells from a solid tumor that: (1) have extensive proliferative capacity; (2) are capable of asymmetric cell division to generate one or more kinds of differentiated progeny with reduced proliferative or developmental potential; and (3) are capable of symmetric cell divisions for self-renewal or self-maintenance. These properties of "cancer stem cells," "CSCs," "tumor stem cells," or "solid tumor stem cells" confer on those cancer stem cells the ability to form palpable tumors upon serial transplantation into an immunocompromised host (e.g., a mouse) compared to the majority of tumor cells that fail to form tumors. Cancer stem cells undergo self-renewal versus differentiation in a chaotic manner to form tumors with abnormal cell types that can change over time as mutations occur.

[0064] The terms "cancer cell" and "tumor cell" and grammatical equivalents refer to the total population of cells derived from a tumor including both non-tumorigenic cells, which comprise the bulk of the tumor cell population, and tumorigenic stem cells also referred to herein as cancer stem cells.

[0065] The term "tumorigenic" refers to the functional features of a solid tumor stem cell including the properties of self-renewal (giving rise to additional tumorigenic cancer stem cells) and proliferation to generate all other tumor cells (giving rise to differentiated and thus non-tumorigenic tumor cells) that allow solid tumor stem cells to form a tumor. These properties of self-renewal and proliferation to generate all other tumor cells confer on cancer stem cells the ability to form palpable tumors upon serial transplantation into an immunocompromised host (e.g., a mouse) compared to non-tumorigenic tumor

cells, which are unable to form tumors upon serial transplantation. It has been observed that non-tumorigenic tumor cells may form a tumor upon primary transplantation into an immunocompromised host (e.g., a mouse) after obtaining the tumor cells from a solid tumor, but those non-tumorigenic tumor cells do not give rise to a tumor upon serial transplantation.

[0066] As used herein an "acceptable pharmaceutical carrier" or "pharmaceutically acceptable carrier" refers to any material that, when combined with an active ingredient of a pharmaceutical composition such as a therapeutic polypeptide, allows the therapeutic polypeptide, for example, to retain its biological activity. In addition, an "acceptable pharmaceutical carrier" does not trigger an immune response in a recipient subject. In some embodiments, the term "pharmaceutical vehicle" is used interchangeably with "pharmaceutical carrier". Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, and various oil/water emulsions. Examples of diluents for aerosol or parenteral administration are phosphate buffered saline or normal (0.9%) saline.

[0067] The term "therapeutically effective amount" refers to an amount of an agent (e.g., a soluble receptor or other drug) effective to "treat" a disease or disorder in a subject or mammal. In the case of cancer, the therapeutically effective amount of the agent (e.g., a soluble receptor) can reduce the number of cancer cells; reduce the tumor size; reduce the frequency of cancer stem cells; inhibit and/or stop cancer cell infiltration into peripheral organs; inhibit and/or stop tumor metastasis; inhibit and/or stop tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. To the extent the agent (e.g., a soluble receptor) prevents growth and/or kills existing cancer cells, it can be referred to as cytostatic and/or cytotoxic.

[0068] As used herein the term "inhibit tumor growth" refers to any mechanism by which tumor cell growth can be inhibited. In certain embodiments, tumor cell growth is inhibited by slowing proliferation of tumor cells. In certain embodiments, tumor cell growth is inhibited by halting proliferation of tumor cells. In certain embodiments, tumor cell growth is inhibited by killing tumor cells. In certain embodiments, tumor cell growth is inhibited by inducing apoptosis of tumor cells. In certain embodiments, tumor cell growth is inhibited by inducing differentiation of tumor cells. In certain embodiments, tumor cell growth is inhibited by depriving tumor cells of nutrients. In certain embodiments, tumor cell growth is inhibited by preventing migration of tumor cells. In certain embodiments, tumor cell growth is inhibited by preventing invasion of tumor cells.

[0069] Terms such as "treating" and "treatment" and "to treat" and "alleviating" and "to alleviate" refer to both 1) therapeutic measures that cure, slow down, lessen symptoms of, and/or halt progression of a diagnosed pathologic condition or disorder, and 2) prophylactic or preventative measures that prevent or slow the development of a targeted pathologic condition or disorder. Thus, those in need of treatment include those who already have the disorder; those prone to have the disorder; and those in whom the disorder is to be prevented. In certain embodiments, a subject is successfully "treated" for cancer

according to the methods of the present invention if the patient shows one or more of the following: a reduction in the number of, or complete absence of, cancer or tumor cells; a reduction in the tumor size; inhibition of, or an absence of, cancer or tumor cell infiltration into peripheral organs including, for example, the spread of cancer into soft tissue and bone; inhibition of, or an absence of, tumor metastasis; inhibition of, or an absence of, tumor or cancer growth; relief of one or more symptoms associated with the specific cancer; reduced morbidity and mortality; improvement in quality of life; reduction in tumorigenicity, tumorgenic frequency, or tumorgenic capacity of a tumor; reduction in the number or frequency of cancer stem cells in the tumor; differentiation of tumorigenic cells to a non-tumorigenic state; or some combination of these effects.

[0070] As used herein, the terms "polynucleotide" and "nucleic acid" refer to a polymer composed of a multiplicity of nucleotide units (ribonucleotide or deoxyribonucleotide or related structural variants) linked via phosphodiester bonds, including but not limited to, DNA or RNA. The term encompasses sequences that include any of the known base analogs of DNA and RNA including, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxyl-methyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethyl-aminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudo-uracil, 1-methylguanine, 1-methylinosine, 2,2-dimethyl-guanine, 2-methyladenine, 2-methylguanine, 3-methyl-cytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxy-amino-methyl-2-thiouracil, beta D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps"; substitution of one or more of the naturally occurring nucleotides with an analog; internucleotide modifications such as uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.); pendant moieties, such as proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.); intercalators (e.g., acridine, psoralen, etc.); chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.); alkylators; modified linkages (e.g., alpha anomeric nucleic acids, etc.); as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH can

be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, alpha-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, heptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), (O)NR2 ("amidate"), P(O)R, P(O)OR', CO or CH2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (--O--) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical.

[0071] As used herein, the term "vector" is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The term "vector" means a construct, which is capable of delivering, and preferably expressing, one or more gene(s) or sequence(s) of interest to a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, phagemid, cosmid or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, and DNA or RNA expression vectors encapsulated in liposomes.

[0072] The terms "polypeptide" and "peptide" and "protein" and "protein fragment" are used interchangeably herein to refer to a polymer of amino acid residues of any length. The terms apply to amino acid polymers in which one or more amino acid residue in the polymer is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. It is understood that, because the polypeptides of this invention are based, at least in part, upon antibodies, in certain embodiments, the polypeptides can occur as single chains or associated chains.

[0073] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function similarly to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, gamma-carboxyglutamate, and O-phosphoserine. Amino acid

analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, e.g., an alpha carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs can have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetic refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function similarly to a naturally occurring amino acid.

[0074] That a polypeptide or other agent "specifically binds" to a protein means that the polypeptide or other agent reacts or associates more frequently, more rapidly, with greater duration, with greater affinity, or with some combination of the above to the protein than with alternative substances, including unrelated proteins. In certain embodiments, "specifically binds" means, for instance, that an agent binds to a protein with a K_D of about 0.1mM or less, but more usually less than about 1μM. In certain embodiments, "specifically binds" means that an agent binds to a protein at times with a K_D of at least about 0.1μM or less, at least about 0.01μM or less, and at other times at least about 1nM or less. Because of the sequence identity between homologous proteins in different species, specific binding can include an agent that recognizes a particular protein such as a Wnt protein in more than one species. Likewise, because of homology between different Wnt proteins in certain regions of the sequences of the Wnts, specific binding can include an polypeptide (or other agent) that recognizes more than one Wnt protein. It is understood that an agent that specifically binds to a first target may or may not specifically bind to a second target. As such, "specific binding" does not necessarily require (although it can include) exclusive binding, i.e. binding to a single target. Thus, an agent may, in certain embodiments, specifically bind to more than one target (e.g., multiple different human Wnts). Generally, but not necessarily, reference to binding means specific binding.

[0075] The terms "identical" or "percent identity" in the context of two or more nucleic acids or polypeptides, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned (introducing gaps, if necessary) for maximum correspondence, not considering any conservative amino acid substitutions as part of the sequence identity. The percent identity may be measured using sequence comparison software or algorithms or by visual inspection. Various algorithms and software that may be used to obtain alignments of amino acid or nucleotide sequences are known in the art. One such non-limiting example of a sequence alignment algorithm is the algorithm described in Karlin et al, 1990, *Proc. Natl. Acad. Sci.*, 87:2264-2268, as modified in Karlin et al., 1993, *PNAS,* 90:5873-5877, and incorporated into the NBLAST and XBLAST programs (Altschul et al., 1991, *Nucleic Acids Res.*, 25:3389-3402). Additional publicly available software programs that can be used to align sequences include, but are not limited to, Gapped BLAST, BLAST-2, WU-BLAST-2 (Altschul et al., 1996, *Methods in Enzymology,*

266:460-480), ALIGN, ALIGN-2 (Genentech, South San Francisco, California), Megalign (DNASTAR), and the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711).

[0076] In some embodiments, two nucleic acids or polypeptides of the invention are substantially identical, meaning they have at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, and in some embodiments at least 95%, 96%, 97%, 98%, 99% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm or by visual inspection. In some embodiments, identity exists over a region of the sequences that is at least about 10, at least about 20, at least about 40-60, at least about 60-80 residues in length or any integral value therebetween. In some embodiments, identity exists over a longer region than 60-80 residues, such as at least about 90-100 residues. In some embodiments, the sequences are substantially identical over the full length of the sequences being compared, such as the coding region of a nucleotide sequence.

[0077] A "conservative amino acid substitution" is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). For example, substitution of a phenylalanine for a tyrosine is a conservative substitution. Preferably, conservative substitutions in the sequences of the polypeptides and other agents of the invention do not abrogate the binding of the polypeptide containing the amino acid sequence, to the target(s), i.e., the one or more Wnts to which the polypeptide or other agent binds. Methods of identifying nucleotide and amino acid conservative substitutions which do not eliminate target binding are well-known in the art (see, e.g., Brummell et al., 1993, *Biochem.,* 32: 1180-87; Kobayashi et al., 1999, *Protein Eng.* 12:879-84; and Burks et al., 1997, *PNAS,* 94:412-17).

[0078] As used herein, "about" refers to plus or minus 10% of the indicated number. For example, "about 10%" indicates a range of 9% to 11%.

[0079] As used in the present disclosure and claims, the singular forms "a" "an" and "the" include plural forms unless the context clearly dictates otherwise.

[0080] It is understood that wherever embodiments are described herein with the language "comprising," otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[0081] The term "and/or" as used in a phrase such as "A and/or B" herein is intended to include both A and B; A or B; A (alone), and B (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following embodiments: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

II. Wnt-binding agents

[0082] The present invention provides agents that bind (e.g., specifically bind) one or more human Wnt proteins (Wnts). These agents are referred to herein as "Wnt-binding agent(s)." In certain embodiments, the agents specifically bind one, two, three, four, five, six, seven, eight, nine, ten, or more Wnt proteins. By way of non-limiting example, the Wnt-binding agent may bind Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt10a, and/or Wnt10b. In certain embodiments, the Wnt-binding agent binds Wnt1, Wnt2, Wnt3, Wnt3a, and Wnt7b.

[0083] In certain embodiments, the Wnt-binding agent is a Wnt antagonist. In certain embodiments, the agent inhibits Wnt-signaling. In some embodiments, the agent inhibits canonical Wnt signaling.

[0084] In certain embodiments, the Wnt-binding agent is a polypeptide. In certain embodiments, the Wnt-binding agent is a soluble receptor.

[0085] In certain embodiments, the Wnt-binding agent comprises the extracellular domain of a FZD receptor. In some embodiments, the Wnt-binding agent comprises a Fri domain of a FZD receptor. In certain embodiments, the FZD receptor is a human FZD receptor. In certain embodiments, the human FZD receptor is FZD1, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9, or FZD10. In some alternative embodiments, the Wnt-binding agent comprises a portion of a SFRP. In some embodiments, the Wnt-binding agent comprises a Fri domain of a SFRP. In certain embodiments, the SFRP is a human SFRP. In some embodiments, the human SFRP is SFRP1, SFRP2, SFRP3, SFRP4, or SFRP5. In other alternative embodiments, the Wnt-binding agent comprises the extracellular domain of a Ror protein. In some embodiments, the Wnt-binding agent comprises a Fri domain of a Ror protein. In certain embodiments, the Ror is a human Ror. In some embodiments, the human Ror is Ror1 or Ror2.

[0086] In certain embodiments, the Wnt-binding agent is a soluble receptor. In some embodiments, the Wnt-binding agent is a soluble protein. In certain embodiments, the Wnt-binding agent is a soluble FZD receptor. Nonlimiting examples of soluble FZD receptors can be found in U.S. Patent No. 7,723,477, which is incorporated by reference herein in its entirety. In certain embodiments, the Wnt-binding agent is a soluble SFRP or a soluble Ror receptor.

[0087] The Fri domain of FZD1 includes approximately amino acids 87-237 of SEQ ID NO:27. The Fri domain of FZD2 includes approximately amino acids 24-159 of SEQ ID NO:28. The Fri domain of FZD3 includes approximately amino acids 23-143 of SEQ ID NO:29. The Fri domain of FZD4 includes approximately amino acids 40-170 of SEQ ID NO:22. The Fri domain of FZD5 includes approximately

amino acids 27-157 of SEQ ID NO:23. The Fri domain of FZD6 includes approximately amino acids 19-146 of SEQ ID NO:24. The Fri domain of FZD7 includes approximately amino acids 33-170 of SEQ ID NO:25. The Fri domain of FZD8 includes approximately amino acids 28-158 of SEQ ID NO:30. The Fri domain of FZD9 includes approximately amino acids 23-159 of SEQ ID NO:31. The Fri domain of FZD10 includes approximately amino acids 21-154 of SEQ ID NO:26. The corresponding, predicted Fri domains for each of the human FZD receptors are provided as SEQ ID NOs:32-41. The minimal, core Fri domain sequences for each of the human FZD receptors (FZD1-10) are provided as SEQ ID NOs:3-12. The minimal, core Fri domain sequences for each of the human SFRPs (SFRP1-5) are provided as SEQ ID NOs:13-17. The minimal, core Fri domain sequences of human Ror1 and Ror2 are provided as SEQ ID NO:58 and SEQ ID NO:59. Those of skill in the art may differ in their understanding of the exact amino acids corresponding to the various Fri domains. Thus the N-terminus or C-terminus of the domains outlined above and herein may extend or be shortened by 1, 2, 3, 4, 5, 6, 7, 8, 9, or even 10 amino acids.

[0088] In certain embodiments, the Wnt-binding agent comprises a Fri domain of a human FZD receptor, or a fragment or variant of the Fri domain that binds one or more human Wnt proteins. In certain embodiments, the human FZD receptor is FZD1, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9, or FZD10. In certain embodiments, the human FZD receptor is FZD4. In certain alternative embodiments, the human FZD receptor is FZD5. In certain additional alternative embodiments, the human FZD receptor is FZD8. In certain embodiments, the FZD is FZD4 and the Wnt-binding agent comprises SEQ ID NO:6 or comprises approximately amino acids 40 to 170 of SEQ ID NO:19. In certain embodiments, the FZD is FZD5 and the Wnt-binding agent comprises SEQ ID NO:7 or comprises approximately amino acids 27-157 of SEQ ID NO:20. In certain embodiments, the FZD is FZD7 and the Wnt-binding agent comprises SEQ ID NO:9 or comprises approximately amino acids 33 to 170 of SEQ ID NO:25. In certain embodiments, the FZD is FZD8 and the Wnt-binding agent comprises SEQ ID NO:10 or comprises approximately amino acids 28-158 of SEQ ID NO:21. In certain embodiments, the FZD is FZD10 and the Wnt-binding agent comprises SEQ ID NO:12 or comprises approximately amino acids 21-154 of SEQ ID NO:26.

[0089] In certain embodiments, the Wnt-binding agent comprises a minimal Fri domain sequence selected from the group consisting of SEQ ID NOs:3-12. In certain embodiments, the Wnt-binding agent comprises a minimal Fri domain sequence selected from the group consisting of SEQ ID NOs:13-17. In certain embodiments, the Wnt-binding agent comprises a minimal Fri domain sequence selected from the group consisting of SEQ ID NO:58 and SEQ ID NO:59.

[0090] In certain embodiments, the Wnt-binding agent comprises a variant of any one of the aforementioned FZD Fri domain sequences that comprises one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc.) conservative substitutions and is capable of binding Wnt(s).

[0091] In certain alternative embodiments, the Wnt-binding agent comprises a Fri domain of a human SFRP, or a fragment or variant of such a Fri domain that binds to one or more human Wnt proteins. For example, in certain embodiments, the agent comprises a minimal SFRP Fri domain sequence selected from the group consisting of SEQ ID NOs:13-17. In certain embodiments, the Wnt-binding agent comprises a variant of any one of the aforementioned SFRP Fri domain sequences that comprises one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc.) conservative substitutions and maintains the ability to bind Wnt(s).

[0092] In certain alternative embodiments, the Wnt-binding agent comprises a Fri domain of a human Ror protein, or a fragment or variant of such a Fri domain that binds to one or more human Wnt proteins. For example, in certain embodiments, the agent comprises a minimal Ror Fri domain sequence selected from the group consisting of SEQ ID NO:58 and SEQ ID NO:59. In certain embodiments, the Wnt-binding agent comprises a variant of any one of the aforementioned Ror Fri domain sequences that comprises one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc.) conservative substitutions and maintains the ability to bind Wnt(s).

[0093] In certain embodiments, the Wnt-binding agent, such as an agent comprising a minimum Fri domain of a human FZD receptor or other soluble FZD receptor, further comprises a human Fc region (e.g., a human IgG1 Fc region). The Fc region can be obtained from any of the classes of immunoglobulin, IgG, IgA, IgM, IgD and IgE. In some embodiments, the Fc region is a wild-type Fc region. In some embodiments, the Fc region is a mutated Fc region. In some embodiments, the Fc region is truncated at the N-terminal end by 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids, (e.g., in the hinge domain). In some embodiments, an amino acid in the hinge domain is changed to hinder undesirable disulfide bond formation. In some embodiments, a cysteine is replaced with a serine to hinder undesirable disulfide bond formation. In certain embodiments, the Fc region comprises or consists of SEQ ID NO:18, SEQ ID NO:42, or SEQ ID NO:43.

[0094] In certain embodiments, a Wnt-binding agent is a fusion protein comprising at least a minimum Fri domain of a FZD receptor, a SFRP or Ror protein and a Fc region. As used herein, a "fusion protein" is a hybrid protein expressed by a nucleic acid molecule comprising nucleotide sequences of at least two genes. In some embodiments, the C-terminus of the first polypeptide is linked to the N-terminus of the immunoglobulin Fc region. In some embodiments, the first polypeptide (e.g., a FZD Fri domain) is directly linked to the Fc region (i.e. without an intervening peptide linker). In some embodiments, the first polypeptide is linked to the Fc region via a peptide linker.

[0095] As used herein, the term "linker" refers to a linker inserted between a first polypeptide (e.g., a FZD component) and a second polypeptide (e.g., a Fc region). In some embodiments, the linker is a peptide linker. Linkers should not adversely affect the expression, secretion, or bioactivity of the polypeptide. Linkers should not be antigenic and should not elicit an immune response. Suitable linkers

are known to those of skill in the art and often include mixtures of glycine and serine residues and often include amino acids that are sterically unhindered. Other amino acids that can be incorporated into useful linkers include threonine and alanine residues. Linkers can range in length, for example from 1-50 amino acids in length, 1-22 amino acids in length, 1-10 amino acids in length, 1-5 amino acids in length, or 1-3 amino acids in length. Linkers may include, but are not limited to, SerGly, GGSG, GSGS, GGGS, $S(GGS)_n$ where n is 1-7, GRA, poly(Gly), poly(Ala), ESGGGGVT (SEQ ID NO:60), LESGGGGVT (SEQ ID NO:61), GRAQVT (SEQ ID NO:62), WRAQVT (SEQ ID NO:63), and ARGRAQVT (SEQ ID NO:64). As used herein, a linker is an intervening peptide sequence that does not include amino acid residues from either the C-terminus of the first polypeptide (e.g., a FZD Fri domain) or the N-terminus of the second polypeptide (e.g., the Fc region).

[0096] FZD receptors, SFRPs and Ror proteins contain a signal sequence that directs the transport of the proteins. Signal sequences (also referred to as signal peptides or leader sequences) are located at the N-terminus of nascent polypeptides. They target the polypeptide to the endoplasmic reticulum and the proteins are sorted to their destinations, for example, to the inner space of an organelle, to an interior membrane, to the cell's outer membrane, or to the cell exterior via secretion. Most signal sequences are cleaved from the protein by a signal peptidase after the proteins are transported to the endoplasmic reticulum. The cleavage of the signal sequence from the polypeptide usually occurs at a specific site in the amino acid sequence and is dependent upon amino acid residues within the signal sequence. Although there is usually one specific cleavage site, more than one cleavage site may be recognized and/or used by a signal peptidase resulting in a non-homogenous N-terminus of the polypeptide. For example, the use of different cleavage sites within a signal sequence can result in a polypeptide expressed with different N-terminal amino acids. Accordingly, in some embodiments, the polypeptides as described herein may comprise a mixture of polypeptides with different N-termini. In some embodiments, the N-termini differ in length by 1, 2, 3, 4, or 5 amino acids. In some embodiments, the polypeptide is substantially homogeneous, i.e., the polypeptides have the same N-terminus. In some embodiments, the signal sequence of the polypeptide comprises one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc.) amino acid substitutions and/or deletions. In some embodiments, the signal sequence of the polypeptide comprises amino acid substitutions and/or deletions that allow one cleavage site to be dominant, thereby resulting in a substantially homogeneous polypeptide with one N-terminus. In some embodiments, the signal sequence is SEQ ID NO:67 (amino acids 1-27 of SEQ ID NO:30). In some embodiments, amino acids 25 and/or 26 of SEQ ID NO:67 are substituted with different amino acids. In some embodiments, amino acids 17, 18, 19, 23, 24, 25, and/or 26 of SEQ ID NO:67 are substituted with different amino acids. In some embodiments, amino acids 17, 23, 24, 25, and 26 of SEQ ID NO:67 are substituted with different amino acids. In some embodiments, amino acid 17 of SEQ ID NO:67 is substituted with a phenylalanine or a leucine. In some embodiments, amino acid 23 of SEQ ID NO:67 is

substituted with a proline. In some embodiments, amino acid 24 of SEQ ID NO:67 is substituted with an isoleucine or a phenylalanine. In some embodiments, amino acid 25 of SEQ ID NO:67 is substituted with a valine, an isoleucine, or an alanine. In some embodiments, amino acid 26 of SEQ ID NO:67 is substituted with a histidine, a tyrosine, or a histidine. In some embodiments, amino acid 25 of SEQ ID NO:67 is substituted with a valine. In some embodiments, amino acid 26 of SEQ ID NO:67 is substituted with a leucine. In some embodiments, the signal sequence of the polypeptide comprises or consists of a sequence selected from the group listed in Table 1.

Table 1

MEWGYLLEVTSLLAALALLQRSSGAAA	SEQ ID NO:67
MEWGYLLEVTSLLAALALLQRSSGALA	SEQ ID NO:68
MEWGYLLEVTSLLAALALLQRSSGVLA	SEQ ID NO:69
MEWGYLLEVTSLLAALLLLQRSPIVHA	SEQ ID NO:70
MEWGYLLEVTSLLAALFLLQRSPIVHA	SEQ ID NO:71
MEWGYLLEVTSLLAALLLLQRSPFVHA	SEQ ID NO:72
MEWGYLLEVTSLLAALLLLQRSPIIYA	SEQ ID NO:73
MEWGYLLEVTSLLAALLLLQRSPIAHA	SEQ ID NO:74

[0097] In certain embodiments, the Wnt-binding agent comprises a first polypeptide comprising a FZD domain component and a Fc region. In some embodiments, the FZD domain component is from FZD1, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9, or FZD10. In some embodiments, the Fc region is from an IgG1 immunoglobulin. In some embodiments, the Wnt-binding agent comprises: (a) a first polypeptide consisting essentially of amino acids selected from the group consisting of: X1 to Y1 of SEQ ID NO:27, X2 to Y2 of SEQ ID NO:28, X3 to Y3 of SEQ ID NO:29, X4 to Y4 of SEQ ID NO:22, X5 to Y5 of SEQ ID NO:23, X6 to Y6 of SEQ ID NO:24, X7 to Y7 of SEQ ID NO:25, X8 to Y8 of SEQ ID NO:30, X9 to Y9 of SEQ ID NO:31, and X10 to Y10 of SEQ ID NO:26; and

(b) a second polypeptide consisting essentially of amino acids A to B of SEQ ID NO:43;

wherein X1 = amino acid 69, 70, 71,72, 73, 74, 75, or 76

Y1 = amino acid 236, 237, 238, 239, 240, 241, 242, or 243

X2 = amino acid 22, 23, 24, 25, 26, 27 or 28

Y2 = amino acid 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171 or 172

X3 = amino acid 18, 19, 20, 21, 22, 23, 24, or 25

Y3 = amino acid 141, 142, 143, 144, 145, 146, 147, 148, or 149

X4 = amino acid 38, 39, 40, 41, or 42

Y4 = amino acid 168, 169, 170, 171, 172, 173, 174, 175 or 176

X5 = amino acid 25, 26, 27, 28 or 29

Y5 = amino acid 155, 156, 157, 158, 159, 160, 161, 162, 163, or 164

X6 = amino acid 19, 20, 21, 22, 23, or 24

Y6 = amino acid 144, 145, 146, 147, 148, 149, 150, 151 or 152

X7 = amino acid 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, or 34

Y7 = amino acid 178, 179, 180, 181, 182, 183, 184, 185, or 186

X8 = amino acid 25, 26, 27, 28, 29, 30, or 31

Y8 = amino acid 156, 157, 158, 159, 160, 161, 162, 163, or 164

X9 = amino acid 21, 22, 23, or 24

Y9 = amino acid 137, 138, 139, 140, 141, 142, 143, 144, 145, or 146

X10 = amino acid 20, 21, 22, 23, 24, or 25

Y10 = amino acid 152, 153, 154, 155, 156, 157, 158, 159, or 160

A = amino acid 1, 2, 3, 4, 5, or 6

B = amino acid 231 or 232.

In some embodiments, the first polypeptide is directly linked to the second polypeptide. In some embodiments, the first polypeptide is linked to the second polypeptide via a peptide linker. In some embodiments, the first polypeptide is linked to the second polypeptide via the peptide linker GRA. A polypeptide (e.g., a first or second polypeptide) that "consists essentially of" certain amino acids or is "consisting essentially of" certain amino acids may, in some embodiments, include one or more (e.g., one, two, three, four or more) additional amino acids at one or both ends, so long as the additional amino acids do not materially affect the function of the Wnt-binding agent.

[0098] In certain embodiments, the Wnt-binding agent comprises: (a) a first polypeptide consisting essentially of amino acids X to Y of SEQ ID NO:30; and (b) a second polypeptide consisting essentially of amino acids A to B of SEQ ID NO:43; wherein the first polypeptide is directly linked to the second polypeptide; and wherein

X = amino acid 25, 26, 27, 28, 29, 30, or 31

Y = amino acid 156, 157, 158, 159, 160, 161, 162, 163, or 164

A = amino acid 1, 2, 3, 4, 5, or 6

B = amino acid 231 or 232.

In some embodiments, the first polypeptide consists essentially of amino acids 25-158 of SEQ ID NO:30. In other embodiments, the first polypeptide consists of amino acids 25-158 of SEQ ID NO:30. In some embodiments, the first polypeptide consists essentially of amino acids 28-158 of SEQ ID NO:30. In other embodiments, the first polypeptide consists of amino acids 28-158 of SEQ ID NO:30. In some embodiments, the first polypeptide consists of amino acids 31-158 of SEQ ID NO:30. In some embodiments, the second polypeptide consists of amino acids 1-232 of SEQ ID NO:43. In some

embodiments, the second polypeptide consists of amino acids 3-232 of SEQ ID NO:43. In some embodiments, the second polypeptide consists of amino acids 6-232 of SEQ ID NO:43. In some embodiments, the first polypeptide is SEQ ID NO:39 and the second polypeptide is SEQ ID NO:43. In some embodiments, the first polypeptide is SEQ ID NO:39 and the second polypeptide is SEQ ID NO:42. In some embodiments, the first polypeptide is SEQ ID NO:39 and the second polypeptide is SEQ ID NO:18.

[0099] In some embodiments, the Wnt-binding agent is a polypeptide comprising a first polypeptide and a second polypeptide, wherein the polypeptides are selected from Table 2.

Table 2

First Polypeptide	Second Polypeptide
Amino acids 25-158 of SEQ ID NO:30	Amino acids 1-232 of SEQ ID NO:43
Amino acids 25-158 of SEQ ID NO:30	Amino acids 1-231 of SEQ ID NO:43
Amino acids 25-158 of SEQ ID NO:30	Amino acids 2-232 of SEQ ID NO:43
Amino acids 25-158 of SEQ ID NO:30	Amino acids 2-231 of SEQ ID NO:43
Amino acids 25-158 of SEQ ID NO:30	Amino acids 3-232 of SEQ ID NO:43
Amino acids 25-158 of SEQ ID NO:30	Amino acids 3-231 of SEQ ID NO:43
Amino acids 25-158 of SEQ ID NO:30	Amino acids 4-232 of SEQ ID NO:43
Amino acids 25-158 of SEQ ID NO:30	Amino acids 4-231 of SEQ ID NO:43
Amino acids 25-158 of SEQ ID NO:30	Amino acids 5-232 of SEQ ID NO:43
Amino acids 25-158 of SEQ ID NO:30	Amino acids 5-231 of SEQ ID NO:43
Amino acids 25-158 of SEQ ID NO:30	Amino acids 6-232 of SEQ ID NO:43
Amino acids 25-158 of SEQ ID NO:30	Amino acids 6-231 of SEQ ID NO:43
Amino acids 26-158 of SEQ ID NO:30	Amino acids 1-232 of SEQ ID NO:43
Amino acids 26-158 of SEQ ID NO:30	Amino acids 1-231 of SEQ ID NO:43
Amino acids 26-158 of SEQ ID NO:30	Amino acids 2-232 of SEQ ID NO:43
Amino acids 26-158 of SEQ ID NO:30	Amino acids 2-231 of SEQ ID NO:43
Amino acids 26-158 of SEQ ID NO:30	Amino acids 3-232 of SEQ ID NO:43
Amino acids 26-158 of SEQ ID NO:30	Amino acids 3-231 of SEQ ID NO:43
Amino acids 26-158 of SEQ ID NO:30	Amino acids 4-232 of SEQ ID NO:43
Amino acids 26-158 of SEQ ID NO:30	Amino acids 4-231 of SEQ ID NO:43
Amino acids 26-158 of SEQ ID NO:30	Amino acids 5-232 of SEQ ID NO:43
Amino acids 26-158 of SEQ ID NO:30	Amino acids 5-231 of SEQ ID NO:43
Amino acids 26-158 of SEQ ID NO:30	Amino acids 6-232 of SEQ ID NO:43
Amino acids 26-158 of SEQ ID NO:30	Amino acids 6-231 of SEQ ID NO:43
Amino acids 27-158 of SEQ ID NO:30	Amino acids 1-232 of SEQ ID NO:43
Amino acids 27-158 of SEQ ID NO:30	Amino acids 1-231 of SEQ ID NO:43
Amino acids 27-158 of SEQ ID NO:30	Amino acids 2-232 of SEQ ID NO:43

Amino acids 27-158 of SEQ ID NO:30	Amino acids 2-231 of SEQ ID NO:43
Amino acids 27-158 of SEQ ID NO:30	Amino acids 3-232 of SEQ ID NO:43
Amino acids 27-158 of SEQ ID NO:30	Amino acids 3-231 of SEQ ID NO:43
Amino acids 27-158 of SEQ ID NO:30	Amino acids 4-232 of SEQ ID NO:43
Amino acids 27-158 of SEQ ID NO:30	Amino acids 4-231 of SEQ ID NO:43
Amino acids 27-158 of SEQ ID NO:30	Amino acids 5-232 of SEQ ID NO:43
Amino acids 27-158 of SEQ ID NO:30	Amino acids 5-231 of SEQ ID NO:43
Amino acids 27-158 of SEQ ID NO:30	Amino acids 6-232 of SEQ ID NO:43
Amino acids 27-158 of SEQ ID NO:30	Amino acids 6-231 of SEQ ID NO:43
Amino acids 28-158 of SEQ ID NO:30	Amino acids 1-232 of SEQ ID NO:43
Amino acids 28-158 of SEQ ID NO:30	Amino acids 1-231 of SEQ ID NO:43
Amino acids 28-158 of SEQ ID NO:30	Amino acids 2-232 of SEQ ID NO:43
Amino acids 28-158 of SEQ ID NO:30	Amino acids 2-231 of SEQ ID NO:43
Amino acids 28-158 of SEQ ID NO:30	Amino acids 3-232 of SEQ ID NO:43
Amino acids 28-158 of SEQ ID NO:30	Amino acids 3-231 of SEQ ID NO:43
Amino acids 28-158 of SEQ ID NO:30	Amino acids 4-232 of SEQ ID NO:43
Amino acids 28-158 of SEQ ID NO:30	Amino acids 4-231 of SEQ ID NO:43
Amino acids 28-158 of SEQ ID NO:30	Amino acids 5-232 of SEQ ID NO:43
Amino acids 28-158 of SEQ ID NO:30	Amino acids 5-231 of SEQ ID NO:43
Amino acids 28-158 of SEQ ID NO:30	Amino acids 6-232 of SEQ ID NO:43
Amino acids 28-158 of SEQ ID NO:30	Amino acids 6-231 of SEQ ID NO:43
Amino acids 25-161 of SEQ ID NO:30	Amino acids 1-232 of SEQ ID NO:43
Amino acids 25-161 of SEQ ID NO:30	Amino acids 1-231 of SEQ ID NO:43
Amino acids 25-161 of SEQ ID NO:30	Amino acids 2-232 of SEQ ID NO:43
Amino acids 25-161 of SEQ ID NO:30	Amino acids 2-231 of SEQ ID NO:43
Amino acids 25-161 of SEQ ID NO:30	Amino acids 3-232 of SEQ ID NO:43
Amino acids 25-161 of SEQ ID NO:30	Amino acids 3-231 of SEQ ID NO:43
Amino acids 25-161 of SEQ ID NO:30	Amino acids 4-232 of SEQ ID NO:43
Amino acids 25-161 of SEQ ID NO:30	Amino acids 4-231 of SEQ ID NO:43
Amino acids 25-161 of SEQ ID NO:30	Amino acids 5-232 of SEQ ID NO:43
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Amino acids 25-161 of SEQ ID NO:30	Amino acids 6-232 of SEQ ID NO:43
Amino acids 25-161 of SEQ ID NO:30	Amino acids 6-231 of SEQ ID NO:43
Amino acids 26-161 of SEQ ID NO:30	Amino acids 1-232 of SEQ ID NO:43
Amino acids 26-161 of SEQ ID NO:30	Amino acids 1-231 of SEQ ID NO:43
Amino acids 26-161 of SEQ ID NO:30	Amino acids 2-232 of SEQ ID NO:43
Amino acids 26-161 of SEQ ID NO:30	Amino acids 2-231 of SEQ ID NO:43
Amino acids 26-161 of SEQ ID NO:30	Amino acids 3-232 of SEQ ID NO:43
Amino acids 26-161 of SEQ ID NO:30	Amino acids 3-231 of SEQ ID NO:43

Amino acids 26-161 of SEQ ID NO:30	Amino acids 4-232 of SEQ ID NO:43
Amino acids 26-161 of SEQ ID NO:30	Amino acids 4-231 of SEQ ID NO:43
Amino acids 26-161 of SEQ ID NO:30	Amino acids 5-232 of SEQ ID NO:43
Amino acids 26-161 of SEQ ID NO:30	Amino acids 5-231 of SEQ ID NO:43
Amino acids 26-161 of SEQ ID NO:30	Amino acids 6-232 of SEQ ID NO:43
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Amino acids 27-161 of SEQ ID NO:30	Amino acids 1-232 of SEQ ID NO:43
Amino acids 27-161 of SEQ ID NO:30	Amino acids 1-231 of SEQ ID NO:43
Amino acids 27-161 of SEQ ID NO:30	Amino acids 2-232 of SEQ ID NO:43
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Amino acids 28-161 of SEQ ID NO:30	Amino acids 6-231 of SEQ ID NO:43
Amino acids 25-164 of SEQ ID NO:30	Amino acids 1-232 of SEQ ID NO:43
Amino acids 25-164 of SEQ ID NO:30	Amino acids 1-231 of SEQ ID NO:43
Amino acids 25-164 of SEQ ID NO:30	Amino acids 2-232 of SEQ ID NO:43
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Amino acids 25-164 of SEQ ID NO:30	Amino acids 4-232 of SEQ ID NO:43
Amino acids 25-164 of SEQ ID NO:30	Amino acids 4-231 of SEQ ID NO:43
Amino acids 25-164 of SEQ ID NO:30	Amino acids 5-232 of SEQ ID NO:43

Amino acids 25-164 of SEQ ID NO:30	Amino acids 5-231 of SEQ ID NO:43
Amino acids 25-164 of SEQ ID NO:30	Amino acids 6-232 of SEQ ID NO:43
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Amino acids 27-164 of SEQ ID NO:30	Amino acids 1-232 of SEQ ID NO:43
Amino acids 27-164 of SEQ ID NO:30	Amino acids 1-231 of SEQ ID NO:43
Amino acids 27-164 of SEQ ID NO:30	Amino acids 2-232 of SEQ ID NO:43
Amino acids 27-164 of SEQ ID NO:30	Amino acids 2-231 of SEQ ID NO:43
Amino acids 27-164 of SEQ ID NO:30	Amino acids 3-232 of SEQ ID NO:43
Amino acids 27-164 of SEQ ID NO:30	Amino acids 3-231 of SEQ ID NO:43
Amino acids 27-164 of SEQ ID NO:30	Amino acids 4-232 of SEQ ID NO:43
Amino acids 27-164 of SEQ ID NO:30	Amino acids 4-231 of SEQ ID NO:43
Amino acids 27-164 of SEQ ID NO:30	Amino acids 5-232 of SEQ ID NO:43
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Amino acids 28-164 of SEQ ID NO:30	Amino acids 1-232 of SEQ ID NO:43
Amino acids 28-164 of SEQ ID NO:30	Amino acids 1-231 of SEQ ID NO:43
Amino acids 28-164 of SEQ ID NO:30	Amino acids 2-232 of SEQ ID NO:43
Amino acids 28-164 of SEQ ID NO:30	Amino acids 2-231 of SEQ ID NO:43
Amino acids 28-164 of SEQ ID NO:30	Amino acids 3-232 of SEQ ID NO:43
Amino acids 28-164 of SEQ ID NO:30	Amino acids 3-231 of SEQ ID NO:43
Amino acids 28-164 of SEQ ID NO:30	Amino acids 4-232 of SEQ ID NO:43
Amino acids 28-164 of SEQ ID NO:30	Amino acids 4-231 of SEQ ID NO:43
Amino acids 28-164 of SEQ ID NO:30	Amino acids 5-232 of SEQ ID NO:43
Amino acids 28-164 of SEQ ID NO:30	Amino acids 5-231 of SEQ ID NO:43
Amino acids 28-164 of SEQ ID NO:30	Amino acids 6-232 of SEQ ID NO:43
Amino acids 28-164 of SEQ ID NO:30	Amino acids 6-231 of SEQ ID NO:43

[00100] In some embodiments, the Wnt-binding agent comprises an amino acid sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:65, and SEQ ID NO:66.

[00101] In certain embodiments, the Wnt-binding agent comprises the sequence of SEQ ID NO:1. In certain alternative embodiments, the agent comprises the sequence of SEQ ID NO:1, comprising one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc.) conservative substitutions. In certain embodiments, the agent comprises a sequence having at least about 90%, about 95%, or about 98% sequence identity with SEQ ID NO:1. In certain embodiments, the variants of SEQ ID NO:1 maintain the ability to bind one or more human Wnts.

[00102] In certain embodiments, the Wnt-binding agent comprises the sequence of SEQ ID NO:46. In some embodiments, the Wnt-binding agent is SEQ ID NO:46. In certain alternative embodiments, the agent comprises the sequence of SEQ ID NO:46, comprising one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc.) conservative substitutions. In certain embodiments, the agent comprises a sequence having at least about 90%, about 95%, or about 98% sequence identity with SEQ ID NO:46. In certain embodiments, the variants of SEQ ID NO:46 maintain the ability to bind one or more human Wnts.

[00103] In certain embodiments, the Wnt-binding agent comprises the sequence of SEQ ID NO:48. In some embodiments, the Wnt-binding agent is SEQ ID NO:48. In certain alternative embodiments, the agent comprises the sequence of SEQ ID NO:48, comprising one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc.) conservative substitutions. In certain embodiments, the agent comprises a sequence having at least about 90%, about 95%, or about 98% sequence identity with SEQ ID NO:48. In certain embodiments, the variants of SEQ ID NO:48 maintain the ability to bind one or more human Wnts.

[00104] In certain embodiments, the Wnt-binding agent comprises the sequence of SEQ ID NO:50. In some embodiments, the Wnt-binding agent is SEQ ID NO:50. In certain alternative embodiments, the agent comprises the sequence of SEQ ID NO:50, comprising one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc.) conservative substitutions. In certain embodiments, the agent comprises a sequence having at least about 90%, about 95%, or about 98% sequence identity with SEQ ID NO:50. In certain embodiments, the variants of SEQ ID NO:50 maintain the ability to bind one or more human Wnts.

[00105] In certain embodiments, the Wnt-binding agent comprises the sequence of SEQ ID NO:53. In some embodiments, the Wnt-binding agent is SEQ ID NO:53. In certain alternative embodiments, the agent comprises the sequence of SEQ ID NO:53, comprising one or more (e.g., one, two, three, four, five,

six, seven, eight, nine, ten, etc.) conservative substitutions. In certain embodiments, the agent comprises a sequence having at least about 90%, about 95%, or about 98% sequence identity with SEQ ID NO:53. In certain embodiments, the variants of SEQ ID NO:53 maintain the ability to bind one or more human Wnts.

[00106] In some embodiments, the Wnt-binding agents as described herein inhibit the growth of a tumor or tumor cells. In some embodiments, the Wnt-binding agents induce cells in a tumor to differentiate. In some embodiments, the Wnt-binding agents induce the expression of differentiation markers on a tumor or tumor cell. In certain embodiments, the Wnt-binding agents reduce the frequency of cancer stem cells in a tumor. In some embodiments, a Wnt-binding agent comprising SEQ ID NO:46 inhibits tumor growth to a greater extent than a Wnt-binding agent comprising SEQ ID NO:1. In some embodiments, a Wnt-binding agent comprising SEQ ID NO:48 inhibits tumor growth to a greater extent than a Wnt-binding agent comprising SEQ ID NO:1. In some embodiments, a Wnt-binding agent comprising SEQ ID NO:50 inhibits tumor growth to a greater extent than a Wnt-binding agent comprising SEQ ID NO:1. In some embodiments, a Wnt-binding agent comprising SEQ ID NO:53 inhibits tumor growth to a greater extent than a Wnt-binding agent comprising SEQ ID NO:1. In some embodiments, a Wnt-binding agent as described herein inhibits tumor growth to a greater extent than a Wnt-binding agent comprising a FZD domain component, a Fc domain and a linker component connecting the FZD domain component and the Fc domain. In some embodiments, the linker component is an intervening peptide linker.

[00107] In certain embodiments, the Wnt-binding agents as described herein inhibit the growth of a Wnt-dependent tumor. In some embodiments, the tumor is a tumor selected from selected from the group consisting of colorectal tumor, colon tumor, pancreatic tumor, lung tumor, ovarian tumor, liver tumor, breast tumor, kidney tumor, prostate tumor, gastrointestinal tumor, melanoma, cervical tumor, bladder tumor, glioblastoma, and head and neck tumor. In certain embodiments, the tumor is a colorectal tumor. In certain embodiments, the tumor is a pancreatic tumor. In certain embodiments, the tumor is a breast tumor.

[00108] In certain embodiments, a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:65 and SEQ ID NO:66 is provided. In certain embodiments, the polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, and SEQ ID NO:53. In some embodiments, a polypeptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, and SEQ ID NO:53. In certain embodiments, the polypeptide comprises the amino acid sequence of SEQ ID NO:50. In some embodiments, the polypeptide is SEQ ID NO:50. In certain embodiments, the

polypeptide comprises the amino acid sequence of SEQ ID NO:53. In some embodiments, the polypeptide is SEQ ID NO:53.

[00109] In certain embodiments, the polypeptide (before signal sequence cleavage) comprises SEQ ID NO:50 and a signal sequence selected from the group consisting of SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, and SEQ ID NO:74. In certain embodiments, the polypeptide (before signal sequence cleavage) comprises SEQ ID NO:50 and a signal sequence selected from the group consisting of SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, and SEQ ID NO:74. In some embodiments, the polypeptide (before signal sequence cleavage) comprises SEQ ID NO:53 and a signal sequence selected from the group consisting of SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, and SEQ ID NO:74. In some embodiments, the polypeptide (before signal sequence cleavage) comprises SEQ ID NO:53 and a signal sequence selected from the group consisting of SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, and SEQ ID NO:74. In some embodiments, the polypeptide comprises SEQ ID NO:71 and SEQ ID NO:50. In some embodiments, the polypeptide comprises SEQ ID NO:71 and SEQ ID NO:53. In some embodiments, the polypeptide comprises SEQ ID NO:75. In some embodiments, the polypeptide consists essentially of SEQ ID NO:75.

[00110] In some embodiments, the polypeptide is a substantially purified polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, and SEQ ID NO:53. In certain embodiments, the substantially purified polypeptide consists of at least 90% of a polypeptide that has an N-terminal sequence of ASA. In some embodiments, the nascent polypeptide comprises a signal sequence selected from the group consisting of SEQ ID NOs:67-74. In some embodiments, the nascent polypeptide comprises a signal sequence of SEQ ID NOs:71. In some embodiments, the nascent polypeptide comprises a signal sequence that results in a substantially homogeneous polypeptide product with one N-terminal sequence.

[00111] In certain alternative embodiments, the agent does not comprise a Fri domain of a FZD receptor.

[00112] In certain embodiments, the Wnt-binding agent is an antibody (e.g., an antibody that specifically binds to one or more Wnt proteins).

[00113] In certain embodiments, the Wnt-binding agent comprises a Fc region of an immunoglobulin. Those skilled in the art will appreciate that the binding agents of this invention will comprise fusion proteins in which at least a portion of the Fc region has been deleted or otherwise altered so as to provide desired biochemical characteristics, such as increased cancer cell localization, increased tumor penetration, reduced serum half-life, or increased serum half-life, when compared with a fusion protein of approximately the same immunogenicity comprising a native or unaltered constant region. Modifications to the Fc region may include additions, deletions, or substitutions of one or more amino acids in one or more domains. The modified fusion proteins disclosed herein may comprise alterations or modifications

to one or more of the two heavy chain constant domains (CH2 or CH3) or to the hinge region. In other embodiments, the entire CH2 domain is removed (ΔCH2 constructs). In some embodiments, the omitted constant region domain is replaced by a short amino acid spacer (e.g., 10 aa residues) that provides some of the molecular flexibility typically imparted by the absent constant region domain.

[00114] In some embodiments, the modified fusion proteins are engineered to link the CH3 domain directly to the hinge region of the antibody. In other embodiments, a peptide spacer is inserted between the hinge region and the modified CH2 and/or CH3 domains. For example, constructs may be expressed wherein the CH2 domain has been deleted and the remaining CH3 domain (modified or unmodified) is joined to the hinge region with a 5-20 amino acid spacer. Such a spacer may be added to ensure that the regulatory elements of the constant domain remain free and accessible or that the hinge region remains flexible. However, it should be noted that amino acid spacers may, in some cases, prove to be immunogenic and elicit an unwanted immune response against the construct. Accordingly, in certain embodiments, any spacer added to the construct will be relatively non-immunogenic so as to maintain the desired biological qualities of the modified antibodies.

[00115] In some embodiments, the modified fusion proteins may have only a partial deletion of a constant domain or substitution of a few or even a single amino acid. For example, the mutation of a single amino acid in selected areas of the CH2 domain may be enough to substantially reduce Fc binding and thereby increase cancer cell localization and/or tumor penetration. Similarly, it may be desirable to simply delete that part of one or more constant region domains that control a specific effector function (e.g., complement C1q binding) to be modulated. Such partial deletions of the constant regions may improve selected characteristics of the antibody (e.g., serum half-life) while leaving other desirable functions associated with the subject constant region domain intact. Moreover, as alluded to above, the constant regions of the disclosed fusion proteins may be modified through the mutation or substitution of one or more amino acids that enhances the profile of the resulting construct. In this respect it may be possible to disrupt the activity provided by a conserved binding site (e.g., Fc binding) while substantially maintaining the configuration and immunogenic profile of the modified antibody. In certain embodiments, the modified fusion proteins comprise the addition of one or more amino acids to the constant region to enhance desirable characteristics such as decreasing or increasing effector function, or provide for more cytotoxin or carbohydrate attachment.

[00116] It is known in the art that the constant region mediates several effector functions. For example, binding of the C1 component of complement to the Fc region of IgG or IgM antibodies (bound to antigen) activates the complement system. Activation of complement is important in the opsonization and lysis of cell pathogens. The activation of complement also stimulates the inflammatory response and can also be involved in autoimmune hypersensitivity. In addition, the Fc region of an antibody can bind to a cell expressing a Fc receptor (FcR). There are a number of Fc receptors which are specific for different

classes of antibody, including IgG (gamma receptors), IgE (epsilon receptors), IgA (alpha receptors) and IgM (mu receptors). Binding of antibody to Fc receptors on cell surfaces triggers a number of important and diverse biological responses including engulfment and destruction of antibody-coated particles, clearance of immune complexes, lysis of antibody-coated target cells by killer cells (antibody-dependent cell-mediated cytotoxicity or ADCC), release of inflammatory mediators, placental transfer, and control of immunoglobulin production.

[00117] In some embodiments, the Wnt-binding agents provide for altered effector functions that, in turn, affect the biological profile of the administered agent. For example, in some embodiments, the deletion or inactivation (through point mutations or other means) of a constant region domain may reduce Fc receptor binding of the circulating modified agent (e.g., Wnt-binding agent) thereby increasing cancer cell localization and/or tumor penetration. In other embodiments, the constant region modifications increase or reduce the serum half-life of the agent. In some embodiments, the constant region is modified to eliminate disulfide linkages or oligosaccharide moieties.

[00118] In certain embodiments, a Wnt-binding agent does not have one or more effector functions normally associated with an Fc region. In some embodiments, the agent has no ADCC activity, and/or no complement-dependent cytotoxicity (CDC) activity. In certain embodiments, the agent does not bind to the Fc receptor and/or complement factors. In certain embodiments, the agent has no effector function.

[00119] In some embodiments, the Wnt-binding agents described herein are modified to reduce immunogenicity. In general, immune responses against completely normal human proteins are rare when these proteins are used as therapeutics. However, although many fusion proteins comprise polypeptides sequences that are the same as the sequences found in nature, several therapeutic fusion proteins have been shown to be immunogenic in mammals. In some studies, a fusion protein comprising a linker has been found to be more immunogenic than a fusion protein that does not contain a linker. Accordingly, in some embodiments, the polypeptides of the invention are analyzed by computation methods to predict immunogenicity. In some embodiments, the polypeptides are analyzed for the presence of T-cell and/or B-cell epitopes. If any T-cell or B-cell epitopes are identified and/or predicted, modifications to these regions (e.g., amino acid substitutions) may be made to disrupt or destroy the epitopes. Various algorithms and software that can be used to predict T-cell and/or B-cell epitopes are known in the art. For example, the software programs SYFPEITHI, HLA Bind, PEPVAC, RANKPEP, DiscoTope, ElliPro and Antibody Epitope Prediction are all publicly available.

[00120] In some embodiments, a cell producing any of the Wnt-binding agents or polypeptides described herein is provided. In some embodiments, a composition comprising any of the Wnt-binding agents or polypeptides described herein is provided. In some embodiments, the composition comprises a polypeptide wherein at least 80%, 90%, 95%, 97%, 98%, or 99% of the polypeptide has an N-terminal sequence of ASA. In some embodiments, the composition comprises a polypeptide wherein 100% of the

polypeptide has an N-terminal sequence of ASA. In some embodiments, the composition comprises a polypeptide wherein at least 80% of the polypeptide has an N-terminal sequence of ASA. In some embodiments, the composition comprises a polypeptide wherein at least 90% of the polypeptide has an N-terminal sequence of ASA. In some embodiments, the composition comprises a polypeptide wherein at least 95% of the polypeptide has an N-terminal sequence of ASA.

[00121] The polypeptides of the present invention can be recombinant polypeptides, natural polypeptides, or synthetic polypeptides. It will be recognized in the art that some amino acid sequences of the invention can be varied without significant effect on the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity. Thus, the invention further includes variations of the polypeptides which show substantial activity or which include regions of FZD proteins, SFRP proteins or Ror proteins such as the protein portions discussed herein. Such mutants include deletions, insertions, inversions, repeats, and type substitutions. As indicated below, guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, et al., 1990, *Science,* 247:1306-10.

[00122] Of course, the number of amino acid substitutions a skilled artisan would make depends on many factors, including those described above. In certain embodiments, the number of substitutions for any given soluble receptor polypeptide will not be more than 50, 40, 30, 25, 20, 15, 10, 5 or 3.

[00123] Fragments or portions of the polypeptides of the present invention can be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments can be employed as intermediates for producing the full-length polypeptides. These fragments or portion of the polypeptides can also be referred to as "protein fragments" or "polypeptide fragments".

[00124] A protein fragment of this invention is a portion, or all, of a protein which is capable of binding to one or more human Wnt proteins (e.g., a human FZD receptor, a human SFRP or a Ror protein). In some embodiments, the fragment has a high affinity for one or more human Wnt proteins. Some fragments of fusion proteins described herein are protein fragments comprising at least part of the extracellular portion of a FZD receptor, a SFRP or the extracellular portion of a Ror protein which contains a binding domain linked to at least part of a constant region of an immunoglobulin (e.g., a Fc region). The binding affinity of the protein fragment can be in the range of about 10^{-11} to 10^{-12} M, although the affinity can vary considerably with fragments of different sizes, ranging from 10^{-7} to 10^{-13} M. In some embodiments, the fragment is about 100 to about 200 amino acids in length and comprises a binding domain linked to at least part of a constant region of an immunoglobulin.

[00125] The Wnt-binding agents of the present invention can be assayed for specific binding by any method known in the art. The immunoassays which can be used include, but are not limited to, competitive and non-competitive assay systems using techniques such as BIAcore analysis, FACS analysis, immunofluorescence, immunocytochemistry, Western blots, radioimmunoassays, ELISA,

"sandwich" immunoassays, immunoprecipitation assays, precipitation reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, and protein A immunoassays. Such assays are routine and well known in the art (see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety).

[00126] For example, the specific binding of a polypeptide to a human Wnt may be determined using ELISA. An ELISA assay comprises preparing antigen, coating wells of a 96 well microtiter plate with antigen, adding the polypeptide (e.g., a Wnt -binding agent) conjugated to a detectable compound such as an enzymatic substrate (e.g. horseradish peroxidase or alkaline phosphatase) to the well, incubating for a period of time and detecting the presence of the agent. In some embodiments, the polypeptide (e.g., Wnt-binding agent) is not conjugated to a detectable compound, but instead a second conjugated antibody that recognizes the polypeptide is added to the well. In some embodiments, instead of coating the well with the antigen, the polypeptide (e.g., Wnt-binding agent) can be coated to the well and a second antibody conjugated to a detectable compound can be added following the addition of the antigen to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art (see e.g. Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 11.2.1).

[00127] The binding affinity of an agent to a Wnt and the off-rate of a binding agent-antigen interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled antigen (e.g., ^{3}H or ^{125}I), or fragment or variant thereof, with the binding agent of interest in the presence of increasing amounts of unlabeled antigen followed by the detection of the antibody bound to the labeled antigen. The affinity of the binding agent against a Wnt and the binding off-rates can be determined from the data by Scatchard plot analysis. In some embodiments, BIAcore kinetic analysis is used to determine the binding on and off rates of agents that bind one or more human Wnts. BIAcore kinetic analysis comprises analyzing the binding and dissociation of antibodies from chips with immobilized Wnt antigens on their surface.

[00128] In certain embodiments, the Wnt-binding agent binds to at least one Wnt with a dissociation constant (K_D) of about 1μM or less, about 100nM or less, about 40nM or less, about 20nM or less, or about 10nM or less.

[00129] In certain embodiments, the Wnt-binding agent (e.g., a FZD8-Fc) is an antagonist of at least one Wnt (i.e., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 Wnts) bound by the agent. In certain embodiments, the agent inhibits at least about 10%, at least about 20%, at least about 30%, at least about 50%, at least about 75%, at least about 90%, or about 100% of one or more activity of the bound human Wnt(s).

[00130] In vivo and in vitro assays for determining whether a Wnt-binding agent (or candidate Wnt-binding agent) inhibits Wnt signaling are known in the art. For example, cell-based, luciferase reporter

assays utilizing a TCF/Luc reporter vector containing multiple copies of the TCF-binding domain upstream of a firefly luciferase reporter gene may be used to measure canonical Wnt signaling levels in vitro (Gazit et al., 1999, *Oncogene* 18; 5959-66). The level of Wnt signaling in the presence of one or more Wnts (e.g., Wnt(s) expressed by transfected cells or provided by Wnt-conditioned media) with the Wnt-binding agent present is compared to the level of signaling without the Wnt-binding agent present. In addition to the TCF/Luc reporter assay, the effect of a Wnt-binding agent (or candidate agent) on canonical Wnt signaling may be measured in vitro or in vivo by measuring the effect of the agent on the level of expression of β-catenin regulated genes, such as c-myc (He et al., *Science*, 281:1509-12 (1998)), cyclin D1 (Tetsu et al., *Nature*, 398:422-6 (1999)) and/or fibronectin (Gradl et al. *Mol. Cell Biol.*, 19:5576-87 (1999)). In certain embodiments, the effect of an agent on Wnt signaling may also be assessed by measuring the effect of the agent on the phosphorylation state of Dishevelled-1, Dishevelled-2, Dishevelled-3, LRP5, LRP6, and/or β-catenin.

[00131] The polypeptides described herein can be produced by any suitable method known in the art. Such methods range from direct protein synthesis methods to constructing DNA sequences that encode polypeptide sequences and expressing those sequences in a suitable host. In some embodiments, a DNA sequence is constructed using recombinant technology by isolating or synthesizing a DNA sequence encoding a wild-type protein of interest. Optionally, the sequence can be mutagenized by site-specific mutagenesis to provide functional analogs thereof. See, e.g., Zoeller et al., 1984, *PNAS*, 81:5662-66 and U.S. Pat. No. 4,588,585. In some embodiments, a DNA sequence is constructed using recombinant technology by isolating more than one DNA sequence encoding two polypeptides of interest and ligating these DNA sequences together to generate a fusion protein. In some embodiments, the fusion of the two polypeptides adds additional amino acids to the junction between the two polypeptides (i.e., the ligation site for the DNA sequences). These additional amino acids are considered a linker. In some embodiments, a peptide linker is inserted between the two polypeptides of the fusion protein.

[00132] In some embodiments, a DNA sequence that encodes a polypeptide of interest may be constructed by chemical synthesis using an oligonucleotide synthesizer. Such oligonucleotides can be designed based on the amino acid sequence of the desired polypeptide. In some embodiments, the oligonucleotides are designed to select codons that are favored in the host cell in which the recombinant polypeptide of interest will be produced. Standard methods can be applied to synthesize a polynucleotide sequence that encodes a polypeptide of interest. For example, a complete amino acid sequence can be used to construct a back-translated gene. Further, a DNA oligomer containing a nucleotide sequence coding for the particular polypeptide can be synthesized. For example, several small oligonucleotides coding for portions of the desired polypeptide can be synthesized and then ligated. The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly. In some embodiments, a nucleotide sequence coding for

the desired fusion protein is synthesized so that the two polypeptides are directly linked without an intervening peptide linker.

[00133] Once assembled (by synthesis, site-directed mutagenesis, recombinant technology, or another method), the polynucleotide sequences encoding a particular polypeptide of interest can be inserted into an expression vector and operatively linked to an expression control sequence appropriate for expression of the polypeptide in a desired host. Proper assembly can be confirmed by nucleotide sequencing, restriction mapping, and/or expression of a biologically active polypeptide in a suitable host. As is well-known in the art, in order to obtain high expression levels of a transfected gene in a host, the gene must be operatively linked to transcriptional and translational expression control sequences that are functional in the chosen expression host.

[00134] In certain embodiments, recombinant expression vectors are used to amplify and express DNA that encode Wnt-binding agents and polypeptides described herein. For example, recombinant expression vectors can be replicable DNA constructs which have synthetic or cDNA-derived DNA fragments encoding a fusion protein comprising a FZD Fri domain and a Fc region, operatively linked to suitable transcriptional or translational regulatory elements derived from mammalian, microbial, viral or insect genes. A transcriptional unit generally comprises an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, transcriptional promoters and/or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences. Regulatory elements can include an operator sequence to control transcription. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants can additionally be incorporated. DNA regions are "operatively linked" when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operatively linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operatively linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operatively linked to a coding sequence if it is positioned so as to permit translation. Generally, operatively linked means contiguous and, in the case of secretory leaders, means contiguous and in reading frame. In some embodiments, structural elements intended for use in yeast expression systems can include a leader sequence enabling extracellular secretion of translated protein by a host cell. In some embodiments, where recombinant protein is expressed without a leader or transport sequence, it can include an N-terminal methionine residue. This residue can optionally be subsequently cleaved from the expressed recombinant protein to provide a final product.

[00135] The choice of an expression control sequence and expression vector depends upon the choice of host. A wide variety of expression host/vector combinations can be employed. Useful expression vectors for eukaryotic hosts include, for example, vectors comprising expression control sequences from SV40,

bovine papilloma virus, adenovirus and cytomegalovirus. Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from E. coli, including pCR1, pBR322, pMB9 and their derivatives, and wider host range vectors, such as M13 and other filamentous single-stranded DNA phages.

[00136] Suitable host cells for expression of a Wnt-binding agent include prokaryotes, yeast, insect, or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram-negative or gram-positive organisms, for example E. coli or Bacilli. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Cell-free translation systems can also be employed. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels et al., 1985, *Cloning Vectors: A Laboratory Manual*, Elsevier, N.Y., the relevant disclosure of which is hereby incorporated by reference.

[00137] Various mammalian or insect cell culture systems are used to express recombinant polypeptides. In some embodiments, expression of recombinant proteins in mammalian cells is preferred because such proteins are generally correctly folded, appropriately modified and completely functional. Examples of suitable mammalian host cell lines include the COS-7 (monkey kidney-derived), L-929 (murine fibroblast-derived), C127 (murine mammary tumor-derived), 3T3 (murine fibroblast-derived), CHO (Chinese hamster ovary-derived), HeLa (human cervical cancer-derived) and BHK (hamster kidney fibroblast-derived) cell lines. Mammalian expression vectors can comprise non-transcribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking non-transcribed sequences, and 5' or 3' non-translated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences. Baculovirus systems for production of heterologous proteins in insect cells are known to those of skill in the art and are reviewed by Luckow and Summers, 1988, *Bio/Technology*, 6:47.

[00138] The proteins produced by a transformed host can be purified according to any suitable method. Such standard methods include chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for protein purification. Affinity tags such as hexahistidine, maltose binding domain, influenza coat sequence, and glutathione-S-transferase can be attached to the protein to allow easy purification by passage over an appropriate affinity column. Isolated proteins can also be physically characterized using such techniques as proteolysis, mass spectrometry (MS), high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and x-ray crystallography.

[00139] In some embodiments, supernatants from expression systems which secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix. In some embodiments, an anion exchange

resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose, or other types commonly employed in protein purification. In some embodiments, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. In some embodiments, a hydroxyapatite (CHT) media can be employed, including but not limited to, ceramic hydroxyapatite. In some embodiments, one or more reversed-phase HPLC steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify a fusion protein. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

[00140] In some embodiments, recombinant protein produced in bacterial culture can be isolated, for example, by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange, and/or size exclusion chromatography steps. HPLC can be employed for final purification steps. Microbial cells employed in expression of a recombinant protein can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

[00141] Methods known in the art for purifying antibodies and other proteins also include, for example, those described in U.S. Patent Appl. Nos. 2008/0312425; 2008/0177048; and 2009/0187005.

[00142] The polypeptides described herein can be further modified to contain additional chemical moieties not normally part of the protein. Those derivatized moieties can improve the solubility, the biological half life, or absorption of the protein. The moieties can also reduce or eliminate any undesirable side effects of the proteins and the like. An overview for those moieties can be found in *Remington: The Science and Practice of Pharmacy*, 21st Edition, University of the Sciences, Philadelphia, 2005.

[00143] The chemical moieties most suitable for derivatization include water soluble polymers. A water soluble polymer is desirable because the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. In some embodiments, the polymer will be pharmaceutically acceptable for the preparation of a therapeutic product or composition. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. The effectiveness of the derivatization can be ascertained by administering the derivative, in the desired form (i.e., by osmotic pump, or by injection or infusion, or, further formulated for oral, pulmonary or other delivery routes), and determining its effectiveness. Suitable water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), dextran, poly(n-vinyl pyrrolidone)-

polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde can have advantages in manufacturing due to its stability in water.

[00144] The number of polymer molecules so attached can vary, and one skilled in the art will be able to ascertain the effect on function. One can mono-derivatize, or can provide for a di-, tri-, tetra- or some combination of derivatization, with the same or different chemical moieties (e.g., polymers, such as different weights of polyethylene glycols). The proportion of polymer molecules to protein (or peptide) molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined by factors such as the desired degree of derivatization (e.g., mono-, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

[00145] The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art. See for example, EP 0401384, the disclosure of which is hereby incorporated by reference (coupling PEG to G-CSF), see also Malik et al., 1992, *Exp. Hematol.*, 20:1028-35 (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol can be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule can be bound. The amino acid residues having a free amino group can include lysine residues and the N-terminal amino acid residue. Those having a free carboxyl group can include aspartic acid residues, glutamic acid residues, and the C-terminal amino acid residue. Sulfhydryl groups can also be used as a reactive group for attaching the polyethylene glycol molecule(s). For therapeutic purposes, attachment at an amino group, such as attachment at the N-terminus or lysine group can be performed. Attachment at residues important for receptor binding should be avoided if receptor binding is desired.

[00146] One can specifically design an amino-terminal chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one can select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) can be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemical modification can be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group

containing polymer is achieved. For example, one can selectively N-terminally pegylate the protein by performing the reaction at a pH which allows one to take advantage of the pKa differences between the epsilon amino group of the lysine residues and that of the alpha amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer to a protein is controlled, e.g., the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. Using reductive alkylation, the water soluble polymer can be of the type described above, and should have a single reactive aldehyde for coupling to the protein. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, can be used.

[00147] Pegylation can be carried out by any of the pegylation reactions known in the art. See, e.g., *Focus on Growth Factors*, 1992, 3: 4-10; EP 0154316, the disclosure of which is hereby incorporated by reference; EP 0401384; and the other publications cited herein that relate to pegylation. The pegylation can be carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water soluble polymer).

[00148] Thus, it is contemplated that soluble receptor polypeptides to be used in accordance with the present invention can include pegylated soluble receptor proteins or variants, wherein the PEG group(s) is (are) attached via acyl or alkyl groups. Such products can be mono-pegylated or poly-pegylated. The PEG groups are generally attached to the protein at the α or ε amino groups of amino acids, but it is also contemplated that the PEG groups could be attached to any amino group attached to the protein, which is sufficiently reactive to become attached to a PEG group under suitable reaction conditions.

[00149] The polymer molecules used in both the acylation and alkylation approaches can be selected from among water soluble polymers as described above. The polymer selected should be modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, so that the degree of polymerization can be controlled as provided for in the present methods. An exemplary reactive PEG aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono C1-C10 alkoxy or aryloxy derivatives thereof (see U.S. Pat. No. 5,252,714). The polymer can be branched or unbranched. For the acylation reactions, the polymer(s) selected should have a single reactive ester group. For the present reductive alkylation, the polymer(s) selected should have a single reactive aldehyde group. Generally, the water soluble polymer will not be selected from naturally occurring glycosyl residues since these are usually made more conveniently by mammalian recombinant expression systems. The polymer can be of any molecular weight, and can be branched or unbranched. One water soluble polymer for use herein is polyethylene glycol. As used herein, polyethylene glycol is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (C1-C10) alkoxy- or aryloxy-polyethylene glycol.

[00150] Other reaction parameters, such as solvent, reaction times, temperatures, etc., and means of purification of products, can be determined case by case based on the published information relating to derivatization of proteins with water soluble polymers (see the publications cited herein). In certain embodiments, the Wnt-binding agent is a polypeptide that is not derived from a human FZD or SFRP. A variety of methods for identifying and producing polypeptides that bind with high affinity to a protein target are known in the art. See, e.g., Skerra, 2007, *Curr. Opin. Biotechnol.*, 18:295-304; Hosse et al., 2006, *Protein Science*, 15:14-27; Gill et al., 2006, *Curr. Opin. Biotechnol.*, 17:653-58; Nygren, 2008, *FEBS J.*, 275:2668-76; and Skerra, 2008, *FEBS J.*, 275:2677-83, each of which is incorporated by reference herein in its entirety. In certain embodiments, phage display technology has been used to identify/produce the Wnt-binding polypeptide. In certain embodiments, the polypeptide comprises a protein scaffold of a type selected from the group consisting of protein A, a lipocalin, a fibronectin domain, an ankyrin consensus repeat domain, and thioredoxin.

[00151] In some embodiments, the Wnt-binding agent is a non-protein molecule. In certain embodiments, the agent is a small molecule. Combinatorial chemistry libraries and techniques useful in the identification of non-protein Wnt-binding agents are known to those skilled in the art. See, e.g., Kennedy et al., 2008, *J. Comb. Chem.*, 10:345-54; Dolle et al, 2007, *J. Comb. Chem.*, 9:855-902; and Bhattacharyya, 2001, *Curr. Med. Chem.*, 8:1383-404, each of which is incorporated by reference herein in its entirety. In certain further embodiments, the agent is a carbohydrate, a glycosaminoglycan, a glycoprotein, or a proteoglycan.

[00152] In certain embodiments, the agent is a nucleic acid aptamer. Aptamers are polynucleotide molecules that are selected (e.g., from random or mutagenized pools) on the basis of their ability to bind to another molecule. In some embodiments, the aptamer comprises a DNA polynucleotide. In certain alternative embodiments, the aptamer comprises an RNA polynucleotide. In certain embodiments, the aptamer comprises one or more modified nucleic acid residues. Methods of generating and screening nucleic acid aptamers for binding to proteins are well known in the art. See, e.g., U.S. Patent Nos. 5,270,163; 5,683,867; 5,763,595; 6,344,321; 7,368,236; 5,582,981; 5,756,291; 5,840,867; 7,312,325; and 7,329,742, International Patent Publication Nos. WO 02/077262 and WO 03/070984, U.S. Patent Application Publication Nos. 2005/0239134; 2005/0124565; and 2008/0227735, each of which is incorporated by reference herein in its entirety.

[00153] In certain embodiments, the Wnt-binding agent has a circulating half-life in mice, cynomolgus monkeys, or humans of at least about 5 hours, at least about 10 hours, at least about 24 hours, at least about 3 days, at least about 1 week, or at least about 2 weeks. In certain embodiments, the Wnt-binding agent is an IgG (e.g., IgG1 or IgG2) antibody that has a circulating half-life in mice, cynomolgus monkeys, or humans of at least about 10 hours, at least about 24 hours, at least about 3 days, at least about 1 week, or at least about 2 weeks. Methods of increasing the half-life of agents such as polypeptides and

antibodies are known in the art. For example, known methods of increasing the circulating half-life of IgG antibodies include the introduction of mutations in the Fc region which increase the pH-dependent binding of the antibody to the neonatal Fc receptor (FcRn) at pH 6.0 (see, e.g., U.S. Pat. Pub. Nos. 2005/0276799, 2007/0148164, and 2007/0122403). Known methods of increasing the circulating half-life of antibody fragments lacking the Fc region include such techniques as pegylation.

[00154] In certain embodiments, the Wnt-binding agents and polypeptides as described herein have a half-life of at least about 50 hours in a rat when administered via the tail vein at a dose ranging from about 2mg/kg to about 10mg/kg. In certain embodiments, the Wnt-binding agent or polypeptide has a half-life of at least about 50 hours in a rat when administered via the tail vein at a dose of about 10mg/kg. In certain embodiments, the Wnt-binding agent or polypeptide has a half-life of at least about 100 hours in a rat when administered via the tail vein at a dose ranging from about 2mg/kg to about 10mg/kg. In certain embodiments, the Wnt-binding agent or polypeptide has a half-life of at least about 100 hours in a rat when administered via the tail vein at a dose of about 10mg/kg. In certain embodiments, the Wnt-binding agent has a half-life of at least about 120 hours in a rat when administered via the tail vein at a dose ranging from about 2mg/kg to about 10mg/kg. In certain embodiments, the Wnt-binding agent has a half-life of at least about 150 hours in a rat when administered via the tail vein at a dose ranging from about 2mg/kg to about 10mg/kg.

[00155] In certain embodiments, the agent is a soluble FZD receptor that comprises a Fri domain of a human FZD receptor (or a fragment or variant of the Fri domain that binds one or more Wnts) and a human Fc region and has a half-life *in vivo* (e.g., in a mouse or rat) that is longer than a soluble FZD receptor comprising the extracellular domain of the FZD receptor and a human Fc region.

[00156] Cells producing the Wnt-binding agents or polypeptides described herein are provided. In some embodiments, the cell produces a soluble Wnt-binding agent which comprises a Fri domain of human FZD8, wherein at least about 80% of the Wnt-binding agent has an N-terminal sequence of ASA. In some embodiments, the cell produces a soluble Wnt-binding agent which comprises a Fri domain of human FZD8, wherein at least about 85%, at least about 90%, at least about 95%, or at least about 98% of the Wnt-binding agent has an N-terminal sequence of ASA. In some embodiments, the cell is a mammalian cell. In some embodiments, the cell produces a Wnt-binding agent which comprises a human Fc region. In some embodiments, the cell produces a Wnt-binding agent which comprises an amino acid sequence selected from the group consisting of SEQ ID NO:53, SEQ ID NO:50, SEQ ID NO:46, SEQ ID NO:48, and SEQ ID NO:1. In some embodiments, the cell produces a Wnt-binding agent which comprises an amino acid sequence of SEQ ID NO:53. In some embodiments, the cell produces a Wnt-binding agent which comprises an amino acid sequence of SEQ ID NO:50.

[00157] Wnt-binding agents produced by the cells described herein are provided.

[00158] Compositions comprising the Wnt-binding agents or polypeptides described herein are also provided. In some embodiments, the composition comprises a soluble Wnt-binding agent which comprises a Fri domain of human FZD8, wherein at least about 80% of the Wnt-binding agent has an N-terminal sequence of ASA. In some embodiments, the composition comprises a soluble Wnt-binding agent which comprises a Fri domain of human FZD8, wherein at least about 85%, at least about 90%, at least about 95%, or at least about 98% of the Wnt-binding agent has an N-terminal sequence of ASA. In some embodiments, the composition comprises a Wnt-binding agent which comprises a human Fc region. In some embodiments, the composition comprises a Wnt-binding agent which comprises an amino acid sequence selected from the group consisting of SEQ ID NO:53, SEQ ID NO:50, SEQ ID NO:46, SEQ ID NO:48, and SEQ ID NO:1. In some embodiments, the composition comprises a Wnt-binding agent which comprises an amino acid sequence of SEQ ID NO:53. In some embodiments, the composition comprises a Wnt-binding agent which comprises an amino acid sequence of SEQ ID NO:50. In some embodiments, the compositions as described herein further comprise a pharmaceutically acceptable carrier.

[00159] Methods of using the compositions comprising the Wnt-binding agents or polypeptides described herein are also provided.

III. Polynucleotides

[00160] In certain embodiments, the invention encompasses polynucleotides comprising polynucleotides that encode a polypeptide that specifically binds a human Wnt protein or a fragment of such a polypeptide. For example, the invention provides a polynucleotide comprising a nucleic acid sequence that encodes a soluble FZD receptor or encodes a fragment of such a soluble receptor. In some embodiments, the invention provides a polynucleotide comprising a nucleic acid sequence that encodes a soluble SFRP, a soluble Ror protein or encodes a fragment of such a soluble protein. In some embodiments, the polynucleotides comprise polynucleotides that encode any of the Wnt-binding agents as described herein. The polynucleotides of the invention can be in the form of RNA or in the form of DNA. DNA includes cDNA, genomic DNA, and synthetic DNA; and can be double-stranded or single-stranded, and if single-stranded can be the coding strand or non-coding (anti-sense) strand.

[00161] In certain embodiments, the polynucleotides are isolated. In certain embodiments, the polynucleotides are substantially pure.

[00162] The invention provides a polynucleotide comprising a polynucleotide that encodes a polypeptide comprising the sequence of SEQ ID NO:1, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:65, SEQ ID NO:66 and SEQ ID NO:75. In some embodiments, the polynucleotide further comprises a polynucleotide that encodes a polypeptide signal sequence selected from the group consisting of SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID

NO:71, SEQ ID NO:72, SEQ ID NO:73, and SEQ ID NO:74. In some embodiments, the polynucleotide further comprises a polynucleotide that encodes a polypeptide signal sequence selected from the group consisting of SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, and SEQ ID NO:74. In some embodiments, the polynucleotide comprises a polynucleotide that encodes a polypeptide having the sequence of SEQ ID NO:71 and SEQ ID NO:50. In some embodiments, the polynucleotide comprises a polynucleotide that encodes a polypeptide having the sequence of SEQ ID NO:71 and SEQ ID NO:53. In some embodiments, the polynucleotide comprises a polynucleotide that encodes a polypeptide having the sequence of SEQ ID NO:75. The invention further provides a polynucleotide comprising the sequence of SEQ ID NO:2.

[00163] The invention provides a polynucleotide comprising a polynucleotide that encodes a polypeptide comprising: a signal sequence selected from the group consisting of SEQ ID NO:71, SEQ ID NO:70, SEQ ID NO:72, SEQ ID NO:73, and SEQ ID NO:74; a Fri domain of human FZD8; and a human Fc region. In some embodiments, the polynucleotide comprises a polynucleotide that encodes a polypeptide comprising a signal sequence of SEQ ID NO:71; a Fri domain of human FZD8; and a human Fc region.

[00164] Also provided is a polynucleotide that comprises a polynucleotide that hybridizes to a polynucleotide having the sequence of SEQ ID NO:2 and/or to a polynucleotide that encodes a polypeptide having the sequence of SEQ ID NO:1, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:65, SEQ ID NO:66 and SEQ ID NO:75. In certain embodiments, the hybridization is under conditions of high stringency.

[00165] In certain embodiments, the polynucleotides comprise the coding sequence for the mature polypeptide joined in the same reading frame to a polynucleotide which aids, for example, in expression and secretion of a polypeptide from a host cell (e.g. a leader sequence or signal sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell). The polypeptide having a leader sequence is a preprotein and can have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides can also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

[00166] In certain embodiments, the polynucleotides comprise the coding sequence for the mature polypeptide joined in the same reading frame to a marker sequence that allows, for example, for purification of the encoded polypeptide. For example, the marker sequence can be a hexahistidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide joined to the marker in the case of a bacterial host, or the marker sequence can be a hemagglutinin (HA) tag derived from the influenza hemagglutinin protein when a mammalian host (e.g., COS-7 cells) is used.

[00167] The present invention further relates to variants of the hereinabove described polynucleotides encoding, for example, fragments, analogs, and derivatives. Fragments or portions of the polynucleotides of the present invention can be used to synthesize full-length polynucleotides of the present invention.

[00168] In certain embodiments, the present invention provides isolated polynucleotides comprising polynucleotides having a nucleotide sequence at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, and in some embodiments, at least 96%, 97%, 98% or 99% identical to a polynucleotide encoding a polypeptide comprising a soluble FZD receptor or other Wnt-binding agent described herein.

[00169] By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence can include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence can be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence can be inserted into the reference sequence. These mutations of the reference sequence can occur at the amino- or carboxy-terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

[00170] The polynucleotide variants can contain alterations in the coding regions, non-coding regions, or both. In some embodiments the polynucleotide variants contain alterations which produce silent amino acid substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. In some embodiments, nucleotide variants are produced by silent substitutions due to the degeneracy of the genetic code. In some embodiments, nucleotide variants comprise nucleotide sequences which result in expression differences (e.g., increased or decreased expression), even though the amino acid sequence is not changed. Polynucleotide variants can be produced for a variety of reasons, e.g., to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by a bacterial host such as E. coli).

[00171] The polynucleotides described herein can be produced by any suitable method known in the art. As described herein in some embodiments, a DNA sequence is constructed using recombinant technology by isolating or synthesizing a DNA sequence encoding a wild-type protein of interest. In some embodiments, a DNA sequence is constructed using recombinant technology by isolating more than one DNA sequence encoding two polypeptides of interest and ligating these DNA sequences together to generate a fusion protein.

[00172] In some embodiments, a DNA sequence may be constructed by chemical synthesis using an oligonucleotide synthesizer. Such oligonucleotides can be designed based on the amino acid sequence of the desired polypeptide. Standard methods can be applied to synthesize a polynucleotide sequence encoding a polypeptide of interest. For example, a complete amino acid sequence can be used to construct a back-translated gene. Further, a DNA oligomer containing a nucleotide sequence coding for the particular polypeptide can be synthesized. For example, several small oligonucleotides coding for portions of the desired polypeptide can be synthesized and then ligated. The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly. In some embodiments, a nucleotide sequence coding for the desired fusion protein is synthesized so that the two polypeptides are directly linked without an intervening peptide linker.

[00173] Once assembled (by synthesis, site-directed mutagenesis, recombinant technology, or another method), the polynucleotide sequences encoding a particular polypeptide of interest can be inserted into an expression vector and operatively linked to an expression control sequence appropriate for expression of the polypeptide in a desired host. Proper assembly can be confirmed by nucleotide sequencing, restriction mapping, and/or expression of a biologically active polypeptide in a suitable host. As is well-known in the art, in order to obtain high expression levels of a transfected gene in a host, the gene must be operatively linked to transcriptional and translational expression control sequences that are functional in the chosen expression host.

[00174] Vectors comprising the polynucleotides described herein are provided. Cells comprising the vectors or polynucleotides described herein are also provided.

IV. Pharmaceutical compositions

[00175] The present invention further provides pharmaceutical compositions comprising agents (e.g., soluble FZD receptors) that bind to one or more Wnt proteins and/or are Wnt antagonists. In some embodiments, the pharmaceutical compositions comprise the Wnt-binding agents and polypeptides as described herein. These pharmaceutical compositions find use in inhibiting tumor cell growth and treating cancer in human patients. In some embodiments, the Wnt-binding agents as described herein find use in the manufacture of a medicament for the treatment of cancer.

[00176] Formulations are prepared for storage and use by combining a purified agent or antagonist of the present invention with a pharmaceutically acceptable carrier, excipient, and/or stabilizer as a sterile lyophilized powder, aqueous solution, etc. (*Remington: The Science and Practice of Pharmacy, 21st Edition*, University of the Sciences, Philadelphia, 2005). Suitable carriers, excipients, or stabilizers comprise nontoxic buffers such as phosphate, citrate, and other organic acids; salts such as sodium chloride; antioxidants including ascorbic acid and methionine; preservatives (e.g. octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride;

benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens, such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight polypeptides (such as less than about 10 amino acid residues); proteins such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; carbohydrates such as monosacchandes, disaccharides, glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN or polyethylene glycol (PEG).

[00177] The pharmaceutical compositions of the present invention can be administered in any number of ways for either local or systemic treatment. Administration can be topical (such as to mucous membranes including vaginal and rectal delivery) such as transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders; pulmonary (e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal); oral; or parenteral including intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial (e.g., intrathecal or intraventricular) administration.

[00178] The therapeutic formulation can be in unit dosage form. Such formulations include tablets, pills, capsules, powders, granules, solutions or suspensions in water or non-aqueous media, or suppositories for oral, parenteral, or rectal administration or for administration by inhalation. In solid compositions such as tablets the principal active ingredient is mixed with a pharmaceutical carrier. Conventional tableting ingredients include corn starch, lactose, sucrose, sorbitol, talc, stearic acid, magnesium stearate, dicalcium phosphate or gums, and other diluents (e.g. water) to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention, or a non-toxic pharmaceutically acceptable salt thereof. The solid preformulation composition is then subdivided into unit dosage forms of the type described above. The tablets, pills, etc., of the novel composition can be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner composition covered by an outer component. Furthermore, the two components can be separated by an enteric layer that serves to resist disintegration and permits the inner component to pass intact through the stomach or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol and cellulose acetate.

[00179] Pharmaceutical formulations include antagonists (e.g., Wnt-binding agents) of the present invention complexed with liposomes (Epstein et al., 1985, *PNAS,* 82:3688; Hwang et al.,1980, *PNAS,* 77:4030; and U.S. Patent Nos. 4,485,045 and 4,544,545). Liposomes with enhanced circulation time are disclosed in U.S. Patent 5,013,556. Liposomes can be generated by the reverse phase evaporation with a lipid composition comprising phosphatidylcholine, cholesterol, and PEG-derivatized

phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter.

[00180] The antagonist (e.g. Wnt-binding agent) can also be entrapped in microcapsules. Such microcapsules are prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions as described in *Remington: The Science and Practice of Pharmacy*, 21st Edition, University of the Sciences, Philadelphia, 2005.

[00181] In addition, sustained-release preparations can be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the agent, which matrices are in the form of shaped articles (e.g., films or microcapsules). Examples of sustained-release matrices include polyesters, hydrogels such as poly(2-hydroxyethyl-methacrylate) or poly(vinylalcohol), polylactides (U.S. Patent No. 3,773,919), copolymers of L-glutamic acid and 7 ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), sucrose acetate isobutyrate, and poly-D-(-)-3-hydroxybutyric acid.

V. Methods of use

[00182] The Wnt-binding agents (including soluble receptors) of the invention are useful in a variety of applications including, but not limited to, therapeutic treatment methods, such as the treatment of cancer. In certain embodiments, the agents are useful for inhibiting Wnt signaling (e.g., canonical Wnt signaling), inhibiting tumor growth, inducing differentiation, reducing tumor volume, reducing cancer stem cell frequency, and/or reducing the tumorigenicity of a tumor. The methods of use may be *in vitro*, *ex vivo*, or *in vivo* methods. In certain embodiments, the Wnt-binding agent or polypeptide is an antagonist of the one or more human Wnt proteins to which it binds.

[00183] In certain embodiments, the Wnt-binding agents or antagonists are used in the treatment of a disease associated with Wnt signaling activation. In particular embodiments, the disease is a disease dependent upon Wnt signaling. In particular embodiments, the Wnt signaling is canonical Wnt signaling. In certain embodiments, the Wnt-binding agents or antagonists are used in the treatment of disorders characterized by increased levels of stem cells and/or progenitor cells.

[00184] In certain embodiments, the disease treated with the Wnt-binding agent or antagonist (e.g., a soluble FZD receptor, SFRP-derived protein, or soluble Ror receptor) is a cancer. In certain embodiments, the cancer is characterized by Wnt-dependent tumors. In certain embodiments, the cancer is characterized by tumors expressing the one or more Wnts to which the Wnt-binding agent (e.g., soluble receptor) binds.

[00185] In certain embodiments, the disease treated with the Wnt-binding agent or antagonist is not a cancer. For example, the disease may be a metabolic disorder such as obesity or diabetes (e.g., type II diabetes) (Jin T., 2008, *Diabetologia*, 51:1771-80). Alternatively, the disease may be a bone disorder such as osteoporosis, osteoarthritis, or rheumatoid arthritis (Corr M., 2008, *Nat. Clin. Pract. Rheumatol.*, 4:550-6; Day et al., 2008, *Bone Joint Surg. Am.*, 90 Suppl 1:19-24). The disease may also be a kidney disorder, such as a polycystic kidney disease (Harris et al., 2009, *Ann. Rev. Med.*, 60:321-37; Schmidt-Ott et al., 2008, *Kidney Int.*, 74:1004-8; Benzing et al., 2007, *J. Am. Soc. Nephrol.*, 18:1389-98). Alternatively, eye disorders including, but not limited to, macular degeneration and familial exudative vitreoretinopathy may be treated (Lad et al., 2009, *Stem Cells Dev.*, 18:7-16). Cardiovascular disorders, including myocardial infarction, atherosclerosis, and valve disorders, may also be treated (Al-Aly Z., 2008, *Transl. Res.*, 151:233-9; Kobayashi et al., 2009, *Nat. Cell Biol.*, 11:46-55; van Gijn et al., 2002, *Cardiovasc. Res.*, 55:16-24; Christman et al., 2008, *Am. J. Physiol. Heart Circ. Physiol.*, 294:H2864-70). In some embodiments, the disease is a pulmonary disorder such as idiopathic pulmonary arterial hypertension or pulmonary fibrosis (Laumanns et al., 2008, *Am. J. Respir. Cell Mol. Biol.*, 2009, 40:683-91; Königshoff et al., 2008 *PLoS ONE*, 3:e2142). In some embodiments, the disease treated with the Wnt-binding agent is a liver disease, such as cirrhosis or liver fibrosis (Cheng et al., 2008, *Am. J. Physiol. Gastrointest. Liver Physiol.*, 294:G39-49).

[00186] The present invention provides methods of treating cancer comprising administering a therapeutically effective amount of a Wnt-binding agent to a subject (e.g., a subject in need of treatment). In certain embodiments, the cancer is a cancer selected from the group consisting of colorectal cancer, pancreatic cancer, lung cancer, ovarian cancer, liver cancer, breast cancer, kidney cancer, prostate cancer, gastrointestinal cancer, melanoma, cervical cancer, bladder cancer, glioblastoma, and head and neck cancer. In certain embodiments, the cancer is pancreatic cancer. In certain embodiments, the cancer is colorectal cancer. In certain embodiments, the cancer is breast cancer. In certain embodiments, the subject is a human.

[00187] The present invention further provides methods for inhibiting tumor growth using the Wnt-binding agents described herein. In certain embodiments, the method of inhibiting tumor growth comprises contacting the tumor or tumor cell with a Wnt-binding agent *in vitro*. For example, an immortalized cell line or a cancer cell line that expresses the targeted Wnt(s) is cultured in medium to which is added the Wnt-binding agent to inhibit tumor cell growth. In some embodiments, tumor cells are isolated from a patient sample such as, for example, a tissue biopsy, pleural effusion, or blood sample and cultured in medium to which is added a Wnt-binding agent to inhibit tumor cell growth.

[00188] In some embodiments, the method of inhibiting tumor growth comprises contacting the tumor or tumor cells with the Wnt-binding agent (e.g., a FZD soluble receptor) *in vivo*. In certain embodiments, contacting a tumor or tumor cell with a Wnt-binding agent is undertaken in an animal model. For

example, Wnt-binding agents may be administered to xenografts expressing one or more Wnts that have been grown in immunocompromised mice (e.g. NOD/SCID mice) to inhibit tumor growth. In certain embodiments, cancer stem cells are isolated from a patient sample such as, for example, a tissue biopsy, pleural effusion, or blood sample and injected into immunocompromised mice that are then administered a Wnt-binding agent to inhibit tumor cell growth. In some embodiments, the Wnt-binding agent is administered at the same time or shortly after introduction of tumorigenic cells into the animal to prevent tumor growth. In some embodiments, the Wnt-binding agent is administered as a therapeutic after the tumorigenic cells have grown to a tumor of a specified size.

[00189] In certain embodiments, the method of inhibiting the growth of a tumor comprises administering to a subject a therapeutically effective amount of a Wnt-binding agent. In certain embodiments, the subject is a human. In certain embodiments, the subject has a tumor or has had a tumor removed.

[00190] The invention also provides methods of reducing cancer stem cell frequency in a tumor comprising cancer stem cells, the method comprising administering a therapeutically effective amount of a Wnt-binding agent to a subject. In addition are provided methods of inducing differentiation of tumor cells in a subject, wherein the method comprises administering a therapeutically effective amount of a Wnt-binding agent to the subject. In some embodiments, methods for inducing expression of differentiation markers in a tumor comprise administering a therapeutically effective amount of a Wnt-binding agent to a subject. In certain embodiments, the subject is a human.

[00191] In certain embodiments, the tumor is a tumor in which Wnt signaling is active. In certain embodiments, the Wnt signaling that is active is canonical Wnt signaling. In certain embodiments, the tumor is a Wnt-dependent tumor. For example, in some embodiments, the tumor is sensitive to axin over-expression. In certain embodiments, the tumor does not comprise an inactivating mutation (e.g., a truncating mutation) in the adenomatous polyposis coli (APC) tumor suppressor gene or an activating mutation in the beta-catenin gene. In certain embodiments, the tumor expresses one or more genes in a Wnt gene signature. In certain embodiments, the cancer for which a subject is being treated involves such a tumor.

[00192] In certain embodiments, the tumor expresses the one or more human Wnt proteins to which the Wnt-binding agent binds. In certain embodiments, the tumor over-expresses the human Wnt(s).

[00193] In certain embodiments, the tumor is a tumor selected from the group consisting of colorectal tumor, pancreatic tumor, lung tumor, ovarian tumor, liver tumor, breast tumor, kidney tumor, prostate tumor, gastrointestinal tumor, melanoma, cervical tumor, bladder tumor, glioblastoma, and head and neck tumor. In certain embodiments, the tumor is a colorectal tumor. In certain embodiments, the tumor is a pancreatic tumor. In certain embodiments, the tumor is a breast tumor.

[00194] The invention also provides a method of inhibiting Wnt signaling in a cell comprising contacting the cell with an effective amount of a Wnt-binding agent. In certain embodiments, the cell is a tumor cell.

In certain embodiments, the method is an *in vivo* method wherein the step of contacting the cell with the agent comprises administering a therapeutically effective amount of the agent to the subject. In some alternative embodiments, the method is an *in vitro* or *ex vivo* method. In certain embodiments, the Wnt signaling that is inhibited is canonical Wnt signaling. In certain embodiments, the Wnt signaling is signaling by Wnt1, Wnt2, Wnt3, Wnt3a, Wnt7a, Wnt7b, and/or Wnt10b. In certain embodiments, the Wnt signaling is signaling by Wnt1, Wnt3a, Wnt7b, and/or Wnt10b.

[00195] In addition, the invention provides a method of reducing the tumorigenicity of a tumor in a subject, comprising administering a therapeutically effective amount of a Wnt-binding agent to the subject. In certain embodiments, the tumor comprises cancer stem cells. In some embodiments, the tumorigenicity of a tumor is reduced by reducing the frequency of cancer stem cells in the tumor. In certain embodiments, the frequency of cancer stem cells in the tumor is reduced by administration of the Wnt-binding agent. In certain embodiments, the agent or antibody is capable of reducing the tumorigenicity of a tumor comprising cancer stem cells in an animal model, such as a mouse xenograft model. In certain embodiments, the number or frequency of cancer stem cells in a tumor is reduced by at least about two-fold, about three-fold, about five-fold, about ten-fold, about 50-fold, about 100-fold, or about 1000-fold. In certain embodiments, the reduction in the number or frequency of cancer stem cells is determined by limiting dilution assay using an animal model. Additional examples and guidance regarding the use of limiting dilution assays to determine a reduction in the number or frequency of cancer stem cells in a tumor can be found, e.g., in International Publication Number WO 2008/042236, U.S. Patent Application Publication No. 2008/0064049, and U.S. Patent Application Publication No. 2008/0178305, each of which is incorporated by reference herein in its entirety.

[00196] Thus, the invention also provides a method of reducing the frequency of cancer stem cells in a tumor comprising cancer stem cells, the method comprising contacting the tumor with an effective amount of a Wnt-binding agent (e.g., a soluble FZD receptor, a soluble Ror receptor or a SFRP-Fc fusion).

[00197] The invention further provides methods of differentiating tumorigenic cells into non-tumorigenic cells comprising contacting the tumorigenic cells with a Wnt-binding agent (for example, by administering the Wnt-binding agent to a subject that has a tumor comprising the tumorigenic cells or that has had such a tumor removed). In certain embodiments, the tumorigenic cells are pancreatic tumor cells. In certain alternative embodiments, the tumorigenic cells are colon tumor cells.

[00198] The use of the Wnt-binding agents described herein to induce the differentiation of cells, including, but not limited to tumor cells, is also provided. For example, methods of inducing cells to differentiate comprising contacting the cells with an effective amount of a Wnt-binding agent (e.g., a soluble FZD receptor, a soluble Ror receptor, or a SFRP-Fc fusion) described herein are envisioned. Methods of inducing cells in a tumor in a subject to differentiate comprising administering a

therapeutically effective amount of a Wnt-binding agent to the subject are also provided. In certain embodiments, the tumor is a pancreatic tumor. In certain other embodiments, the tumor is a colon tumor.

[00199] Methods of treating a disease or disorder in a subject, wherein the disease or disorder is associated with Wnt signaling activation and/or is characterized by an increased level of stem cells and/or progenitor cells are further provided. In some embodiments, the treatment methods comprise administering a therapeutically effective amount of the Wnt-binding agent to the subject. In certain embodiments, the Wnt signaling is canonical Wnt signaling.

[00200] The Wnt-binding agents or antagonists are administered as an appropriate pharmaceutical composition to a human patient according to known methods. Suitable methods of administration include, but are not limited to, intravenous (administration as a bolus or by continuous infusion over a period of time), intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

[00201] In certain embodiments, in addition to administering the Wnt-binding agent, the method or treatment further comprises administering a second therapeutic agent (e.g. an anti-cancer agent) prior to, concurrently with, and/or subsequently to administration of the Wnt-binding agent. Pharmaceutical compositions comprising the Wnt-binding agent and the second therapeutic agent are also provided.

[00202] It will be appreciated that the combination of a Wnt-binding agent and a second therapeutic agent may be administered in any order or concurrently. In selected embodiments, the Wnt-binding agents will be administered to patients that have previously undergone treatment with the second therapeutic agent. In certain other embodiments, the Wnt-binding agent and the second therapeutic agent will be administered substantially simultaneously or concurrently. For example, a subject may be given the Wnt-binding agent while undergoing a course of treatment with the second therapeutic agent (e.g., chemotherapy). In certain embodiments, the Wnt-binding agent will be administered within 1 year of the treatment with the second therapeutic agent. In certain alternative embodiments, the Wnt-binding agent will be administered within 10, 8, 6, 4, or 2 months of any treatment with the second therapeutic agent. In certain other embodiments, the Wnt-binding agent will be administered within 4, 3, 2, or 1 week of any treatment with the second therapeutic agent. In some embodiments, the Wnt -binding agent will be administered within 5, 4, 3, 2, or 1 days of any treatment with the second therapeutic agent. It will further be appreciated that the two agents or treatment may be administered to the subject within a matter of hours or minutes (i.e., substantially simultaneously).

[00203] Combination therapy with at least two therapeutic agents often uses agents that work by different mechanisms of action, although this is not required. Combination therapy using agents with different mechanisms of action may result in additive or synergetic effects. Combination therapy may allow for a lower dose of each agent than is used in monotherapy, thereby reducing toxic side effects. Combination therapy may decrease the likelihood that resistant cancer cells will develop. In some embodiments,

combination therapy comprises a therapeutic agent that affects (e.g., inhibits or kills) non-tumorigenic cells and a therapeutic agent that affects (e.g., inhibits or kills) tumorigenic CSCs.

[00204] Useful classes of therapeutic (e.g., anti-cancer) agents include, for example, antitubulin agents, auristatins, DNA minor groove binders, DNA replication inhibitors, alkylating agents (e.g., platinum complexes such as cis-platin, mono(platinum), bis(platinum) and tri-nuclear platinum complexes and carboplatin), anthracyclines, antibiotics, antifolates, antimetabolites, chemotherapy sensitizers, duocarmycins, etoposides, fluorinated pyrimidines, ionophores, lexitropsins, nitrosureas, platinols, performing compounds, purine antimetabolites, puromycins, radiation sensitizers, steroids, taxanes, topoisomerase inhibitors, vinca alkaloids, or the like. In certain embodiments, the second therapeutic agent is an antimetabolite, an antimitotic, a topoisomerase inhibitor, or an angiogenesis inhibitor.

[00205] Therapeutic agents that may be administered in combination with the Wnt-binding agents include chemotherapeutic agents. Thus, in some embodiments, the method or treatment involves the combined administration of a Wnt-binding agent of the present invention and a chemotherapeutic agent or cocktail of multiple different chemotherapeutic agents. Treatment with a Wnt-binding agent can occur prior to, concurrently with, or subsequent to administration of chemotherapies. Chemotherapies contemplated by the invention include chemical substances or drugs which are known in the art and are commercially available, such as gemcitabine, irinotecan, doxorubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, paclitaxel, methotrexate, cisplatin, melphalan, vinblastine, and carboplatin. Combined administration can include co-administration, either in a single pharmaceutical formulation or using separate formulations, or consecutive administration in either order but generally within a time period such that all active agents can exert their biological activities simultaneously. Preparation and dosing schedules for such chemotherapeutic agents can be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992).

[00206] Chemotherapeutic agents useful in the instant invention also include, but are not limited to, alkylating agents such as thiotepa and cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan, and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamime; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, caminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-

5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK.; razoxane; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL) and docetaxel (TAXOTERE), chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Chemotherapeutic agents also include anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and antiandrogens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

[00207] In certain embodiments, the chemotherapeutic agent is a topoisomerase inhibitor. Topoisomerase inhibitors are chemotherapy agents that interfere with the action of a topoisomerase enzyme (e.g., topoisomerase I or II). Topoisomerase inhibitors include, but are not limited to, doxorubicin HCl, daunorubicin citrate, mitoxantrone HCl, actinomycin D, etoposide, topotecan HCl, teniposide (VM-26), and irinotecan. In certain embodiments, the second therapeutic agent is irinotecan. In certain embodiments, the tumor to be treated is a colorectal tumor and the second therapeutic agent is a topoisomerase inhibitor, such as irinotecan. In some embodiments, the Wnt-binding agent comprises SEQ ID NO:53 and the second therapeutic agent is irinotecan. In some embodiments, the Wnt-binding agent comprises SEQ ID NO:75 and the second therapeutic agent is irinotecan.

[00208] In certain embodiments, the chemotherapeutic agent is an anti-metabolite. An anti-metabolite is a chemical with a structure that is similar to a metabolite required for normal biochemical reactions, yet different enough to interfere with one or more normal functions of cells, such as cell division. Anti-metabolites include, but are not limited to, gemcitabine, fluorouracil, capecitabine, methotrexate sodium, ralitrexed, pemetrexed, tegafur, cytosine arabinoside, thioguanine, 5-azacytidine, 6-mercaptopurine, azathioprine, 6-thioguanine, pentostatin, fludarabine phosphate, and cladribine, as well as pharmaceutically acceptable salts, acids, or derivatives of any of these. In certain embodiments, the second therapeutic agent is gemcitabine. In certain embodiments, the tumor to be treated is a pancreatic tumor and the second therapeutic agent is an anti-metabolite (e.g., gemcitabine). In some embodiments, the Wnt-binding agent comprises SEQ ID NO:53 and the second therapeutic agent is gemcitabine. In some embodiments, the Wnt-binding agent comprises SEQ ID NO:75 and the second therapeutic agent is gemcitabine.

[00209] In certain embodiments, the chemotherapeutic agent is an antimitotic agent, including, but not limited to, agents that bind tubulin. By way of non-limiting example, the agent comprises a taxane. In certain embodiments, the agent comprises paclitaxel or docetaxel, or a pharmaceutically acceptable salt, acid, or derivative of paclitaxel or docetaxel. In certain embodiments, the agent is paclitaxel (TAXOL), docetaxel (TAXOTERE), albumin-bound paclitaxel (ABRAXANE), DHA-paclitaxel, or PG-paclitaxel. In certain alternative embodiments, the antimitotic agent comprises a vinka alkaloid, such as vincristine, binblastine, vinorelbine, or vindesine, or pharmaceutically acceptable salts, acids, or derivatives thereof. In some embodiments, the antimitotic agent is an inhibitor of Eg5 kinesin or an inhibitor of a mitotic kinase such as Aurora A or Plk1. In certain embodiments where the chemotherapeutic agent administered in combination with the Wnt-binding agent or polypeptide comprises an antimitotic agent, the cancer or tumor being treated is breast cancer or a breast tumor. In certain embodiments, the tumor to be treated is a breast tumor and the second therapeutic agent is paclitaxel. In some embodiments, the Wnt-binding agent comprises SEQ ID NO:53 and the second therapeutic agent is paclitaxel. In some embodiments, the Wnt-binding agent comprises SEQ ID NO:75 and the second therapeutic agent is paclitaxel.

[00210] In certain embodiments, the treatment involves the combined administration of a Wnt-binding agent of the present invention and radiation therapy. Treatment with the Wnt-binding agent can occur prior to, concurrently with, or subsequent to administration of radiation therapy. Any dosing schedules for such radiation therapy can be used as determined by the skilled practitioner.

[00211] In some embodiments, the second therapeutic agent comprises an antibody. Thus, treatment can involve the combined administration of Wnt-binding agents of the present invention with antibodies against tumor-associated antigens including, but not limited to, antibodies that bind to EGFR, ErbB2, HER2, DLL4, Notch, and/or VEGF. Exemplary, anti-DLL4 antibodies are described, for example, in U.S. Patent No. 7,750,124, incorporated by reference herein in its entirety. Additional anti-DLL4

antibodies are described in, e.g., International Patent Publication Nos. WO 2008/091222 and WO 2008/0793326, and U.S. Patent Application Publication Nos. US 2008/0014196, US 2008/0175847, US 2008/0181899, and US 2008/0107648, each of which is incorporated by reference herein in its entirety. In certain embodiments, the second therapeutic agent is an antibody that is an angiogenesis inhibitor (e.g., an anti-VEGF antibody). In some embodiments, the Wnt-binding agent comprises SEQ ID NO:53 and the second therapeutic agent is an anti-VEGF antibody. In some embodiments, the Wnt-binding agent comprises SEQ ID NO:75 and the second therapeutic agent is an anti-VEGF antibody. In certain embodiments, the second therapeutic agent is an inhibitor of Notch signaling. In some embodiments, the second therapeutic agent is an anti-Notch antibody. Exemplary anti-Notch antibodies are described, for example, in U.S. Patent Application Publication No. US 2008/0131434, incorporated by reference herein in its entirety. In certain embodiments, the second therapeutic agent is bevacizumab (AVASTIN), trastuzumab (HERCEPTIN), panitumumab (VECTIBIX), or cetuximab (ERBITUX). Combined administration can include co-administration, either in a single pharmaceutical formulation or using separate formulations, or consecutive administration in either order but generally within a time period such that all active agents can exert their biological activities simultaneously.

[00212] Furthermore, treatment can include administration of one or more cytokines (e.g., lymphokines, interleukins, tumor necrosis factors, and/or growth factors) or can be accompanied by surgical removal of tumor or cancer cells or any other therapy deemed necessary by a treating physician.

[00213] For the treatment of the disease, the appropriate dosage of an agent of the present invention depends on the type of disease to be treated, the severity and course of the disease, the responsiveness of the disease, whether the agent is administered for therapeutic or preventative purposes, previous therapy, patient's clinical history, and so on all at the discretion of the treating physician. The agent can be administered one time or over a series of treatments lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved (e.g. reduction in tumor size). Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient and will vary depending on the relative potency of an individual agent. The administering physician can easily determine optimum dosages, dosing methodologies and repetition rates. In certain embodiments, dosage is from 0.01μg to 100mg per kg of body weight, and can be given once or more daily, weekly, monthly or yearly. In certain embodiments, the dosage of the soluble receptor or other Wnt-binding agent is from about 0.1mg to about 20 mg per kg of body weight. In certain embodiments, the Wnt-binding agent is given once every week. In certain embodiments, the Wnt-binding agent is given once every two weeks or once every three weeks. The treating physician can estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues.

[00214] The present invention further provides methods of screening agents for efficacy in inhibiting Wnt signaling, for anti-tumor activity, and/or activity against cancer stem cells. In certain embodiments, the

method comprises comparing the level of one or more differentiation markers and/or one or more stemness markers in a first solid tumor (e.g., a solid tumor comprising cancer stem cells) that has been exposed to the agent to the level of the one or more differentiation markers in a second solid tumor that has not been exposed to the agent. In some embodiments, the method comprises: (a) exposing a first solid tumor, but not a second solid tumor, to the agent; (b) assessing the level of one or more differentiation markers and/or one or more stemness markers in the first and second solid tumors; and (c) comparing the level of the one or more differentiation markers in the first tumor and the level of the one or more differentiation markers in the second solid tumor. In certain embodiments, the (a) increased levels of the one or more differentiation markers in the first solid tumor relative to the levels of the one or more differentiation markers in the second solid tumor indicates anti-tumor (or anti-cancer stem cell) activity; and (b) decreased levels of the one or more stemness markers indicate anti-tumor (or anti-cancer stem cell) activity. In certain embodiments, the agent binds one or more Wnt proteins. In certain embodiments, the agent is a FZD soluble receptor. In certain methods, the agent is an antibody, such as an anti-Wnt antibody.

[00215] Additional methods for screening agents include, but are not limited to, methods comprising comparing the levels of one or more differentiation markers in a first solid tumor that has been exposed to an agent to the levels of the one or more differentiation markers in a second solid tumor that has not been exposed to the agent. In certain embodiments, the methods include comprising (a) exposing a first solid tumor, but not a second solid tumor, to the agent; (b) assessing the levels of one or more differentiation markers in the first and second solid tumors; and (c) comparing the levels of the one or more differentiation markers in the first tumor to the levels of the one or more differentiation markers in the second solid tumor. In certain embodiments, the agent is a Wnt-binding agent. In certain embodiments, the agent is an inhibitor of the canonical Wnt signaling pathway. In certain embodiments, the agent inhibits binding of one or more human Wnt proteins to one or more human FZD receptors. In certain embodiments, increased levels of one or more differentiation markers in the first solid tumor relative to levels of one or more differentiation markers in the second solid tumor indicates efficacy against solid tumor stem cells (CSCs). In certain alternative embodiments, decreased levels of one or more differentiation markers (i.e., negative markers for differentiation) in the first solid tumor relative to the levels of one or more differentiation markers in the second solid tumor indicates efficacy against solid tumor stem cells.

[00216] In certain embodiments, the solid tumor in the screening method is a pancreatic tumor. In certain embodiments, the solid tumor is a pancreatic tumor and the one or more differentiation markers may comprise one or more mucins (e.g., Muc16), one or more cytokeratins (e.g., CK20) and/or chromogranin A (CHGA).

[00217] In certain alternative embodiments, the solid tumor in the screening method is a colon tumor. In some embodiments, the solid tumor is a colon tumor and the one or more differentiation markers may comprise one or more cytokeratins (e.g., cytokeratin 7 or CK20).

[00218] In certain embodiments, the one or more stemness markers used in the screening methods described herein comprise ALDH1A1, APC, AXIN2, BMI1, CD44, FGF1, GJB1, GJB2, HES1, JAG1, LGR5, LHX8, MYC, NANOG, NEUROD1, NEUROG2, NOTCH1, NOTCH2, NOTCH3, NOTCH4, PROCR, RARRES1, RARRES3, RBP2, SOX1, SOX2, ASCL2,TDGF1, OLFM4, MSI1, DASH1, EPHB3 and/or EPHB4. In certain embodiments, two or more stemness markers, three or more stemness markers, four or more stemness markers, five or more stemness markers, six or more, or ten or more stemness markers are selected from the group consisting of ALDH1A1, APC, AXIN2, BMI1, CD44, FGF1, GJB1, GJB2, HES1, JAG1, LGR5, LHX8, MYC, NANOG, NEUROD1, NEUROG2, NOTCH1, NOTCH2, NOTCH3, NOTCH4, PROCR, RARRES1, RARRES3, RBP2, SOX1, SOX2, ASCL2,TDGF1, OLFM4, MSI1, DASH1, EPHB3 and EPHB4.

[00219] In certain embodiments, the one or more differentiation markers used in the screening methods comprise ALDOB, BMP2, BMP7, BMPR1B, CEACAM5, CEACAM6, CDX1, CDX2, CLCA2, COL1A2, COL6A1, CHGA, CSTA, CST4, CK20, DAB2, FABP4, GST1, KRT4, KRT7, KRT15, KRT17, KRT20, LAMA1, MUC3A, MUC4, MUC5AC, MUC5B, MUC13, MUC15, MUC16, MUC17, NDRG2, PIP, PLUNC, SPRR1A, REG4, VSIG1, and/or XAF1. In certain embodiments two or more, three or more, four or more, five or more, six or more, or ten or more differentiation markers used in the screening methods are selected from the group consisting of ALDOB, BMP2, BMP7, BMPR1B, CEACAM5, CEACAM6, CDX1, CDX2, CLCA2, COL1A2, COL6A1, CHGA, CSTA, CST4, CK20, DAB2, FABP4, GST1, KRT4, KRT7, KRT15, KRT17, KRT20, LAMA1, MUC3A, MUC4, MUC5AC, MUC5B, MUC13, MUC15, MUC16, MUC17, NDRG2, PIP, PLUNC, SPRR1A, REG4, VSIG1, and XAF1.

[00220] Other potential differentiation markers for pancreas and colon as well as other tumor types are known to those skilled in the art. In addition, the usefulness of potential differentiation markers in a screening method can be readily assessed by one skilled in the art by treating the desired tumor type with one or more of the soluble FZD receptors describe herein such as FZD8-Fc and then assessing for changes in expression of the marker by the treated tumor relative to control. Non-limiting examples of such methods, can for instance, be found in the specific Examples below.

[00221] The present invention further provides methods for producing soluble Wnt-binding agents. In certain embodiments, the method comprises producing a soluble Wnt-binding agent which comprises a Fri domain of human FZD8 in a cell, wherein at least 80% of the Wnt-binding agent has an N-terminal sequence of ASA, the method comprising using a signal sequence selected from the group consisting of: SEQ ID NO:71, SEQ ID NO:70, SEQ ID NO:72, SEQ ID NO:73, and SEQ ID NO:74 for production of

the Wnt-binding agent. In some embodiments of the method, the signal sequence is SEQ ID NO:71. In some embodiments, at least about 90%, at least about 95%, or at least about 98% of the Wnt-binding agent has an N-terminal sequence of ASA. In some embodiments, the cell is a mammalian cell. In some embodiments, the Wnt-binding agent comprises a human Fc region. In some embodiments, the Wnt-binding agent comprises an amino acid sequence selected from the group consisting of: SEQ ID NO:53, SEQ ID NO:50, SEQ ID NO:46, SEQ ID NO:48, and SEQ ID NO:1. In some embodiments, the Wnt-binding agent comprises SEQ ID NO:53. In some embodiments, the Wnt-binding agent comprises SEQ ID NO:50. In some embodiments, the cell comprises a polynucleotide comprising a polynucleotide that encodes a polypeptide having the sequence of SEQ ID NO:75.

EXAMPLES

[00222] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

EXAMPLE 1

Production of FZD8-Fc

[00223] cDNA encoding FZD8-Fc (54F03) was subcloned into a pEE14.4 expression vector (Lonza) digested with HindIII and EcoRI. After cloning, the pEE14.4-FZD8-Fc DNA was linearized by digestion with PvuI and subsequently introduced into GS-CHOK1 cells by electroporation using standard procedures. Stable clones expressing FZD8-Fc were obtained, and expanded in serum free medium. FZD8-Fc was purified by affinity capture using a protein A-conjugated resin. SDS-PAGE analysis revealed greater than 98% purity and endotoxin level was lower than 1 EU/mg protein.

[00224] The amino acid sequence of FZD8-Fc is SEQ ID NO:1 and the polynucleotide sequence encoding FZD8-Fc is SEQ ID NO:2.

EXAMPLE 2

Pharmacokinetics of FZD8-Fc in rat

[00225] The pharmacokinetics of FZD8-Fc (54F03) were assessed in rats in a two week pharmacokinetics (PK) study using doses of 2mg/kg and 10mg/kg. Sprague Dawley rats, five males in each group, were dosed with FZD8-Fc via the tail vein at 2mg/kg or 10mg/kg and followed for two weeks with samples collected at the time points 1, 24, 48, 72, 96, 168, 240, and 336 hours. At each time point, 1ml of blood was collected into potassium-EDTA tubes and centrifuged. The plasma supernatants were collected and frozen until the samples were analyzed.

[00226] The level of FZD8-Fc fusion protein present in the plasma at each time point was quantified and the half-life of FZD8-Fc was calculated for the two doses. As shown in Figure 1, the half-life of FZD8-Fc was estimated to be 163 hours at 2mg/kg and to be 157 hours at 10mg/kg.

EXAMPLE 3

Anti-tumor activity of FZD8-Fc in pancreatic tumor model

[00227] *Inhibition of tumor growth by FZD8-Fc in pancreatic tumor model.* The anti-tumor activity of FZD8-Fc (54F03) was evaluated in the PN4 pancreas tumor xenograft model. Dissociated OMP-PN4 cells (50,000 per animal) were injected subcutaneously into 6-8 week old male NOD/SCID mice. Tumor growth was monitored weekly and tumor measurements were initiated once tumors were palpable. On day 50, mice with average tumor volumes of 137mm^3 were randomized into 4 groups of 10 animals each. Animals were injected with either control antibody, FZD8-Fc (15 mg/kg), gemcitabine (2 mg/kg) or a combination of FZD8-Fc and gemcitabine. Administration of the FZD8-Fc and gemcitabine was performed via injection into the intra-peritoneal cavity, once weekly (gemcitabine) or twice weekly (FZD8-Fc). Tumors were measured twice a week and tumor volume was determined using the formula ½(a x b^2); where a = length, and b = breadth. Data are expressed as mean and mean ± S.E.M. Group means were compared using Student's two-tailed, unpaired t test. Probability (p) values of < 0.05 were interpreted as significantly different.

[00228] Treatment with FZD8-Fc resulted in a 66% reduction in tumor growth, as shown in Figure 2 ($p < 0.001$). Furthermore, treatment with FZD8-Fc and gemcitabine resulted in a 29% reduction of tumor growth relative to treatment with FZD8-Fc alone ($p = 0.04$ vs. FZD8-Fc alone) (Figure 2). Thus, FZD8-Fc demonstrated anti-tumor growth activity in the PN4 pancreas tumor model as a single agent as well as in combination with gemcitabine.

[00229] *Reduction of CD44hi population in PN4 tumors treated with FZD8-Fc.* Control and treated tumors from the OMP-PN4 xenograft study described above were harvested at the end of the study (day 85). The tumors were processed and dissociated into single cells. Single cell suspensions derived from 5 tumors of each treatment group were pooled, and the pooled samples were then incubated on ice for 30 min with antibodies that bind mouse cells selectively (α-mouse CD45-biotin 1:200 dilution and rat α-mouse H2Kd-biotin 1:100 dilution, BioLegend, San Diego, CA), followed by addition of streptavidin-labeled magnetic beads (Invitrogen, Carlsbad, CA). Mouse cells were removed with the aid of a magnet. For analysis of human cell surface markers, the single tumor cell suspension was stained with anti-ESA (Biomeda, Hayward, CA) and anti-CD44 (BD Biosciences, San Jose, CA) antibodies which were directly conjugated to fluorochromes. Dead cells were excluded by using the viability dye DAPI. Flow cytometry was performed using a FACS Aria instrument (Becton Dickinson, Franklin Lakes, NJ). Side scatter and forward scatter profiles were used to eliminate cell clumps.

[00230] Analysis of the tumors treated with control antibody revealed that 12.7% of the bulk tumor population expressed both ESA and CD44 at high levels. The double positive population was not significantly affected by treatment with gemcitabine alone (13.9%) as shown in Figure 3, but treatment with either FZD8-Fc or the combination of FZD8-Fc with gemcitabine reduced the double positive population (1.9% and 1.7% respectively).

[00231] *Analysis of FZD8-Fc-treated PN4 tumors by limiting dilution assay.* Limiting dilution assays (LDA) can be used to assess the effect of therapeutic agents on solid tumor cancer stem cells and on the tumorigenicity of a tumor comprising the cancer stem cells. Such assays can be used to determine the frequency of cancer stem cells in tumors from animals treated with the FZD8-Fc fusion protein or other agent and to compare that frequency to the frequency of cancer stem cells in tumors from control animals.

[00232] Control and treated tumors from the PN4 xenograft study described above were harvested at the end of the study (day 85). The tumors were processed and dissociated into single cells. Single cell suspensions derived from 5 tumors of each treatment group were pooled, and the pooled samples were then incubated on ice for 30 min with antibodies that bind mouse cells selectively (α-mouse CD45-biotin 1:200 dilution and rat α-mouse H2Kd-biotin 1:100 dilution, BioLegend, San Diego, CA), followed by addition of streptavidin-labeled magnetic beads (Invitrogen, Carlsbad, CA). The mouse cells were removed with the aid of a magnet. The human cells in the suspension were harvested, counted, and stained for cell surface markers and appropriate cell doses (30, 90, and 270 cells) in FACS buffer were mixed in a 1:1 mixture with Matrigel and injected subcutaneously in NOD/SCID mice (10 mice per cell dose per treatment group). Tumors are allowed to grow for up to 4 months.

[00233] At the desired time point, the percentage of mice with detectable tumors was determined in all groups injected with FZD8-Fc-treated tumor cells and compared to the percentage of mice with detectable tumors in the controls. For example, the number of mice injected with 125 control antibody-treated tumor cells that have detectable tumors is determined and compared to the number of mice injected with 125 FZD8-Fc treated tumor cells that have detectable tumors.

[00234] On day 75 after injection of the cells, tumor take rates in the various groups were as follows: control – 7 mice out of 30 mice; FZD8-Fc – 3 mice out of 30 mice; gemcitabine – 7 mice out of 30 mice; FZD8-Fc and gemcitabine – 0 mice out of 30 mice (Figure 4). The reduced tumor take rate in the FZD8-Fc and in the combination treated groups indicated that the cancer stem cell frequency was reduced in PN4 pancreatic tumors by FZD8-Fc. The evidence from the assessment of both, CD44 expression and limiting dose dilution analysis revealed that FZD8-Fc treatment reduces cancer stem cell frequency in PN4 pancreatic tumors.

[00235] The cancer stem cell (CSC) frequency can be calculated using L-CalcTM software (StemCell Technologies Inc.; www.stemcell.com). Briefly, based on Poisson statistics, exactly one cancer stem cell exists among the known number of injected cells if 37% of the animals fail to develop tumors. The CSC

frequency for the control antibody treated group was 1:280, the CSC frequency for the gemcitabine treated group was 1:476, the CSC frequency for the FZD8-Fc treated group was 1:881, and the CSC frequency for the group treated with a combination of FZD8-Fc and gemcitabine was calculated to be lower than 1:3763. This number could not be accurately determined because the tumor take rate in this group was zero, even at the highest cell dose.

EXAMPLE 4

Increased cell differentiation of pancreatic tumors by FZD8-Fc

[00236] *Increased cell differentiation of PN4 and PN8 tumors with FZD8-Fc treatment.* Control and treated tumors from the OMP-PN4 xenograft study described above (Example 3) were harvested at the end of the study (day 85). Tumors were fixed in formalin, embedded in paraffin, and tumor sections of 4μm thickness were cut. After deparaffinization and hydration, the sections were treated with aqueous acetic acid for 5 minutes at room temperature. The sections were then treated with 1% alcian blue in 3% aqueous acetic acid for 30 minutes and washed with water. Sections were counter-stained in neutral fast red, dehydrated and mounted. Using this method, sialomucins in the tissue samples stain blue and the background appears as pink or red.

[00237] The treatment of PN4 tumors with FZD8-Fc caused an increase in cells expressing sialomucins as compared to tumors treated with control antibody or gemcitabine (Fig. 5, where the sialomucins appear as a dark gray). The combination treatment of FZD8-Fc and gemcitabine also increased the expression of sialomucins in PN4 pancreatic tumors. Therefore, FZD8-Fc treatment of PN4 tumors increased the frequency of mucin-expressing differentiated cells. Similar results were seen in the PN8 pancreatic tumor xenograft model (Fig. 6).

[00238] *Increased cell differentiation of PN13 tumors with FZD8-Fc treatment.* The cell differentiation capability of FZD8-Fc was also evaluated in the OMP-PN13 pancreas tumor xenograft model. Dissociated OMP-PN13 cells (50,000 per animal) were injected subcutaneously into 6-8 week old male NOD/SCID mice. Tumor growth was monitored weekly and tumor measurements were initiated once tumors were palpable. On day 40 mice with average tumor volume of 114 mm^3 were randomized into 4 groups of 10 animals each. Animals were injected with either control antibody or FZD8-Fc (15mg/kg). Administration of the FZD8-Fc and control antibody was performed via injection into the intraperitoneal cavity, twice weekly. After 19 days of treatment, the tumors were excised and immunohistochemistry analysis was performed using standard techniques. Briefly, tumors were fixed in formalin, embedded in paraffin, and tumor sections of 4μm thickness were cut. After deparaffinization and hydration, the tumor sections were subjected to an antigen retrieval process in Tris buffer (pH 9.5). Sections were incubated with hydrogen peroxide (Sigma-Aldrich, St Louis, MO) for 10 minutes to block endogenous peroxidases. Anti-Ki67 antibody (Dako, clone MIB-1) at 1:200 dilution in blocking buffer (3% NHS, 1% BSA, 0.1%

Tween-20, in PBS) was added to each section and incubated for 1 hour. Slides were rinsed 3 times in PBST for 5 minutes each. Anti-mouse secondary antibody conjugated with HRP (ImmPRESS™ anti-mouse, Vector Laboratories Inc., Burlingame, CA) was added to the slides and incubated for 30 minutes. After multiple washes with PBST, Vector Nova Red substrate (Vector Laboratories Inc., Burlingame, CA) was added for localization of Ki67 antigen. The sections were treated with aqueous acetic acid for 5 minutes at room temperature. The sections were then treated with 1% alcian blue in 3% aqueous acetic acid for 30 min and washed with water. Sections were counter-stained in neutral fast red, dehydrated and mounted. Using this method, sialomucins in the tissue samples stain blue and proliferating cells are marked dark red.

[00239] The treatment of PN13 tumors with FZD8-Fc resulted in an increase in cells expressing sialomucins as compared to tumors treated with control antibody. Treatment of PN13 tumors with FZD8-Fc also resulted in a decrease in proliferating cells as denoted by expression of Ki67. Figure 7 shows a clear decrease in the number of proliferating cells (identified by black spots) in the tissue from FZD8-Fc treated tumors. Therefore treatment of PN13 tumors with FZD8-Fc decreased cell proliferation, and increased frequency of mucin expressing differentiated cells.

[00240] *Increased Muc16 staining in Pn13 tumors with FZD8-Fc.* Tumor sections from PN13 tumors treated with control antibody or FZD8-Fc were obtained and treated as described above. In this example, anti-Muc16 (Abcam, Cambridge, MA) antibody in blocking buffer at 1:200 dilution (3% NHS, 1% BSA, 0.1% Tween-20 in PBS) was added to each section and incubated for 1 hour. The bound antibody was detected using the immunohistochemistry protocol described above.

[00241] The treatment of PN13 tumors with FZD8-Fc resulted in an increase in cells expressing Muc16 as compared to tumors treated with control antibody (Figure 8, dark staining).

[00242] *Increased CK20 staining in Pn13 tumors with FZD8-Fc.* Tumor sections were obtained and treated as described above. In this example, anti-CK20 (clone Ks20.8, Dako, Carpinteria, CA) antibody in blocking buffer at 1:200 dilution (3% NHS, 1% BSA, 0.1% Tween-20, in PBS) was added to each section and incubated for 1 hour. The bound antibody was detected using the immunohistochemistry protocol described above.

[00243] The treatment of PN13 tumors with FZD8-Fc resulted in an increase in cells expressing CK20 as compared to tumors treated with control antibody (Figure 9, dark staining).

EXAMPLE 5

Inhibition of breast tumor growth *in vivo* by FZD8-Fc

[00244] Dissociated PE13 breast tumor cells (50,000 per animal) were injected subcutaneously into the mammary fat pads of NOD/SCID mice. Mice were monitored weekly and tumors were allowed to grow until they were approximately 106mm^3. On day 27 post cell injection the mice were randomized into four

treatment groups (n = 10 mice/group) and treated with control antibody, FZD8-Fc (54F03), taxol or a combination of FZD8-Fc and taxol. Taxol was administered intraperitoneally at a dose of 7.5mg/kg once a week and FZD8-Fc was administered intraperitoneally at a dose of 5mg/kg twice a week. Tumors were measured on the days indicated in Figure 10.

[00245] Treatment with FZD8-Fc was observed to reduce tumor growth by 20% ($p = 0.002$) as a single agent relative to the control antibody group. In addition, treatment with the combination of FZD8-Fc and taxol reduced tumor growth by 55% ($p = 0.003$) as compared to taxol treatment alone (Figure 10).

EXAMPLE 6

Inhibition of colon tumor growth *in vivo* by FZD8-Fc

[00246] The effect of multiple doses and dosing regimen of FZD8-Fc (54F03) on the growth of C28 colon tumor xenografts was analyzed. Dissociated C28 cells (10,000 per animal) were injected subcutaneously into 6-8 week old male NOD/SCID mice. On day 2, mice were randomized into 6 groups of 10 animals each. Animals were injected with either control antibody or FZD8-Fc at doses of 1.5mg/kg (twice a week), 5mg/kg (once and twice a week) and 15mg/kg (once and twice a week). Administration of the antibody and FZD8-Fc was performed via injection into the intraperitoneal cavity. Tumor growth was monitored weekly and tumor measurements were initiated once tumors were palpable. Tumors were measured twice a week and tumor volume was determined as described herein.

[00247] Treatment with 15mg/kg of FZD8-Fc (twice weekly) resulted in 83% reduction in tumor growth over treatment with the control antibody, as shown in Figure 11A ($p < 0.001$). Furthermore, treatment with FZD8-Fc at the lowest dose evaluated (1.5mg/kg administered twice a week) also resulted in a 52% reduction of growth over control antibody treatment group (Figure 11A). Thus, FZD8-Fc demonstrated anti-tumor growth activity in the OMP-C28 colon tumor model as a single agent in a dose dependent manner.

[00248] The effect of FZD8-Fc in combination with a chemotherapeutic agent on the growth of C28 colon tumor xenografts was analyzed. Dissociated C28 colon tumor cells (10,000 cells) were injected subcutaneously into 6-8 week old male NOD/SCID mice. Tumors were allowed to grow for 21 days until they reached an average volume of $128mm^3$. The mice were randomized (n = 10 per group) and treated with FZD8-Fc (54F03) (15mg/kg once a week), irinotecan (15mg/kg once a week), a combination of FZD8-Fc and irinotecan or a control antibody. Administration of the FZD8-Fc, irinotecan and control antibody was performed via injection into the intraperitoneal cavity. Tumor growth was monitored and tumor volumes were measured with electronic calipers at the indicated time points. Data are expressed as mean ± S.E.M.

[00249] As shown in Figure 11B, treatment with FZD8-Fc as a single agent (-▲-) resulted in 66% reduction in tumor growth over treatment with the control antibody (-■-) ($p < 0.001$). Furthermore,

treatment with FZD8-Fc in combination with irinotecan (-●-) resulted in a 76% reduction of growth over control antibody treatment group ($p < 0.001$), which was greater than either agent alone. Thus, FZD8-Fc demonstrated anti-tumor growth activity in the OMP-C28 colon tumor model as a single agent, as well as in combination with a chemotherapeutic agent.

EXAMPLE 7

Increased cell differentiation of colon tumors by FZD8-Fc

[00250] *Increased CK20 staining in C28 tumors with FZD8-Fc.* Control and treated tumors from the C28 xenograft study described above (Example 6) were harvested at the end of the study. The tumors were excised and immunohistochemistry analysis was performed using standard techniques. Briefly, tumors were fixed in formalin, embedded in paraffin, and tumor sections of 4um thickness were cut. After deparaffinization and hydration, the tumor sections were subjected to an antigen retrieval process in Tris buffer (pH 9.5). Sections were incubated with hydrogen peroxide (Sigma-Aldrich, St Louis, MO) for 10 minutes to block endogenous peroxidases. Anti-CK20 (clone Ks20.8, Dako, Carpinteria, CA) antibody in blocking buffer at 1:200 dilution (3% NHS, 1% BSA, 0.1% Tween-20, in PBS) was added to each section and incubated for 1 hour. Slides were rinsed 3 times in PBST for 5 minutes each. Anti-mouse secondary antibody conjugated with HRP (ImmPRESS™ anti-mouse Ig, Vector Laboratories Inc., Burlingame, CA) was added to the slides and incubated for 30 minutes. After multiple washes with PBST, Vector Nova Red substrate (Vector Laboratories Inc., Burlingame, CA) was added for localization of CK20 antigen.

[00251] The treatment of C28 tumors with FZD8-Fc resulted in an increase in cells expressing CK20 as compared to tumors treated with control antibody (Figure 12, dark staining).

EXAMPLE 8

Anti-tumor activity of FZD8-Fc in pancreatic tumor model

[00252] *Inhibition of tumor growth by FZD8-Fc in PN21 pancreatic tumor model.* The anti-tumor activity of FZD8-Fc (54F03) was evaluated in the PN21 pancreas tumor xenograft model. Dissociated OMP-PN21 cells (50,000 per animal) were injected subcutaneously into 6-8 week old male NOD/SCID mice. Tumor growth was monitored weekly and tumor measurements were initiated once tumors were palpable. On day 36, mice with average tumor volumes of $144mm^3$ were randomized into 4 groups of 9 animals each. Animals were injected with control antibody, FZD8-Fc (15mg/kg), gemcitabine (2mg/kg) or a combination of FZD8-Fc and gemcitabine. Administration of the FZD8-Fc and gemcitabine was performed via injection into the intraperitoneal cavity, once weekly. Tumors were measured twice a week and tumor volume was determined as described herein.

[00253] Treatment with FZD8-Fc resulted in a 66% reduction in tumor growth as compared to control, as shown in Figure 13A ($p < 0.001$). Furthermore, treatment with a combination of FZD8-Fc and

gemcitabine resulted in a greater reduction of tumor growth compared to either agent alone ($p = 0.001$ vs. gemcitabine). Thus, FZD8-Fc demonstrated anti-tumor growth activity in the PN21 pancreas tumor model as a single agent as well as in combination with gemcitabine.

[00254] *Analysis of FZD8-Fc-treated PN21 tumors by limiting dilution assay.* As described above in Example 3, a limiting dilution assays was used to assess the effect of treatment with FZD8-Fc alone or in combination with gemcitabine on solid tumor cancer stem cells in the PN21 pancreatic tumor model.

[00255] Control and treated tumors from the PN21 xenograft study described above were harvested at the end of the study. The tumors were processed and dissociated into single cells. Single cell suspensions derived from 5 tumors of each treatment group were pooled, and the pooled samples were then incubated on ice for 30 min with antibodies that bind mouse cells selectively (α-mouse CD45-biotin 1:200 dilution and rat α-mouse H2Kd-biotin 1:100 dilution, BioLegend, San Diego, CA), followed by addition of streptavidin-labeled magnetic beads (Invitrogen, Carlsbad, CA). The mouse cells were removed with the aid of a magnet. The human cells in the suspension were harvested, counted, and stained for cell surface markers and appropriate cell doses (30, 90, and 270 cells) in FACS buffer were mixed in a 1:1 mixture with Matrigel and injected subcutaneously in NOD/SCID mice (10 mice per cell dose per treatment group). Tumors are allowed to grow for up to 4 months.

[00256] At the desired time point, the percentage of mice with detectable tumors was determined in all groups injected with treated tumor cells and compared to the percentage of mice with detectable tumors in control treated cells. For example, the number of mice injected with 125 control antibody-treated tumor cells that have detectable tumors is determined and compared to the number of mice injected with 125 FZD8-Fc treated tumor cells that have detectable tumors.

[00257] On day 72 after injection of the cells, tumor take rates in the various groups were determined and the cancer stem cell frequency was calculated using L-CalcTM software (StemCell Technologies Inc.; www.stemcell.com). As shown in Figure 13B, treatment with FZD8-Fc reduced cancer stem cell frequency to 1:976, approximately a four-fold reduction as compared to treatment with the control antibody. In contrast, treatment with gemcitabine slightly increased cancer stem cell frequency. Significantly, treatment with a combination of FZD8-Fc and gemcitabine reduced the cancer stem cell frequency to 1:5472, almost a 25-fold reduction as compared to treatment with control. Surprisingly, treatment with the combination of FZD8-Fc and gemcitabine reduced the cancer stem cell frequency approximately 5.5-fold greater than FZD8-Fc treatment alone and despite the fact that gemcitabine appeared to actually increase the cancer stem cell frequency.

EXAMPLE 9

[00258] *Increased cell differentiation of PN21 tumors with FZD8-Fc.* The cell differentiation capability of FZD8-Fc was also evaluated in the OMP-PN21 pancreas tumor xenograft model. PN21 tumors from

studies described in Example 8 were harvested and fixed in formalin, embedded in paraffin, and tumor sections of 4μm thickness were cut. After deparaffinization and hydration, the tumor sections were subjected to an antigen retrieval process in Tris buffer (pH 9.5). Sections were incubated with hydrogen peroxide (Sigma-Aldrich, St Louis, MO) for 10 minutes to block endogenous peroxidases. Anti-Ki67 antibody (clone MIB-1, Dako, Carpinteria, CA) in blocking buffer (3% NHS, 1% BSA, 0.1% Tween-20 in PBS) at 1:200 dilution was added to each section and incubated for 1 hour. Slides were rinsed 3 times in PBST for 5 minutes each. Anti-mouse secondary antibody conjugated with HRP (ImmPRESS™ anti-mouse, Vector Laboratories Inc., Burlingame, CA) was added to the slides and incubated for 30 minutes. After multiple washes with PBST, Vector Nova Red substrate (Vector Laboratories Inc., Burlingame, CA) was added for localization of Ki67 antigen. The sections were treated with aqueous acetic acid for 5 minutes at room temperature. The sections were then treated with 1% alcian blue in 3% aqueous acetic acid for 30 minutes and washed with water. Sections were counter-stained in neutral fast red, dehydrated and mounted. Using this method, sialomucins in the tissue samples stain blue and the background appears as pink or red.

[00259] The treatment of PN21 tumors with FZD8-Fc resulted in an increase in cells expressing sialomucins as compared to tumors treated with control antibody (Figure 14, dark gray staining). The treatment of PN21 tumors with FZD8-Fc or FZD-Fc in combination with gemcitabine resulted in an increase in cells expressing sialomucins as compared to tumors treated with control antibody or gemcitabine alone (Figure 15). Treatment of PN21 tumors with FZD8-Fc or the combination of FZD8-Fc and gemcitabine also resulted in a decrease in proliferating cells denoted by expression of Ki67. Therefore treatment of PN21 tumors with FZD8-Fc, either alone or in combination with gemcitabine, decreased cell proliferation, and increased frequency of mucin-expressing differentiated cells.

EXAMPLE 10

Production of FZD8-Fc variants

[00260] *Production of FZD8-Fc variants.* FZD8-Fc variants were produced at DNA2.0 (Menlo Park, CA). DNA2.0 synthesized and assembled short single-stranded oligonucleotides to produce the different FZD8-Fc variant proteins, 54F05, 54F08, 54F09, 54F12, 54F13, 54F14, 54F15, 54F16, 54F17, 54F18, 54F19, 54F20, 54F21 and 54F22. The assembled oligonucleotides were subsequently cloned and sequence verified.

EXAMPLE 11

Inhibition of Wnt signaling by FZD8-Fc variants

[00261] The ability of the FZD8-Fc variants to block or inhibit activation of the Wnt signaling pathway was determined *in vitro* using a luciferase reporter assay. STF293 cells were cultured in DMEM

supplemented with antibiotics and 10% FCS. The STF293 cells are stably transfected with a reporter vector containing seven copies of the TCF transcriptional response element linked to a promoter upstream of a firefly luciferase reporter gene. This construct measures the activity of the canonical Wnt signaling pathway. The cells were added to cultures plates at 10,000 cells per well. After an overnight incubation the FZD8-Fc variants or control mouse JAG1-Fc were added in combination with Wnt3a-conditioned medium. The FZD8-Fc variants and JAG1-Fc were used at concentrations of 20, 4, 0.8, 0.16, 0.03, 0.006, 0.0012, and 0.0003ug/ml. The cells were incubated in 25% Wnt3A-conditioned medium that had been prepared from L cells that stably express Wnt3a. After overnight incubation (approximately 18 hrs), luciferase levels were measured using a Steady-Glo® luciferase assay kit (Promega, Madison, WI).

[00262] The blocking activity of the FZD8-Fc variants was determined and is presented in Table 3 as relative activity as compared to the same reference standard run in each assay which was set at 100%.

Table 3

FZD8-Fc Variant	% Relative Activity
54F03	107, 143
54F05	167
54F08	137
54F09	156, 157
54F12	52
54F13	103
54F14	125
54F15	128
54F16	125

EXAMPLE 12

Pharmacokinetics of FZD8-Fc variants in rats

[00263] The pharmacokinetics of several FZD8-Fc variants were assessed in rats in a two week pharmacokinetics (PK) study. The FZD8-Fc variants evaluated were 54F03, 54F09, 54F12, 54F13, 54F15 and 54F16. Sprague Dawley rats, five males in each group, were dosed with FZD8-Fc variants via the tail vein at 10mg/kg and followed for two weeks with samples collected at time points 1, 24, 48, 72, 96, 168, 240, and 336 hours. At each time point, 1ml of blood was collected into potassium-EDTA tubes and centrifuged. The plasma supernatants were collected and frozen until the samples were analyzed.

[00264] The level of FZD8-Fc variant protein present in the plasma at each time point was quantified and the half-life of each FZD8-Fc variant was calculated. The half-life of the FZD8-Fc variants is shown in Table 4.

Table 4

FZD8-Fc Variant	$t_{1/2}$ in hours	Fc Region
54F03	162	IgG1

54F09	136	IgG1
54F12	152	IgG2
54F13	268	IgG2
54F15	109	IgG1
54F16	154	IgG1

EXAMPLE 13

Pharmacokinetics of FZD8-Fc variants in cynomolgus monkeys

[00265] Four young adult/adult male naïve cynomolgus monkeys were randomly divided into two groups of two, and administered an intravenous (IV) bolus of FZD-Fc variant 54F15 or variant 54F16 at a dose of 30mg/kg. Twice daily (a.m. and p.m.) animals were observed for mortality and signs of pain and distress. Cageside observations for general health and appearance were done once daily. On the day of dosing, each animal was observed at approximately 1 and 4 hours post-dose for mortality and signs of pain and distress. Any unusual observations noted throughout the duration of the study were recorded. Body weights were taken on the day of dose administration and at the end of blood collection. Blood (approximately 0.5ml) was collected from a femoral vein via syringe and needle and transferred into tubes containing EDTA K3 anticoagulant pre-dose and at 1, 6, 12, 24, 48, 72, 96, 168, 240, and 336 hours post-dose for PK analysis. In addition, blood (approximately 0.5ml) was collected from a femoral vein via syringe and needle and transferred into tubes containing no anticoagulant pre-dose and at 336 hours post-dose for anti-drug antibody (ADA) analysis. Plasma supernatants were collected and frozen until the samples were analyzed. HTRF (homogeneous time resolved fluorescence) immunoassays were performed to determine the FZD-Fc concentration in animal plasma samples for PK analysis and the concentration of anti-drug antibody in serum. FZD-Fc concentration in plasma versus time was analyzed by non-compartmental analysis (NCA) with Phoenix™ WinNonlin® Version 6.0, using a bolus IV administration model.

[00266] The level of FZD8-Fc variants present in the plasma at each time point was quantified (Figure 16) and the half-life of FZD8-Fc variants 54F15 and 54F16 was calculated. As shown in Table 5, the half-life of FZD8-Fc variant 54F15 was estimated to be 102 hours at 30mg/kg and the half-life of FZD-Fc8 variant 54F16 was estimated to be 137 hours at 30mg/kg.

Table 5

Animal ID	$T_{1/2\lambda z}$ (hr)	$AUC_{0\text{-last}}$ (ng*hr/ml)	$AUC_{0\text{-}\infty}$ (ng*hr/ml)	AUC % Extrap	Vz (ml)	Cl (ml/hr)
FZD8-Fc 54F15						

C43064	102.3	28642771.4	31231858.7	8.3	141.7	0.960
C43061	102.5	31904918.2	34846234.1	8.4	127.3	0.861
Mean	102.4	30273845	33039046	8.35	134.5	0.9105
FZD8-Fc 54F16						
C43066	139.0	29088083.0	35470395.6	18.0	169.6	0.846
C43076	134.1	35934159.0	43449944.6	17.3	133.6	0.690
Mean	136.6	32511121	39460170	17.65	151.6	0.768

EXAMPLE 14

Inhibition of colon tumor growth *in vivo* by FZD8-Fc variants

[00267] Dissociated C28 colon tumor cells (10,000 cells) were injected subcutaneously into 6-8 week old male NOD/SCID mice. Tumors were allowed to grow for 28 days until they reached an average volume of 145mm^3. The mice were randomized (n = 9 per group) and treated with FZD8-Fc variants or a control antibody at a dose of 15mg/kg twice a week. Administration of the FZD8-Fc variants and control antibody was performed via injection into the intraperitoneal cavity. Tumor growth was monitored and tumor volumes were measured with electronic calipers at the indicated time points. Data are expressed as mean ± S.E.M.

[00268] Variants 54F12 and 54F13 had no apparent effect upon tumor growth, with tumor volumes substantially the same as the tumors in mice treated with control antibody. In contrast, treatment with variants 54F03, 54F09, 54F15 and 54F16 resulted in approximately 56%, 70%, 64% and 70% reduction (respectively) in tumor growth as compared to treatment with the control antibody, as shown in Figure 17. Thus, the anti-tumor growth activity of the FZD8-Fc variants appeared to be affected by the amino acid sequence at the junction between the FZD8 portion and the Fc portion. In addition, anti-tumor growth activity appeared to be affected by the source of the Fc region, as both variants that are IgG2 fusion proteins (54F12 and 54F13), did not inhibit tumor growth in this model.

EXAMPLE 15

Inhibition of pancreatic tumor growth *in vivo* by FZD8-Fc variants

[00269] Dissociated PN4 pancreatic tumor cells (10,000 cells) were injected subcutaneously into 6-8 week old male NOD/SCID mice. Tumors were allowed to grow for 36 days until they reached an average volume of 112mm^3. The mice were randomized (n = 10 per group) and treated with FZD8-Fc variants or a control antibody at a dose of 15mg/kg twice a week. Administration of the FZD8-Fc variants and control antibody was performed via injection into the intraperitoneal cavity. Tumor growth was

monitored and tumor volumes were measured with electronic calipers at the indicated time points. Data are expressed as mean ± S.E.M.

[00270] Variants 54F12 and 54F13 reduced tumor growth less than 20% as compared with tumors in mice treated with control antibody. Treatment with FZD8-Fc variants 54F03, 54F09, 54F15 and 54F16 reduced tumor growth approximately 20% to 60% as compared to treatment with the control antibody. As shown in Figure 18, variants 54F09 and 54F16 reduced tumor growth by the greatest amount, 45% ($p < 0.001$) and 60% ($p < 0.001$), respectively. Thus, the anti-tumor growth activity of the FZD8-Fc variants appeared to be affected by the amino acid sequence at the junction between the FZD8 portion and the Fc portion. As seen in the Example 14, anti-tumor growth activity also appeared to be affected by the source of the Fc region, as both variants that are IgG2 fusion proteins (54F12 and 54F13) had weaker anti-tumor activity than the FZD8-Fc variants that are IgG1 fusion proteins.

[00271] Pancreatic tumor cells from the tumor-bearing mice described above were harvested and minced into approximately $1mm^3$ fragments, followed by enzymatic digestion at 1 gram per 10ml of 300 µg/ml collagenase and 200 U/ml DNase I for 2 hours at 37°C/5% CO_2 with intermittent mixing with a 10ml pipet to disperse cells. Digestion was stopped by adding an equal volume of FACS buffer (1x Hanks Buffered Saline Solution (HBSS), 2% heat-inactivated Fetal Calf Serum (FCS) and 2mM HEPES pH 7.4). Cells were filtered through 40µm nylon filters and collected by centrifugation at 150 xg for 5 minutes. Red blood cells were lysed in a hypotonic buffer containing ammonium chloride for 2 minutes on ice, and the cells were washed again with excess FACS buffer, and resuspended with FACS buffer at $1x10^7$ cells/ml.

[00272] The freshly prepared single cell suspensions were stained for 20 minutes on ice with biotinylated anti-mouse H-2Kd (clone SF1-1.1, Biolegend, San Diego, CA) at 5µg/ml, biotinylated anti-mouse CD45 (30-F11, Biolegend) at 2.5µg/ml, and streptavidin-PerCP-Cy5.5 (eBioscience, San Diego, CA) at 1:200 dilution. Unbound antibody was removed by washing twice with 10 volume of FACS buffer. For analysis of human cell surface markers, the single tumor cell suspension was stained with anti-ESA-FITC (Miltenyi Biotec, Auburn, CA) at 1:50 dilution, anti-human CD44-PE-Cy7 (eBioscience, San Diego, CA) at 1:100 dilution, and anti-human CD201-PE (BD Biosciences) at 1:5 dilution. The cells are washed, and resuspended in FACS buffer containing 2.5ug/ml of 4'-6-Diamidino-2-phenylindole (DAPI). Cells stained with a single fluorescent color were used for instrument calibration. Any remaining mouse cells (positive for H-2Kd and CD45) and dead cells (DAPI-positive) were excluded during cell sorting. Cell doublets and clumps were excluded using doublet discrimination gating.

[00273] As shown in Figure 19, treatment of tumor-bearing mice with FZD8-Fc variants 54F03 and 54F16 decreased the percentage of $CD44^{hi}$ cells as compared to mice treated with the control antibody. Although the percentage of $CD201^+CD44^+$ cells was small, treatment of tumor-bearing mice with FZD8-Fc variants 54F03 and 54F16 decreased the percentage of $CD201^+CD44^+$ cells as compared to mice treated with the control antibody. CD44 has been shown to be to marker of tumorigenic cells (e.g. cancer stem cells). In

addition, in some embodiments, cells that are $CD44^{hi}CD201^{+}$ have been found to be more tumorigenic than $CD44^{hi}CD201^{-}$ cells. Thus, it is important that FZD8-Fc variants were capable of decreasing the percentage of both $CD44^{hi}$ and $CD44^{hi}CD201^{+}$ cell populations, thereby decreasing the percentage or number of tumorigenic cells in the treated mice.

EXAMPLE 16

Characterization of N-termini

[00274] The correct signal sequence cleavage site in the FZD8 protein is predicted to be between amino acid 25 (an alanine) and amino acid 26 (an alanine); cleavage at this site leaves an N-termini of ASA. Analysis by mass spectrometry was used to determine the mass of FZD8-Fc proteins as compared to the theoretical mass of the FZD8-Fc protein cleaved at the predicted site.

[00275] Additional FZD8-Fc variants with modified signal sequences were produced at DNA2.0 (Menlo Park, CA) as described above. DNA2.0 synthesized and assembled short single-stranded oligonucleotides to produce the FZD8-Fc variant proteins, 54F23 to 54F35. The assembled oligonucleotides were subsequently cloned and sequence verified.

[00276] Plasmid DNA of each FZD8-Fc variant was prepared using QIAGEN maxi-prep kits following the manufacturer's protocol. Expression of each variant was done using FreeStyle™ MAX reagent (Life Technologies) and 293FS cells. Cells were grown to log phase and diluted to $1x10^6$ cell/ml. For each reaction, 315ug of plasmid DNA was diluted into 5ml of OptiMEM Pro. In a different tube, 315ul of FreeStyle™ MAX reagent was diluted into 5ml of OptiMem Pro. Plasmid DNA was complexed with the FreeStyle™ MAX reagent by adding the diluted reagent dropwise to the DNA, followed by an incubation of 15 minutes at room temperature. The DNA-reagent complex was then added to 250ml of 293FS cells. Expression reactions were allowed to grow for 7-10 days, at which time, they were harvested by centrifugation and filtration. Each Fzd8-Fc variant was purified by affinity purification using a 5ml HiTrap MAbSelect SURE column. Briefly, harvested media were passed through columns that had been equilibrated with binding buffer. The columns were washed with binding buffer to remove unbound material, and then FZD8-Fc protein was eluted with elution buffer. Eluted samples were then dialyzed into a buffer suitable for mass spectrometry.

[00277] Approximately 250 µg of each FZD8-Fc sample was reduced with Tris (2-carboxyethyl) phosphine (TCEP) for 30 minutes at 37°C to separate heavy and light chains of the antibody. Reduced samples were then alkylated by treatment with iodoacetamide for 30 minutes at 37°C. Samples were passed over NAP-5 columns (GE Health Care) to change the buffer to 10 mM Tris-HCL (pH 7.4). Buffer exchange was followed deglycosylation with endoglycosidase PNGase F. Samples were incubated overnight at 37°C at a ratio of 1:200 (enzyme:sample). The reactions were stopped by addition of acid. The reduced, alkylated, and deglycosylated FZD8-Fc samples were loaded into vials for liquid

chromatography-mass spectrometry (LC/MS) analysis using a Waters UPLC™ and electrospray-QToF mass spectrometer. Mass calibration of each sample analysis using cesium trifluoroacetic acid ion clusters was conducted with a Waters LockSpray™ dual electrospray ion source.

[00278] As shown in Figure 20A, a FZD8-Fc protein (54F16) with a signal sequence that was the same sequence as the native sequence was produced as a heterogeneous mixture in regard to the N-terminal sequence. A proportional of the protein present in sample was equivalent in mass to a protein cleaved at amino acids 25 and 26 (peak at 41704.0) with an N-terminal sequence of ASA. However, greater than 50% of the protein was present in a form with a different mass (peak at 41918.2). This peak most likely represents a protein cleaved at amino acids 22 and 23; cleavage at this site leaves an N-terminal sequence of AAAASA (SEQ ID NO:76).

[00279] FZD8-Fc variants with signal sequences SEQ ID NO:68 to SEQ ID NO:74 were generated, purified from cell culture, and analyzed by mass spectrometry as described above. It was observed that variants with signal sequences SEQ ID NO:70 to SEQ ID NO:74 produced an almost completely homogeneous protein sample (several representative results are shown in Figures 20B-20E). FZD8-Fc variant 54F26 which comprises SEQ ID NO:53 with signal sequence SEQ ID NO:71 was present predominantly (greater than 95%) as a protein cleaved at amino acids 25 and 26 (peak 41930.1) with an N-terminal sequence of ASA (Fig. 20B). Similar results were also seen with a variant comprising SEQ ID NO:53 and signal sequence SEQ ID NO:72 (54F28, Fig. 20C), a variant comprising SEQ ID NO:53 and signal sequence SEQ ID NO:73 (54F30, Fig. 20D) and a variant comprising SEQ ID NO:53 and signal sequence SEQ ID NO:74 (54F32, Fig. 20E). Similar results were observed with proteins produced from transient and stable transfections.

EXAMPLE 17

Inhibition of colon tumor growth *in vivo* by FZD8-Fc variants

[00280] Dissociated C28 colon tumor cells (10,000 cells) were injected subcutaneously into 6-8 week old male NOD/SCID mice. Tumors were allowed to grow for 56 days until they reached an average volume of 175mm^3. The mice were randomized (n = 10 per group) and treated with FZD8-Fc constructs 54F03, 54F23, 54F26, or a control antibody at a dose of 15mg/kg twice a week. Administration of the FZD8-Fc variants and control antibody was performed via injection into the intraperitoneal cavity. Tumor growth was monitored and tumor volumes were measured with electronic calipers at the indicated time points. Data are expressed as mean ± S.E.M.

[00281] FZD8-Fc variants 54F23 and 54F26 are produced as predominantly a homogenous protein with an N-terminus of amino acids ASA, while 54F03 is produced as a heterogeneous protein mixture with N-termini of amino acids ASA and AAAASA. As shown in Figure 21 treatment with FZD8-Fc variants

54F03, 54F23, and 54F26 reduced tumor growth 48%, 57% and 52%, respectively, as compared to treatment with the control antibody after three weeks of treatment.

[00282] All publications, patents, patent applications, internet sites, and accession numbers/database sequences (including both polynucleotide and polypeptide sequences) cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, internet site, or accession number/database sequence were specifically and individually indicated to be so incorporated by reference.

SEQUENCES

FZD8-Fc amino acid sequence-variant 54F03 (without predicted signal sequence; the "GRA" linker sequence between the FZD8 sequence and the Fc sequence of the fusion protein is underlined) (SEQ ID NO:1)

ASAKELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFF
LCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNPDTL
CMDYNRTDLTTGRADKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV
SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ
PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
K

FZD8-Fc coding sequence (nucleotides encoding FZD8-derived sequences are underlined) (SEQ ID NO:2)

ATGGAGTGGGGTTACCTGTTGGAAGTGACCTCGCTGCTGGCCGCCTTGGCGCTGCTGCAG
CGCTCTAGCGGCGCTGCGGCCGCCTCGGCCAAGGAGCTGGCATGCCAAGAGATCACCGTG
CCGCTGTGTAAGGGCATCGGCTACAACTACACCTACATGCCCAATCAGTTCAACCACGAC
ACGCAAGACGAGGCGGGCCTGGAGGTGCACCAGTTCTGGCCGCTGGTGGAGATCCAGTGC
TCGCCCGATCTCAAGTTCTTCCTGTGCAGCATGTACACGCCCATCTGCCTAGAGGACTAC
AAGAAGCCGCTGCCGCCCTGCCGCTCGGTGTGCGAGCGCGCCAAGGCCGGCTGCGCGCCG
CTCATGCGCCAGTACGGCTTCGCCTGGCCCGACCGCATGCGCTGCGACCGGCTGCCCGAG
CAAGGCAACCCTGACACGCTGTGCATGGACTACAACCGCACCGACCTAACCACCGGGCGC
GCCGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCA
GTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTC
ACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTG
GACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACG
TACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTAC
AAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCC
AAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACC
AAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTG
GAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGAC
TCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAG
GGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACACAGAAG
AGCCTCTCCCTGTCTCCGGGTAAATGA

Minimum FZD and SFRP Fri domain sequences

h-FZD1 amino acids 116-227 (SEQ ID NO:3)

CQPISIPLCTDIAYNQTIMPNLLGHTNQEDAGLEVHQFYPLVKVQCSAELKFFLCSMYAP
VCTVLEQALPPCRSLCERARQGCEALMNKFGFQWPDTLKCEKFPVHGAGELC

h-FZD2 amino acids 39-150 (SEQ ID NO:4)

CQPISIPLCTDIAYNQTIMPNLLGHTNQEDAGLEVHQFYPLVKVQCSPELRFFLCSMYAP
VCTVLEQAIPPCRSICERARQGCEALMNKFGFQWPERLRCEHFPRHGAEQIC

h-FZD3 amino acids 28-133 (SEQ ID NO:5)

CEPITLRMCQDLPYNTTFMPNLLNHYDQQTAALAMEPFHPMVNLDCSRDFRPFLCALYAP
ICMEYGRVTLPCRRLCQRAYSECSKLMEMFGVPWPEDMECSRFPDC

h-FZD4 amino acids 48-161 (SEQ ID NO:6)

CDPIRISMCQNLGYNVTKMPNLVGHELQTDAELQLTTFTPLIQYGCSSQLQFFLCSVYVP
MCTEKINIPIGPCGGMCLSVKRRCEPVLKEFGFAWPESLNCSKFPPQNDHNHMC

h-FZD5 amino acids 33-147 (SEQ ID NO:7)
CQEITVPMCRGIGYNLTHMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLRFFLCSMYTP
ICLPDYHKPLPPCRSVCERAKAGCSPLMRQYGFAWPERMSCDRLPVLGRDAEVLC

h-FZD6 amino acids 24-129 (SEQ ID NO:8)
CEPITVPRCMKMAYNMTFFPNLMGHYDQSIAAVEMEHFLPLANLECSPNIETFLCKAFVP
TCIEQIHVVPPCRKLCEKVYSDCKKLIDTFGIRWPEELECDRLQYC

h-FZD7 amino acids 49-160 (SEQ ID NO:9)
CQPISIPLCTDIAYNQTILPNLLGHTNQEDAGLEVHQFYPLVKVQCSPELRFFLCSMYAP
VCTVLDQAIPPCRSLCERARQGCEALMNKFGFQWPERLRCENFPVHGAGEIC

h-FZD8 amino acids 35-148 (SEQ ID NO:10)
CQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFFLCSMYTP
ICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNPDTLC

h-FZD9 amino acids 39-152 (SEQ ID NO:11)
CQAVEIPMCRGIGYNLTRMPNLLGHTSQGEAAAELAEFAPLVQYGCHSHLRFFLCSLYAP
MCTDQVSTPIPACRPMCEQARLRCAPIMEQFNFGWPDSLDCARLPTRNDPHALC

h-FZD10 amino acids 34-147 (SEQ ID NO:12)
CQPIEIPMCKDIGYNMTRMPNLMGHENQREAAIQLHEFAPLVEYGCHGHLRFFLCSLYAP
MCTEQVSTPIPACRVMCEQARLKCSPIMEQFNFKWPDSLDCRKLPNKNDPNYLC

h-SFRP1 amino acids 57-165 (SEQ ID NO:13)
CVDIPADLRLCHNVGYKKMVLPNLLEHETMAEVKQQASSWVPLLNKNCHAGTQVFLCSLF
APVCLDRPIYPCRWLCEAVRDSCEPVMQFFGFYWPEMLKCDKFPEGDVC

h-SFRP2 amino acids 40-152 (SEQ ID NO:14)
CKPIPANLQLCHGIEYQNMRLPNLLGHETMKEVLEQAGAWIPLVMKQCHPDTKKFLCSLF
APVCLDDLDETIQPCHSLCVQVKDRCAPVMSAFGFPWPDMLECDRFPQDNDLC

h-SFRP3 amino acids 35-147 (SEQ ID NO:15)
CEPVRIPLCKSLPWNMTKMPNHLHHSTQANAILAIEQFEGLLGTHCSPDLLFFLCAMYAP
ICTIDFQHEPIKPCKSVCERARQGCEPILIKYRHSWPENLACEELPVYDRGVC

h-SFRP4 amino acids 24-136 (SEQ ID NO:16)
CEAVRIPMCRHMPWNITRMPNHLHHSTQENAILAIEQYEELVDVNCSAVLRFFFCAMYAP
ICTLEFLHDPIKPCKSVCQRARDDCEPLMKMYNHSWPESLACDELPVYDRGVC

h-SFRP5 amino acids 53-162 (SEQ ID NO:17)
CLDIPADLPLCHTVGYKRMRLPNLLEHESLAEVKQQASSWLPLLAKRCHSDTQVFLCSLF
APVCLDRPIYPCRSLCEAVRAGCAPLMEAYGFPWPEMLHCHKFPLDNDLC

Fc sequences

Human IgG_1 Fc region (SEQ ID NO:18)
DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK
GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS
DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Human IgG_1 Fc region (SEQ ID NO:42)
KSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW
YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV
LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Human IgG_1 Fc region (SEQ ID NO:43)
EPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT
ISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Human IgG_2 Fc region (SEQ ID NO:44)
CVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVE
VHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQP
REPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGS
FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

FZD Fri domain sequences

Human FZD4 Fri domain (predicted signal sequence underlined) (SEQ ID NO:19)
MLAMAWRGAGPSVPGAPGGVGLSLGLLLQLLLLLGPARGFGDEEERRCDPIRISMCQNLG
YNVTKMPNLVGHELQTDAELQLTTFTPLIQYGCSSQLQFFLCSVYVPMCTEKINIPIGPC
GGMCLSVKRRCEPVLKEFGFAWPESLNCSKFPPQNDHNHMCMEGPGDEEV

Human FZD5 Fri domain (predicted signal sequence underlined) (SEQ ID NO:20)
MARPDPSAPPSLLLLLLAQLVGRAAAASKAPVCQEITVPMCRGIGYNLTHMPNQFNHDTQ
DEAGLEVHQFWPLVEIQCSPDLRFFLCSMYTPICLPDYHKPLPPCRSVCERAKAGCSPLM
RQYGFAWPERMSCDRLPVLGRDAEVLCMDYNRSEATT

Human FZD8 Fri domain (predicted signal sequence underlined) (SEQ ID NO:21)
MEWGYLLEVTSLLAALALLQRSSGAAAASAKELACQEITVPLCKGIGYNYTYMPNQFNHD
TQDEAGLEVHQFWPLVEIQCSPDLKFFLCSMYTPICLEDYKKPLPPCRSVCERAKAGCAP
LMRQYGFAWPDRMRCDRLPEQGNPDTLCMDYNRTDLTT

Human FZD1 Fri domain amino acid sequence without predicted signal sequence (SEQ ID NO:32; amino acids 87-237 of SEQ ID NO:27)
QQPPPPPQQQQSGQQYNGERGISVPDHGYCQPISIPLCTDIAYNQTIMPNLLGHTNQEDA
GLEVHQFYPLVKVQCSAELKFFLCSMYAPVCTVLEQALPPCRSLCERARQGCEALMNKFG
FQWPDTLKCEKFPVHGAGELCVGQNTSDKGT

Human FZD2 Fri domain amino acid sequence without predicted signal sequence SEQ ID NO:33; amino acids 24-159 of SEQ ID NO:28)
QFHGEKGISIPDHGFCQPISIPLCTDIAYNQTIMPNLLGHTNQEDAGLEVHQFYPLVKVQ
CSPELRFFLCSMYAPVCTVLEQAIPPCRSICERARQGCEALMNKFGFQWPERLRCEHFPR
HGAEQICVGQNHSEDG

Human FZD3 Fri domain amino acid sequence without predicted signal sequence (SEQ ID NO:34; amino acids 23-143 of SEQ ID NO:29)
HSLFSCEPITLRMCQDLPYNTTFMPNLLNHYDQQTAALAMEPFHPMVNLDCSRDF
RPFLCALYAPICMEYGRVTLPCRRLCQRAYSECSKLMEMFGVPWPEDMECSRFPDCDEPY
PRLVDL

Human FZD4 Fri domain amino acid sequence without predicted signal sequence e (SEQ ID NO:35; amino acids 40-170 of SEQ ID NO:22)
FGDEEERRCDPIRISMCQNLGYNVTKMPNLVGHELQTDAELQLTTFTPLIQYGCSSQLQF
FLCSVYVPMCTEKINIPIGPCGGMCLSVKRRCEPVLKEFGFAWPESLNCSKFPPQNDHNH
MCMEGPGDEEV

Human FZD5 Fri domain amino acid sequence without predicted signal sequence (SEQ ID NO:36; amino acids 27-157 of SEQ ID NO:23)
ASKAPVCQEITVPMCRGIGYNLTHMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLRFFL
CSMYTPICLPDYHKPLPPCRSVCERAKAGCSPLMRQYGFAWPERMSCDRLPVLGRDAEVL
CMDYNRSEATT

Human FZD6 Fri domain amino acid sequence without predicted signal sequence (SEQ ID NO:37; amino acids 19-146 of SEQ ID NO:24)
HSLFTCEPITVPRCMKMAYNMTFFPNLMGHYDQSIAAVEMEHFLPLANLECSPNIETFLC
KAFVPTCIEQIHVVPPCRKLCEKVYSDCKKLIDTFGIRWPEELECDRLQYCDETVPVTFD
PHTEFLG

Human FZD7 Fri domain amino acid sequence without predicted signal sequence (SEQ ID NO:38; amino acids 33-170 of SEQ ID NO:25)
QPYHGEKGISVPDHGFCQPISIPLCTDIAYNQTILPNLLGHTNQEDAGLEVHQFYPLVKV
QCSPELRFFLCSMYAPVCTVLDQAIPPCRSLCERARQGCEALMNKFGFQWPERLRCENFP
VHGAGEICVGQNTSDGSG

Human FZD8 Fri domain amino acid sequence without predicted signal sequence (SEQ ID NO:39; amino acids 28-158 of SEQ ID NO:30)
ASAKELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFF
LCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNPDTL
CMDYNRTDLTT

Human FZD9 Fri domain amino acid sequence without predicted signal sequence (SEQ ID NO:40; amino acids 23-159 of SEQ ID NO:31)
LEIGRFDPERGRGAAPCQAVEIPMCRGIGYNLTRMPNLLGHTSQGEAAAELAEFAPLVQY
GCHSHLRFFLCSLYAPMCTDQVSTPIPACRPMCEQARLRCAPIMEQFNFGWPDSLDCARL
PTRNDPHALCMEAPENA

Human FZD10 Fri domain amino acid sequence without predicted signal sequence (SEQ ID NO:41; amino acids 21-154 of SEQ ID NO:26)
ISSMDMERPGDGKCQPIEIPMCKDIGYNMTRMPNLMGHENQREAAIQLHEFAPLVEYGCH
GHLRFFLCSLYAPMCTEQVSTPIPACRVMCEQARLKCSPIMEQFNFKWPDSLDCRKLPNK
NDPNYLCMEAPNNG

FZD extracellular domain (ECD) sequences

Human FZD1 ECD with signal sequence (SEQ ID NO:27)
MAEEEAPKKSRAAGGGASWELCAGALSARLAEEGSGDAGGRRRPPVDPRRLARQLLLLLW
LLEAPLLLGVRAQAAGQGPGQGPGPGQQPPPPPQQQQSGQQYNGERGISVPDHGYCQPIS
IPLCTDIAYNQTIMPNLLGHTNQEDAGLEVHQFYPLVKVQCSAELKFFLCSMYAPVCTVL
EQALPPCRSLCERARQGCEALMNKFGFQWPDTLKCEKFPVHGAGELCVGQNTSDKGTPTP
SLLPEFWTSNPQHGGGGHRGGFPGGAGASERGKFSCPRALKVPSYLNYHFLGEKDCGAPC

EPTKVYGLMYFGPEELRFSRT

Human FZD2 ECD with signal sequence (SEQ ID NO:28)
MRPRSALPRLLLPLLLLPAAGPAQFHGEKGISIPDHGFCQPISIPLCTDIAYNQTIMPNL
LGHTNQEDAGLEVHQFYPLVKVQCSPELRFFLCSMYAPVCTVLEQAIPPCRSICERARQG
CEALMNKFGFQWPERLRCEHFPRHGAEQICVGQNHSEDGAPALLTTAPPPGLQPGAGGTP
GGPGGGGAPPRYATLEHPFHCPRVLKVPSYLSYKFLGERDCAAPCEPARPDGSMFFSQEE
TRFARLWILT

Human FZD3 ECD with signal sequence (SEQ ID NO:29)
MAMTWIVFSLWPLTVFMGHIGGHSLFSCEPITLRMCQDLPYNTTFMPNLLNHYDQQTAAL
AMEPFHPMVNLDCSRDFRPFLCALYAPICMEYGRVTLPCRRLCQRAYSECSKLMEMFGVP
WPEDMECSRFPDCDEPYPRLVDLNLAGEPTEGAPVAVQRDYGFWCPRELKIDPDLGYSFL
HVRDCSPPCPNMYFRREELSFARY

Human FZD4 ECD with signal sequence (SEQ ID NO:22)
MLAMAWRGAGPSVPGAPGGVGLSLGLLLQLLLLLGPARGFGDEEERRCDPIRISMCQNLG
YNVTKMPNLVGHELQTDAELQLTTFTPLIQYGCSSQLQFFLCSVYVPMCTEKINIPIGPC
GGMCLSVKRRCEPVLKEFGFAWPESLNCSKFPPQNDHNHMCMEGPGDEEVPLPHKTPIQP
GEECHSVGTNSDQYIWVKRSLNCVLKCGYDAGLYSRSAKEFTDI

Human FZD5 ECD with signal sequence (SEQ ID NO:23)
MARPDPSAPPSLLLLLLAQLVGRAAAASKAPVCQEITVPMCRGIGYNLTHMPNQFNHDTQ
DEAGLEVHQFWPLVEIQCSPDLRFFLCSMYTPICLPDYHKPLPPCRSVCERAKAGCSPLM
RQYGFAWPERMSCDRLPVLGRDAEVLCMDYNRSEATTAPPRPFPAKPTLPGPPGAPASGG
ECPAGGPFVCKCREPFVPILKESHPLYNKVRTGQVPNCAVPCYQPSFSADERT

Human FZD6 ECD with signal sequence (SEQ ID NO:24)
MEMFTFLLTCIFLPLLRGHSLFTCEPITVPRCMKMAYNMTFFPNLMGHYDQSIAAVEMEH
FLPLANLECSPNIETFLCKAFVPTCIEQIHVVPPCRKLCEKVYSDCKKLIDTFGIRWPEE
LECDRLQYCDETVPVTFDPHTEFLGPQKKTEQVQRDIGFWCPRHLKTSGGQGYKFLGIDQ
CAPPCPNMYFKSDELEFAKSFIGTVSI

Human FZD7 ECD with signal sequence (SEQ ID NO:25)
MRDPGAAAPLSSLGLCALVLALLGALSAGAGAQPYHGEKGISVPDHGFCQPISIPLCTDI
AYNQTILPNLLGHTNQEDAGLEVHQFYPLVKVQCSPELRFFLCSMYAPVCTVLDQAIPPC
RSLCERARQGCEALMNKFGFQWPERLRCENFPVHGAGEICVGQNTSDGSGGPGGGPTAYP
TAPYLPDLPFTALPPGASDGRGRPAFPFSCPRQLKVPPYLGYRFLGERDCGAPCEPGRAN
GLMYFKEEERRFARL

Human FZD8 ECD with signal sequence (SEQ ID NO:30)
MEWGYLLEVTSLLAALALLQRSSGAAAASAKELACQEITVPLCKGIGYNYTYMPNQFNHD
TQDEAGLEVHQFWPLVEIQCSPDLKFFLCSMYTPICLEDYKKPLPPCRSVCERAKAGCAP
LMRQYGFAWPDRMRCDRLPEQGNPDTLCMDYNRTDLTTAAPSPPRRLPPPPPGEQPPSGS
GHGRPPGARPPHRGGGRGGGGGDAAAPPARGGGGGGKARPPGGGAAPCEPGCQCRAPMVS
VSSERHPLYNRVKTGQIANCALPCHNPFFSQDERAFT

Human FZD9 ECD with signal sequence (SEQ ID NO:31)
MAVAPLRGALLLWQLLAAGGAALEIGRFDPERGRGAAPCQAVEIPMCRGIGYNLTRMPNL
LGHTSQGEAAAELAEFAPLVQYGCHSHLRFFLCSLYAPMCTDQVSTPIPACRPMCEQARL
RCAPIMEQFNFGWPDSLDCARLPTRNDPHALCMEAPENATAGPAEPHKGLGMLPVAPRPA
RPPGDLGPGAGGSGTCENPEKFQYVEKSRSCAPRCGPGVEVFWSRRDKDF

Human FZD10 ECD with signal sequence (SEQ ID NO:26)
MQRPGPRLWLVLQVMGSCAAISSMDMERPGDGKCQPIEIPMCKDIGYNMTRMPNLMGHEN
QREAAIQLHEFAPLVEYGCHGHLRFFLCSLYAPMCTEQVSTPIPACRVMCEQARLKCSPI
MEQFNFKWPDSLDCRKLPNKNDPNYLCMEAPNNGSDEPTRGSGLFPPLFRPQRPHSAQEH
PLKDGGPGRGGCDNPGKFHHVEKSASCAPLCTPGVDVYWSREDKRFA

FZD8-Fc variants
FZD8-Fc variant 54F03 amino acid sequence (without predicted signal sequence; alternative cleavage) (SEQ ID NO:45)
AAAASAKELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDL
KFFLCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNP
DTLCMDYNRTDLTTGRADKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVV
VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES
NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL
SPGK

FZD8-Fc variant 54F09 amino acid sequence (without predicted signal sequence) (SEQ ID NO:46)
ASAKELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFF
LCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNPDTL
CMDYNRTDLTTAAPSPPDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVV
VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES
NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL
SPGK

FZD8-Fc variant 54F09 amino acid sequence (without predicted signal sequence; alternative cleavage) (SEQ ID NO:47)
AAAASAKELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDL
KFFLCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNP
DTLCMDYNRTDLTTAAPSPPDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT
CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVE
WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS
LSLSPGK

FZD8-Fc variant 54F15 amino acid sequence (without predicted signal sequence) (SEQ ID NO:48)
ASAKELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFF
LCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNPDTL
CMDYNRTDLTTAAPDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV
SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ
PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
K

FZD8-Fc variant 54F15 amino acid sequence (without predicted signal sequence; alternative cleavage) (SEQ ID NO:49)
AAAASAKELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDL
KFFLCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNP
DTLCMDYNRTDLTTAAPDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVV
VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES
NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL

SPGK

FZD8-Fc variant 54F16, 54F17, 54F18, 54F23, 54F25, 54F27, 54F29, 54F31, and 54F34 amino acid sequence (without predicted signal sequence) (SEQ ID NO:50)
ASAKELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFF
LCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNPDTL
CMDYNRTDLTTKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV
SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ
PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
K

FZD8-Fc variant 54F16 amino acid sequence (without predicted signal sequence; alternative cleavage) (SEQ ID NO:51)
AAAASAKELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDL
KFFLCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNP
DTLCMDYNRTDLTTKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVV
VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES
NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL
SPG

FZD8-Fc variant 54F16 amino acid sequence (with signal sequence) (SEQ ID NO:52)
MEWGYLLEVTSLLAALALLQRSSGAAAASAKELACQEITVPLCKGIGYNYTYMPNQFNHD
TQDEAGLEVHQFWPLVEIQCSPDLKFFLCSMYTPICLEDYKKPLPPCRSVCERAKAGCAP
LMRQYGFAWPDRMRCDRLPEQGNPDTLCMDYNRTDLTTKSSDKTHTCPPCPAPELLGGPS
VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST
YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT
KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQ
GNVFSCSVMHEALHNHYTQKSLSLSPGK

FZD8-Fc variant 54F19, 54F20, 54F24, 54F26, 54F28, 54F30, 54F32, 54F34 and 54F35 amino acid sequence (without predicted signal sequence) (SEQ ID NO:53)
ASAKELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFF
LCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNPDTL
CMDYNRTDLTTEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVV
DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS
NKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESN
GQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS
PGK

FZD8-Fc variant 54F19 amino acid sequence (without predicted signal sequence; alternative cleavage) (SEQ ID NO:54)
ALAASAKELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDL
KFFLCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNP
DTLCMDYNRTDLTTEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC
VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL
SLSPGK

FZD8-Fc variant 54F20 amino acid sequence (without predicted signal sequence; alternative cleavage) (SEQ ID NO:55)
VLAASAKELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDL

KFFLCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNP
DTLCMDYNRTDLTTEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC
VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL
SLSPGK

FZD8-Fc variant 54F34 amino acid sequence (without predicted signal sequence) (SEQ ID NO:65)
KELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFFLCS
MYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNPDTLCMD
YNRTDLTTEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKA
LPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

FZD8-Fc variant 54F33 amino acid sequence (without predicted signal sequence) (SEQ ID NO:66)
KELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFFLCS
MYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMRCDRLPEQGNPDTLCMD
YNRTDLTTKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
APIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN
NYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Human ROR1 ECD with signal sequence (SEQ ID NO:56)
MHRPRRRGTRPPLLALLAALLLAARGAAAQETELSVSAELVPTSSWNISSELNKDSYLTL
DEPMNNITTSLGQTAELHCKVSGNPPPTIRWFKNDAPVVQEPRRLSFRSTIYGSRLRIRN
LDTTDTGYFQCVATNGKEVVSSTGVLFVKFGPPPTASPGYSDEYEEDGFCQPYRGIACAR
FIGNRTVYMESLHMQGEIENQITAAFTMIGTSSHLSDKCSQFAIPSLCHYAFPYCDETSS
VPKPRDLCRDECEILENVLCQTEYIFARSNPMILMRLKLPNCEDLPQPESPEAANCIRIG
IPMADPINKNHKCYNSTGVDYRGTVSVTKSGRQCQPWNSQYPHTHTFTALRFPELNGGHS
YCRNPGNQKEAPWCFTLDENFKSDLCDIPACDSKDSKEKNKMEILY

Human ROR2 ECD with signal sequence (SEQ ID NO:57)
MARGSALPRRPLLCIPAVWAAAALLLSVSRTSGEVEVLDPNDPLGPLDGQDGPIPTLKGY
FLNFLEPVNNITIVQGQTAILHCKVAGNPPPNVRWLKNDAPVVQEPRRIIIRKTEYGSRL
RIQDLDTTDTGYYQCVATNGMKTITATGVLFVRLGPTHSPNHNFQDDYHEDGFCQPYRGI
ACARFIGNRTIYVDSLQMQGEIENRITAAFTMIGTSTHLSDQCSQFAIPSFCHFVFPLCD
ARSRTPKPRELCRDECEVLESDLCRQEYTIARSNPLILMRLQLPKCEALPMPESPDAANC
MRIGIPAERLGRYHQCYNGSGMDYRGTASTTKSGHQCQPWALQHPHSHHLSSTDFPELGG
GHAYCRNPGGQMEGPWCFTQNKNVRMELCDVPSCSPRDSSKMG

h-ROR1 minimal Fri domain (SEQ ID NO:58)
CQPYRGIACARFIGNRTVYMESLHMQGEIENQITAAFTMIGTSSHLSDKCSQFAIPSLCH
YAFPYCDETSSVPKPRDLCRDECEILENVLCQTEYIFARSNPMILMRLKLPNCEDLPQPE
SPEAANC

h-ROR2 minimal Fri domain (SEQ ID NO:59)
CQPYRGIACARFIGNRTIYVDSLQMQGEIENRITAAFTMIGTSTHLSDQCSQFAIPSFCH
FVFPLCDARSRTPKPRELCRDECEVLESDLCRQEYTIARSNPLILMRLQLPKCEALPMPE
SPDAANC

Linker (SEQ ID NO:60)
ESGGGGVT

Linker (SEQ ID NO:61)
LESGGGGVT

Linker (SEQ ID NO:62)
GRAQVT

Linker (SEQ ID NO:63)
WRAQVT

Linker (SEQ ID NO:64)
ARGRAQVT

Signal Sequence (SEQ ID NO:67)
MEWGYLLEVTSLLAALALLQRSSGAAA

Signal Sequence (SEQ ID NO:68)
MEWGYLLEVTSLLAALALLQRSSGALA

Signal Sequence (SEQ ID NO:69)
MEWGYLLEVTSLLAALALLQRSSGVLA

Signal Sequence (SEQ ID NO:70)
MEWGYLLEVTSLLAALLLLQRSPIVHA

Signal Sequence (SEQ ID NO:71)
MEWGYLLEVTSLLAALFLLQRSPIVHA

Signal Sequence (SEQ ID NO:72)
MEWGYLLEVTSLLAALLLLQRSPFVHA

Signal Sequence (SEQ ID NO:73)
MEWGYLLEVTSLLAALLLLQRSPIIYA

Signal Sequence (SEQ ID NO:74)
MEWGYLLEVTSLLAALLLLQRSPIAHA

FZD8-Fc variant 54F26 with signal sequence (SEQ ID NO:75)
MEWGYLLEVTSLLAALFLLQRSPIVHAASAKELACQEITVPLCKGIGYNYTYMPNQFNHD
TQDEAGLEVHQFWPLVEIQCSPDLKFFLCSMYTPICLEDYKKPLPPCRSVCERAKAGCAP
LMRQYGFAWPDRMRCDRLPEQGNPDTLCMDYNRTDLTTEPKSSDKTHTCPPCPAPELLGG
PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN
STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDE
LTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGK

N-terminal sequence (SEQ ID NO:76)
AAAASA

WHAT IS CLAIMED IS:

1. A Wnt-binding agent comprising:
(a) a first polypeptide consisting of SEQ ID NO:39; and
(b) a second polypeptide comprising SEQ ID NO:43;
wherein the first polypeptide is directly linked to the second polypeptide.

2. The Wnt-binding agent of claim 1, wherein the second polypeptide consists of SEQ ID NO:43.

3. A Wnt-binding agent comprising:
(a) a first polypeptide comprising amino acids X to Y of SEQ ID NO:30; and
(b) a second polypeptide comprising SEQ ID NO:43;
wherein the first polypeptide is directly linked to the second polypeptide; and
wherein X = amino acid 25, 26, 27, or 28; and
Y = amino acid 158, 159, 160, 161, 162, 163, or 164.

4. A Wnt-binding agent comprising the amino acid sequence of SEQ ID NO:53.

5. A Wnt-binding agent consisting of SEQ ID NO:53.

6. A polypeptide comprising the Wnt-binding agent of any one of claims 1 to 5, and further comprising the amino acid sequence of SEQ ID

NO:72.

7. A polypeptide consisting of SEQ ID NO:72 and SEQ ID NO:53.

8. A cell producing the Wnt-binding agent of any one of claims 1 to 5.

9. A cell comprising the polypeptide of claim 6 or claim 7.

10. A composition comprising the Wnt-binding agent of any one of claims 1 to 5, wherein at least about 80% of the Wnt-binding agent has an N-terminal amino acid sequence of ASA.

11. A pharmaceutical composition comprising the Wnt-binding agent of any one of claims 1 to 5, and a pharmaceutically acceptable carrier.

12. A polynucleotide comprising a polynucleotide that encodes the Wnt-binding agent of any one of claims 1 to 5.

13. A polynucleotide comprising a polynucleotide that encodes the polypeptide of claim 6 or claim 7.

14. A cell comprising the polynucleotide of claim 12 or claim 13.

15. Use of the Wnt-binding agent of any one of claims 1 to 5 in the manufacture of a medicament for the treatment of cancer.

16. Use of the Wnt-binding agent of any one of claims 1 to 5 in the manufacture of a medicament for treatment of a disease associated with Wnt signaling activation.

17. The use of the Wnt-binding agent of claim 15 or claim 16, wherein the treatment comprises a second therapeutic agent.

18. A method of producing the Wnt-binding agent of any one of claims 1 to 5, wherein at least about 80% of the Wnt-binding agent has an N-terminal amino acid sequence of ASA, the method comprising using a signal sequence of SEQ ID NO:72 for production of the Wnt-binding agent.

19. A polypeptide comprising:
(a) a signal sequence of SEQ ID NO:72;
(b) a Fri domain of human FZD8; and
(c) a human Fc region.

20. A polynucleotide comprising a polynucleotide that encodes the polypeptide of claim 19.

21. A cell comprising the polynucleotide of claim 20.

22. A cell comprising the polypeptide of claim 19.

23. A method of producing a soluble Wnt-binding agent which comprises a Fri domain of human FZD8 in a cell, wherein at least about 80% of the Wnt-binding agent has an N-terminal amino acid sequence of ASA, the method comprising using a signal sequence of SEQ ID NO:72 for production of the Wnt-binding agent.

24. A soluble Wnt-binding agent produced by the method of claim 23.

25. A composition comprising a soluble Wnt-binding agent which comprises a Fri domain of human FZD8, wherein at least about 80% of the Wnt-binding agent has an N-terminal sequence of ASA.