(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 August 2017 (10.08.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/136558 A1

(51) International Patent Classification:
C07K 14/705 (2006.01)

(21) International Application Number:
PCT/US2017/016226

(22) International Filing Date:
2 February 2017 (02.02.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/291,346 4 February 2016 (04.02.2016) US

(71) Applicants: **CURIS, INC.** [US/US]; 4 Maguire Road, Lexington, MA 02421 (US). **GENENTECH, INC.** [US/US]; 1 DNA Way, South San Francisco, CA 94080 (US).

(72) Inventors: **SHARPE, Hayley**; 1 DNA Way, South San Francisco, CA 94080 (US). **GENDREAU, Steven**; 1 DNA Way, South San Francisco, CA 94080 (US).

(74) Agents: **RONES, Melissa S.** et al.; Ropes & Gray LLP, Prudential Tower, 800 Boylston Street, Boston, MA 02199 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2017/136558 A1

(54) Title: MUTANT SMOOTHENED AND METHODS OF USING THE SAME

(57) Abstract: The emergence of mutations in tyrosine kinases following treatment of cancer patients with molecular-targeted therapy represents a major mechanism of acquired drug resistance. Here, mutations in the serpentine receptor, Smoothened (SMO) are described, which result in resistance to a Hedgehog (Hh) pathway inhibitor, such as in medulloblastoma. Amino acid substitutions in conserved residues of SMO maintain Hh signaling, but result in the inability of the Hh pathway inhibitor, GDC-0449, to suppress the pathway. In some embodiments, the disclosure provides for novel mutant SMO proteins and nucleic acids and for screening methods to detect SMO mutations and methods to screen for drugs that specifically modulate mutant SMO exhibiting drug resistance.

MUTANT SMOOTHENED AND METHODS OF USING THE SAME

RELATED APPLICATION

This application claims priority to United States provisional application serial number 62/291,346, filed February 4, 2016. The disclosure of the foregoing application is hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Molecular-targeted cancer therapeutics have shown impressive activity in the clinic. Some of the best noted examples include the tyrosine kinase inhibitors imatinib in Philadelphia chromosome-positive chronic myelogenous leukemia (CML) or KIT / PDGFR-mutant gastrointestinal stromal tumors (GISTs) and erlotinib in EGFR-mutant non-small cell lung cancer (NSCLC) (Krause, D.S. and R.A. Van Etten (2005) *N. Engl. J. Med.* 353(2):172-187). Treatment with these agents has led to dramatic anti-tumor responses in patient populations harboring these molecular abnormalities. However, despite the impressive initial clinical responses, most patients eventually progress due to the acquisition of drug resistance (Engelman, J.A. and J. Settleman (2008) *Curr. Opin. Genet. Dev.* 18(1):73-79). Identification of mechanisms of resistance have consequently opened the door to more rational drug combinations and the development of "second-generation" inhibitors that can potentially overcome or avoid the emergence of resistance.

Medulloblastoma is a primitive neuroectodermal tumor of the cerebellum that represents the most common brain malignancy in children (Polkinghorn, W.R. and N.J. Tarbell (2007) *Nat. Clin. Pract. Oncol.* 4(5):295-304). One form of treatment for medulloblastoma is adjuvant radiation therapy. Despite improvements in survival rates, adjuvant radiation is associated with debilitating side effects, thus supporting the need for new molecular targeted therapies.

The Hedgehog (Hh) signaling pathway has been directly implicated in the pathogenesis of medulloblastoma. Constitutive Hh signaling, most often due to underlying loss of function mutations in the inhibitory receptor PTCH1, has been demonstrated in approximately 30% of sporadic cases (Zurawel, R.H. *et al.* (2000) *Genes Chromosomes Cancer* 27(1):44-51; Kool, M. *et al.* (2008) *PLoS ONE* 3(8):e3088; Dellovade, T. *et al.*

(2006) *Annu. Rev. Neurosci.* 29:539; Rubin, L.L. and F.J. de Sauvage (2006) *Nat. Rev. Drug Discov.* 5:1026). Mice heterozygous for Ptch1 (Ptch1$^{+/-}$) can spontaneously develop medulloblastoma and treatment with Hh pathway inhibitors results in tumor elimination and prolonged survival (Goodrich, L.V. *et al.* (1997) *Science* 277(5329):1109-1113; Romer, J.T. *et al.* (2004) *Cancer Cell* 6(3):229-240). However, it has recently been observed that a patient treated with the novel Hh pathway inhibitor, GDC-0449 initially showed a dramatic response to treatment (Charles M. Rudin *et al.* (2009) *N. Engl. J. Med.* (submitted)), only to fail to have a durable response to treatment and a relapse of the tumor.

BCC is the most common human cancer and is predominantly driven by hyperactivation of the Hh pathway (Oro et al., 1997; Xie et al., 1998). The association between Hh signaling and cancer was first discovered in patients with Gorlin or basal cell nevus syndrome (BCNS), who are highly susceptible to medulloblastoma (MB) and BCC. These patients generally possess heterozygous germline mutations in *Patched 1* (*PTCH1*), which encodes a receptor for Hh ligands (Hahn et al., 1996; Johnson et al., 1996). Hh ligand binding relieves PTCH1 suppression of the serpentine transmembrane (TM) signal transducer Smoothened (SMO). The vast majority of sporadic BCCs are driven by inactivating mutations and loss of heterozygosity (LOH) in *PTCH1*, with most of the remainder harboring activating mutations in *SMO* (Reifenberger et al., 2005). SMO promotes the activation and nuclear localization of GLI transcription factors by inhibition of Suppressor of fused (SUFU) and Protein kinase A (PKA). SUFU negatively regulates the Hh pathway by binding and sequestering GLI transcription factors in the cytoplasm (Stone et al., 1999). Loss-of-function mutations in *SUFU* are also associated with Gorlin Syndrome (Pastorino et al., 2009; Smith et al., 2014; Taylor et al., 2002). Approximately 50% of sporadic BCCs also have *TP53* mutations (Jayaraman et al., 2014).

Several Hh pathway inhibitors (HPIs) are currently under clinical investigation for both BCC and MB (Amakye et al., 2013). Vismodegib, previously known as GDC-0449, is a SMO inhibitor approved for the treatment of metastatic and locally advanced BCC (Sekulic et al., 2012). The majority of BCC patients treated with vismodegib experience a clinical benefit, including both complete and partial responses (Sekulic et al., 2012).

However, a preliminary estimate suggests that up to 20% of advanced BCC patients develop resistance to vismodegib within the first year of treatment (Chang and Oro, 2012). To date, the only functionally characterized mechanism of acquired resistance to vismodegib in the clinic came from a patient with metastatic MB. A SMO-D473H mutation was detected in a biopsy from a relapsed metastatic tumor and was shown to abrogate drug binding in vitro

(Yauch et al., 2009). Four other clinical SMO mutations were recently reported in vismodegib-resistant BCC, but were not examined functionally (Brinkhuizen et al., 2014; Pricl et al., 2014). Several resistance mechanisms to SMO inhibitors have been delineated from preclinical models, including additional SMO mutations, amplification of downstream Hh pathway components such as GLI2, and activation of bypass signaling pathways including phosphatidylinositol 3-kinase (PI3K) kinase and atypical protein kinase C ι/λ (aPKC-ι/λ) (Atwood et al., 2013; Buonamici et al., 2010; Dijkgraaf et al., 2011). However, it remains unclear which mechanisms drive resistance in patients.

There is an urgent need in the art to identify additional GDC-0449-resistant mutant SMO proteins and to find compounds that modulate SMO activity in such mutant SMO proteins to overcome drug resistance upon treatment with GDC-0449. There is further a need to a method to diagnose patients who may be resistant to treatment either through natural variation of their SMO genotype or through acquired mutation and resistance.

SUMMARY OF THE DISCLOSURE

The present disclosure relates, in certain embodiments, to isolated mutant SMO nucleic acids and proteins, such as those related to chemotherapeutic resistance of tumors and methods of screening for compounds that bind to SMO mutants, or modulate SMO activity, and to cancer diagnostics and therapies and in particular to the detection of mutations that are diagnostic and/or prognostic and treatment of drug-resistant tumors.

In some embodiments, the disclosure provides for a nucleic acid molecule, such as an isolated nucleic acid molecule, encoding a mutant SMO protein comprising an amino acid sequence that is at least 95% identical to SEQ ID NO:1 wherein the amino acid sequence comprises an amino acid other than glycine at amino acid 529. In some embodiments, the mutant SMO protein comprises the amino acid sequence of SEQ ID NO:2 wherein the amino acid sequence comprises a serine (S) at amino acid 529. In some embodiments, the nucleic acid molecule comprises a parental nucleic acid sequence of SEQ ID NO:3, wherein the sequence contains a mutation that alters the sequence encoding amino acid 529 to encode a different amino acid.

In some embodiments, the disclosure provides for a nucleic acid probe capable of specifically hybridizing to nucleic acid encoding a mutated SMO protein or fragment thereof incorporating a mutation in the sequence encoding amino acid 529. In some embodiments, the probe is complementary to the nucleic acid encoding the mutated SMO or the fragment

thereof. In some embodiments, the probe has a length of about 10 to about 50 nucleotides. In some embodiments, the probe comprises a detectable label.

In some embodiments, the disclosure provides for an isolated mutant SMO protein comprising an amino acid sequence that is at least 95% identical to SEQ ID NO: 2 wherein the amino acid sequence comprises an amino acid other than glycine at amino acid 529. In some embodiments, the protein comprises the amino acid sequence of SEQ ID NO: 2 wherein the amino acid sequence comprises an amino acid other thanglycine at amino acid 529. In some embodiments, the amino acid sequence comprises serine (S) at amino acid 529.

In some embodiments, the disclosure provides for an isolated antibody that specifically binds to any of the mutant SMO proteins disclosed herein, wherein the antibody does not bind wild-type SMO having a glycine at amino acid 529. In some embodiments, the antibody is a monoclonal antibody, a chimeric antibody, a humanized antibody, a single chain antibody or an antigen-binding fragment thereof. In some ebodiments, the antibody is conjugated to a cytotoxic agent. In some embodiments, the antibody is conjugated to a detectable label. In some embodiments, the antibody inhibits SMO activity.

In some embodiments, the disclosure provides for a method of identifying at least one SMO mutation in a sample comprising contacting nucleic acid from the sample with a nucleic acid probe that is capable of specifically hybridizing to nucleic acid encoding a mutated SMO protein, or fragment thereof incorporating a mutation that alters the sequence encoding amino acid 529 to an amino acid other than glycine, and detecting the hybridization. In some embodiments, the probe is detectably labeled. In some embodiments, the probe is an antisense oligomer. In some embodiments, the SMO gene or a fragment thereof in the nucleic acid the sample is amplified and contacted with the probe.

In some embodiments, the disclosure provides for a method for identifying a tumor in a human subject that is or becomes resistant to treatment with GDC-0449 comprising determining the presence of a mutated SMO gene or mutated SMO protein in a sample of the tumor, wherein the mutated SMO gene encodes a SMO protein comprising a mutation at amino acid 529, and wherein the SMO protein comprises a mutation at amino acid 529, whereby the presence of the mutated SMO gene or mutated SMO protein indicates that the tumor is resistant to treatment with a GDC-0449. In some embodiments, the method further comprises treating the subject having a tumor that is not or is no longer susceptible to treatment with GDC-0449 with a compound that binds the mutated SMO. In some embodiments, the presence or absence of the mutation is determined by examining a nucleic

acid sample. In some embodiments, the presence or absence of the mutation is determined by examining a protein sample.

In some embodiments, the disclosure provides for a method of screening for compounds that inhibit signaling of a mutant SMO protein that incorporates a mutation at amino acid 529 comprising contacting the mutant SMO with a test compound and detecting binding of the compound to the mutant SMO whereby binding of the test compound to mutant SMO indicates that the test compound is an inhibitor of mutant SMO.

In some embodiments, the disclosure provides for a method of screening for compounds that inhibit signaling of a mutant SMO protein that incorporates a mutation at amino acid 529 comprising contacting a cell that expresses the mutant SMO with a test compound and detecting activity of Gli in the cell whereby the presence of Gli activity indicates that the test compound is not an inhibitor of mutant SMO.

In some embodiments, the disclosure provides for a method of inhibiting proliferation or growth of a cell having aberrant hedgehog signaling, comprising administering to the cell a bromodomain inhibitor, wherein the cell expresses a smoothened protein having a mutation at amino acid position 529 of SEQ ID NO: 1. In some embodiments, the cell is in a subject. In some embodiments, the cell is a cancer cell. In some embodiments, the cell further comprises a *SUFU* mutation. In some embodiments, the cell is a human cell, wherein the cell comprises a 10q deletion mutation that results in the loss of a copy of the *SUFU* gene. In some embodiments, the 10q deletion further results in the loss of a copy of the *PTEN* gene. In some embodiments, the bromodomain inhibitor is I-BET762, JQ1 or JQ2.

In some embodiments, the disclosure provides for a method of identifying a hedgehog pathway inhibitor, wherein the method comprises: contacting a cell with an amount of a test agent, wherein the cell is responsive to hedgehog protein or has increased hedgehog signaling and/or activation of the hedgehog signaling pathway, and wherein the cell expresses any of the mutant SMO proteins disclosed herein, and determining, as compared to a control, whether the test agent inhibits hedgehog signaling in the cell, wherein if the test agent inhibits hedgehog signaling in the cell relative to the control, then the test agent is identified as a hedgehog pathway inhibitor. In some embodiments, the ability of the test agent to inhibit hedgehog signaling in the cell is determined using a Gli1 expression assay.

In some embodiments, the disclosure provides for a method of identifying a hedgehog pathway inhibitor, wherein the method comprises: contacting a cell with an amount of a test agent, wherein the cell is responsive to hedgehog protein or has increased hedgehog signaling and/or activation of the hedgehog signaling pathway, and wherein the cell expresses any of

the mutant SMO proteins disclosed herein, and determining, as compared to a control, whether the test agent inhibits growth and/or proliferation of the cell, wherein if the test agent inhibits growth and/or proliferation of the cell relative to the control, then the test agent is identified as a hedgehog pathway inhibitor. In some embodiments, the control is a cell expressing a wildtype SMO protein. In some embodiments, the control is a cell expressing the same mutant SMO proteins as the cell contacted with the test agent, wherein the control is treated with a control agent to which the mutant SMO protein is partially or completely resistant. In some embodiments, the control agent is vismodegib, LY2940680, LDE225 and/or compound 5. In some embodiments, the test agent binds to mutant SMO protein but not wildtype SMO protein. In some embodiments, the test agent binds to both the mutant SMO protein and wildtype SMO protein. In some embodiments, the test agent is more effective in inhibiting the hedgehog signaling pathway in a cell expressing mutant SMO protein than in a cell expressing wildtype SMO protein. In some embodiments, the test agent is more effective in inhibiting growth and/or proliferation of a cell expressing mutant SMO protein than of a cell expressing wildtype SMO protein.

In some embodiments, the disclosure provides for a vector comprising any of the nucleic acids disclosed herein.

In some embodiments, the disclosure provides for a host cell comprising any of the vectors disclosed herein.

In some embodiments, the disclosure provides for a host cell comprising and capable of expressing any of the vectors disclosed herein.

In some embodiments, the disclosure provides for a method of identifying a hedgehog pathway inhibitor, wherein the method comprises: a) contacting a cell with an amount of a test agent, wherein the cell is responsive to hedgehog protein or has increased hedgehog signaling and/or activation of the hedgehog signaling pathway, and wherein the cell expresses any of the vectors disclosed herein, and b) determining, as compared to a control, whether the test agent inhibits hedgehog signaling in the cell, wherein if the test agent inhibits hedgehog signaling in the cell relative to the control, then the test agent is identified as a hedgehog pathway inhibitor. In some embodiments, the ability of the test agent to inhibit hedgehog signaling in the cell is determined using a Gli1 expression assay.

In some embodiments, the disclosure provides for a method of identifying a hedgehog pathway inhibitor, wherein the method comprises: a) contacting a cell with an amount of a test agent, wherein the cell is responsive to hedgehog protein or has increased hedgehog signaling and/or activation of the hedgehog signaling pathway, and wherein the cell expresses

any of the vectors disclosed herein, and b) determining, as compared to a control, whether the test agent inhibits growth and/or proliferation of the cell, wherein if the test agent inhibits growth and/or proliferation of the cell relative to the control, then the test agent is identified as a hedgehog pathway inhibitor.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects and advantages of the disclosure will be apparent upon consideration of the following detailed description, taken in conjunction with the accompanying drawings, in which like reference characters refer to like parts throughout, and in which:

Figure 1 lists characteristics of the mBCC patients described herein and treated with vismodegib.

Figure 2 shows the mutational load of each tumor biopsy sample taken from each patient. "-P" indicates a progression sample, "-A" indicates an archival sample, and "-i" or "-ii" indicate a first or second biopsy sample, respectively.

Figure 3 shows the genomic alterations detected using in the FoundationOne panel conducted on each tumor biopsy sample taken from each patient.

Figure 4 shows the amino acid changes and corresponding allele frequencies of mutations in the SMO gene observed in tumor biopsy samples taken from each patient. AA=amino acid; AF=allele frequency; ND=not detected; NR=not relevant a=previously reported to be associated with resistance to vismodegib; b=previously reported to confer pathway activation in vitro. "-P" indicates a progression sample, "-A" indicates an archival sample, and "-i" or "-ii" indicate a first or second biopsy sample, respectively.

Figure 5 shows a multiple sequence alignment of protein sequences from a given region of Frizzled (FZD) and SMO proteins from a selection of vertebrates and insects.

Figure 6 shows the vismodegib binding pocket of SMO highlighting residues associated with vismodegib resistance as well as a previously unassociated residue, G529.

Figure 7 shows the results of a vismodegib dose response experiment comparing luciferase reporter activity in C3H10T1/2 cells co-transfected with 400 ng SMO-WT or SMO-G529S expressing constructs, and 400 ng of 9x-Gli-BS and 200 ng of pRL-TK. Data plotted are mean ± SD of triplicates.

DETAILED DESCRIPTION

It is a discovery of the present disclosure that mutational events associated with resistance to chemotherapy for hedgehog-dependent tumors occur in Smoothened (SMO) which impart resistance of the tumors to treatment with compounds that inhibit hedgehog signaling such as cyclopamine and GDC-0449. The present disclosure provides compositions and methods that are useful as prognostics, diagnostics and therapeutics for cancer that is dependent on Hedgehog signaling.

The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* 3rd. edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; *Current Protocols in Molecular Biology* (F. M. Ausubel, *et al.* eds., (2003)); the series *Methods in Enzymology* (Academic Press, Inc.): *PCR 2: A Practical Approach* (M. J. MacPherson, B. D. Hames and G. R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) *Antibodies, A Laboratory Manual*, and *Animal Cell Culture* (R. I. Freshney, ed. (1987)); *Oligonucleotide Synthesis* (M. J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J. E. Cellis, ed., 1998) Academic Press; *Animal Cell Culture* (R. I. Freshney), ed., 1987); *Introduction to Cell and Tissue Culture* (J. P. Mather and P. E. Roberts, 1998) Plenum Press; *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J. B. Griffiths, and D. G. Newell, eds., 1993-8) J. Wiley and Sons; *Handbook of Experimental Immunology* (D. M. Weir and C. C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J. M. Miller and M. P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis *et al.*, eds., 1994); *Current Protocols in Immunology* (J. E. Coligan *et al.*, eds., 1991); *Short Protocols in Molecular Biology* (Wiley and Sons, 1999); *Immunobiology* (C. A. Janeway and P. Travers, 1997); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal Antibodies: A Practical Approach* (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and *Cancer: Principles and Practice of Oncology* (V. T. DeVita *et al.*, eds., J.B. Lippincott Company, 1993). Cited references are incorporated by reference in their entirety.

For purposes of interpreting this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and vice versa.

In the event that any definition set forth below conflicts with any document incorporated herein by reference, the definition set forth below shall control.

Before continuing to describe the present disclosure in further detail, it is to be understood that this disclosure is not limited to specific compositions or process steps, as such may vary. It must be noted that, as used in this specification and the appended claims, the singular form "a", "an" and "the" include plural referents unless the context clearly dictates otherwise.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure is related. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this disclosure.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

It is convenient to point out here that "and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue 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 polymer. As used herein, the term "polypeptide," "peptide" and "protein" encompass, at least, any of the mutant SMO proteins, variants or fragments thereof described herein.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.* bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater than 99% by weight; (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "VH." The variable domain of the light chain may be referred to as "VL." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions (HVRs) both in the light-chain and the heavy-chain

variable domains. The more highly conserved portions of variable domains are called the framework regions (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a beta-sheet configuration, connected by three HVRs, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The HVRs in each chain are held together in close proximity by the FR regions and, with the HVRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, Fifth Edition, National Institute of Health, Bethesda, MD (1991)). The constant domains are not involved directly in the binding of an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas *et al. Cellular and Mol. Immunology*, 4th ed. (W.B. Saunders, Co., 2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain an Fc region.

A "naked antibody" for the purposes herein is an antibody that is not conjugated to a cytotoxic moiety or radiolabel.

"Antibody fragments" comprise a portion of an intact antibody, , and in some embodiments, comprise the antigen binding region thereof. Examples of antibody fragments

include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three HVRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six HVRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three HVRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

"Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv, see, *e.g.*, Pluckthün, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., (Springer-Verlag, New York, 1994), pp. 269-315.

The term "diabodies" refers to antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too

short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404,097; WO 1993/01161; Hudson *et al.*, *Nat. Med.* 9:129-134 (2003); and Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson *et al.*, *Nat. Med.* 9:129-134 (2003).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible mutations, *e.g.*, naturally occurring mutations, that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. In certain embodiments, such a monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity *in vivo*, to create a multispecific antibody, *etc.*, and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this disclosure. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins.

The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present disclosure may be made by a variety of techniques, including, for example, the hybridoma method (*e.g.*, Kohler and Milstein, *Nature*, 256:495-97 (1975); Hongo *et al.*, *Hybridoma*, 14 (3): 253-260 (1995), Harlow *et al.*, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier,

N.Y., 1981)), recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567), phage-display technologies (see, *e.g.*, Clackson *et al.*, *Nature*, 352: 624-628 (1991); Marks *et al.*, *J. Mol. Biol.* 222: 581-597 (1992); Sidhu *et al.*, *J. Mol. Biol.* 338(2): 299-310 (2004); Lee *et al.*, *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34):12467-12472 (2004); and Lee *et al.*, *J. Immunol. Methods* 284(1-2): 119-132(2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, *e.g.*, WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits *et al.*, *Nature* 362: 255-258 (1993); Bruggemann *et al.*, *Year in Immunol.* 7:33 (1993); U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks *et al.*, *Bio/Technology* 10: 779-783 (1992); Lonberg *et al.*, *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild *et al.*, *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (see, *e.g.*,U.S. Patent No. 4,816,567; and Morrison *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)). Chimeric antibodies include PRIMATIZED® antibodies wherein the antigen-binding region of the antibody is derived from an antibody produced by, *e.g.*, immunizing macaque monkeys with the antigen of interest.

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. In one embodiment, a humanized antibody is a human immunoglobulin (recipient antibody) in which residues from a HVR of the recipient are replaced by residues from a HVR of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and/or capacity. In some instances, FR residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications may be made to further refine antibody

performance. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin, and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see, *e.g.*, Jones *et al., Nature* 321:522-525 (1986); Riechmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also, *e.g.*, Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994); and U.S. Pat. Nos. 6,982,321 and 7,087,409.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art, including phage-display libraries. Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks *et al.*, *J. Mol. Biol.*, 222:581 (1991). Also available for the preparation of human monoclonal antibodies are methods described in Cole *et al.*, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner *et al.*, *J. Immunol.*, 147(1):86-95 (1991). See also van Dijk and van de Winkel, *Curr. Opin. Pharmacol.*, 5: 368-74 (2001). Human antibodies can be prepared by administering the antigen to a transgenic animal that has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, *e.g.*, immunized xenomice (see, *e.g.*, U.S. Pat. Nos. 6,075,181 and 6,150,584 regarding XENOMOUSE™ technology). See also, for example, Li *et al., Proc. Natl. Acad. Sci. USA*, 103:3557-3562 (2006) regarding human antibodies generated via a human B-cell hybridoma technology.

The term "hypervariable region," "HVR," or "HV," when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). In native antibodies, H3 and L3 display the most diversity of the six HVRs, and H3 in particular is believed to play a unique role in conferring fine specificity to antibodies. See, *e.g.*, Xu *et al., Immunity* 13:37-45 (2000); Johnson and Wu, in *Methods in Molecular Biology* 248:1-25 (Lo, ed., Human Press, Totowa, NJ, 2003). Indeed, naturally occurring camelid antibodies consisting of a heavy chain only are functional

and stable in the absence of light chain. See, *e.g.*, Hamers-Casterman *et al.*, *Nature* 363:446-448 (1993); Sheriff *et al.*, *Nature Struct. Biol.* 3:733-736 (1996).

A number of HVR delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). Chothia refers instead to the location of the structural loops (Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). The AbM HVRs represent a compromise between the Kabat HVRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The "contact" HVRs are based on an analysis of the available complex crystal structures. The residues from each of these HVRs are noted below.

Loop	C	Contact
L1	LLL	L30-L36
L2	LLL	L46-L55
L3	LLL	L89-L96
H1	H31-H35B	H26-H35B H H30 (Kabat Numbering)
H1	HHH	H30 (Chothia Numbering)
H2	HHH	H47-H58
H3	H95-H102	H95-H102 H9 H93-H101

HVRs may comprise "extended HVRs" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 or 89-96 (L3) in the VL and 26-35 (H1), 50-65 or 49-65 (H2) and 93-102, 94-102, or 95-102 (H3) in the VH. The variable domain residues are numbered according to Kabat *et al.*, supra, for each of these definitions.

"Framework" or "FR" residues are those variable domain residues other than the HVR residues as herein defined.

The term "variable domain residue numbering as in Kabat" or "amino acid position numbering as in Kabat," and variations thereof, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat *et al.*, *supra*. Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or HVR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (*e.g.* residues 82a, 82b, and 82c, *etc.* according to Kabat) after

heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The Kabat numbering system is generally used when referring to a residue in the variable domain (approximately residues 1-107 of the light chain and residues 1-113 of the heavy chain) (e.g, Kabat *et al.*, *Sequences of Immunological Interest.* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The "EU numbering system" or "EU index" is generally used when referring to a residue in an immunoglobulin heavy chain constant region (*e.g.*, the EU index reported in Kabat *et al., supra*). The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody. Unless stated otherwise herein, references to residue numbers in the variable domain of antibodies means residue numbering by the Kabat numbering system. Unless stated otherwise herein, references to residue numbers in the constant domain of antibodies means residue numbering by the EU numbering system (*e.g.*, see United States Provisional Application No. 60/640,323, Figures for EU numbering).

An "affinity matured" antibody is one with one or more alterations in one or more HVRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). In one embodiment, an affinity matured antibody has nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies may be produced using certain procedures known in the art. For example, Marks *et al. Bio/Technology* 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of HVR and/or framework residues is described by, for example, Barbas *et al. Proc Nat. Acad. Sci. USA* 91:3809-3813 (1994); Schier *et al. Gene* 169:147-155 (1995); Yelton *et al. J. Immunol.* 155:1994-2004 (1995); Jackson *et al., J. Immunol.* 154(7):3310-9 (1995); and Hawkins *et al, J. Mol. Biol.* 226:889-896 (1992).

A "blocking" antibody or an "antagonist" antibody is one which inhibits or reduces biological activity of the antigen it binds. Certain blocking antibodies or antagonist antibodies substantially or completely inhibit the biological activity of the antigen.

An "agonist antibody," as used herein, is an antibody which partially or fully mimics at least one of the functional activities of a polypeptide of int

"Growth inhibitory" antibodies are those that prevent or reduce proliferation of a cell expressing an antigen to which the antibody binds. For example, the antibody may prevent or reduce proliferation of cancer cells that express Smo or mutant *in vitro* and/or *in vivo*.

Antibodies that "induce apoptosis" are those that induce programmed cell death as determined by standard apoptosis assays, such as binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies).

Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (*e.g.* B cell receptor); and B cell activation.

The term "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain, including native sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during production or purification of the antibody, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody. Accordingly, a composition of intact antibodies may comprise antibody populations with all K447 residues removed, antibody populations with no K447 residues removed, and antibody populations having a mixture of antibodies with and without the K447 residue.

A "functional Fc region" possesses an "effector function" of a native sequence Fc region. Exemplary "effector functions" include C1q binding; CDC; Fc receptor binding; ADCC; phagocytosis; down regulation of cell surface receptors (*e.g.* B cell receptor; BCR), *etc.* Such effector functions generally require the Fc region to be combined with a binding domain (*e.g.*, an antibody variable domain) and can be assessed using various assays as disclosed, for example, in definitions herein.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgG1 Fc region (non-A and A allotypes); native sequence human IgG2 Fc region; native sequence human IgG3 Fc region; and native sequence human IgG4 Fc region as well as naturally occurring variants thereof.

A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification, and, in some

embodiments, one or more amino acid substitution(s). In some embodiments, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, *e.g.* from about one to about ten amino acid substitutions, and, in some embodiments, from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will in some embodiments possess at least about 80% homology with a native sequence Fc region and/or with an Fc region of a parent polypeptide, and in some embodiments at least about 90% homology therewith, and in some embodiments at least about 95% homology therewith.

"Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. In some embodiments, an FcR is a native human FcR. In some embodiments, an FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of those receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see, *e.g.*, Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed, for example, in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel *et al.*, *Immunomethods* 4:25-34 (1994); and de Haas *et al.*, *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein.

The term "Fc receptor" or "FcR" also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al.*, *J. Immunol.* 117:587 (1976) and Kim *et al.*, *J. Immunol.* 24:249 (1994)) and regulation of homeostasis of immunoglobulins. Methods of measuring binding to FcRn are known (see, *e.g.*, Ghetie and Ward., *Immunol. Today* 18(12):592-598 (1997); Ghetie *et al.*, *Nature Biotechnology*, 15(7):637-640 (1997); Hinton *et al.*, *J. Biol. Chem.* 279(8):6213-6216 (2004); WO 2004/92219 (Hinton *et al.*).

Binding to human FcRn *in vivo* and serum half life of human FcRn high affinity binding polypeptides can be assayed, *e.g.*, in transgenic mice or transfected human cell lines expressing human FcRn, or in primates to which the polypeptides with a variant Fc region are

administered. WO 2000/42072 (Presta) describes antibody variants with improved or diminished binding to FcRs. See also, *e.g.*, Shields *et al. J. Biol. Chem.* 9(2):6591-6604 (2001).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. In certain embodiments, the cells express at least FcγRIII and perform ADCC effector function(s). Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells, and neutrophils. The effector cells may be isolated from a native source, *e.g.*, from blood.

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (*e.g.* NK cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII, and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 or U.S. Patent No. 6,737,056 (Presta), may be performed. Useful effector cells for such assays include PBMC and NK cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in an animal model such as that disclosed in Clynes *et al. PNAS (USA)* 95:652-656 (1998).

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass), which are bound to their cognate antigen. To assess complement activation, a CDC assay, *e.g.*, as described in Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996), may be performed. Polypeptide variants with altered Fc region amino acid sequences (polypeptides with a variant Fc region) and increased or decreased C1q binding capability are described, *e.g.*, in US Patent No. 6,194,551 B1 and WO 1999/51642. See also, *e.g.*, Idusogie *et al. J. Immunol.* 164: 4178-4184 (2000).

The term "Fc region-comprising antibody" refers to an antibody that comprises an Fc region. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc

region may be removed, for example, during purification of the antibody or by recombinant engineering of the nucleic acid encoding the antibody. Accordingly, a composition comprising an antibody having an Fc region according to this disclosure can comprise an antibody with K447, with all K447 removed, or a mixture of antibodies with and without the K447 residue.

"Binding affinity" generally refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (*e.g.*, an antibody) and its binding partner (*e.g.*, an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (*e.g.*, antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present disclosure. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

In one embodiment, the "Kd" or "Kd value" according to this disclosure is measured by a radiolabeled antigen binding assay (RIA) performed with the Fab version of an antibody of interest and its antigen as described by the following assay. Solution binding affinity of Fabs for antigen is measured by equilibrating Fab with a minimal concentration of (^{125}I)-labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (see, *e.g.*, Chen, *et al.*, *J. Mol. Biol.* 293:865-881(1999)). To establish conditions for the assay, MICROTITER® multi-well plates (Thermo Scientific) are coated overnight with 5 µg/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbent plate (Nunc #269620), 100 pM or 26 pM [^{125}I]-antigen are mixed with serial dilutions of a Fab of interest (*e.g.*, consistent with assessment of the anti-VEGF antibody, Fab-12, in Presta *et al.*, *Cancer Res.* 57:4593-4599 (1997)). The Fab of interest is then incubated overnight; however, the incubation may continue for a longer period (*e.g.*, about 65 hours) to ensure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature (*e.g.*, for one hour). The solution is then removed and the plate washed eight times with 0.1% TWEEN-20™ in PBS. When the

plates have dried, 150 µl/well of scintillant (MICROSCINT-20™; Packard) is added, and the plates are counted on a TOPCOUNT™ gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of maximal binding are chosen for use in competitive binding assays.

According to another embodiment, the Kd or Kd value is measured by using surface plasmon resonance assays using a BIACORE®-2000 or a BIACORE®-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIACORE, Inc.) are activated with *N*-ethyl-*N'*- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8, to 5 µg/ml (~0.2 µM) before injection at a flow rate of 5 µl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% TWEEN-20™ surfactant (PBST) at 25°C at a flow rate of approximately 25 µl/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIACORE® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (Kd) is calculated as the ratio k_{off}/k_{on}. See, *e.g.*, Chen *et al.*, *J. Mol. Biol.* 293:865-881 (1999). If the on-rate exceeds 10^6 M^{-1} s^{-1} by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophometer (Aviv Instruments) or a 8000-series SLM-AMINCO™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

An "on-rate," "rate of association," "association rate," or "k_{on}" according to this disclosure can also be determined as described above using a BIACORE®-2000 or a BIACORE®-3000 system (BIAcore, Inc., Piscataway, NJ).

The term "substantially similar" or "substantially the same," as used herein, denotes a sufficiently high degree of similarity between two numeric values (for example, one associated with an antibody of the disclosure and the other associated with a reference/comparator antibody), such that one of skill in the art would consider the difference

between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (*e.g.*, Kd values). The difference between said two values is, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, and/or less than about 10% as a function of the reference/comparator value.

The phrase "substantially reduced," or "substantially different," as used herein, denotes a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (*e.g.*, Kd values). The difference between said two values is, for example, greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, and/or greater than about 50% as a function of the value for the reference/comparator molecule.

"Purified" means that a molecule is present in a sample at a concentration of at least 95% by weight, or at least 98% by weight of the sample in which it is contained.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is separated from at least one other nucleic acid molecule with which it is ordinarily associated, for example, in its natural environment. An isolated nucleic acid molecule further includes a nucleic acid molecule contained in cells that ordinarily express the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

An "isolated" protein is a protein that is separated from at least one other cellular component with which it is ordinarily associated, for example, in its natural environment. In some embodiments, an "isolated" protein is a protein expressed in a cell in which the protein is not normally expressed. In some embodiments, the isolated protein is a recombinant protein.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be

integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors," or simply, "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector.

"Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. In some embodiments, the nucleic acid is a cDNA molecule, or fragment thereof. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may comprise modification(s) made after synthesis, such as conjugation to a label. 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, for example, those with uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, *etc.*) and with charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, *etc.*), those containing pendant moieties, such as, for example, proteins (*e.g.*, nucleases, toxins, antibodies, signal peptides, ply-L-lysine, *etc.*), those with intercalators (*e.g.*, acridine, psoralen, *etc.*), those containing chelators (*e.g.*, metals, radioactive metals, boron, oxidative metals, *etc.*), those containing alkylators, those with modified linkages (*e.g.*, alpha anomeric nucleic acids, *etc.*), as well as unmodified forms of the polynucleotides(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 or semi-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, α-anomeric sugars,

epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs, and basic 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)NR_2 ("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. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

"Oligonucleotide," as used herein, generally refers to short, generally single-stranded, generally synthetic polynucleotides that are generally, but not necessarily, less than about 200 nucleotides in length. The terms "oligonucleotide" and "polynucleotide" are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides.

The term "Smo," or "SMO" or "smoothened" as used interchangeably herein, refers to any native smoothened protein or nucleic acid from any vertebrate source, including mammals such as primates (*e.g.* humans) and rodents (*e.g.*, mice and rats), unless otherwise indicated. The term encompasses "full-length," unprocessed SMO as well as any form of SMO that results from processing in the cell. The term also encompasses naturally occurring variants of SMO, *e.g.*, splice variants or allelic variants. In some embodiments, "mutant SMO" or "mutant SMO polypeptide" or "mutant SMO protein" as used herein, refers to SMO having a mutation in the seventh transmembrane of SMO at position 529 of human SMO. In some embodiments, "mutant SMO" or "mutant SMO polypeptide" or "mutant SMO protein" as used herein, refers to a smoothened polypeptide comprising a mutation at the amino acid position corresponding to position 529 of SEQ ID NO: 1 or 2. In some embodiments, "mutant SMO" or "mutant SMO polypeptide" or "mutant SMO protein" as used herein, refers to a smoothened polypeptide comprising a mutation at the amino acid position corresponding to position 529 of SEQ ID NO: 1 or 2, and at least one additional mutation at any one or more of the amino acids corresponding to positions 241, 281, 321, 408, 412, 459, 469, 473, 518, 533 and/or 535 of SEQ ID NO: 1. In some embodiments, the mutation at the amino acid position corresponding to position 529 is a G529S substitution. In some embodiments, the at least one additional mutation corresponds to any one or more of T241M, W281C, V321M, I408V, A459V, C469Y, D473H, E518K, E518A S533N, and/or

W535L. Similarly, a mutant SMO protein is described as having variation at any one or more of the foregoing positions of wildtype human SMO. The disclosure contemplates that any of the mutant polypeptides or nucleic acids described herein can be described relative to a sequence identifier or described relative to wildtype human SMO. Moreover, mutants can be described relative to SEQ ID NO: 1 or described relative to any of the other sequence identifiers.

In some embodiments, as used herein, "treatment" (and variations such as "treat" or "treating") refers to clinical intervention in an attempt to alter the natural course of the individual or cell being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the disclosure are used to delay development of a disease or disorder or to slow the progression of a disease or disorder. In some embodiments, as used herein, "treating" or "treatment" or "alleviation" refers to improving, alleviating, and/or decreasing the severity of one or more symptoms of a condition being treated. By way of example, treating cancer refers to improving (improving the patient's condition), alleviating, delaying or slowing progression or onset, decreasing the severity of one or more symptoms of cancer. For example, treating cancer includes any one or more of: decreasing tumor size, decreasing rate of tumor size increase, halting increase in size, decreasing the number of metastases, decreasing pain, increasing survival, and increasing progression free survival.

"Treating" or "treatment" or "alleviation" refers to improving, alleviating, and/or decreasing the severity of one or more symptoms of a condition being treated. By way of example, treating cancer refers to improving (improving the patient's condition), alleviating, delaying or slowing progression or onset, decreasing the severity of one or more symptoms of cancer. For example, treating cancer includes any one or more of: decreasing tumor size, decreasing rate of tumor size increase, halting increase in size, decreasing the number of metastases, decreasing pain, increasing survival, and increasing progression free survival. "Diagnosing" refers to the process of identifying or determining the distinguishing characteristics of a disease or tumor. In the case of cancer, the process of diagnosing is sometimes also expressed as staging or tumor classification based on severity or disease progression.

"Diagnosing" refers to the process of identifying or determining the distinguishing characteristics of a disease or tumor. In the case of cancer, the process of diagnosing is sometimes also expressed as staging or tumor classification based on severity or disease progression.

An "individual," "subject," or "patient" is a vertebrate, such as a human. In certain embodiments, the vertebrate is a mammal. Mammals include, but are not limited to, farm animals (such as cows), sport animals, pets (such as cats, dogs, and horses), primates, mice and rats. In certain embodiments, a mammal is a human.

The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of the active ingredient to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. Such formulations may be sterile. In certain embodiments, the pharmaceutical formulation is pyrogen free.

A "sterile" formulation is aseptic or free from all living microorganisms and their spores. An "effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

A "therapeutically effective amount" of a substance/molecule of the disclosure may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance/molecule, to elicit a desired response in the individual. A therapeutically effective amount encompasses an amount in which any toxic or detrimental effects of the substance/molecule are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount would be less than the therapeutically effective amount.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents a cellular function and/or causes cell death or destruction. The term is intended to include radioactive isotopes (*e.g.*, At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu), chemotherapeutic agents (*e.g.*, methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents, enzymes and fragments thereof such as nucleolytic enzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, and the various antitumor or anticancer agents disclosed below.

Other cytotoxic agents are described below. A tumoricidal agent causes destruction of tumor cells.

A "toxin" is any substance capable of having a detrimental effect on the growth or proliferation of a cell.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN®); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, chlorophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gamma1I and calicheamicin omegaI1 (see, *e.g.*, Nicolaou *et al.*, *Angew. Chem Intl. Ed. Engl.*, 33: 183-186 (1994)); CDP323, an oral alpha-4 integrin inhibitor; dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including ADRIAMYCIN®, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, doxorubicin HCl liposome injection (DOXIL®), liposomal doxorubicin TLC D-99 (MYOCET®), peglylated liposomal doxorubicin (CAELYX®), and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins,

peplomycin, porfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate, gemcitabine (GEMZAR®), tegafur (UFTORAL®), capecitabine (XELODA®), an epothilone, 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; 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; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2'-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoid, *e.g.*, paclitaxel (TAXOL®), albumin-engineered nanoparticle formulation of paclitaxel (ABRAXANE™), and docetaxel (TAXOTERE®); chloranbucil; 6-thioguanine; mercaptopurine; methotrexate; platinum agents such as cisplatin, oxaliplatin (*e.g.*, ELOXATIN®), and carboplatin; vincas, which prevent tubulin polymerization from forming microtubules, including vinblastine (VELBAN®), vincristine (ONCOVIN®), vindesine (ELDISINE®, FILDESIN®), and vinorelbine (NAVELBINE®); etoposide (VP-16); ifosfamide; mitoxantrone; leucovorin; novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid, including bexarotene (TARGRETIN®); bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), etidronate (DIDROCAL®), NE-58095, zoledronic acid/zoledronate (ZOMETA®), alendronate (FOSAMAX®), pamidronate (AREDIA®), tiludronate (SKELID®), or risedronate (ACTONEL®); troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in aberrant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-

R); vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; topoisomerase 1 inhibitor (*e.g.*, LURTOTECAN®); rmRH (*e.g.*, ABARELIX®); BAY439006 (sorafenib; Bayer); SU-11248 (sunitinib, SUTENT®, Pfizer); perifosine, COX-2 inhibitor (*e.g.* celecoxib or etoricoxib), proteosome inhibitor (*e.g.* PS341); bortezomib (VELCADE®); CCI-779; tipifarnib (R11577); orafenib, ABT510; Bcl-2 inhibitor such as oblimersen sodium (GENASENSE®); pixantrone; EGFR inhibitors (see definition below); tyrosine kinase inhibitors (see definition below); serine-threonine kinase inhibitors such as rapamycin (sirolimus, RAPAMUNE®); farnesyltransferase inhibitors such as lonafarnib (SCH 6636, SARASAR™); and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone; and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU and leucovorin.

Chemotherapeutic agents as defined herein include "anti-hormonal agents" or "endocrine therapeutics" which act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of cancer. They may be hormones themselves, including, but not limited to: anti-estrogens with mixed agonist/antagonist profile, including, tamoxifen (NOLVADEX®), 4-hydroxytamoxifen, toremifene (FARESTON®), idoxifene, droloxifene, raloxifene (EVISTA®), trioxifene, keoxifene, and selective estrogen receptor modulators (SERMs) such as SERM3; pure anti-estrogens without agonist properties, such as fulvestrant (FASLODEX®), and EM800 (such agents may block estrogen receptor (ER) dimerization, inhibit DNA binding, increase ER turnover, and/or suppress ER levels); aromatase inhibitors, including steroidal aromatase inhibitors such as formestane and exemestane (AROMASIN®), and nonsteroidal aromatase inhibitors such as anastrazole (ARIMIDEX®), letrozole (FEMARA®) and aminoglutethimide, and other aromatase inhibitors include vorozole (RIVISOR®), megestrol acetate (MEGASE®), fadrozole, and 4(5)-imidazoles; lutenizing hormone-releasing hormone agonists, including leuprolide (LUPRON® and ELIGARD®), goserelin, buserelin, and tripterelin; sex steroids, including progestines such as megestrol acetate and medroxyprogesterone acetate, estrogens such as diethylstilbestrol and premarin, and androgens/retinoids such as fluoxymesterone, all transretionic acid and fenretinide; onapristone; anti-progesterones; estrogen receptor down-regulators (ERDs); anti-androgens such as flutamide, nilutamide and bicalutamide; and

pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell (such as a cell expressing SMO) either *in vitro* or *in vivo*. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of cells (such as a cell expressing SMO) in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topoisomerase II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in Mendelsohn and Israel, eds., *The Molecular Basis of Cancer*, Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami *et al.* (W.B. Saunders, Philadelphia, 1995), *e.g.*, p. 13. The taxanes (paclitaxel and docetaxel) are anticancer drugs both derived from the yew tree. Docetaxel (TAXOTERE®, Rhone-Poulenc Rorer), derived from the European yew, is a semisynthetic analogue of paclitaxel (TAXOL®, Bristol-Myers Squibb). Paclitaxel and docetaxel promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization, which results in the inhibition of mitosis in cells.

A "mutant Smo antagonist" is a compound that inhibits the biological activity of a SMO having an amino acid substitution at the amino acid position corresponding to amino acid 529 of human SMO that changes the wild-type amino acid at this position to any other amino acid. In some embodiments, the biological activity of SMO is the ability to transduce a signal upon stimulation with hedgehog to activation of Gli transcription factor.

The term "hedgehog pathway inhibitor," as used herein, is intended to refer to an agent that is capable of inhibiting hedgehog signaling in a cell. In particular embodiments, the hedgehog antagonist is capable of inhibiting hedgehog signaling in a cell that expresses any of the mutant SMO proteins described herein. In some embodiments, the hedgehog pathway inhibitor is capable of inhibiting hedgehog signaling in a cell that expresses a smoothened polypeptide comprising a mutation at one or more amino acids corresponding to 529 of SEQ ID NO: 1 (e.g., to the corresponding position in wildtype human SMO). In some embodiments, the hedgehog pathway inhibitor is capable of inhibiting hedgehog signaling in a cell that expresses a smoothened polypeptide comprising a G529S mutation.

I. Nucleic Acids

The nucleic acids of the disclosure include isolated mutant SMO-encoding sequences. In some embodiments, the nucleic acids encode a mutant SMO protein that is partially or fully resistant to vismodegib. In some embodiments, the nucleic acid encodes a mutant SMO protein that is partially or fully resistant to vismodegib in a cell having an additional mutation in a gene encoding a protein in the hedgehog signaling pathway. In some embodiments, the additional mutation is any of the patched and/or SUFU mutations described herein.

In some embodiments, the disclosure provides for an isolated nucleic acid molecule encoding a mutant SMO protein wherein said amino acid sequence of the protein comprises an amino acid other than glycine at the amino acid position corresponding to position 529 of the wildtype SMO amino acid sequence. In some embodiments nucleic acids comprise a sequence that is at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the nucleic acid sequence of SEQ ID NO: 3 and which contain at least one mutation such that the nucleic acid encodes a SMO polypeptide comprising an amino acid other than glycine (G) at the amino acid position corresponding to amino acid position 529 of SEQ ID NO: 1. In some embodiments, the nucleic acid encodes serine (S) at the amino acid position corresponding to position 529 of SEQ ID NO: 1. In some embodiments, the nucleic acid has at least one mutation from the parental wild-type SMO at a nucleotide position corresponding to nucleotide position 1585, 1586, and/or 1587 of SEQ ID NO: 3. In some embodiments, the percent identity is 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% with SEQ ID NO: 3 providing that there is at least one mutation at a nucleotide position corresponding to positions 1585, 1586, and/or 1587 of SEQ ID NO: 3.

In some embodiments, the disclosure provides for an isolated nucleic acid molecule encoding a mutant SMO protein, wherein the amino acid sequence of the protein comprises an amino acid other than glycine at the amino acid position corresponding to position 529 of the wildtype SMO amino acid sequence, and wherein the amino acid sequence further comprises at least one amino acid substitution at any one or more of the amino acid positions corresponding to 241, 281, 321, 408, 412, 459, 469, 473, 518, 533 and/or 535 of the wildtype SMO amino acid sequence. In some embodiments, the nucleic acid molecule comprises a sequence that is at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the nucleic acid sequence of SEQ ID NO: 3 and which contain at least one mutation such that the nucleic acid encodes a SMO polypeptide comprising an amino acid other than glycine (G) at the amino acid position corresponding to

nucleotide position 529 of SEQ ID NO: 1, and wherein the polypeptide further comprises an amino acid sequence having at least one mutation at any one or more of the amino acid positions corresponding to 241, 281, 321, 408, 412, 459, 469, 473, 518, 533 and/or 535 of SEQ ID NO: 1. In some embodiments, the nucleic acid molecules comprise a sequence that is at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the nucleic acid sequence of SEQ ID NO: 3, the nucleic acid encodes serine (S) at the amino acid position corresponding to position 529 of SEQ ID NO: 1, and the nucleic acid encodes a polypeptide having any one or more of the following substitutions: T241M, W281C, V321M, I408V, A459V, C469Y, D473H, E518K, E518A S533N, and/or W535L. The disclosure also contemplates fragments of such nucleic acids that span the region of the mutations described above in fragments that are at least 20 nucleotides in length. In some embodiments, the nucleotide fragments are 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 nucleotides in length. The fragments may be any length that spans the region of the mutations described above up to the full length mutant SMO-encoding nucleic acid molecule. Isolated mutant SMO and fragments thereof may be used, for example, for hybridization, to generate primers and probes for the prognostic and diagnostic assays of the disclosure, and for expression in recombinant systems (such as to generate mutant SMO protein or portions thereof for use as immunogens and for use in assays of the disclosure as described herein).

The disclosure provides nucleic acid probes which may be used to identify the mutant SMO nucleic acid molecule in the methods of the disclosure. Nucleic acid samples derived from tissue suspected of having a mutant SMO or from tissue wherein the status of SMO is unknown may be screened using a specific probe for mutant SMO using standard procedures, such as described in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory Press, NY, 1989). Alternatively, the nucleic acid encoding SMO may be amplified from the tissue and probed with a specific probe of the disclosure to determine the presence of absence of mutant SMO. PCR methodology is well known in the art (Sambrook *et al.*, *supra*; Dieffenbach *et al.*, PCR PRIMER: A LABORATORY MANUAL, Cold Spring Harbor Laboratory Press, NY, 1995).

Nucleotide sequences (or their complement) encoding mutant SMO have various applications in the art of molecular biology, including uses as hybridization probes, and in the generation of anti-sense RNA and DNA probes. Mutant SMO-encoding nucleic acid will also be useful for the preparation of mutant SMO polypeptides by the recombinant techniques

described herein, wherein those mutant SMO polypeptides may find use, for example, in the preparation of anti-mutant SMO antibodies as described herein.

The full-length mutant SMO nucleic acids, or portions thereof, may be used as hybridization probes for identifying mutant SMO.

Optionally, the length of the probes will be about 20 to about 50 bases. The hybridization probes may be derived from at least the mutant region of the full length mutant SMO nucleotide sequence.

By way of example, a screening method will comprise isolating the coding region of mutant SMO using the known DNA sequence to synthesize a selected probe of about 40 bases. Hybridization probes may be labeled by a variety of labels, including radionucleotides such as ^{32}P or ^{35}S, or enzymatic labels such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems. Labeled probes having a sequence complementary to that of the mutant SMO gene of the present disclosure can be used to screen libraries of human cDNA, genomic DNA or mRNA to determine which members of such libraries the probe hybridizes to. Hybridization products may be resolved on polyacrylamide gels. In addition, the SMO mutations may be determined using the method described in the Examples. Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook *et al.*, *supra*.

Sequences identified in such library screening methods can be compared and aligned to the known sequences for SMO and mutant SMO. Sequence identity at the seventh transmembrane domain can be determined using methods known in the art.

Other useful fragments of the SMO-encoding nucleic acids include antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target mutant SMO mRNA (sense) or mutant SMO DNA (antisense) sequences. Antisense or sense oligonucleotides, according to the present disclosure, comprise a fragment of the coding region of mutant SMO DNA containing the mutation region. Such a fragment generally comprises at least about 14 nucleotides, and, in some embodiments, from about 14 to 30 nucleotides. The ability to derive an antisense or a sense oligonucleotide, based upon a cDNA sequence encoding a given protein is described in, for example, Stein and Cohen (1988) *Cancer Res.* 48:2659 and van der Krol *et al.* (1988) *BioTechniques* 6:958.

In some embodiments, the disclosure provides for nucleic acids capable of inhibiting expression of any of the mutant SMO nucleic acids described herein. Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of duplexes

that block transcription or translation of the target sequence by one of several means, including enhanced degradation of the duplexes, premature termination of transcription or translation, or by other means. Such methods are encompassed by the present disclosure. The antisense oligonucleotides thus may be used to block expression of mutant SMO proteins, wherein those mutant SMO proteins may play a role in the resistance of cancer in mammals to chemotherapeutics such as GDC-0449. Antisense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages, such as those described in WO 91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable *in vivo* (i.e., capable of resisting enzymatic degradation) but retain sequence specificity to be able to bind to target nucleotide sequences.

Specific examples of antisense compounds useful for inhibiting expression of mutant SMO proteins include oligonucleotides containing modified backbones or non-natural internucleoside linkages. Oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides. In some embodiment, modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotri-esters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and borano-phosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. In some embodiments, oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage *i.e.* a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included. Representative United States patents that teach the preparation of phosphorus-containing linkages include, but are not limited to, U.S. Patent Nos.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799;

5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, each of which is herein incorporated by reference.

In some embodiments, the nucleic acid comprises modified nucleotides or modified oligonucleotide backbones. In some embodiments, modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH.sub.2 component parts. Representative United States patents that teach the preparation of such oligonucleosides include, but are not limited to: U.S. Patent Nos.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, each of which is herein incorporated by reference.

In some embodiments of antisense oligonucleotides, both the sugar and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Patent Nos.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen *et al.*(1991) *Science* 254:1497-1500.

In some embodiments, antisense oligonucleotides incorporate phosphorothioate backbones and/or heteroatom backbones, and in particular -CH_2-NH-O-CH_2-, -CH_2-N(CH_3)-O-CH_2- (known as a methylene (methylimino) or MMI backbone), -CH_2-O-N(CH_3)-CH_2-, -

CH_2-N(CH_3)-N(CH_3)-CH_2- and -O-N(CH_3)-CH_2-CH_2- (wherein the native phosphodiester backbone is represented as -O-P-O-CH_2-) described in the above referenced U.S. Patent No. 5,489,677, and the amide backbones of the above referenced U.S. Patent No. 5,602,240. In some embodiments, antisense oligonucleotides have morpholino backbone structures of the above-referenced U.S. Patent No. 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. In some embodiments, oligonucleotides comprise one of the following at the 2' position: OH; F; O-alkyl, S-alkyl, or N-alkyl; O-alkenyl, S-alkeynyl, or N-alkenyl; O-alkynyl, S-alkynyl or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C1 to C10 alkyl or C2 to C10 alkenyl and alkynyl. In some embodiments, the oligonucleotides are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. In some embodiments, antisense oligonucleotides comprise one of the following at the 2' position: C1 to C10 lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2 CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. In some embodiments, a modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.* (1995) *Helv. Chim. Acta* 78:486-504) *i.e.*, an alkoxyalkoxy group. In some embodiments, a modification includes 2'-dimethylaminooxyethoxy, *i.e.*, a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.*, 2'-O-CH_2-O-CH_2-N(CH_2).

In some embodiments, a modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is, in some embodiments, a methelyne $(-CH_2-)_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

In some embodiments, modifications include 2'-methoxy (2'-O-CH3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-allyl (2'-CH_2-CH=CH_2), 2'-O-allyl (2'-O-CH_2-CH=CH_2) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo

(down) position. In some embodiments, a 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Patent Nos.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, each of which is herein incorporated by reference in its entirety.

In some embodiments, oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C=C-CH_3 or -CH_2-C=CH) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine (1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (*e.g.*, 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in U.S. Patent No. 3,687,808, those disclosed in THE CONCISE ENCYCLOPEDIA OF POLYMER SCIENCE AND ENGINEERING, Kroschwitz, J.I., ed., John Wiley & Sons, 1990, pp.

858-859, and those disclosed by Englisch *et al.*, ANGEWANDTE CHEMIE, INTERNATIONAL EDITION, Wiley-VCH, Germany, 1991, 30:613. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the disclosure. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 °C. (Sanghvi *et al.* ANTISENSE RESEARCH AND APPLICATIONS, CRC Press, Boca Raton, 1993, pp. 276-278) and are possible base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications. Representative U.S. patents that teach the preparation of modified nucleobases include, but are not limited to: U.S. Patent No. 3,687,808, as well as U.S. Patent Nos.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; 5,681,941 and 5,750,692, each of which is herein incorporated by reference.

Another modification of antisense oligonucleotides involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the disclosure can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the disclosure include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, cation lipids, phospholipids, cationic phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this disclosure, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this disclosure, include groups that improve oligomer uptake, distribution, metabolism or excretion. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6553-6556), cholic acid (Manoharan *et al.* (1994) *Bioorg. Med. Chem. Lett.* 4:1053-1060), a thioether, *e.g.*, hexyl-S-tritylthiol (Manoharan *et al.* (1992) *Ann. N.Y. Acad. Sci.* 660:306-309; Manoharan *et al.* (1993) *Bioorg. Med. Chem. Lett.* 3:2765-2770), a thiocholesterol (Oberhauser *et al.* (1992) *Nucl. Acids Res.* 20:533-538), an aliphatic chain, *e.g.*, dodecandiol

or undecyl residues (Saison-Behmoaras *et al.* (1991) *EMBO J.* 10:1111-1118; Kabanov *et al.* (1990) *FEBS Lett.* 259:327-330; Svinarchuk *et al.* (1993) *Biochimie* 75:49-54, a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan *et al.* (1995) *Tetrahedron Lett.* 36:3651-3654; Shea *et al.* (1990) *Nucl. Acids Res.* 18:3777-3783), a polyamine or a polyethylene glycol chain (Manoharan *et al.* (1995) *Nucleosides & Nucleotides* 14:969-973), or adamantane acetic acid (Manoharan *et al.* (1995) *Tetrahedron Lett.* 36:3651-3654), a palmityl moiety (Mishra *et al.* (1995) *Biochim. Biophys. Acta* 1264:229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety. Oligonucleotides of the disclosure may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in U.S. Patent Nos.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928; 5,688,941 and 6,656,730, each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present disclosure also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this disclosure, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, *i.e.*, a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a

substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Chimeric antisense compounds of the disclosure may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. In some embodiments, chimeric antisense oligonucleotides incorporate at least one 2' modified sugar (*e.g.*, 2'-O-$(CH_2)_2$-O-CH_3) at the 3' terminal to confer nuclease resistance and a region with at least 4 contiguous 2'-H sugars to confer RNase H activity. Such compounds have also been referred to in the art as hybrids or gapmers. In some embodiments, gapmers have a region of 2' modified sugars (*e.g.*, 2'-O-$(CH_2)_2$-O-CH_3) at the 3'-terminal and at the 5' terminal separated by at least one region having at least 4 contiguous 2'-H sugars and, in some embodiments, incorporate phosphorothioate backbone linkages. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S. Patent Nos.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, each of which is herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this disclosure may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives. The compounds of the disclosure may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S. Patent Nos.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619;

5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

Other examples of sense or antisense oligonucleotides include those oligonucleotides which are covalently linked to organic moieties, such as those described in WO 90/10048, and other moieties that increase affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents or metal complexes may be attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oligonucleotide for the target nucleotide sequence.

Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, $CaPO_4$-mediated DNA transfection, electroporation, or by using gene transfer vectors such as Epstein-Barr virus. In one embodiment, an antisense or sense oligonucleotide is inserted into a suitable retroviral vector. A cell containing the target nucleic acid sequence is contacted with the recombinant retroviral vector, either *in vivo* or *ex vivo*. Suitable retroviral vectors include, but are not limited to, those derived from the murine retrovirus M-MuLV, N2 (a retrovirus derived from M-MuLV), or the double copy vectors designated DCT5A, DCT5B and DCT5C (see WO 90/13641).

Sense or antisense oligonucleotides also may be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. In some embodiments, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

Alternatively, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is, in some embodiments, dissociated within the cell by an endogenous lipase.

Antisense or sense RNA or DNA molecules are generally at least about 5 nucleotides in length, alternatively at least about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200,

210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, or 1000 nucleotides in length, wherein in this context the term "about" means the referenced nucleotide sequence length plus or minus 10% of that referenced length.

Nucleotide sequences encoding a mutant SMO can also be used to construct hybridization probes for mapping the gene which encodes that SMO and for the genetic analysis of individuals with genetic disorders. The nucleotide sequences provided herein may be mapped to a chromosome and specific regions of a chromosome using known techniques, such as *in situ* hybridization, linkage analysis against known chromosomal markers, and hybridization screening with libraries.

A potential mutant SMO antagonist is an antisense RNA or DNA construct prepared using antisense technology, where, *e.g.*, an antisense RNA or DNA molecule acts to block directly the translation of mRNA by hybridizing to targeted mRNA and preventing protein translation. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example nucleic acids encoding mutant SMO herein, are used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix - see Lee *et al.* (1979) *Nucl. Acids Res.* 6:3073; Cooney *et al.* (1988) *Science* 241:456; Dervan *et al.* (1991) *Science* 251:1360), thereby preventing transcription and the production of mutant SMO. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the mutant SMO (Okano (1991) *Neurochem.* 56:560); OLIGODEOXYNUCLEOTIDES AS ANTISENSE INHIBITORS OF GENE EXPRESSION, CRC Press, Boca Raton, FL, 1988). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the mutant SMO. When antisense DNA is used, oligodeoxyribonucleotides derived from the translation-initiation site, *e.g.*, between about -10 and +10 positions of the target gene nucleotide sequence, may be used in some embodiments.

Any of the nucleic acids are suitable for use in expressing mutant SMO proteins and identifying natural targets or binding partners for the expressed mutant smoothened proteins (*e.g.*, a smoothened protein having a G529S mutation relative to wildtype SMO, such as wildtype human SMO). The nucleic acids may also be used to study mutant smoothened

bioactivity, to purify mutant smoothened and its binding partners from various cells and tissues, and to identify additional components of the hedgehog signaling pathway.

II. Small Molecules

Potential antagonists of mutant SMO include small molecules that bind to the site occupied in wild-type SMO by GDC-0449, thereby blocking the biological activity of mutant SMO. Examples of small molecules include, but are not limited to, small peptides or peptide-like molecules, *e.g.*, soluble peptides, and synthetic non-peptidyl organic or inorganic compounds.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. Ribozymes act by sequence-specific hybridization to the complementary target RNA, followed by endonucleolytic cleavage. Specific ribozyme cleavage sites within a potential RNA target can be identified by known techniques. For further details see, *e.g.*, Rossi (1994) *Current Biology*, 4:469-471, and PCT publication No. WO 97/33551 (published September 18, 1997).

Nucleic acid molecules in triple-helix formation used to inhibit transcription should be single-stranded and composed of deoxynucleotides. The base composition of these oligonucleotides is designed such that it promotes triple-helix formation via Hoogsteen base-pairing rules, which generally require sizeable stretches of purines or pyrimidines on one strand of a duplex. For further details see, *e.g.*, PCT publication No. WO 97/33551, *supra*.

These small molecules can be identified by any one or more of the screening assays discussed hereinabove and/or by any other screening techniques well known for those skilled in the art.

III. Proteins

The disclosure provides isolated mutant SMO proteins. Wild-type human SMO is shown in SEQ ID NO: 1. In some embodiments, the mutant SMO proteins are partially or fully resistant to vismodegib. In some embodiments, the mutant SMO proteins are partially or fully resistant to vismodegib in a cell having an additional mutation in a gene encoding a protein in the hedgehog signaling pathway. In some embodiments, the additional mutation is any of the patched and/or SUFU mutations described herein.

In some embodiments, the disclosure provides for an isolated mutant SMO protein comprising an amino acid sequence, wherein the amino acid sequence comprises an amino acid other than glycine at the amino acid position corresponding to position 529 of the

wildtype SMO amino acid sequence. In some embodiments, the SMO protein comprises an amino acid sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 1, provided that there is a substitution at amino acid position 529. In some embodiments, the SMO protein comprises an amino acid sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 1, provided that the amino acid sequence comprises an amino acid other than glycine (G) at the amino acid position corresponding to position 529 of SEQ ID NO: 1. In some embodiments, the SMO protein comprises an amino acid sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 1, provided that the SMO protein comprises a serine (S) at the amino acid position corresponding to position 529 of SEQ ID NO: 1.

In some embodiments, the disclosure provides for an isolated mutant SMO protein comprising an amino acid sequence, wherein the amino acid sequence of the protein comprises an amino acid other than glycine at the amino acid position corresponding to position 529 of the wildtype SMO amino acid sequence, and wherein the amino acid sequence further comprises at least one amino acid substitution at any one or more of the amino acid positions corresponding to 241, 281, 321, 408, 412, 459, 469, 473, 518, 533 and/or 535 of the wildtype SMO amino acid sequence. In some embodiments, the SMO protein comprises an amino acid sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 1, provided that there is a substitution at amino acid position 529, and wherein the protein further comprises at least one additional mutation at any one or more of the amino acid positions corresponding to 241, 281, 321, 408, 412, 459, 469, 473, 518, 533 and/or 535 of SEQ ID NO: 1. In some embodiments, the SMO protein comprises an amino acid sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 1, provided that the amino acid sequence comprises an amino acid other than glycine (G) at the amino acid position corresponding to position 529 of SEQ ID NO: 1, and wherein the amino acid sequence further comprises any one or more of the following substitutions: T241M, W281C, V321M, I408V, A459V, C469Y, D473H, E518K, E518A, S533N, and/or W535L. In some embodiments, the SMO protein comprises an amino acid sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 1, provided that the amino acid sequence comprises a serine (S) at the amino acid position corresponding

to position 529 of SEQ ID NO: 1, and wherein the amino acid sequence further comprises any one or more of the following substitutions: T241M, W281C, V321M, I408V, A459V, C469Y, D473H, E518K, E518A, S533N, and/or W535L. In particular embodiments, the disclosure provides for a SMO protein comprising an amino acid sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 1, provided that the amino acid sequence comprises an amino acid other than glycine (G), *e.g.*, a serine (S), at the amino acid position corresponding to position 529 of SEQ ID NO: 1, and wherein the amino acid sequence further comprises an amino acid other than valine (V), *e.g.*, a methionine (M), at the amino acid position corresponding to position 321 of SEQ ID NO: 1.

In some embodiments, the mutant human SMO is shown in SEQ ID NO:2 wherein amino acid 529 is shown as "Xaa" which, with respect to this application stands for any amino acid other than glycine (G). In some embodiments, the Xaa is serine (S).

In some embodiments, any of the mutant SMO proteins lack the N-terminal methionine corresponding to position 1 of any of SEQ ID NOs; 1 or 2

Mutant SMO and fragments thereof may be produced in recombinant systems as is well known in the art using the mutant SMO nucleic acids described herein. Such nucleic acids may be incorporated into expression vectors as are well-known in that art and transfected into host cells, which may be prokaryotic or eukaryotic cells depending on the proposed use of the protein. Full length or fragments of mutant SMO (in which the fragments contain at least a seventh transmembrane domain of SMO and position 529 of human SMO.) may be used as immunogens to produce antibodies of the disclosure, or to purify antibodies of the disclosure, for example.

In some embodiments, the SMO protein or fragment thereof has at least one of the same biological activities of a wildtype SMO polypeptide (*e.g.*, a SMO protein having the amino acid sequence of SEQ ID NO: 1). In some embodiments, a mutant SMO protein (*e.g.*, a SMO protein having a mutation at an amino acid position corresponding to amino acid 529 of SEQ ID NO: 1) has increased basal biological activity as compared to wildtype SMO protein (*e.g.*, a SMO protein having the amino acid sequence of SEQ ID NO: 1). By the terms "biological activity", "bioactivity" or "functional" is meant the ability of the SMO protein or fragment thereof to carry out at least one of the functions associated with wildtype SMO proteins, for example, transducing the hedgehog signaling pathway and/or inducing Gli1 expression. In certain embodiments, the SMO protein binds kinesin motor protein

Costal-2. The terms "biological activity", "bioactivity", and "functional" are used interchangeably herein.

In some embodiments, any of the SMO proteins (*e.g.*, any of the mutant SMO proteins described herein) is capable of transducing hedgehog signaling. By the terms "has the ability" or "is capable of" is meant the recited protein will carry out the stated bioactivity under suitable conditions (*e.g.*, physiological conditions or standard laboratory conditions). In certain embodiments, the term "can" may be used to describe this ability (e.g., "can bind" or "binds" to a given sequence). For example, if a SMO protein (*e.g.*, any of the mutant SMO proteins described herein) has the ability or is capable of facilitating hedgehog signaling, the SMO protein is capable of facilitating hedgehog signaling in a cell under normal physiological conditions. One of ordinary skill in the art would understand what conditions would be needed to test whether a polypeptide has the ability or is capable of carrying out a recited bioactivity.

In some embodiments, the SMO and mutant SMO proteins described herein comprise a smoothened gain-of-function mutation. In some embodiments, the gain-of-function smoothened mutation results in a constitutively active smoothened protein. In certain embodiments, the mutation in Smoothened comprises a mutation at any of the specific positions, such as position corresponding to a particular position in SEQ ID NO: 1, as set forth above with respect to the screening assay. *See, e.g.*, WO 2011/028950; WO2012047968 and WO 2015/120075, each of which is incorporated by reference. In certain embodiments, the mutation is a mutation at a position corresponding to position 529 of SEQ ID NO: 1. In some embodiments, the smoothened mutation has a mutation that renders it resistant to certain smoothened inhibitors.

In some embodiments, any of the SMO proteins described herein (*e.g.*, any of the mutant SMO proteins described herein) is fused to another agent. In some embodiments, the SMO protein is fused to another polypeptide.

Any of the mutant SMO proteins described herein are suitable for use in identifying natural targets or binding partners for mutant smoothened proteins (*e.g.*, a smoothened protein having a G529S mutationeither alone or in combination with any one or more of T241M, W281C, V321M, I408V, A459V, C469Y, D473H, E518K, E518A, S533N, and/or W535L). The mutant SMO proteins may also be used to study mutant smoothened bioactivity, to purify mutant smoothened and its binding partners from various cells and tissues, and to identify additional components of the hedgehog signaling pathway.

IV. Antibodies

A. Anti-mutant SMO Antibodies

In one aspect, the disclosure provides antibodies that bind to SMO, particularly mutant SMO. In some embodiments, any of the antibodies disclosed herein specifically bind any of the mutant SMO polypeptides described herein. For example, a mutant SMO polypeptide comprises an epitope specifically bound by antibodies of the disclosure. In some embodiments, the antibodies specifically bind SMO protein that comprises an amino acid sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 1, provided that there is a mutation at an amino acid position corresponding to positions 529 of SEQ ID NO: 1. In some embodiments, the antibodies do not specifically bind a SMO protein having the amino acid sequence of SEQ ID NO: 1 or preferentially bind a mutant SMO protein in comparison to a SMO protein having the amino acid sequence of SEQ ID NO: 1 (e.g., binding is selective for a mutant SMO protein). In some embodiments, the antibodies do not bind a SMO protein that lacks a mutation at any one of the amino acid positions corresponding to positions 529 of SEQ ID NO: 1.

In one embodiment, an anti-SMO antibody is a monoclonal antibody. In one embodiment, an anti-SMO antibody is an antibody fragment, *e.g.*, a Fab, Fab'-SH, Fv, scFv, or $(Fab')_2$ fragment. In one embodiment, an anti-mutant SMO antibody is a chimeric, humanized, or human antibody. In one embodiment, an anti-SMO antibody is purified. In certain embodiments, a composition is a pharmaceutical formulation for the treatment of cancer.

1. Antibody Fragments

The present disclosure encompasses antibody fragments. Antibody fragments may be generated by traditional means, such as enzymatic digestion, or by recombinant techniques. In certain circumstances there are advantages of using antibody fragments, rather than whole antibodies. The smaller size of the fragments allows for rapid clearance, and may lead to improved access to solid tumors. For a review of certain antibody fragments, see Hudson *et al.* (2003) *Nat. Med.* 9:129-134.

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al.*, *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992); and Brennan *et al.*, *Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. Fab, Fv and ScFv antibody fragments can all be expressed

in and secreted from *E. coli*, thus allowing the facile production of large amounts of these fragments. Antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al.*, *Bio/Technology* 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Fab and $F(ab')_2$ fragment with increased *in vivo* half-life comprising salvage receptor binding epitope residues are described in U.S. Pat. No. 5,869,046. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In certain embodiments, an antibody is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Pat. Nos. 5,571,894; and 5,587,458. Fv and scFv are the only species with intact combining sites that are devoid of constant regions; thus, they may be suitable for reduced nonspecific binding during *in vivo* use. scFv fusion proteins may be constructed to yield fusion of an effector protein at either the amino or the carboxy terminus of an scFv. See *Antibody Engineering*, ed. Borrebaeck, *supra*. The antibody fragment may also be a "linear antibody", *e.g.*, as described in U.S. Pat. No. 5,641,870, for example. Such linear antibodies may be monospecific or bispecific.

2. Humanized Antibodies

The disclosure encompasses humanized antibodies. Various methods for humanizing non-human antibodies are known in the art. For example, a humanized antibody can have one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.* (1986) *Nature* 321:522-525; Riechmann *et al.* (1988) *Nature* 332:323-327; Verhoeyen *et al.* (1988) *Science* 239:1534-1536), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies can be important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened

against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework for the humanized antibody. See, *e.g.*, Sims *et al.* (1993) *J. Immunol.* 151:2296; Chothia *et al.* (1987) *J. Mol. Biol.* 196:901. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies. See, *e.g.*, Carter *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89:4285; Presta *et al.* (1993) *J. Immunol.*, 151:2623.

It is further generally desirable that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to one method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

3. Human Antibodies

Human antibodies of the disclosure can be constructed by combining Fv clone variable domain sequence(s) selected from human-derived phage display libraries with known human constant domain sequence(s) as described above. Alternatively, human monoclonal antibodies of the disclosure can be made by the hybridoma method. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described, for example, by Kozbor *J. Immunol.*, 133: 3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner *et al.*, *J. Immunol.*, 147: 86 (1991).

It is now possible to produce transgenic animals (*e.g.* mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al.*, *Proc. Natl. Acad. Sci USA*, 90: 2551 (1993); Jakobovits *et al.*, *Nature*, 362: 255 (1993); Bruggermann *et al.*, *Year in Immunol.*, 7: 33 (1993).

Gene shuffling can also be used to derive human antibodies from non-human, *e.g.* rodent, antibodies, where the human antibody has similar affinities and specificities to the starting non-human antibody. According to this method, which is also called "epitope imprinting", either the heavy or light chain variable region of a non-human antibody fragment obtained by phage display techniques as described herein is replaced with a repertoire of human V domain genes, creating a population of non-human chain/human chain scFv or Fab chimeras. Selection with antigen results in isolation of a non-human chain/human chain chimeric scFv or Fab wherein the human chain restores the antigen binding site destroyed upon removal of the corresponding non-human chain in the primary phage display clone, *i.e.* the epitope governs (imprints) the choice of the human chain partner. When the process is repeated in order to replace the remaining non-human chain, a human antibody is obtained (see PCT WO 93/06213 published April 1, 1993). Unlike traditional humanization of non-human antibodies by CDR grafting, this technique provides completely human antibodies, which have no FR or CDR residues of non-human origin.

4. Bispecific Antibodies

Bispecific antibodies are monoclonal antibodies that have binding specificities for at least two different antigens. In certain embodiments, bispecific antibodies are human or humanized antibodies. In certain embodiments, one of the binding specificities is for SMO and the other is for any other antigen. In certain embodiments, bispecific antibodies may bind to two different epitopes of SMO. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express SMO. These antibodies possess a SMO-binding arm and an arm which binds a cytotoxic agent, such as, *e.g.*, saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, *Nature*, 305: 537 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829 published May 13, 1993, and in Traunecker *et al.*, *EMBO J.*, 10: 3655 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion, for example, is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. In certain embodiments, the first heavy-chain constant region (CH1), containing the site necessary for light chain binding, is present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In one embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh *et al.*, *Methods in Enzymology*, 121:210 (1986).

According to another approach, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.* alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/00373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking method. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al.*, *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby *et al.*, *J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells,

as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al.*, *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the VH and VL domains of one fragment are forced to pair with the complementary VL and VH domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al. J. Immunol.* 147: 60 (1991).

5. Multivalent Antibodies

A multivalent antibody may be internalized (and/or catabolized) faster than a bivalent antibody by a cell expressing an antigen to which the antibodies bind. The antibodies of the present disclosure can be multivalent antibodies (which are other than of the IgM class) with three or more antigen binding sites (*e.g.* tetravalent antibodies), which can be readily produced by recombinant expression of nucleic acid encoding the polypeptide chains of the antibody. The multivalent antibody can comprise a dimerization domain and three or more antigen binding sites. In certain embodiments, the dimerization domain comprises (or consists of) an Fc region or a hinge region. In this scenario, the antibody will comprise an Fc region and three or more antigen binding sites amino-terminal to the Fc region. In certain embodiments, a multivalent antibody comprises (or consists of) three to about eight antigen binding sites. In one such embodiment, a multivalent antibody comprises (or consists of) four antigen binding sites. The multivalent antibody comprises at least one polypeptide chain (for example, two polypeptide chains), wherein the polypeptide chain(s) comprise two or

more variable domains. For instance, the polypeptide chain(s) may comprise VD1-(X1)n -VD2-(X2)n -Fc, wherein VD1 is a first variable domain, VD2 is a second variable domain, Fc is one polypeptide chain of an Fc region, X1 and X2 represent an amino acid or polypeptide, and n is 0 or 1. For instance, the polypeptide chain(s) may comprise: VH-CH1-flexible linker-VH-CH1-Fc region chain; or VH-CH1-VH-CH1-Fc region chain. The multivalent antibody herein may further comprise at least two (for example, four) light chain variable domain polypeptides. The multivalent antibody herein may, for instance, comprise from about two to about eight light chain variable domain polypeptides. The light chain variable domain polypeptides contemplated here comprise a light chain variable domain and, optionally, further comprise a CL domain.

6. Single-Domain Antibodies

In some embodiments, an antibody of the disclosure is a single-domain antibody. A single-domain antibody is a single polyeptide chain comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody (Domantis, Inc., Waltham, MA; *see, e.g.*, U.S. Patent No. 6,248,516 B1). In one embodiment, a single-domain antibody consists of all or a portion of the heavy chain variable domain of an antibody.

7. Antibody Variants

In some embodiments, amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the antibody may be prepared by introducing appropriate changes into the nucleotide sequence encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid alterations may be introduced in the subject antibody amino acid sequence at the time that sequence is made.

A useful method for identification of certain residues or regions of the antibody that are possible locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) *Science*, 244:1081-1085. Here, a residue or group of target residues are identified (*e.g.*, charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (*e.g.*, alanine or polyalanine) to affect the

interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed immunoglobulins are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (*e.g.* for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

In certain embodiments, an antibody of the disclosure is altered to increase or decrease the extent to which the antibody is glycosylated. Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of a carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition or deletion of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that one or more of the above-described tripeptide sequences (for N-linked glycosylation sites) is created or removed. The alteration may also be made by the addition, deletion, or substitution of one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. See, *e.g.*, Wright *et al.* (1997) *TIBTECH* 15:26-32. The oligosaccharide may include various carbohydrates, *e.g.*, mannose, N-acetyl glucosamine

(GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the "stem" of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody of the disclosure may be made in order to create antibody variants with certain improved properties.

For example, antibody variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. Such variants may have improved ADCC function. See, *e.g.*, US Patent Publication Nos. US 2003/0157108 (Presta, L.); US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Examples of publications related to "defucosylated" or "fucose-deficient" antibody variants include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO2005/053742; WO2002/031140; Okazaki *et al. J. Mol. Biol.* 336:1239-1249 (2004); Yamane-Ohnuki *et al. Biotech. Bioeng.* 87: 614 (2004). Examples of cell lines capable of producing defucosylated antibodies include Lec13 CHO cells deficient in protein fucosylation (Ripka *et al. Arch. Biochem. Biophys.* 249:533-545 (1986); US Pat Appl No US 2003/0157108 A1, Presta, L; and WO 2004/056312 A1, Adams *et al.*, especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, *FUT8,* knockout CHO cells (see, *e.g.*, Yamane-Ohnuki *et al. Biotech. Bioeng.* 87: 614 (2004); Kanda, Y. *et al., Biotechnol. Bioeng.*, 94(4):680-688 (2006); and WO2003/085107).

Antibodies variants are further provided with bisected oligosaccharides, *e.g.*, in which a biantennary oligosaccharide attached to the Fc region of the antibody is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, *e.g.*, in WO 2003/011878 (Jean-Mairet *et al.*); US Patent No. 6,602,684 (Umana *et al.*); and US 2005/0123546 (Umana *et al.*). Antibody variants with at least one galactose residue in the oligosaccharide attached to the Fc region are also provided. Such antibody variants may have improved CDC function. Such antibody variants are described, *e.g.*, in WO 1997/30087 (Patel *et al.*); WO 1998/58964 (Raju, S.); and WO 1999/22764 (Raju, S.).

In certain embodiments, an antibody variant comprises an Fc region with one or more amino acid substitutions which further improve ADCC, for example, substitutions at positions 298, 333, and/or 334 of the Fc region (Eu numbering of residues). Such substitutions may occur in combination with any of the variations described above.

In certain embodiments, the disclosure contemplates an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for many applications in which the half life of the antibody *in vivo* is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. In certain embodiments, the Fc activities of the antibody are measured to ensure that only the desired properties are maintained. *In vitro* and/or *in vivo* cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express Fc(RIII only, whereas monocytes express Fc(RI, Fc(RII and Fc(RIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991). Non-limiting examples of *in vitro* assays to assess ADCC activity of a molecule of interest is described in U.S. Patent No. 5,500,362 (see, *e.g.* Hellstrom, I., *et al. Proc. Nat'l Acad. Sci. USA* 83:7059-7063 (1986)) and Hellstrom, I *et al.*, *Proc. Nat'l Acad. Sci. USA* 82:1499-1502 (1985); 5,821,337 (see Bruggemann, M. *et al.*, *J. Exp. Med.* 166:1351-1361 (1987)). Alternatively, non-radioactive assays methods may be employed (see, for example, ACTI™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA; and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in a animal model such as that disclosed in Clynes *et al. Proc. Nat'l Acad. Sci. USA* 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity. To assess complement activation, a CDC assaymay be performed (see, for example, Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996); Cragg, M.S. *et al.*, *Blood* 101:1045-1052 (2003); and Cragg, M.S. and M.J. Glennie, *Blood* 103:2738-2743 (2004)). FcRn binding and *in vivo* clearance/half life determinations can also be performed using methods known in the art (see, for example, Petkova, S.B. *et al.*, *Int'l. Immunol.* 18(12):1759-1769 (2006)).

Other antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions." More substantial changes, denominated "exemplary

substitutions" are provided in Table 1, or as further described below in reference to amino acid classes. Amino acid substitutions may be introduced into an antibody of interest and the products screened, *e.g.*, for a desired activity, such as improved antigen binding, decreased immunogenicity, improved ADCC or CDC, *etc.*

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Modifications in the biological properties of an antibody may be accomplished by selecting substitutions that affect (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, New York (1975)):

(1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)

(2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)

(3) acidic: Asp (D), Glu (E)

(4) basic: Lys (K), Arg (R), His(H)

Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Such substituted residues also may be introduced into the conservative substitution sites or, into the remaining (non-conserved) sites.

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have modified (*e.g.*, improved) biological properties relative to the parent antibody from which they are generated. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated using phage display-based affinity maturation techniques. Briefly, several hypervariable region sites (*e.g.* 6-7 sites) are mutated to generate all possible amino acid substitutions at each site. The antibodies thus generated are displayed from filamentous phage particles as fusions to at least part of a phage coat protein (*e.g.*, the gene III product of M13) packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.* binding affinity). In order to identify candidate hypervariable region sites for modification, scanning mutagenesis (*e.g.*, alanine scanning) can be performed to identify hypervariable region residues contributing significantly to antigen binding.

Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues are candidates for substitution according to techniques known in the art, including those elaborated herein. Once such variants are generated, the panel of variants is subjected to screening using techniques known in the art, including those described herein, and variants with superior properties in one or more relevant assays may be selected for further development.

Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.

It may be desirable to introduce one or more amino acid modifications in an Fc region of antibodies of the disclosure, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (*e.g.*, a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (*e.g.* a substitution) at one or more amino acid positions including that of a hinge cysteine.

In accordance with this description and the teachings of the art, it is contemplated that in some embodiments, an antibody of the disclosure may comprise one or more alterations as compared to the wild type counterpart antibody, *e.g.* in the Fc region. These antibodies would nonetheless retain substantially the same characteristics required for therapeutic utility as compared to their wild type counterpart. For example, it is thought that certain alterations can be made in the Fc region that would result in altered (*i.e.*, either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), *e.g.*, as described in WO99/51642. See also Duncan & Winter, *Nature* 322:738-40 (1988); U.S. Patent No. 5,648,260; U.S. Patent No. 5,624,821; and WO94/29351 concerning other examples of Fc region variants. WO00/42072 (Presta) and WO 2004/056312 (Lowman) describe antibody variants with improved or diminished binding to FcRs. The content of these patent publications are specifically incorporated herein by reference. See, also, Shields *et al. J. Biol. Chem.* 9(2): 6591-6604 (2001). Antibodies with increased half lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al.*, *J. Immunol.* 117:587 (1976) and Kim *et al.*, *J. Immunol.* 24:249 (1994)), are described in US2005/0014934A1 (Hinton *et al.*). These antibodies comprise an Fc region

with one or more substitutions therein which improve binding of the Fc region to FcRn. Polypeptide variants with altered Fc region amino acid sequences and increased or decreased C1q binding capability are described in US patent No. 6,194,551B1, WO99/51642. The contents of those patent publications are specifically incorporated herein by reference. See, also, Idusogie *et al. J. Immunol.* 164: 4178-4184 (2000).

In another aspect, the disclosure provides antibodies comprising modifications in the interface of Fc polypeptides comprising the Fc region, wherein the modifications facilitate and/or promote heterodimerization. These modifications comprise introduction of a protuberance into a first Fc polypeptide and a cavity into a second Fc polypeptide, wherein the protuberance is positionable in the cavity so as to promote complexing of the first and second Fc polypeptides. Methods of generating antibodies with these modifications are known in the art, *e.g.*, as described in U.S. Pat. No. 5,731,168.

In yet another aspect, it may be desirable to create cysteine engineered antibodies, *e.g.*, "thioMAbs," in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, as described further herein. In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 (Kabat numbering) of the light chain; A118 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc region.

8. Antibody Derivatives

The antibodies of the present disclosure can be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. In some embodiments, the moieties suitable for derivatization of the antibody are water soluble polymers. Non-limiting examples of 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), and dextran or 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 may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or

unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer are attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, *etc.*

In another embodiment, conjugates of an antibody and nonproteinaceous moiety that may be selectively heated by exposure to radiation are provided. In one embodiment, the nonproteinaceous moiety is a carbon nanotube (Kam *et al.*, *Proc. Natl. Acad. Sci. USA* 102: 11600-11605 (2005)). The radiation may be of any wavelength, and includes, but is not limited to, wavelengths that do not harm ordinary cells, but which heat the nonproteinaceous moiety to a temperature at which cells proximal to the antibody-nonproteinaceous moiety are killed.

B. Certain Methods of Making Antibodies

1. Certain Hybridoma-Based Methods

Monoclonal antibodies of the disclosure can be made using the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), and further described, *e.g.*, in Hongo *et al.*, *Hybridoma*, 14 (3): 253-260 (1995), Harlow *et al.*, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981), and Ni, *Xiandai Mianyixue*, 26(4):265-268 (2006) regarding human-human hybridomas.

Additional methods include those described, for example, in U.S. Pat. No. 7,189,826 regarding production of monoclonal human natural IgM antibodies from hybridoma cell lines. Human hybridoma technology (Trioma technology) is described in Vollmers and Brandlein, *Histology and Histopathology*, 20(3):927-937 (2005) and Vollmers and Brandlein, *Methods and Findings in Experimental and Clinical Pharmacology*, 27(3):185-91 (2005).

For various other hybridoma techniques, see, *e.g.*, US 2006/258841; US 2006/183887 (fully human antibodies), US 2006/059575; US 2005/287149; US 2005/100546; US 2005/026229; and U.S. Pat. Nos. 7,078,492 and 7,153,507. An exemplary protocol for producing monoclonal antibodies using the hybridoma method is described as follows. In one embodiment, a mouse or other appropriate host animal, such as a hamster, is immunized to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Antibodies are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of a polypeptide comprising

mutant SMO or a fragment thereof, and an adjuvant, such as monophosphoryl lipid A (MPL)/trehalose dicrynomycolate (TDM) (Ribi Immunochem. Research, Inc., Hamilton, MT). A polypeptide comprising mutant SMO or a fragment thereof may be prepared using methods well known in the art, such as recombinant methods, some of which are further described herein. Serum from immunized animals is assayed for anti-mutant SMO antibodies, and booster immunizations are optionally administered. Lymphocytes from animals producing anti-mutant SMO antibodies are isolated. Alternatively, lymphocytes may be immunized *in vitro*.

Lymphocytes are then fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. See, *e.g.*, Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986). Myeloma cells may be used that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Exemplary myeloma cells include, but are not limited to, murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium, *e.g.*, a medium that contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells. In some embodiments, serum-free hybridoma cell culture methods are used to reduce use of animal-derived serum such as fetal bovine serum, as described, for example, in Even *et al.*, *Trends in Biotechnology*, 24(3), 105-108 (2006).

Oligopeptides as tools for improving productivity of hybridoma cell cultures are described in Franek, *Trends in Monoclonal Antibody Research*, 111-122 (2005). Specifically, standard culture media are enriched with certain amino acids (alanine, serine, asparagine, proline), or with protein hydrolyzate fractions, and apoptosis may be significantly suppressed

by synthetic oligopeptides, constituted of three to six amino acid residues. The peptides are present at millimolar or higher concentrations.

Culture medium in which hybridoma cells are growing may be assayed for production of monoclonal antibodies that bind to mutant SMO. The binding specificity of monoclonal antibodies produced by hybridoma cells may be determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoadsorbent assay (ELISA). The binding affinity of the monoclonal antibody can be determined, for example, by Scatchard analysis. See, *e.g.*, Munson *et al., Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods. See, *e.g.*, Goding, *supra*. Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, hybridoma cells may be grown *in vivo* as ascites tumors in an animal. Monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. One procedure for isolation of proteins from hybridoma cells is described in US 2005/176122 and U.S. Pat. No. 6,919,436. The method includes using minimal salts, such as lyotropic salts, in the binding process and, in some embodiments, also using small amounts of organic solvents in the elution process.

2. Certain Library Screening Methods

Antibodies of the disclosure can be made by using combinatorial libraries to screen for antibodies with the desired activity or activities. For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics. Such methods are described generally in Hoogenboom *et al.* in *Methods in Molecular Biology* 178:1-37 (O'Brien *et al.*, ed., Human Press, Totowa, NJ, 2001). For example, one method of generating antibodies of interest is through the use of a phage antibody library as described in Lee *et al.*, J. Mol. Biol. (2004), 340(5):1073-93.

In principle, synthetic antibody clones are selected by screening phage libraries containing phage that display various fragments of antibody variable region (Fv) fused to phage coat protein. Such phage libraries are panned by affinity chromatography against the desired antigen. Clones expressing Fv fragments capable of binding to the desired antigen are adsorbed to the antigen and thus separated from the non-binding clones in the library.

The binding clones are then eluted from the antigen, and can be further enriched by additional cycles of antigen adsorption/elution. Any of the antibodies of the disclosure can be obtained by designing a suitable antigen screening procedure to select for the phage clone of interest followed by construction of a full length antibody clone using the Fv sequences from the phage clone of interest and suitable constant region (Fc) sequences described in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3.

In certain embodiments, the antigen-binding domain of an antibody is formed from two variable (V) regions of about 110 amino acids, one each from the light (VL) and heavy (VH) chains, that both present three hypervariable loops (HVRs) or complementarity-determining regions (CDRs). Variable domains can be displayed functionally on phage, either as single-chain Fv (scFv) fragments, in which VH and VL are covalently linked through a short, flexible peptide, or as Fab fragments, in which they are each fused to a constant domain and interact non-covalently, as described in Winter *et al.*, *Ann. Rev. Immunol.*, 12: 433-455 (1994). As used herein, scFv encoding phage clones and Fab encoding phage clones are collectively referred to as "Fv phage clones" or "Fv clones."

Repertoires of VH and VL genes can be separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be searched for antigen-binding clones as described in Winter *et al.*, *Ann. Rev. Immunol.*, 12: 433-455 (1994). Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned to provide a single source of human antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths *et al.*, *EMBO J*, 12: 725-734 (1993). Finally, naive libraries can also be made synthetically by cloning the unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement *in vitro* as described by Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992).

In certain embodiments, filamentous phage is used to display antibody fragments by fusion to the minor coat protein pIII. The antibody fragments can be displayed as single chain Fv fragments, in which VH and VL domains are connected on the same polypeptide chain by a flexible polypeptide spacer, *e.g.* as described by Marks *et al.*, *J. Mol. Biol.*, 222: 581-597 (1991), or as Fab fragments, in which one chain is fused to pIII and the other is secreted into the bacterial host cell periplasm where assembly of a Fab-coat protein structure

which becomes displayed on the phage surface by displacing some of the wild type coat proteins, *e.g.* as described in Hoogenboom *et al.*, *Nucl. Acids Res.*, 19: 4133-4137 (1991).

In general, nucleic acids encoding antibody gene fragments are obtained from immune cells harvested from humans or animals. If a library biased in favor of anti-mutant SMO clones is desired, the subject is immunized with mutant SMO to generate an antibody response, and spleen cells and/or circulating B cells other peripheral blood lymphocytes (PBLs) are recovered for library construction. In one embodiment, a human antibody gene fragment library biased in favor of anti-mutant SMO clones is obtained by generating an anti-mutant SMO antibody response in transgenic mice carrying a functional human immunoglobulin gene array (and lacking a functional endogenous antibody production system) such that mutant SMO immunization gives rise to B cells producing human antibodies against mutant SMO. The generation of human antibody-producing transgenic mice is described below.

Additional enrichment for anti-mutant SMO reactive cell populations can be obtained by using a suitable screening procedure to isolate B cells expressing mutant SMO-specific membrane bound antibody, *e.g.*, by cell separation using mutant SMO affinity chromatography or adsorption of cells to fluorochrome-labeled mutant SMO followed by flow-activated cell sorting (FACS).

Alternatively, the use of spleen cells and/or B cells or other PBLs from an unimmunized donor provides a better representation of the possible antibody repertoire, and also permits the construction of an antibody library using any animal (human or non-human) species in which mutant SMO is not antigenic. For libraries incorporating *in vitro* antibody gene construction, stem cells are harvested from the subject to provide nucleic acids encoding unrearranged antibody gene segments. The immune cells of interest can be obtained from a variety of animal species, such as human, mouse, rat, lagomorpha, luprine, canine, feline, porcine, bovine, equine, and avian species, *etc.*

Nucleic acid encoding antibody variable gene segments (including VH and VL segments) are recovered from the cells of interest and amplified. In the case of rearranged VH and VL gene libraries, the desired DNA can be obtained by isolating genomic DNA or mRNA from lymphocytes followed by polymerase chain reaction (PCR) with primers matching the 5' and 3' ends of rearranged VH and VL genes as described in Orlandi *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 86: 3833-3837 (1989), thereby making diverse V gene repertoires for expression. The V genes can be amplified from cDNA and genomic DNA, with back primers at the 5' end of the exon encoding the mature V-domain and forward

primers based within the J-segment as described in Orlandi *et al.* (1989) and in Ward *et al.*, *Nature*, 341: 544-546 (1989). However, for amplifying from cDNA, back primers can also be based in the leader exon as described in Jones *et al.*, *Biotechnol.*, 9: 88-89 (1991), and forward primers within the constant region as described in Sastry *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 86: 5728-5732 (1989). To maximize complementarity, degeneracy can be incorporated in the primers as described in Orlandi *et al.* (1989) or Sastry *et al.* (1989). In certain embodiments, library diversity is maximized by using PCR primers targeted to each V-gene family in order to amplify all available VH and VL arrangements present in the immune cell nucleic acid sample, *e.g.* as described in the method of Marks *et al.*, *J. Mol. Biol.*, 222: 581-597 (1991) or as described in the method of Orum *et al.*, *Nucleic Acids Res.*, 21: 4491-4498 (1993). For cloning of the amplified DNA into expression vectors, rare restriction sites can be introduced within the PCR primer as a tag at one end as described in Orlandi *et al.* (1989), or by further PCR amplification with a tagged primer as described in Clackson *et al.*, *Nature*, 352: 624-628 (1991).

Repertoires of synthetically rearranged V genes can be derived *in vitro* from V gene segments. Most of the human VH-gene segments have been cloned and sequenced (reported in Tomlinson *et al.*, *J. Mol. Biol.*, 227: 776-798 (1992)), and mapped (reported in Matsuda *et al.*, *Nature Genet.*, **3**: 88-94 (1993)); these cloned segments (including all the major conformations of the H1 and H2 loop) can be used to generate diverse VH gene repertoires with PCR primers encoding H3 loops of diverse sequence and length as described in Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992). VH repertoires can also be made with all the sequence diversity focused in a long H3 loop of a single length as described in Barbas *et al.*, *Proc. Natl. Acad. Sci. USA*, 89: 4457-4461 (1992). Human Vκ and Vλ segments have been cloned and sequenced (reported in Williams and Winter, *Eur. J. Immunol.*, 23: 1456-1461 (1993)) and can be used to make synthetic light chain repertoires. Synthetic V gene repertoires, based on a range of VH and VL folds, and L3 and H3 lengths, will encode antibodies of considerable structural diversity. Following amplification of V-gene encoding DNAs, germline V-gene segments can be rearranged *in vitro* according to the methods of Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992).

Repertoires of antibody fragments can be constructed by combining VH and VL gene repertoires together in several ways. Each repertoire can be created in different vectors, and the vectors recombined *in vitro*, *e.g.*, as described in Hogrefe *et al.*, *Gene*, 128: 119-126 (1993), or *in vivo* by combinatorial infection, *e.g.*, the loxP system described in Waterhouse *et al.*, *Nucl. Acids Res.*, 21: 2265-2266 (1993). The *in vivo* recombination approach exploits

the two-chain nature of Fab fragments to overcome the limit on library size imposed by *E. coli* transformation efficiency. Naive VH and VL repertoires are cloned separately, one into a phagemid and the other into a phage vector. The two libraries are then combined by phage infection of phagemid-containing bacteria so that each cell contains a different combination and the library size is limited only by the number of cells present (about 10^{12} clones). Both vectors contain *in vivo* recombination signals so that the VH and VL genes are recombined onto a single replicon and are co-packaged into phage virions. These huge libraries provide large numbers of diverse antibodies of good affinity (K_d^{-1} of about 10^{-8} M).

Alternatively, the repertoires may be cloned sequentially into the same vector, *e.g.* as described in Barbas *et al.*, *Proc. Natl. Acad. Sci. USA*, 88: 7978-7982 (1991), or assembled together by PCR and then cloned, *e.g.* as described in Clackson *et al.*, *Nature*, 352: 624-628 (1991). PCR assembly can also be used to join VH and VL DNAs with DNA encoding a flexible peptide spacer to form single chain Fv (scFv) repertoires. In yet another technique, "in cell PCR assembly" is used to combine VH and VL genes within lymphocytes by PCR and then clone repertoires of linked genes as described in Embleton *et al.*, *Nucl. Acids Res.*, 20: 3831-3837 (1992).

The antibodies produced by naive libraries (either natural or synthetic) can be of moderate affinity (K_d^{-1} of about 10^6 to 10^7 M^{-1}), but affinity maturation can also be mimicked *in vitro* by constructing and reselecting from secondary libraries as described in Winter *et al.* (1994), *supra*. For example, mutation can be introduced at random *in vitro* by using error-prone polymerase (reported in Leung *et al.*, *Technique*, **1**: 11-15 (1989)) in the method of Hawkins *et al.*, *J. Mol. Biol.*, 226: 889-896 (1992) or in the method of Gram *et al.*, *Proc. Natl. Acad. Sci USA*, 89: 3576-3580 (1992). Additionally, affinity maturation can be performed by randomly mutating one or more CDRs, *e.g.* using PCR with primers carrying random sequence spanning the CDR of interest, in selected individual Fv clones and screening for higher affinity clones. WO 9607754 (published 14 March 1996) described a method for inducing mutagenesis in a complementarity determining region of an immunoglobulin light chain to create a library of light chain genes. Another effective approach is to recombine the VH or VL domains selected by phage display with repertoires of naturally occurring V domain variants obtained from unimmunized donors and screen for higher affinity in several rounds of chain reshuffling as described in Marks *et al.*, *Biotechnol.*, 10: 779-783 (1992). This technique allows the production of antibodies and antibody fragments with affinities of about 10^{-9} M or less.

Screening of the libraries can be accomplished by various techniques known in the art. For example, mutant SMO can be used to coat the wells of adsorption plates, expressed on host cells affixed to adsorption plates or used in cell sorting, or conjugated to biotin for capture with streptavidin-coated beads, or used in any other method for panning phage display libraries.

The phage library samples are contacted with immobilized mutant SMO under conditions suitable for binding at least a portion of the phage particles with the adsorbent. Normally, the conditions, including pH, ionic strength, temperature and the like are selected to mimic physiological conditions. The phages bound to the solid phase are washed and then eluted by acid, *e.g.* as described in Barbas *et al.*, *Proc. Natl. Acad. Sci USA*, 88: 7978-7982 (1991), or by alkali, *e.g.* as described in Marks *et al.*, *J. Mol. Biol.*, 222: 581-597 (1991), or by mutant SMO antigen competition, *e.g.* in a procedure similar to the antigen competition method of Clackson *et al.*, *Nature*, 352: 624-628 (1991). Phages can be enriched 20-1,000-fold in a single round of selection. Moreover, the enriched phages can be grown in bacterial culture and subjected to further rounds of selection.

The efficiency of selection depends on many factors, including the kinetics of dissociation during washing, and whether multiple antibody fragments on a single phage can simultaneously engage with antigen. Antibodies with fast dissociation kinetics (and weak binding affinities) can be retained by use of short washes, multivalent phage display and high coating density of antigen in solid phase. The high density not only stabilizes the phage through multivalent interactions, but favors rebinding of phage that has dissociated. The selection of antibodies with slow dissociation kinetics (and good binding affinities) can be promoted by use of long washes and monovalent phage display as described in Bass *et al.*, *Proteins*, 8: 309-314 (1990) and in WO 92/09690, and a low coating density of antigen as described in Marks *et al.*, *Biotechnol.*, 10: 779-783 (1992).

It is possible to select between phage antibodies of different affinities, even with affinities that differ slightly, for mutant SMO. However, random mutation of a selected antibody (*e.g.* as performed in some affinity maturation techniques) is likely to give rise to many mutants, most binding to antigen, and a few with higher affinity. With limiting mutant SMO, rare high affinity phage could be competed out. To retain all higher affinity mutants, phages can be incubated with excess biotinylated mutant SMO, but with the biotinylated mutant SMO at a concentration of lower molarity than the target molar affinity constant for mutant SMO. The high affinity-binding phages can then be captured by streptavidin-coated paramagnetic beads. Such "equilibrium capture" allows the antibodies to be selected

according to their affinities of binding, with sensitivity that permits isolation of mutant clones with as little as two-fold higher affinity from a great excess of phages with lower affinity. Conditions used in washing phages bound to a solid phase can also be manipulated to discriminate on the basis of dissociation kinetics.

Anti-mutant SMO clones may be selected based on activity. In certain embodiments, the disclosure provides anti-mutant SMO antibodies that bind to living cells that naturally express mutant SMO, such as GDC-0449-resistant tumor cells. In one embodiment, the disclosure provides anti-mutant SMO antibodies that bind to the same region as that bound by GDC-0449 in wild type SMO. Fv clones corresponding to such anti-mutant SMO antibodies can be selected by (1) isolating anti-mutant SMO clones from a phage library as described above, and optionally amplifying the isolated population of phage clones by growing up the population in a suitable bacterial host; (2) selecting mutant SMO and a second protein against which blocking and non-blocking activity, respectively, is desired; (3) adsorbing the anti-mutant SMO phage clones to immobilized mutant SMO; (4) using an excess of the second protein to elute any undesired clones that recognize mutant SMO-binding determinants which overlap or are shared with the binding determinants of the second protein; and (5) eluting the clones which remain adsorbed following step (4). Optionally, clones with the desired blocking/non-blocking properties can be further enriched by repeating the selection procedures described herein one or more times.

DNA encoding hybridoma-derived monoclonal antibodies or phage display Fv clones of the disclosure is readily isolated and sequenced using conventional procedures (*e.g.* by using oligonucleotide primers designed to specifically amplify the heavy and light chain coding regions of interest from hybridoma or phage DNA template). Once isolated, the DNA can be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of the desired monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of antibody-encoding DNA include Skerra *et al.*, *Curr. Opinion in Immunol.*, 5: 256 (1993) and Pluckthun, *Immunol. Revs*, 130: 151 (1992).

DNA encoding the Fv clones of the disclosure can be combined with known DNA sequences encoding heavy chain and/or light chain constant regions (*e.g.* the appropriate DNA sequences can be obtained from Kabat *et al.*, supra) to form clones encoding full or partial length heavy and/or light chains. It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions,

and that such constant regions can be obtained from any human or animal species. An Fv clone derived from the variable domain DNA of one animal (such as human) species and then fused to constant region DNA of another animal species to form coding sequence(s) for "hybrid," full length heavy chain and/or light chain is included in the definition of "chimeric" and "hybrid" antibody as used herein. In certain embodiments, an Fv clone derived from human variable DNA is fused to human constant region DNA to form coding sequence(s) for full- or partial-length human heavy and/or light chains.

DNA encoding anti-mutant SMO antibody derived from a hybridoma of the disclosure can also be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of homologous murine sequences derived from the hybridoma clone (*e.g.* as in the method of Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 81: 6851-6855 (1984)). DNA encoding a hybridoma- or Fv clone-derived antibody or fragment can be further modified by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In this manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of the Fv clone or hybridoma clone-derived antibodies of the disclosure.

3. Vectors, Host Cells, and Recombinant Methods

Antibodies may also be produced using recombinant methods. For recombinant production of an anti-mutant SMO antibody, nucleic acid encoding the antibody is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the antibody may be readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

a) Signal sequence component

An antibody of the disclosure may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is, in some embodiments, a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected, in some embodiments,is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process a native antibody signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected,

for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, α factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

b) Origin of replication

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

c) Selection gene component

Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up antibody-encoding nucleic acid, such as DHFR, glutamine synthetase (GS), thymidine kinase, metallothionein-I and -II, *e.g.*, primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, *etc.*

For example, cells transformed with the DHFR gene are identified by culturing the transformants in a culture medium containing methotrexate (Mtx), a competitive antagonist of DHFR. Under these conditions, the DHFR gene is amplified along with any other co-transformed nucleic acid. A Chinese hamster ovary (CHO) cell line deficient in endogenous DHFR activity (*e.g.*, ATCC CRL-9096) may be used.

Alternatively, cells transformed with the GS gene are identified by culturing the transformants in a culture medium containing L-methionine sulfoximine (Msx), an inhibitor of GS. Under these conditions, the GS gene is amplified along with any other co-transformed nucleic acid. The GS selection/amplification system may be used in combination with the DHFR selection/amplification system described above.

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding an antibody of interest, wild-type DHFR gene, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.*, kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, *Nature*, 282:39 (1979)). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics*, 85:12 (1977). The presence of the *trp*1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Alternatively, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, *Bio/Technology*, 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer *et al.*, *Bio/Technology*, 9:968-975 (1991).

d) Promoter component

Expression and cloning vectors generally contain a promoter that is recognized by the host organism and is operably linked to nucleic acid encoding an antibody. Promoters suitable for use with prokaryotic hosts include the *phoA* promoter , β-lactamase and lactose

promoter systems, alkaline phosphatase promoter, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding an antibody.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoter sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phospho-fructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Antibody transcription from vectors in mammalian host cells can be controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus, Simian Virus 40 (SV40), or from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine

papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Reyes *et al.*, *Nature* 297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the Rous Sarcoma Virus long terminal repeat can be used as the promoter.

e) Enhancer element component

Transcription of a DNA encoding an antibody of this disclosure by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature* 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the antibody-encoding sequence, but is, in some embodiments, located at a site 5' from the promoter.

f) Transcription termination component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

g) Selection and transformation of host cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, *e.g.*, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*, *e.g.*, *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and

Streptomyces. One possible *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

Full length antibody, antibody fusion proteins, and antibody fragments can be produced in bacteria, in particular when glycosylation and Fc effector function are not needed, such as when the therapeutic antibody is conjugated to a cytotoxic agent (*e.g.*, a toxin) that by itself shows effectiveness in tumor cell destruction. Full length antibodies have greater half life in circulation. Production in *E. coli* is faster and more cost efficient. For expression of antibody fragments and polypeptides in bacteria, see, *e.g.*, U.S. 5,648,237 (Carter *et al.*), U.S. 5,789,199 (Joly *et al.*), U.S. 5,840,523 (Simmons *et al.*), which describes translation initiation region (TIR) and signal sequences for optimizing expression and secretion. *See also* Charlton, *Methods in Molecular Biology, Vol. 248* (B.K.C. Lo, ed., Humana Press, Totowa, NJ, 2003), pp. 245-254, describing expression of antibody fragments in *E. coli*. After expression, the antibody may be isolated from the *E. coli* cell paste in a soluble fraction and can be purified through, *e.g.*, a protein A or G column depending on the isotype. Final purification can be carried out similar to the process for purifying antibody expressed *e.g.*, in CHO cells.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, *e.g.*, *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g.*, *Neurospora, Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*. For a review discussing the use of yeasts and filamentous fungi for the production of therapeutic proteins, see, *e.g.*, Gerngross, *Nat. Biotech.* 22:1409-1414 (2004).

Certain fungi and yeast strains may be selected in which glycosylation pathways have been "humanized," resulting in the production of an antibody with a partially or fully human glycosylation pattern. *See, e.g.*, Li *et al.*, *Nat. Biotech.* 24:210-215 (2006) (describing humanization of the glycosylation pathway in *Pichia pastoris*); and Gerngross *et al.*, *supra*.

Suitable host cells for the expression of glycosylated antibody are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present disclosure, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, duckweed (*Lemnaceae*), alfalfa (*M. truncatula*), and tobacco can also be utilized as hosts. *See, e.g.*, US Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants).

Vertebrate cells may be used as hosts, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)) ; baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al., Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2). Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including $DHFR^-$ CHO cells (Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); and myeloma cell lines such as NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for antibody production, see, *e.g.*, Yazaki and Wu, *Methods in Molecular Biology, Vol. 248* (B.K.C. Lo, ed., Humana Press, Totowa, NJ, 2003), pp. 255-268.

Host cells are transformed with the above-described expression or cloning vectors for antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

h) Culturing the host cells

The host cells used to produce an antibody of this disclosure may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al., Meth. Enz.* 58:44 (1979), Barnes *et al., Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

i) Purification of antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Carter *et al., Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, hydrophobic interaction chromatography, gel

electrophoresis, dialysis, and affinity chromatography. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark *et al., J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss *et al.*(1986) *EMBO J.* 5:1567-1575). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl) benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, in some embodiments, performed at low salt concentrations (*e.g.*, from about 0-0.25M salt).

In general, various methodologies for preparing antibodies for use in research, testing, and clinical are well-established in the art, consistent with the above-described methodologies and/or as deemed appropriate by one skilled in the art for a particular antibody of interest.

C. Immunoconjugates

The disclosure also provides immunoconjugates (interchangeably referred to as "antibody-drug conjugates," or "ADCs") comprising an antibody conjugated to one or more cytotoxic agents, such as a chemotherapeutic agent, a drug, a growth inhibitory agent, a toxin (*e.g.*, a protein toxin, an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (*i.e.*, a radioconjugate).

Immunoconjugates have been used for the local delivery of cytotoxic agents, *i.e.*, drugs that kill or inhibit the growth or proliferation of cells, in the treatment of cancer (Lambert, J. (2005) *Curr. Opinion in Pharmacology* 5:543-549; Wu et al (2005) *Nature Biotechnology* 23(9):1137-1146; Payne, G. (2003) i 3:207-212; Syrigos and Epenetos (1999) *Anticancer Research* 19:605-614; Niculescu-Duvaz and Springer (1997) *Adv. Drug Deliv.*

Rev. 26:151-172; U.S. Pat. No. 4,975,278). Immunoconjugates allow for the targeted delivery of a drug moiety to a tumor, and intracellular accumulation therein, where systemic administration of unconjugated drugs may result in unacceptable levels of toxicity to normal cells as well as the tumor cells sought to be eliminated (Baldwin *et al.*, *Lancet* (Mar. 15, 1986) pp. 603-05; Thorpe (1985) "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in *Monoclonal Antibodies '84: Biological And Clinical Applications* (A. Pinchera *et al.*, eds) pp. 475-506. Both polyclonal antibodies and monoclonal antibodies have been reported as useful in these strategies (Rowland *et al.*, (1986) *Cancer Immunol. Immunother.* 21:183-87). Drugs used in these methods include daunomycin, doxorubicin, methotrexate, and vindesine (Rowland *et al.*, (1986) *supra*). Toxins used in antibody-toxin conjugates include bacterial toxins such as diphtheria toxin, plant toxins such as ricin, small molecule toxins such as geldanamycin (Mandler et al (2000) *J. Nat. Cancer Inst.* 92(19):1573-1581; Mandler et al (2000) *Bioorganic & Med. Chem. Letters* 10:1025-1028; Mandler et al (2002) *Bioconjugate Chem.* 13:786-791), maytansinoids (EP 1391213; Liu *et al.*, (1996) *Proc. Natl. Acad. Sci. USA* 93:8618-8623), and calicheamicin (Lode et al (1998) *Cancer Res.* 58:2928; Hinman et al (1993) *Cancer Res.* 53:3336-3342). The toxins may exert their cytotoxic effects by mechanisms including tubulin binding, DNA binding, or topoisomerase inhibition. Some cytotoxic drugs tend to be inactive or less active when conjugated to large antibodies or protein receptor ligands.

ZEVALIN® (ibritumomab tiuxetan, Biogen/Idec) is an antibody-radioisotope conjugate composed of a murine IgG1 kappa monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes and 111In or 90Y radioisotope bound by a thiourea linker-chelator (Wiseman et al (2000) *Eur. Jour. Nucl. Med.* 27(7):766-77; Wiseman et al (2002) *Blood* 99(12):4336-42; Witzig et al (2002) *J. Clin. Oncol.* 20(10):2453-63; Witzig et al (2002) *J. Clin. Oncol.* 20(15):3262-69). Although ZEVALIN has activity against B-cell non-Hodgkin's Lymphoma (NHL), administration results in severe and prolonged cytopenias in most patients. MYLOTARG™ (gemtuzumab ozogamicin, Wyeth Pharmaceuticals), an antibody-drug conjugate composed of a huCD33 antibody linked to calicheamicin, was approved in 2000 for the treatment of acute myeloid leukemia by injection (*Drugs of the Future* (2000) 25(7):686; US Patent Nos. 4970198; 5079233; 5585089; 5606040; 5693762; 5739116; 5767285; 5773001). Cantuzumab mertansine (Immunogen, Inc.), an antibody-drug conjugate composed of the huC242 antibody linked via the disulfide linker SPP to the maytansinoid drug moiety, DM1, is advancing into Phase II

trials for the treatment of cancers that express CanAg, such as colon, pancreatic, gastric, and other cancers. MLN-2704 (Millennium Pharm., BZL Biologics, Immunogen Inc.), an antibody-drug conjugate composed of the anti-prostate specific membrane antigen (PSMA) monoclonal antibody linked to the maytansinoid drug moiety, DM1, is under development for the potential treatment of prostate tumors. The auristatin peptides, auristatin E (AE) and monomethylauristatin (MMAE), synthetic analogs of dolastatin, were conjugated to chimeric monoclonal antibodies cBR96 (specific to Lewis Y on carcinomas) and cAC10 (specific to CD30 on hematological malignancies) (Doronina et al (2003) *Nature Biotechnol.* 21(7):778-784) and are under therapeutic development.

In certain embodiments, an immunoconjugate comprises an antibody and a chemotherapeutic agent or other toxin. Chemotherapeutic agents useful in the generation of immunoconjugates are described herein (*e.g.*, above). Enzymatically active toxins and fragments thereof that can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes. See, *e.g.*, WO 93/21232 published October 28, 1993. A variety of radionuclides are available for the production of radioconjugated antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y, and ^{186}Re. Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.*, Science, 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, maytansinoids, dolastatins, aurostatins, a trichothecene, and CC1065, and the derivatives of these toxins that have toxin activity, are also contemplated herein.

1. Maytansine and maytansinoids

In some embodiments, the immunoconjugate comprises an antibody (full length or fragments) conjugated to one or more maytansinoid molecules.

Maytansinoids are mitototic inhibitors which act by inhibiting tubulin polymerization. Maytansine was first isolated from the east African shrub Maytenus serrata (U.S. Patent No. 3,896,111). Subsequently, it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters (U.S. Patent No. 4,151,042). Synthetic maytansinol and derivatives and analogues thereof are disclosed, for example, in U.S. Patent Nos. 4,137,230; 4,248,870; 4,256,746; 4,260,608; 4,265,814; 4,294,757; 4,307,016; 4,308,268; 4,308,269; 4,309,428; 4,313,946; 4,315,929; 4,317,821; 4,322,348; 4,331,598; 4,361,650; 4,364,866; 4,424,219; 4,450,254; 4,362,663; and 4,371,533.

Maytansinoid drug moieties are attractive drug moieties in antibody drug conjugates because they are: (i) relatively accessible to prepare by fermentation or chemical modification, derivatization of fermentation products, (ii) amenable to derivatization with functional groups suitable for conjugation through the non-disulfide linkers to antibodies, (iii) stable in plasma, and (iv) effective against a variety of tumor cell lines.

Immunoconjugates containing maytansinoids, methods of making same, and their therapeutic use are disclosed, for example, in U.S. Patent Nos. 5,208,020, 5,416,064 and European Patent EP 0 425 235 B1, the disclosures of which are hereby expressly incorporated by reference. Liu *et al.*, Proc. Natl. Acad. Sci. USA 93:8618-8623 (1996) described immunoconjugates comprising a maytansinoid designated DM1 linked to the monoclonal antibody C242 directed against human colorectal cancer. The conjugate was found to be highly cytotoxic towards cultured colon cancer cells, and showed antitumor activity in an *in vivo* tumor growth assay. Chari *et al.*, Cancer Research 52:127-131 (1992) describe immunoconjugates in which a maytansinoid was conjugated via a disulfide linker to the murine antibody A7 binding to an antigen on human colon cancer cell lines, or to another murine monoclonal antibody TA.1 that binds the HER-2/neu oncogene. The cytotoxicity of the TA.1-maytansinoid conjugate was tested *in vitro* on the human breast cancer cell line SK-BR-3, which expresses 3 x 105 HER-2 surface antigens per cell. The drug conjugate achieved a degree of cytotoxicity similar to the free maytansinoid drug, which could be increased by increasing the number of maytansinoid molecules per antibody molecule. The A7-maytansinoid conjugate showed low systemic cytotoxicity in mice.

Antibody-maytansinoid conjugates are prepared by chemically linking an antibody to a maytansinoid molecule without significantly diminishing the biological activity of either

the antibody or the maytansinoid molecule. See, *e.g.*, U.S. Patent No. 5,208,020 (the disclosure of which is hereby expressly incorporated by reference). An average of 3-4 maytansinoid molecules conjugated per antibody molecule has shown efficacy in enhancing cytotoxicity of target cells without negatively affecting the function or solubility of the antibody, although even one molecule of toxin/antibody would be expected to enhance cytotoxicity over the use of naked antibody. Maytansinoids are well known in the art and can be synthesized by known techniques or isolated from natural sources. Suitable maytansinoids are disclosed, for example, in U.S. Patent No. 5,208,020 and in the other patents and nonpatent publications referred to hereinabove. In some embodiments, maytansinoids are maytansinol and maytansinol analogues modified in the aromatic ring or at other positions of the maytansinol molecule, such as various maytansinol esters.

There are many linking groups known in the art for making antibody-maytansinoid conjugates, including, for example, those disclosed in U.S. Patent No. 5,208,020 or EP Patent 0 425 235 B1, Chari *et al.*, Cancer Research 52:127-131 (1992), and U.S. Patent Application No. 10/960,602, filed Oct. 8, 2004, the disclosures of which are hereby expressly incorporated by reference. Antibody-maytansinoid conjugates comprising the linker component SMCC may be prepared as disclosed in U.S. Patent Application No. 10/960,602, filed Oct. 8, 2004. The linking groups include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups, or esterase labile groups, as disclosed in the above-identified patents, disulfide and thioether groups may be used in some embodiments. Additional linking groups are described and exemplified herein.

Conjugates of the antibody and maytansinoid may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). In some embodiments, coupling agents include N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Carlsson *et al.*, Biochem. J. 173:723-737 (1978)) and N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP) to provide for a disulfide linkage.

The linker may be attached to the maytansinoid molecule at various positions, depending on the type of the link. For example, an ester linkage may be formed by reaction

with a hydroxyl group using conventional coupling techniques. The reaction may occur at the C-3 position having a hydroxyl group, the C-14 position modified with hydroxymethyl, the C-15 position modified with a hydroxyl group, and the C-20 position having a hydroxyl group. In one embodiment, the linkage is formed at the C-3 position of maytansinol or a maytansinol analogue.

2. Auristatins and dolastatins

In some embodiments, the immunoconjugate comprises an antibody conjugated to dolastatins or dolostatin peptidic analogs and derivatives, the auristatins (US Patent Nos. 5635483; 5780588). Dolastatins and auristatins have been shown to interfere with microtubule dynamics, GTP hydrolysis, and nuclear and cellular division (Woyke et al (2001) Antimicrob. Agents and Chemother. 45(12):3580-3584) and have anticancer (US 5663149) and antifungal activity (Pettit et al (1998) Antimicrob. Agents Chemother. 42:2961-2965). The dolastatin or auristatin drug moiety may be attached to the antibody through the N (amino) terminus or the C (carboxyl) terminus of the peptidic drug moiety (WO 02/088172).

Exemplary auristatin embodiments include the N-terminus linked monomethylauristatin drug moieties DE and DF, disclosed in "Monomethylvaline Compounds Capable of Conjugation to Ligands", US Ser. No. 10/983,340, filed Nov. 5, 2004, the disclosure of which is expressly incorporated by reference in its entirety.

Typically, peptide-based drug moieties can be prepared by forming a peptide bond between two or more amino acids and/or peptide fragments. Such peptide bonds can be prepared, for example, according to the liquid phase synthesis method (see E. Schröder and K. Lübke, "The Peptides", volume 1, pp 76-136, 1965, Academic Press) that is well known in the field of peptide chemistry. The auristatin/dolastatin drug moieties may be prepared according to the methods of: US 5635483; US 5780588; Pettit et al (1989) J. Am. Chem. Soc. 111:5463-5465; Pettit et al (1998) Anti-Cancer Drug Design 13:243-277; Pettit, G.R., *et al.* Synthesis, 1996, 719-725; and Pettit et al (1996) J. Chem. Soc. Perkin Trans. 1 5:859-863. See also Doronina (2003) Nat Biotechnol 21(7):778-784; "Monomethylvaline Compounds Capable of Conjugation to Ligands", US Ser. No. 10/983,340, filed Nov. 5, 2004, hereby incorporated by reference in its entirety (disclosing, *e.g.*, linkers and methods of preparing monomethylvaline compounds such as MMAE and MMAF conjugated to linkers).

3. Calicheamicin

In other embodiments, the immunoconjugate comprises an antibody conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. For the preparation

of conjugates of the calicheamicin family, see U.S. patents 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, 5,877,296 (all to American Cyanamid Company). Structural analogues of calicheamicin which may be used include, but are not limited to, γ1I, α2I, α3I, N-acetyl-γ1I, PSAG and θI1 (Hinman *et al.*, Cancer Research 53:3336-3342 (1993), Lode *et al.*, Cancer Research 58:2925-2928 (1998) and the aforementioned U.S. patents to American Cyanamid). Another anti-tumor drug that the antibody can be conjugated is QFA which is an antifolate. Both calicheamicin and QFA have intracellular sites of action and do not readily cross the plasma membrane. Therefore, cellular uptake of these agents through antibody mediated internalization greatly enhances their cytotoxic effects.

4. Other cytotoxic agents

Other antitumor agents that can be conjugated to the antibodies include BCNU, streptozoicin, vincristine and 5-fluorouracil, the family of agents known collectively LL-E33288 complex described in U.S. patents 5,053,394, 5,770,710, as well as esperamicins (U.S. patent 5,877,296).

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published October 28, 1993.

The present disclosure further contemplates an immunoconjugate formed between an antibody and a compound with nucleolytic activity (*e.g.*, a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

For selective destruction of the tumor, the antibody may comprise a highly radioactive atom. A variety of radioactive isotopes are available for the production of radioconjugated antibodies. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu. When the conjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example tc99m or I123, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

The radio- or other labels may be incorporated in the conjugate in known ways. For example, the peptide may be biosynthesized or may be synthesized by chemical amino acid synthesis using suitable amino acid precursors involving, for example, fluorine-19 in place of hydrogen. Labels such as $tc^{99}m$ or I^{123}, Re^{186}, Re^{188} and In^{111} can be attached via a cysteine residue in the peptide. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker et al (1978) Biochem. Biophys. Res. Commun. 80: 49-57) can be used to incorporate iodine-123. "Monoclonal Antibodies in Immunoscintigraphy" (Chatal,CRC Press 1989) describes other methods in detail.

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.*, Science 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari *et al.*, Cancer Research 52:127-131 (1992); U.S. Patent No. 5,208,020) may be used.

The compounds expressly contemplate, but are not limited to, ADC prepared with cross-linker reagents: BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (*e.g.*, from Pierce Biotechnology, Inc., Rockford, IL., U.S.A). See pages 467-498, 2003-2004 Applications Handbook and Catalog.

5. Preparation of antibody drug conjugates

In the antibody drug conjugates (ADC), an antibody (Ab) is conjugated to one or more drug moieties (D), *e.g.* about 1 to about 20 drug moieties per antibody (p = 1 to about 20), through a linker (L). The ADC of the formula shown below may be prepared by several routes, employing organic chemistry reactions, conditions, and reagents known to those

skilled in the art, including: (1) reaction of a nucleophilic group of an antibody with a bivalent linker reagent, to form Ab-L, via a covalent bond, followed by reaction with a drug moiety D; and (2) reaction of a nucleophilic group of a drug moiety with a bivalent linker reagent, to form D-L, via a covalent bond, followed by reaction with the nucleophilic group of an antibody. Additional methods for preparing ADC are described herein.

$Ab–(L–D)_p$

The linker may be composed of one or more linker components. Exemplary linker components include 6-maleimidocaproyl ("MC"), maleimidopropanoyl ("MP"), valine-citrulline ("val-cit"), alanine-phenylalanine ("ala-phe"), p-aminobenzyloxycarbonyl ("PAB"), N-Succinimidyl 4-(2-pyridylthio) pentanoate ("SPP"), N-Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1 carboxylate ("SMCC'), and N-Succinimidyl (4-iodo-acetyl) aminobenzoate ("SIAB"). Additional linker components are known in the art and some are described herein. See also "Monomethylvaline Compounds Capable of Conjugation to Ligands", US Ser. No. 10/983,340, filed Nov. 5, 2004, the contents of which are hereby incorporated by reference in its entirety.

In some embodiments, the linker may comprise amino acid residues. Exemplary amino acid linker components include a dipeptide, a tripeptide, a tetrapeptide or a pentapeptide. Exemplary dipeptides include: valine-citrulline (vc or val-cit), alanine-phenylalanine (af or ala-phe). Exemplary tripeptides include: glycine-valine-citrulline (gly-val-cit) and glycine-glycine-glycine (gly-gly-gly). Amino acid residues which comprise an amino acid linker component include those occurring naturally, as well as minor amino acids and non-naturally occurring amino acid analogs, such as citrulline. Amino acid linker components can be designed and optimized in their selectivity for enzymatic cleavage by a particular enzymes, for example, a tumor-associated protease, cathepsin B, C and D, or a plasmin protease.

Nucleophilic groups on antibodies include, but are not limited to: (i) N-terminal amine groups, (ii) side chain amine groups, *e.g.* lysine, (iii) side chain thiol groups, *e.g.* cysteine, and (iv) sugar hydroxyl or amino groups where the antibody is glycosylated. Amine, thiol, and hydroxyl groups are nucleophilic and capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups. Certain antibodies have reducible interchain disulfides, *i.e.* cysteine bridges. Antibodies may be

made reactive for conjugation with linker reagents by treatment with a reducing agent such as DTT (dithiothreitol). Each cysteine bridge will thus form, theoretically, two reactive thiol nucleophiles. Additional nucleophilic groups can be introduced into antibodies through the reaction of lysines with 2-iminothiolane (Traut's reagent) resulting in conversion of an amine into a thiol. Reactive thiol groups may be introduced into the antibody (or fragment thereof) by introducing one, two, three, four, or more cysteine residues (*e.g.*, preparing mutant antibodies comprising one or more non-native cysteine amino acid residues).

Antibody drug conjugates may also be produced by modification of the antibody to introduce electrophilic moieties, which can react with nucleophilic substituents on the linker reagent or drug. The sugars of glycosylated antibodies may be oxidized, *e.g.* with periodate oxidizing reagents, to form aldehyde or ketone groups which may react with the amine group of linker reagents or drug moieties. The resulting imine Schiff base groups may form a stable linkage, or may be reduced, *e.g.* by borohydride reagents to form stable amine linkages. In one embodiment, reaction of the carbohydrate portion of a glycosylated antibody with either glactose oxidase or sodium meta-periodate may yield carbonyl (aldehyde and ketone) groups in the protein that can react with appropriate groups on the drug (Hermanson, Bioconjugate Techniques). In another embodiment, proteins containing N-terminal serine or threonine residues can react with sodium meta-periodate, resulting in production of an aldehyde in place of the first amino acid (Geoghegan & Stroh, (1992) *Bioconjugate Chem.* 3:138-146; US 5362852). Such aldehyde can be reacted with a drug moiety or linker nucleophile.

Likewise, nucleophilic groups on a drug moiety include, but are not limited to: amine, thiol, hydroxyl, hydrazide, oxime, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide groups capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups.

Alternatively, a fusion protein comprising the antibody and cytotoxic agent may be made, *e.g.*, by recombinant techniques or peptide synthesis. The length of DNA may comprise respective regions encoding the two portions of the conjugate either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the conjugate.

In yet another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pre-targeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation

using a clearing agent and then administration of a "ligand" (*e.g.*, avidin) which is conjugated to a cytotoxic agent (*e.g.*, a radionucleotide). A variety of radionuclides are available for the production of radioconjugated antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y, and ^{186}Re.

V. Methods

A. Diagnostic methods and methods of detection of mutant SMO with antibodies

In one aspect, antibodies of the disclosure are useful for detecting the presence of mutant SMO in a biological sample. The term "detecting" as used herein encompasses quantitative or qualitative detection. In certain embodiments, a biological sample comprises a cell or tissue, such as tumor tissue.

In one aspect, the disclosure provides a method of detecting the presence of mutant SMO in a biological sample. In certain embodiments, the method comprises contacting the biological sample with an anti-mutant SMO antibody under conditions permissive for binding of the anti-mutant SMO antibody to mutant SMO, and detecting whether a complex is formed between the anti-mutant SMO antibody and mutant SMO.

In one aspect, the disclosure provides a method of diagnosing a disorder associated with expression of mutant SMO or a condition, such as drug resistance, associated with expression of mutant SMO. In certain embodiments, the method comprises contacting a test cell with an anti-mutant SMO antibody; determining the level of expression (either quantitatively or qualitatively) of mutant SMO by the test cell by detecting binding of the anti-mutant SMO antibody to mutant SMO; and comparing the level of expression of mutant SMO by the test cell with the level of expression of mutant SMO by a control cell (*e.g.*, a normal cell of the same tissue origin as the test cell or a cell that expresses wild-type SMO at levels comparable to such a normal cell), wherein a higher level of expression of mutant SMO by the test cell as compared to the control cell indicates the presence of a disorder associated with increased expression of mutant SMO. In certain embodiments, the test cell is obtained from an individual suspected of having a disorder associated with increased expression of mutant SMO. In certain embodiments, the disorder is a cell proliferative disorder, such as a cancer or a tumor. It is appreciated that in, for example, a tumor sample, there may be heterogeneity in SMO expression. Thus, it is appreciated that in a sample only a subset of cells in the sample may express the mutant SMO, and such expression is sufficient to, for example, be associated with drug resistance. Accordingly, evaluating expression includes evaluating expression in a sample and detecting mutant SMO protein in a subset of cells in a sample.

Exemplary disorders that may be diagnosed or in which drug resistance can be evaluated using an antibody of the disclosure include, but are not limited to medulloblastoma, pancreatic cancer basal cell carcinoma.

Certain other methods can be used to detect binding of antibodies to mutant SMO. Such methods include, but are not limited to, antigen-binding assays that are well known in the art, such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, fluorescent immunoassays, protein A immunoassays, and immunohistochemistry (IHC).

In certain embodiments, antibodies are labeled. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, *e.g.*, through an enzymatic reaction or molecular interaction. Exemplary labels include, but are not limited to, the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, *e.g.*, firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases, *e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

In certain embodiments, antibodies are immobilized on an insoluble matrix. Immobilization may entail separating an anti-mutant SMO antibody from any mutant SMO that remains free in solution. This conventionally is accomplished by either insolubilizing the anti-mutant SMO antibody before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich *et al.*, U.S. 3,720,760), or by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the anti-mutant SMO antibody after formation of a complex between the anti-mutant SMO antibody and mutant SMO, *e.g.*, by immunoprecipitation.

It is understood that any of the above embodiments of diagnosis or detection may be carried out using an immunoconjugate of the disclosure in place of or in addition to an anti-mutant SMO antibody.

B. Methods of detecting mutant SMO with nucleic acid probes

In one aspect, nucleic acid probes as described herein are useful for detecting the presence of mutant SMO nucleic acid in a biological sample. The term "detecting" as used herein encompasses quantitative or qualitative detection. In certain embodiments, a biological sample comprises a cell or tissue, such as tumor tissue.

In one aspect, the disclosure provides a method of detecting the presence of mutant SMO-encoding nucleic acid in a biological sample. In certain embodiments, the method comprises contacting nucleic acid from the biological sample with a probe as described herein and hybridizing the probe to the nucleic acid under conditions permissive for hybridization under stringent conditions, and detecting whether a complex is formed between the probe and the nucleic acid sample.

The mutant SMO-encoding nucleic acid may be detected using any methodology known in the art including, but not limited to the use of probes as described herein, or by PCR amplification, rtPCR sequencing, single strand conformational polymorphism (SSCP), differential restriction digestion of DNA, hybridization, or any other method known in the art.

In these methods, detection of a mutant SMO as described herein in a cell indicates the presence of a disorder associated with increased expression of mutant SMO (*i.e.*, resistance to treatment with a Smo inhibitor such as GDC-0449). In certain embodiments, the test cell is obtained from an individual suspected of having a resistant tumor associated with expression of mutant SMO. As detailed above, it is appreciated that mutations may be in a subset of cells from a sample, such as a subset of cells from a tumor sample.

Exemplary disorders that may be diagnosed using an antibody of the disclosure include, but are not limited to medulloblastoma, pancreatic cancer basal cell carcinoma.

C. Methods of detecting mutant SMO in cell based assays

Mutant SMO may be detected in cell based assays as known in the art including, but not limited to binding of a mutant SMO-detecting antibody to the surface of a cell sample, such as a tumor sample *in vitro* Immunohistochemical staining of histological preparations of tumor samples or tissue suspected of containing mutant SMO. Functional assays in which a tissue sample is contacted with GDC-0449 and hedgehog to determine whether Hh signaling occurs (*e.g.*, by measuring activation of pathway components, expression of Gli, and the like). Any functional assay using the Hh signaling pathway that can be disrupted using GDC-0449 may be used in the method of the disclosure to determine the presence and activity of a mutant SMO.

D. Methods of screening for compounds that bind to mutant SMO

In some embodiments, the disclosure provides for a method of screening for a hedgehog pathway inhibitor that is capable of inhibiting hedgehog signaling in a cell that expresses any of the mutant SMO proteins disclosed herein. In some embodiments the screen is of single agents or a discrete number of agents. In some embodiments, the screen is of pools of agents. In some embodiments, the screen is high-throughput screening. In some embodiments, the screen is of a library or libraries of compounds (*e.g.*, libraries of small molecules, libraries of antisense oligonucleotides, or libraries of antibodies or peptides). In some embodiments, screening may involve a primary assay alone or a primary assay and one or more secondary assays. In some embodiments, the agents can be assessed in an assay (*e.g.*, a hedgehog signaling assay (*e.g.*, by using any of the *Gli1* expression assays described herein or known in the art to examine Gli1 nucleic acid or protein expression in response to an agent), a mutant SMO protein binding assay (*e.g.*, by using any of the mutant SMO binding assays described herein), a cell proliferation assay (*e.g.*, by using any of the cell proliferation assays described herein or known in the art). Use in screening assays is an exemplary use for the mutant SMO proteins and nucleic acids of the disclosure (e.g., a mutant SMO protein can be used in a cell free or cell based assay; a mutant SMO nucleic acid can be provided in a vector and used to express a mutant SMO protein in host cells or a host organism suitable for a screeening assay.

The disclosure provides a method for screening for compounds that bind to mutant SMO. Without being held to any particular mode of operation, it is expected that much in the way that GDC-0449 binds wild-type SMO and doesn't bind mutant SMO, a compound which acts as an inhibitor of mutant SMO would bind mutant SMO. Thus, one may express the mutant SMO protein or a fragment thereof, such as a fragment comprising all or a portion of transmembrane domain 6 (TM6), and run binding assays using a library of compounds by any means known in the art. Also one may use a smaller library of compounds represented by variations of GDC-0449 using a modeling approach based on potential contact points of GDC-0449 and then modeling similar contact points for mutant SMO and variations of GDC-0449. Such modeling programs and algorithms may be any that are known in the art. Compounds that bind mutant SMO and wild-type SMO may be identified that are inhibitors of both wild-type and mutant SMO. Alternatively, compounds may be discovered that bind to mutant SMO, but which do not bind to wild-type SMO and therefore are inhibitors only for mutant SMO. In certain embodiments, binding and/or some other readout (e.g., hedgehog signaling) are assessed and compare to that for wildtype SMO or a suitable control (e.g., empty vector).

In one embodiment, the compounds to be screened are small molecule compounds such as variants of GDC-0449. In other embodiments, the compounds that bind mutant SMO are antibodies that specifically recognize an epitope that is in the same region as the binding site of GDC-0449 to wild-type SMO. In one embodiment the antibody binds to a region in the amino-terminal portion of TM7 of mutant SMO and inhibits mutant SMO activity.

Compounds may alternatively, or additionally be screened for their ability to inhibit mutant SMO activity. In these embodiments, one may assess the ability of these compounds to inhibit hedgehog signaling in cells expressing mutant SMO. These assays may be performed in cells that have a hedgehog signaling pathway intact but which express a recombinant SMO bearing the mutation in place of, or in addition to wild-type SMO. In these assays one determines the ability of the cell to have active hedgehog signaling when incubated with hedgehog in the presence or ansence of the candidate inhibitor. If hedgehog signaling is inhibited in the presence of the candidate compound, such compound is a hedgehog inhibitor. In some embodiments the cells express both wild-type and mutant SMO and are incubated with GDC-0449 and a candidiate inhibitor. In other embodiments, the cells express only mutant SMO and may be incubated with Hh and the candidate inhibitor alone (*i.e.*, in the absence of GDC-0449). The compound is an inhibitor of mutant SMO if Hh signaling is reduced or inhibited in such cells.

In some embodiments, the disclosure provides for a method of identifying a hedgehog pathway inhibitor, wherein the method comprises: contacting a cell with an amount of a test agent, wherein the cell is responsive to hedgehog protein or has increased hedgehog signaling and/or activation of the hedgehog signaling pathway, and wherein the cell expresses any of the mutant SMO proteins described herein, and b) determining, as compared to a control, whether the test agent inhibits hedgehog signaling in the cell, wherein if the test agent inhibits hedgehog signaling in the cell relative to the control, then the test agent is identified as a hedgehog pathway inhibitor. In some embodiments, the control (or basis for comparison) is a cell expressing a wildtype SMO protein (*e.g*, a SMO protein having the amino acid sequence of SEQ ID NO: 1). In some embodiments, the control is a cell expressing the same mutant SMO proteins as the cell contacted with the test agent, wherein the control is untreated or treated with a control agent to which the mutant SMO protein is partially or completely resistant. In some embodiments, the control agent is vismodegib, LY2940680, LDE225 and/or compound 5. In some embodiments, the test agent binds to mutant SMO protein but not wildtype SMO protein. In some embodiments, the test agent binds to both the mutant SMO protein and wildtype SMO protein. In some embodiments, the test agent is more

effective in inhibiting hedgehog signaling in a cell expressing mutant SMO protein than in a cell expressing wildtype SMO protein.

In some embodiments, the disclosure provides for a method of identifying a hedgehog pathway inhibitor, wherein the method comprises: contacting a cell with an amount of an agent, wherein the cell is responsive to hedgehog protein or has increased hedgehog signaling and/or activation of the hedgehog signaling pathway, and wherein the cell expresses any of the mutant SMO proteins described herein, and b) determining, as compared to a control, whether the agent inhibits growth and/or proliferation of the cell, wherein if the agent inhibits growth and/or proliferation of the cell relative to the control, then the agent is identified as a hedgehog pathway inhibitor. In some embodiments, the control is a cell expressing a wildtype SMO protein (*e.g*, a SMO protein having the amino acid sequence of SEQ ID NO: 1). In some embodiments, the control is a cell expressing the same mutant SMO proteins as the cell contacted with the test agent, wherein the control is untreated or treated with a control agent to which the mutant SMO protein is partially or completely resistant. In some embodiments, the control agent is vismodegib, LY2940680, LDE225 and/or compound 5. In some embodiments, the test agent binds to mutant SMO protein but not wildtype SMO protein. In some embodiments, the test agent binds to both the mutant SMO protein and wildtype SMO protein. In some embodiments, the test agent is more effective in inhibiting growth and/or proliferation of a cell expressing mutant SMO protein than of a cell expressing wildtype SMO protein.

In some embodiments, the cell used in the screening methods described herein is in culture. In some embodiments, the agent contacted with the cells in the culture is sufficient to inhibit, partially or entirely, hedgehog signaling in at least 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of cells in a cell culture. In some embodiments, the agent contacted with the cells in the culture is sufficient to reduce the rate of proliferation of a cell and/or rate of survival of at least 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of cells in a cell culture, wherein the cells are expressing or overexpressing hedgehog or have active hedgehog signaling.

In other embodiments, the cell is in an animal. In some embodiments, the animal is a mammal or other vertebrate. In some embodiments, the animal is post-natal. In some embodiments, the animal is pediatric. In some embodiments, the animal is adult. When referring to cells in vitro, the cells may be of any vertebrate species, such as a mammal, such as rodent, hamster, or human. In vitro or in vivo, a cell may be a cancer cell, such as a primary cancer cell, a metastatis cancer cell, or a cancer cell line. In some embodiments, the

cell is a medullablastoma cell. In some embodiments, the cell is a basal cell carcinoma cell. In some embodiments, the cell is a nevoid basal cell carcinoma cell. In some embodiments, the cell is a Gorlin's Syndrome cell.

In some embodiments, the cell comprises one or more mutations in a hedgehog signaling pathway gene. In some embodiments, the one or more mutations are in patched. In some embodiments, the patched mutation is loss-of-function mutation. In some embodiments, the one or more mutations are in smoothened. In some embodiments, the smoothened mutation is a smoothened gain-of-function mutation. In some embodiments, the gain-of-function smoothened mutation results in a constitutively active smoothened protein. In some embodiments, the one or more mutations are in *suppressor-of-fused*, and the cell has suppressor-of-fused (SuFu) loss-of-function. In some embodiments, the SuFu mutation results in a partial loss-of-function of SuFu activity. In some embodiments, the SuFu mutation results in a full loss-of-function in SuFu activity. In some embodiments, the SuFu mutation confers resistance to vismodegib.

In some embodiments, the agent tested in any of the screening methods described herein is a small molecule. In other embodiments, the agent is a polypeptide. In other embodiments, the agent is an siRNA antagonist.

In some embodiments of any of the screening methods described herein, the mutant SMO DNA is exogenously expressed in a cell. In some embodiments, the mutant SMO DNA is stably expressed in the cell. In some embodiments, the mutant SMO DNA is transiently expressed in the cell.

The growth inhibitory effects of the various hedgehog pathway inhibitors useable in the disclosure may be assessed by methods known in the art, e.g., using cells which express a mutant SMO polypeptide either endogenously or following transfection with the respective mutant SMO gene. For example, appropriate tumor cell lines and cells transfected with mutant SMO-encoding DNA may be treated with the hedgehog pathway inhibitors of the disclosure at various concentrations for a few days (e.g., 2-7 days) and stained with crystal violet, MTT or analyzed by some other colorimetric or luciferase-based (eg CellTiterGlo) assay. Another method of measuring proliferation would be by comparing ^{3}H-thymidine uptake by the cells treated in the presence or absence of such hedgehog pathway inhibitors. After treatment, the cells are harvested and the amount of radioactivity incorporated into the DNA quantitated in a scintillation counter. Appropriate positive controls include treatment of a selected cell line with a growth inhibitory antibody or small molecule known to inhibit growth of that cell line. Growth inhibition of tumor cells in vivo can be determined in

various ways known in the art. In some embodiments, the tumor cell is one that has one or more mutations in a hedgehog pathway signaling gene. In some embodiments, such hedgehog pathway inhibitors will inhibit cell proliferation of a hedgehog-expressing tumor cell in vitro or in vivo by about 10-25% , by about 25-100%, by about 30-100%, by about 50-100%, or by about or 70-100% compared to the untreated tumor cell. Growth inhibition can be measured at a hedgehog pathway inhibitor concentration of about 0.5 to 30 μg/ml, about 0.5 nM to 200 nM, about 200 nM to 1μM, about 1 μM to 5 μM, or about 5 μM to 10 μM, in cell culture, where the growth inhibition is determined 1-10 days after exposure of the tumor cells to the antagonist. The antagonist is growth inhibitory in vivo if administration of antagonist and/or agonist at about 1 μg/kg to about 100 mg/kg body weight results in reduction in tumor size or reduction of tumor cell proliferation within about 5 days to 3 months from the first administration of the antibody or small molecule antagonist, in some embodiments, within about 5 to 30 days.

In some embodiments, to select for hedgehog pathway inhibitors which induce cell death, loss of membrane integrity as indicated by, e.g., propidium iodide (PI), trypan blue or 7AAD uptake may be assessed relative to control. A PI uptake assay can be performed in the absence of complement and immune effector cells. In some embodiments, mutant SMO protein-expressing expressing tumor cells are incubated with medium alone or medium containing the appropriate hedgehog pathway inhibitor. The cells are incubated for a 3 day time period. Following each treatment, cells are washed and aliquoted a into 35 mm strainer-capped 12 x 75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN® flow cytometer and FACSCONVERT® CellQuest software (Becton Dickinson), or any other device used by the skilled worker for analyses. Those hedgehog pathway inhibitors that induce statistically significant levels of cell death as determined by PI uptake may then be selected.

In some embodiments, to screen for hedgehog pathway inhibitors which bind to an epitope on a mutant SMO polypeptide, a routine cross-blocking assay such as that described in Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. This assay can be used to determine if a test antibody, polypeptide, oligopeptide or other organic molecule binds the same site or epitope as a known hedgehog pathway inhibitor. Alternatively, or additionally, epitope mapping can be performed by methods known in the art. For example, the mutant SMO protein sequence can be mutagenized such as by alanine scanning or by making chimerae with immunologically

distinct GPCR proteins, to identify contact residues. The mutant antigen is initially tested for binding with polyclonal antibody to ensure proper folding. In a different method, peptides corresponding to different regions of a mutant SMO protein can be used in competition assays with the test antibodies or with a test antibody and an antibody with a characterized or known epitope.

In some embodiments, the mutant SMO protein or the candidate hedgehog pathway inhibitor agent is immobilized on a solid phase, e.g., on a microliter plate, by covalent or non-covalent attachments. Non-covalent attachment generally is accomplished by coating the solid surface with a solution of the mutant SMO protein or candidate hedgehog signaling agent and drying. Alternatively, an immobilized antibody, e.g., a monoclonal antibody, specific for the target portion of mutant SMO to be immobilized can be used to anchor it to a solid surface. The assay may be performed by adding the non-immobilized component, which may be labeled by a detectable label, to the immobilized component, e.g., the coated surface containing the anchored component. When the reaction is complete, the non-reacted components may be removed, e.g., by washing, and complexes anchored on the solid surface are detected. When the originally non-immobilized component carries a detectable label, the detection of label immobilized on the surface indicates that complexing occurred. Where the originally non-immobilized component does not carry a label, complexing can be detected, for example, by using a labeled antibody specifically binding the immobilized complex.

If the candidate hedgehog pathway inhibitor interacts with but does not bind directly to a hedgehog signaling polypeptide identified herein, its interaction with that polypeptide can be assayed by methods well known for detecting protein-protein interactions. Such assays include traditional approaches, such as, e.g., cross-linking, co-immunoprecipitation, and co-purification through gradients or chromatographic columns. In addition, protein-protein interactions can be monitored by using a yeast-based genetic system described by Fields and co-workers (Fields and Song, Nature (London). 340:245-246 (1989); Chien et al, Proc. Natl. Acad. Sci. USA, 88:9578-9582 (1991)) as disclosed by Chevray and Nathans, Proc. Natl. Acad. Sci. USA. 89: 5789-5793 (1991). Many transcriptional activators, such as yeast GAL4, consist of two physically discrete modular domains, one acting as the DNA-binding domain, the other one functioning as the transcription- activation domain. The yeast expression system described in the foregoing publications (generally referred to as the "two-hybrid system") takes advantage of this property, and employs two hybrid proteins, one in which the target protein is fused to the DNA-binding domain of GAL4, and another, in which candidate activating proteins are fused to the activation domain. The expression of a GAL1-

LacZ reporter gene under control of a GAL4-activated promoter depends on reconstitution of GAL4 activity via protein-protein interaction. Colonies containing interacting polypeptides are detected with a chromogenic substrate for β-galactosidase. A complete kit (MATCHMAKER™) for identifying protein-protein interactions between two specific proteins using the two- hybrid technique is commercially available from Clontech. This system can also be extended to map protein domains involved in specific protein interactions as well as to pinpoint amino acid residues that are crucial for these interactions.

The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays, and cell-based assays, which are well characterized in the art.

Agents that interfere with the interaction of hedgehog signaling polypeptide and other intra- or extracellular components (*e.g.*, Costal-2) can be tested by means well-known by the skilled worker. In some embodiments, a reaction mixture is prepared containing the mutant SMO polypeptide and an intra- or extracellular component under conditions and for a time allowing for the interaction and binding of the two products. In some embodiments, to test the ability of a candidate compound to inhibit binding, the reaction is run in the absence and in the presence of the test compound. In addition, a placebo may be added to a third reaction mixture, to serve as positive control. The binding (complex formation) between the test compound and the intra- or extracellular component present in the mixture is monitored as described hereinabove. The formation of a complex in the control reaction(s) but not in the reaction mixture containing the test agent indicates that the test agent interferes with the interaction of the test compound and its reaction partner.

The disclosure contemplates methods for identifying hedgehog pathway inhibitors using any one or combination of the foregoing assay steps. In other words various screening assays can be combined to identify antagonists having, for example, a particular activity or to confirm that an agent that antagonizes mutant SMO in one assay also inhibits hedgehog signaling in an independent assay. For any assay or method of identification, results may be compared to one or more appropriate controls, including positive and/or negative controls.

For any of the foregoing assay methods for screening and/or identifying hedgehog pathway inhibitors, agents may be screened singly or in pools. Agents may be screened from a library of agents or a set of candidate agents. Suitable agents that may be screened include, but are not limited to, antibodies, antibody fragments, peptides, antisense oligonucleotides, RNAi and small molecules (*e.g.*, a bromodomain inhibitor).

In some embodiments, the cell used in any of the screening methods disclosed herein comprises one or more mutations in a gene that results in an activation or increase hedgehog signaling. In some embodiments, the one or more mutations are in the patched gene resulting in a patched loss of function. In some embodiments, the one or more mutations in the patched gene result in a mutant gene that encodes a patched protein having one or more of the following mutations: S616G, fs251, E380*, Q853*, W926*, P1387S, sp2667, Q501H, fs1017, fs108, or A1380V.

In some embodiments, the one or more mutations in a gene that results in an activation or increase hedgehog signaling are in *smoothened*, and the cell has a smoothened mutation. In some embodiments, the smoothened mutation is a smoothened gain-of-function mutation. In some embodiments, the gain-of-function smoothened mutation results in a constitutively active smoothened protein. *See, e.g.*, WO 2011/028950; WO2012047968 and WO 2015/120075, each of which is incorporated by reference.

In some embodiments, the smoothened protein comprises a mutation at a position corresponding to position 529 of SEQ ID NO: 1. In some embodiments, the mutation is a G529S at position 529 or at that corresponding position in SEQ ID NO: 1. In some embodiments, the smoothened protein comprises a mutation at a position corresponding to position 529 of SEQ ID NO: 1, and at least one additional mutation. In some embodiments, the additional smoothened mutation is a mutation at a position corresponding to position 241 of SEQ ID NO: 1, such as a T241M mutation at position 241 or at a position corresponding to that position of SEQ ID NO: 1. In some embodiments, the additional smoothened mutation is a mutation at a position corresponding to position 281 of SEQ ID NO: 1, such as a W281C mutation at position 281 or at a position corresponding to that position of SEQ ID NO: 1. In some embodiments, the additional smoothened mutation is a mutation at a position corresponding to position 321 of SEQ ID NO: 1, such as a V321M mutation at position 321 or at a position corresponding to that position of SEQ ID NO: 1. In some embodiments, the additional smoothened mutation is a mutation at a position corresponding to position 408 of SEQ ID NO: 1, such as a I408V mutation at position 408 or at a position corresponding to that position of SEQ ID NO: 1. In some embodiments, the additional smoothened mutation is a mutation at a position corresponding to position 412 of SEQ ID NO: 1, such as a L412F mutation at position 412 or at a position corresponding to that position of SEQ ID NO: 1. In some embodiments, the additional smoothened mutation is a mutation at a position corresponding to position 459 of SEQ ID NO: 1, such as a A459V mutation at position 459 or at a position corresponding to that position of SEQ ID NO: 1. In some embodiments, the

additional smoothened mutation is a mutation at a position corresponding to position 469 of SEQ ID NO: 1, such as a C469Y mutation at position 469 or at a position corresponding to that position of SEQ ID NO: 1. In some embodiments, the additional smoothened mutation is a mutation at a position corresponding to position 473 of SEQ ID NO: 1, such as a D473H mutation at position 473 or at a position corresponding to that position of SEQ ID NO: 1. In some embodiments, the additional smoothened mutation is a mutation at a position corresponding to position 518 of SEQ ID NO: 1, such as a E518K or E518A mutation at position 518 or at a position corresponding to that position of SEQ ID NO: 1. In some embodiments, the additional smoothened mutation is a mutation at a position corresponding to position 533 of SEQ ID NO: 1, such as a S533N mutation at position 533 or at a position corresponding to that position of SEQ ID NO: 1. In some embodiments, the additional smoothened mutation is a mutation at a position corresponding to position 535 of SEQ ID NO: 1, such as a W535L mutation at position 535 or at that corresponding position of SEQ ID NO: 1. In some embodiments, the additional smoothened mutation is a mutation at a position corresponding to position 562 of SEQ ID NO: 1, such as a R562Q mutation at position 562 or at a position corresponding to that position of SEQ ID NO: 1. In some embodiments, the smoothened mutation has an alternative mutation that renders it resistant to certain smoothened inhibitors.

In some embodiments, the one or more mutations are in a *hedgehog* gene and result in overexpression of a hedgehog protein. In some embodiments, the overexpressed hedgehog protein is Sonic hedgehog protein. In some embodiments, the overexpressed hedgehog protein is Indian hedgehog protein. In some embodiments, the overexpressed hedgehog protein is Desert hedgehog protein.

In some embodiments, the one or more mutations are in *suppressor-of-fused*, and the cell has suppressor-of-fused (SuFu or SUFU) loss-of-function. In some embodiments, the results in a loss-of-function in SuFu activity. In some embodiments, the SuFu mutation is in a medulloblastoma, meningioma, adenoid cystic carcinoma, basal cell carcinoma and rhabdomyosarcoma cancer cell. In some embodiments, the SuFu mutation is any of the mutations described in Brugieres et al., 2012, JCO, 30(17):2087-2093, which is incorporated herein in its entirety. In some embodiments, the SuFu mutation is any of the mutations described in Tables 1 or 2 or any of the mutations described in Brugieres et al., 2012, JCO, 30(17):2087-2093 , which is incorporated herein in its entirety.

Table 2: Germline SUFU Mutations

Age at Diagnosis of MB	Histologic Subtype	Associated Symptoms	Inheritance of Mutation	Mutation
4 years	Desmoplastic	Developmental delay	NA	Loss of contiguous genes at 10q
		Frontal bossing, hypertelorism		IVS1—1A—>T
NA	Desmoplastic	None	NA	143insA
NA	Desmoplastic	Meningioma in radiation field	NA	
8 months	MBEN	Macrocrania, palmar and plantar pits	Inherited	c.1022 + 1G>A
<1 month	MBEN	None	Inherited	c.72delC
<3 months	MBEN	None	Inherited	c.72delC
<1 months	MBEN	None	Inherited	c.72insC
6-12 months	Desmoplastic/nodular	None	Inherited	c.72insC
<6 months	Desmoplastic/nodular	None	Inherited	c.72insC
12-24 months	MB NOS	None	Inherited	c.72insC
22 months	Desmoplastic/nodular	None	NA	c.846insC
23 months	Desmoplastic/nodular	None	NA	c.1022 + 1G>A

Abbreviations: MB, medulloblastoma; MBEN, MB with extensive nodularity; NA, not available; NOS, not otherwise specified.

Table 3. Germline Pathogenic *SUFU* Mutations

Exon/Intron	Type of Mutation	Nucleotide Change (In SEQ ID NO: 5)	Consequence (In SEQ ID NO: 4)	Tumor Analysis

Intron 1	Splice → frameshift	c.182 + 3A>T	p.Thr55fs	Not available
Exon 2	Frameshift	c.294_295dupCT	p.Tyr99fs	Not available
Intron 2	Splice → frameshift	c.318-10delT	p.Phe107fs	Loss of wild-type allele
Exon 3	Large duplication	c.318-?_454+?dup	p.Glu106-?_Glu152+?dup	UV (c.1022 + 5G>A)
Exon 3	Missense	c.422T>G	p.Met141Arg	Not available
Exon 9	Nonsense	c.1123C>T	p.Gln375X	Not available
Exon 9	Frameshift	c.1149_1150dupCT	p.Cys384fs	Loss of wild-type allele
Intron 10	Splice → frameshift	c.1297-1G>C	p.?	Not available

Abbreviation: UV, unknown variant.

In some embodiments, the SuFu mutation comprises a mutation at a position corresponding to any of the following amino acid positions in SEQ ID NO: 4: position 15, 184, 123, 295, 187. In certain embodiments, the SuFu mutation comprises any one or more of: P15L, Q184X, R123C, L295fs, or P187L, where the mutation is at that position or at the position corresponding to the stated position in SEQ ID NO: 4. In some embodiments, the SuFU mutation is any of the mutations corresponding to c.1022+1G>A (IVS8-1G>T), c.72delC, c.72insC, 143insA, c.846insC, or IVS1-1A->T of SEQ ID NO: 5. In some embodiments, the SuFu mutation is any of the mutations described in Taylor et al (2002) Nat Genet 31:306-310 (*e.g.*, IVS8-1G>T (=c.1022 +1G>A), 1129del, P15L and Ng's two (all

+LOH)); Slade et al (2011) Fam Cancer 10:337-342, 2011 (*e.g.*, c.1022 +1G>A; c.848insC); Pastorino et al (2009) Am J Med Genet A 149A:1539-1543 (*e.g.*, c.1022 +1G>A); Ng et al (2005) Am J Med Genet A 134:399-403 (*e.g.*, 143insA; IVS1-1A>T); Kijima et al (2012) Fam Cancer 11: 565-70 (*e.g.*, c.550C>T (Q184X)); Aavikko et al (2012) Am J Hum Genet 91: 520–526 (*e.g.*, c.367C>T (R123C)); Stephens et al (2013) J Clin Invest 123: 2965-2968 (*e.g.*, x881_882insG (L295fs)); or Reifenberger et al (2005) Brit J Dermatology 152: 43–51 (*e.g.*, c560C>T (P187L)).

In some embodiments, the cell is a human cell and has a chromosome 10 duplication and/or a deletion of a portion of 10q, wherein said portion contains *SUFU* and *PTEN*. In some embodiments, the cell comprises a Fs1017 *SUFU* mutation.

In some embodiments, the cell used in any of the screening methods described herein is a cell in which the hedgehog signaling pathway is active. In some embodiments, the cell is a cell in which the hedgehog signaling pathway is constitutively active. In some embodiments, the cell is a cell that has been stimulated with hedgehog protein or hedgehog agonist. In some embodiments, the activity of the hedgehog pathway in a cell is determined by monitoring Gli1 levels or activity in a Gli-luciferase reporter assay.

In some embodiments, the cell used in any of the screening methods described herein is a cell in culture. In some embodiments, the disclosure provides for a method comprising contacting a culture comprising a plurality of cells. In some embodiments, the cell is in a vertebrate. In some embodiments, the cell is in a mammal, and contacting the cell comprises administering the hedgehog signaling inhibitor to the mammal. In some embodiments, the mammal is a human subject. In some embodiments, the cell is a cancer cell and/or the mammal is a mammal diagnosed with cancer. In some embodiments, the cancer cell is a cancer cell selected from the group consisting of: a colon, lung, prostate, skin, blood, liver, kidney, breast, bladder, bone, brain, medulloblastoma, sarcoma, basal cell carcinoma, gastric, ovarian, esophageal, pancreatic, or testicular cancer cell. In some embodiments, the cancer cell is a medulloblastoma cell, a basal cell carcinoma cell, or a nevoid basal cell cell carcinoma cell (Gorlin syndrome cell).

In certain embodiments, once an agent is identified as a hedgehog pathway inhibitor, the agent can then be formulated and further evaluated in a cell or animal-based assay. For example, the agent can be tested in a cell or animal-based cancer model to evaluate efficacy as an anti-cancer agent.

VI. Methods of Treatment

In some embodiments, the present disclosure relates to methods of modulating a differentiation state, survival, and/or proliferation of a cell expressing a smoothened protein having any of the smoothened mutations described herein. In some embodiments, the cell is in a subject (*e.g.*, a human patient). In some embodiments, the cell is in culture, and the method comprises an *in vitro* method. In certain embodiments, the cell is a cancer cell. In certain embodiments, the cell is characterized by unwanted or abnormal cell proliferation. In some embodiments, the cell comprises or has been predetermined to express a smoothened protein comprising any of the smoothened mutations described herein. In certain embodiments, the cell has been predetermined to express a smoothened polypeptide comprising a mutation, relative to wild type human SMO, at an amino acid corresponding to 529 of SEQ ID NO: 1. In some embodiments, the cell has been predetermined to express a smoothened polypeptide comprising at least two mutations, wherein at least one of the mutations is at an amino acid corresponding to amino acid position 529 of SEQ ID NO: 1, and wherein the polypeptide further comprises a mutation at any one or more of the amino acid positions corresponding to 241, 281, 321, 408, 412, 459, 469, 473, 518, 533 and/or 535 of SEQ ID NO: 1. In some embodiments, the cell expresses a smoothened polypeptide comprising a G529S mutation of SEQ ID NO: 1, and optionally any of the following substitutions: T241M, W281C, V321M, I408V, A459V, C469Y, D473H, E518K, E518A S533N, and/or W535L.

In some embodiments, the disclosure provides for a method of reducing hedgehog signaling in a cell, wherein the cell expresses a smoothened protein having any of the smoothened mutations described herein, wherein the cell is responsive to hedgehog protein or comprises one or more mutations in a hedgehog signaling pathway gene (e.g., a component of the hedgehog signaling pathway), wherein the one or more mutations results in increased hedgehog signaling and/or activation of the hedgehog signaling pathway in the absence of ligand, wherein the method comprises the step of contacting the cell with an effective amount of an agent, wherein the agent is a hedgehog pathway inhibitor. In some embodiments, the agent is a compound that selectively binds and inhibits the mutant smoothened protein. In some embodiments, the agent inhibits a component of the hedgehog signaling pathway that acts downstream of the mutant smoothened protein in the cell. In other embodiments, the agent is a bromodomain inhibitor.

In some embodiments, the disclosure provides for a method of treating a subject having a cancer with an anti-cancer therapeutic agent, wherein said subject comprises and/or has been determined to express a mutant SMO protein, wherein said mutant SMO protein has

an amino acid other than glycine at position corresponding to position 529 of SEQ ID NO: 1. In some embodiments, the disclosure provides for a method of inhibiting hedgehog signaling in a cell, wherein the cell expresses a mutant SMO protein having an amino acid other than glycine at the position corresponding to position 529 of SEQ ID NO: 1. In some embodiments, the disclosure provides for a method of diagnosing a subject having a cancer, comprising the steps of: a) obtaining a sample from the subject, b) testing said sample for the presence of a nucleic acid encoding a mutant SMO protein having an amino acid other than glycine at the position corresponding to position 529 of SEQ ID NO: 1, wherein if said sample comprises said mutant SMO protein, said subject has cancer. In some embodiments, the cancer is a basal cell carcinoma. In some embodiments, the mutant SMO protein has a serine at the amino acid position corresponding to amino acid position 529 of SEQ ID NO: 1. In some embodiments, the cancer comprises a smoothened protein having an additional mutation at at least one amino acid position selected from the group of amino acid positions corresponding to 241, 281, 321, 408, 412, 459, 469, 473, 518, 533 and/or 535 of SEQ ID NO: 1.

In some embodiments, the disclosure provides for a method of inhibiting unwanted growth, proliferation or survival of a cell, wherein the cell expresses a smoothened protein having any of the smoothened mutations described herein, wherein the cell is responsive to hedgehog protein or comprises one or more mutations in a hedgehog signaling pathway gene, wherein the one or more mutations results in increased hedgehog signaling and/or activation of the hedgehog signaling pathway in the absence of ligand, wherein the method comprises the step of contacting the cell with an effective amount of an agent, wherein the agent is a hedgehog pathway inhibitor. In some embodiments, the agent is an agent that selectively binds and inhibits the mutant smoothened protein. In some embodiments, the agent inhibits a component of the hedgehog signaling pathway that acts downstream of the mutant smoothened protein in the cell. In some embodiments, the agent is a bromodomain inhibitor.

In some embodiments, the disclosure provides for a method of inhibiting growth, proliferation or survival of a tumor cell, wherein the tumor cell expresses a smoothened protein having any of the smoothened mutations described herein, wherein the cell is responsive to hedgehog protein or comprises one or more mutations in a hedgehog signaling pathway gene, wherein the one or more mutations results in increased hedgehog signaling and/or activation of the hedgehog signaling pathway in the absence of ligand, wherein the method comprises the step of contacting the cell with an effective amount of an agent, wherein the agent is a hedgehog pathway inhibitor. In some embodiments, the agent is an

agent that selectively binds and inhibits the mutant smoothened protein. In some embodiments, the agent inhibits a component of the hedgehog signaling pathway that acts downstream of the mutant smoothened protein in the cell. In other embodiments, the agent is a bromodomain inhibitor. In some embodiments, the method comprises administering an agent to a patient in need thereof.

In some embodiments, the cell treated with any of the methods disclosed herein comprises one or more mutations in a gene that results in an activation or increase hedgehog signaling. In some embodiments, the one or more mutations are in the patched gene resulting in a patched loss of function. In some embodiments, the one or more mutations in the patched gene result in a mutant gene that encodes a patched protein having one or more of the following mutations: S616G, fs251, E380*, Q853*, W926*, P1387S, sp2667, Q501H, fs1017, fs108, or A1380V.

In some embodiments, the one or more mutations in a gene that results in an activation or increase hedgehog signaling are in *smoothened*, and the cell has a smoothened mutation. In some embodiments, the smoothened mutation is a smoothened gain-of-function mutation. In some embodiments, the gain-of-function smoothened mutation results in a constitutively active smoothened protein. *See, e.g.*, WO 2011/028950, WO2012047968 and WO 2015/120075, each of which is incorporated by reference. In some embodiments, the smoothened mutation is a mutation at the amino acid position corresponding to position 529 of SEQ ID NO: 1, such as a G529S mutation at position 529 or a corresponding position of SEQ ID NO: 1. In some embodiments, the SMO protein comprises an amino acid sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 1, provided that there is a substitution at amino acid position 529, and wherein the protein further comprises at least one additional mutation at any one or more of the amino acid positions corresponding to 241, 281, 321, 408, 412, 459, 469, 473, 518, 533 and/or 535 of SEQ ID NO: 1. In some embodiments, the SMO protein comprises an amino acid sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 1, provided that the amino acid sequence comprises an amino acid other than glycine (G) at the amino acid position corresponding to position 529 of SEQ ID NO: 1, and wherein the amino acid sequence further comprises any one or more of the following substitutions: T241M, W281C, V321M, I408V, A459V, C469Y, D473H, E518K, E518A, S533N, and/or W535L.

In some embodiments, the one or more mutations are in a *hedgehog* gene and result in overexpression of a hedgehog protein. In some embodiments, the overexpressed hedgehog

protein is Sonic hedgehog protein. In some embodiments, the overexpressed hedgehog protein is Indian hedgehog protein. In some embodiments, the overexpressed hedgehog protein is Desert hedgehog protein.

In some embodiments, the one or more mutations are in *suppressor-of-fused*, and the cell has suppressor-of-fused (SuFu or SUFU) loss-of-function. In some embodiments, the results in a loss-of-function in SuFu activity. In some embodiments, the SuFu mutation is in a medulloblastoma, meningioma, adenoid cystic carcinoma, basal cell carcinoma and rhabdomyosarcoma cancer cell. In some embodiments, the SuFu mutation is any of the mutations described in Brugieres et al., 2012, JCO, 30(17):2087-2093, which is incorporated herein in its entirety.

In some embodiments, the SuFu mutation is any of the mutations described in Tables 2 or 3 or any of the mutations described in Brugieres et al., 2012, JCO, 30(17):2087-2093 , which is incorporated herein in its entirety.

Table 2: Germline SUFU Mutations

Age at Diagnosis of MB	**Histologic Subtype**	**Associated Symptoms**	**Inheritance of Mutation**	**Mutation**
4 years	Desmoplastic	Developmental delay	NA	Loss of contiguous genes at 10q
		Frontal bossing, hypertelorism		IVS1—1A—>T
NA	Desmoplastic	None	NA	143insA
NA	Desmoplastic	Meningioma in radiation field	NA	
8 months	MBEN	Macrocrania, palmar and plantar pits	Inherited	c.1022 + 1G>A
<1 month	MBEN	None	Inherited	c.72delC
<3 months	MBEN	None	Inherited	c.72delC
<1 months	MBEN	None	Inherited	c.72insC
6-12 months	Desmoplastic/nodular	None	Inherited	c.72insC

<6 months	Desmoplastic/nodular	None	Inherited	c.72insC
12-24 months	MB NOS	None	Inherited	c.72insC
22 months	Desmoplastic/nodular	None	NA	c.846insC
23 months	Desmoplastic/nodular	None	NA	c.1022 + 1G>A

Abbreviations: MB, medulloblastoma; MBEN, MB with extensive nodularity; NA, not available; NOS, not otherwise specified.

Table 3. Germline Pathogenic *SUFU* Mutations

Exon/Intron	Type of Mutation	Nucleotide Change (In SEQ ID NO: 5)	Consequence (In SEQ ID NO: 4)	Tumor Analysis
Intron 1	Splice → frameshift	c.182 + 3A>T	p.Thr55fs	Not available
Exon 2	Frameshift	c.294_295dupCT	p.Tyr99fs	Not available
Intron 2	Splice → frameshift	c.318-10delT	p.Phe107fs	Loss of wild-type allele
Exon 3	Large duplication	c.318-?_454+?dup	p.Glu106-?_Glu152+?dup	UV (c.1022 + 5G>A)
Exon 3	Missense	c.422T>G	p.Met141Arg	Not available
Exon 9	Nonsense	c.1123C>T	p.Gln375X	Not available
Exon 9	Frameshift	c.1149_1150dupCT	p.Cys384fs	Loss of wild-type allele

Intron 10	Splice → frameshift	c.1297-1G>C	p.?	Not available

Abbreviation: UV, unknown variant.

In some embodiments, the SuFu mutation comprises a mutation at a position corresponding to any of the following amino acid positions in SEQ ID NO: 4: position 15, 184, 123, 295, 187. In certain embodiments, the SuFu mutation comprises any one or more of: P15L, Q184X, R123C, L295fs, or P187L, where the mutation is at that position or at the position corresponding to the stated position in SEQ ID NO: 4. In some embodiments, the SuFU mutation is any of the mutations corresponding to c.1022+1G>A (IVS8-1G>T), c.72delC, c.72insC, 143insA, c.846insC, or IVS1-1A->T of SEQ ID NO: 5. In some embodiments, the SuFu mutation is any of the mutations described in Taylor et al (2002) Nat Genet 31:306-310 (*e.g.*, IVS8-1G>T (=c.1022 +1G>A), 1129del, P15L and Ng's two (all +LOH)); Slade et al (2011) Fam Cancer 10:337-342, 2011 (*e.g.*, c.1022 +1G>A; c.848insC); Pastorino et al (2009) Am J Med Genet A 149A:1539-1543 (*e.g.*, c.1022 +1G>A); Ng et al (2005) Am J Med Genet A 134:399-403 (*e.g.*, 143insA; IVS1-1A>T); Kijima et al (2012) Fam Cancer 11: 565-70 (*e.g.*, c.550C>T (Q184X)); Aavikko et al (2012) Am J Hum Genet 91: 520–526 (*e.g.*, c.367C>T (R123C)); Stephens et al (2013) J Clin Invest 123: 2965-2968 (*e.g.*, x881_882insG (L295fs)); or Reifenberger et al (2005) Brit J Dermatology 152: 43–51 (*e.g.*, c560C>T (P187L)).

In some embodiments, the cell is a human cell and has a chromosome 10 duplication and/or a deletion of a portion of 10q, wherein said portion contains *SUFU* and *PTEN*. In some embodiments, the cell comprises a Fs1017 *SUFU* mutation.

In some embodiments, the cell treated with any of the methods described herein is a cell in which the hedgehog signaling pathway is active. In some embodiments, the cell is a cell in which the hedgehog signaling pathway is constitutively active. In some embodiments, the cell is a cell that has been stimulated with hedgehog protein or hedgehog agonist. In some embodiments, the activity of the hedgehog pathway in a cell is determined by monitoring Gli1 levels or activity in a Gli-luciferase reporter assay.

In some embodiments, the cell treated with any of the methods described herein is a cell in culture. In some embodiments, the disclosure provides for a method comprising contacting a culture comprising a plurality of cells. In some embodiments, the cell is in a

vertebrate. In some embodiments, the cell is in a mammal, and contacting the cell comprises administering the hedgehog signaling inhibitor to the mammal. In some embodiments, the mammal is a human subject. In some embodiments, the cell is a cancer cell and/or the mammal is a mammal diagnosed with cancer. In some embodiments, the cancer cell is a cancer cell selected from the group consisting of: a colon, lung, prostate, skin, blood, liver, kidney, breast, bladder, bone, brain, medulloblastoma, sarcoma, basal cell carcinoma, gastric, ovarian, esophageal, pancreatic, or testicular cancer cell. In some embodiments, the cancer cell is a medulloblastoma cell, a basal cell carcinoma cell, or a nevoid basal cell cell carcinoma cell (Gorlin syndrome cell).

In some embodiments, the hedgehog pathway inhibitor used in any of the methods disclosed herein is a polynucleotide molecule that inhibits the expression of any of the mutant smoothened proteins described herein. In some embodiments, the polynucleotide molecule is an antisense oligonucleotide that specifically hybridizes to a nucleic acid encoding any of the mutant smoothened proteins disclosed herein. In some embodiments, the antisense molecule does not hybridize to a nucleic acid that encodes a wildtype smoothened protein (*e.g.*, a nucleic acid that encodes a protein having the sequence of SEQ ID NO: 1). In some embodiments, the hedgehog pathway inhibitor is a RNAi antagonist that targets the mRNA transcript encoding any of the mutant smoothened polypeptides disclosed hIn some embodiments, the RNAi antagonist is an siRNA. In some embodiments, the siRNA is 19-23 nucleotides in length. In some embodiments, the siRNA is double stranded, and includes short overhang(s) at one or both ends. In some embodiments, the siRNA targets an mRNA transcript encoding any of the mutant smoothened polypeptides disclosed herein. In some embodiments, the RNAi or siRNA does not target an mRNA transcript that encodes a wildtype smoothened protein (*e.g.*, a nucleic acid that encodes a protein having the sequence of SEQ ID NO: 1). In some embodiments, the RNAi comprises an shRNA.

In some embodiments, the hedgehog pathway inhibitor used in any of the methods disclosed herein is a small molecule that specifically binds to any of the mutant smoothened polypeptides described herein. In some embodiments, the small molecule binds to a polypeptide that acts downstream of smoothened in the hedgehog signaling pathway. In some embodiments, the small molecule binds to a polypeptide in a pathway distinct from the hedgehog signaling pathway. In some embodiments, the small molecule is a bromodomain inhibitor. In some embodiments, the bromodomain inhibitor is a BRD4 inhibitor. In some embodiments, the bromodomain inhibitor is any of the bromodomain inhibitors described in Ciceri et al., 2014, Nature Chemical Biology, 10:305-312; Muller et al., 2014, Med Chem

Commun, 5:288-296; Garnier et al., 2014, 24(2):185-199, which are each incorporated herein in their entirety. In some embodiments, the bromodomain inhibitor is I-BET762, JQ1, JQ2, BRD4 by BI-2536 and TG-101348.

In some embodiments, the hedgehog pathway inhibitor used in any of the methods disclosed herein is an antibody that specifically binds to any of the mutant smoothened polypeptides described herein. In some embodiments, the antibody binds to a polypeptide that acts downstream of smoothened in the hedgehog signaling pathway. In some embodiments, the antibody is a monoclonal antibody.

In some embodiments, the cell contacted with an agent according to any of the methods described herein is also contacted with an additional inhibitor of the hedgehog signaling pathway (e.g., a HPI). In some embodiments, the additional inhibitor of the hedgehog signaling pathway is a veratrum-type steroidal alkaloid. In some embodiments, the veratrum-type steroidal alkaloid is cyclopamine, or KAAD-cyclopamine or any functional derivatives thereof (*e.g.*, IPI-269609 or IPI-926). In some embodiments, the veratrum-type steroidal alkaloid is jervine, or any functional derivatives thereof. In some embodiments, the additional inhibitor is vismodegib, sonidegib, BMS-833923, PF-04449913, or LY2940680, or any functional derivatives thereof. In some embodiments the additional inhibitor is a smoothened inhibitor chemically unrelated to veratrum alkaloids or vismodegib, including but not limited to: sonidegib, BMS-833923, PF-04449913, LY2940680, Erivedge, BMS-833923 (XL319), LDE225 (Erismodegib), PF-04449913, NVP-LDE225, NVP-LEQ506, TAK-441, XL-319, LY-2940680, SEN450, Itraconazole, MRT-10, MRT-83, or PF-04449913.). In some embodiments, the additional inhibitor is any of the compounds disclosed in Amakye, et al., Nature Medicine, 19(11):1410-1422 (which is incorporated herein in its entirety). In some embodiments, the additional inhibitor of the hedgehog signaling pathway is an antibody. In some embodiments, the antibody is an antibody that binds, such as specifically binds, hedgehog proteins. In some embodiments, the additional inhibitor of the hedgehog signaling pathway is an RNAi antagonist.

Subjects in need of treatment or diagnosis include those already with aberrant hedgehog signaling as well as those prone to having or those in whom aberrant hedgehog signaling is to be prevented. For example, a subject or mammal is successfully "treated" for aberrant hedgehog signaling if, according to the method of the present disclosure, after receiving a hedgehog pathway inhibitor, the patient shows observable and/or measurable reduction in or absence of one or more of the following: reduction in the number of tumor cells or absence of such cells; reduction in the tumor size; inhibition (i.e., slow to some extent

and, in some embodiments, stop) of tumor cell infiltration into peripheral organs including the spread of cancer into soft tissue and bone; inhibition (i.e., slow to some extent and, in some embodiments, stop) of tumor metastasis; inhibition, to some extent, of tumor growth; and/or relief to some extent, of one or more of the symptoms associated with the specific cancer; reduced morbidity and mortality, and improvement in quality of life issues. To the extent such hedgehog pathway inhibitors may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. Reduction of these signs or symptoms may also be felt by the patient. Additionally, successful exposure to the hedgehog pathway inhibitor (particularly in cases where no tumor response is measurable) can be monitored by Gli1 expression either in skin punch biopsies or hair follicles (as done for vismodegib).

In certain embodiments, the subject treated with any of the hedgehog pathway inhibitors disclosed herein expresses a mutant smoothened protein that is resistant to vismodegib. In some embodiments, the subject expresses a smoothened protein comprising any of the smoothened mutations described herein. In certain embodiments the subject expresses a smoothened polypeptide comprising a mutation at an amino acid corresponding to 529 of SEQ ID NO: 1. In some embodiments the subject expresses a smoothened polypeptide comprising a mutation at an amino acid corresponding to G529S of SEQ ID NO: 1. In some embodiments, the subject expresses a smoothened polypeptide comprising a mutation at an amino acid corresponding to 529 of SEQ ID NO: 1, wherein the polypeptide further comprises at least one additional mutation at any one or more of the amino acid positions corresponding to 241, 281, 321, 408, 412, 459, 469, 473, 518, 533 and/or 535 of SEQ ID NO: 1. In some embodiments, the subject expresses a smoothened polypeptide comprising a G529S mutation of SEQ ID NO: 1, and wherein the polypeptide further comprises any one or more of the following substitutions: T241M, W281C, V321M, I408V, A459V, C469Y, D473H, E518K, E518A, S533N, and/or W535L. In some embodiments, prior to being treated with any of the treatment methods described herein, the subject has been determined to express a smoothened protein comprising any of the smoothened mutations described herein. In certain embodiments, prior to being treated with any of the treatment methods described herein, the subject has been determined to express a smoothened polypeptide comprising a mutation at an amino acid corresponding to 529 of SEQ ID NO: 1. In some embodiments, prior to being treated with any of the treatment methods described herein, the subject has been determined to express a smoothened polypeptide comprising a mutation at an amino acid corresponding to G529S of SEQ ID NO: 1. In some embodiments, prior to being treated with any of the treatment methods described herein, the subject has

been determined to express a smoothened polypeptide comprising a mutation at an amino acid corresponding to 529 of SEQ ID NO: 1, wherein the polypeptide further comprises at least one additional mutation at any one or more of the amino acid positions corresponding to 241, 281, 321, 408, 412, 459, 469, 473, 518, 533 and/or 535 of SEQ ID NO: 1. In some embodiments, prior to being treated with any of the treatment methods described herein, the subject has been determined to express a smoothened polypeptide comprising a G529S mutation of SEQ ID NO: 1, wherein the polypeptide further comprises any one or more of the following substitutions: T241M, W281C, V321M, I408V, A459V, C469Y, D473H, E518K, E518A, S533N, and/or W535L.

The above parameters for assessing successful treatment and improvement in the disease are readily measurable by routine procedures familiar to a physician. For cancer therapy, efficacy can be measured, for example, by assessing the time to disease progression (TTP) and/or determining the response rate (RR). Metastasis can be determined by staging tests and tests for calcium level and other enzymes to determine the extent of metastasis. CT scans can also be done to look for spread to regions outside of the tumor or cancer. The disclosure described herein relating to the process of prognosing, diagnosing and/or treating involves the determination and evaluation of, for example, Gli1 expression.

"Mammal" for purposes of the treatment of, alleviating the symptoms of or diagnosis of a disease (*e.g.*, cancer) refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, ferrets, etc. In some embodiments, the mammal is human. In some embodiments, the mammal is post-natal. In some embodiments, the mammal is pediatric. In some embodiments, the mammal is adult.

Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

In certain embodiments, a hedgehog pathway inhibitor is used in the treatment of a cancer selected from any of the cancers described herein or a cancer in which one or more cells of a tumor comprises a mutation in a hedgehog pathway component, such as any of the mutations described herein. It should be generally appreciated and is specifically noted herein that tumors comprise cells that may have a level of heterogeneity. Accordingly, not all cells in a tumor necessarily comprise a particular deleterious mutation. Accordingly, the disclosure contemplates methods in which a cancer or tumor being treated comprises cells having a mutation in a component of the hedgehog pathway, such as any of the mutations described herein, even if such a mutation is not present in every cell of the tumor.

It is further contemplated that use of hedgehog pathway inhibitors may be specifically targeted to disorders where the affected tissue and/or cells exhibit high hedgehog pathway activation. Expression of Gli genes activated by the hedgehog signaling pathway, including Gli1 and Gli2, most consistently correlate with hedgehog signaling across a wide range or tissues and disorders, while Gli3 is somewhat less so. The *Gli* genes encode transcription factors that activate expression of many genes needed to elicit the full effects of hedgehog signaling. However, the Gli3 transcription factors can also act as a repressor of hedgehog effector genes, and therefore, expression of Gli3 can cause a decreased effect of the hedgehog signaling pathway. Whether Gli3 acts as a transcriptional activator or repressor depends on post-translational events, and therefore it is expected that methods for detecting the activating form (versus the repressing form) of Gli3 protein (such as western blotting) would also be a reliable measure of hedgehog pathway activation. The Gli1 gene is strongly expressed in a wide array of cancers, hyperplasias and immature lungs, and serves as a marker for the relative activation of the hedgehog pathway. In addition, tissues such as immature lung, that have high Gli gene expression, are strongly affected by hedgehog inhibitors. Accordingly, it is contemplated that the detection of Gli gene expression may be used as a powerful predictive tool to identity tissues and disorders that will particularly benefit from treatment with a hedgehog antagonist. In some embodiments, Gli1 expression levels are detected, either by direct detection of the transcript or by detection of protein levels or activity. Transcripts may be detected using any of a wide range of techniques that depend primarily on hybridization or probes to the Gli1 transcripts or to cDNAs synthesized therefrom. Well known techniques include Northern blotting, reverse-transcriptase PCR and microarray analysis of transcript levels. Methods for detecting Gli protein levels include Western blotting, immunoprecipitation, two-dimensional polyacrylamide gel electrophoresis (2D SDS- PAGE – in some embodiments compared against a standard wherein the position of the Gli proteins has been determined), and mass spectroscopy. Mass spectroscopy may be coupled with a series of purification steps to allow high-throughput identification of many different protein levels in a particular sample. Mass spectroscopy and 2D SDS-PAGE can also be used to identify post-transcriptional modifications to proteins including proteolytic events, ubiquitination, phosphorylation, lipid modification, etc. Gli activity may also be assessed by analyzing binding to substrate DNA or in vitro transcriptional activation of target promoters. Gel shift assay, DNA footprinting assays and DNA-protein crosslinking assays are all methods that may be used to assess the presence of a protein capable of binding to GU

binding sites on DNA. J Mol. Med 77(6):459-68 (1999); Cell 100(4): 423-34 (2000); Development 127(19): 4923-4301 (2000).

Because Gli1 is so ubiquitously expressed during hedgehog activation, any degree of Gli1 overexpression should be useful in determining that a hedgehog pathway inhibitor will be an effective therapeutic. In some embodiments, Gli1 should be expressed at a level at least twice as high as in a normal control cell/tissue/subject. In some embodiments, Gli1 expression is four, six, eight or ten times as high as in a normal cell/tissue/subject.

In certain embodiments, Gli1 transcript levels are measured, and diseased or disordered tissues showing abnormally high Gli1 levels are treated with a hedgehog pathway inhibitor. In other embodiments, the condition being treated is known to have a significant correlation with aberrant activation of the hedgehog pathway, even though a measurement of Gli1 expression levels is not made in the tissue being treated. Premature lung tissue, lung cancers (e.g., adeno carcinomas, bronco-alveolar adenocarcinoma, small cell carcinomas), breast cancers (e.g., inferior ductal carcinomas, inferior lobular carcinomas, tubular carcinomas), prostate cancers (e.g., adenocarcinomas), and benign prostatic hyperplasias all show strongly elevated Gli1 expression levels in certain cases. Accordingly, Gli1 expression levels are a powerful diagnostic device to determine which of these tissues should be treated with a Hedgehog pathway inhibitor. In addition, there is substantial correlative evidence that cancers of the urothelial cells (e.g., bladder cancer, other urogenital cancers) will also have elevated gli-1 levels in certain cases. For example, it is known that loss of heterozygosity on chromosome 9q22 is common in bladder cancers. The Ptch1 gene is located at this position and Ptch1 loss of function is probably a partial cause of hyperproliferation, as in many other cancer types. Accordingly, such cancers would also show high Gli1 expression and would be particularly amenable to treatment with a hedgehog antagonist.

In certain embodiments, any of the hedgehog pathway inhibitors described herein are used for treating a subject having a tumor having a ptch-1 and/or ptch-2 mutation, *e.g.*, a patched-1 or patched-2 loss of function mutation. Expression of ptch-1 and ptch-2 is also activated by the hedgehog signaling pathway, but not typically to the same extent as gli genes, and as a result are inferior to the gli genes as markers of hedgehog pathway activation. In certain tissues, only one of ptch-1 or ptch-2 is expressed although the hedgehog pathway is highly active. For example, in testicular development, desert hedgehog plays an important role and the hedgehog pathway is activated, but only ptc-2 is expressed. Accordingly, these genes may be individually unreliable as markers for hedgehog pathway activation, although

simultaneous measurement of both genes is contemplated as a more useful indicator for tissues to be treated with a hedgehog antagonist.

In light of the broad involvement of hedgehog signaling in the formation of ordered spatial arrangements of differentiated tissues in vertebrates, the hedgehog pathway inhibitors of the present disclosure could be used in a process for generating and/or maintaining an array of different vertebrate tissue both in vitro and in vivo. The Hedgehog pathway inhibitor, can be, as appropriate, any of the preparations described above.

In some embodiments, the hedgehog pathway inhibitors can be used as part of a treatment regimen for malignant medulloblastoma and other primary CNS malignant neuroectodermal tumors. Medulloblastoma, a primary brain tumor, is the most common brain tumor in children. A medulloblastoma is a primitive neuroectodermal (PNET) tumor arising in the posterior fossa. They account for approximately 25% of all pediatric brain tumors. Histologically, they are small round cell tumors commonly arranged in a true rosette, but may display some differentiation to astrocytes, ependymal cells or neurons. PNETs may arise in other areas of the brain including the pineal gland (pineoblastoma) and cerebrum. Those arising in the supratentorial region generally have a worsened prognosis.

Medulloblastom/PNETs are known to recur anywhere in the CNS after resection, and can even metastasize to bone. Pretreatment evaluation should therefore include and examination of the spinal cord to exclude the possibility of "dropped metastases". Gadolinium-enhanced MRI has largely replaced myelography for this purpose, and CSF cytology is obtained postoperatively as a routine procedure.

In some embodiments, the hedgehog pathway inhibitors are used as part of a treatment program for ependymomas. Ependymomoas account for approximately 10% of the pediatric brain tumors in children. Grossly, they are tumors that arise from the ependymal lining of the ventricles and microscopically form rosettes, canals, and perivascular rosettes. In the CHOP series of 51 children reported with epenymomas, ¾ were histologically benign, approximately 2/3 arose from the region of the 4th ventricule, and one third presented in the supratentorial region. Age at presentation peaks between birth and 4 years. The median age is about 5 years. Because so many children with this disease are babies, they often require multimodal therapy.

In some embodiments, the hedgehog pathway inhibitors of the present disclosure, based on the involvement of hedgehog signaling in various tumors, or expression of hedgehog or its receptors in such tissues during development, can be used to inhibit growth of a tumor having dysregulated hedgehog activity. Such tumors include, but are not limited to:

tumors related to Gorlin's syndrome (e.g., medulloblastoma, meningioma, etc.), tumors associated with a ptch mutation (e.g., hemangiona, rhabdomyosarcoma, etc.), tumors resulting from Gli1 amplification (e.g., glioblastoma, sarcoma, etc.), tumors resulting from Smo dysfunction (e.g., basal cell carcinoma, etc.), tumors connected with TRC8, a ptc homolog (e.g., renal carcinoma, thyroid carcinoma, etc.), Ext-1 related tumors (e.g., bone cancer, etc.), Sft/x-induced tumors (e.g., lung cancer, chondrosarcomas, etc.), tumors overexpressing a hedgehog protein, and other tumors (e.g., breast cancer, urogenital cancer (e.g.,, kidney, bladder, ureter, prostate, etc.), adrenal cancer, gastrointestinal cancer (e.g., stomach, intestine, etc.).

In some embodiments, the hedgehog pathway inhibitors of the present disclosure may also be used to treat several forms of cancer. These cancers include, but are not limited to: prostate cancer, bladder cancer, lung cancer (including small cell and non-small cell), colon cancer, kidney cancer, liver cancer, breast cancer, cervical cancer, endometrial or other uterine cancer, ovarian cancer, testicular cancer, cancer of the penis, cancer of the vagina, cancer of the urethra, gall bladder cancer, esophageal cancer, or pancreatic cancer. Additional cancer types include cancer of skeletal or smooth muscle, stomach cancer, cancer of the small intestine, cancer of the salivary gland, anal cancer, rectal cancer, thyroid cancer, parathyroid cancer, pituitary cancer, and nasopharyngeal cancer. Further exemplary forms of cancer which can be treated with the hedgehog antagonists of the present disclosure include cancers comprising hedgehog expressing cells. Still further exemplary forms of cancer which can be treated with the hedgehog antagonists of the present disclosure include cancers comprising Gli expressing cells. In one embodiment, the cancer is not characterized by a mutation in patched-1. In some embodiments, the cancer is characterized by a smoothened and/or SuFu mutation.

In certain embodiments, the hedgehog pathway inhibitors may be used to treat a subject having basal cell carcinoma. In particular embodiments, the basal cell carcinoma is nevoid basal cell carcinoma. In particular embodiments, the subject has Gorlin's Syndrome.

The foregoing are merely exemplary of *in vitro* and *in vivo* uses for hedgehog pathway inhibitors of the disclosure. Hedgehog pathway inhibitors are also suitable for use in identifying natural targets or binding partners for mutant smoothened proteins (*e.g.*, a smoothened protein having a G529S mutation, alone or in combination with any one or more of T241M, W281C, V321M, I408V, A459V, C469Y, D473H, E518K, E518A S533N, and/or W535L mutations), to study mutant smoothened bioactivity, to purify mutant smoothened

and its binding partners from various cells and tissues, and to identify additional components of the hedgehog signaling pathway.

In certain embodiments, the hedgehog pathway inhibitor is any of the antibodies disclosed. An antibody of the disclosure may be used in, for example, *in vitro*, *ex vivo*, and *in vivo* therapeutic methods. In one aspect, the disclosure provides methods for treating cancer, inhibiting unwanted cellular proliferation, inhibiting metastasis of cancer and inducing apoptosis of tumor cells either *in vivo* or *in vitro*, the method comprising exposing a cell to an antibody of the disclosure under conditions permissive for binding of the antibody to mutant SMO. In certain embodiments, the cell is a myelogenous leukemia cell, a lung cancer cell, a gastric cancer cell, a breast cancer cell, a prostate cancer cell, a renal cell cancer cell, and a glioblastoma cell. In one embodiment, an antibody of the disclosure can be used for inhibiting an activity of mutant SMO, the method comprising exposing mutant SMO to an antibody of the disclosure such that the activity of mutant SMO is inhibited.

In one aspect, the disclosure provides methods for treating cancer comprising administering to an individual an effective amount of an antibody of the disclosure. In certain embodiments, a method for treating cancer comprises administering to an individual an effective amount of a pharmaceutical formulation comprising an antibody of the disclosure and, optionally, at least one additional therapeutic agent, such as those provided below.

Antibodies of the disclosure can be used either alone or in combination with other compositions in a therapy. For instance, an antibody of the disclosure may be co-administered with at least one additional therapeutic agent and/or adjuvant. In certain embodiments, an additional therapeutic agent is an anti-VEGF antibody.

Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate formulations), and separate administration, in which case, administration of the antibody of the disclosure can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant. Antibodies of the disclosure can also be used in combination with radiation therapy.

In one embodiment, an antibody of the disclosure is used in a method for binding mutant SMO in an individual suffering from a disorder associated with increased mutant SMO expression and/or activity, the method comprising administering to the individual the antibody such that mutant SMO in the individual is bound. In one embodiment, the mutant SMO is human mutant SMO, and the individual is human.

An antibody of the disclosure (and any additional therapeutic agent or adjuvant) can be administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the antibody is suitably administered by pulse infusion, particularly with declining doses of the antibody. Dosing can be by any suitable route, *e.g.* by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

The location of the binding target of an antibody of the disclosure may be taken into consideration in preparation and administration of the antibody. When the binding target is an intracellular molecule, certain embodiments of the disclosure provide for the antibody or antigen-binding fragment thereof to be introduced into the cell where the binding target is located. In one embodiment, an antibody of the disclosure can be expressed intracellularly as an intrabody. The term "intrabody," as used herein, refers to an antibody or antigen-binding portion thereof that is expressed intracellularly and that is capable of selectively binding to a target molecule, as described, *e.g.*, in Marasco, *Gene Therapy* 4: 11-15 (1997); Kontermann, *Methods* 34: 163-170 (2004); U.S. Patent Nos. 6,004,940 and 6,329,173; U.S. Patent Application Publication No. 2003/0104402, and PCT Publication No. WO2003/077945. See also, for example, WO96/07321 published March 14, 1996, concerning the use of gene therapy to generate intracellular antibodies.

Intracellular expression of an intrabody may be effected by introducing a nucleic acid encoding the desired antibody or antigen-binding portion thereof (lacking the wild-type leader sequence and secretory signals normally associated with the gene encoding that antibody or antigen-binding fragment) into a target cell. One or more nucleic acids encoding all or a portion of an antibody of the disclosure can be delivered to a target cell, such that one or more intrabodies are expressed which are capable of binding to an intracellular target polypeptide and modulating the activity of the target polypeptide. Any standard method of introducing nucleic acids into a cell may be used, including, but not limited to, microinjection, ballistic injection, electroporation, calcium phosphate precipitation, liposomes, and transfection with retroviral, adenoviral, adeno-associated viral and vaccinia vectors carrying the nucleic acid into a cell.

In certain embodiments, nucleic acid (optionally contained in a vector) may be introduced into a patient's cells by *in vivo* and *ex vivo* methods. In one example of *in vivo* delivery, nucleic acid is injected directly into the patient, *e.g.*, at the site where therapeutic

intervention is required. In a further example of *in vivo* delivery, nucleic acid is introduced into a cell using transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Chol, for example). For review of certain gene marking and gene therapy protocols, see Anderson *et al.*, *Science* 256:808-813 (1992), and WO 93/25673 and the references cited therein. In an example of *ex vivo* treatment, a patient's cells are removed, nucleic acid is introduced into those isolated cells, and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are implanted into the patient (see, *e.g.*, U.S. Patent Nos. 4,892,538 and 5,283,187). A commonly used vector for *ex vivo* delivery of a nucleic acid is a retroviral vector.

In another embodiment, internalizing antibodies are provided. Antibodies can possess certain characteristics that enhance delivery of antibodies into cells, or can be modified to possess such characteristics. Techniques for achieving this are known in the art. For example, cationization of an antibody is known to facilitate its uptake into cells (see, *e.g.*, U.S. Patent No. 6,703,019). Lipofections or liposomes can also be used to deliver the antibody into cells. Where antibody fragments are used, the smallest inhibitory fragment that specifically binds to the target protein may be advantageous. For example, based upon the variable-region sequences of an antibody, peptide molecules can be designed that retain the ability to bind the target protein sequence. Such peptides can be synthesized chemically and/or produced by recombinant DNA technology. See, *e.g.*, Marasco *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 7889-7893 (1993).

Entry of antibodies into target cells can be enhanced by other methods known in the art. For example, certain sequences, such as those derived from HIV Tat or the Antennapedia homeodomain protein are able to direct efficient uptake of heterologous proteins across cell membranes. See, *e.g.*, Chen *et al.*, *Proc. Natl. Acad. Sci. USA* (1999), 96:4325-4329.

When the binding target of an antibody is located in the brain, certain embodiments of the disclosure provide for the antibody to traverse the blood-brain barrier. Several art-known approaches exist for transporting molecules across the blood-brain barrier, including, but not limited to, physical methods, lipid-based methods, stem cell-based methods, and receptor and channel-based methods.

Physical methods of transporting an antibody across the blood-brain barrier include, but are not limited to, circumventing the blood-brain barrier entirely, or by creating openings in the blood-brain barrier. Circumvention methods include, but are not limited to, direct

injection into the brain (see, *e.g.*, Papanastassiou *et al.*, *Gene Therapy* 9: 398-406 (2002)), interstitial infusion/convection-enhanced delivery (see, *e.g.*, Bobo *et al.*, *Proc. Natl. Acad. Sci. USA* 91: 2076-2080 (1994)), and implanting a delivery device in the brain (see, *e.g.*, Gill *et al.*, *Nature Med.* 9: 589-595 (2003); and Gliadel Wafers™, Guildford Pharmaceutical).

Methods of creating openings in the barrier include, but are not limited to, ultrasound (see, *e.g.*, U.S. Patent Publication No. 2002/0038086), osmotic pressure (*e.g.*, by administration of hypertonic mannitol (Neuwelt, E. A., *Implication of the Blood-Brain Barrier and its Manipulation*, Vols 1 & 2, Plenum Press, N.Y. (1989)), permeabilization by, *e.g.*, bradykinin or permeabilizer A-7 (see, *e.g.*, U.S. Patent Nos. 5,112,596, 5,268,164, 5,506,206, and 5,686,416), and transfection of neurons that straddle the blood-brain barrier with vectors containing genes encoding the antibody (see, *e.g.*, U.S. Patent Publication No. 2003/0083299).

Lipid-based methods of transporting an antibody across the blood-brain barrier include, but are not limited to, encapsulating the antibody in liposomes that are coupled to antibody binding fragments that bind to receptors on the vascular endothelium of the blood-brain barrier (see, *e.g.*, U.S. Patent Application Publication No. 20020025313), and coating the antibody in low-density lipoprotein particles (see, *e.g.*, U.S. Patent Application Publication No. 20040204354) or apolipoprotein E (see, *e.g.*, U.S. Patent Application Publication No. 20040131692).

Stem-cell based methods of transporting an antibody across the blood-brain barrier entail genetically engineering neural progenitor cells (NPCs) to express the antibody of interest and then implanting the stem cells into the brain of the individual to be treated. *See* Behrstock *et al.* (2005) *Gene Ther.* 15 Dec. 2005 advanced online publication (reporting that NPCs genetically engineered to express the neurotrophic factor GDNF reduced symptoms of Parkinson disease when implanted into the brains of rodent and primate models).

Receptor and channel-based methods of transporting an antibody across the blood-brain barrier include, but are not limited to, using glucocorticoid blockers to increase permeability of the blood-brain barrier (see, *e.g.*, U.S. Patent Application Publication Nos. 2002/0065259, 2003/0162695, and 2005/0124533); activating potassium channels (see, *e.g.*, U.S. Patent Application Publication No. 2005/0089473), inhibiting ABC drug transporters (see, *e.g.*, U.S. Patent Application Publication No. 2003/0073713); coating antibodies with a transferrin and modulating activity of the one or more transferrin receptors (see, *e.g.*, U.S. Patent Application Publication No. 2003/0129186), and cationizing the antibodies (see, *e.g.*, U.S. Patent No. 5,004,697).

Antibodies of the disclosure would be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The antibody need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibody present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as described herein, or about from 1 to 99% of the dosages described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

For the prevention or treatment of disease, the appropriate dosage of an antibody of the disclosure (when used alone or in combination with one or more other additional therapeutic agents) will depend on the type of disease to be treated, the type of antibody, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (*e.g.* 0.1mg/kg-10mg/kg) of antibody can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the antibody would be in the range from about 0.05 mg/kg to about 10 mg/kg. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 4.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, *e.g.* every week or every three weeks (*e.g.* such that the patient receives from about two to about twenty, or *e.g.* about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. An exemplary dosing regimen comprises administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the antibody. However, other

dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

It is understood that any of the above therapeutic methods may be carried out using an immunoconjugate of the disclosure in place of or in addition to an anti-mutant SMO antibody.

5

VII. Pharmaceutical Formulations

In some embodiments, any of the hedgehog pathway inhibitors described herein or hedgehog pathway inhibitors in accordance with the disclosure may be formulated in a pharmaceutical composition.

10 Pharmaceutical compositions of the hedgehog pathway inhibitors used in accordance with the present disclosure may be prepared for storage by mixing the agent(s) having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington: The Science of Practice of Pharmacy. 20th edition, Gennaro, A. et al., Ed., Philadelphia College of Pharmacy and Science (2000)), in the form of lyophilized 15 formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as acetate, Tris, phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or 20 benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates 25 including glucose, mannose, or dextrins; chelating agents such as EDTA; tonicifiers such as trehalose and sodium chloride; sugars such as sucrose, mannitol, trehalose or sorbitol; surfactant such as polysorbate; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN®, PLURONICS® or polyethylene glycol (PEG).

30 In some embodiments, any of the formulations of hedgehog pathway inhibitors in accordance with the present disclosure and/or described herein may also contain more than one active compound as necessary for the particular indication being treated, in some embodiments, those with complementary activities that do not adversely affect each other. It should be recognized that, in certain embodiments, a hedgehog pathway inhibitor and a

second active agent are formulated together (e.g., a formulation or composition contains both agents). In other embodiments, the two (or more) active agents are formulated separately such that the separate formulations can be marketed, sold, stored, and used together or separately. When formulated separately, the disclosure contemplates that they can be administered at the same or differing times and, in certain embodiments, may be combined and administered simultaneously.

For example, in addition to the preceding therapeutic agent(s), it may be desirable to include in the formulation, an additional antibody, e.g., a second such therapeutic agent, or an antibody to some other target (e.g., a growth factor that affects the growth of a tumor). In some embodiments, it may be desirable to include in the formulation a hedgehog inhibitor (*e.g.*, robotkinin). Alternatively, or additionally, the composition may further comprise a chemotherapeutic agent, cytotoxic agent, cytokine, growth inhibitory agent, anti- hormonal agent, and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended. In some embodiments, the additional active compound is a steroidal alkaloid. In some embodiments, the steroidal alkaloid is cyclopamine, or KAAD-cyclopamine or jervine or any functional derivative thereof (*e.g.*, IPI-269609 or IPI-926). In some embodiments, the additional active compound is vismodegib, sonidegib, BMS-833923, PF-04449913, or LY2940680 or any derivative thereof. In some embodiments, the additional active compound is any of the compounds disclosed in Amakye, et al., Nature Medicine, 19(11):1410-1422 (whichi is incorporated herein in its entirety). In some embodiments the additional active compound is another smoothened inhibitor chemically unrelated to veratrum alkaloids or vismodegib, including but not limited to: Erivedge, BMS-833923 (XL319), LDE225 (Erismodegib), PF-04449913, NVP-LDE225, NVP-LEQ506, TAK-441, XL-319, LY-2940680, SEN450, Itraconazole, MRT-10, MRT-83, or PF-04449913). As noted above, the disclosure contemplates formulations in which a second active agent is formulated together with a hedgehog pathway inhibitor (e.g., as a single formulation comprising two active agents), as well as embodiments in which the two active agents are present in two separate formulations or compositions.

In some embodiments, any of the hedgehog pathway inhibitors of the disclosure, such as those described herein, may also be entrapped in microcapsules 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. Such techniques are disclosed in Remington: The Science and Practice of Pharmacy, supra.

In some embodiments, any of the hedgehog pathway inhibitors of the disclosure are formulated in sustained-release preparations. Suitable examples of sustained- release preparations include semi-permeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ 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), and poly-D(-)-3-hydroxybutyric acid.

The amount of the compositions of the disclosure for use in the methods of the present disclosure can be determined by standard clinical techniques and may vary depending on the particular indication or use. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

In certain embodiments, compositions of the disclosure, including pharmaceutical preparations, are non-pyrogenic. In other words, in certain embodiments, the compositions are substantially pyrogen free. In one embodiment the formulations of the disclosure are pyrogen-free formulations that are substantially free of endotoxins and/or related pyrogenic substances. Endotoxins include toxins that are confined inside a microorganism and are released only when the microorganisms are broken down or die. Pyrogenic substances also include fever-inducing, thermostable substances (glycoproteins) from the outer membrane of bacteria and other microorganisms. Both of these substances can cause fever, hypotension and shock if administered to humans. Due to the potential harmful effects, even low amounts of endotoxins must be removed from intravenously administered pharmaceutical drug solutions. The Food & Drug Administration ("FDA") has set an upper limit of 5 endotoxin units (EU) per dose per kilogram body weight in a single one hour period for intravenous drug applications (The United States Pharmacopeial Convention, Pharmacopeial Forum 26 (1):223 (2000)). When therapeutic proteins are administered in relatively large dosages and/or over an extended period of time (e.g., such as for the patient's entire life), even small amounts of harmful and dangerous endotoxin could be dangerous. In certain specific embodiments, the endotoxin and pyrogen levels in the composition are less then 10 EU/mg,

or less then 5 EU/mg, or less then 1 EU/mg, or less then 0.1 EU/mg, or less then 0.01 EU/mg, or less then 0.001 EU/mg.

In some embodiments, the hedgehog pathway inhibitors are formulated in sterile formulations. This is readily accomplished by filtration through sterile filtration membranes.

IX. Articles of Manufacture and Kits

In some embodiments, the hedgehog pathway inhibitors of the present disclosure, such as the hedgehog pathway inhibitors disclosed herein are prepared in an article of manufacture. Similarly, polypeptides and nucleic acids of the disclosure, such as mutant SMO polypeptides, may be prepared as an article of manufacture. In some embodiments, the article of manufacture comprises a container and a label or package insert on or associated with the container indicating a use for the inhibition in whole or in part of hedgehog signaling, or alternatively for the treatment of a disorder or condition resulting from activation of the hedgehog signaling pathway. In other embodiments, the article of manufacture comprises a container and a label or package insert on or associated with the container indicating a use in a screening assay. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. In some embodiments, the container holds a composition which is effective for treating the cancer condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is a hedgehog pathway inhibitor. The label or package insert will further comprise instructions for administering the hedgehog pathway inhibitor or for use the SMO polypeptide or nucleic acid or vector or host cell. Additionally, the article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

In some embodiments, kits are provided that are useful for various other purposes, e.g., for mutant SMO protein-expressing cell killing assays, for purification or immunoprecipitation of hedgehog signaling polypeptide from cells. For isolation and purification of mutant SMO protein, the kit can contain the respective mutant SMO protein-binding reagent coupled to beads (e.g., sepharose beads). Kits can be provided which contain such molecules for detection and quantitation of mutant SMO protein in vitro, e.g., in an

ELISA or a Western blot. In some embodiments, as with the article of manufacture, the kit comprises a container and a label or package insert on or associated with the container. In some embodiments, the container holds a composition comprising at least one such hedgehog pathway inhibitor reagent useable with the disclosure. In some embodiments, additional containers may be included that contain, e.g., diluents and buffers, control antibodies. In some embodiments, the label or package insert may provide a description of the composition as well as instructions for the intended in vitro or diagnostic use.

EXAMPLES

The disclosure now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present disclosure, and are not intended to limit the disclosure.

Example 1: ***Mutational analysis of vismodegib-resistant Basal Cell Carcinomas.***

Clinical responses to targeted therapies (e.g., cancer therapies) can be short-lived due to the acquisition of genetic alterations that confer drug resistance. Identification of resistance mechanisms will guide novel therapeutic strategies. Inappropriate Hh signaling is linked to several cancers, including basal cell carcinoma (BCC). Loss-of-function mutations in PTCH (~90%) and activating mutations in SMO (~10%) are the primary drivers in BCC. Clinical mechanisms of resistance to vismodegib (GDC-0449) were identified by assessing vismodegibsensitivity and mutation status of BCCs from patients using the FoundationOne™ next-generation sequencing (NGS) platform. Figure 1 lists characteristics of the mBCC (metastatic basal cell carcinoma) patients treated with vismodegib.

As shown in Figure 2, median exon coverage for each tumor biopsy specimen ranged from 460- to 921-fold coverage. The rate of somatic mutation in the BCCs ranged from 3.99-63.89, which is higher relative to that observed in other cancers (Lawrence et al., 2013). Analysis of the somatic mutation spectrum revealed a predominance of C to T nucleotide transition mutations, indicative of UV light-induced mutagenesis (Miller, J. Mol. Bio. 1985).

As shown in Figure 3, mutations in MYCN (P44L/F, P60L, P237L), LRP1B, PTCH1, SMO and TERT (promoter) were the most commonly detected mutational variants observed. No mutant alleles for SUFU or GLI1 were observed. However several genes known to be found in patent samples with acquired resistance to vismodegib (PRKACA, GLI2, and GLI3 (Sharpe et al., Cancer Cell 2015), are not contained in the FoundationOne™ panel.

Example 2: ***SMO Variant analysis identifies novel G529S mutation***

Mutations in SMO were observed in 5 of the 7 post-progression specimens (from 4 of 5 patients) that were collected. SMO mutations that have been described to confer resistance to vismodegib (V321M and T241M; Sharpe et al., Cancer Cell 2015) were observed in 3/4 samples that contained a SMO mutation (Figure 4).

One novel SMO mutation, G529S, was identified in a post progression biopsy. The G529 amino acid is a highly conserved residue located outside of the drug binding pocket (DBP) in the 7th transmembrane domain (TM7) of SMO, suggesting that this residue is functionally relevant (Figure 5). Based on computational modeling, G529 is spatially adjacent to residues that, when mutated, are known to be oncogenic or confer resistance to vismodegib (Figure 6). Without wishing to be bound by theory, these mutations may disrupt helix packing, leading to increased conformational flexibility of SMO, and thereby reduce the affinity for antagonists (Sharpe et al, Cancer Cell 2015). Consistent with this hypothesis, *in vitro* experiments demonstrated that the SMO G529S mutant had increased basal activity and reduced sensitivity to vismodegib (Figure 7).

Patient 002 appeared to acquire two mutations in SMO in residues known to confer resistance to vismodegib (T241M, V321M; Figure 4). These mutations appeared to have been acquired during disease progression, as these mutations were not detected in the pre-treatment biopsy. These observations confirm that SMO mutations are the most common somatic genetic alterations responsible for vismodegib resistance in patients with metastatic BCC.

As discussed above, the progression biopsy sample from patient 002 contained the T241M and V321M mutations that were present at similar allele frequencies, while the progression biopsy from patient 011 contained mutations in G529S and V321M at different allele frequencies (Figure 4). In the cases where two separate biopsies were collected contemporaneously at progression (patient/sample IDs 011-P-i, 011-P-ii, 005-P-i, and 005-P-ii), there was discordance in the detection of SMO mutations as well as the respective allele frequencies in the time matched, paired samples. For example, in patient 011, the V321M mutation was detected in only one of the paired biopsies and, further, the allele frequency of the V321M mutation varied (11% and 39%) between the contemporaneously collected progression specimens. Without wishing to be bound by theory, these observations are consistent with the outgrowth of two distinct resistant subclones and supports the notion of genetic heterogeneity in drug resistance.

Materials and Methods for Examples 1 and 2

Patient treatment histories

008: 72-year-old female patient with metastatic basal cell carcinoma (mBCC). No prior surgery for BCC was reported. Prior systemic therapy for metastatic BCC included the following agent: anthracycline chemotherapy. Sites of metastases at the time of screening included soft tissue in the right hemi pelvis. Measurable lesions were identified on the skin/soft tissue next to the right OS ilium, next to the right femur and ventral surface of the OS sacrum. Non-measurable lesions were identified on the bone destruction region in OS ilium and OS sacrum. The patient received her first dose of 150 mg vismodegib Study Day 1. The patient received a total of 20 cycles and was on SD during this time. On Study Day 673, an overall response assessment showed disease progression and study treatment with vismodegib was discontinued on Study Day 763. Based on the confirmed last dose of study drug, administration of vismodegib ended on Study Day 763 due to disease progression.

001: 77 year old male patient with metastatic basal cell carcinoma (mBCC). Prior surgery for BCC included 6 skin neoplasm excisions. No prior topical or systemic therapy for BCC was reported. Sites of metastases at the time of screening included skin of head. Measurable lesions were identified on skin of head (lymph nodes [besides trachea and infracarinal]). The patient received his first dose of 150 mg vismodegib on Study Day 1. The patient presented a PR on target lesions on Study day 397 and was in PR until cycle 19. On Study Day 516, an overall response assessment revealed disease progression. Based on confirmed last dose of study drug, administration of vismodegib ended on Study Day 532 due to progression of disease.

002: 55-year-old female patient with metastatic basal cell carcinoma (mBCC). Prior surgery for BCC included skin neoplasm excision. No prior topical or systemic therapy for BCC was reported. Sites of metastases at the time of screening (24-Jan-2012) included bone. Measurable lesions were identified on lung (S 5 right side and S 10 left side), os sacrum, vertebral 9 rib, right femur, occipital, and lymph nodes (mediastenum, retrocaval). The patient received her first dose of 150 mg vismodegib on Study Day 1. The patient presented with a PR on cycle 7 on Study day 172. The patient continue in PR until cycle 15. On Study Day 399, an overall response assessment showed disease progression. Based on confirmed last dose of study drug, administration of vismodegib ended onStudy Day 533 due to disease progression.

011: 52 year old male patient with metastatic basal cell carcinoma (mBCC). No prior surgery for BCC was reported. No prior topical or systemic therapy for BCC was reported.

Sites of metastases at the time of screening included bone and lung. Measurable lesion was identified on the skin of trunk. The patient received his first dose of 150 mg vismodegib on Study Day 1. The patient was on stable disease during his treatment (cycle 11) on Study day 282. In a new evaluation on Study Day 310, an overall response assessment revealed progressive disease. Vismodegib was permanently discontinued at this date due to disease progression, with the last dose administered on the same day Study Day 310.

005: 65-year-old female patient with metastatic basal cell carcinoma (mBCC). Prior surgery for BCC included skin neoplasm excision. Prior radiation directed to head and neck (total dose: 50 Gy). No prior topical or systemic therapy for BCC was reported. Sites of metastasis at the time of screening included neck, sternum and left clavicle. Measurable lesions were identified on neck (supraclavicular region), lung (segment 10 and 3). Non-measurable lesion was identified on sternocleidomastoid muscle and bone (left clavicle and sternum). The patient received her first dose of 150 mg vismodegib on Study Day 1. During the treatment, the patient was on SD until cycle 13. On Study Day 336, an overall response assessment showed progressive disease with new lesions in lungs (S10 and S3 region) and sites of locally advanced disease included skin of neck (sternum and left clavicle). Based on confirmed last dose of study drug, administration of vismodegib ended on Study Day 309.

Genomic profiling

FoundationOne™ genomic profiling was conducted as per the service provider's protocol (Foundation Medicine, Cambridge, MA).

GLI-Luciferase reporter assay

C3H10T½ cells (ATCC) were seeded into six-well plates at 1.75 x 10E5 cells/well in DMEM High Glucose with 4mM glutamine, 10 mM Hepes pH 7.2 and 10% FBS. After 16 hours, cells were transfected with 400 ng of expression construct, 400 ng of 9x-Gli-BS and 200 ng of pRL-TK per well using GeneJuice Transfection Reagent (Novagen). Six hours later, cells from one well were trypsinized and redistributed over four wells of a 12-well plate. After 16 hours the FBS content of the culture medium was reduced to 0.5% to induce formation of primary cilia, and small molecule Hh inhibitors were added at indicated concentrations. Firefly luciferase activity was determined 24 hours later with the Dual-Glo Luciferase Assay System (Promega) and read using a Wallac EnVision plate reader (Perkin Elmer). Values were divided by renilla luciferase activities to normalize for transfection efficiency. Individual experiments were carried out in duplicate or triplicate and repeated at least once. Dose response data were fit to a 4-parameter equation in GraphPad Prism:

$$Y = 1 + \frac{1-E}{\left(1+10^{((\log IC_{50}-X)H)}\right)},$$

where "Y" is normalized, *Gli*-luciferase signal or normalized thymidine incorporation calculated as a fraction of control that did not include inhibitor, and "X" is the inhibitor concentration. The top values were constrained to be equal for each sample. "H" is the Hill Slope.

Cited References

- Brinkhuizen, T., Reinders, M.G., van Geel, M., Hendriksen, A.J., Paulussen, A.D., Winnepenninckx, V.J., Keymeulen, K.B., Soetekouw, P.M., van Steensel, M.A., and Mosterd, K. (2014). Acquired resistance to the Hedgehog pathway inhibitor vismodegib due to smoothened mutations in treatment of locally advanced basal cell carcinoma. J. Am. Acad. Dermatol. 71, 1005.
- Horn S, Figl A, Rachakonda PS, Fischer C, Sucker A, Gast A, Kadel S, Moll I, Nagore E, Hemminki K, Schadendorf D, Kumar R. (2013). TERT promoter mutations in familial and sporadic melanoma. Science 339, 959.
- Miller, J.H. (1985). Mutagenic specificity of ultraviolet light. J. Mol. Biol. 182, 45–65
- Priel, S., Cortelazzi, B., Dal Col, V., Marson, D., Laurini, E., Fermeglia, M., Licitra, L., Pilotti, S., Bossi, P., and Perrone, F. (2014). Smoothened (SMO) receptor mutations dictate resistance to vismodegib in basal cell carcinoma. Mol. Oncol. 9, 389.
- Sharpe HJ, Pau G, Dijkgraaf GJ, Basset-Seguin N, Modrusan Z, Januario T1, Tsui V, Durham AB, Dlugosz AA, Haverty PM, Bourgon R, Tang JY, Sarin KY, Dirix L, Fisher DC, Rudin CM, Sofen H, Migden MR, Yauch RL, de Sauvage FJ. (2015). Genomic analysis of smoothened inhibitor resistance in basal cell carcinoma. Cancer Cell 27, 327.
- Vinagre J, Almeida A, Pópulo H, Batista R, Lyra J, Pinto V, Coelho R, Celestino R, Prazeres H, Lima L, Melo M, da Rocha AG, Preto A, Castro P, Castro L, Pardal F, Lopes JM, Santos LL, Reis RM, Cameselle-Teijeiro J, Sobrinho-Simões M, Lima J,

Máximo V, Soares P. (2013). Frequency of TERT promoter mutations in human cancers. Nat Commun. 4, 2185.

- Wang, C., Wu, H., Katritch, V., Han, G.W., Huang, X.P., Liu, W., Siu, F.Y., Roth, B.L., Cherezov, V., and Stevens, R.C. (2013). Structure of the human smooth- ened receptor bound to an antitumour agent. Nature 497, 338.
- Amakye, D., Jagani, Z., and Dorsch, M. (2013). Unraveling the therapeutic potential of the Hedgehog pathway in cancer. Nature medicine *19*, 1410-1422.
- Atwood, S. X., Li, M., Lee, A., Tang, J. Y., and Oro, A. E. (2013). GLI activation by atypical protein kinase C iota/lambda regulates the growth of basal cell carcinomas. Nature *494*, 484-488.
- Brastianos, P. K., Horowitz, P. M., Santagata, S., Jones, R. T., McKenna, A., Getz, G., Ligon, K. L., Palescandolo, E., Van Hummelen, P., Ducar, M. D., *et al.* (2013). Genomic sequencing of meningiomas identifies oncogenic SMO and AKT1 mutations. Nature genetics *45*, 285-289.
- Brinkhuizen, T., Reinders, M. G., van Geel, M., Hendriksen, A. J., Paulussen, A. D., Winnepenninckx, V. J., Keymeulen, K. B., Soetekouw, P. M., van Steensel, M. A., and Mosterd, K. (2014). Acquired resistance to the Hedgehog pathway inhibitor vismodegib due to smoothened mutations in treatment of locally advanced basal cell carcinoma. Journal of the American Academy of Dermatology.
- Buonamici, S., Williams, J., Morrissey, M., Wang, A., Guo, R., Vattay, A., Hsiao, K., Yuan, J., Green, J., Ospina, B., *et al.* (2010). Interfering with resistance to smoothened antagonists by inhibition of the PI3K pathway in medulloblastoma. Sci Transl Med *2*, 51ra70.
- Chang, A. L., and Oro, A. E. (2012). Initial assessment of tumor regrowth after vismodegib in advanced Basal cell carcinoma. Archives of dermatology *148*, 1324-1325.
- Clark, V. E., Erson-Omay, E. Z., Serin, A., Yin, J., Cotney, J., Ozduman, K., Avsar, T., Li, J., Murray, P. B., Henegariu, O., *et al.* (2013). Genomic analysis of non-NF2 meningiomas reveals mutations in TRAF7, KLF4, AKT1, and SMO. Science *339*, 1077- 1080.
- Das Thakur, M., and Stuart, D. D. (2013). The evolution of melanoma resistance reveals therapeutic opportunities. Cancer research *73*, 6106-6110.

- Dijkgraaf, G. J., Alicke, B., Weinmann, L., Januario, T., West, K., Modrusan, Z., Burdick, D., Goldsmith, R., Robarge, K., Sutherlin, D., *et al.* (2011). Small molecule inhibition of GDC-0449 refractory smoothened mutants and downstream mechanisms of drug resistance. Cancer research *71*, 435-444.
- Gether, U., Ballesteros, J. A., Seifert, R., Sanders-Bush, E., Weinstein, H., and Kobilka, B. K. (1997). Structural instability of a constitutively active G protein-coupled receptor. Agonist-independent activation due to conformational flexibility. The Journal of biological chemistry *272*, 2587-2590.
- Gonzalez-Perez, A., and Lopez-Bigas, N. (2011). Improving the assessment of the outcome of nonsynonymous SNVs with a consensus deleteriousness score, Condel. American journal of human genetics *88*, 440-449.
- Greenman, C. D., Bignell, G., Butler, A., Edkins, S., Hinton, J., Beare, D., Swamy, S., Santarius, T., Chen, L., Widaa, S., *et al.* (2010). PICNIC: an algorithm to predict absolute allelic copy number variation with microarray cancer data. Biostatistics *11*, 164-175.
- Hahn, H., Wicking, C., Zaphiropoulous, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A. B., Gillies, S., *et al.* (1996). Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. Cell *85*, 841-851.
- Inukai, M., Toyooka, S., Ito, S., Asano, H., Ichihara, S., Soh, J., Suehisa, H., Ouchida, M., Aoe, K., Aoe, M., *et al.* (2006). Presence of epidermal growth factor receptor gene T790M mutation as a minor clone in non-small cell lung cancer. Cancer research *66*, 7854-7858.
- Jayaraman, S. S., Rayhan, D. J., Hazany, S., and Kolodney, M. S. (2014). Mutational landscape of basal cell carcinomas by whole-exome sequencing. The Journal of investigative dermatology *134*, 213-220.
- Johnson, R. L., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W., Bonifas, J. M., Quinn, A. G., Myers, R. M., Cox, D. R., Epstein, E. H., Jr., and Scott, M. P. (1996). Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science *272*, 1668-1671.
- Kandoth, C., McLellan, M. D., Vandin, F., Ye, K., Niu, B., Lu, C., Xie, M., Zhang, Q., McMichael, J. F., Wyczalkowski, M. A., *et al.* (2013). Mutational landscape and significance across 12 major cancer types. Nature *502*, 333-339.

- Katritch, V., Cherezov, V., and Stevens, R. C. (2013). Structure-function of the G protein-coupled receptor superfamily. Annual review of pharmacology and toxicology *53*, 531-556.
- Kijima, C., Miyashita, T., Suzuki, M., Oka, H., and Fujii, K. (2012). Two cases of nevoid basal cell carcinoma syndrome associated with meningioma caused by a PTCH1 or SUFU germline mutation. Fam Cancer *11*, 565-570.
- Kool, M., Jones, D. T., Jager, N., Northcott, P. A., Pugh, T J., Hovestadt, V., Piro, R. M., Esparza, L. A., Markant, S. L., Remke, M., *et al.* (2014). Genome Sequencing of SHH Medulloblastoma Predicts Genotype-Related Response to Smoothened Inhibition. Cancer cell *25*, 393-405.
- Lackner, M. R., Wilson, T. R., and Settleman, J. (2012). Mechanisms of acquired resistance to targeted cancer therapies. Future Oncol *8*, 999-1014.
- Lee, Y., Kawagoe, R., Sasai, K., Li, Y., Russell, H. R., Curran, T., and McKinnon, P. J. (2007). Loss of suppressor-of-fused function promotes tumorigenesis. Oncogene *26*, 6442-6447.
- Long, J., Li, B., Rodriguez-Blanco, J., Pastori, C., Volmar, C. H., Wahlestedt, C., Capobianco, A., Bai, F., Pei, X. H., Ayad, N. G., and Robbins, D. J. (2014). The BET bromodomain inhibitor I-BET151 acts downstream of Smoothened to abrogate the growth of Hedgehog driven cancers. The Journal of biological chemistry.
- LoRusso, P. M., Rudin, C. M., Reddy, J. C., Tibes, R., Weiss, G. J., Borad, M. J., Hann, C. L., Brahmer, J. R., Chang, I., Darbonne, W. C., *et al.* (2011). Phase I trial of hedgehog pathway inhibitor vismodegib (GDC-0449) in patients with refractory, locally advanced or metastatic solid tumors. Clinical cancer research : an official journal of the American Association for Cancer Research *17*, 2502-2511.
- Metcalfe, C., Alicke, B., Crow, A., Lamoureux, M., Dijkgraaf, G. J., Peale, F., Gould, S. E., and de Sauvage, F. J. (2013). PTEN loss mitigates the response of medulloblastoma to Hedgehog pathway inhibition. Cancer research *73*, 7034-7042.
- Miller, J. H. (1985). Mutagenic specificity of ultraviolet light. J Mol Biol *182*, 45-65.
- Nedelcu, D., Liu, J., Xu, Y., Jao, C., and Salic, A. (2013). Oxysterol binding to the extracellular domain of Smoothened in Hedgehog signaling. Nature chemical biology *9*, 557-564.
- Negrini, S., Gorgoulis, V. G., and Halazonetis, T. D. (2010). Genomic instability—an evolving hallmark of cancer. Nature reviews Molecular cell biology *11*, 220-228.

- Nik-Zainal, S., Van Loo, P., Wedge, D. C., Alexandrov, L. B., Greenman, C. D., Lau, K. W., Raine, K., Jones, D., Marshall, J., Ramakrishna, M., *et al.* (2012). The life history of 21 breast cancers. Cell *149*, 994-1007.
- Oro, A. E., Higgins, K. M., Hu, Z., Bonifas, J. M., Epstein, E. H., Jr., and Scott, M. P. (1997). Basal cell carcinomas in mice overexpressing sonic hedgehog. Science *276*, 817-821.
- Pastorino, L., Ghiorzo, P., Nasti, S., Battistuzzi, L., Cusano, R., Marzocchi, C., Garre, M. L., Clementi, M., and Scarra, G. B. (2009). Identification of a SUFU germline mutation in a family with Gorlin syndrome. Am J Med Genet A *149A*, 1539-1543.
- Pesz, K. A., Bieniek, A., Makowska, I., and Sasiadek, M. M. (2013). Basal cell carcinoma of the skin: whole genome screening by comparative genome hybridization revisited. Journal of cutaneous pathology *40*, 25-29.
- Pricl, S., Cortelazzi, B., Dal Col, V., Marson, D., Laurini, E., Fermeglia, M., Licitra, L.,
- Pilotti, S., Bossi, P., and Perrone, F. (2014). Smoothened (SMO) receptor mutations dictate resistance to vismodegib in basal cell carcinoma. Molecular oncology.
- Reifenberger, J., Wolter, M., Knobbe, C. B., Kohler, B., Schonicke, A., Scharwachter, C., Kumar, K., Blaschke, B., Ruzicka, T., and Reifenberger, G. (2005). Somatic mutations in the PTCH, SMOH, SUFUH and TP53 genes in sporadic basal cell carcinomas. Br J Dermatol *152*, 43-51.
- Reifenberger, J., Wolter, M., Weber, R. G., Megahed, M., Ruzicka, T., Lichter, P., and Reifenberger, G. (1998). Missense mutations in SMOH in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. Cancer research *58*, 1798-1803.
- Sasai, K., Romer, J. T., Lee, Y., Finkelstein, D., Fuller, C., McKinnon, P. J., and Curran, T. (2006). Shh pathway activity is down-regulated in cultured medulloblastoma cells: implications for preclinical studies. Cancer research *66*, 4215-4222.
- Sekulic, A., Migden, M. R., Oro, A. E., Dirix, L., Lewis, K. D., Hainsworth, J. D., Solomon, J. A., Yoo, S., Arron, S. T., Friedlander, P. A., *et al.* (2012). Efficacy and safety of vismodegib in advanced basal-cell carcinoma. The New England journal of medicine *366*, 2171-2179.

- Smith, M. J., Beetz, C., Williams, S. G., Bhaskar, S. S., O'Sullivan, J., Anderson, B., Daly, S. B., Urquhart, J. E., Bholah, Z., Oudit, D., *et al.* (2014). Germline Mutations in SUFU Cause Gorlin Syndrome-Associated Childhood Medulloblastoma and Redefine the Risk Associated With PTCH1 Mutations. Journal of clinical oncology : official journal of the American Society of Clinical Oncology.
- Stjernqvist, S., Ryden, T., and Greenman, C. D. (2011). Model-integrated estimation of normal tissue contamination for cancer SNP allelic copy number data. Cancer informatics *10*, 159-173.
- Stone, D. M., Murone, M., Luoh, S., Ye, W., Armanini, M. P., Gurney, A., Phillips, H., Brush, J., Goddard, A., de Sauvage, F. J., and Rosenthal, A. (1999). Characterization of the human suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli. Journal of cell science *112 (Pt 23)*, 4437-4448.
- Svard, J., Heby-Henricson, K., Persson-Lek, M., Rozell, B., Lauth, M., Bergstrom, A., Ericson, J., Toftgard, R., and Teglund, S. (2006). Genetic elimination of Suppressor of fused reveals an essential repressor function in the mammalian Hedgehog signaling pathway. Developmental cell *10*, 187-197.
- Sweeney, R. T., McClary, A. C., Myers, B. R., Biscocho, J., Neahring, L., Kwei, K. A., Qu, K., Gong, X., Ng, T., Jones, C. D., *et al.* (2014). Identification of recurrent SMO and BRAF mutations in ameloblastomas. Nature genetics *46*, 722-725.
- Tang, Y., Gholamin, S., Schubert, S., Willardson, M. I., Lee, A., Bandopadhayay, P., Bergthold, G., Masoud, S., Nguyen, B., Vue, N., *et al.* (2014). Epigenetic targeting of Hedgehog pathway transcriptional output through BET bromodomain inhibition. Nature medicine *20*, 732-740.
- Taylor, M. D., Liu, L., Raffel, C., Hui, C. C., Mainprize, T. G., Zhang, X., Agatep, R., Chiappa, S., Gao, L., Lowrance, A., *et al.* (2002). Mutations in SUFU predispose to medulloblastoma. Nature genetics *31*, 306-310.
- Wang, C., Wu, H., Katritch, V., Han, G. W., Huang, X. P., Liu, W., Siu, F. Y., Roth, B. L., Cherezov, V., and Stevens, R. C. (2013). Structure of the human smoothened receptor bound to an antitumour agent. Nature *497*, 338-343.
- Wang, G. Y., So, P. L., Wang, L., Libove, E., Wang, J., and Epstein, E. H., Jr. (2011). Establishment of murine basal cell carcinoma allografts: a potential model for preclinical drug testing and for molecular analysis. The Journal of investigative dermatology *131*, 2298-2305.

- Wechsler-Reya, R. J., and Scott, M. P. (1999). Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. Neuron *22*, 103-114.
- Wong, H., Alicke, B., West, K. A., Pacheco, P., La, H., Januario, T., Yauch, R. L., de Sauvage, F. J., and Gould, S. E. (2011). Pharmacokinetic-pharmacodynamic analysis of vismodegib in preclinical models of mutational and ligand-dependent Hedgehog pathway activation. Clinical cancer research : an official journal of the American Association for Cancer Research *17*, 4682-4692.
- Xie, J., Murone, M., Luoh, S. M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J. M., Lam, C. W., Hynes, M., Goddard, A., *et al.* (1998). Activating Smoothened mutations in sporadic basal-cell carcinoma. Nature *391*, 90-92.
- Yauch, R. L., Dijkgraaf, G. J., Alicke, B., Januario, T., Ahn, C. P., Holcomb, T., Pujara, K., Stinson, J., Callahan, C. A., Tang, T., *et al.* (2009). Smoothened mutation confers resistance to a Hedgehog pathway inhibitor in medulloblastoma. Science *326*, 572-574.
- Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. Genome biology *11*, R106.
- DePristo, M. A., Banks, E., Poplin, R., Garimella,K.V.,Maguire,J.R.,Hartl, C., Philippakis, A. A., del Angel, G., Rivas, M. A., Hanna, M., *et al.* (2011). A framework for variation discovery and genotyping using next---generation DNA sequencing data. Nature genetics *43*, 491---498.
- Diskin, S. J., Li, M., Hou, C., Yang, S., Glessner, J., Hakonarson, H.,Bucan, M., Maris, J. M., and Wang, K. (2008). Adjustment of genomic waves in signal intensities from whole---genome SNP genotyping platforms. Nucleic acids research *36*, e126.
- Filippakopoulos,P.,Qi,J.,Picaud,S.,Shen,Y.,Smith,W.B.,Fedorov,O.,Morse,E.M.,Keates,T.,Hickman,T.T.,Felletar,*et.al.*(2010). Selective Inhibition.of BET bromodomains. Nature *468*, 1067---1073.
- Forbes, S. A., Bindal, N., Bamford, S., Cole, C., Kok, C. Y., Beare, D., Jia, M., Shepherd, R., Leung, K., Menzies, A., *et al.* (2011). COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. Nucleic acids research *39*, D945---950.
- Forbes, S. A., Tang, G., Bindal, N., Bamford, S., Dawson, E., Cole, C., Kok, Y., Jia, M., Ewing, R., Menzies, A., *et al.* (2010). COSMIC (the Catalogue of Somatic

Mutations in Cancer): a resource to investigate acquired mutations in human cancer. Nucleic acids research *38*, D652-657.

- Gonzalez---Perez, A., and Lopez---Bigas, N. (2011). Improving the assessment of the outcome of nonsynonymous SNVs with a consensus deleteriousness score, Condel. American journal of human genetics *88*, 440-449.
- Harfe, B. D., Scherz, P. J., Nissim, S., Tian, H., McMahon, A. P., and Tabin, C. J. (2004). Evidence for an expansion---based temporal Shh gradient in specifying vertebrate digit identities. Cell *118*, 517-528.
- Jonkers, J., Meuwissen, R., van der Gulden, H., Peterse, H., van der Valk, M., and Berns, A. (2001). Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nature genetics *29*, 418-425
- Kasper, M., Jaks, V., Are, A., Bergstrom, A., Schwager, A., Svard, J., Teglund S., Barker, N. and Toftgard, R. (2011). Wounding enhances epidermal tumorigenesis by recruiting hair follicle keratinocytes. Proceedings of the National Academy of Sciences of the United States of America *108*, 4099-4104.
- Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H.A., Gu, H., Ng, L. L., Palmiter, R. D., Hawrylycz, M. J., Jones, A. R., *et al.* (2010). Nature neuroscience *13*, 133-140.
- Morgan, M., et al. (2009). Bioinformatics *25*, 2607-2608.
- Murone, M. et al,. (1999). Current biology: CB *9*, 76-84.
- Rudin, C et al., (2012). Nature genetics *44*, 1111-1116.
- Sherry, S et al., (2001). Nucleic acids research *29*, 308-311.
- Tibshirani, R., and Wang, P. (2008). Biostatistics *9*, 18-29.
- Venkatraman, E. et al., (2007). Bioinformatics *23*, 657-663.
- Wu, T. et al., (2010). Bioinformatics *26*, 873-881.

INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

While specific embodiments of the subject disclosure have been discussed, the above specification is illustrative and not restrictive. Many variations of the disclosure will become

apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the disclosure should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations. The foregoing Examples are for illustrative purposes only and should not be construed to limit the scope of the disclosure which is defined by the appended claims.

SEQUENCE LISTING

SEQ ID NO: 1 -- Human wildtype smoothened amino acid sequence (GenBank Accesion No. NP_005622.1)

Met Ala Ala Ala Arg Pro Ala Arg Gly Pro Glu Leu Pro Leu Leu Gly Leu Leu Leu Leu Leu
Leu Leu Gly Asp Pro Gly Arg Gly Ala Ala Ser Ser Gly Asn Ala Thr Gly Pro Gly Pro Arg
Ser Ala Gly Gly Ser Ala Arg Arg Ser Ala Ala Val Thr Gly Pro Pro Pro Pro Leu Ser His Cys
Gly Arg Ala Ala Pro Cys Glu Pro Leu Arg Tyr Asn Val Cys Leu Gly Ser Val Leu Pro Tyr
Gly Ala Thr Ser Thr Leu Leu Ala Gly Asp Ser Asp Ser Gln Glu Glu Ala His Gly Lys Leu
Val Leu Trp Ser Gly Leu Arg Asn Ala Pro Arg Cys Trp Ala Val Ile Gln Pro Leu Leu Cys Ala
Val Tyr Met Pro Lys Cys Glu Asn Asp Arg Val Glu Leu Pro Ser Arg Thr Leu Cys Gln Ala
Thr Arg Gly Pro Cys Ala Ile Val Glu Arg Glu Arg Gly Trp Pro Asp Phe Leu Arg Cys Thr
Pro Asp Arg Phe Pro Glu Gly Cys Thr Asn Glu Val Gln Asn Ile Lys Phe Asn Ser Ser Gly
Gln Cys Glu Val Pro Leu Val Arg Thr Asp Asn Pro Lys Ser Trp Tyr Glu Asp Val Glu Gly
Cys Gly Ile Gln Cys Gln Asn Pro Leu Phe Thr Glu Ala Glu His Gln Asp Met His Ser Tyr Ile
Ala Ala Phe Gly Ala Val Thr Gly Leu Cys Thr Leu Phe Thr Leu Ala Thr Phe Val Ala Asp
Trp Arg Asn Ser Asn Arg Tyr Pro Ala Val Ile Leu Phe Tyr Val Asn Ala Cys Phe Phe Val
Gly Ser Ile Gly Trp Leu Ala Gln Phe Met Asp Gly Ala Arg Arg Glu Ile Val Cys Arg Ala Asp
Gly Thr Met Arg Leu Gly Glu Pro Thr Ser Asn Glu Thr Leu Ser Cys Val Ile Ile Phe Val Ile
Val Tyr Tyr Ala Leu Met Ala Gly Val Val Trp Phe Val Val Leu Thr Tyr Ala Trp His Thr Ser
Phe Lys Ala Leu Gly Thr Thr Tyr Gln Pro Leu Ser Gly Lys Thr Ser Tyr Phe His Leu Leu Thr
Trp Ser Leu Pro Phe Val Leu Thr Val Ala Ile Leu Ala Val Ala Gln Val Asp Gly Asp Ser Val
Ser Gly Ile Cys Phe Val Gly Tyr Lys Asn Tyr Arg Tyr Arg Ala Gly Phe Val Leu Ala Pro Ile
Gly Leu Val Leu Ile Val Gly Gly Tyr Phe Leu Ile Arg Gly Val Met Thr Leu Phe Ser Ile Lys
Ser Asn His Pro Gly Leu Leu Ser Glu Lys Ala Ala Ser Lys Ile Asn Glu Thr Met Leu Arg Leu
Gly Ile Phe Gly Phe Leu Ala Phe Gly Phe Val Leu Ile Thr Phe Ser Cys His Phe Tyr Asp Phe
Phe Asn Gln Ala Glu Trp Glu Arg Ser Phe Arg Asp Tyr Val Leu Cys Gln Ala Asn Val Thr
Ile Gly Leu Pro Thr Lys Gln Pro Ile Pro Asp Cys Glu Ile Lys Asn Arg Pro Ser Leu Leu Val
Glu Lys Ile Asn Leu Phe Ala Met Phe Gly Thr Gly Ile Ala Met Ser Thr Trp Val Trp Thr Lys
Ala Thr Leu Leu Ile Trp Arg Arg Thr Trp Cys Arg Leu Thr Gly Gln Ser Asp Asp Glu Pro
Lys Arg Ile Lys Lys Ser Lys Met Ile Ala Lys Ala Phe Ser Lys Arg His Glu Leu Leu Gln Asn
Pro Gly Gln Glu Leu Ser Phe Ser Met His Thr Val Ser His Asp Gly Pro Val Ala Gly Leu Ala
Phe Asp Leu Asn Glu Pro Ser Ala Asp Val Ser Ser Ala Trp Ala Gln His Val Thr Lys Met Val
Ala Arg Arg Gly Ala Ile Leu Pro Gln Asp Ile Ser Val Thr Pro Val Ala Thr Pro Val Pro Pro
Glu Glu Gln Ala Asn Leu Trp Leu Val Glu Ala Glu Ile Ser Pro Glu Leu Gln Lys Arg Leu

Gly Arg Lys Lys Lys Arg Arg Lys Arg Lys Lys Glu Val Cys Pro Leu Ala Pro Pro Pro Glu Leu His Pro Pro Ala Pro Ala Pro Ser Thr Ile Pro Arg Leu Pro Gln Leu Pro Arg Gln Lys Cys Leu Val Ala Ala Gly Ala Trp Gly Ala Gly Asp Ser Cys Arg Gln Gly Ala Trp Thr Leu Val Ser Asn Pro Phe Cys Pro Glu Pro Ser Pro Pro Gln Asp Pro Phe Leu Pro Ser Ala Pro Ala Pro Val Ala Trp Ala His Gly Arg Arg Gln Gly Leu Gly Pro Ile His Ser Arg Thr Asn Leu Met Asp Thr Glu Leu Met Asp Ala Asp Ser Asp Phe

SEQ ID NO: 2 -- Human smoothened amino acid sequence having mutation at amino acid position 529 of SMO

Met Ala Ala Ala Arg Pro Ala Arg Gly Pro Glu Leu Pro Leu Leu Gly Leu Leu Leu Leu Leu Leu Leu Gly Asp Pro Gly Arg Gly Ala Ala Ser Ser Gly Asn Ala Thr Gly Pro Gly Pro Arg Ser Ala Gly Gly Ser Ala Arg Arg Ser Ala Ala Val Thr Gly Pro Pro Pro Pro Leu Ser His Cys Gly Arg Ala Ala Pro Cys Glu Pro Leu Arg Tyr Asn Val Cys Leu Gly Ser Val Leu Pro Tyr Gly Ala Thr Ser Thr Leu Leu Ala Gly Asp Ser Asp Ser Gln Glu Glu Ala His Gly Lys Leu Val Leu Trp Ser Gly Leu Arg Asn Ala Pro Arg Cys Trp Ala Val Ile Gln Pro Leu Leu Cys Ala Val Tyr Met Pro Lys Cys Glu Asn Asp Arg Val Glu Leu Pro Ser Arg Thr Leu Cys Gln Ala Thr Arg Gly Pro Cys Ala Ile Val Glu Arg Glu Arg Gly Trp Pro Asp Phe Leu Arg Cys Thr Pro Asp Arg Phe Pro Glu Gly Cys Thr Asn Glu Val Gln Asn Ile Lys Phe Asn Ser Ser Gly Gln Cys Glu Val Pro Leu Val Arg Thr Asp Asn Pro Lys Ser Trp Tyr Glu Asp Val Glu Gly Cys Gly Ile Gln Cys Gln Asn Pro Leu Phe Thr Glu Ala Glu His Gln Asp Met His Ser Tyr Ile Ala Ala Phe Gly Ala Val Thr Gly Leu Cys Thr Leu Phe Thr Leu Ala Thr Phe Val Ala Asp Trp Arg Asn Ser Asn Arg Tyr Pro Ala Val Ile Leu Phe Tyr Val Asn Ala Cys Phe Phe Val Gly Ser Ile Gly Trp Leu Ala Gln Phe Met Asp Gly Ala Arg Arg Glu Ile Val Cys Arg Ala Asp Gly Thr Met Arg Leu Gly Glu Pro Thr Ser Asn Glu Thr Leu Ser Cys Val Ile Ile Phe Val Ile Val Tyr Tyr Ala Leu Met Ala Gly Val Val Trp Phe Val Val Leu Thr Tyr Ala Trp His Thr Ser Phe Lys Ala Leu Gly Thr Thr Tyr Gln Pro Leu Ser Gly Lys Thr Ser Tyr Phe His Leu Leu Thr Trp Ser Leu Pro Phe Val Leu Thr Val Ala Ile Leu Ala Val Ala Gln Val Asp Gly Asp Ser Val Ser Gly Ile Cys Phe Val Gly Tyr Lys Asn Tyr Arg Tyr Arg Ala Gly Phe Val Leu Ala Pro Ile Gly Leu Val Leu Ile Val Gly Gly Tyr Phe Leu Ile Arg Gly Val Met Thr Leu Phe Ser Ile Lys Ser Asn His Pro Gly Leu Leu Ser Glu Lys Ala Ala Ser Lys Ile Asn Glu Thr Met Leu Arg Leu Gly Ile Phe Gly Phe Leu Ala Phe Gly Phe Val Leu Ile Thr Phe Ser Cys His Phe Tyr Asp Phe Phe Asn Gln Ala Glu Trp Glu Arg Ser Phe Arg Asp Tyr Val Leu Cys Gln Ala Asn Val Thr Ile Gly Leu Pro Thr Lys Gln Pro Ile Pro Asp Cys Glu Ile Lys Asn Arg Pro Ser Leu Leu Val Glu Lys Ile Asn Leu Phe Ala Met Phe Gly Thr Xaa Ile Ala Met Ser Thr Trp Val Trp Thr Lys

Ala Thr Leu Leu Ile Trp Arg Arg Thr Trp Cys Arg Leu Thr Gly Gln Ser Asp Asp Glu Pro Lys Arg Ile Lys Lys Ser Lys Met Ile Ala Lys Ala Phe Ser Lys Arg His Glu Leu Leu Gln Asn Pro Gly Gln Glu Leu Ser Phe Ser Met His Thr Val Ser His Asp Gly Pro Val Ala Gly Leu Ala Phe Asp Leu Asn Glu Pro Ser Ala Asp Val Ser Ser Ala Trp Ala Gln His Val Thr Lys Met Val Ala Arg Arg Gly Ala Ile Leu Pro Gln Asp Ile Ser Val Thr Pro Val Ala Thr Pro Val Pro Pro Glu Glu Gln Ala Asn Leu Trp Leu Val Glu Ala Glu Ile Ser Pro Glu Leu Gln Lys Arg Leu Gly Arg Lys Lys Lys Arg Arg Lys Arg Lys Lys Glu Val Cys Pro Leu Ala Pro Pro Pro Glu Leu His Pro Pro Ala Pro Ala Pro Ser Thr Ile Pro Arg Leu Pro Gln Leu Pro Arg Gln Lys Cys Leu Val Ala Ala Gly Ala Trp Gly Ala Gly Asp Ser Cys Arg Gln Gly Ala Trp Thr Leu Val Ser Asn Pro Phe Cys Pro Glu Pro Ser Pro Pro Gln Asp Pro Phe Leu Pro Ser Ala Pro Ala Pro Val Ala Trp Ala His Gly Arg Arg Gln Gly Leu Gly Pro Ile His Ser Arg Thr Asn Leu Met Asp Thr Glu Leu Met Asp Ala Asp Ser Asp Phe

SEQ ID NO: 3(WT SMO) atggccgctg cccgcccagc gcgggggccg gagctcccgc tcctggggct gctgctgctg ctgctgctgg gggacccggg ccgggggggcg gcctcgagcg ggaacgcgac cgggcctggg cctcggagcg cgggcgggag cgcgaggagg agcgcggcgg tgactggccc tccgccgccg ctgagccact gcggccgggc tgcccctgc gagccgctgc gctacaacgt gtgcctgggc tcggtgctgc cctacggggc cacctccaca ctgctggccg gagactcgga ctcccaggag gaagcgcacg gcaagctcgt gctctggtcg ggcctccgga atgccccccg ctgctgggca gtgatccagc ccctgctgtg tgccgtatac atgcccaagt gtgagaatga ccgggtggag ctgcccagcc gtaccctctg ccaggccacc cgaggcccct gtgccatcgt ggagagggag cggggctggc ctgacttcct gcgctgcact cctgaccgct tccctgaagg ctgcacgaat gaggtgcaga acatcaagtt caacagttca ggccagtgcg aagtgccctt ggttcggaca gacaacccca agagctggta cgaggacgtg gagggctgcg gcatccagtg ccagaacccg ctcttcacag aggctgagca ccaggacatg cacagctaca tcgcggcctt cggggccgtc acgggcctct gcacgctctt cacctggcc acattcgtgg ctgactggcg gaactcgaat cgctaccctg ctgttattct cttctacgtc aatgcgtgct tctttgtggg cagcattggc tggctggccc agttcatgga tggtgcccgc cgagagatcg tctgccgtgc agatggcacc atgaggcttg gggagcccac ctccaatgag actctgtcct gcgtcatcat ctttgtcatc gtgtactacg ccctgatggc tggtgtggtt tggtttgtgg tcctcaccta tgcctggcac acttccttca aagccctggg caccacctac cagcctctct cgggcaagac ctcctacttc cacctgctca cctggtcact ccccttgtc ctcactgtgg caatccttgc tgtggcgcag gtggatgggg actctgtgag tggcatttgt tttgtgggct acaagaacta ccgataccgt gcgggcttcg tgctggcccc aatcggcctg gtgctcatcg tgggaggcta cttcctcatc cgaggagtca tgactctgtt ctccatcaag agcaaccacc ccgggctgct gagtgagaag gctgccagca agatcaacga gaccatgctg cgcctgggca tttttggctt cctggccttt ggctttgtgc tcattacctt cagctgccac ttctacgact tcttcaacca ggctgagtgg gagcgcagct tccgggacta tgtgctatgt caggccaatg tgaccatcgg gctgcccacc aagcagccca tccctgactg tgagatcaag aatcgcccga gccttctggt ggagaagatc aacctgtttg ccatgtttgg aactggcatc gccatgagca cctgggtctg gaccaaggcc acgctgctca

tctggaggcg tacctggtgc aggttgactg ggcagagtga cgatgagcca aagcggatca agaagagcaa gatgattgcc aaggccttct ctaagcggca cgagctcctg cagaacccag gccaggagct gtccttcagc atgcacactg tgtcccacga cgggcccgtg gcgggcttgg cctttgacct caatgagccc tcagctgatg tctcctctgc ctgggcccag catgtcacca agatggtggc tcggagagga gccatactgc cccaggatat ttctgtcacc cctgtggcaa ctccagtgcc cccagaggaa caagccaacc tgtggctggt tgaggcagag atctccccag agctgcagaa gcgcctgggc cggaagaaga agaggaggaa gaggaagaag gaggtgtgcc cgctggcgcc gccccctgag cttcaccccc ctgcccctgc ccccagtacc attcctcgac tgcctcagct gccccggcag aaatgcctgg tggctgcagg tgcctgggga gctggggact cttgccgaca gggagcgtgg accctggtct ccaacccatt ctgcccagag cccagtcccc ctcaggatcc atttctgccc agtgcaccgg cccccgtggc atgggctcat ggccgccgac agggcctggg gcctattcac tcccgcacca acctgatgga cacagaactc atggatgcag actcggactt ctga

SEQ ID NO: 4- human Suppressor of Fused (SuFu) amino acid sequence (GenBank Accesion No. NM_016169.2)

MAELRPSGAPGPTAPPAPGPTAPPAFASLFPPGLHAIYGECRRLYPDQPNPLQV TAIVKYWLGGPDPLDYVSMYRNVGSPSANIPEHWHYISFGLSDLYGDNRVHEFTGT DGPSGFGFELTFRLKRETGESAPPTWPAELMQGLARYVFQSENTFCSGDHVSWHSPL DNSESRIQHMLLTEDPQMQPVQTPFGVVTFLQIVGVCTEELHSAQQWNGQGILELLR TVPIAGGPWLITDMRRGETIFEIDPHLQERVDKGIETDGSNLSGVSAKCAWDDLSRPP EDDEDSRSICIGTQPRRLSGKDTEQIRETLRRGLEINSKPVLPPINPQRQNGLAHDRAPS RKDSLESDSSTAIIPHELIRTRQLESVHLKFNQESGALIPLCLRGRLLHGRHFTYKSITG DMAITFVSTGVEGAFATEEHPYAAHGPWLQILLTEEFVEKMLEDLEDLTSPEEFKLPK EYSWPEKKLKVSILPDVVFDSPLH

SEQ ID NO: 5- human Suppressor of Fused (SuFu) cDNA sequence (GenBank Accession No. NM_016169.2)

CGCCGTGCGCAGGCGCGGAGCTAGACCTCGCTGCAGCCCCCATCGCCTCG GGGAGTCTCACCCACCGAGTCCGCCCGCTGGCCCGTCAGTGCTCTCCCCGTCGTT TGCCCTCTCCAGTTCCCCCAGTGCCTGCCCTACGCACCCCGATGGCGGAGCTGCG GCCTAGCGGCGCCCCCGGCCCCACCGCGCCCCCGGCCCCTGGCCCGACTGCCCCC CCGGCCTTCGCTTCGCTCTTTCCCCCGGGACTGCACGCCATCTACGGAGAGTGCC GCCGCCTTTACCCTGACCAGCCGAACCCGCTCCAGGTTACCGCTATCGTCAAGTA CTGGTTGGGTGGCCCAGACCCCTTGGACTATGTTAGCATGTACAGGAATGTGGGG AGCCCTTCTGCTAACATCCCCGAGCACTGGCACTACATCAGCTTCGGCCTGAGTG ATCTCTATGGTGACAACAGAGTCCATGAGTTTACAGGAACAGATGGACCTAGTG

GTTTTGGCTTTGAGTTGACCTTTCGTCTGAAGAGAGAAACTGGGGAGTCTGCCCC
ACCAACATGGCCCGCAGAGTTAATGCAGGGCTTGGCACGATACGTGTTCCAGTC
AGAGAACACCTTCTGCAGTGGGGACCATGTGTCCTGGCACAGCCCTTTGGATAAC
AGTGAGTCAAGAATTCAGCACATGCTGCTGACAGAGGACCCACAGATGCAGCCC
GTGCAGACACCCTTTGGGGTAGTTACCTTCCTCCAGATCGTTGGTGTCTGCACTG
AAGAGCTACACTCAGCCCAGCAGTGGAACGGGCAGGGCATCCTGGAGCTGCTGC
GGACAGTGCCTATTGCTGGCGGCCCCTGGCTGATAACTGACATGCGGAGGGGAG
AGACCATATTTGAGATCGATCCACACCTGCAAGAGAGAGTTGACAAAGGCATCG
AGACAGATGGCTCCAACCTGAGTGGTGTCAGTGCCAAGTGTGCCTGGGATGACC
TGAGCCGGCCCCCCGAGGATGACGAGGACAGCCGGAGCATCTGCATCGGCACAC
AGCCCCGGCGACTCTCTGGCAAAGACACAGAGCAGATCCGGGAGACCCTGAGGA
GAGGACTCGAGATCAACAGCAAACCTGTCCTTCCACCAATCAACCCTCAGCGGC
AGAATGGCCTCGCCCACGACCGGGCCCCGAGCCGCAAAGACAGCCTGGAAAGTG
ACAGCTCCACGGCCATCATTCCCCATGAGCTGATTCGCACGCGGCAGCTTGAGAG
CGTACATCTGAAATTCAACCAGGAGTCCGGAGCCCTCATTCCTCTCTGCCTAAGG
GGCAGGCTCCTGCATGGACGGCACTTTACATATAAAAGTATCACAGGTGACATG
GCCATCACGTTTGTCTCCACGGGAGTGGAAGGCGCCTTTGCCACTGAGGAGCATC
CTTACGCGGCTCATGGACCCTGGTTACAAATTCTGTTGACCGAAGAGTTTGTAGA
GAAAATGTTGGAGGATTTAGAAGATTTGACTTCTCCAGAGGAATTCAAACTTCCC
AAAGAGTACAGCTGGCCTGAAAAGAAGCTGAAGGTCTCCATCCTGCCTGACGTG
GTGTTCGACAGTCCGCTACACTAGCCTGGGCTGGGCCCTGCAGGGGCCAGCAGG
GAGCCCAGCTGCTCCCCAGTGACTTCCAGTGTAACAGTTGTGTCAACGAGATCTC
CACAAATAAAAGGACAAGTGTGAGGAAGACTGCGCAGTGCCACCCCGCAGCCCA
GTGGGGTGCCATGCACAGGCCACAGGCCCTCCACCTCACCTCCAGCTCAGGGGC
CGCACCCCGCCGCTGGCTAAGCCTTGTGACCCATCAGGCCAGTGAGTGGGCAAA
TGCGGACCCTCCCTGCCTGCAGCCTGCACAGATTCTGGTTTGAGGTTTGACTCTG
GACCCTGGCTGTGCCCCTAGGTGGAGACAGCCCTCTTTCTCACCTACCCCCTGCC
GCACAGCCCAGCAGGAGGGAGGCGGACAGCCAGATGCAGAGCGAGTGGATGCA
CTTCCCAGCTCATCTCTGGAAGCCTTTGCTACTCAAGCTCCTCTGGCCGCGGAAC
AATTCCTCTGATCATGTTTGGTTTTCTTCTTCCTTATTTTATTTTGTAGAAACCGGG
TGGTATTTTATTGCTCTGCAAAGATGTCCAGAAGCCATGTATATAATGTTTTTTAA
ACAGAACTTCATTCCCCGTTGAACTTTCGCATTCTCTGACAGAGGCCTAGGGCTG
TATCTCTCCCTGGGCTGCCACCAGAGAAGGTGCTTGGTGTTCGCCTGCCAGCCCA
GAGCCCTGGAGGAGCCGGCTGCACAGAGAGGCTTTTCTTCCCAGCTGGGCCTGAT

GGAGCCCGGGGCAGGGGGAGAGTAGAGACACTCCCTTGTGCAGCTTTGAGCCTA
GTTTAGCTGGGGCCAGGGAGGGGTGCTACTGTTTTCCAAGTGAATGGGTCTCAAA
GACTTGGTGACCCCAGCCTCATCTTCTAGGCCTTTTCCATCCAACCAGGCCTACCT
GGGAGAGGGTGAGGTTCAGCACATCACACACCATCCCCACTGTCATTCAGGGCC
TGGGTCTCCAGCTCTGTAACCAGTCCTGTCCCATTTCCTCAGTCCCTGGGCCTCCC
AGCCTTCAGGCTGTAGGGCTGCCTTACTAAAATTGAAAAAT
CCACCTCTTAACATCTCTTTCACTTTGGTTTTGCTAACACTGCTCTCTGCTGCCCTC
CCATCCTCCCTGTATCCATTCATGCCCTATCTTTCATTCTCCACTCCTAATCCCTCT
CCTTTCTGGCATCCTGGCCTCTCGTGGTCCTCAGCCCCTCACCCCCAGTACTGCAG
ATCTCACAGTTTGCCTTCCAGAAGCCAGCCTATCTCTAGCCCATGGTTTTGGAGTT
CCTCTCGGGTTATCTCCCACGCCTGACCTGGAACCAGCAAGCCCCTTTCCTGCCTT
CTTACCCCCAACTCTAGGGATGGGACTGTTACAATACTTCAAGATCACTCTTTAC
ACCTCTTCAAAGCAAAGTCATGACAATGCAGGGCTCCTCATTGCTCCCATCTGCC
TCTGCTGCACACACAGGCACCAGCAGGGATGCCACAGGAGTGCCCACAGGGTGC
AGGACTCCACTGATGAGAGATCCAGCCAAAGAGCTGCCCCCAGGGGTATGAGGG
CACCAGCTGGGTTCTCCAGGGAGCAGGAGTTGGACCTCCATGGAGCCACTAGGC
CTGGCCTCCTCTACACATCCCCAGGGCTATCTGGTTAATTCCATCAAGCTCAGAG
TTAAAAGGCATATCAGCCTGGAGTATTTGGGAGAGACTGGCTGCAGATCCCCGC
CAGCCAAGATGCAAGCCACTCGGGACCTGATGTCGGCAGCTGTGCCTCTACTGCC
CTGAGGACTTACCAGAGGGAGCCCTACTGGCCTTCCCCCACCACAGCAGCCCTGC
CTGTGAAGCTCTTGTTTCTGACATTTCACAGGCAGAGAGGTGCCATCAGTTCGCC
TCCATTCCTTGCCACCATGACCAGCCTCTCCCTGAACTCTCTCTTGCTCGGGACCT
GCCTGAGGGCTCCCTGCTGCAGTTCGCCGTACTTCCATCTGCTGGGTGCCTCCATC
GTTGGTTGGGTGGGGATGGGGCATTTTCTGAGCTAAGCTTTGTCATTAGTTTGTG
AAGCACCTGGTCAGCAACCTGCCCCAGACCTGGAGGGTCTTTGTGGACTGAAGG
TAGACACCAGCCAGCATGGTGGCCCTGTTCTGGGGGAGCAGGGTAAGGCAGGAG
GAAGTGGGTGAGCTCCGAGATGATGAGCACATGAAGCCTGTGGCCCCTTCGTAC
CTGCAATATGTCAGGAGCCTCACGCTCACCCAAGATCCTGCAGGGGCCAGGCTCC
ATCTCACTGGCTCTGAGGGCAGGACAGGGTATCACACATTTCTCACCAGGCCTCC
TTTCCTATGGGCATTGGTGCCTCCCAGAGGTTTCTTGGGCTGCTGGCTGGTGAGA
GAGGACCCTTAAAGAAGATCAAGCCAAGCTGACCTTGGACCCTGTCCAGCACAG
CTTCTGGCACAGGATGCTTGGTGAATGTACCCTTTCTTTCCCTCCCTGCAGCTCTG
AGGGAGCCCCTGACCTTGTAGTGGGTGGAGGAGGTAAGGGGCCTCCCTCCCTAA
ATCTGCCTCTTCTGCAAGCTACTTGGAGACTTGCCTAGTTGTACCCACCCCTCCAG

GTCCCTGGTGCTAGAGCTTCTGAGAAGGGCCTTTCCCTTTCCTCTTTGCCTGCTAT
ATAAGGCAGGCTCCTGTGGCTCTGCTGGCTCAGTGTGGGCTGCAGGAGGACTGC
AGACTCAGCTGCAATTCTGAGGGGGGTTTGGGAGGCTTGTGCGAGGTCTCAGGC
CTGTGTGGGGAGCTGGTGCCTCTTCCTGCCCGTATCTTTCTCTTCCAAGGGCAGTG
CTCCAAGGCAGGGACTGGAGAAGCCAAGGGGAGAGTCTAAAAGGGCTAGAGCA
TTTTTAAAAATAGACACAGGGTCTTGGGACTGGGGTTTCGGATTGAGTTGCAAGC
AGGGAGAAAACCTGAAGGTCGGTGCCCCTATGGGGCTGACCAGTAGAGAATTTC
CTTTACTGTATTTTTGTGTCTGGTCTTCCCTTTCTGGCTTCTAGGACATCCATGCCA
GGTGAGGTGCCTGGGTCCCTGTTACAAGTCAGGAGCCCTGTAGGGAGACCCCTCC
TTTTGTACAAGTACCTGAATGCTGCGACAAGCAGATTTTTGTAAAATTTTATATTA
GTTTTTAATGTCAGTGGCGACTCGGTTCCTGGGGCTGCAGCCAGCCTGGGACTTT
TGTAAGAATTTTTGGGTGACTCACTTAGATGTCGTTTCCTTCTTGCCCCCTCTTCC
TCTCTGTAATCTAAGTGCATTAAACATCTTTGCAG

What is claimed is:

1. An isolated nucleic acid molecule encoding a mutant SMO protein comprising an amino acid sequence that is at least 95% identical to SEQ ID NO:1 wherein said amino acid sequence comprises an amino acid other than glycine at amino acid 529.

2. The isolated nucleic acid molecule of claim 1 wherein the mutant SMO protein comprises the amino acid sequence of SEQ ID NO:2 wherein said amino acid sequence comprises a serine (S) at amino acid 529.

3. The isolated nucleic acid molecule of claim 1 comprising a parental nucleic acid sequence of SEQ ID NO:3, wherein said sequence contains a mutation that alters the sequence encoding amino acid 529 to encode a different amino acid.

4. A nucleic acid probe capable of specifically hybridizing to nucleic acid encoding a mutated SMO protein or fragment thereof incorporating a mutation in the sequence encoding amino acid 529.

5. The probe of claim 4 wherein said probe is complementary to said nucleic acid encoding the mutated SMO or said fragment thereof.

6. The probe of claim 4 having a length of about 10 to about 50 nucleotides.

7. The probe of claim 4 further comprising a detectable label.

8. An isolated mutant SMO protein comprising an amino acid sequence that is at least 95% identical to SEQ ID NO: 2 wherein said amino acid sequence comprises an amino acid other than glycine at amino acid 529.

9. The isolated mutant SMO protein of claim 8 comprising the amino acid sequence of SEQ ID NO: 2 wherein said amino acid sequence comprises an amino acid other thanglycine at amino acid 529.

10. The isolated mutant SMO protein of claim 8 or 9 wherein said amino acid sequence comprises serine (S) at amino acid 529.

11. An isolated antibody that specifically binds to the mutant SMO protein of any of claims 8 to 10, wherein said antibody does not bind wild-type SMO having a glycine at amino acid 529.

12. The antibody of claim 11, wherein said antibody is a monoclonal antibody, a chimeric antibody, a humanized antibody, a single chain antibody or an antigen-binding fragment thereof.

13. The antibody of claim 11 or 12, wherein said antibody is conjugated to a cytotoxic agent.

14. The antibody of claim 11 or 12, wherein said antibody is conjugated to a detectable label.

15. The antibody of any of claims 11 to 14, wherein said antibody inhibits SMO activity.

16. A method of identifying at least one SMO mutation in a sample comprising contacting nucleic acid from said sample with a nucleic acid probe that is capable of specifically hybridizing to nucleic acid encoding a mutated SMO protein, or fragment thereof incorporating a mutation that alters the sequence encoding amino acid 529 to an amino acid other than glycine, and detecting said hybridization.

17. The method of claim 16 wherein said probe is detectably labeled.

18. The method of claim 16 wherein said probe is an antisense oligomer.

19. The method of claim 16 wherein the SMO gene or a fragment thereof in said nucleic acid said sample is amplified and contacted with said probe.

20. A method for identifying a tumor in a human subject that is or becomes resistant to treatment with GDC-0449 comprising determining the presence of a mutated SMO gene or mutated SMO protein in a sample of said tumor, wherein said mutated SMO gene encodes a SMO protein comprising a mutation at amino acid 529, and wherein said

SMO protein comprises a mutation at amino acid 529, whereby the presence of said mutated SMO gene or mutated SMO protein indicates that said tumor is resistant to treatment with a GDC-0449.

21. The method of claim 20 further comprising treating said subject having a tumor that is not or is no longer susceptible to treatment with GDC-0449 with a compound that binds said mutated SMO.

22. The method of claim 20 wherein the presence or absence of said mutation is determined by examining a nucleic acid sample.

23. The method of claim 20 wherein the presence or absence of said mutation is determined by examining a protein sample.

24. A method of screening for compounds that inhibit signaling of a mutant SMO protein that incorporates a mutation at amino acid 529 comprising contacting said mutant SMO with a test compound and detecting binding of said compound to said mutant SMO whereby binding of said test compound to mutant SMO indicates that said test compound is an inhibitor of mutant SMO.

25. A method of screening for compounds that inhibit signaling of a mutant SMO protein that incorporates a mutation at amino acid 529 comprising contacting a cell that expresses said mutant SMO with a test compound and detecting activity of Gli in said cell whereby the presence of Gli activity indicates that said test compound is not an inhibitor of mutant SMO.

26. A method of inhibiting proliferation or growth of a cell having aberrant hedgehog signaling, comprising administering to said cell a bromodomain inhibitor, wherein said cell expresses a smoothened protein having a mutation at amino acid position 529 of SEQ ID NO: 1.

27. The method of claim 26, wherein the cell is in a subject.

28. The method of claim 26 or 27, wherein the cell is a cancer cell.

29. The method of claim 28, wherein the cell further comprises a *SUFU* mutation.

30. The method of claim 29, wherein the cell is a human cell, and wherein said cell comprises a 10q deletion mutation that results in the loss of a copy of the *SUFU* gene.

31. The method of claim 30, wherein the 10q deletion further results in the loss of a copy of the *PTEN* gene.

32. The method of any of claims 26-31, wherein the bromodomain inhibitor is I-BET762, JQ1 or JQ2.

33. A method of identifying a hedgehog pathway inhibitor, wherein the method comprises: contacting a cell with an amount of a test agent, wherein the cell is responsive to hedgehog protein or has increased hedgehog signaling and/or activation of the hedgehog signaling pathway, and wherein the cell expresses the mutant SMO protein of any of claims 8-10, and determining, as compared to a control, whether the test agent inhibits hedgehog signaling in the cell, wherein if the test agent inhibits hedgehog signaling in the cell relative to the control, then the test agent is identified as a hedgehog pathway inhibitor.

34. The method of claim 33, wherein the ability of the test agent to inhibit hedgehog signaling in the cell is determined using a Gli1 expression assay.

35. A method of identifying a hedgehog pathway inhibitor, wherein the method comprises: contacting a cell with an amount of a test agent, wherein the cell is responsive to hedgehog protein or has increased hedgehog signaling and/or activation of the hedgehog signaling pathway, and wherein the cell expresses the mutant SMO protein of any of 8-10, and determining, as compared to a control, whether the test agent inhibits growth and/or proliferation of the cell, wherein if the test agent inhibits growth and/or proliferation of the cell relative to the control, then the test agent is identified as a hedgehog pathway inhibitor.

36. The method of any of claims 33-35, wherein the control is a cell expressing a wildtype SMO protein.

37. The method of any of claims 33-35, wherein the control is a cell expressing the same mutant SMO proteins as the cell contacted with the test agent, wherein the control is

treated with a control agent to which the mutant SMO protein is partially or completely resistant.

38. The method of claim 37, wherein the control agent is vismodegib, LY2940680, LDE225 and/or compound 5.

39. The method of any of claims 33-38, wherein the test agent binds to mutant SMO protein but not wildtype SMO protein.

40. The method of any of claims 33-38, wherein the test agent binds to both the mutant SMO protein and wildtype SMO protein.

41. The method of claim 33 or 34, wherein the test agent is more effective in inhibiting the hedgehog signaling pathway in a cell expressing mutant SMO protein than in a cell expressing wildtype SMO protein.

42. The method of claim 35, wherein the test agent is more effective in inhibiting growth and/or proliferation of a cell expressing mutant SMO protein than of a cell expressing wildtype SMO protein.

43. A vector comprising the nucleic acid of any of claims 1-3.

44. A host cell comprising the vector of claim 43.

45. A host cell comprising and capable of expressing the vector of claim 43.

46. A method of identifying a hedgehog pathway inhibitor, wherein the method comprises: a) contacting a cell with an amount of a test agent, wherein the cell is responsive to hedgehog protein or has increased hedgehog signaling and/or activation of the hedgehog signaling pathway, and wherein the cell expresses the vector of claim 43, and b) determining, as compared to a control, whether the test agent inhibits hedgehog signaling in the cell, wherein if the test agent inhibits hedgehog signaling in the cell relative to the control, then the test agent is identified as a hedgehog pathway inhibitor.

47. The method of claim 46, wherein the ability of the test agent to inhibit hedgehog signaling in the cell is determined using a Gli1 expression assay.

48. A method of identifying a hedgehog pathway inhibitor, wherein the method comprises: a) contacting a cell with an amount of a test agent, wherein the cell is responsive to hedgehog protein or has increased hedgehog signaling and/or activation of the hedgehog signaling pathway, and wherein the cell expresses the vector of claim 43, and b) determining, as compared to a control, whether the test agent inhibits growth and/or proliferation of the cell, wherein if the test agent inhibits growth and/or proliferation of the cell relative to the control, then the test agent is identified as a hedgehog pathway inhibitor.

Figure 1

Patient ID	Best Response	Final Dose of Vismodegib – Study Day	Reason for Discontinuation	Number of Post-Progression Biopsy Samples	Pre-treatment Sample Provided?
008	Stable Disease	763	Progressive Disease	1	No
001	Partial Response	532	Progressive Disease	1	Yes
002	Partial Response	533	Progressive Disease	1	Yes
011	Stable Disease	310	Progressive Disease	2	No
005	Stable Disease	309	Progressive Disease	2	No

Figure 2

Patient/Sample ID	Type	Median Exon Coverage	Mutations Detected	Mutation Load Per MB	C>T	C>A	C>G	T>C	T>A	T>G
008-P	Progression	921	5	3.99	3	0	1	1	0	0
001-P	Progression	615	80	63.89	71	1	1	2	1	1
001-A	Archival	903	78	62.29	69	1	1	2	1	1
002-P	Progression	460	15	11.98	9	0	1	3	0	0
002-A	Archival	908	14	11.18	8	0	1	3	0	0
011-P-i	Progression	900	16	12.78	10	0	0	5	0	0
011-P-ii	Progression	920	15	11.98	9	0	0	5	0	0
005-P-i	Progression	885	13	10.38	10	0	1	2	0	0
005-P-ii	Progression	853	16	12.78	13	0	2	1	0	0

Figure 3A

PATIENT / SAMPLE ID	Source	MYCN	LRP1B	PTCH1	SMO	TERT	CHEK2	MTOR	TP53BP1	ATM	FLT3
008-P	Progression										
001-P	Pre-Treatment	o	o		o		o	o	o	o	o
001-A	Progression	o	o		o	o	o	o	o	o	o
002-P	Pre-Treatment	o	o	o		o	o	o	o		
002-A	Progression	o	o	o	o		o	o	o		
011-P-i	Progression	o		o	o	o				o	o
011-P-ii	Progression	o		o	o	o				o	o
005-P-i	Progression	o	o	o		o					
005-P-ii	Progression	o	o	o	o	o					

o Variant detected

Figure 3B

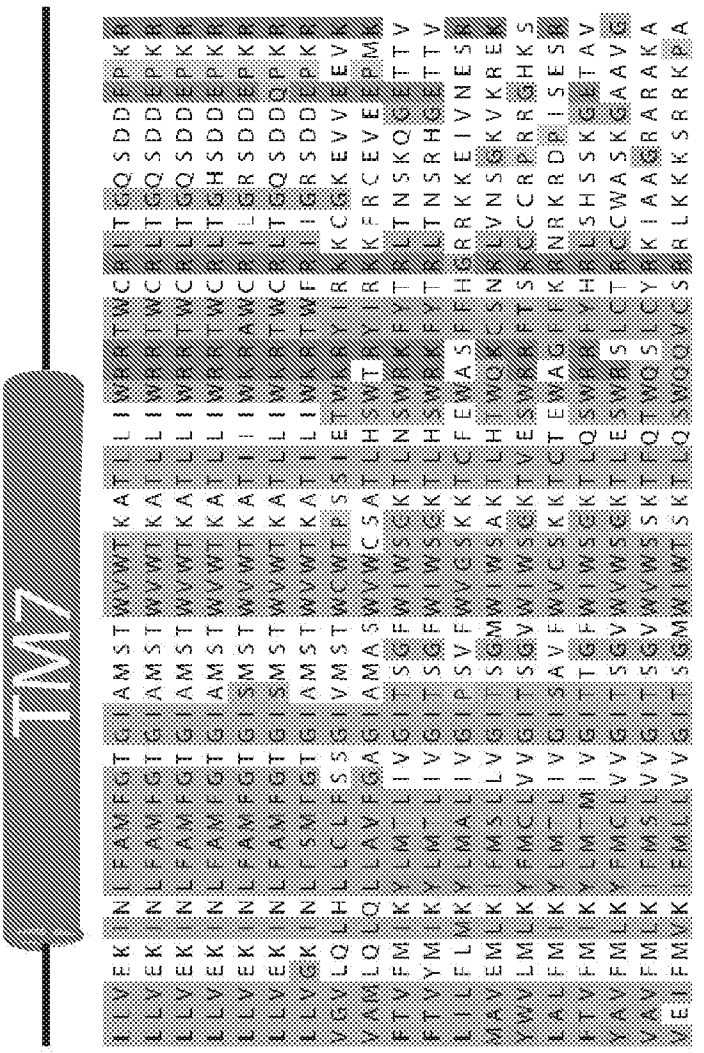
PATIENT / SAMPLE ID	Source	KDR	SMARCA4	TP53	BRCA1	CDH5	EPHA3	GRIN2A	MLL	NSD1	PTPRD
008-P	Progression										
001-P	Pre-Treatment	o	o	o		o	o	o	o	o	o
001-A	Progression	o	o	o		o	o	o	o	o	o
002-P	Pre-Treatment				o						
002-A	Progression				o						
011-P-i	Progression	o	o								
011-P-ii	Progression	o	o								
005-P-i	Progression			o							
005-P-ii	Progression			o							

o Variant detected

Figure 4

Patient / Sample ID	Source	AA change	SMO Mutation AF
008-P	Progression	ND	NR
001-P	Progression	W535L[b]	31
001-A	Pre-Treatment	W535L[b]	15
002-P	Progression	T241M[a, b], V321M[a, b]	8,6
002-A	Pre-Treatment	ND	NR
011-P-i	Progression	G529S, V321M[a, b]	11, 27
011-P-ii	Progression	G529S	39
005-P-i	Progression	ND	NR
005-P-ii	Progression	V321M[a, b]	23

Figure 5



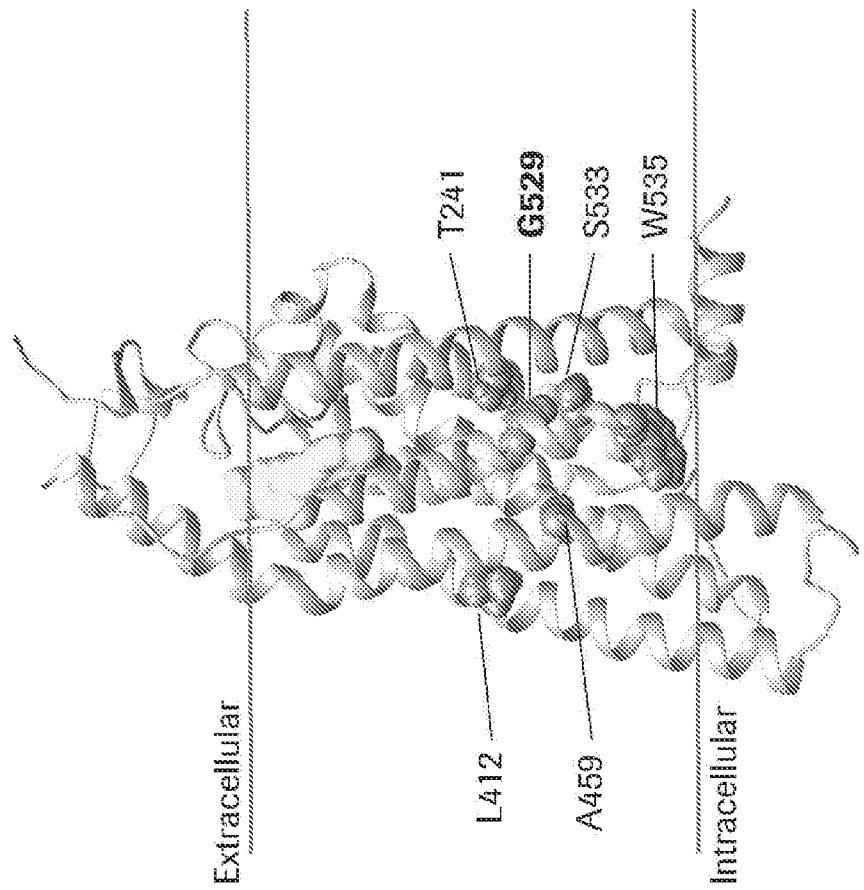

Extracellular
T241
G529
S533
W535
L412
A459
Intracellular

Figure 6

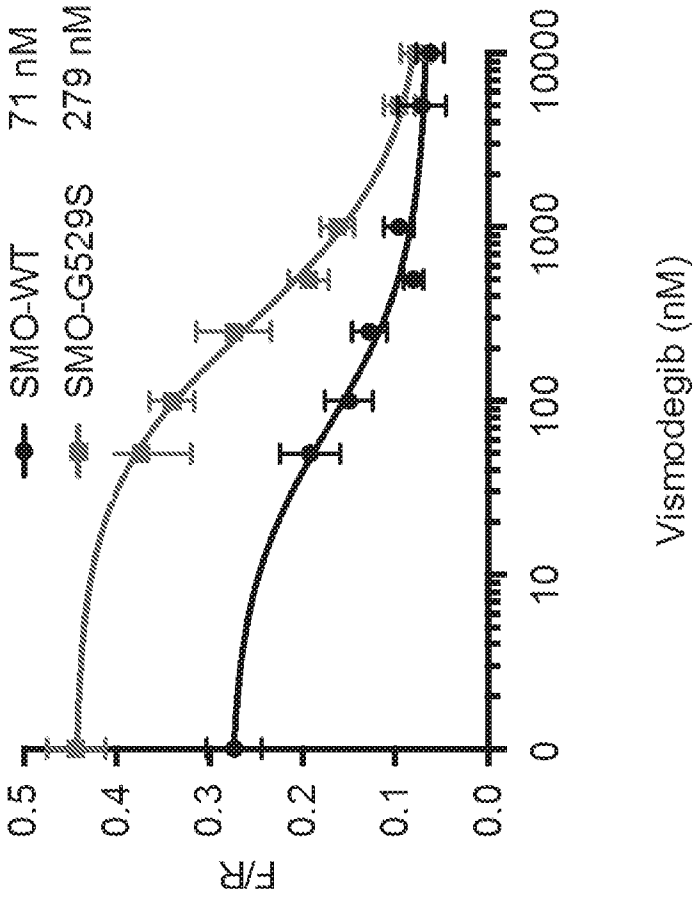

IC50
71 nM
279 nM
SMO-WT
SMO-G529S
F/R
0.5
0.4
0.3
0.2
0.1
0.0
0
10
100
1000
10000
Vismodegib (nM)

Figure 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/016226

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/705
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2015/120075 A2 (GENENTECH INC [US]; CURIS INC [US]) 13 August 2015 (2015-08-13) page 151, line 29 - page 152, line 14 claims 1-4 -----	1-48
A	SHARPE HAYLEY J ET AL: "Genomic Analysis of Smoothened Inhibitor Resistance in Basal Cell Carcinoma", March 2015 (2015-03), CANCER CELL, VOL. 27, NR. 3, PAGE(S) 327-341, XP002768930, ISSN: 1535-6108(print) page 331, right-hand column, paragraph 3 - page 333, left-hand column, paragraph 3 -----	1-48

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 April 2017	12/06/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Barnas, Christoph

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2017/016226

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015120075 A2	13-08-2015	AU 2015214264 A1	04-08-2016
		CA 2937539 A1	13-08-2015
		EP 3102197 A2	14-12-2016
		JP 2017506072 A	02-03-2017
		US 2017044232 A1	16-02-2017
		WO 2015120075 A2	13-08-2015