

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))

(54) Title: G-PROTEIN COUPLED RECEPTORS IN HEDGEHOG SIGNALING

(57) Abstract: The disclosure provides methods for identifying TPRA40 antagonists and agonists. The method further provides methods for inhibiting hedgehog signaling and/or inhibiting unwanted cell proliferation, such as unwanted cell proliferation caused, in whole or in part, by hyperactive hedgehog signaling, using a TPRA40 antagonist.
G-Protein Coupled Receptors in Hedgehog Signaling

RELATED APPLICATIONS

This application claims the benefit of priority to United States provisional application 65/934,620, filed January 31, 2014. The foregoing disclosure is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on January 22, 2015, is named CIBT-224-W01___SL.txt and is 37,643 bytes in size.

BACKGROUND OF THE DISCLOSURE

Members of the Hedgehog (Hh) family of signaling molecules mediate many important short- and long-range patterning processes during invertebrate and vertebrate embryonic, fetal, and adult development. Hedgehog activity exerts its effects on cells and tissues via the hedgehog signaling pathway.

Hedgehog signaling occurs through the interaction of a hedgehog protein (e.g., in mammals, Shh, Dhh, or Ihh) with the hedgehog receptor, patched (Ptch), and the co-receptor Smoothened (Smo). There are two mammalian homoigbs of Ptch, Ptch-1 and Ptch-2, both of which are 12 transmembrane proteins containing a sterol sensing domain (Motoyama et al, Nature Genetics JL8: 104-106 (1998), Carpenter et al, P.N.A.S. (U.S.A.) 95(23): 13630-40 (1998). The interaction of Hh with Ptch triggers a signaling cascade that results in the regulation of transcription by zinc-finger transcription factors of the Gli family.

In humans, the Hh signaling cascade is initiated in the target cell by the Hh ligand binding to the 12-span transmembrane protein Patched1. In the absence of a Hh ligand, Patched inhibits the activity of the seven-transmembrane-span receptor-like protein, Smoothened.

Binding of Hh to Patched results in the loss of Patched activity and the consequent activation of Smoothened, which transduces the Hh signal to the cytoplasm (Taipale et al., 2002, Nature 418:}
The Hh signal is transmitted via an alteration of the balance between the activator and repressor forms of the Gli family of zinc-finger transcription factors. Hh signaling occurs in the nonmotile cilia to which the Smoothened protein and other downstream pathway components transit to activate the Gli transcription factors (Rubin and de Sauvage, 2006, Nat Rev Drug Discov 5: 1026-1033: Corbit et al., 2005, Nature 437: 1018-1021; Huangfu and Anderson, 2005, Proc Natl Acad Sci U S A 102: 11325-1 1330: Huangfu et al., 2003, Nature 426: 83-87). The Gli transcription factors exist as three separate zinc-finger proteins, Glil and Gii2 functioning as transcriptional activators and Gii3 functioning mainly as a transcriptional repressor (Ruiz I Altaba, 1997, Cell 90: 193-196). The expression of Gli is highly dependent upon active Hh signaling and thus Gli expression is often used as a readout of pathway activation. In the absence of a Hh ligand, Patched blocks Smoothened activity and full length Gli proteins are proteolytically processed to generate the repressor GLiR, largely derived from Gli3, which represses Hh target genes. Hh binding to Patched relieves Smoothened inhibition, promotes generation of the activator GLI, largely contributed by Gli 2 and the subsequent expression of the Hh target genes, including positive feedback by Gli 1. Gli activation is regulated at several different levels via phosphorylation by inhibitors such as SuFu, Ren, protein kinase A (PKA), glycogen synthase kinase 3β (G8K3p) and activators such as Dyrkl, Ras and Akt (Varjosalo and Taipale, 2007, J Cell Sci 120: 3-6; Ferretti et al., 2005, Trends Mol Med 11: 537-545; For Review See, Gupta, 2010, Ther Adv Med Oncol. 2(4): 237-250). The Hedgehog signaling pathway has been extensively studied and one of skill in the art readily understands what is meant by a component or gene implicated in the hedgehog pathway and what is meant by hedgehog signaling, and hedgehog signal transduction. Numerous components of the hedgehog pathway that participate in the transduction of signal or regulation of signaling are known. Exemplary components include: Sonic hedgehog. Patched!, Gli1, Gli2, Gli3, protein kinase A (PKA), Suppressor of Fused (SuFu), and Smoothened.

Malignant tumors (cancers) are the second leading cause of death in the United States, after heart disease (Boring et al., CA Cancel J. Clin. 43:7 (1993)). Cancer is an example of unwanted cell proliferation and is characterized by the increase in the number of abnormal, or neoplastic, cells derived from a normal tissue which proliferate to form a tumor mass or otherwise proliferate unchecked by proper control. Cancer may be further characterized by the invasion of adjacent tissues by these neoplastic tumor cells, and the generation of malignant cells...
which eventually spread via the blood or lymphatic system to regional lymph nodes and to
distant sites via a process called metastasis. In a cancerous state, a cell proliferates under
conditions in which normal cells would not grow. Cancer manifests itself in a wide variety of
forms, characterized by different degrees of invasiveness and aggressiveness.

Hedgehog signaling has been implicated in a wide variety of cancers and carcinogenesis.
Specifically, increased or misregulated hedgehog signaling activity has been implicated in cancer,
and a current product approved for the treatment of locally advanced or metastatic basal cell
carcinoma, vismodigib (Erivedge®), is an inhibitor of hedgehog signaling. Vismodigib is a
smoothened inhibitor, and thus, inhibits hedgehog signaling at the level of smoothened. For
example, mutations in components of the hedgehog signaling pathway (e.g., mutations in
hedgehog signaling pathway genes) that lead to misregulated hedgehog signaling activity, such
as signaling in the absence of hedgehog ligand, have been identified in certain cancers. For
example, loss-of-function mutations in Patched!, gain-of-function mutations in Smoothened, and
loss-of-function mutations in Suppressor of Fused lead to misregulated hedgehog signaling and
have been identified in cancers. These are exemplary of mutations in components of the
hedgehog pathway (e.g., are exemplary of mutations in a hedgehog signaling pathway gene).

TPRA40 (also known as TPRA1 and GPR175) is an orphan G-protein coupled receptor
whose physiological functions were previously unknown. TPRA40 is a 40 kDa protein having
seven transmembrane domains (Fujimoto et al., 2001, Biochim Biophys Acta, 1518(1-2): 173-7)
and an 84-amino acid cytoplasmic region (Fujimoto et al.). TPRA40 has been shown to be
expressed during oxidative stress, aging and under certain pathophysiological conditions (AM et
al., 2008, J Cell Physiol, 217(1): 194-206). In addition, it has been proposed that Sjogren
syndrome Nuclear Autoantigen of 14 kDa (Ssna1, also known as NA14), an autoantigen of
Sjogren's syndrome, co-immunoprecipitates with TPRA40 in an immunoprecipitation assay,
suggesting a direct or indirect interaction between these two proteins, at least under conditions of
overexpression (AM et al.). To date, however, the role of TPRA40 in any particular signaling
pathway was unknown. In addition, it was previously unknown whether TPRA40 was
associated with any disease conditions, such as cancer.

Given the role of hedgehog signaling in a variety of cancers, identification of components
of the hedgehog signaling pathway suitable as targets for drug development represents a.
significant advance. The present disclosure provides a novel component of the hedgehog signaling pathway suitable as a target for drug development.

SUMMARY OF THE DISCLOSURE

The disclosure provides methods for inhibiting cell proliferation, such as unwanted cell proliferation, in a cell in vitro or in vivo. The disclosure is based on appreciation and understanding of the role of TPRA40 as a novel component of the hedgehog signaling pathway that endogenously functions as a positive regulator of hedgehog signaling. As such, the disclosure provides methods for inhibiting hedgehog signaling in cells, such as any of the cells described herein, using a TPRA40 antagonist. In certain embodiments, the disclosure provides methods for inhibiting cell proliferation, such as unwanted cell proliferation, in a cell in vitro or in vivo using a TPRA40 antagonist. Suitable cells in which proliferation may be inhibited include cells that are responsive to hedgehog protein and/or in which hedgehog signaling is active or hyperactive, as well as cells comprising one or more mutations in a hedgehog pathway gene (e.g., comprising one or more mutations in a component of the hedgehog signaling pathway) or otherwise determined to exhibit hedgehog signaling activity.

Moreover, the disclosure provides various methods for screening for and/or identifying agents suitable as TPRA40 antagonists. Identified agents may then be used in vitro or in vivo.

Moreover, the disclosure provides various methods for screening for and/or identifying agents suitable as TPRA40 agonists. Identified agents may then be used in vitro or in vivo.

In one aspect, the disclosure provides for a method of reducing hedgehog signaling in a cell, for example, in a cell that is responsive to hedgehog protein or exhibits active or hyperactive hedgehog signaling activity. In certain embodiments, the cell is responsive to hedgehog protein or comprises one or more mutations in a hedgehog signaling pathway gene (e.g., a mutation in a component of the hedgehog 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 a TPRA40 antagonist. In certain embodiments, the cell exhibits active or hyperactive hedgehog signaling.

In some aspects, the disclosure provides for a method of inhibiting unwanted growth, proliferation or survival of a cell, for example, in a cell that is responsive to hedgehog protein or
exhibits active or hyperactive hedgehog signaling activity. In certain embodiments, the cell is responsive to hedgehog protein or comprises one or more mutations in a hedgehog signaling pathway gene (e.g., a mutation in a component of the hedgehog 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 a TPRA40 antagonist. In certain embodiments, the cell exhibits active or hyperactive hedgehog signaling.

In some aspects, the disclosure provides for a method of inhibiting growth, proliferation or survival of a tumor cell, for example, in a cell that is responsive to hedgehog protein or exhibits active or hyperactive hedgehog signaling activity. In certain embodiments, 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 a TPRA40 antagonist. In certain embodiments, the cell exhibits active or hyperactive hedgehog signaling. In certain embodiments, the cell exhibits hyperactive hedgehog signaling.

In some aspects, the disclosure provides for a method of inhibiting unwanted growth, proliferation or survival of a cell, wherein the cell comprises one or more mutations in suppressor-of-fused, in which one or more mutations result in the cell having suppressor-of-fused loss-of-function, wherein the one or more mutations results in increased hedgehog signaling and/or activation of the hedgehog signaling pathway in the absence of ligand (e.g., the absence of hedgehog protein), wherein the method comprises the step of contacting the cell with an effective amount of a TPRA40 antagonist.

In some aspects, the disclosure provides for a method of inhibiting growth, proliferation or survival of a tumor cell, wherein the cell comprises one or more mutations in suppressor-of-fused resulting in the cell having suppressor-of-fused loss-of-function, 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 a TPRA40 antagonist.

The following may apply, in certain embodiments, to any of the methods disclosed herein. In certain embodiments of any of the methods disclosed herein, hedgehog signaling is
hyperactive in the cell. In certain embodiments, the hedgehog signaling is overactive because
the cell or an adjacent cell overexpressed hedgehog protein (e.g., where the cell is a cancer cell
or a cell in a tumor, the tumor cell or stroma overexpress a hedgehog protein). In certain
embodiments, the cell comprises one or more mutations in a hedgehog signaling pathway gene
(e.g., one or more mutations in a component of the hedgehog a signaling pathway). In certain
embodiments, the cell comprises one or more mutations in a component of the hedgehog
signaling pathway. In certain embodiments, the one or more mutations are in *smoothened*, and
the cell has a smoothened gain-of-function. In certain embodiments, the gain-of-function
smoothened mutation results in a coconstitutively active Smoothened protein. In certain
embodiments, the one or more mutations are in *patched*, and the cell has a patched loss-of-
function. In certain embodiments, the one or more mutations result in overexpression of a
hedgehog protein or, as noted above, hedgehog protein is overexpressed in the cell or in an
adjacent cell, despite the fact that the cell does not have a mutation in a hedgehog gene and/or in
a component of the hedgehog pathway. In certain embodiments, the overexpressed hedgehog
protein is Sonic hedgehog protein. In certain embodiments, the overexpressed hedgehog protein
is Indian hedgehog protein. In certain embodiments, the overexpressed hedgehog protein is
Desert hedgehog protein. In certain embodiments, the one or more mutations are in *suppressor-
of-fitsed*, and the cell has suppressor-of-fused loss-of-function. In certain embodiments, prior to
contacting the cell with the TPRA40 antagonist, the cell is determined to have one or more
mutations in a hedgehog signaling pathway gene or otherwise determined to exhibit hedgehog
signaling activity, such as determined to have active or hyperactive hedgehog signaling. In
certain embodiments, the cell is determined to exhibit hedgehog signaling activity by measuring
*GUI or Patched* levels.

The following may apply, in certain embodiments, to any of the methods disclosed herein.

In certain embodiments of any of the methods described herein, the cell is a cell in culture. In
certain embodiments of any of the methods described herein, the method comprises contacting a
culture comprising a plurality of cells. In certain embodiments, the cell is in a vertebrate, and
contacting the cell comprises administering the TPRA40 antagonist to the vertebrate. In certain
embodiments, the vertebrate is a human subject. In certain embodiments, the cell is a vertebrate
cell, such as a mammalian cell. In certain embodiments, the cell is a cancer cell or cancer cell
line.
The following may apply, in certain embodiments, to any of the methods disclosed herein. In certain embodiments of any of the methods described herein, the cell is a cancer cell and/or the vertebrate is a vertebrate diagnosed with cancer. In certain 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, rhabdomyosarcoma, basal cell carcinoma, gastric, ovarian, esophageal, pancreatic, or testicular cancer cell. In certain embodiments, the cancer cell is a cancer cell selected from the group consisting of: a medulloblastoma, meningioma, adenoid cystic carcinoma, basal cell carcinoma and rhabdomyosarcoma cancer cell.

The following may apply, in certain embodiments, to any of the methods disclosed herein. In certain embodiments of the disclosure, the TPRA40 antagonist is a polynucleotide molecule that inhibits the expression of TPRA40. For example, in certain embodiments the TPRA40 antagonist is a polynucleotide that binds to (e.g., targets) TPRA40. In certain embodiments, the polynucleotide molecule is an antisense oligonucleotide that hybridizes to a TPRA40 transcript to inhibit expression of TPRA40. In certain embodiments, the TPRA40 antagonist is an RNAi (e.g., an RNAi molecule) that targets the TPRA40 mRNA transcript. In certain embodiments, the RNAi molecule comprises an siRNA. In certain embodiments, the siRNA is 19-23 nucleotides in length. In certain embodiments, the siRNA is double stranded, and includes short overhang(s) at one or both ends. In certain embodiments, the RNAi comprises an shRNA. In certain embodiments, the siRNA targets TPRA40 mRNA transcript. In certain embodiments, the siRNA comprises one or more of the nucleotide sequences selected from: SEQ ID NOs: 16-23. In certain embodiments, the TPRA40 antagonist is a small molecule that binds to TPRA40, such as a small molecule that binds to and inhibits an activity of TPRA40. In certain embodiments, the TPRA40 antagonist is an antibody that binds to TPRA40 protein. In certain embodiments, the antibody is a monoclonal antibody. In certain embodiments, the TPRA40 antagonist is a polypeptide antagonist.

The following may apply, in certain embodiments, to any of the methods disclosed herein. In certain embodiments, regardless of the method or the particular TPRA40 antagonist used, the method comprises contacting the cell (at the same or a differing time) with an additional antagonist of the hedgehog signaling pathway (e.g., generically a hedgehog pathway inhibitor - HPI). In certain embodiments, the additional antagonist of the hedgehog signaling pathway is a
veratrum-type steroidal alkaloid. In certain embodiments, the veratrum-type steroidal alkaloid is
cytopamine or KAAD-cyclopamine or a derivative thereof (e.g., IPI-269609 or IPI-926/saridegib). In certain embodiments, the veratrum-type steroidal alkaloid is jervine, IPI-269609 or IPI-926. In certain embodiments, the antagonist is a non-veratrum-type synthetic
small molecule inhibitor of Smoothened (e.g., the HPI is a small molecule inhibitor of
Smoothened). In certain embodiments, the antagonist is Erivedge (vismodegib), 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 certain
embodiments, the additional antagonist of the hedgehog signaling pathway is an antibody. In
certain embodiments, the antibody is an antibody that specifically binds Sonic, Indian or Desert
dohedgehog protein. In certain embodiments, the additional antagonist of the hedgehog pathway is
a hedgehog inhibitor (e.g., the HPI is a hedgehog inhibitor - an inhibitor of hedgehog protein).
In certain embodiments, the hedgehog inhibitor is robotkinin. In certain embodiments, the HPI
(e.g., hedgehog pathway inhibitor; antagonist of the hedgehog signaling pathway) is selected
from the group consisting of: vismodegib, sonidegib, BMS-833923, PF-04449913, and
LY2940680. In certain embodiments, the additional antagonist of the hedgehog signaling
pathway is an RNAi antagonist.

In another aspect, the disclosure provides for a method of screening for a TPRA40
antagonist, wherein the method comprises: a) contacting a cell that expresses TPRA40, adenylvl
cyclase and a reporter (e.g., a report construct, such as a reporter that indicates adenylvl cyclase
activity) with an adenylvl cyclase activator and an agent; b) determining, as compared to an
untreated control, whether the agent rescues the adenylvl cyclase activity suppressed by TPRA40
expression, wherein if the agent increases adenylvl cyclase activity relative to the non-agent
treated TPRA40 expressing cells, then the agent is identified as a TPRA40 antagonist. In certain
embodiments, the untreated control is the same type of cell (e.g., expressing TPRA40, adenylvl
cyclase, and a reporter), but the untreated control is not contacted with the agent. In certain
embodiments, TPRA40 is expressed in the cell exogenously, such as by transfecting or
transforming the cell with a vector expressing TPRA40. Optionally, the cell may also
endogenously express TPRA40. In certain embodiments, the cell is contacted with the activator
and the agent simultaneously, concurrently, or consecutively.
In another aspect, the disclosure provides for a method of identifying a TPRA40 antagonist, comprising: a) providing a cell that expresses TPRA40 and that expresses a reporter gene capable of indicating adenylyl cyclase activity; b) contacting the cell with an activator of adenylyl cyclase and with an agent, wherein the cells are contacted with the activator and the agent simultaneously, concurrently, or consecutively; and c) determining, as compared to a control, whether the agent rescues adenylyl cyclase activity induced by the activator, wherein if the agent increases the adenylyl cyclase activity relative to the control, then the agent is identified as a TPRA40 antagonist. In certain embodiments, the untreated control is the same type of cell (e.g., expressing TPRA40 and the reporter), but the untreated control is not contacted with the agent. In certain embodiments, TPRA40 is expressed in the cell exogenously, such as by transfecting or transforming the cell with a vector expressing TPRA40. Optionally, the cell may also endogenously express TPRA40. In certain embodiments, the cell is contacted with the activator and the agent simultaneously, concurrently, or consecutively.

In another aspect, the disclosure provides for a method of screening for an agent for inhibiting the proliferation, growth or survival of a cancer cell. For example, the method comprises: a) screening for an agent that binds to TPRA40 protein, reduces expression of TPRA40, inhibits transport of TPRA40 protein to the plasma membrane or to primary cilia, prevents activation of TPRA40 or uncouples TPRA40 from God; b) contacting a cancer cell with an amount of the agent identified in step a), wherein the cancer 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, and c) determining, as compared to a control, whether the agent inhibits the proliferation or growth of the cancer cell, wherein if the agent inhibits cell proliferation or growth relative to the control, then an agent that inhibits the proliferation or growth of the cancer cell is identified. Methods in which any one of the specific activities recited in step (a) are recited as the screen are expressly contemplated.

In another aspect, the disclosure provides for a method of screening for an agent for inhibiting hedgehog signaling in a cell, wherein the method comprises: a) screening for an agent that binds to TPRA40 protein, reduces expression of TPRA40, inhibits transport of TPRA40 protein to the plasma membrane or to primary cilia, prevents activation of TPRA40 or uncouples TPRA40 from God; b) contacting a cell with an amount of the agent identified in step a), 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, and e) determining, as compared to a control, whether the agent inhibits hedgehog signaling in the cell, wherein if the agent inhibits hedgehog signaling in the cell relative to the control, then an agent that inhibits hedgehog signaling is identified. Methods in which any one of the specific activities recited in step (a.) are recited as the screen are expressly contemplated.

In another aspect, the disclosure provides for a method of identifying a TPRA40 antagonist, wherein the method comprises: a) screening for an agent that binds to TPRA40 protein; b) contacting a cell with an amount of the agent identified in step a), 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, and c) determining, as compared to a control, whether the agent that binds to TPRA40 protein also inhibits hedgehog signaling in the cell, wherein if the agent inhibits hedgehog signaling in the cell relative to the control, then the agent is identified as a TPRA40 antagonist.

The following may apply, in certain embodiments, to any of the screening methods disclosed herein, as well as any other method where context indicates. In certain embodiments of any of the screening methods described herein, the cell is in culture. In certain embodiments, the cell is in an animal. In certain embodiments, the cell is a vertebrate cell, such as a rodent, hamster, or human cell. In certain embodiments, the cell is a cancer cell, such as from a primary tumor or a cancer cell line. In certain embodiments, the hedgehog signaling is overactive because the cell or an adjacent cell overexpressed hedgehog protein (e.g., where the cell is a cancer cell or a cell in a tumor, the tumor cell or stroma overexpress a hedgehog protein). In certain embodiments, the cell comprises one or more mutations in a hedgehog signaling pathway gene (e.g., a mutation in a component of the hedgehog signaling pathway). In certain embodiments, the one or more mutations are in smoothened, and the cell has a smoothened gain-of-function. In certain embodiments, the gain-of-function smoothened mutation results in a constitutively active smoothened protein. In certain embodiments, the one or more mutations are in patched!, and the cell has a patched loss-of-function. In certain embodiments, the tumor
overexpresses a hedgehog protein. In certain embodiments, the one or more mutations are in
*suppressor-of-fused*, and the cell has suppressor-of-fused loss-of-function.

The following may apply, in certain embodiments, to any of the screening methods disclosed herein, as well as any other method where context indicate. In certain embodiments, the agent for use in any of the screening methods described herein is a polypeptide or an antibody. In certain embodiments, the agent is a small molecule. In certain embodiments, the agent is an siRNA or shRNA that decreases TPRA40 transcription. In certain embodiments, the agent binds TPRA40 protein. In other words, the methods are suitable for use in screening to identify TPRA40 antagonists that are, for example, polypeptides, including antibodies, or small molecules, or polynucleotides. In certain embodiments, the agent is identified in step a) using a yeast two-hybrid screen. In certain embodiments, the agent is identified in step a) using a high throughput binding or activity screen of a small molecule library. In certain embodiments, the agent inhibits transport of the TPRA40 protein to the plasma membrane or to primary cilia. In certain embodiments, the agent is by a method comprising the steps of: i) contacting a cell expressing TPRA40 with an agent, and ii) determining the localization of TPRA40 in the first cell expressing TPRA40 using immunohistochemistry. In certain embodiments, the agent is identified by a method comprising the steps of: i) contacting a cell expressing TPRA40 with an agent; and ii) determining the levels of TPRA40 in a plasma membrane or ciliary membrane fraction. In certain embodiments, the agent reduces expression of TPRA40 protein or RNA. In certain embodiments, the agent is identified by a method comprising: i) contacting a cell expressing TPRA40 with an agent; and ii) determining activity of TPRA40 in the cell using a &7/-luciferase reporter or adenylate cyclase reporter assay. In certain embodiments, the agent is identified in step a) by a method comprising: i) contacting a cell expressing TPRA40 with an agent; and ii) determining the expression of TPRA40 in the cell by RT-PCR. In certain embodiments, the agent is identified in step a) by a method comprising: i) contacting a cell expressing TPRA40 with an agent; and ii) determining the expression of TPRA40 in the cell using Northern Blot analysis of TPRA40 RNA or Western Blot, flow cytometry, immunofluorescence or immunohistochemistry analysis of TPRA40 protein.

In certain embodiments of any of the screening methods disclosed herein, the cell is treated with a compound that induces adenylyl cyclase activity (e.g., an activator of adenylyl cyclase or an adenylate cyclase activator) prior to step a).
In some embodiments of any of the screening methods disclosed herein, the reporter gene is used in order to determine whether adenyl cyclase activity has been rescued by an agent and/or to evaluate an increase in adenyl cyclase activity. In some embodiments, the reporter gene is a luciferase gene controlled by a cAMP response element.

In some embodiments of any of the screening methods disclosed herein, the compound that induces adenyl cyclase activity is forskolin, 8-bromo-cAMP or dibutyryl-cAMP.

The following may apply, in certain embodiments, to any of the screening methods disclosed herein, as well as any other method where context indicate. In some embodiments of any of the screening methods disclosed herein, the agent is further assessed in an assay for hedgehog signaling. In some embodiments, the assay for hedgehog signaling comprises the steps of: i. contacting a cell with an amount of the agent, 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, and ii. determining, as compared to a control, whether the agent inhibits hedgehog signaling in the cell, wherein if the agent inhibits hedgehog signaling in the cell relative to the control, then an agent that inhibits hedgehog signaling is identified.

The following may apply, in certain embodiments, to any of the screening methods disclosed herein, as well as any other method where context indicate. In certain embodiments of any of the screening methods disclosed herein, TPRA40 is expressed in a cell exogenously (e.g., by transfecting or transforming the cell with a vector expressing TPR40). In certain embodiments, TPRA40 is stably expressed in the cell. In certain embodiments, TPRA4Q is transiently expressed in the cell. In certain embodiments, the cell is transformed with a vector expressing TPRA40 protein. In certain embodiments, the reporter gene is a luciferase gene controlled by a cAMP response element. In certain embodiments, the activator is forskolin, 8-bromo-cAMP or dibutyryl-cAMP. Even when TPRA40 is expressed using exogenous means (e.g., introducing TPRA40) it is also contemplated that the cell may optionally express endogenous TPRA40. When providing TPRA40 using exogenous means, the TPRA40 may be from the same species as the cell or from a different species (e.g., use a vector to express human TPRA40 in a murine cell or use a vector to express human TPRA40 in a human cell).
The disclosure specifically contemplates that any of the embodiments described above may be combined with any other embodiment, as well as with any aspect of the disclosure. Moreover, these aspects and embodiments may be combined with each other, as well as with embodiments described in the detailed description.

Also contemplated are methods for screening for TPRA40 agonists. The disclosure contemplates that any of the foregoing assays for identifying agents that act as TPRA40 antagonists and/or inhibit hedgehog signaling may be used, similarly but evaluating for the opposite effect or read-out, to identify agents that act as TPRA40 agonists and/or promote hedgehog signaling. Such assays are explicitly contemplated and are summarized below.

Embodiments described above and herein that describe appropriate cells, assay reagents, reporters, activators and the like are applicable to agonist assays and are expressly contemplated.

In certain aspects, the disclosure provides for a method of screening for a TPRA40 agonist, wherein the method comprises: a) contacting a cell that expresses TPRA40, adenylyl cyclase and a reporter with an agent; b) determining, as compared to an untreated control, whether the agent suppresses the adenylyl cyclase activity, wherein if the agent suppresses adenylyl cyclase activity relative to the non-agent treated TPRA40 expressing cells, then the agent is identified as a TPRA40 agonist.

In another aspect, the disclosure provides for a method of identifying a TPRA40 agonist, comprising: a) providing a cell that expresses TPRA40 and that expresses a reporter gene capable of indicating adenylyl cyclase activity; b) contacting the cell with an activator of adenylyl cyclase and with an agent, wherein the cells are contacted with the activator and the agent simultaneously, concurrently, or consecutively; and c) determining, as compared to a control, whether the agent suppresses adenylyl cyclase activity induced by the activator, wherein if the agent suppresses the adenylyl cyclase activity relative to the control, then the agent is identified as a TPRA40 agonist.

In another aspect, the disclosure provides for a method of screening for an agent for inducing hedgehog signaling in a cell, wherein said method comprises: a) screening for an agent that binds to TPRA40 protein, induces expression of TPRA40, facilitates transport of TPRA40 protein to the plasma membrane or to primary cilia, induces activation of TPRA40 or couples it with Gai; b) contacting a cell with an amount of the agent identified in step a), and c) determining, as compared to a control, whether said agent induces hedgehog signaling in said
cell, wherein if said agent induces hedgehog signaling in said cell relative to the control, then an agent that induces hedgehog signaling is identified.

In another aspect, the disclosure provides for a method of identifying a TPRA40 agonist, wherein said method comprises: a) screening for an agent that binds to TPRA40 protein; b) contacting a cell with an amount of the agent identified in step a), and c) determining, as compared to a control, whether said agent that binds to TPRA40 protein also induces hedgehog signaling in said cell, wherein if said agent induces hedgehog signaling in said cell relative to the control, then the agent is identified as a TPRA40 agonist. In certain embodiments, the agent is a small molecule. In certain embodiments, the agent is a polypeptide. In certain embodiments, the agent is a polynucleotide.

In another aspect, a TPRA40 agonist may be used in methods of promoting hedgehog signaling in a cell, and such methods are contemplated.

The disclosure contemplates all combinations of any of the foregoing aspects and embodiments, as well as combinations with any of the embodiments set forth in the detailed description and examples. For example, any of the TPRA40 antagonists described generally or specifically herein (e.g., TPRA40 antagonists of the disclosure) may be used in any of the methods described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show that TPRA40 depletion inhibited Hh- and Snio-stimulated &Gf-luciferase activity in S12 cells. (A) S12 cells were depleted of murine 1f8S (by 72%), Ssnal (by 75%) and TPRA40 (by 80%) by 50nM siRNA transfection (with pools of four siRNAs per target) for 72 hours, the last 24 hours of which they were incubated in serum-free media with (black; right bar in each set of bars) or without (grey; left bar in each set of bars) 200ng/ml octyl-Shh to stimulate Hh signaling. &Gf-luciferase activity was measured versus renilla-luciferase (as a measure of viability) and the data were expressed as a % of the non-targeting control siRNA (siNTC) +Hh. The mean and standard deviation of four independent experiments are shown. *, p <0.05; **, p < 0.01 ; ***, p < 0.001 (student's unpaired t-test). (B) S12 cells were treated as in (A) except treated with DMSO control or stimulated with 100nM of the Smoothened, small molecule agonist Hh1.2 (HhAgl .2) instead of octyl-Shh (black; right bar in each set of bars). Results are expressed as a percentage of non-targeting control (siNTC), agonist (HhAgl. 2)
treated cells (black; right bar in each set of bars). TPRA40 depletion using siRNA inhibited both Hh and Smoothened agonist stimulated Gli-luciferase activity by approximately 40%.

Figures 2A and 2B show that TPRA40 depletion inhibited Hh-stimulated GUI induction in S12 cells. (A) S12 cells were depleted of TPRA40 by siRNA treatment and stimulated with 200ng/ml octylated Sonic Hedgehog protein (black; Hh; right bar in each set) or without Hedgehog protein (grey; left bar in each set) as in Figure 1, but analyzed by qRT-PCR for endogenous murine GUI expression instead of G/Muciferase activity. The mean and standard deviation of three independent experiments was plotted and showed an approximately 50% reduction in Hedgehog pathway stimulation in TPRA40 siRNA treated cells (i.e., TPRA40-deficient cells). (B) Cells were treated as in (A) but lysed and analyzed by western blotting with a Gli1-specific monoclonal antibody, then reprobed for tubulin (55kDa) as a loading control. The Hh-induced upregulation of Gli1 protein (~150 kDa) was also diminished by TPRA40 knockdown (a representative blot of two independent experiments is shown).

Figures 3A-C show that three of the four individual siRNA components of the siTPRA40 pool are active and reduce TPRA40 expression. (A) The four siRNAs to TPRA40 that make up the pool (siRNAs #9-12) were transfected individually into S12 cells at 25nM and the Gli-luciferase activity measured as in Figure 1A except the data were normalized to siNTC +Hh as 1. Mean and standard deviation of three experiments are shown. siRNAs 10-12 inhibited Gli-luciferase stimulation similarly to the 100nM pool! (by 34-40%), while siRNA #9 did not. (B) S12 cells were treated as in (A) but endogenous Gli1 levels were measured by qRT-PCR. 25nM siRNA #9 again had no effect, while siRNAs 10-12 inhibited Gli1 induction, albeit less effectively than 100nM of the 4 siRNA pool. Data from a single experiment is shown. (C) TPRA40 gene expression levels by qRT-PCR were decreased by more than 80% by the siRNA pool and individual siRNAs 9 and 10; by about 60% by siRNA #11 and by about 70% by siRNA #12. Hedgehog stimulation had no effect on TPRA40 levels (in the non-targeting control (NTC)-transfected cells), consistent with the conclusion that TPRA40 is not itself a hedgehog pathway target gene. Mean and standard deviations of triplicate samples from a single experiment is shown. In Figure 3B and 3C, results in the presence of hedgehog treatment are shown in black (the right bar in each set) and the results in the absence of hedgehog treatment are shown in grey (the left bar in each set).
Figure 4 shows that depletion of TPRA40 inhibited hedgehog signaling in Daoy medulloblastoma cells. Daoy medulloblastoma cells exhibit constitutively active hedgehog signaling (in the absence of Hh ligand) as monitored by hGli1 siRNA levels. This constitutive signaling can be decreased by treatment with 0.5 or 2 µM Cycodamine, a well-known Smothingen inhibitor. TPRA40 depletion by transfection of 50nM siRNA pool to hTPRA40 also reduced hGUI levels and, when combined with cycodamine, further reduced hGli1 levels. Mean and standard deviations of triplicate samples from a single experiment is shown. For each of the DMSO or cycodamine treatment groups, data for cells treated with the TPRA40 siRNA pool is shown by the right-hand bar (black).

Figures 5A-C show that TPRA40 acts downstream of Ptch1 and SuFu. (A) Schematic of key hedgehog pathway components, with positive regulators Shh (Sonic Hedgehog), Srao (Smothingen) and Gli activators (Gli-A) in normal font and negative regulators Ptc (Patched 1), SuFu (Suppressor of Fused), PKA (Protein Kinase A) and Gli repressors (Gli-R) in bold type. (B-C) Depletion of the negative regulator Ptch1 (B, data normalized to siPtch1) or depletion of the negative regulator SuFu (C, data normalized to siSuFu) stimulated Gli-luciferase activity in S12 cells in the absence of ligand (Sonic hedgehog protein). This stimulation of hedgehog signaling activity was partially rescued by co-depletion of TPRA40. Mean and standard deviation of 3 independent experiments is shown.

Figure 6 shows that TPRA40 does not act downstream of PKA. S12 cells were treated with DMSO vehicle control or 80 µM cell permeable 14-22 amide (a PKA inhibitor) for 24 hours in the absence of stimulation with Hedgehog (e.g., ligand) following 48 hours of treatment with siRNAs to TPRA40 (black/right bar) or non-targeting control (grey/left bar). TPRA40 depletion does not rescue PKA inhibition, suggesting that TPRA40 acts at the level of, or upstream of, PKA.

Figures 7A and 7B show that TPRA40 knockdown increased levels of Gli3 repressor. (A) Western blot of Gli3 using monoclonal antibody 6F5. Data is shown for the following: culture in the presence or absence of 24 hour Hedgehog treatment and TPRA40 or Ift88 depletion, with tubulin 1A2 as a loading control. (B) Quantitation of 3 westerns of Gii3FL and Gli3R normalized to tubulin (mean and standard deviation normalized to NTC -Hh). Hedgehog stimulation for 24 hours inhibits PKA activity and attenuates GH3R production, which requires primary cilia (as evidenced by the increase in Gli3R levels following Ift88 depletion). TPRA40
knockdown increased both the baseline level of Gli3R (by about 25%) and the level remaining after Hh stimulation (by 2 fold).

Figures 8A-8B show the characterization of antibodies to endogenous TPRA40 by western blotting of S12 cells. S12 cells transfected with NTC or TPRA40 siRNAs for 48 hours were serum starved in the presence or absence of Hedgehog protein for 24 hours (72 hours total knockdown), then lysed and subjected to western blotting. (A) Custom-made rabbit anti-TPRA40 C-terminal antibody 12569B (generated by YenZym) was the antibody used in this experiment. This antibody detects a single band of about 55kDa that is not affected by Hh treatment (i.e., is obsen'ed regardless of whether the cells are cultured in the presence or absence of Hedgehog), but disappears following TPRA40 depletion using siRNAs. These results indicate that this antibody is specific for TPRA40. (B) A mouse anti-TPRA40 antibody 6H2 (commercially available from Santa Cruz) was the antibody used in this experiment. This antibody also detects TPRA40. However, this antibody appears less clean and also recognizes a couple of smaller non-TPRA40 proteins, albeit to a lesser extent.

Figures 9A-9B show that endogenous TPRA40 localizes to primary cilia of S12 cells in a Hedgehog-dependent fashion. (A) S12 cells were fixed and processed for immunofluorescence using anti-TPRA40 monoclonal antibody 6H2 (left panels and red channel) and rabbit anti-Aril 3b antibody (a marker for primary cilia in the middle and green channel) following 24 hours serum starvation alone (upper panel) or with Hedgehog stimulation (lower panel). Nuclei stained with DAPI are in blue. (B) Counting the number of Aril3b-positive cilia for TPRA40 staining revealed TPRA40 was found in only about 20% of cilia in the absence of Hedgehog stimulation, but accumulated in 60% of cilia following overnight Hedgehog stimulation. This level of accumulation to cilia is very similar to the extent of accumulation of Smoothened (mean and SD of 2 experiments is shown).

Figure 10 shows that TPRA40 expression inhibited cAMP production in a CRE-luciferase reporter assay. (A) Schematic of a model of TPRA40 as a modulator of cAMP levels in a cell. (B) 293T cells expressing CRE (cAMP Response ElementLuciferase along with GFP (negative control, white diamonds) or a TPRA40 expression construct (black squares) were treated with varying doses of Forskolin, a potent activator of Adenyl Cyclase, thus increasing cAMP levels inside the cells. The graph shows that exogenous expression of TPRA40 suppressed the CRE-reporter activity in a dose dependent manner, suggesting that this GPCR is
coupled to Gα(i), which inhibits cAMP production. The mean and standard deviation of four independent experiments normalized to 20μM forskolin in GFP-transfected cells is shown.

Figure 11A shows that S12 cells transfected with siRNAs to Gα(i)l show reduced Hedgehog signaling compared to NTC treated cells. Knockdown of Gα(i)l with siRNAs decreased Gli-luciferase activity in Hedgehog-treated S12 cells by about 50%, consistent with increased cAMP production stimulating more PKA activity and Gli3R production. Co-expression of siTPRA40 along with siGαα(i)l did not rescue reporter activity compared to siGαα(i)l alone, suggesting that TPRA40 functions at the level of or upstream of Gαα(i)l. Mean and SD of 3 independent experiments is shown. Figure 11B shows that Gli3 depletion is partially rescued by TPRA40 knockdown in S12 cells. S12 cells depleted of Gli3 by siRNA show active Hedgehog signaling in the absence of ligand due to loss of Gli3 repressor (the Gli-luciferase signal is initiated by Gli2 activator). TPRA40 depletion partially inhibits the signal in GH3-depleted cells. Mean and SD of Gli-luciferase signals from 5 independent experiments (in the absence of Hedgehog ligand) were expressed as a percentage of siGli3 alone. Figure 11C shows that TPRA40 depletion does not prevent Gli3 accumulation at cilia tips. The images on the left show a representative immunofluorescence analysis of S12 cells serum starved overnight and treated for one hour (lower two panels) or not (upper two panels) with Hedgehog prior to fixation. Cells were co-stained for the ciliary marker acetylated tubulin (left column and green channel in merged right column) and Gli3 (middle column and red channel in merge). Depletion of TPRA40 by siRNA treatment does not prevent the Hedgehog-dependent accumulation of Gli3 at cilia tips (bottom row). Arrows show the tips of the primary cilia. The number of cilia with and without Gli3 at the tips was counted for each condition and plotted as a percentage of total cilia in the graph (the mean and SD of three independent experiments is shown). The grey bars (the left of each pair of bars) show that less than 10% of cilia have Gli3 at the tips in the absence of Hedgehog stimulation, while the black bars (the right of each pair of bars) show approximately 80% of cilia have Gli3 at their tips irrespective of the presence of TPRA40.

Figure 12 provides a working model for TPRA40 function as a positive regulator of Hedgehog signaling.

Figure 13 shows an alignment of the amino acid sequences of mouse and human TPRA40. Human TPRA40 (top, 373 amino acids, predicted MW 41034 Da; Swissprot Q86W33) is aligned with mouse TPRA40 (middle, 369 amino acids, predicted MW 40560 Da; Swissprot Q99MU1)
and zebrafish TPRA1 (bottom, 378 amino acids, predicted MW 41685 Da; Swissprot Q4V8X0) using the Align program in GSeqWeb. Identical amino acids are colored, and the positions (predicted by Swissprot) of the 7 transmembrane (tni) domains typical of GPCRs are underlined in blue. Mouse and human TPRA share 91.4% identity and 94.1% similarity at the protein level. Zebrafish TPRA1 is 70.0% and 68.7% identical (79.3% and 78.0% similar) to human and mouse TPRA40, respectively, suggesting an evolutionarily conserved function. As expected for a GPCR, the N-terminus is luminal/extracellular and the C-terminus is cytoplasmic. This topography was verified by FACS with epitope tags at each end of the protein (data not shown).

10 DETAILED DESCRIPTION OF THE DISCLOSURE

1. Overview

Hedgehog (Hh) signaling plays an essential role in vertebrate embryonic development, affecting tissue patterning of many organs. All key components of this signaling pathway traffic through primary cilia, with the GPCR GPR161 and Hedgehog receptor Patched exiting cilia, while the GPCR-like protein Smoothened and the SuFu/Gli complex accumulate in cilia in response to Hedgehog ligand. Here we identify a novel orphan G-protein-coupled receptor, TPRA40 (also known as TPRA1 and GPR175), that also localizes to primary cilia upon Hh stimulation and positively regulates Hh signaling downstream of Smoothened. TPRA40 knockout decreases Hh signaling activity as evidenced by decreased Hh-stimulated activity in a (7/i-luciferase reporter (murine S32 cell) assay and decreased elevation of the endogenous early transcriptional target GUI. Furthermore, TPRA40 depletion reduces constitutive (cyclopamine-sensitive) GUI expression in human medulloblastoma (Daoy) cells. Epistasis experiments in S12 cells indicate that TPRA40 acts at the level of Protein Kinase A downstream of SuFu and may be coupled to G alpha(i) since its overexpression inhibits forskolin-mediated cAMP production in CRE-luciferase reporter 293 cells. These data support a role for TPRA40 as a novel positive regulator of the Hh signaling pathway.

The present disclosure is based upon the identification of TPRA40 as a novel component of the hedgehog signaling pathway and as positive regulator of Hedgehog signaling. Based on this identification, the present disclosure provides assays for screening to identify agents that antagonize TPRA40 expression and/or activity and/or localization (e.g., TPRA40 antagonists). TPRA40 antagonists, such as those so identified, may be used to inhibit hedgehog signaling in
any of a number of in vivo and in vitro settings, such as in cells with hyperproliferation or otherwise characterized by unwanted cell proliferation and/or in cells with hyperactive hedgehog signaling (e.g., such as due to a mutation in a component of the hedgehog signaling pathway or due to stimulation by hedgehog protein overexpressed by the cell or by an adjacent cell).

Moreover, the present disclosure provides examples of TPRA40 antagonists, including working examples of specific antagonists and generic and specific examples of other antagonists and classes of antagonists (collectively, "TPRA40 antagonists of the disclosure" or "TPRA40 antagonists described herein"), and provides numerous methods for using TPRA40 antagonists of the disclosure. The disclosure provides numerous methods of using TPRA40 antagonists in vitro and/or in vivo. In certain embodiments, an agent identified as a TPRA40 antagonist using any of the screening assays provided herein may be used in any of the methods of inhibiting cell proliferation and/or hedgehog signaling provided herein.

Thus, it is specifically contemplated that the TPRA40 antagonists of the present disclosure will not only interfere with aspects of hedgehog signal transduction activity (e.g., inhibit hedgehog signaling or decreasing hedgehog signaling), but will likewise be capable of changing the fate of a cell or tissue that is affected by hedgehog signaling, such as cells undergoing normal development or disease states that are characterized by aberrant (e.g., over-expressing) hedgehog signaling. More specifically, the TPRA40 antagonists described herein may be used for inhibiting hedgehog signaling that can occur either (i) as active, wild-type hedgehog signaling or (ii) as a result of hyperactivation of the hedgehog pathway, such as due to mutation or excess hedgehog protein. Disorders resulting from hyperactivation of the hedgehog pathway can be attributed to mutations arising in hedgehog signaling components or inappropriate activation or stimulation that does not result from a mutation or lesion in a hedgehog signaling component such as overexpression of hedgehog ligand(s) (e.g., overexpression of Sonic Hedgehog). One of skill in the art will readily recognize that TPRA40 antagonists are suitable for the treatment of conditions or disorders characterized by hyperactive hedgehog signaling as well as modifying the cell fate during development by suppression of hedgehog signaling.

The present disclosure also provides assays for screening to identify agents that agonize TPRA40 expression and/or activity and/or localization (e.g., TPRA40 agonists). TPRA40 agonists so identified may be used to promote hedgehog signaling in any of a number of in vivo
and/or in vitro settings. It is also specifically contemplated that TPRA40 agonists of the present disclosure can be used to promote hedgehog signal transduction and be capable of changing the fate of a cell or tissue that is affected by hedgehog signaling, such as cells undergoing normal development or disease states that are characterized by aberrant (i.e., under-expressing) hedgehog signaling. Agonists may be useful in promoting hair growth and/or studying hair growth. Merely by way of example, agonists are also useful as reagents for stem cell biology, cell proliferation, cell differentiation, and to study hedgehog signaling.

II. Definitions

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.

A "TPRA40 polypeptide," includes both "native sequence TPRA40 polypeptides" and a TPRA40 polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 91%,
92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of any of SEQ ID NOs: 1-3, but which retains a biological activity of a native TPRA40 polypeptide. In certain embodiments, TPRA40 polypeptides of the disclosure retain a biological activity of native TPRA40, such as the ability to positively regulate hedgehog signaling, the ability to localize to cilia in response to stimulation with hedgehog ligand, and the ability to modulate adenylate cyclase activity. In certain embodiments, the TPRA40 polypeptide varies by about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, or about 20 amino acid residues in length from the corresponding native sequence polypeptide (e.g., SEQ ID NO: 1, 2, or 3). Alternatively, in certain embodiments, the TPRA40 polypeptide has no more than one amino acid substitution, such as a conservative substitution, as compared to the corresponding native polypeptide sequence, alternatively no more than 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid substitutions, such as conservative substitutions, as compared to the native polypeptide sequence.

A "native sequence TPRA40 polypeptide" comprises a polypeptide having the same amino acid sequence as the corresponding TPRA40 polypeptide derived from nature. Such native sequence TPRA40 polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence TPRA40 polypeptide" specifically encompasses naturally-occurring truncated or secreted forms of the specific TPRA40 polypeptide (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the polypeptide. In one embodiment, the native sequence TPRA40 polypeptides disclosed herein are mature or full-length native sequence polypeptides corresponding to the sequence of any of SEQ ID NOs: 1-3, in the presence or absence of the N-terminal methionine.

"TPRA40 polypeptide variant" means a TPRA40 polypeptide, preferably active forms thereof, as defined herein, having at least about 80% amino acid sequence identity with a full-length native TPRA40 polypeptide sequence, respectively, as disclosed herein, and variant forms thereof lacking one or more of the C-terminal domain, an extracellular domain, a cytoplasmic domain, an N-terminal domain or any other fragment of a full length native sequence TPRA40 polypeptide, such as those referenced herein. Such variant polypeptides include, for instance, polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the full-length native amino acid sequence. In a specific aspect, such variant polypeptides will have at least about 80% amino acid sequence identity, alternatively at least
about 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,
96%, 97%, 98%, or 99% amino acid sequence identity, to a full-length native sequence TPRA40
polypeptide (e.g., any of SEQ ID NOs: 1-3), as disclosed herein, and variant forms thereof
lacking an extracellular domain, or any other fragment of a full length native sequence TPRA40
polypeptide, such as those disclosed herein. In certain embodiments, such variant polypeptides
will vary at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100
amino acids in length from the corresponding native sequence. Alternatively, in certain
embodiments, the TPRA40 polypeptide has no more than one amino acid substitution, such as a
conservative substitution, as compared to the corresponding native polypeptide sequence,
alternatively no more than 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid substitutions, such as
conservative substitutions, as compared to the native polypeptide sequence.

"Percent (%) amino acid sequence identity" with respect to the TPRA40 polypeptide
sequences identified herein is defined as the percentage of amino acid residues in a candidate
sequence that are identical with the amino acid residues in the specific TPRA40 polypeptide
sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the
maximum percent sequence identity, and not considering any conservative substitutions as part
of the sequence identity. Alignment for purposes of determining percent amino acid sequence
identity can be achieved in various ways that are within the skill in the art, for instance, using
publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign
(DNASTAR) software. Those skilled in the art can determine appropriate parameters for
measuring alignment, including any algorithms needed to achieve maximal alignment over the
full length of the sequences being compared. For purposes herein, however, % amino acid
sequence identity values are generated using the sequence comparison computer program
ALIGN-2 sequence comparison computer program was authored by Genentech, Inc. and the
source code has been filed with user documentation in the U.S. Copyright Office, Washington
D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU5 10087. The
ALIGN-2 program is publicly available through Genentech, Inc., South San Francisco,
California. The ALIGN-2 program should be compiled for use on a UNIX operating system,
preferably digital UNIX V4.0D. All sequence comparison parameters used with ALIGN-2 are
set by the ALIGN-2 program and do not vary.
"TPRA40 variant polynucleotide" or "TPRA40 variant nucleic acid sequence" means a nucleic acid molecule which encodes a TPRA40 polypeptide, preferably active forms thereof, as defined herein, and which have at least about 80% nucleic acid sequence identity with a nucleotide acid sequence encoding a full-length native sequence TPRA40 polypeptide sequence identified herein, or any other fragment of the respective full-length TPRA40 polypeptide sequence as identified herein (such as those encoded by a nucleic acid that represents only a portion of the complete coding sequence for a full-length TPRA40 polypeptide).

Ordinarily, TPRA40 variant polynucleotide will have at least about 80% nucleic acid sequence identity, alternatively at least about 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity with a nucleic acid sequence encoding the respective full-length native sequence TPRA40, or any other fragment of the respective full-length TPRA40 polypeptide sequence identified herein. Such variant polynucleotides do not encompass the native nucleotide sequence. Ordinarily, such variant polynucleotides vary at least about 50 nucleotides in length from the native sequence polypeptide, alternatively the variance can be at least about 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, 601, 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.

"Percent (%) nucleic acid sequence identity" with respect to TPRA40 polypeptide encoding nucleic acid sequences identified herein, is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in the TPRA40 nucleic acid sequence of interest, respectively, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. For purposes herein, however, % nucleic acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The
ALIGN-2 sequence comparison computer program was authored by Genentech, Inc. and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXUS 10087. The align-2 program is publicly available through Genentech, Inc., South San Francisco, California. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters used with ALIGN-2 are set by the ALIGN-2 program and do not vary. In situations where ALIGN-2 is employed for nucleic acid sequence comparisons, the % nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given nucleic acid sequence D (which can alternatively be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence D) is calculated as follows:

\[
\text{Identity} = \frac{W}{Z} \times 100
\]

where W is the number of nucleotides scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of C and D, and where Z is the total number of nucleotides in D. It will be appreciated that where the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

In other embodiments, TPRA40 variant polynucleotides are nucleic acid molecules that encode TPRA40 polypeptides, and which are capable of hybridizing, preferably under stringent hybridization and wash conditions, to nucleotide sequences encoding a full-length TPRA40 polypeptide, as disclosed herein. Such variant polypeptides may be those that are encoded by such variant polynucleotides.

"Isolated" means identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for an agent.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is
operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

"Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995).

"Stringent conditions" or "high stringency conditions", as defined herein, may be identified by those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) overnight hybridization in a solution that employs 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with a 10 minute wash at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) followed by a 10 minute high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.
"Moderately stringent conditions" may be identified as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Press, 1989, and include the use of washing solution and hybridization conditions (e.g., temperature, ionic strength and %SDS) less stringent that those described above. An example of moderately stringent conditions is overnight incubation at 37°C in a solution comprising: 20% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1 x SSC at about 37-50°C. The ordinarily skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

"Active" or "activity", when context indicates such term is used to refer to a TPRA40 polypeptide, refers to form(s) of a TPRA40 polypeptide which retain a biological and/or an immunological activity of native or naturally-occurring TPRA40 polypeptide, wherein "biological" activity refers to a biological function caused by a native or naturally-occurring TPRA40 polypeptide, other than the ability to induce the production of an antibody against an antigenic epitope possessed by a native or naturally-occurring TPRA40 polypeptide, and an "immunological" activity refers to the ability to induce the production of an antibody against an antigenic epitope possessed by a native or naturally-occurring TPRA40 polypeptide. Exemplary biological activities of TPRA40 are described herein. "Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterforts such as sodium; and/or nonionic surfactants such as TWEEN®, polyethylene glycol (PEG), and PLURONICS®. Pharmaceutical compositions or formulations,
such as compositions or formulations of the disclosure comprising a TPRA40 antagonist of the disclosure, may be formulated with one or more carriers and/or excipients.

By "solid phase" or "solid support" is meant a non-aqueous matrix to which a molecule that binds TPRA40 polypeptide of the present disclosure, or to which a TPRA40 polypeptide fragment (e.g., the C-terminal region of the TPRA40 polypeptide) can adhere or attach. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate: in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles.

An "effective amount" of a TPRA40 antagonist agent is an amount sufficient to inhibit, partially or entirely, hedgehog signaling that is dependent upon stimulation from hedgehog or that is due to one or more mutations in a gene in the hedgehog signaling pathway (e.g. smoothened or patched). Alternatively or additionally, an effective amount of TPRA40 antagonist is an amount sufficient to reduce the rate of proliferation of a cell and/or rate of survival of a cell and/or the rate of growth of a cell that is expressing or overexpressing hedgehog or that has active or hyperactive hedgehog signaling. Alternatively or additionally, an effective amount of TPRA40 antagonist is an amount sufficient to decrease or halt the growth, proliferation, and/or survival of a tumor, such as a tumor responsive to hedgehog signaling, characterized by hyperactive hedgehog signaling, or comprising a mutation in one or more components of the hedgehog signaling pathway. An "effective amount" may be determined empirically and in a routine manner, in relation to this purpose. In some embodiments, the effective amount is determined with respect to the amount of a TPRA40 antagonist 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 and/or to inhibit hedgehog signaling in a cell by at least 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%. In some embodiments, the effective amount is determined with respect to the amount of a TPRA40 antagonist sufficient to reduce the rate of proliferation of a cell and/or rate of survival and/or rate of growth 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 some embodiments, the effective amount is determined with respect to the amount of a TPRA40 antagonist sufficient to reduce \( G_{li} \) expression in at least 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of cells in a cell culture and/or to inhibit hedgehog signaling in a cell by at 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%. The term "effective amount" also refers to a TPRA40 antagonist or other drug effective to "treat" a disease or disorder in a subject or mammal. In the case of hedgehog signaling, the effective amount of the drug will improve aberrant hedgehog signaling such that it is closer to normal physiological levels; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) the infiltration of tumor cells into peripheral tissue or organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the tumor or cancer. See the definition herein of "treating". To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic.

A "growth inhibitory amount" of a TPRA40 antagonist is an amount capable of inhibiting the growth of a cell, especially tumor, e.g., cancer cell, either in vitro or in vivo. For purposes of inhibiting neoplastic cell growth, such an amount may be determined empirically and in a routine manner.

A "cytotoxic amount" of a TPRA40 antagonist is an amount capable of causing the destruction of a cell, especially a tumor cell, e.g., cancer cell, either in vitro or in vivo. For purposes of inhibiting neoplastic cell growth may be determined empirically and in a routine manner.

The term "TPRA40 antagonist" refers to an agent that inhibits the expression and/or activity and/or localization of TPRA40 by: (i) binding to TPRA40 DNA, RNA, or protein, and/or (ii) disrupting the interaction between TPRA40 and a TPRA40 target or binding partner, and/or (iii) modulating TPRA40 activity downstream of Suppressor of Fused (SuFu) or Smoothened (Smo). In certain embodiments, the activity of TPRA40 that is decreased or inhibited by the TPRA40 antagonist is the activity of TPRA40 as a positive regulator of hedgehog signaling (e.g., the TPRA40 antagonist inhibits or antagonizes hedgehog signaling). In some embodiments, the TPRA40 antagonist inhibits the transport of TPRA40 polypeptide to the plasma membrane and/or cilia. In some embodiments, the TPRA40 antagonist inhibits a biological function of the TPRA40 polypeptide. In some embodiments, the TPRA40 antagonist inhibits expression of
TPRA40 RNA or protein. In some embodiments, the TPRA40 antagonist prevents interaction between TPRA40 polypeptide with the Gaipha-i protein. In some embodiments, the TPRA40 antagonist inhibits TPRA40-mediated inhibition of Protein Kinase A. In some embodiments, the TPRA40 antagonist induces an increase in cAMP levels in a cell. The disclosure provides working examples of agents that are TPRA40 antagonists, as well as numerous other specific and generic examples of such TPRA40 antagonist agents and categories of agents (collectively, "TPRA40 antagonists of the disclosure" or "a TPRA40 antagonists of the disclosure").

The term "TPRA40 antagonist" expressly includes, in certain embodiments, TPRA40 polypeptide variants (e.g. TPRA40 polypeptide variants that bind the ligand bound by TPRA40 or that interact with Ssnal but do not promote hedgehog signaling), anti-TPRA40 antibodies (e.g., antibodies that bind to the C-terminal 84 amino acids of TPRA40 polypeptide or to any of the extracellular portions of the TPRA40 polypeptide), TPRA40-binding antibody fragments thereof, TPRA40 antigen binding fragments, TPRA40-binding oligopeptides (e.g., oligopeptides that bind to the ligand binding site of TPRA40 to prevent ligand binding, or that bind to the C-terminal region of TPRA40), polynucleotides that inhibit TPRA40 expression (e.g., TPRA40 sense/antisense nucleic acid and/or TPRA40 RNAi), and/or TPRA40 binding small organic molecules (e.g., small organic molecules that bind to the ligand binding site of TPRA40 and prevent ligand binding or that bind to TPRA40 and interfere with protein-protein interactions or trafficking). A "TPRA40 antagonist polypeptide" includes an anti-TPRA40 antibody, an antagonist TPRA40 chimeric polypeptide and a TPRA40 binding oligopeptide. Methods for identifying TPRA40 antagonists may comprise contacting the TPRA40 polypeptide, including a cell expressing it, with a candidate agonist or antagonist molecule and measuring a detectable change in one or more biological activities normally associated with the TPRA40 polypeptide, e.g., ability to inhibit adenyl cyclase activity (e.g., also known in the art and referred to interchangeably as adenylate cyclase activity) and/or to promote hedgehog signaling. In certain embodiments, the TPRA40 antagonist is an antagonist of hedgehog signaling.

The term "TPRA40 agonist" refers to an agent that activates, maintains or potentiates the expression and/or activity of TPRA40 and/or facilitates the transport of TPRA40 to the plasma membrane or cilia by: (i) binding to TPRA40 DNA, RNA, or protein, and/or (ii) facilitating the interaction between TPRA40 and a TPRA40 target or binding partner, and/or (iii) modulating
TPRA40 activity downstream of Suppressor of Fused (SuFu) or Smoothened (Smo). In certain embodiments, the TPRA40 agonist promotes (e.g., agonizes) hedgehog signaling.

A TPRA40 antagonist "which binds" a target of interest, e.g. TPRA40, is one that binds the target with sufficient affinity so as to be a useful diagnostic, prognostic and/or therapeutic agent. In certain embodiments, the antagonist does not significantly cross-react with other proteins. Moreover, the term "specific binding" or "specifically binds to" or is "specific for" or "specifically targets", means binding that is measurably different from a non-specific interaction. This concept may be similarly used when referring to binding or targeting of a nucleic acid antagonist to its target. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding can be determined by competition with a control molecule that is similar to the target, for example, an excess of non-labeled target. In this case, specific binding is indicated if the binding of the labeled target to a probe is competitively inhibited by excess unlabeled target. In one embodiment, such terms refer to binding where a molecule binds to a particular polypeptide or epitope on a particular polypeptide without substantially binding to any other polypeptide or polypeptide epitope. Alternatively, such terms can be described by a molecule having a Kd for the target of at least about $10^{-4}$ M, $10^{-5}$ M, $10^{-6}$ M, $10^{-7}$ M, $50^{-8}$ M, $10^{-9}$ M, $10^{-10}$ M, $10^{-11}$ M, $10^{-12}$ M, or greater.

A 'TPRA40- hyperactive tumor or cancer" produces excessive levels of TPRA40, such that hedgehog signaling is active or hyperactive, such that a TPRA40 hedgehog antagonist can bind thereto or otherwise target and have a therapeutic effect with respect to the tumor.

A tumor that "overexpresses" hedgehog or in which hedgehog signaling is "hyperactive" is one which has significantly higher levels of hedgehog at the cell surface thereof, or that produces and secretes, compared to a noncancerous cell of the same tissue type, or that has more active hedgehog signaling and/or has hedgehog signaling that is not dependent on the presence of Hedgehog protein, as compared to a noncancerous cell of the same tissue type. Such overexpression or hyperactivity may result from gene amplification or by increased transcription or translation of certain hedgehog pathway genes or by mutation in a component of the hedgehog signaling pathway. In some embodiments, the hyperactive hedgehog signaling is because the cell or an adjacent cell overexpresses hedgehog protein, such as Sonic hedgehog protein. In
some embodiments, the hyperactive hedgehog signaling is due to a mutation in a gene in the hedgehog pathway (e.g., a component). In some embodiments, the mutated gene is in any of the patched, smoothened, or SuFu, genes. In certain embodiments, hyperactive hedgehog signaling is because the cell comprises a smoothened gain-of-function mutation. In certain embodiments, hyperactive hedgehog signaling is because the cell comprises a Suppressor of Fused (SuFu) loss-of-function mutation. In certain embodiments, the cell comprises one or more mutations in a hedgehog pathway gene, and hyperactive hedgehog signaling is signaling that is not dependent on the presence and/or concentration of hedgehog ligand.

The "growth state" of a cell refers to the rate of proliferation of the cell and/or the state of differentiation of the cell. An "altered growth state" is a growth state characterized by an abnormal rate of proliferation, e.g., a cell exhibiting an increased or decreased rate of proliferation relative to a normal cell.

The term "hedgehog" or "hedgehog polypeptide" (Hh) is used herein to refer generic-ally to any of the mammalian homologs of the Drosophila hedgehog, i.e., sonic hedgehog (SHh), desert hedgehog (SHh) or Indian hedgehog (IHh). The term may be used to describe protein or nucleic acid.

The terms "hedgehog signaling pathway", "hedgehog pathway", "hedgehog signaling" and "hedgehog signal transduction pathway" as used herein, interchangeably to refer to the signaling cascade mediated by hedgehog and its receptors (e.g., patched, patched-2) and which results in changes of gene expression and other phenotypic changes typical of hedgehog activity. The hedgehog pathway may, in certain embodiments, be activated or potentiated in the absence of hedgehog through activation of a downstream component (e.g., overexpression of Smoothened, loss of function of patched, loss of function of Suppressor of fused, gain of function of smoothened, or transfections with Smoothened or Patched mutants to result in constitutive activation with activate hedgehog signaling in the absence of hedgehog). The transcription factors of the Gl family are often used as markers or indicators of hedgehog pathway activation. Hedgehog signaling is dysregulated in some cancers, sometimes due to mutations in a hedgehog pathway gene.

The term "Hh signaling component" or "component of the hedgehog signaling pathway" refers to gene and/or protein products that participate in the Hh signaling pathway (e.g., Shh, Smo, Ptc, Gli, and SuFu). A Hh signaling component frequently materially or substantially
affects the transmission of the Hh signal in cells or tissues, thereby affecting the downstream
gene expression levels and/or other phenotypic changes associated with hedgehog pathway
activation.

Each Hh signaling component, depending on their biological function and effects on the
final outcome of the downstream gene activation or expression, can be classified as either
positive or negative regulators. A positive regulator is a Hh signaling component that positively
affects the transmission of the Hh signal, e.g., stimulates downstream biological events when Hh
is present (e.g., stimulates or promotes GUI expression). A negative regulator is a Hh signaling
component that negatively affects the transmission of the Hh signal, e.g., inhibits downstream
biological events when Hh is present (e.g., inhibits or decreases Gli1 expression). In some
embodiments, a Hh signaling component may be determined to be a positive or negative
regulator of Hh signaling by inhibiting or expressing/overexpressing/activating the signaling
component and monitoring GUI transcription. In these embodiments, if inhibition of the
signaling component results in increased GUI transcription, then the signaling component is a
negative regulator of Hh signaling, and if inhibition of the signaling component results in
decreased GUI transcription, then the signaling component is a positive regulator of Hh signaling.
And, if expression/overexpression/activation of a signaling component results in increased GUI
transcription, then the signaling component is a positive regulator of Hh signaling, and if
expression/overexpression/activation of a signaling component results in decreased GUI
transcription, then the signaling component is a negative regulator of Hh signaling.

The term "patched loss-of-function" refers to an aberrant modification or mutation of a
Ptch gene, or a decreased expression level of the gene, which results in a phenotype that
resembles contacting the cell with a hedgehog protein, e.g., aberrant activation of a hedgehog
pathway. The loss-of-function may include a loss of the ability of the ptch gene product to
regulate the expression level of the transcription factors Gli1, Gli2 and/or Gli3.

The term "proliferating" and "proliferation" refer to a cell or cells undergoing mitosis.

The term "smoothened gain-of-function" refers to an aberrant modification or mutation of a
Smo gene, or in the ability of a Ptch gene product to bind to Smo and thereby suppress
hedgehog signaling, which results in a phenotype that resembles activating the hedgehog
pathway with hedgehog, e.g., aberrant activation of a hedgehog pathway.
III. TPRA40 Antagonists

i- TPRA40 and TPRA40 Antagonists

TPRA40 (also known as TPRA1 or GPR175) is an orphan G-protein coupled receptor whose physiological functions were previously unknown. TPRA40 is a 40 kDa protein having seven transmembrane domains (Fujimoto et al., 2001, Biochim Biophys Acta, 1518(1-2): 173-7) and an 84-amino acid cytoplasmic region (Fujimoto et al.). TPRA40 has been shown to be expressed during oxidative stress, aging and under certain pathophysiological conditions (Aki et al., 2008, J Cell Physiol, 217(1): 194-206). The role of TPRA40 in any particular signaling pathway was previously unknown. In addition, it was previously unknown whether TPRA40 was associated with any disease conditions, such as cancer, although it may be elevated in obese and diabetic mice models (Yang et al., 1999, Endocrinol 140: 2859). The present disclosure demonstrates for the first time a role for TPRA40 as a component of the hedgehog signaling pathway. Specifically, the disclosure demonstrates that TPRA40 is a positive regulator of hedgehog signaling, and that inhibition of TPRA40 activity results in inhibition of the hedgehog signaling pathway. In addition, the present disclosure provides data showing that TPRA40 acts downstream of both Smoothened and SuFu, and that TPRA40 interacts with a Galphai protein to inhibit adenylyl cyclase. As aberrant hedgehog signaling is involved in a number of disorders (e.g., cancer, psoriasis and trichosis), TPRA40 antagonists would be useful in treating these diseases, in modulating hedgehog signaling in vitro and/or in vivo, and in modulating cell growth, proliferation, and or survival in vitro and/or in vivo.

The present disclosure provides TPRA40 antagonists. As defined herein, TPRA40 antagonist refers to an agent that inhibits the expression and/or activity and/or localization of TPRA40 by: (i) binding to TPRA40 DNA, RNA, or protein, and/or (ii) disrupting the interaction between TPRA40 and a TPRA40 target or binding partner, and/or (iii) modulating TPRA40 activity downstream of Suppressor of Fused (SuFu) or Smoothened (Smo). In other words, a TPRA40 antagonist of the disclosure inhibits the expression and/or activity and/or localization of TPRA40 to antagonize one or more biological activities of native TPRA4Q. In certain embodiments, the activity of TPRA40 that is decreased or inhibited by the TPRA40 antagonist is the activity of TPRA40 as a positive regulator of hedgehog signaling (e.g., the TPRA40 antagonist inhibits or antagonizes hedgehog signaling). In some embodiments, the TPRA40 antagonist inhibits the transport of TPRA40 polypeptide to the plasma membrane and/or cilia. In
some embodiments, the TPRA40 antagonist inhibits a biological function of the TPRA40 polypeptide. In some embodiments, the TPRA40 antagonist inhibits expression of TPRA40 RNA or protein. In some embodiments, the TPRA40 antagonist prevents interaction between TPRA40 polypeptide with the Galpha-i protein. In some embodiments, the TPRA40 antagonist inhibits TPRA40-mediated inhibition of Protein Kinase A. In some embodiments, the TPRA40 antagonist induces an increase in cAMP levels in a cell. The disclosure provides working examples of agents that are TPRA40 antagonists, as well as numerous other specific and generic examples of such TPRA40 antagonist agents and categories of agents (collectively, "TPRA40 antagonists of the disclosure" or "a TPRA40 antagonist of the disclosure").

The term expressly includes TPRA40 polypeptides variants (e.g. TPRA40 polypeptide variants that bind the ligand bound by TPRA40 but do not promote downstream hedgehog signaling), anti-TPRA40 antibodies (e.g., antibodies that bind to an epitope within the C-terminal 84 amino acids of TPRA40 polypeptide or to an epitope that includes a portion of the C-terminal 84 amino acids of TPRA40, or an antibody to any extracellular portion of the TPRA40 polypeptide), TPRA40-binding antibody fragments thereof, TPRA40-binding antigen binding fragments, TPRA40-binding oligopeptides (e.g., oligopeptides that bind to the ligand binding site of TPRA40 to prevent ligand binding, or that bind to the C-terminal region of TPRA40), TPRA40 sense/antisense nucleic acid, TPRA40 binding small molecules (e.g., small organic molecules that bind to TPRA40 and inhibit TPRA40 activity), and/or polynucleotides that inhibit TPRA40 expression (e.g., RNAi, antisense oligonucleotides).

In some embodiments, the TPRA40 antagonist inhibits TPRA40 bioactivity. In some embodiments, TPRA40 bioactivity refers to its role in facilitating hedgehog signaling. In some embodiments, TPRA40 bioactivity refers to the ability to promote G\(\text{UI}\) expression. In some embodiments, TPRA40 bioactivity is inhibition of adenyly cyclase. In some embodiments, TPRA40 bioactivity is inhibition of Protein Kinase A. As such, in some embodiments, the TPRA40 antagonists inhibit the hedgehog signaling pathway, inhibit G\(\text{UI}\) expression and/or release the inhibition of adenyly cyclase and/or Protein Kinase A.

In some embodiments, the TPRA40 antagonist is for use in treating a cell. In some embodiments, the cell is responsive to hedgehog protein or comprises one or more mutations in a hedgehog signaling pathway gene (e.g., one or more mutations in 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. In some embodiments, the TPRA40 antagonist is contacted with the cell in an amount effective to inhibit hedgehog signaling, inhibit \textit{GUI} expression and/or release the inhibition of adenyllyl cyclase and/or Protein Kinase A. In some embodiments, the effective amount of the TPRA40 antagonist needed to inhibit hedgehog signaling or inhibit \textit{GUI} expression is determined by assessing the amount of TPRA40 antagonist needed to reduce Gli1 protein levels (\textit{e.g.}, by SDS-PAGE) and/or Gli1 mRNA transcript levels (\textit{e.g.}, by RT-PCR or Northern Blot) in the treated cell or cells. In some embodiments, the effective amount of the TPRA40 antagonist inhibits Gli1 protein levels and/or Gli1 mRNA transcript by at least 10\%, at least 20\%, at least 30\%, at least 40\%, at least 50\%, at least 60\%, at least 70\%, at least 80\%, at least 90\%, or at least 100\% as compared to an untreated control cell or cells. In some embodiments, the effective amount of the TPRA40 antagonist needed to release the inhibition of adenyllyl cyclase and/or Protein Kinase A activity is monitored by assessing cAMP levels in the treated cell or cells. In some embodiments, the effective amount of the TPRA40 antagonist increases cAMP levels in the treated cell or cells by at least 10\%, at least 20\%, at least 30\%, at least 40\%, at least 50\%, at least 60\%, at least 70\%, at least 80\%, at least 90\%, or at least 100\%, at least 150\%, or at least 200\% as compared to an untreated control cell or cells. In some embodiments, the effective amount of the TPRA40 antagonist needed to release the inhibition of adenyllyl cyclase and/or Protein Kinase A activity is monitored by assessing the expression levels of a gene under the control of a cAMP Response Element (CRE) in the treated cell or cells. In some embodiments, the effective amount of the TPRA40 antagonist increases expression levels of the CRE-controlled gene by at least 10\%, at least 20\%, at least 30\%, at least 40\%, at least 50\%, at least 60\%, at least 70\%, at least 80\%, at least 90\%, or at least 100\%, at least 150\%, or at least 200\% as compared to an untreated control cell or cells. In some embodiments, the gene under the control of the cAMP Response Element is a transgenic reporter gene. In some embodiments, the reporter gene is luciferase. In some embodiments, the cell or cell contacted with the TPRA40 antagonist is treated with an agent to induce adenyllyl cyclase (\textit{e.g.,} forskolin, 8-bromo-cAMP or dibutryl-cAMP) as well as contacting the cell or cells with the TPRA40 antagonist.

In some embodiments, the TPRA40 antagonist inhibits processes associated with active hedgehog signaling, \textit{e.g.,} cell proliferation. In some embodiments, the effects of the TPRA40 antagonist on cell proliferation may be monitored by using any of the standard cell proliferation
assays known in the art. In some embodiments, the assay measures the rate of DNA synthesis in cell populations (e.g., using \(^{3}\)H-TdR Proliferation or BrDU incorporation assays). In some embodiments, the assay measures cell viability (e.g., by using an MTT, XTT, CelTiterGlo or WST-1 assay). In some embodiments, the assay measures plasma membrane damage/leakage (e.g., by means of a trypan blue exclusion assay or propidium idodide exclusion assay).

In some embodiments, the TPRA40 antagonist inhibits the growth and/or proliferation of tumor cells. In some embodiments, compositions for use in treatment comprise growth inhibitory amounts of at least one type of TPRA40 antagonist (e.g., anti-TPRA antibody), so as to inhibit growth of tumor cells by greater than 20%, preferably from about 20% to about 50%, and even more preferably, by greater than 50% (e.g., from about 50% to about 100%) as compared to the appropriate control. A "growth inhibitory" amount of a TPRA40 antagonist is one which results in measurable growth inhibition of cancer cells expressing/overexpressing hedgehog and/or expressing/overexpressing the TPRA40 polypeptide. In one embodiment, growth inhibition can be measured at a molecule concentration of about 0.1 to 30 \(\mu\)g/ml or about 0.5 iM to 200 tiM in cell culture, where the growth inhibition is determined 1-10 days after exposure of the tumor cells to the antibody. Growth inhibition of tumor cells in vivo can be determined in various ways such as is described in the Experimental Examples section below. An amount of any of the TPRA40 antagonists disclosed herein is growth inhibitory in vivo if administration of such molecule at about 1 jig/kg to about 100 mg/kg body weight results in reduction in tumor size or tumor cell proliferation within about 5 days to 3 months from the first administration of the antibody, preferably within about 5 to 30 days.

In one embodiment, the TPRA40 antagonists disclosed herein induce apoptosis. A TPRA40 antagonist which "induces apoptosis" is one which induces programmed cell death of a cell (e.g., a tumor cell) as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dialation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies) or by monitoring caspase activity or cleavage. The cell is usually one which overexpresses a hedgehog polypeptide and/or that has active hedgehog signaling. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering; and nuclear/chroniatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid
cells; caspase activity can be assayed using caspase substrate kits or by western blotting for cleaved caspases or PARP. In some embodiments, the TPRA40 antagonist which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cells in an annexin binding assay.

A TPRA40 antagonist which "induces cell death" is one which causes a viable cell (e.g., a tumor or cancer cell) to become nonviable. Such a cell is one which has active hedgehog signaling and/or which expresses a hedgehog polypeptide (and in some cases overexpresses it) and which expresses a TPRA40 polypeptide (and in some cases overexpresses it) as compared to a non-diseased cell. The ability to induce cell death can be assessed, for example, relative to untreated cells by suitable techniques, such as loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. Cytotechnology 17:1-11 (1995)) or 7AAD. In some embodiments, cell death-inducing TPRA40 antagonists are those which induce PI uptake in the PI uptake assay.

In some embodiments, the TPRA40 antagonist may be used to treat a subject in need thereof, comprising the step of administering the TPRA40 antagonist to the subject. In some embodiments, the TPRA40 antagonists may be used to treat a subject suffering from any of the diseases or disorders described herein. In some embodiments, any of the TPRA40 antagonists described herein may be used for inhibiting hedgehog signaling or inhibiting cell proliferation (particularly unwanted cell proliferation), growth, or increasing survival in a subject in need thereof. In some embodiments, the TPRA40 is for use in treating unwanted cell proliferation in a subject. In some embodiments, the unwanted cell proliferation (or unwanted cell growth) is cancer. In some embodiments, the TPRA40 antagonist is for use in inhibiting unwanted angiogenesis in a subject. In some embodiments, the unwanted angiogenesis is associated with a tumor. In some embodiments, the TPRA40 antagonist is for use in treating a skin disease (e.g., psoriasis, acne) in a subject. In some embodiments, the TPRA40 antagonist is an antibody (e.g., an antibody, antigen binding fragment or an immunoconjugate), a TPRA40-binding oligopeptide, a TPRA40 polypeptide variant, a polynucleotide antagonist (e.g., a sense/antisense nucleic acid or an RNAi molecule), or a small molecule. The disclosure contemplates, in certain embodiments, that any of the TPRA40 antagonists described herein (TPRA40 antagonists of the disclosure) may be suitable for use in any of the in vitro or in vivo methods of the disclosure.
The disclosure provides numerous examples of specific agents and categories of agents that are TPRA40 antagonists (e.g., TPRA40 antagonists of the disclosure). The disclosure contemplates numerous methods for using TPRA40 antagonists in vitro or in vivo, as well as using TPRA40 antagonists in assays and as reagents to identify other components of the hedgehog signaling pathway and/or natural ligands or binders of TPRA40. The disclosure contemplates that any of the TPRA40 antagonists of the disclosure (e.g., specific antagonists or categories of antagonists) may be used in any of the methods described herein. The disclosure contemplates that any of the structural and functional features of TPRA40 antagonists may be combined and used in combination with any of the features of the methods described herein.

ii. Screening for TPRA40 Hedgehog Antagonists

In some embodiments, the disclosure provides for a method of screening for a TPRA40 antagonist. 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 of candidate 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, any of the agents identified as TPRA40 antagonists in the screening methods described herein can be further assessed in an additional assay (e.g., a hedgehog signaling assay (e.g., by using any of the GU1 expression assays described herein or known in the art to examine Glil nucleic acid or protein expression in response to an agent), a TPRA40 activity assay (e.g., by using any of the adenyiyl cyclase assays described herein or known in the art), a TPRA40 binding assay (e.g., by using any of the TPRA40 binding assays described herein, such as with FACS antibody screen), a cell proliferation assay (e.g., by using any of the cell proliferation assays described herein or known in the art).

In some embodiments, the disclosure provides for a method of screening for a TPRA40 antagonist, wherein the method comprises: a) contacting a cell that expresses TPRA40 and adenyiyl cyclase with an agent; b) determining, as compared to an untreated control, whether the agent rescues (e.g., increases; relieves inhibition) adenyiyl cyclase activity, wherein if the agent rescues (e.g., increases) adenyiyl cyclase activity relative to the control, then an agent is
identified as a TPRA40 antagonist. In some embodiments, the disclosure provides for a method of identifying a TPRA40 inhibitor or antagonist, comprising: a) providing a cell that expresses TPRA40 and that expresses a reporter construct to indicate adenylyl cyclase activity; b) contacting the cell with an activator of adenylyl cyclase and with an agent, wherein the cells are contacted with the activator and the agent simultaneously, concurrently, or consecutively; and c) determining, as compared to a control, whether the agent rescues (e.g., increases; relieves inhibition) the adenylyl cyclase activity induced by the activator, wherein if the agent increases the adenylyl cyclase activity relative to the non-agent treated control, then the agent is identified as a TPRA40 inhibitor or antagonist. In some embodiments, the cell or cells used in this method are contacted with a compound that induces adenylyl cyclase activity (e.g., forskolin, 8-bromo-cAMP or dibutyryl-cAMP) prior to step a). In some embodiments, a reporter gene is used in order to determine whether adenylyl cyclase activity has been inhibited by an agent. In some embodiments, the reporter gene is a luciferase gene controlled by a cAMP response element (CRE). In these embodiments, inhibition of adenylyl cyclase by active TPRA40 will result in a reduction in cAMP levels and, therefore, a corresponding reduction in luciferase expression. An agent that inhibits TPRA40 would permit adenylyl cyclase to regain activity (e.g., in the presence of forskolin, 8-bromo-cAMP or dibutyryl-cAMP), thereby resulting in an increase in cAMP levels and luciferase expression. In some embodiments, the agent identified in step b) is further tested to determine whether or not it binds to TPRA40. In some embodiments, the identified in step b) is further tested to determine whether or not it inhibits hedgehog signaling. In some embodiments, the agent identified in step b) is further tested to determine whether or not it inhibits proliferation, growth or survival of a cancer cell.

In some embodiments, the disclosure provides for a method of identifying a TPRA40 inhibitor, wherein the method comprises: a) screening for an agent that binds to TPRA40 protein; b) contacting a cell with an amount of the agent identified in step a), 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, and c) determining, as compared to a control, whether the agent that binds to TPRA40 protein also inhibits hedgehog signaling in the cell, wherein if the agent inhibits hedgehog signaling in the cell relative to the control, then the agent is identified as a TPRA40 inhibitor.
In some embodiments, the disclosure provides for a method of screening for an agent for inhibiting the proliferation, growth or survival of a cancer cell, wherein the method comprises: a) screening for an agent that binds to TPRA40 protein, reduces expression of TPRA40, inhibits transport of TPRA40 protein to the plasma membrane or to primary cilia, prevents activation of TPRA40 or uncouples TPRA40 from Gai, or inhibits TPRA40 inhibition of adenylyl cyclase; b) contacting a cancer cell with an amount of the agent identified in step a), wherein the cancer 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, and c) determining, as compared to a control, whether the agent inhibits the proliferation or growth of the cancer cell, wherein if the agent inhibits cell proliferation or growth relative to the control, then an agent that inhibits the proliferation or growth of the cancer cell is identified.

In some embodiments, the disclosure provides for a method of screening for an agent for inhibiting hedgehog signaling in a cell, wherein the method comprises: a) screening for an agent that binds to TPRA40 protein, reduces expression of TPRA40, inhibits transport of TPRA40 protein to the plasma membrane or to primary cilia, prevents activation of TPRA40 or uncouples TPRA40 from Gai, or inhibits TPRA40 inhibition of adenylyl cyclase; b) contacting a cell with an amount of the agent identified in step a), 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, and c) determining, as compared to a control, whether the agent inhibits hedgehog signaling in the cell, wherein if the agent inhibits hedgehog signaling in the cell relative to the control, then an agent that inhibits hedgehog signaling is identified.

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 metastasis cancer cell, or a cancer cell line.

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 TPRA40. 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 smoothened mutation is a mutation at a position corresponding to position 535 of SEQ ID NO: 42. In certain embodiments, the mutation is a mutation at a position corresponding to position 562 of SEQ ID NO: 42. In certain embodiments, the mutation is W535L at position 535 or at that corresponding position in SEQ ID NO: 42. In some embodiments, the smoothened mutation is a mutation corresponding to position R562Q of SEQ ID NO: 42 (a R562Q mutation at position 562 or at a position corresponding to position 562 of SEQ ID NO: 42. In some embodiments, the smoothened mutation is a mutation at a position corresponding to position 412 of SEQ ID NO: 42, such as a L412F at such a position of SEQ ID NO: 42. In some embodiments, the smoothened mutation has a mutation that renders it resistant to certain smoothened inhibitors. In some embodiments, the smoothened protein comprises an amino acid alteration at amino acid position 518 of SEQ ID NO: 42 or at a position corresponding to position 518 of SEQ ID NO: 42. In some embodiments, the amino acid alteration is E518K or E518A substitution at the amino acid position corresponding to amino acid position 518 of SEQ ID NO: 42. In some embodiments, the smoothened protein comprises an amino acid alteration at amino acid position 473 of SEQ ID NO: 42 or at a position corresponding to position 473 of SEQ ID NO: 42. In some embodiments, the amino acid alteration is the substitution of aspartic acid with any of histidine, glycine, phenylalanine, tyrosine, leucine, isoleucine, proline, serine threonine, methionine, glutamiiie, or asparagine at
the amino acid position corresponding to amino acid position 473 of SEQ ID NO: 42. See, e.g., WO 2011/028950 and WO2012047968, each of which is incorporated by reference.

In some embodiments, unknown events result in overexpression of a hedgehog protein. For example, hedgehog protein may be overexpressed in the cell or in an adjacent cell. 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 supressor-of-fused, and the cell has suppressor-of-fused (SuFu) loss-of-function. In some embodiments, the SuFu mutation results in a loss-of-function in SuFu activity. In some embodiments, the SuFu mutation is in a medulloblastoma, meningioma, adenocystic carcinoma, basal cell carcinoma and rhabdomyosarcoma cancer cell. 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 1: Germline SUFU Mutations

<table>
<thead>
<tr>
<th>Age at Diagnosis of MB</th>
<th>Histologic Subtype</th>
<th>Associated Symptoms</th>
<th>Inheritance of Mutation</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 years</td>
<td>Desmoplastic</td>
<td>Developmental delay</td>
<td>NA</td>
<td>Loss of contiguous genes at 10q IVS1--1A →T</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Frontal bossing, hypertelorism</td>
<td></td>
<td>143insA</td>
</tr>
<tr>
<td>NA</td>
<td>Desmoplastic</td>
<td>None</td>
<td>NA</td>
<td>c.1022 + 1G&gt;A</td>
</tr>
<tr>
<td>NA</td>
<td>Desmoplastic</td>
<td>Meningioma in radiation field</td>
<td>NA</td>
<td>c.72delC</td>
</tr>
<tr>
<td>8 months</td>
<td>MBEN</td>
<td>Macrocrania, palmar and plantar pits</td>
<td>Inherited</td>
<td>c.1022 + 1G&gt;A</td>
</tr>
<tr>
<td>&lt; 1 month</td>
<td>MBEN</td>
<td>None</td>
<td>inherited</td>
<td>c.72delC</td>
</tr>
<tr>
<td>&lt; 3 months</td>
<td>MBEN</td>
<td>None</td>
<td>Inherited</td>
<td>c.72delC</td>
</tr>
</tbody>
</table>
**Table 2**, Germline Pathogenic *SUFU* Mutations

<table>
<thead>
<tr>
<th>Exon/Intron</th>
<th>Type of Mutation</th>
<th>Nucleotide Change (In SEQ ID NO: 44)</th>
<th>Consequence (In SEQ ID NO: 43)</th>
<th>Tumor Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Intron 1</td>
<td>Splice → frameshift</td>
<td>c.182 + 3A&gt;T</td>
<td>p.ThrSSfs</td>
<td>Not available</td>
</tr>
<tr>
<td>2 Exon 2</td>
<td>Frameshift</td>
<td>c.294_295dupCT</td>
<td>p.Tyr99fs</td>
<td>Not available</td>
</tr>
<tr>
<td>3 Intron 2</td>
<td>Splice → frameshift</td>
<td>c.318-?_454+?dup</td>
<td>p.Glu06-?_Glu52-?dup</td>
<td>UV (c.1022 + 5G&gt;A)</td>
</tr>
<tr>
<td>4 Exon 3</td>
<td>Large duplication</td>
<td>c.1123C&gt;T</td>
<td>p.Metl41Arg</td>
<td>Not available</td>
</tr>
<tr>
<td>5 Exon 9</td>
<td>Nonsense</td>
<td>c.1149_?1SOdupCT</td>
<td>p.Gln375X</td>
<td>Not available</td>
</tr>
<tr>
<td>6 Exon 9</td>
<td>Frameshift</td>
<td>c.1297-1G&gt;C</td>
<td>p..*</td>
<td>Not available</td>
</tr>
</tbody>
</table>

Abbreviation: LJV, unknown variant.
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: 44. 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 →+1(3)G>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.550OT (Q184X)); Aavikko et al (2012) Am J Hum Genet 91: 520-526 (e.g., c.3670T (R123Q); 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., C560OT (P1871,))

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, the agent tested in any of the screening methods described herein binds a TPRA40 protein. In some embodiments, the agent that binds TPRA40 is identified or confirmed using a yeast two-hybrid screen. In some embodiments, the agent that binds TPRA40 is identified or confirmed using high throughput binding screen of a small molecule library. In some embodiments, the agent that binds TPRA40 is identified or confirmed using a co-immunoprecipitation assay. In some embodiments, the agent that binds TPRA40 is identified or confirmed by labeling the agent (e.g., with a fluorescent label or radiolabel) and detecting whether the labeled agent binds to TPRA40.

In some embodiments, the agent tested in any of the screening methods described herein inhibits transport of the TPRA40 protein to the plasma membrane or to primary cilia, prevents activation of TPRA40 or uncouples TPRA40 from Gαd. In some embodiments, an agent that inhibits transport of the TPRA40 protein is identified or confirmed by utilizing a method comprising the steps of: i) contacting a cell expressing TPRA40 with an agent, ii) determining the localization of TPRA40 in the first cell expressing TPRA40 using immunofluorescence. In some embodiments, the TPRA40 is tagged or labeled (e.g., fluorescently or radiolabeled) in order to monitor its localization in a cell. In some embodiments, an agent that inhibits transport of the TPRA40 protein is identified or confirmed by utilizing a method comprising the steps of: i)
contacting a cell expressing TPRA40 with an agent; and ii) determining the levels of TPRA40 in a plasma membrane or ciliary membrane fraction. In some embodiments, the levels of TPRA40 are determined by fractionation of cell components and determining the levels of TPRA40 in each component (e.g., by SDS PAGE analysis).

In some embodiments, the agent tested in any of the screening methods described herein reduces expression of TPRA40 protein or RNA. In some embodiments, an agent that reduces expression of TPRA40 protein or RNA is identified or confirmed by a method comprising the steps of: i) contacting a cell expressing TPRA40 with an agent; and ii) determining the expression of TPRA40 in the cell using a CRE-luciferase or G/z-luciferase reporter assay. In some embodiments, an agent that reduces expression of TPRA40 protein or RNA is identified or confirmed by a method comprising the steps of: i) contacting a cell expressing TPRA40 with an agent; and ii) determining the expression of TPRA40 in the cell by RT-PCR. In some embodiments, an agent that reduces expression of TPRA40 protein or RNA is identified or confirmed by a method comprising the steps of: i) contacting a cell expressing TPRA40 with an agent; and ii) determining the expression of TPRA40 in the cell using Northern Blot analysis of TPRA40 RNA or Western Blot or immunofluorescence analysis of TPRA40 protein.

In some embodiments, the agent tested in any of the screening methods described herein inhibits TPRA40 inhibition of adenylyl cyclase. In some embodiments, an agent that reduces expression of TPRA40 protein or RNA is identified or confirmed by using a reporter gene assay. In some embodiments, the reporter gene is a luciferase gene controlled by a cAMP response element (CRE) and is stimulated by compounds that increase cAMP levels, such as forskolin, 8-bromo-cAMP or dibutryl-cAMP. In these embodiments, inhibition of adenylyl cyclase by active TPRA40 (in the presence of forskolin) will result in a reduction in cAMP levels and, therefore, a corresponding reduction in luciferase expression. An agent that inhibits TPRA40 would permit adenylyl cyclase to regain activity (e.g., in the presence of forskolin), thereby resulting in an increase in cAMP levels and luciferase expression.

In some embodiments, the agent identified in any of the screening methods described herein is further assessed in an assay for hedgehog signaling. In some embodiments, wherein the assay for hedgehog signaling comprises the steps of: i) contacting a cell with an amount of the agent, 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, and ii. determining, as compared to a control, whether the agent inhibits hedgehog signaling in the cell, wherein if the agent inhibits hedgehog signaling in the cell relative to the control, then an agent that inhibits hedgehog signaling is identified.

In some embodiments of any of the screening methods described herein, the TPRA40 DNA is exogenously expressed in a cell. In some embodiments, the TPRA40 DNA is stably expressed in the cell. In some embodiments, the TPRA40 DNA is transiently expressed in the cell. In some embodiments, the cell is an S12 cell. In some embodiments, the cell is a 293T cell.

The growth inhibitory effects of the various TPRA40 antagonists useable in the disclosure may be assessed by methods known in the art, e.g., using cells which express a TPRA40 polypeptide either endogenously or following transfection with the respective TPRA40 gene. For example, appropriate tumor cell lines and cells transfected with TPRA40-encoding DNA may be treated with the TPRA40 antagonists 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 (e.g. 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 TPRA40 antagonists. 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. Preferably, the tumor cell is one that has one or more mutations in a hedgehog pathway signaling gene. Preferably, such TPRA40 antagonists 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 70-100% compared to the untreated tumor cell. Growth inhibition can be measured at a TPRA40 antagonist concentration of about 0.5 to 30 μg/mL, about 0.5 nM to 200 nM, about 200 nM to 1 μM, 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 mg/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, preferably within about 5 to 30 days.

In some embodiments, to select for TPRA40 antagonists 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. TPRA40 polypeptide-expressing expressing tumor cells are incubated with medium alone or medium containing the appropriate TPRA40 antagonist. The cells are incubated for a 3 day time period. Following each treatment, cells are washed and aliquoted 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® CzUQuest software (Becton Dickinson), or any other device used by the skilled worker for analyses. Those TPRA40 antagonists that induce statistically significant levels of cell death as determined by PI uptake may then be selected.

In some embodiments, to screen for TPRA40 hedgehog antagonists which bind to an epitope on a TPRA40 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 TPRA40 antagonist. Alternatively, or additionally, epitope mapping can be performed by methods known in the art. For example, the TPRA40 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 TPRA40 polypeptide 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 TPRA40 polypeptide or the candidate TPRA40 hedgehog antagonist 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 TPRA40 or candidate agent and drying. Alternatively, an immobilized antibody, e.g., a monoclonal antibody, specific for the target portion of TPRA40 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 TPRA40 antagonist interacts with but does not bind directly to another TPRA40 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 TPRA40 polypeptide and other intra- or extracellular components (e.g., Ssnal/NA14) can be tested by means well-known by the skilled worker. In some embodiments, a reaction mixture is prepared containing the TPRA40 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 TPRA40 antagonists 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 TPRA40 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 TPRA40 antagonists, 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.

In certain embodiments, once an agent is identified as a TPRA40 antagonist, 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.
The foregoing systems for screening for TPRA40 antagonists can also be used to screen for TPRA40 agonists, such as small molecules that bind to TPRA40 and agonize its activity. The disclosure contemplates that any of the assays described herein to screen for antagonists can be adapted for screening for agonists by changing the relevant read-out (e.g., identifying as agonists an agent that promotes hedgehog signaling rather than identifying as an antagonist an agent that inhibits hedgehog signaling).

In certain embodiments, the disclosure provides for a method of screening for a TPRA40 agonist, wherein the method comprises: a) contacting a cell that expresses TPRA40, adenyl cyclase and a reporter with an agent; b) determining, as compared to an untreated control, whether the agent suppresses (e.g., decreases) the adenyl cyclase activity, wherein if the agent suppresses adenyl cyclase activity relative to the non-agent treated TPRA40 expressing cells, then the agent is identified as a TPRA40 agonist. In certain embodiments, the disclosure provides for a method of identifying a TPRA40 agonist, comprising: a) providing a cell that expresses TPRA40 and that expresses a reporter gene capable of indicating adenyl cyclase activity; b) contacting the cell with an activator of adenyl cyclase and with an agent, wherein the cells are contacted with the activator and the agent simultaneously, concurrently, or consecutively; and c) determining, as compared to a control, whether the agent suppresses (e.g., decreases) adenyl cyclase activity induced by the activator, wherein if the agent suppresses (e.g., decreases) the adenyl cyclase activity relative to the control, then the agent is identified as a TPRA40 agonist. In certain embodiments, the disclosure provides for a method of screening for an agent for inducing hedgehog signaling in a cell, wherein said method comprises: a) screening for an agent that binds to TPRA40 protein, induces expression of TPRA40, facilitates transport of TPRA40 protein to the plasma membrane or to primary cilia, induces activation of TPRA40 or couples it with God; b) contacting a cell with an amount of the agent identified in step a), and c) determining, as compared to a control, whether said agent induces (e.g., increases) hedgehog signaling in said cell, wherein if said agent induces (e.g., increases) hedgehog signaling in said cell relative to the control, then an agent that induces (e.g., increases) hedgehog signaling is identified. In certain embodiments, the disclosure provides for a method of identifying a TPRA40 agonist, wherein said method comprises: a) screening for an agent that binds to TPRA40 protein; b) contacting a cell with an amount of the agent identified in step a), and c) determining, as compared to a control, whether said agent that binds to TPRA40 protein
also induces (e.g., increases) hedgehog signaling in said cell, wherein if said agent induces (e.g., increases) hedgehog signaling in said cell relative to the control, then the agent is identified as a TPRA40 agonist. In certain embodiments, the agent is a small molecule. In certain embodiments, the agent is a polypeptide. In certain embodiments, the agent is a polynucleotide.

iii. Exemplary TPRA40 Antagonists

Below is provided a description of exemplary TPRA40 antagonists and categories of TPRA40 antagonists. Such antagonists, as well as any of the TPRA40 antagonists described generally or specifically herein may be used in any of the methods of the disclosure (e.g., TPRA40 antagonists of the disclosure). The disclosure contemplate that any of the TPRA40 antagonists of the disclosure can be described using any combination of functional and structural features described herein.

A. Anti-TPRA40 antibodies.

In one embodiment, the present disclosure provides the use of anti-TPRA40 antibodies, which may find use herein as therapeutic, diagnostic and/or prognostic agents (e.g., for determining the severity of and/or prognosing the disease course of a hedgehog pathway-hyperactive tumor or cancer). Antibodies that may be used for such purposes include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies. In some embodiments, the term "antibodies" may refer to antigen-binding fragments. Thus, in certain embodiments, a TPRA40 antagonist of the disclosure comprises an anti-TPRA40 antibody. Exemplary antibodies that function as TPRA40 antagonists include: (i) an antibody that binds to TPRA40 and prevents binding to ligand; (ii) an antibody that binds to TPRA40 and prevents transport to cilia; (iii) an antibody that binds to TPRA40 and prevents binding to Sema/NA-14. In other embodiments, a TPRA40 antagonist is an antibody that does not bind directly to TPRA40 but prevents transport of TPRA40 to cilia or blocks a protein-protein interaction necessary for native TPRA40 function, expression or localization.

The term "anti-TPRA40 antibody" is used in the broadest sense and covers, for example, anti-TPRA40 monoclonal antibodies, anti-TPRA40 antibody compositions with polypeptidic specificity, polyclonal antibodies, single chain anti-TPRA40 antibodies, multispecific antibodies (e.g., bispecific) and antigen binding fragments (see below) of all of the above enumerated antibodies as long as they exhibit the desired biological or immunological activity. Such
antibodies bind to TPRA40. The term "immunoglobulin" (Ig) is used interchangeably with antibody herein. In the case of TPRA40 antagonists which are anti-TPRA40 antibodies, such antibodies bind to TPRA40 to inhibit an activity (e.g., to antagonize a function).

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 that are purified away from the isolated antibody are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In certain embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more 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 a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using 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. The basic 4-chain antibody unit is a heterotetrameric glycoprotein composed of two identical light (L) chains and two identical heavy (H) chains (an IgM antibody consists of 5 of the basic heterotetramer unit along with an additional polypeptide called J chain, and therefore contain 10 antigen binding sites, while secreted IgA antibodies can polymerize to form polyvalent assemblages comprising 2-5 of the basic 4-chain units along with J chain). In the case of IgGs, the 4-chain unit is generally about 150,000 daltons. Each L chain is linked to an H chain by one covalent disulfide bond, while the two H chains are linked to each other by one or more disulfide bonds depending on the H chain isotype. Each H and L chain also has regularly spaced intrachain disulfide bridges. Each H chain has at the N-terminus, a variable domain (VH) followed by three constant domains (CH) for each of the a and γ chains and four CH domains for μ and ε isotypes. Each L chain has at the N-terminus, a variable domain (VL) followed by a constant domain (CL) at its other end. The VL is aligned with the VH and the CL is aligned with the first constant domain of the heavy chain (CHI). Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains. The pairing of a VH and VL together forms a single antigen-binding site. For the structure and properties of the different classes of antibodies, see, e.g., Basic and Clinical Immunology, 8th edition, Daniel P.

The L chain from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda, based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains (CH), immunoglobulins can be assigned to different classes or isotypes. There are five classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, having heavy chains designated \( \alpha \), \( \delta \), \( \epsilon \), \( \gamma \), and \( \mu \), respectively. The \( \gamma \) and \( \alpha \) classes are further divided into subclasses on the basis of relatively minor differences in CH sequence and function, e.g., humans express the following subclasses: IgGl, IgG2, IgG3, IgG4, IgAl, and IgA2. In some embodiments, the antibody disclosed herein is any of the classes or subclasses of immunoglobulins described herein.

The term "variable", with respect to domains of an immunoglobulin, refers to the fact that certain segments of the variable domains differ extensively in sequence among antibodies. The V domain mediates antigen binding and define specificity of a particular antibody for its particular antigen. However, the variability is not evenly distributed across the approximately 110-amino acid span of the variable domains. Instead, the V regions consist of relatively invariant stretches called framework regions (FRs) of 15-30 amino acids separated by shorter regions of extreme variability called "hypervariable regions" that are each 9-12 amino acids long. The variable domains of native heavy and light chains each comprise four FRs, largely adopting a \( \beta \)-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the \( \beta \)-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions 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, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region generally comprises amino acid residues from a "complementarity determining region" or "CDR" (e.g. if the Kabat system is used, around about residues 24-34 (LI), 50-56 (L2) and 89-97 (L3) in the VL,
and around about residues 31-35B (HI), 50-65 (H2) and 95-102 (H3) in the VH (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (e.g. if the Chothia system is used, around about residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the VL, and 26-32 (HI), 52A-55 (H2) and 96-101 (H3) in the VH (Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)).


"Chimeric" antibodies (immunoglobulins) have a portion of the heavy and/or light chain 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 (U.S. Patent No. 4,816,567; and Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984)). Humanized antibody as used herein is a subset of chimeric antibodies.

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient or acceptor antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance such as binding affinity. Generally, the 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 FR regions are those of a human immunoglobulin sequence although the FR regions may include one or more amino acid substitutions that improve binding affinity. The number of these amino acid substitutions in the FR are typically no more than 6 in the H chain, and in the L chain, no more than 3. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., Nature 321:522-525 (1986); Reichmann et al., Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992).

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments (including scFv); diabodies; linear antibodies (see U.S. Patent No. 5,641,870, Example 2; Zapata et al., Protein Eng. 8(10): 1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

In certain embodiments, the TPRA40 antagonist is an anti-TPRA40 antibody. Regardless of the specific type of antibody (e.g., chimeric, murine, humanized, human, a human antibody made in non-human cells, etc.) or the method used to make it, suitable TPRA40 antagonists can
be readily identified by generating a panel of antibodies that bind TPRA40 and testing to identify the antibodies having the desired function (e.g., antagonist activity).

In some embodiments, the anti-TPRA40 antibodies for use as TPRA40 antagonists bind to the putative extracellular N-terminal portion of the TPRA40 polypeptide (e.g., any of the amino acids corresponding to amino acids 1-47 of SEQ ID NO: 1). In some embodiments, the anti-TPRA40 antibodies bind to the putative intracellular C-terminal portion of the TPRA40 protein (e.g., any of the amino acids corresponding to amino acids 286-373 of SEQ ID NO: 1).

In some embodiments, the anti-TPRA40 antibodies bind to any of the putative extracellular portions of TPRA40 (e.g., any of the amino acids corresponding to amino acids 97-122, 172-191, or 261-264 of SEQ ID NO: 1). In some embodiments, the anti-TPRA40 antibodies bind to any of the putative intracellular portions of TPRA40 (e.g., any of the amino acids corresponding to amino acids 70-74, 144-150, and 213-239). In certain embodiments, the anti-TPRA40 antibodies bind to the C-terminal region of a TPRA40 protein and prevents TPRA40 from interacting with another protein, e.g., Ssna/NA14. In some embodiments, the anti-TPRA40 antibodies bind to a ligand-binding site of TPRA40 and does not activate hedgehog signaling downstream of TPRA40, but rather, sterically blocks a ligand from binding to TPRA40. In some embodiments, the anti-TPRA40 antibodies bind to the TPRA40 to sterically block an interaction between TPRA40 and a G protein (e.g., a G-alpha-i protein).

Suitable antibodies may be polyclonal antibodies or monoclonal antibodies.

1. Polyclonal Antibodies

In some embodiments, the antibodies of the present disclosure are polyclonal antibodies. In some embodiments, polyclonal antibodies are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen (especially when synthetic peptides are used) to a protein that is immunogenic in the species to be immunized. For example, the antigen can be conjugated to keyhole limpet hemocyanin (KLH), serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor, using a bifunctional or derivatizing agent, e.g., maleimidobenzoxy sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl₂, or R¹N=C=NR, where R and R¹ are different alkyl groups.
In particular embodiments, animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later, the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later, the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably-used to enhance the immune response.

2. Monoclonal Antibodies

In some embodiments of the present disclosure, the antibodies described herein are monoclonal antibodies. Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., Nature, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567). In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as described above to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Given the high homology (94.1%) between human and mouse TPRA40, it may be useful to use TPRA40 knockout mice for immunization in order to increase the chances of an immune response. Alternatively, lymphocytes may be immunized in vitro. In some embodiments, after immunization, lymphocytes are isolated and then fused with a myeloma cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Coding, Monoclonal Antibodies: Principles and Practice, pp. 59-103 (Academic Press, 1986)). There are numerous other approaches for screening to identify antibodies of a desired specificity known in the art, including phage display.

In certain embodiments, the hybridoma cells described herein are seeded and grown in a suitable culture medium which medium preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells (also referred to as fusion partner). For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the selective culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.
In certain embodiments, fusion partner myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a selective medium that selects against the unfused parental cells. Preferred myeloma cell lines are 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 and derivatives e.g., X63-Ag8-653 cells available from the American Type Culture Collection, Manassas, Virginia, 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); and Brodeur et al, Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis described in Munson et al., Anal. Biochem.. 107:220 (1980).

Once hybridoma cells that produce antibodies of the desired specificity, affinity, and/or activity are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Coding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal e.g., by i.p. injection of the cells into mice. The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such as, for example, affinity chromatography (e.g., using protein A or protein G-Sepharose) or ion-exchange chromatography, hydroxylapatite chromatography, gel electrophoresis, dialysis, etc. DNA encoding the monoclonal antibodies is 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 murine antibodies).

The hybridoma method is one way to make antibodies which can be readily tested for the desired activity, and hybridomas expressing an antibody having the desired activity are a source
of DNA encoding that antibody. However, many other methods are known in the art. Once isolated, the DNA may 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 antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra ex al, Curr. Opinion in Immunol., 5:256-262 (1993) and Pluckthun, Immunol. Revs. 130:151-188 (1992). In a further embodiment, monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al, Nature, 348:552-554 (1990). Clackson et al, Nature. 352:624-628 (1991) and Marks et al, J. Mol. Biol. 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al, Bio/Technology, 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al, Nuc. Acids. Res. 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies. The DNA that encodes the antibody may be modified to produce chimeric or fusion antibody polypeptides, for example, by substituting human heavy chain and light chain constant domain (CH and QJ sequences for the homologous murine sequences (U.S. Patent No. 4,816,567; and Morrison, et al, Proc. Natl Acad. ScL USA, 81:6851 (1984)), or by fusing the immunoglobulin coding sequence with all or part of the coding sequence for a non-immunoglobulin polypeptide (heterologous polypeptide). The non- immunoglobulin polypeptide sequences can substitute for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

3. Human and Humanized Antibodies

In some embodiments of the present disclosure, the antibodies for use as TPRA40 antagonists are human or humanized antibodies. Numerous methods exist in the art for the making of human and humanized antibodies.

4. Antibody fragments
In some embodiments, the present disclosure provides for TPRA40 antagonists that are antibody fragments. In certain circumstances, there are advantages of using antibody fragments, rather than whole antibodies. The smaller size of the fragments allows for rapid clearance, while retaining similar antigen binding specificity of the corresponding full length molecule, and may lead to improved access to target tissues, e.g., solid tumors.

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 a salvage receptor binding epitope residues are described in U.S. Patent No. 5,869,046. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Patent No. 5,751,894; and U.S. Patent No. 5,587,458. Fv and sFv are the only species with intact combining sites that are devoid of constant regions; thus, they are suitable for reduced nonspecific binding during in vivo use. sFv fusion proteins may be constructed to yield fusion of an effector protein at either the amino or the carboxy terminus of an sFv. See Antibody Engineering, ed. Borrebæck, supra. The antibody fragment may also be a “linear antibody”, e.g., as described in U.S. Patent 5,641,870, for example. Such linear antibody fragments may be monospecific or bispecific.

B. TPRA40 Binding Oligopeptides

In some embodiments, TPRA40 binding oligopeptides are examples of TPRA40 antagonists of the disclosure. TPRA40 binding oligopeptides of the present disclosure are oligopeptides that bind, preferably specifically, to a TPRA40 polypeptide, as described herein. In some embodiments, the TPRA40 binding oligopeptide binds to the putative extracellular N-
terminal portion of the TPRA40 polypeptide (e.g., any of the amino acids corresponding to amino acids 1-47 of SEQ ID NO: 1). In some embodiments, the TPRA40 binding oligopeptides bind to the putative intracellular C-terminal portion of the TPRA40 protein (e.g., any of the amino acids corresponding to amino acids 286-373 of SEQ ID NO: 1). In some embodiments, the TPRA40 binding oligopeptide binds to any of the putative extracellular portions of TPRA40 (e.g., any of the amino acids corresponding to amino acids 97-122, 172-191, or 261-264 of SEQ ID NO: 1). In some embodiments, the TPRA40 binding oligopeptide binds to any of the putative intracellular portions of TPRA40 (e.g., any of the amino acids corresponding to amino acids 70-74, 144-150, and 213-239). In certain embodiments, the TPRA40 binding oligopeptide binds to the C-terminal region of a TPRA40 protein and prevents TPRA40 from interacting with another protein, e.g., Ssna1/NA14. In some embodiments, the TPRA40 binding oligopeptide binds to a ligand-binding site of TPRA40 and does not activate hedgehog signaling downstream of TPRA40, but rather, sterically blocks a ligand from binding to TPRA40. In some embodiments, the TPRA40 binding oligopeptide binds to the TPRA40 to sterically block an interaction between TPRA40 and a G protein (e.g., a G-α/pha-i protein).

In some embodiments, the TPRA40 binding oligopeptides disclosed herein may be chemically synthesized using known oligopeptide synthesis methodology or may be prepared and purified using recombinant technology. TPRA40 binding oligopeptides may be about 5 amino acids 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, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 amino acids in length or more, and such oligopeptides are capable of binding, preferably specifically, to a TPRA40 polypeptide, respectively, as described herein. TPRA40 binding oligopeptides may be identified without undue experimentation using well known techniques. In this regard, it is noted that techniques for screening oligopeptide libraries for oligopeptides that are capable of specifically binding to a polypeptide target are well known in the art (see, e.g., U.S. Patent Nos. 5,556,762, 5,750,373, 4,708,871, 4,833,092, 5,223,409, 5,403,484, 5,571,689, 5,663,143; PCX Publication Nos. WO 84/03506 and WO84/03564; Geysen et al, Proc. Natl. Acad. Sci. U.S.A., 81:3998-4002 (1984); Geysen et al, Proc. Natl. Acad. Sci. U.S.A., 82:178-182 (1985); Geysen et al, in Synthetic Peptides as Antigens. 130-149 (1986);


Many other improvements and variations of the basic phage display concept have now been developed. These improvements enhance the ability of display systems to screen peptide libraries for binding to selected target molecules and to display functional proteins with the
potential of screening these proteins for desired properties. Combinatorial reaction devices for
phage display reactions have been developed (WO 98/14277) and phage display libraries have
been used to analyze and control bimolecular interactions (WO 98/20169; WO 98/20159) and
properties of constrained helical peptides (WO 98/20036). WO 97/35196 describes a method of
isolating an affinity ligand in which a phage display library is contacted with one solution in
which the ligand will bind to a target molecule and a second solution in which the affinity ligand
will not bind to the target molecule, to selectively isolate binding ligands. WO 97/46251
describes a method of biopanning a random phage display library with an affinity purified
antibody and then isolating binding phage, followed by a niicropanning process using micropiate
wells to isolate high affinity binding phage. The use of Staphylococcus aureus protein A as an
affinity tag has also been reported (Li et al. (1998) MoT Biotech., 9:187). WO 97/47314
describes the use of substrate subtraction libraries to distinguish enzyme specificities using a
combinatorial library which may be a phage display library. A method for selecting enzymes
suitable for use in detergents using phage display is described in WO 97/09446. Additional
methods of selecting specific binding proteins are described in U.S. Patent Nos. 5,498,538,
5,432,018, and WO 98/15833.

Methods of generating peptide libraries and screening these libraries are also disclosed in
U.S. Patent Nos. 5,723,286, 5,432,018, 5,580,717, 5,427,908, 5,498,530, 5,770,434, 5,734,018,
5,698,426, 5,763,592, and 5,723,323.

The disclosure contemplates that, in certain embodiments, a TPRA40 antagonist for use
in the methods of the disclosure comprises a TPRA40 oligopeptide, such as any of the TPRA40
oligopeptides described herein. TPRA40 oligopeptides having the desired activity as a TPRA40
antagonist may be readily selected using, for example, any of the assays described herein to
confirm that a TPRA40 oligopeptide has the desired function of a TPRA40 antagonist of the
disclosure.

C. TPRA40 Polypeptide Variants

In some embodiments, the TPRA40 antagonists are TPRA40 polypeptide variants. In
certain embodiments the polypeptide variant is a dominant negative. Such variants can be
prepared by introducing appropriate nucleotide changes into the encoding DNA, and/or by
synthesis of the desired polypeptide. Those skilled in the art will appreciate that amino acid
changes may alter post-translational processes of these molecules, such as changing the number
or position of glycosylation sites or altering the membrane anchoring characteristics. Variations in amino acid sequence can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Patent No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the amino acid sequence that results in a change in the amino acid sequence as compared with the native sequence. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the amino acid sequence of interest. Guidance in determining which amino acid residue may be inserted, substituted or deleted without adversely affecting the desired activity may be found by comparing the sequence of the amino acid sequence of interest with homologous known protein molecules and minimizing the number of amino acid sequence changes made in regions of high homology. Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with a serine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of about 1 to 5 amino acids. The variation allowed may be determined by systematically making insertions, deletions or substitutions of amino acids in the sequence and testing the resulting variants for activity exhibited by the full-length or mature native sequence.

Fragments of the various TPRA40 polypeptides are provided herein. Such fragments may be truncated at the N-terminus or C-termminus, or may lack internal residues, for example, when compared with a full length native protein. Such fragments which lack amino acid residues that are not essential for a desired biological activity are also useful with the disclosed methods.

In some embodiments, the TPRA40 fragments may be used as inhibitors of hedgehog signaling. For example, a fragment of TPRA40 that interacts with a TPRA40 binding partner, but which does not inhibit adenyl cyclase, may be used as a TPRA antagonist. In some embodiments, the fragment of TPRA40 is a soluble C-terminal portion of the TPRA40 polypeptide (e.g., a soluble polypeptide comprising a portion of the amino acids corresponding to amino acids 286-373 of SEQ ID NO: 1). In some embodiments, the fragment of TPRA40 is a soluble N-terminal portion of the TPRA40 polypeptide (e.g., a soluble polypeptide comprising a portion of the amino acids corresponding to amino acids 1-47 of SEQ ID NO: 1). In some embodiments, the fragment of TPRA40 comprises a soluble portion of any of the putative
extracellular portions of TPRA40 (e.g., a soluble polypeptide comprising at least a portion of any of the amino acids corresponding to amino acids 70-74, 97-122, 144-150, 172-191, 213-239 or 261-264 of SEQ ID NO: 1). In some embodiments, the fragments comprise at least 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, or 90 amino acids of the full-length TPRA40 amino acid sequence.

The above polypeptide fragments may be prepared by any of a number of conventional techniques. In some embodiments, desired peptide fragments may be chemically synthesized. An alternative approach involves generating such fragments by enzymatic digestion, e.g., by treating the protein with an enzyme known to cleave proteins at sites defined by particular amino acid residues, or by digesting the DNA with suitable restriction enzymes and isolating the desired fragment. Yet another suitable technique involves isolating and amplifying a DNA fragment encoding the desired fragment by polymerase chain reaction (PCR). Oligonucleotides that define the desired termini of the DNA fragment are employed at the 5' and 3' primers in the PCR. Preferably, such fragments share at least one biological and/or immunological activity with the corresponding full length molecule.

In particular embodiments, conservative substitutions of interest are shown in Table 3 under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 3, or as further described below in reference to amino acid classes, are introduced and the products screened in order to identify the desired variant.

TABLE 3
Substantial modifications in function or immunological identity of the TPRA40 polypeptides are accomplished by selecting substitutions that differ significantly in their effect on maintaining (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. Naturally occurring residues are divided into groups based on common side-chain properties:

1. hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;
2. neutral hydrophobic: Cys, Ser, Thr; Asn; Gin
3. acidic: Asp, Glu;
4. basic: His, Lys, Arg;
5. residues that influence chain orientation: Giy, Pro; and
6. aromatic: Trp, Tyr, Phe.

In some embodiments, 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, more preferably, into the remaining (non-conserved) sites.

In some embodiments, the variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PGR mutagenesis.


<table>
<thead>
<tr>
<th>Original Residue</th>
<th>Exemplary Substitutions</th>
<th>Preferred Substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala (A)</td>
<td>Val; Leu; Ile</td>
<td>Val</td>
</tr>
<tr>
<td>Arg (B)</td>
<td>Lys; Gin; Asn</td>
<td>Lys</td>
</tr>
<tr>
<td>Asn (N)</td>
<td>Gln; His; Asp; Lys; Arg</td>
<td>Gln</td>
</tr>
<tr>
<td>Asp (D)</td>
<td>Glu; Asn</td>
<td>Glu</td>
</tr>
<tr>
<td>Cys (C)</td>
<td>Ser; Ala</td>
<td>Ser</td>
</tr>
<tr>
<td>Gin (Q)</td>
<td>Asn; Glu</td>
<td>Asn</td>
</tr>
<tr>
<td>Glu (E)</td>
<td>Asp; Gln</td>
<td>Asp</td>
</tr>
<tr>
<td>Gly (G)</td>
<td>Pro; Ala</td>
<td>Ala</td>
</tr>
<tr>
<td>His (H)</td>
<td>Asn; Gin; Lys; Arg</td>
<td>Arg</td>
</tr>
<tr>
<td>Ile (I)</td>
<td>Leu; Val; Met; Ala; Phe; Norleucine</td>
<td>Ile</td>
</tr>
<tr>
<td>Leu (L)</td>
<td>Norleucine; Ile; Val; Met; Ala; Phe</td>
<td>Arg</td>
</tr>
<tr>
<td>Lys (K)</td>
<td>Arg; Gin; Asn</td>
<td>Arg</td>
</tr>
<tr>
<td>Met (M)</td>
<td>Leu; Phe; Ile</td>
<td>Leu</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>Trp; Leu; Val; Ile; Ala; Tyr</td>
<td>Leu</td>
</tr>
<tr>
<td>Pro (P)</td>
<td>Ala</td>
<td>Ala</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>Thr</td>
<td>Thr</td>
</tr>
<tr>
<td>Thr (T)</td>
<td>Val; Ser</td>
<td>Ser</td>
</tr>
<tr>
<td>Trp (W)</td>
<td>Tyr; Phe</td>
<td>Tyr</td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>Trp; Phe; Thr; Ser</td>
<td>Phe</td>
</tr>
<tr>
<td>Val (V)</td>
<td>Ile; Leu; Met; Phe; Ala; Norleucine</td>
<td>Leu</td>
</tr>
</tbody>
</table>

In some embodiments, scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant [Cunningham and Wells, Science, 244:1081-1085 (1989)]. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins. (W.H. Freeman & Co., N. Y.); Chothia, J. MoT. Biol.. 150: 1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Any cysteine residue not involved in maintaining the proper conformation of the TPRA40 polypeptides also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to such a molecule to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

In one embodiment, the 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 improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g., 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene DI product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g., binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis 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 target polypeptide. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Nucleic acid molecules encoding amino acid sequence variants of TPRA40 polypeptides may be 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, PGR mutagenesis, and cassette mutagenesis of a native sequence or an earlier prepared variant.

D. Modifications of TPRA40 polypeptides

In one embodiment, the TPRA40 antagonist comprises a fusion of any of the TPRA40 polypeptides disclosed herein (e.g., TPRA40 chimeric polypeptides) with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl-terminus of such antibody or polypeptide. The presence of such epitope-tagged forms of such antibodies or polypeptides can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables such antibodies or polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag. Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol. 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6): 547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., Biotechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al, Science. 255:192-194 (1992)]; an a-tubulin epitope peptide [Skinner et al, J. Biol. Cheiru 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al, Proc. Natl. Acad. Sci. USA. 87:6393-6397 (1990)].
In an alternative embodiment, the TPRA40 antagonist may comprise a fusion of the TPRA40 polypeptides with an immunoglobulin or a particular region of an immunoglobulin (e.g., Fc domain). For a bivalent form of the TPRA40 antagonist (also referred to as an "immunoadhesin"), such a fusion could be to the Fc region of an IgG molecule. The Ig fusions preferably include the substitution of a soluble (transmembrane domain deleted or inactivated) form of a preceding antibody or polypeptide in the place of at least one variable region within an Ig molecule. In a particularly preferred embodiment, the immunoglobulin fusion includes the hinge, CH2 and CHS, or the hinge, CH1, CH2 and CHS regions of an IgGl molecule. For the production of immunoglobulin fusions see also US Patent No. 5,428,130 issued June 27, 1995.

E. Preparation of TPRA40 polypeptides

The disclosure herein provides for the production of TPRA40 polypeptides (e.g., TPRA40 antagonist polypeptides) in some embodiments, by culturing cells transformed or transfected with a vector containing nucleic acid such antibodies, polypeptides and oligopeptides. It is, of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare any of the antibodies, polypeptides and oligopeptides disclosed herein. For instance, the appropriate amino acid sequence, or portions thereof, may be produced by direct peptide synthesis using solid-phase techniques [see, e.g., Stewart et al., Solid-Phase Peptide Synthesis, W.H. Freeman Co., San Francisco, CA (1969); Merrifield, J. Am. Chem. Soc, 85:2149-2154 (1963)]. In vitro protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be accomplished, for instance, using an Applied Biosystems Peptide Synthesizer (Foster City, CA) using the manufacturer's instructions. Various portions of any of the antibodies, polypeptides or oligopeptides described herein may be chemically synthesized separately and combined using chemical or enzymatic methods to produce the desired product.

1. Isolation of DNA Encoding TPRA40 polypeptides

In some embodiments, DNA encoding a TPRA40 polypeptide may be obtained from a cDNA library prepared from tissue believed to possess such antibody, polypeptide or oligopeptide mRNA and to express it at a detectable level. Accordingly, DNA encoding such polypeptides can be conveniently obtained from a cDNA library prepared from human tissue, a genomic library or by known synthetic procedures (e.g., automated nucleic acid synthesis). Libraries can be screened with probes (such as oligonucleotides of at least about 20-80 bases)
designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). Alternatively, PCR methodology may be used. [Sambrook et al, supra: Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)]. Alternatively, TPRA40 cDNA can simply be purchased from commercial sources (e.g. from Open Biosystems’ mammalian gene collection (ThermoScientific)).

Techniques for screening a cDNA library are well known in the art. In some embodiments, the oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. In some embodiments, the oligonucleotide solubilized such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like \(^{32}\)P-labeled ATP, biotinylation or enzyme labeling. 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 other known sequences deposited and available in public databases such as GenBank or other private sequence databases. Sequence identity (at either the amino acid or nucleotide level) within defined regions of the molecule or across the full-length sequence can be determined using methods known in the art and as described herein.

In some embodiments, nucleic acid having protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

2. Selection and Transformation of Host Cells

In some embodiments, host cells are transfected or transformed with expression or cloning vectors described herein for TPRA40 polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the
productivity of cell cultures can be found in Mammalian Cell Biotechnology: A Practical Approach. M. Butler, ed. (ERL Press, 1991) and Sambrook et al., supra.

Methods of eukaryotic cell transfection and prokaryotic cell transformation are known to the ordinarily skilled artisan, for example, CaCl$_2$, CaPO$_4$, liposome-mediated and electroporation. Depending on the host cell used, transformation is performed using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes. Infection with Agrobacterium tumefaciens is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) can be employed. General aspects of mammalian cell host system transfections have been described in U.S. Patent No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. B act., 130:946 (1977) and Hsiao et al. Proc. Natl. Acad. Sci. (USA). 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, or any other methods available to the skilled worker may also be used. For various techniques for transforming mammalian cells, see Keown et al, Methods in Enzymology. 185:527-537 (1990) and Mansour et al, Nature, 336:348-352 (1988).

Suitable host cells for cloning or expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteraeae such as E. coli. Various E. coli strains are publicly available, such as E. coli K12 strain MM294 (ATCC 31,446); E. coli X1776 (ATCC 31,537); E. coli strain W3110 (ATCC 27,325) and K572 (ATCC 53,635). Other suitable prokaryotic host cells include Enterobacteraeae 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. These examples are illustrative rather than limiting. Strain W3110 is one particularly preferred host or parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the
host cell secretes minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins endogenous to the host, with examples of such hosts including E. coli W3110 strain 1A2, which has the complete genotype tonA; E. coli W3110 strain 9E4, which has the complete genotype tonA ptr3; E. coli W3110 strain 27C7 (ATCC 55,244), which has the complete genotype tonA ptrSphoA E1 5 (argF-lac)169 degP ompTkan'; E. coli W3110 strain 37D6, which has the complete genotype tonA ptrSphoA E1 5 (argF-lac)169 degP ompTrbs? UvG kan'; E. coli W3110 strain 40B4, which is strain 37D6 with a non-kanamycin resistant degP deletion mutation; and an E. coli strain having mutant peripiasmie protease disclosed in U.S. Patent No. 4,946,783 issued 7 August 1990.

Alternatively, in vitro methods of cloning, e.g., PGR or other nucleic acid polymerase reactions, are suitable. Full length antibody, antibody fragments, and antibody fusion proteins 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) and the immunoconjugate 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), and U.S. 5,840,523 (Simmons et al.) which describes translation initiation region (TIR) and signal sequences for optimizing expression and secretion, these patents incorporated herein by reference. After expression, the antibody is 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 in suitable cells (e.g., CHO cells).

In some embodiments, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for vectors encoding TPRA40 polypeptides. Saccharomyces cerevisiae is a commonly used lower eukaryotic host microorganism. Others include Schizosaccharomyces pombe (Beach and Nurse, Nature, 290: 140 [1981]; EP 139,383 published 2 May 1985); Kiyuveromyces hosts (U.S. Patent No. 4,943,529; Fleer et al., Bio/Technology. 9:968-975 (1991)) such as, e.g., K. lactis (MW98-8C, CBS683, CBS4574; Louvencourt et al, J. Bacterid.. 154(2):737-742 [1983]), K.fragilis (ATCC 12,424), K.bulgarcus (ATCC 16,045), K. wickeramii (ATCC 24,178), K.waitii (ATCC 56,500), K. drosophilaram (ATCC 36,906; Vanden Berg et al., Bio/Technology, 8:135 (1990)), K. thermotolerans, and K. marxianus; yarrowia
(EP 402,226); Pichia pastoris (EP 183,070; Sreekrishna et al, J. Basic Microbiol., 28:265-278 [1988]); Candida; Trichoderma reesia (EP 244,234); Neurospora crassa (Case et al, Proc. Natl. Acad. Sci. USA, 76:5259-5263 [1979]); Schwanniomyces such as Schwanniomyces occideiitalis (EP 394,538 published 31 October 1990); and filamentous fungi such as, e.g., Neurospora, Penicillium, Tolypocladium (WO 91/00357 published 10 January 1991), and Aspergillus hosts such as A. nidulans (Ballance et al, Biochem. Biophys. Res. Commun. 112:284-289 [1983]; Tilburn et al, Gene, 26:205-221 [1983]; Yelton et al, Proc. Natl. Acad. Sci. USA, 81: 1470-1474 [1984]) and A. niger (Kelly and Hynes, EMBO J., 4:475-479 [1985]). Methyloptropic yeasts are suitable herein and include, but are not limited to, yeast capable of growth on methanol selected from the genera consisting of Hansenula, Candida, Kloeckera, Pichia, Saccharomyces, Tomlopsis, and Rhodotorula. A list of specific species that are exemplary of this class of yeasts may be found in C. Anthony, The Biochemistry of Methyloptrophs. 269 (1982).

In some embodiments, suitable host cells for the expression of glycosylated TPRA40 polypeptide production are derived from multicellular organisms. Examples of invertebrate cells include insect cells such as Drosophila S2 and Spodoptera Sf9, as well as plant cells, such as cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as Spodoptera fugiperda (caterpillar), Aedes aegypti (mosquito), Aedes albopictus (mosquito), Drosophila melanogaster (fruitfly), and Bombyx mod have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-I 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.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. In some embodiments, the TPRA40 polypeptides are produced in vertebrate cells. Examples of useful mammalian host cell lines are monkey kidney CVI 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 (BHK21, ATCC CCL 10); Chinese hamster ovary cells (CHO, Urlaub et al, Proc. Natl. Acad. Sci. USA 77:4216 (1980)); mouse Sertoli cells (TM4, Mather, Biol. Reprod. 23:243-25 I (1980)); monkey kidney cells (CVI 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).

In some embodiments, host cells are transformed with the above-described expression or cloning vectors for TPRA40 polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

3. Selection and Use of a Replicable Vector

In some embodiments, the nucleic acid (e.g., cDNA or genomic DNA) encoding any of the TPRA40 polypeptides disclosed herein may be inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan. The TPRA40 polypeptide may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be an epitope tag or affinity tag to enable purification, but should not contain an N-terminal signal sequence because TPRA40 does not have one of its own. Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. 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.

In some embodiments, expression and cloning vectors will 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.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up nucleic acid encoding the desire protein, such as DHFR or thymidine kinase. In some embodiments, an appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al, Proe. Natl. Acad. Sci. USA, 77:4216 (1980). A suitable selection gene for use in yeast is the trp gene present in the yeast plasmid YRp7 [Stinchcomb et al, Nature, 282:39 (1979); Kingsman et al, Gene. 7:141 (1979); Tschemper et al, Gene, 10:157 (1980)]. The trp J 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-I [Jones, Genetics, 85:12 (1977)].

Expression and cloning vectors usually contain a promoter operably linked to the nucleic acid sequence encoding the desired amino acid sequence, in order to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems [Chang et al, Nature, 275:615 (1978); Goeddel et al, Nature. 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res.. 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al, Proc. Natl. Acad. Sci. USA. 80:21-25 (5983)]. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the desired protein sequence.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al, J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al, J. Adv. Enzyme Reg., 7: 149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphogluco 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.

DNA Transcription in mammalian host cells is controlled, for example, by promoters
obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504
published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian
sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40),
from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin
promoter, and from heat-shock promoters, provided such promoters are compatible with the host
cell systems. Transcription of a DNA encoding the TPRA40 polypeptide may be increased by
inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA,
usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Many
enhancer sequences are now known from mammalian genes (globin, elastase, albumin, a-
fetoprotein, and insulin). In some embodiments, an enhancer from a eukaryotic cell virus will be
used. 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. The enhancer may be spliced into the vector at a
position 5' or 3' to the coding sequence of the preceding amino acid sequences, but is preferably
located at a site 5' from the promoter.

In some embodiments, 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 the respective
antibody, polypeptide or oligopeptide described in this section.

Still other methods, vectors, and host cells suitable for adaptation to the synthesis of the
respective antibody, polypeptide or oligopeptide in recombinant vertebrate cell culture are
4. Culturing the Host Cells

In some embodiments, the host cells used to produce the TPRA40 polypeptides 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. Biocliem. 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(TM) 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.

5. Detecting Gene Amplification/Expression

In some embodiments, gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA. 77:5201 -5205 (1980)], dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

In some embodiments, gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either
monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies suitable for the present method may be prepared against a native sequence polypeptide or oligopeptide, or against exogenous sequence fused to DNA and encoding a specific antibody epitope of such a polypeptide or oligopeptide.

6. Protein Purification

In some embodiments, any of the TPRA40 polypeptides disclosed herein may be recovered from culture medium or from host cell lysates. If membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or by enzymatic cleavage. In some embodiments, cells employed in expression of the preceding can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents.

In some embodiments, it may be desirable to purify any of the TPRA polypeptides described herein produced by a host cell. The following procedures are examples of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; protein A Sepharose columns to remove contaminants such as IgG; and metal chelating columns to bind epitope-tagged forms of the desired molecules. Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, Methods in Enzymology, 182 (1990); Scopes, Protein Purification: Principles and Practice. Springer-Verlag, New York (1982). The purification step(s) selected will depend, for example, on the nature of the production process used and the particular antibody, polypeptide or oligopeptide produced for the claimed methods.

In some embodiments, when using recombinant techniques, the TPRA40 polypeptide can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If TPRA40 polypeptides are produced intracellularly, as a first step, the particulate debris, either host cells or iysed 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, superaatants 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.

In some embodiments, purification can occur using, for example, hydroxyiapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. 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 (Lmdmark et al., J. Immunol. Meth. 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., EMBO J. 5:15671575 (1986)). In some embodiments, the matrix to which the affinity ligand is attached is agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrene divinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a CHS domain, the Bakerbond ABX(TM)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(TM) chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocussing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

In some embodiments, 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, the chromatography step is performed at low salt concentrations (e.g., from about 0-0.25M salt).

F. Polynucleotide TPRA40 Antagonists

In some embodiments, the TPRA40 antagonist is a polynucleotide that inhibits expression of TPRA40.
In some embodiments, the TPRA40 antagonist is a sense/antisense oligonucleotide. Molecules that would be expected to inhibit TPRA40, and therefore inhibit or antagonize hedgehog signaling, include fragments of the respective TPRA40-encoding nucleic acids such as antisense or sense oligonucleotides ("TPRA40 sense/antisense NA"). Such nucleic acids comprise a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to the respective target (a) TPRA40 mRNA (sense) or (b) TPRA40 DNA (antisense) sequences. TPRA40 sense/antisense NA comprise a fragment of the coding region of the TPRA40 RNA or DNA. 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 (Cancer Res. 48:2659, 1988) and van der Kooi et al. (BioTechniques 6:958, 1988).

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 TPRA40 sense/antisense NA may be used to block the respective expression of: TPRA40 polypeptides, wherein those TPRA40 polypeptides may play a role in the activation or amplification of hedgehog signaling. In some embodiments, the TPRA40 sense/antisense NA may 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; Nucleic acid with such 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. The TPRA40 sense/antisense NA 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, CA). Any other means for such synthesis hitherto 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. Patents that teach the preparation of such
uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.
Fat. Nos. 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932;
5 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170;
10 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 hereinafter
incorporated by reference.

Other examples of TPRA40 sense/antisense NA suitable for use in the present disclosure
include those oligonucleotides which are covalently linked to organic moieties, such as those
described in WO 90/10048, and other moieties that increases the 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.

Although antisense oligonucleotides may function via any of a number of mechanisms, in
certain embodiments, the antisense conjugates promote degradation of the TPRA40 mRNA
transcript. In certain embodiments, the antisense conjugates promote RNaseH-mediated
degradation of the TPRA40 mRNA transcript. Note, however, that the disclosure contemplates
that a given antisense conjugate may have multiple effects. Thus, as long as an antisense
conjugate promotes RNaseH-mediated degradation, it may also have other effects (e.g.,
identification of a mechanism does not imply that such mechanism is the sole mechanism by
which the antisense conjugate impacts a cell or transcript).

One type of antisense oligonucleotide contemplated when some level of RNaseH-
mediated degradation is desired, are antisense oligonucleotides sometimes termed "gapmers".
The oligonucleotides have a central portion that is flanked by two wing portions (e.g., wing-
central portion-wing). The central portion has nucleotide content and chemistry capable of
promoting RNaseH-mediated degradation when hybridized to RNA. For example, the central
portion comprises at least 7 nucleotides of DNA and/or modified nucleotides that retain the
ability to promote RNaseH-mediated degradation when hybridized to RNA, such as
phosphorothioate nucleotides. The central portion may also contain a mixture of DNA and
modified nucleotides, including mixtures of different modified nucleotides. Alternatively, the
central portion may contain only DNA nucleotides or only modified nucleotides.
In contrast to the central portion, the wing portions are not intended to mediate \textit{R}N\textit{A}se\textit{H}-mediated degradation. Rather, the wing portions are intended to improve the stability, half-life, or specificity of the oligonucleotides. Wing portions may include, for example, one or more modified nucleotides (including combinations) selected from: locked nucleic acid (LNA) nucleotides, 2'-O-methoxyethyl nucleotides, 2-O-methyl nucleotides, peptide nucleic acids, and the like. For any of these modified nucleotides provided in the wing portion, the modified nucleotides may be modified DNA or modified RNA nucleotides.

In some embodiments, the antisense oligonucleotide is a morpholino molecule that sterically blocks the binding of a protein or nucleic acid to a target RNA or DNA sequence. In some embodiments, the morpholino also triggers degradation of the target RNA or DNA sequence. In some embodiments, the morpholino molecule binds to TPRA40 RNA. In some embodiments, the morpholino molecule comprises 20-30 nucleotides. In other embodiments, the morpholino molecule comprises 23-27 nucleotides. In other embodiments, the morpholino molecule comprises 25 nucleotides.

In some embodiments, the antisense oligonucleotides of the present disclosure include a nucleotide analog having a constrained furanose ring conformation, such as Locked Nucleic Acids (LNAs). In LNAs, a 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety.

In some embodiments, in modified oligonucleotide, both the sugar and the internucleoside linkage, \textit{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 mimic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (FNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an amineoethylglycine 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 FNA nucleotides include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA nucleotides can be found in Nielsen et al., Science, 1991, 254, 14974500.
Antisense or sense oligonucleotides suitable for use in the present disclosure may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, CaP04-mediated DNA transfection, electroporation, or by using gene transfer vectors such as Epstein-Barr virus. In a preferred procedure, 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 retroviruses M-MuLV, N2 (a retrovirus derived from M-MuLV), or the double copy vectors designated DCT5A, DCT5B and DCT5C (see WO 90/13641).

In some embodiments, any of the sense or antisense oligonucleotides disclosed herein 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.

In some embodiments, any of the sense or antisense oligonucleotides disclosed herein 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 preferably 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.
In some embodiments, the polynucleotide TPRA40 antagonist is an interfering RNA or RNAi molecule.

An RNAi is RNA of 10 to 50 nucleotides in length which reduces expression of a target gene, wherein portions of the strand are sufficiently complementary (e.g. having at least 80% identity to the target gene). The method of RNA interference refers to the target-specific suppression of gene expression (i.e., "gene silencing"), occurring at a post-transcriptional level (e.g., translation), and includes all posttranscriptional and transcriptional mechanisms of RNA mediated inhibition of gene expression, such as those described in P.D. Zamore, Science 296: 1265 (2002) and Hannan and Rossi, Nature 431; 371-378 (2004). As used herein, RNAi can be in the form of small interfering RNA (siRNA), short hairpin RNA (shRNA), and/or micro RNA (miRNA).

Such RNAi molecules are often a double stranded RNA complexes that may be expressed in the form of separate complementary or partially complementary RNA strands. Methods are well known in the art for designing double-stranded RNA complexes. For example, the design and synthesis of suitable shRNA and siRNA may be found in Sandy et at, BioTechniques 39: 215-224 (2005). An "RNA coding region" is a nucleic acid that can serve as a template for the synthesis of an RNA molecule, such as a double-stranded RNA complex. Preferably, the RNA coding region is a DNA sequence.

A "small interfering RNA" or siRNA is a double stranded RNA (dsRNA) duplex of 10 to 50 nucleotides in length which reduces expression of a target gene, wherein portions of the first strand is sufficiently complementary (e.g. having at least 80% identity to the target gene). siRNAs are designed specifically to avoid the anti-viral response characterized by elevated interferon synthesis, nonspecific protein synthesis inhibition and RNA degradation that often results in suicide or death of the cell associated with the use of RNAi in mammalian cells. Paddison et al., Proc Natl Acad Sci USA 99(3): 1443-8. (2002). In some embodiments, the siRNA molecule comprises the nucleotide sequence of any SEQ ID NOs: 16-23.

The term "hairpin" refers to a looping RNA structure of 7-20 nucleotides. A "short hairpin RNA" or shRNA is a single stranded RNA 10 to 50 nucleotides in length characterized by a hairpin turn which reduces expression of a target gene, wherein portions of the RNA strand are sufficiently complementary (e.g. having at least 80% identity to the target gene). The term
"stem-loop" refers to a pairing between two regions of the same molecule base-pair to form a double helix that ends in a short unpaired loop, giving a lollipop-shaped structure.

A "micro RNA" (previously known as stRNA) is a single stranded RNA of about 10 to 70 nucleotides in length that are initially transcribed as pre-miRNA characterized by a "stem-loop" structure, which are subsequently processed into mature miRNA after further processing through the RNA-induced silencing complex (RISC).

An "RNA coding region" is a nucleic acid that can serve as a template for the synthesis of an RNA molecule, such as a double-stranded RNA complex. In some embodiments, the RNA coding region is a DNA sequence.

In some embodiments, the RNA coding region encodes a double-stranded RNA complex (e.g., siRNA, miRNA, shRNA) that is capable of down-regulating the expression of a particular gene or genes. In some embodiments, a double-stranded RNA complex is expressed in the form of an RNA molecule having a stem-loop or a so-called "hairpin" structure. As used herein, "hairpin" structure encompasses shRNAs and miRNAs. In some embodiments, a double-stranded RNA complex is expressed in the form of separate complementary or partially complementary RNA strands. Methods are well-known in the art for designing double-stranded RNA complexes, eg, siRNA, miRNA, and shRNAs. For example, resources and citations describing the design of effective shRNA and siRNA are found in Sandy et al, BioTechniques 39:215-224 (2005). It is understood that the sequences of a double-stranded RNA complex may be of natural origin or may be synthetic.

In some embodiments, the RNA complex comprises a double-stranded region corresponding to a region of a gene to be down-regulated is expressed in the cell. One strand of the RNA double-stranded region is substantially identical (typically at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical) in sequence to the sequence of the coding region targeted for down regulation. The other strand of the double-stranded region (interchangeably termed "RNA double-stranded region) is complementary to the sequence of the coding region targeted for down regulation, or partially complementary to the coding region targeted for down regulation (typically at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to the complement of the coding region targeted). It is understood that the double-stranded region can be formed by two separate RNA stranded, or by the self-complementary portions of a single RNA having a hairpin structure. In some
embodiments, the double-stranded region is generally at least about 15 nucleotides in length and, in some embodiments, is about 15 to about 30 nucleotides in length. However, a significantly longer double-stranded region can be used effectively in some organisms. In some embodiments, the double-stranded region is between about 19 and 22 nucleotides in length. The double-stranded region is preferably identical to the target nucleotide sequence over this region. When the coding region to be down regulated is in a family of highly conserved genes, the sequence of the RNA double-stranded region can be chosen with the aid of sequence comparison to target only the desired gene. On the other hand, if there is sufficient identity among a family of homologous genes within an organism, a double-stranded can be designed that would down regulate a plurality of genes simultaneously. In some embodiments, a single RNA coding region in the construct serves as a template for the expression of a self-complementary hairpin RNA, comprising a sense region, a loop region and an antisense region. The sense and antisense regions are each preferably about 15 to about 30 nucleotides in length. The loop region preferably is about 2 to about 15 nucleotides in length, more preferably from about 4 to about 9 nucleotides in length. Following expression the sense and antisense regions form a duplex.

In some embodiments, the disclosure provides for siRNA molecules comprising a nucleotide sequence that is at least 85, 90, 95, 96, 97, 98, 99 or 100% identical to any of the nucleotide sequences of SEQ ID NOs: 16-23. In some embodiments, the siRNA molecule does not comprise the nucleotide sequence of SEQ ID NO: 16.

In another embodiment, the vector comprises two RNA coding regions. The first coding region is a template for the expression of a first RNA and the second coding region is a template for the expression of a second RNA. Following expression, the first and second RNAs form a duplex. The retroviral construct preferably also comprises a first Pbl III promoter operably linked to the first RNA coding region and a second Pol II promoter operably linked to the second RNA coding region.

It is understood that, in certain embodiments, a vector of the disclosure can encompass nucleic acid sequences sufficient to form more than RNA coding region that inhibit expression of distinct target genes. In this embodiment, simultaneous inhibition of distinct target genes can be accomplished with a single vector of the disclosure. The number of different RNA complex transcripts that can be expressed simultaneously is limited only by the packaging capacity of the vector (if a viral vector is used) and adjacent promoters, including any of the promoters
described below, can be selected to eliminate or minimize interference and allow for efficient simultaneous inhibition of multiple target genes. The inhibition of multiple RNA construct transcripts of adjacent promoters, for example, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, or 10 or more adjacent promoters allows the user to generate a desire phenotype that develops only when several coding regions (e.g., genes) are targeted simultaneously and enables manipulation and elucidation of complex genetic systems.

G. Small Molecules

In some embodiments, the TPRA40 antagonist is a small molecule, such as a small organic molecule. A "small molecule" or "small organic molecule" is defined herein to have a molecular weight below about 1000, below about 900, below about 800, below about 700, below about 600 or below about 500 Daltons. In some embodiments, the TPRA40 antagonist is a small molecule that binds to TPRA40 and inhibits hedgehog signaling. In some embodiments, the TPRA40 antagonist is a small molecule that binds to TPRA40 and prevents TPRA40 from interacting with a binding partner. In some embodiments, the TPRA40 antagonist is a small molecule that binds to TPRA40 and inhibits TPRA40 localization to the plasma membrane or to cilia.

It should be noted that the disclosure also provides TPRA40 agonists. In certain embodiments, the TPRA40 agonist is a small molecule that binds to TPRA40 and promotes hedgehog signaling.

IV. Diagnostic Methods of Using TPRA40 Antagonists

In some embodiments, the present disclosure relates to methods of modulating a differentiation state, survival, and/or proliferation of a cell. 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 disclosure provides for a method of reducing hedgehog signaling in a cell, 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 a TPRA40 antagonist.

In some embodiments, the disclosure provides for a method of inhibiting unwanted growth, proliferation or survival of a cell, 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 a TPRA40 inhibitor.

In some embodiments, the disclosure provides for a method of inhibiting growth, proliferation or survival of a tumor cell, 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 a TPRA40 inhibitor.

In some embodiments, the cell treated with any of the methods disclosed herein comprises one or more mutations in a hedgehog signaling pathway gene. In some embodiments, the one or more mutations are in the TPRA40 gene.

In some embodiments, the one or more mutations 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 consilium syn active smoothened protein. In some embodiments, the smoothened mutation comprises a mutation corresponding to position W535L of SEQ ID NO: 42. In some embodiments, the smoothened mutation comprises a mutation corresponding to position R562Q of SEQ ID NO: 42. In some embodiments, the smoothened mutation comprises a mutation corresponding to position L412F of SEQ ID NO: 42. In some embodiments, the smoothened mutation has a mutation that renders it resistant to certain smoothened inhibitors. In some embodiments, the smoothened protein comprises an amino acid alteration at amino acid position 518 of SEQ ID NO: 42. In some embodiments, the amino acid alteration is E518K or E518A substitution at the amino acid position corresponding to amino acid position 518 of SEQ ID NO: 42. In some embodiments, the smoothened protein comprises an amino acid alteration at amino acid position 473 of SEQ ID NO: 42. In some embodiments, the amino acid alteration is the
substitution of aspartic acid with any of histidine, glycine, phenylalanine, tyrosine, leucine, isoleucine, proline, serine threonine, methionine, glutamine, or asparagine at the amino acid position corresponding to amino acid position 473 of SEQ ID NO: 42. 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: 42, as set forth above with respect to the screening assay. See, e.g., WO 2011/028950 and WO2012047968, each of which is incorporated by reference. In some embodiments, the smoothened mutation is a mutation at a position corresponding to position 535 of SEQ ID NO: 42. In certain embodiments, the mutation is a mutation at a position corresponding to position 562 of SEQ ID NO: 42. In certain embodiments, the mutation is W535L at position 535 or at that corresponding position in SEQ ID NO: 42. In some embodiments, the smoothened mutation is a mutation corresponding to position R562Q of SEQ ID NO: 42 (a R562Q mutation at position 562 or at a position corresponding to position 562 of SEQ ID NO: 42. In some embodiments, the smoothened mutation is a mutation at a position corresponding to position 412 of SEQ ID NO: 42, such as a L412F at such a position of SEQ ID NO: 42. In some embodiments, the smoothened mutation has a mutation that renders it resistant to certain smoothened inhibitors. In some embodiments, the smoothened protein comprises an amino acid alteration at amino acid position 518 of SEQ ID NO: 42 or at a position corresponding to position 518 of SEQ ID NO: 42. In some embodiments, the amino acid alteration is E518K or E518A substitution at the amino acid position corresponding to amino acid position 518 of SEQ ID NO: 42. In some embodiments, the smoothened protein comprises an amino acid alteration at amino acid position 473 of SEQ ID NO: 42 or at a position corresponding to position 473 of SEQ ID NO: 42.

In some embodiments, the one or more mutations are in patched, and the cell has a patched loss-of-function. 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) 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 comprises any of the mutations indicated in Tables 1 and 2. In some embodiments, the SuFu mutation comprises a mutation at a position corresponding to any of the following amino acid position in SEQ ID NO: 43: 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: 43. In some embodiments, the SuFu mutation is any of the mutations corresponding to e.1022+1G>A (TVS8-1G>T), e.72delC, c.72insC, 143insA, e.846insC, or IVS1-1 A>T of SEQ ID NO: 44. 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), 129del, 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-IA>T); Kijima et al (2012) Fam Cancer 11: 565-70 (e.g., c.550OT (Q184X)); Aavikko et al (2012) Am J Hum Genet 91: 520-526 (e.g., c.367C>T (R123C)); Stephens et al (2053) 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, prior to contacting the cell with the TPRA40 inhibitor, the cell is determined to have one or more mutations in a hedgehog signaling pathway gene.

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 G arises 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 comprises 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
TPRA40 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, medullioblastoma, sarcoma, basal cell carcinoma, gastric, ovarian, esophageal, pancreatic, or testicular cancer cell.

In some embodiments, the TPRA40 antagonist used in any of the methods disclosed herein is a polynucleotide molecule that inhibits the expression of SsnaI/NA14 or TPRA40. In some embodiments, the polynucleotide molecule is an antisense oligonucleotide that hybridizes to a NA14 transcript to inhibit expression of NA14. In some embodiments, the TPRA40 antagonist is a RNAi antagonist that targets the NA14 or TPRA40 mRNA transcript. In some embodiments, the TPRA40 antagonist is not an RNAi antagonist that does not target NA14 transcript. In 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 NA14 mRNA transcript. In some embodiments, the siRNA does not target NA14 mRNA transcript. In some embodiments, the siRNA targets TPRA40 mRNA transcript. In some embodiments, the siRNA comprises one or more of the nucleotide sequences selected from: SEQ ID NOs: 16-23. In some embodiments, the siRNA comprises one or more of the nucleotide sequences selected from SEQ ID NOs: 17-23. In some embodiments, the RNAi comprises an shRNA.

In some embodiments, the TPRA40 antagonist used in any of the methods disclosed herein is a small molecule that binds to TPRA40.

In some embodiments, the TPRA40 antagonist used in any of the methods disclosed herein is an antibody that binds to TPRA40 protein. In some embodiments, the antibody is a monoclonal antibody.

In some embodiments, the TPRA40 antagonist used in any of the methods disclosed herein is a polypeptide antagonist. In some embodiments, the polypeptide antagonist is a dominant negative SsnaI/NA14 protein. In some embodiments, the dominant negative NA14 protein is capable of binding TPRA40 but is incapable of binding microtubules. In some embodiments, the dominant negative NA14 protein lacks an N-terminal coiled-coil motif. In some embodiments, the TPRA40 antagonist is not a dominant negative NA14 protein.
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 steroidal alkaloid, 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.

In some embodiments, the present disclosure provides for methods of diagnosing a disease based on increased expression of TPRA40. In some embodiments, the present disclosure provides for methods of treating a subject (e.g., a human) in need thereof by administering to the subject a TPRA40 antagonist.

In some embodiments, a determination of G1/S expression and/or TPRA40 expression/overexpression or of increased TPRA40 activity in a cell is indicative that the cell is a neoplastic cell. In some embodiments, a determination of TPRA40 expression/overexpression or of increased TPRA40 activity in a biological sample obtained from a subject is indicative that the subject comprises a neoplasm. To determine TPRA40 expression in a tumor or cancer, various diagnostic assays are available. In one embodiment, TPRA40 polypeptide overexpression may be analyzed by immunohistochemistry (IHC). Paraffin embedded tissue sections from a tumor biopsy may be subjected to an IHC assay and accorded a TPRA40 protein staining intensity criteria. In one embodiment, the staining intensity criteria is set up as follows:
Score O - no staining is observed or membrane staining is observed in less than 10% of tumor cells.

Score 1+ - a faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane.

Score 2+ - a weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.

Score 3+ - a moderate to strong complete membrane staining is observed in more than 10% of the tumor cells.

In this embodiment, those tumors with 0 or 1+ scores for TPRA40 polypeptide expression may be characterized as underexpressing, or not overexpressing TPRA40, whereas those tumors with 2+ or 3+ scores may be characterized as overexpressing TPRA40. In some embodiments, FISH assays such as the INFORM® (sold by Ventana, Arizona) or PATHVISION™ (Vysis, Illinois) may be carried out on formalin-fixed, paraffin-embedded tumor tissue to determine the extent (if any) of TPRA40 overexpression in the tumor. TPRA40 overexpression or amplification may be evaluated using an in vivo diagnostic assay, e.g., by administering a molecule (such as an antibody, oligopeptide or organic molecule) which binds the molecule to be detected and is tagged with a detectable label (e.g., a radioactive isotope or a fluorescent label) and externally scanning the patient for localization of the label.

"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.

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 therapeutic amount of a TPRA40 antagonist, 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 preferably 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 preferably 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 TPRA40 hedgehog antagonists 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 TPRA40 antagonist (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).

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 TPRA40 and hedgehog amplification and 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 some embodiments, the TPRA40 antagonists described herein are for use in treating cancer in a subject. In some embodiments, depending on the stage of the cancer, cancer
treatment involves one or a combination of the following therapies: surgery to remove the
cancerous tissue, radiation therapy, and chemotherapy. In some embodiments, therapy
comprising of administering TPRA40 antagonists may be especially desirable in elderly patients
who do not tolerate the toxicity and side effects of chemotherapy well and in metastatic disease
where radiation therapy has limited usefulness. In some embodiments, the TPRA40 antagonists
of the present inventive method may also be used to alleviate hedgehog and/or TPRA40
overexpressing cancers upon initial diagnosis of the disease or during relapse. For therapeutic
applications, such TPRA40 antagonists can, in some embodiments, be used in combination with,
before or after application of other conventional agents and/or methods for the treatment of a
tumor, e.g., hormones, antiangiogens, or radiolabeled compounds, or with surgery, cryotherapy,
radiotherapy and/or chemotherapy. In some embodiments, the TPRA40 antagonist is
administered to a subject in combination with a chemotherapeutic agent and/or a growth
inhibitory agent and/or a HPL

Exemplary HPis are described herein and include, for example, smoothened inhibitors
and hedgehog inhibitors. Exemplary chemotherapeutic agents are known in the art and include,
for example, hydroxyureataxanes (such as paclitaxel and doxetaxel) and/or anthracycline
antibiotics; alkylating agents such as diiotepa and CYTOXAN® cyclophosphamide; alkyl
sulfonates such as busulfan, imposulfan and piposulfan; aziridines such as benzodopa,
carboquone, meturedopa, and uredopa; ethylenimines and methylarnelamines including
altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and
trimethylolomelamine; acetogains (especially bullatacin and bullatacinone); delta-9-
tetrahydrocannabinoi (dronabinol, MARINOL®); beta-iapachone; lapachol; colchicines; betulinic
acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-I
(irinotecan, CAMPTOSAR®), acetycamptothecin, scopolectin, and 9-aminocamptothecin);
bryostatin; cailystatin; 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, chlorphosphamide, estramustine, ifosfamide,
mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, iiovembichin, phenesterine,
prednimustine, trofosfamide, uracil mustard; nitrosoresas such as earmustine, chlorozotocin,
fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gammaI and calicheamicin omegaII (see, e.g., Ag[etal]., Chem Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinoniyein, carabiciii, c[a]pijinomycin, carzinophilin, chromomyciiis, dactinomycin, daunorubicin, detombicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifuridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testo!actone; anti-adrenals such as aminoglutethimide, mitotane, tri!ostane; folic acid replenisher such as frolmic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; 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(R) polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichiorotriethylamine; trichothecenes (especially T-2 toxin, verrarracin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FELDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacvtosine; arabinoside ("Ara-C"); thiotepa; taxoids, e.g., TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, NX), ABRAXANE® Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), and TAXOTERE® doxetaxel (Rli[delta]iie-Poulenc Rorer, Antony, France); chloranbucii; gemcitabine (GEMZAR®); 6-
thioguanine; niercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin;
vinblastine (VELBAN®); platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine
(ONCOVIN®); oxaliplatin; leucovorin; vinorelbine (NAVELBINE®); novantrone; edatrexate;
daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000;
difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine (XELODA®);
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.

Also included in the definition of chemotherapeutic agent are anti-hormonal agents that
act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of
cancer, and are often in the form of systemic, or whole-body treatment. They may be hormones
themselves. Examples include anti-estrogens and selective estrogen receptor modulators
(SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), EVI8TA:
raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY1 17018, onapristone, and
FARESTON® toremifene; anti-progesterones; estrogen receptor down-regulators (ERDs); agents
that function to suppress or shut down the ovaries, for example, leutinizing hormone-releasing
hormone (LHRH) agonists such as LUPRON® and ELIGARD® leuprolide acetate, goserelin
acetate, buserelin acetate and tripterel; other anti-androgens such as flutamide, mlutamide and
bicahrtamide; and aromatase inhibitors that inhibit the enzyme aromatase, which regulates
estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles,
aminogluthethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestanie,
fadrozole, RFVISOR® vorozole, FEMARA® ietrozole, and ARIMDDEX® anastrozole. In
addition, such definition of chemotherapeutic agents includes bisphosphonates such as
clodronate (for example, BONEFOS® or OSTAC®), DIDROCAL® etidronate, NE-58095,
ZOMETA® zoledronic acid/zoledronate, FOSAMAX® alendronate, AREOLA® pamidronate,
SKELiD® tiludronate, or ACTONEL® risedronate; as well as troxicitabine (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, LEUVECTTN® vaccine, and VAXID® vaccine; LURTOTECAN® topoisomerase 1 inhibitor; ABARELDC® rmRH; lapatinib ditosylate (an ErbB-2 and EGFR dual tyrosine kinase small-molecule inhibitor also known as GW572016); and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially a hedgehog responsive, a hedgehog expressing (and in some cases overexpressing) or TPRA40-expressing (and in some cases overexpressing) cell, either in vitro or in vivo. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of hedgehog-expressing cells 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 H 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 The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995). The taxanes or hydroxyureataxanes (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 (IA XOL®, Bristol-Myers Squibb). These molecules promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization, which results in the inhibition of mitosis in cells.

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, Phytoiaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated antibodies. Examples include \(^{212}\)Bi, \(^{131}\)I, \(^{131}\)In,
Conjugates of the antibody and cytotoxic agent are made using a variety of functional protein-coupling agents such as Nsuccinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of iodoesters (such as dimethyl adipimide HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoiiiumbenzoyl)- ethylenediamine), disiocypates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoiO-2,4-dinitrobenzene). For example, ricin immunotoxin can be prepared as described in Vitetta et al., Science, 238: 1098 (1987). Carbon-14-labeled l-isothioeyanatobenzyl-S-methylditiyiylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/1 1026. Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, maytansinoids, a trichothene, and CC 1065, and the derivatives of these toxins that have toxin activity, are also contemplated herein.

Chemotherapeutic drugs such as TAXGTERE® (docetaxel), TAXGL® (palictaxel), estramustine and mitoxantrone are used in treating cancer, in particular, in good risk patients. In particular, combination therapy with palictaxel and modified derivatives (see, e.g., EP0600517) is contemplated. In some embodiments, the TPRA40 antagonist is administered with a therapeutically effective dose of any of the chemotherapeutic agents disclosed herein (see definition above). In another embodiment, the TPRA40 antagonist is administered in conjunction with chemotherapy to enhance the activity and efficacy of the chemotherapeutic agent, e.g., paclitaxel. The Physicians' Desk Reference (PDR) discloses dosages of these agents that have been used in treatment- of various cancers. The dosing regimen and dosages of these aforementioned chemotherapeutic drugs that are therapeutically effective will depend on the particular cancer being treated, the extent of the disease and other factors familiar to the physician of skill in the art and can be determined by the physician.

In some embodiments, the TPRA40 antagonists described herein are for used in treating an adenocarcinoma. "Adenocarcinoma" refers to a malignant tumor originating in the glandular epithelium.

In some embodiments, the TPRA40 antagonists described herein are for used in treating a carcinoma. "Carcinoma" refers to a malignant growth derived from epithelial cells that tends to metastasize to other areas of the body. Examples include "basal cell carcinoma" - an epithelial
tumor of the skin that, while seldom metastasizing, can result in local invasion and destruction; "squamous cell carcinoma" - tumors arising from squamous epithelium and having cuboid cells; "carcinosarcoma" - malignant tumors comprising both carcinomatous and sarcomatous tissues; "adenocystic carcinoma" - tumors characterized by large epithelial masses containing round gland-like spaces or cysts, frequently containing mucus, that are bordered by layers of epithelial cells; - "epidermoid carcinoma" - see squamous cell carcinoma; "nasopharyngeal carcinoma" - malignant tumor arising in the epithelial lining of the space behind the nose; "renal cell carcinoma" - tumor in the renal parenchyma composed of tubular cells in varying arrangements. Additional carcinomatous epithelial growth include "papillomas", which are benign tumors derived from the epithelium and having papillomavirus as a causative agent; and "epidermoidomas", which are cerebral of meningeal tumors formed by inclusion of ectodermal elements at the time of closure of the neural groove.

In some embodiments, any of the TPRA40 antagonists disclosed herein are administered to a human patient, in accord with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intracranial, intracerebrospinal, intrarticular, intrathecal, intravenous, intraarterial, subcutaneous, oral, topical, or inhalation routes. Other therapeutic regimens may be combined with the administration of the foregoing TPRA40 antagonists. In some embodiments, the combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. In some embodiments, combined therapy results in a synergistic therapeutic effect. In some embodiments, the TPRA40 antagonist is coadministered (simultaneously or consecutively) with a second hedgehog pathway inhibitor, such as a smoothenec inhibitor or a hedgehog inhibitor (e.g., robotkinin). In some embodiments, the TPRA40 antagonist is coadministered with 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 TPRA40 antagonist is coadministered with vismodegib, sonidegib, BMS-833923, PF-04449913, or LY2940680 or any derivative thereof. In some embodiments, the TPRA40 antagonist is coadministered with any of the compounds disclosed in Amakye, et al., Nature Medicine, 19(11):1410-1422 (wluchi is incorporated herein in its entirety). In some embodiments the antagonist is coadministered with
another smoothened inhibitor chemically unrelated to veratrum alkaloids or vismodegib, including but not limited to: sonidegib, BMS-833923, PF-04449913, LY2940680, 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 therapeutic treatment methods of the present disclosure involves the combined administration of the preceding TPRA40 antagonist and one or more of the chemotherapeutic agents or growth inhibitory agents described herein, including co-administration of cocktails of different chemotherapeutic agents. Example chemotherapeutic agents are disclosed herein. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M.C. Perry, Williams & Wilkms, Baltimore, MD (1992). For the prevention or treatment of disease, the dosage and mode of administration will be chosen by the physician according to known criteria. The appropriate dosage of TPRA40 antagonists will depend on the type of disease to be treated, the severity and course of the disease, whether administration is for preventive or therapeutic purposes, previous therapy (including) the patient's clinical history and response, and the discretion of the attending physician. The preceding TPRA40 antagonists may be suitably administered to the patient at one time or over a series of treatments. Administration may occur by intravenous infusion or by subcutaneous injections or orally in the case of certain small molecule inhibitors. Suitable dose will depend on the agent and the particular use and is determined. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The progress of this therapy can be readily monitored by conventional methods and assays and based on criteria known to the physician or other persons of skill in the art. For any of the methods of the disclosure, it is contemplated that a TPRA40 antagonist can be administered as a monotherapy or in combination with one or more other therapeutic agents.

Aside from administration of TPRA40 polypeptide antagonists to a patient, the present disclosure contemplates administration of the TPRA40 antagonist by gene therapy. Such administration of a nucleic acid encoding the TPRA40 hedgehog polypeptide antagonists is encompassed by the expression "administering a therapeutically effective amount of a TPRA40
antagonist”. See, for example, WQ96/07321 published March 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

There are two major approaches to getting such nucleic acid (optionally contained in a vector) into the patient's cells; in vivo and ex vivo. In some embodiments, in vivo delivery of the nucleic acid involves injection directly into the patient, usually at the site where the antibody is required. In some embodiments, for ex vivo treatment, the patient's cells are removed, the nucleic acid is introduced into these 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). There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells in vitro, or in vivo in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for ex vivo delivery of the gene is a retroviral vector. The currently preferred in vivo nucleic acid transfer techniques include 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-Choi, for example). For review of the currently known gene marking and gene therapy protocols see Anderson et al., Science 256:808-813 (1992). See also WO 93/25673 and the references cited therein.

As hedgehog is known to stimulate angiogenesis, it follows based on the teachings herein that TPRA40, which antagonizes hedgehog signaling activity, would antagonize hedgehog mediated processes, (e.g., angiogenesis) particularly when some level of hedgehog activity is necessary for angiogenesis. In some embodiments, the disclosure provides for a method of modulating angiogenesis in a subject by administering to the subject a therapeutically effective amount of a TPRA40 antagonist. In some embodiments, the disclosure provides a method of treating any of the angiogenesis-related conditions, disorders and/or diseases disclosed herein.

In some embodiments, any of the TPRA40 antagonists described herein inhibits angiogenesis. Angiogenesis is fundamental to many disorders. Persistent, unregulated angiogenesis occurs in a range of disease states, tumor metastases and abnormal growths by
endothelial cells. The vasculature created as a result of angiogenic processes supports the pathological damage seen in these diseases.

In some embodiments, any of the TPRA40 antagonists described herein are used for treating a disease associated with or resulting from angiogenesis by inhibiting angiogenesis. Diseases associated with or resulting from angiogenesis include: ocular neovascular disease, age-related macular degeneration, diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, neovascular glaucoma, retrointental fibroplasias, epidemic keratoconjunctivitis, Vitamin A deficiency, contact lens overwear, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, Sjogren's syndrome, acne rosacea, phlyctenulosis, syphilis, Mycobacteria infections, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, Herpes simplex infections, Herpes zoster infections, protozoan infections, Kaposi's sarcoma, Mooren's ulcer, Terrien's marginal degeneration, marginal keratolysis, rheumatoid arthritis, systemic lupus, polyarteritis, trauma, Wegener's granulomatosis, sarcoidosis, scleritis, Stevens-Johnson syndrome, pemphigoid radial keratotomy, corneal graph rejection, rheumatoid arthritis, systemic lupus, polyarteritis, trauma, Wegener's granulomatosis, sarcoidosis, scleritis, Stevens-Johnson syndrome, pemphigoid radial keratotomy, corneal graph rejection, rheumatoid arthritis, osteoarthritis chronic inflammation (e.g., ulcerative colitis or Crohn's disease), hemangioma. Osier-Weber Rendu disease, and hereditary hemorrhagic telangiectasia.

In some embodiments, any of the TPRA40 antagonists described herein may be used for treating cancer, such as by inhibiting angiogenesis, as angiogenesis is known to play a critical role in cancer. A tumor cannot expand without a blood supply to provide nutrients and remove cellular wastes. Tumors in which angiogenesis is important include solid tumors such as rhabdomyosarcomas, retinoblastoma, Ewing sarcoma, neuroblastoma, osteosarcoma, and benign tumors such as acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas.

Angiogenic factors have been found associated with several solid tumors, and preventing angiogenesis could halt the growth of these tumors and the resultant damage to the animal due to the presence of the tumor. Angiogenesis is also associated with blood-born tumors such as leukemias, any of various acute or chronic neoplastic diseases of the bone marrow in which unrestrained proliferation of white blood cells occurs, usually accompanied by anemia, impaired blood clotting, and enlargement of the lymph nodes, liver, and spleen. It is believed that
angiogenesis plays a role in the abnormalities in the bone marrow that give rise to leukemia-like tumors.

In some embodiments, any of the TPRA40 antagonists described herein may be used for inhibiting metastasis, such as by inhibiting angiogenesis. Initially, angiogenesis is important in the vascularization of the tumor which allows cancerous cells to enter the blood stream and to circulate throughout the body. After the tumor cells have left the primary site, and have settled into the secondary, metastatic site, angiogenesis must occur before the new tumor can grow and expand. Therefore, prevention of angiogenesis could lead to the prevention of metastasis of tumors and possibly contain the neoplastic growth at the primary site. In other embodiments, the TPRA40 antagonists are useful for inhibiting metastasis, regardless of mechanism of action (e.g., regardless of whether that inhibition is due to inhibition of angiogenesis).

Angiogenesis is also involved in normal physiological processes such as reproduction and wound healing. Angiogenesis is an important step in ovulation and also in implantation of the blastula after fertilization. In some embodiments, prevention of angiogenesis using any of the TPRA antagonists described herein could be used to induce amenorrhea, to block ovulation or to prevent implantation by the blastula.

In certain embodiments, a TPRA40 antagonist 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 TPRA40 antagonists 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 of 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 PGR and microarray analysis of transcript levels. Methods for detecting Gli protein levels include Western blotting, immunoprecipitation, two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE - preferably 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 Gli 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 TPRA40 antagonist 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 TPRA40 antagonist. 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 TPRA40 antagonist. 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 Ptc1 gene is located at this position and Ptc1 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 TPRA40 antagonists described herein are used for treating a subject having a tumor having a ptc-1 and/or ptc-2 mutation, e.g., a ptc-1 or ptc-2 loss of function mutation. Expression of ptc-1 and ptc-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 ptc-1 or ptc-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 certain embodiments, any of the TPRA40 antagonists described herein may be used for treating a cell, tumor or subject having a smootherened mutation. In some embodiments, the
smoothened mutation results in a constitutively active smoothened protein. In some embodiments, the smoothened mutation is a mutation corresponding to position W535L of SEQ ID NO: 42. In some embodiments, the smoothened mutation is a mutation corresponding to position R562Q of SEQ ID NO: 42. In some embodiments, the smoothened mutation has a mutation that renders it resistant to certain smoothened inhibitors. In some embodiments, the smoothened protein comprises an amino acid alteration at amino acid position 518 of SEQ ID NO: 42. In some embodiments, the amino acid alteration is E518K or E518A substitution at the amino acid position corresponding to amino acid position 518 of SEQ ID NO: 42. In some embodiments, the smoothened protein comprises an amino acid alteration at amino acid position 473 of SEQ ID NO: 42. In some embodiments, the amino acid alteration is the substitution of aspartic acid with any of histidine, glycine, phenylalanine, tyrosine, leucine, isoleucine, proline, serine threonine, methionine, glutamine, or asparagine at the amino acid position corresponding to amino acid position 473 of SEQ ID NO: 42. See, e.g., WO 2011/028950 and WO201 2047968, each of which is incorporated by reference.

In certain embodiments, any of the TPRA40 antagonists described herein may be used for treating a cell, tumor or subject having a SuFu mutation. In some embodiments, the SuFu mutation results in a loss-of-function in SuFu activity. In some embodiments, the SuFu mutation is in a medulloblastoma, meninogia, adenoid cystic carcinoma, basal cell carcinoma or 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 indicated in Tables 1 and 2. In some embodiments, the SuFu mutation is any of the mutations corresponding to P15L, Q184X, R123C, L295fs, or P187L of SEQ ID NO: 43. In some embodiments, the SuFu mutation is any of the mutations corresponding to c.1022+1G>A (IVS8-10T), c.72delC, c.72insC, 143insA, c.846insC, or IVS1-1A->T of SEQ ID NO: 44. 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.1 022 +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.550OT (Q184X)); Aavikko et al (2012) Am J Hum Genet 91: 520-526 (e.g.,
c.3670T (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 light of the broad involvement of hedgehog signaling in the formation of ordered spatial arrangements of differentiated tissues in vertebrates, the TPRA40 antagonists 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 TPRA40 antagonist, can be, as appropriate, any of the preparations described above.

In some embodiments, the TPRA40 antagonists of the present disclosure are further applicable to cell culture techniques wherein reduction in hedgehog signaling is desirable. Use of the present method may be in culture of, for example, neuronal stem cells, such as in the use of such cultures for the generation of new neurons and glia. In some embodiments, these cultures can be contacted with TPRA40 antagonists in order to alter the rate of proliferation or neuronal stem cells in the culture and/or alter the rate of differentiation, or to maintain the integrity of a culture of certain terminally differentiated neuronal cells. In one embodiment, the TPRA40 antagonists can be used in culture of certain neuron types (e.g., sensory neurons, motor neurons). Such neuronal cultures can be used as convenient assay systems as well as sources of implantable cells for therapeutic treatments.

Stern cells useful for use in any of the methods of the present disclosure are generally known. For example, several neural crest cells have been identified, some of which are multipotent and likely represent uncommitted neural crest cells, and others of which can generate only one type of cell, such as sensory neurons, and likely represent committed progenitor cells. The role of TPRA40 antagonists employed in the present method to culture such stem cells can be to regulate differentiation of the uncommitted progenitor, or to regulate further restriction of the developmental fate of a committed progenitor, or to regulate further restriction of the developmental fate of a committed progenitor cell towards becoming a terminally differentiated neuronal cell. For example, the present method can be used in vitro to regulate the differentiation of neural crest cells into glial cells, Schwann cells, chromaffin cells, cholinergic, sympathetic or parasympathetic neurons, as well as peptidergic and serotonergic neurons. The TPRA40 antagonist can be used alone, or in combination with other neurotrophic factors that act to more particularly enhance a particular differentiation fate of the neuronal progenitor cell. In addition to use of the TPRA40 antagonists in combination with implantation of cell cultures,
another aspect of the present disclosure relates to the therapeutic application of TPRA40 antagonists to regulate the growth state of neurons and other neuronal cells in both the central nervous system and the peripheral nervous system.

In some embodiments, the TPRA40 antagonists can be used in the treatment of neoplastic or hyperplastic transformation such as may occur in the central nervous system. For instance, the TPRA40 antagonists can be utilized to cause such transformed cells to become either post-mitotic or apoptotic. The TPRA40 antagonists may, therefore, be used as part of a treatment for, e.g., malignant gliomas, meningiomas, medulloblastomas, neuroectodermal tumors, and ependymomas.

In some embodiments, the TPRA40 antagonists 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.

Medulloblastoma/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 MR] has largely replaced myelography for this purpose, and CSF cytology is obtained postoperatively as a routine procedure.

In some embodiments, the TPRA40 antagonists are used as part of a treatment program for ependymomas. Ependymomas 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 ependymomas, ¾ were histologically benign, approximately 2/3 arose from the region of the 4th ventricle, 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 TPRA40 antagonists can be used in cell culture and therapeutic method relating to the generation and maintenance of non-neural tissue. Such uses are contemplated as a result of the involvement of hedgehog signaling components (e.g., ptc, hedgehog, smo, fused, SuFu, Cos-2, etc.) in ni morphogenic signals of other vertebrate organogenic pathways, such as endodermal patterning, and mesodermal and endodermal differentiation.

As hedgehog signaling, especially ptc, hedgehog, and smo othened, are involved in controlling the development of stem cells responsible for formation of the digestive tract, liver, lungs, and other organs derived from the primitive gut. Shh is the inductive signal from the endoderm to the mesoderm, which is critical to gut morphogenesis. Therefore, in some embodiments, the TPRA40 antagonists can be employed for regulating the development and maintenance of an artificial liver that can have multiple metabolic functions of a nonnal liver. In one embodiment, the TPRA40 antagonists can be used to regulate functions of a normal liver. In one embodiment, the TPRA40 antagonists can be used to regulate the proliferation and differentiation of digestive tube stem cells to form hepatocyte cultures which can be used to populate extracellular matrices, or which can be encapsulated in biocompatible polymers, to form both implantable and extracorporeal artificial livers.

In some embodiments, the TPRA40 antagonists can be employed therapeutically to regulate such organs after physical, chemical or pathological insult. For instance, therapeutics comprising any of the TPRA40 antagonists described herein can be used in liver repair subsequent to a partial hepactectomy.

In some embodiments, the TPRA40 antagonists of the present disclosure can be used in the treatment of hyperplastic and neoplastic disorders affecting pancreatic tissue, especially those characterized by aberrant proliferation of pancreatic cells. For instance, pancreatic cancers are marked by abnormal proliferation of pancreatic cells, which can result in alterations of insulin secretory capacity of the pancreas. For example, certain pancreatic hyperplasias, such as pancreatic carcinomas, can result in hypoinsulinemia due to dysfunction of β-cells or decreased islet cell mass. Moreover, manipulation of hedgehog signaling properties at different points may be useful as part of a strategy for reshaping/repairing pancreatic tissue both in vivo and in vitro. In one embodiment, the present disclosure makes use of the apparent involvement of ptc, hedgehog and smo othened in regulating the development of pancreatic tissue. In general, the TPRA40 antagonists can be employed therapeutically to regulate the pancreas after physical,
chemical or pathological insult. In some embodiments, the TPRA40 antagonists can be applied to cell culture techniques, and in particular, may be employed to enhance the initial generation of prosthetic pancreatic tissue devices. Manipulation of proliferation and differentiation of pancreatic tissue, such as through using TPRA40 antagonists, can provide a means for more carefully controlling the characteristics of a cultured tissue. In an exemplary embodiment, the TPRA40 antagonists can be used to augment production of prosthetic devise which require β-islet cells, such as may be used in the encapsulation devices described in, for example, as described in U.S.P. 4,892,538, 5,106,627, 4,391,909 and 4,353,888. Early progenitor cells to the pancreatic islets are multipotential, and apparently coactivate all the islet-specific genes from the time they first appear. As development proceeds, expression of islet-specific hormones, such as insulin, becomes restricted to the pattern of expression characteristic of mature islet cells. The phenotype of mature islet cells, however, is not stable in culture, as reappearance of embryonal traits in mature β-cells can be observed. By utilizing any of the TPRA40 antagonists described herein, the differentiation path or proliferative index of the cells can be regulated.

In some embodiments, the TPRA40 antagonists of the present disclosure may also be used as part of a treatment of lung carcinoma and adenocarcinoma, and other proliferative disorders involving the lung epithelia. It has been shown that Shh is expressed in human lung squamous carcinoma and adenocarcinoma cells. Fujita et al., Biochem. Biophys. Res.Commun. 238: 658 (1997). The expression of Shh was also detected in the human lung squamous carcinoma tissues, but not in the normal lung tissue of the same patient. They also observed that Shh stimulates the incorporation of BrdU into the carcinoma cells and stimulates their cell growth, while anti-Shh-H inhibited their cell growth. These results suggest that ptc, hedgehog, and/or smootheneci is involved in cell growth of such transformed lung tissue and therefore indicates that the subject can be used as part of a treatment of lung carcinoma and adenocarcinomas, and other proliferative disorders involving the lung epithelia.

In some embodiments, the TPRA40 antagonists 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 ptc mutation (e.g., hemangiona, rhabdomyosarcoma, etc.), tumors resulting from Glil amplification (e.g.,
glioblastoma, sarcoma, etc.), tumors resulting from Smo dysfunction (e.g., basal cell carcinoma, etc.), tumors connected with TRC8, a ptc homoiog (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 TPRA40 antagonists 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.

For example, the pharmaceutical preparations of the TPRA40 antagonists of the disclosure are intended for the treatment of hyperplastic conditions, such as keratosis, as well as for the treatment of neoplastic epidermal conditions such as those characterized by a high proliferation rate for various skin cancers, e.g., squamous cell carcinoma. In some embodiments, the TPRA40 antagonists of the disclosure can also be used in the treatment of autoimmune diseases affecting the skin, in particular, of dermatological diseases involving morbid proliferation and/or keratinization of the epidermis, as for example, caused by psoriasis or atopic dermatosis. Many common diseases of the skin, such as psoriasis, squamous cell carcinoma, keratoacanthioma and actinic keratosis are characterized by localized abnormal proliferation and growth. For example, in psoriasis, which is characterized by scaly, red, elevated plaques on the skin, the keratinocytes are known to proliferate much more rapidly than normal and to
differentiate less completely. In one embodiment, the preparations of the TPRA40 antagonists of the present disclosure are suitable for the treatment of dermatological ailments linked to keratinization disorders causing abnormal proliferation of skin cells, which disorder may be marked by either inflammatory or non-inflammatory components. In some embodiments, the TPRA40 antagonists that promote quiescence or differentiation can be used to treat varying forms of psoriasis, e.g., cutaneous, mucosal or ungual. Psoriasis, as described above, is typically characterized by epidermal keratinocytes that display marked proliferative activation and differentiation along a "regenerative" pathway. Treatment with such TPRA40 antagonists according to the present disclosure can be used to reverse the pathological epidermal activation and can provide a basis for sustained remission of the disease.

A variety of other keratotic lesions are also candidates for treatment with the TPRA40 antagonists of the present disclosure. Actinic keratoses, for example, are superficial inflammatory premalignant tumors arising on sun-exposed and irradiated skin. The lesions are erythematous to brown with variable scaling. Current therapies include excisional and cryosurgery. These treatments are painful, however, and often produce cosmetically unacceptable scarring. In some embodiments, treatment of keratosis, such as actinic keratosis, can include application, preferably topical, of a TPRA40 antagonist composition in amounts sufficient to inhibit hyperproliferation of epidermal/epidermoid cells of the lesion.

Acne represents yet another dermatologic ailment which may be treated by the TPRA40 antagonists of the present disclosure. Acne vulgaris, a multifactor disease most commonly occurring in teenagers and young adults, is characterized by the appearance of inflammatory and noninflammatory lesions on the face and upper trunk. The basic defect which gives rise to acne vulgaris is hypercornification of the duct of a hyperactive sebaceous gland. Hypercornification blocks the normal mobility of skin and follicle microorganisms, and in so doing, stimulates the release of lipases by Propinobacterian acnes and Staphylococcus epidermidis bacteria and Pitrosporum ovale, a yeast. In some embodiments, treatment with an antiproliferative TPRA40 antagonist, particularly topical preparations, may be useful for preventing the transitional features of the ducts, e.g., hypercornification, which lead to lesion formation. The subject treatment may further include, for example, antibiotics, retinoids and antiandrogens.

In some embodiments, the TPRA40 antagonists of the present disclosure may also be used in a method treating various forms of dermatitis. Dermatitis is a descriptive term referring
to poorly demarcated lesions that are either pruritic, erythematous, scaly, blistered, weeping, fissured or crusted. These lesions arise from any of a wide variety of causes. The most common types of dermatitis are atopic, contact and diaper dermatitis. For example, seborrheic dermatitis is a chronic, usually pruritic, dermatitis with erythema, dry, moist, or greasy scaling, and yellow-crusted patches on various areas, especially the scalp, with exfoliation of an excessive amount of dry scales. In some embodiments, the TPRA40 antagonists may also be used in the treatment of stasis dermatitis, an often chronic, usually eczematous dermatitis. Actinic dermatitis is a dermatitis that due to exposure to actinic radiation such as that from the sun, ultraviolet waves, or x- or gamma-radiation. According to the present disclosure, the TPRA40 antagonists can be used in the treatment and/or prevention of certain symptoms of dermatitis caused by unwanted proliferation of epithelial cells. Such therapies for these various forms of dermatitis can also include topical and systemic corticosteroids, antipruritics, and antibiotics. Additional skin ailments that may be treated with the TPRA40 antagonists of the present disclosure include disorders specific to non-humans, such as mange.

The foregoing are merely exemplary of in vitro and in vivo uses for TPRA40 antagonists of the disclosure. TPRA40 antagonists are also suitable for use in identifying natural targets or binding partners for TPRA40, to study TPRA40 bioactivity, to purify TPRA40 and its binding partners from various cells and tissues, and to identify additional components of the hedgehog signaling pathway.

As detailed herein, the disclosure also provides for methods of screening to identify TPRA40 agonists. TPRA40 agonists can be used in methods of promoting hedgehog signaling in vitro and/or in vivo. TPRA40 agonists are also suitable in subjects in need thereof and can be administered as described herein. In some embodiments, the disclosure provides for a method of administering any of the TPRA40 agonists described herein, such as a small molecule agonist, to a subject in need thereof. In some embodiments, the subject has Down's Syndrome, ischemic heart disease, or alopecia. See, e.g., Sci Transl Med. 2013 Sep 4;5(201):201ral20. doi: 10.126/scitranslmed.3005983; Pharmazie. 2012 Jun;67(6):475-81; Cardiovasc Res. 2012 Sep 1;95(4):507-16. doi: 10.1093/cvr/cvs216; and J Invest Dermatol. 2005 Oct;125(4):638-46. In some embodiments, the subject is a pre-term baby that has been or is being treated with glucocorticoids, and the subject is administered the TPRA40 agonist in order to prevent nerve damage. See, e.g., Sci Transl Med. 2011 Oct 19;3(105):105ral04. doi: 10.126/scitranslmed.3002731.
Exemplary TPRA40 agonists are small molecule that hind to TPRA40. Also contemplated is overexpression of TPRA40 as a TPRA40 agonist.

V. Methods of Administration

Various delivery systems are known and can be used to administer TPRA40 antagonists of the disclosure to cells or subjects. Such delivery systems are similarly applicable to TPRA40 agonists. Any such methods of administration may be used in the context of any of the methods of use described herein and/or in the context of any of the TPRA40 antagonists of the disclosure, such as the TPRA40 antagonists described herein. Methods of introduction can be enteral or parenteral, including but not limited to, intradermal, intramuscular, intraperitoneal, intramyocardial, intravenous, subcutaneous, pulmonary, intranasal, intraocular, epidural, superficial/topical (e.g., a cream) and oral routes. The route of introduction may be selected based on, for example, the particular TPRA40 antagonist used (e.g., small molecule versus protein) and the intended use/purpose of administration. When administering to subjects (such as to cells in a subject), a TPRA40 antagonist may be administered by any convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. When administered to cells in culture, TPRA40 antagonist may be added to culture media, delivered into cells via a viral vector, delivered into cells by transfection, electroporation, or transformation, optionally, as part of a vector, and the like. Similarly, delivery via a viral or other vector may be used to facilitate delivering into cells in vivo.

In certain embodiments, a TPRA40 antagonist is administered intravenously. In certain embodiments, it may be desirable to administer the TPRA40 antagonist locally to the area in need of treatment (e.g., to the site of a tumor); this may be achieved, for example, and not by way of limitation, by local infusion or injection during surgery, by means of a catheter, or by means of an implant, the implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, fibers, or commercial skin substitutes.

Note that the disclosure contemplates methods in which a TPRA40 antagonist of the disclosure is administered, at the same or different times, with a second TPRA40 antagonist and/or a second therapeutic agent. Administration may be by the same of differing routes of
administration. For example, the disclosure contemplates a regimen in which a TPRA40 antagonist of the disclosure is administered intravenously and a second therapeutic agent is administering orally.

The foregoing applies to any of the TPRA40 antagonists, compositions, and methods described herein. The disclosure specifically contemplates any combination of the features of such TPRA40 antagonists, compositions, and methods (alone or in combination) with the features described for the various pharmaceutical compositions and routes of administration described in this section and in the section provided below. The formulations provided below are merely exemplary, and TPRA40 antagonists for use in the subject methods can be formulated as appropriate for the intended use and route of administration.

VI. Pharmaceutical Formulations

In some embodiments, any of the TPRA40 antagonists described herein or TPRA40 antagonists in accordance with the disclosure may be formulated in a pharmaceutical composition. Similarly, pharmaceutical formulations as described herein as applicable to TPRA40 agonists.

Pharmaceutical compositions of the TPRA40 antagonists 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 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 octadecyltrimethylbenzyi ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (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 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).

In some embodiments, any of the formulations of TPRA antagonists 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, preferably those with complementary activities that do not adversely affect each other. It should be recognized that, in certain embodiments, a TPRA40 antagonist 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 (which is incorporated herein in its entirety). In some embodiments the additional active compound is another smoothened inhibitor chemically unrelated to veratum 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 TPRA40 antagonist (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 TPRA antagonists 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-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, naiio-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 TPRA40 antagonists 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(vmylalcho!)!), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and \(\gamma\) ethyl-L-glutarnate, non-degradable ethylenne-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT* (injectable microspheres composed of lactic acid-glyceric acid copolymer and leupro!ide acetate), and poliy-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-pyro genie. 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 Pharmacopeia! Convention, Pharmacopeia! 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 TPRA40 antagonists are formulated in sterile formulations. This is readily accomplished by filtration through sterile filtration membranes.

VII. Articles of Manufacture and Kits

In some embodiments, the TPRA40 antagonists of the present disclosure, such as the TPRA40 antagonists disclosed herein are prepared in 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. 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 TPRA40 antagonist. The label or package insert will further comprise instructions for administering the TPRA40 antagonist. 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 TPRA40-expressing cell killing assays, for purification or immunoprecipitation of TPRA40
polypeptide from cells. For isolation and purification of TPRA40, the kit can contain the
respective TPRA40-binding reagent coupled to beads (e.g., sepharose beads). Kits can be
provided which contain such molecules for detection and quantitation of TPRA40 polypeptide 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
TPRA40 antagonist 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.

Exemplification

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: siRNA Knockdown of TPRA40 Partially Inhibits Hedgehog Signaling

Pools of siRNAs were used to knockdown expression of TPRA40 or Ssnal (a gene
previously shown to be involved in the Hedgehog signaling pathway; Lai et al., 2011, Mol Biol
Cell 22: 1104) in the presence or absence of an agent that stimulates hedgehog signaling, and the
impact on hedgehog signaling was assessed in S12 cells comprising a 6i/i-luciferase reporter.
TPRA40 depletion inhibited, by approximately 40%, Hedgehog signaling stimulated by either
Sonic hedgehog protein or by a small molecule Smoothened agonist. This supports the
conclusion that TPRA40 is a positive regulator of the hedgehog signaling pathway, and that it
acts downstream of Smoothened.
Briefly, for the experiments summarized in Figure 1A, S12 cells were depleted of murine Lft88 (by 72%), Ssna1 (by 75%) and TPRA40 (by 80%) using siRNAs. Cells were transfected with 50nM siRNA (with pools of four siRNAs per target, per standard practice) for 72 hours, the last 24 hours of which they were incubated in serum-free media in the presence or absence of 200ng/ml octyl-Shh to stimulate hedgehog signaling. (In Figure 1A, the results following incubation with Shh are shown in black - the right bar in each set of bars; the results following incubation without Shh are shown in grey - the left bar in each set of bars). G/7-luciferase activity was measured versus renilla-luciferase (as a measure of viability) and the data were expressed as a % of the non-targeting control siRNA (siNTC) +Hh. The mean and standard deviation of four independent experiments are shown. *, p <0.05; **, p ≤ 0.01; ***, p ≤ 0.001 (student's unpaired t-test). The efficiency of each siRNA was measured by qRT-PCR analysis of the corresponding gene.

For the experiments summarized in Figure 1B, S12 cells were treated as in (A) except hedgehog signaling was stimulated with 100ngM of the Smoothened, small molecule agonist Hhl.2 instead of octyl-Shh (black; right bar in each set of bars) or vehicle control (DMSO) (grey; left bar in each set of bars). The results were expressed as a percentage of stimulated control (NTC) cells.

Example 2: siRNA Knockdown of TPRA40 Reduced Hedgehog Induced Gli mRNA and Protein Expression in S12 Cells

TPRA40 depletion using siRNA inhibited hedgehog-stimulated Gli1 induction in S12 cells. The inhibition in Gli1 induction was observed at both the mRNA and protein level, as both Gli1 mRNA and Gli1 protein expression were reduced following TPRA40 depletion. Figure 2A summarizes the results of experiments in which Gli1 mRNA was evaluated. Briefly, S12 cells were depleted of TPRA40 by siRNA treatment and stimulated with 200ng/ml Sonic hedgehog protein (black; Hh; right bar in each set) or serum-starved in the absence of hedgehog protein stimulation (grey; left bar in each set) as in Example 1, but analyzed by qRT-PCR for endogenous murine Gli1 expression (instead of Gli1-luciferase activity, as was done in Example 1). The mean and standard deviation of three independent experiments was plotted and showed an approximately 50% reduction in hedgehog pathway stimulation, as measured by change in endogenous Gli1 expression, in TPRA40 siRNA treated (i.e., TPRA40-deficient) cells. Figure
2B summarizes the results of experiments in which Glil protein levels were evaluated. Briefly, cells were treated as in 2A but lysed and analyzed by western blotting with a Glil-specific monoclonal antibody, then reprobed for tubulin (55kDa) as a loading control. The Hh-induced upregulation of Glil protein (~150 kDa) was also diminished by TPRA40 knockdown (a representative blot of two independent experiments is shown).

Example 3: Analysis of the Individual Components of the TPRA40 siRNA Pool Confirmed that the Observed Effects on TPRA40 Expression and Activity are Not Off-Target Effects

Figures 3A-C show that three of the four individual siRNA components of the siTPRA40 pool are active and reduce TPRA40 expression. Moreover, the active, individual siRNAs cause approximately the same level of inhibition as the pool, consistent with the conclusion that the observed effects on TPRA40 and hedgehog signaling are specific and don't represent off-target effects.

Figure 3A summarizes experiments in which the four siRNAs to TPRA40 that make up the pool (siRNAs #9-12) were transfected individually into S12 cells at 25nM and the Glil-luciferase activity was measured, as described in Example 1, except the data were normalized to siNTC -Hh as 1. As in the experiments depicted in Figure 1A, Sonic hedgehog protein was added to stimulate hedgehog signaling. Mean and standard deviation of three experiments are shown. siRNAs #10-12 inhibited Gli-luciferase stimulation similarly to the 100nM pool (by 34-40%), while siRNA #9 did not.

Figure 3B shows the results of an experiment in which S12 cells were treated as in (A) but endogenous Gli1 (murine Gli1; mGli expression in S12 cells) levels were measured by qRT-PCR. 25nM siRNA #9 again had no effect, while siRNAs #10-12 inhibited Gli1 induction, albeit less effectively than 100nM of the 4 siRNA pool. Data from a single experiment is shown.

Figure 3C shows the results of an experiment in which TPRA40 gene expression levels (murine TPRA40; mTPRA40 expression in S12 cells), assayed by qRT-PCR, were decreased by more than 80% by the siRNA pool and individual siRNAs #9 and 10; by about 60% by siRNA #11 and by about 70% by siRNA #12. In addition, we observed that hedgehog stimulation had no effect on TPRA40 levels (in the non-targeting control (NTC)-transfected cells), consistent with the conclusion that TPRA40 is not itself a hedgehog pathway target gene. Data from a single experiment is shown. In Figures 3B and 3C, results in the presence of hedgehog treatment are
shown in black (the right bar in each set) and the results in the absence of hedgehog treatment are shown in grey (the left bar in each set).

Example 4: siRNA Knockdown of TPRA40 Inhibited Endogenous Hedgehog Signaling in Medulloblastoma Cells.

Figure 4 summarizes results showing that depletion of TPRA40 using siRNA inhibited hedgehog signaling in Daoy medulloblastoma cells. These results are consistent with the conclusion that TPRA40 acts downstream of the activating mutation in Daoy cells. Moreover, this experiment demonstrated that modulation of TPRA40 regulates Hedgehog signaling not only in normal cells (such as S12 cells), but also in cancer cells.

Daoy medulloblastoma cells are a human cell type that exhibits constitutively active Hh signaling (in the absence of Hh ligand), as monitored by hGli1 mRNA levels. This constitutive signaling can be decreased by treatment with 0.5 or 2 μM Cyclopamine, a well-known Smoothened inhibitor. TPRA40 depletion (depletion of endogenous, human TPRA40 expressed in these medulloblastoma cells) by transfection of 50nM of the siRNA pool to hTPRA40 also reduced hGli1 levels and, when combined with cyclopamine, further reduced hGli1 levels (See Figure 4). Data from a single experiment is shown. For each of the DMSO or cyclopamine treatment groups, data for cells treated with the TPRA40 siRNA pool is shown by the right-hand bar (black).

Example 5: Ordering TPRA40 in the Hedgehog Signaling Pathway

Figure 5A provides a schematic representation of certain key Hedgehog pathway components, with positive regulators shown in normal font and negative regulators shown in bold font. The following positive regulators are depicted: Shh (Sonic hedgehog), Smo (Smoothened) and Gli activators (i.e. Gli1, Gli2-fuli length or Gli3-full length). The following negative regulators (in bold font) are depicted: Ptc (Patched 1), SuFu (Suppressor of Fused) and PKA (Protein Kinase A) and Gli repressors (truncated Gli2 or truncated Gli3). Arrows denote a positive effect of the upstream gene, while bars denote an inhibitory effect of the upstream gene on the downstream one. PKA phosphorylates (depicted by the letter P) Gli2 and Gli3-fuli length (FL), which initiates their cleavage into repressor forms (Gli-R). We conducted a series of
experiments to orient TPRA40 in the Hedgehog signaling pathway, relative to other pathway players.

Figures 5B and C summarize experiments designed to order TPRA40 in the hedgehog signaling pathway and show that TPRA40 acts downstream of Ptchl and SuFu. The experiments were conducted in S12 cells in the absence of stimulation with Hedgehog (ligand). As shown, depletion using siRNAs of the negative regulator Ptchl (B, data normalized to siPtchl) or depletion of the negative regulator SuFu (C, data normalized to siSuFu) stimulated G/7-luciferase activity in S12 cells in the absence of ligand. This stimulation of hedgehog signaling activity was partially rescued by co-depletion of TPRA40 using the siRNA pool in both cases. These experiments are consistent with the conclusion that TPRA40 acts downstream of Ptchl and SuFu in the Hh pathway.

Figure 6 shows that TPRA40 does not act downstream of PKA; but rather, acts upstream of or at the same level as PKA (protein kinase A). This experiment was conducted in S12 cells in the absence of stimulation with hedgehog. S12 cells were treated with either DMSO vehicle control or 80 μM cell permeable 14-22 amide (inhibitor of PKA) for 24 hours in the absence of Hedgehog (ligand) following 48 hours treatment with siRNAs to TPRA40 (depicted by black/right bar for each set of bars in Figure 6) or non-targeting control (depicted by grey/left bar). TPRA40 depletion using the siRNA pool to murine TPRA40 described above did not rescue the 14-22 amide induced G/Muciferase activity. In other words, TPRA40 depletion did not rescue PKA inhibitor-mediated stimulation of the pathway. This is consistent with the conclusion that TPRA40 acts upstream of or at the level of PKA.

**Example 6: siRNA Knockdown of TPRA40 increased Gli3 Processing**

Figure 7 shows that TPRA40 knockdown modestly increased levels of Gii3 repressor. To help interpret the results of these experiments, it is useful to note what typically occurs as part of the Hedgehog signaling pathway. Briefly, full length Gli3 (GH3-FL, ~190kDa) is cleaved into a repressor form (GH3R, ~80 kDa) in the absence of Hedgehog via PKA phosphorylation (followed by GSK3β and CK1 phosphorylation, which recruits pTRCP and SCF/Cull to degrade the C-terminal half of Gli3).

Figure 7A (left hand side of figure) presents Western blot analysis of Gli3 using monoclonal antibody 6F5 with and without 24 hour Hedgehog treatment and in the presence or
absence of siRNA depletion of TPRA40 or Ift88. The tubulin antibody 1A2 was used as a
loading control. Figure 7B (two panels on right hand side of figure) depicts quantitation of three
westerns of GH3FL and Gli3R normalized to tubulin. Hedgehog stimulation for 24 hours
inhibits PKA activity and attenuates Gli3R production, which requires primary cilia (as
evidenced by the increase in Gli3R levels following Ift88 depletion). TPRA40 knockdown
increased both the baseline level of Gli3R (by about 25%) and the level remaining after
Hedgehog stimulation (by 2 fold). These results are consistent with less Hh pathway induction
in the presence of Hh, as well as more PKA activity in the absence of Hh. In Figure 7B, for each
set of bars indicative of results observed following treatment with a given pool of siRNAs, the
results observed in the presence of Hedgehog treatment are shown in black (right bar) and the
results observed in the absence of Hedgehog are shown in grey (left bar), normalized to NTC in
the absence of Hh.

Example 7: Characterization of Anti-TPRA40 Antibodies in S12 Cells

Two TPRA40 antibodies were obtained and characterized. Figures 8A and 8B show the
characterization of these antibodies to endogenous TPRA40 by western blotting of S12 cells. 812
cells transfected with NTC or TPRA40 siRNAs for 48 hours were serum starved in the presence
or absence of Hedgehog for 24 hours (72 hours total knockdown), then lysed and subjected to
western blotting.

Panel A shows the results obtained using a custom-made rabbit anti-TPRA40 C-terminal
antibody 12569B (generated by YenZym; see material and methods below for additional
information). This antibody detected a single band of about 55kDa that is not affected by Hh
treatment (i.e., the band is observed regardless of whether the cells are cultured in the presence
or absence of Hedgehog protein), but disappears following TPRA40 depletion using siRNAs.

These results indicate that this antibody is specific for TPRA40.

Panel B shows results obtained using the mouse anti-TPRA40 antibody 6H2
(commercially available from Santa Cruz). This antibody also detected TPRA40. However, this
antibody appeared less clean and also recognized a couple of smaller non-TPRA40 proteins,
albeit to a lesser extent. The predicted molecular weight from the sequence is 40,560 Da plus N-
glycosylation (molecular weight decreases to -35,000 Da following PNGaseF treatment [data
not shown]).
Example 8: TPRA40 Localizes to Cilia in a Hedgehog-Dependent Manner

Figures 9A and 9B show that endogenous TPRA40 localizes to primary cilia of S12 cells in a Hedgehog-dependent fashion. (A) S12 cells were fixed and processed for immunofluorescence using anti-TPRA40 monoclonal antibody 6H2 (left panel and red channel in merge) and rabbit anti-Aril 3b antibody (a marker for primary cilia in the middle panel and green channel in merge) following 24 hours serum starvation alone (upper panel: in the absence of Hedgehog stimulation) or with Hedgehog stimulation (lower panel: +Hh). Nuclei stained with DAPI are in blue in the merged right panel. (B) Counting the number of Aril3b-positive cilia for TPRA40 staining revealed that TPRA40 was found in only about 20% of cilia in the absence of Hedgehog stimulation, but accumulated in 60% of cilia following overnight Hedgehog stimulation. This level of accumulation to cilia is very similar to the extent of accumulation of Smoothened (mean and SD of 2 experiments is shown). In Figure 9B, for each of the TPRA40 and Smo data sets, data obtained in the presence of Hedgehog stimulation is shown in black (right bar in each set of bars) and data obtained in the absence of Hedgehog stimulation is shown in grey (left bar in each set of bars).

Example 9: TPRA40 is a GPCR

Figure 10 summarizes experiments designed to characterize this orphan GPCR. TPRA40 expression inhibited cAMP production in a CRE-luciferase reporter assay. The diagram on the left depicts the assay set up, comprising 293T cells stably expressing CRE (cAMP Response Elementj-luciferase transiently transfected with GFP (negative control, not shown) or a TPRA40 (squiggle traversing the plasma membrane 7 times) expression construct were treated with varying doses of Forskolin, a potent activator of Adenyiyl Cyclase, thus increasing cAMP levels inside the cells. If the transfected GPCR is coupled to Galpha(s, stimulatory), the CRE-luciferase activity should increase more than in control GFP-transfected cells upon Forskolin treatment, whereas it if it coupled to Galpha(i, inhibitory), the CRE-luciferase activity should increase less than in GFP-transfected cells. The graph on the right shows that exogenous expression of TPRA40 suppressed the CRE-reporter activity in a dose dependent manner, suggesting that this GPCR is coupled to Galpha(i), which inhibits cAMP production. The mean
and standard deviation of four independent experiments normalized to 20µM forskolin in GFP-transfected cells is shown.

Example 10: Epistasis Analysis of TPRA40 Relative to Galpha(i)

Figure 11A shows that S12 cells transfected with siRNAs to Galpha (i) show reduced Hh signaling compared to NTC treated cells. Knockdown of Galpha(i) with siRNAs decreased Gli-luciferase activity in Hh-treated S12 cells by about 50%, consistent with increased cAMP production (due to relief of inhibition by Galpha(i) depletion) stimulating more PKA activity and Gli3R production. Co expression of siTPRA40 along with siGalpha (i) did not rescue reporter activity compared to siGalpha(i) alone, suggesting that TPRA40 functions at the level of or upstream of Galpha (i). Mean and SD of 3 independent experiments are shown.

Figure 11B shows that Gli3 depletion is partially rescued by TPRA40 knockdown in S12 cells. S12 cells depleted of Gli3 by siRNA show active Hedgehog signaling in the absence of ligand due to loss of Gli3 repressor (the Gli-luciferase signal is initiated by Gli2 activator).

TPRA40 depletion partially inhibits the signal in Gli3-depleted cells. The sequences of the murine Gli3 siRNAs in the pool are:

- GAACAAACCCIJAGUCACAGGA (SEQ ID NO: 45)
- GCUCUACCGUGCAGAAUA (SEQ ID NO: 46)
- GCUUGUGCUGAAAAAUU (SEQ ID NO: 47)

Figure 11C shows that TPRA40 depletion does not prevent Gli3 accumulation at cilia tips. Depletion of TPRA40 by siRNA treatment does not prevent the Hedgehog-dependent accumulation of Gli3 at cilia tips (bottom row). Arrows show the tips of the primary cilia.

Figure 11C shows that less than 10% of cilia have Gli3 at the tips in the absence of Hedgehog stimulation, while the black bars (the right of each pair of bars) show approximately 80% of cilia have Gli3 at their tips irrespective of the presence of TPRA40. This suggests that the regulation of Gli3 processing by TPRA40 and PKA occurs after the initial translocation of Gli3 to primary cilia.

Example 11: Working Model for TPRA40 in Hedgehog Signaling Pathway
Figure 12 provides a working model for TPRA40 function as a positive regulator of Hedgehog signaling. This model is provided merely to facilitate discussion of the pathway, and the inventors do not intend to be bound by theory. The claimed disclosure is fully operative and does not rely on the accuracy of the details of this model. Left panel: in the absence of Hedgehog protein (ligand), Patched 1 (Ptchl, red multipass protein) in the primary cilium suppresses the activity of extracellular Smoothened (Smo, green GPCR) and prevents its localization to cilia. TPRA40 (brown GPCR) is not present in cilia (its depiction on the plasma membrane is speculative) and so does not interact with ciliary Galpha(i) (Gai, blue circle), leading to high local concentrations of cyclic AMP (cAMP). cAMP stimulates protein kinase A (PKA, yellow oval; asterisks denote active forms of all proteins) to phosphorylate full length Gli3 and Gli2 (Gli-FL, red/green rectangle), which primes further GU3FL phosphorylation by CK1 (white oval) and GSK3β (purple oval). Phospho-Gli3 becomes a substrate for PTRCP binding, which recruits 8CF ubiquitin ligase (not shown) to promote proteasome-dependent cleavage of Gli3 into its repressor form (Gii-R, red rectangle). Gli3-R represses transcriptional activation of Hh pathway target genes, thus the pathway is off. Right panel: in the presence of Hedgehog stimulation, Ptcch is removed from cilia, allowing Smo to enter cilia and become activated. This leads to accumulation of TPRA40 in cilia, where it interacts with Gai to inhibit local cAMP production, preventing PKA activity and Gli3 cleavage. The SuFu-GliFL complex accumulates at the tips of primary cilia, dissociates, and activated Gli-FL (Gli-A) exits cilia and enters the nucleus to promote transcription of Hh target genes such as GUI. Optionally, TPRA40 undergoes an activation step (like Smoothened) in addition to ciliary translocation in order to exert its activity.

Example 12: TPRA40 Amino Acid Sequence Information

Figure 13 shows an alignment of the amino acid sequences of mouse and human TPRA40. Human TPRA40 (top, 373 amino acids, predicted MW 41034 Da; Swissprot Q86W33) is aligned with mouse TPRA40 (middle, 369 amino acids, predicted MW 40560 Da; Swissprot Q99MU1) and zebrafish TPRA1 (bottom, 378 amino acids, predicted MW 41685 Da; Swissprot Q4V8X0) using the Align program in GSeqWeb. Identical amino acids are colored, and the positions (predicted by Swissprot) of the 7 transmembrane (tm) domains typical of GPCRs are underlined in blue. Mouse and human TPRA share 91.4% identity and 94.1% similarity at the protein level.
Zebrafish TPRA1 is 70.0% and 68.7% identical (79.3% and 78.0% similar) to human and mouse
TPRA40, respectively, suggesting an evolutionarily conserved function. As expected for a
GPCR, the N-terminus is luminal/extracellular and the C-terminus is cytoplasmic. This
topography was verified by FACS with epitope tags at each end of the protein (data not shown).

Materials and Methods:
The following methods were used in the experiments described in the Examples.

siPvNA transfection.

Pools of pre-designed ON-TARGETplus siRNAs for murine TPRA40 (GPR175 siRNAs
09-12), Smo, G-alpha(i), 1FT88, or siGenome for murine Ssna! were purchased from
Dharmacon Inc. (Lafayette, CO). S12 cells were seeded into 96-well white wall clear-bottom
plates at 6 x 10^3 cells/well; 8-well LabTekII microscope slides at 2 x 10^4 cells/well; or 60mm
plates at 4 x 10^5 cells/well and reverse-transfected with 50nM final siRNA pools, following 20
minutes preincubation of siRNA pool with DharmaFECT-2 (Dharmacon) in Opti-MEM (Gibco)
at room temperature. For epistasis experiments, two siRNA pools at 50nM each were used. For
testing the four individual components of the pool, each siTPRA40 siRNA was transfected at
25nM. After 48 hours, cells were shifted into 0.5% FBS media to promote ciliogenesis for an
additional 16-24 hours to promote ciliation (± Hh for indicated times).

The following siRNAs were used in these examples (either individually or as a member
of a pool of siRNAs). These siRNAs were double-stranded and prepared in accordance with
manufacturer's protocols. For each of these examples, the sequence for one strand is provided
below:

Mouse TPRA40 siRNA #9:GGAGGAGUUUCUACGUGUA (SEQ ID NO: 16)
Mouse TPRA40 siRNA #10:CGGCAGUUCUGGCUGCUCA (SEQ ID NO: 17)
Mouse TPRA40 siRNA #11:CGACAGUUGCUGACAAGAU (SEQ ID NO: 18)
Mouse TPRA40 siRNA #12:GCCAUUGAGCUGAGUCUGA (SEQ ID NO: 19)
Human TPRA40 siRNA #6:GGUCAGCUCCUCGUUCUUC (SEQ ID NO: 20)
Human TPRA40 siRNA #7:GAGAGUAAGUCCAGCAUCA (SEQ ID NO: 21)
Human TPRA40 siRNA #8:GGAGGAGCUUCUACGUGUA (SEQ ID NO: 22)
Human TPRA40 siRNA #9:CCACAACCUUCCUGUACUU (SEQ ID NO: 23)
Mouse Galpha(i) siRNA #1:GAAUAGC ACAGCCAAAUU (SEQ ID NO: 24)
Mouse Galpha(i)l  siRNA #2:GGAUGAUGCUCGCCAACUU  (SEQ ID NO: 25)
Mouse Galpha(i)l  siRNA #3:UAACAGACGUCCCGGACU  (SEQ ID NO: 26)
Mouse Galpha(i)l  siRNA #4:GAAGAGGAGUGUAAGCAGU  (SEQ ID NO: 27)
Mouse Smo siRNA #1: CAAUUGGCUGUCUGCUUAU  (SEQ ID NO: 28)
Mouse Smo siRNA #2:GAGCGUAGCUUCCGGGACU  (SEQ ID NO: 29)
Mouse Smo siRNA #3:GGAGUAGUCUGGUUCGUGG  (SEQ ID NO: 30)
Mouse Smo siRNA #4:GCUACAAGACUAUCGGUA  (SEQ ID NO: 31)
Mouse Ifit88 siRNA#5:GUAGCUAGCUGCUUUAGAA  (SEQ ID NO: 32)
Mouse Ifit88 siRNA#6:GCUUGGAGCUUAUUACAUU  (SEQ ID NO: 33)
Mouse Ifit88 siRNA#7:CGGAGAUCACGCGCUUAUUACAUU  (SEQ ID NO: 34)
Mouse Ifit88 siRNA#8:GGAGGAGCUGACUAAUUACAUU  (SEQ ID NO: 35)
Mouse Ssnal siRNA #1: GAACCGACUAAACCCGACC  (SEQ ID NO: 36)
Mouse Ssnal siRNA #2:GCAACGAGUUCGACCGGAC  (SEQ ID NO: 37)
Mouse Ssnal siRNA #3:GGAGUUGUGUCAGAAGCGA  (SEQ ID NO: 38)
Mouse Ssnal siRNA #4:ACGAGUUGUGUCAGAAGCGA  (SEQ ID NO: 39)

S12 Gli-luciferase assay.

S12 cells, which are 10^6/2 fibroblasts stably transfected with 8x Gli-binding sites fused to a luciferase reporter (Frank-Kamenetsky et al., 2002), were plated at 6,000 cells/well of a white-walled clear-bottomed 96-well plate (Costar 3610) in regular growth medium (HG (high glucose)-DMEM, 10% FBS, 1% glutamine) and reverse transfected with siRNAs. After 48 hours, the medium was changed to 0.5% serum HG-DMEM ± 200 ng/ml octyl-Shh (Genentech) and incubated for another 24 hours to stimulate hedgehog signaling. PKA was inhibited using 80 µM cell permeable 14-22 amide (Tocris Biosciences). Gli-luciferase activity was measured using an HTS-Steady Lite luciferase detection kit (Perkin Elmer) and a TopCount luminometer; multiple assays were carried out, each in triplicate. Data were fit to a 4-parameter sigmoidal equation, from which the IC50 was derived using Kaleidagraph (Synergy Software) and were corrected for cell viability using cellTiter Glo (Promega).

Real-Time quantitative PGR (qPCR).
Total RNA was extracted from cells using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. On-column genomic DNA digestion was performed with RNase-Free DNase Set (Qiagen). cDNA synthesis from total RNA was conducted using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with random hexamer primers. Quantitative PGR reactions were performed in triplicate on an ABI PRISAd™ 7500 Sequence detection system (Applied Biosystems) using murine ribosomal protein L19 (mRPL19) as the endogenous control. Gene expression was calculated using the relative quantification (2\(^{-\Delta\Delta C_{t}}\)) method. PGR primers and Taqman probes (5' FAM and 3' TAMRA-labeled) are as follows:

10 Murine Glil

mGlil 5' primer: GCA GTG GGT AAC ATG AGT GTC T (SEQ ID NO: 4)
mGlil 3' primer: AGG CAC TAG AGT TGA GGA ATT GT (SEQ ID NO: 5)
mGlil probe: CTC TCC AGG CAG AGA CCC CAG C (SEQ ID NO: 6)

15 Human Glil

hGlil 5' primer: CGC TGC GAA AAC ATG TCA AG (SEQ ID NO: 7)
hGlil 3' primer: CCA CGG TGC CGT TTG GT (SEQ ID NO: 8)
hGlil probe: CAG TGC ATG GTC CTG ACG CCC A (SEQ ID NO: 9)

20 Murine TPRA40

mTPRA40 5' primer: TGC AGG AGG CCA ATG GAA (SEQ ID NO: 10)
mTPRA40 3' primer: GGG CTC ACT GAT ATT GGA TGC T (SEQ ID NO: 11)
mTPRA40 probe: ACA GCG TGG CCA CCG CCC (SEQ ID NO: 12)

25 Human TPRA40

hTPRA40 5' primer: CCT GGT CTA CTC TCT GGT GGT CAT (SEQ ID NO: 13)
KTTPRA40 3' primer: CCG AGA AGG CAG GGA GAT G (SEQ ID NO: 14)
KTTPRA40 probe: CCC AAG ACC CCG CTG AAG GAG C (SEQ ID NO: 15)

30 Antibodies.

Purchased antibodies were anti-Glii mouse monoclonal L42B10 (Cell Signaling Technology cat #2643); anti-TPRA40 mouse monoclonal 6H2 (Santa Cruz Sc-134350); rabbit anti-Aril 3b (Protemtech 171 1-1-AP); mouse anti-acetylated tubulin 6-1 IB-1 (Sigma T6793); mouse anti-tyrosinated tubulin 1A2 (Sigma T9028); mouse anti-gamma tubulin GTU-88 (Sigma T6657). Anti-GH3 mouse monoclonal 6F5 (Wen et al, 2010) was made at Genentech. Rabbit polyclonals 10322B to the C-terminal 110 aa of murine Smoothened and 12965B to a peptide
comprising the C-terminal 19 aa of murine TPRA40 (CHTGSINSTDSERWKA1NA-COOH) were raised at YenZym (South San Francisco, CA).

**Western blotting.**

Cells were lysed in RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) containing freshly added phosphatase inhibitor cocktails I and Ii (Sigma), Complete protease inhibitor cocktail (Roche), and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). Equal amounts of protein (as measured using the BCA kit (Pierce)) were separated on 4-12% reducing Tris-Glycine gels prior to transfer to nitrocellulose membranes (Invitrogen) and blocking in 5% w/v milk in TBST (Tris-buffered saline with 0.05% Tween 20). Anti-Gli3 6F5 (Wen et al. 2010) was used at 5μg/ml, mouse anti-TPRA40 at 1:1000 and rabbit anti-TPRA40 at 1:1000 overnight at 4 °C. Detection was accomplished using HRP-conjugated anti-rabbit (Jackson Immunoresearch) or anti-mouse secondary antibodies (GE Healthcare) and ChemiGlow (alpha-Innotech). Blots were reprobed for protein loading using mouse anti-tubulin 1A2 at 1:10,000 followed by HRP-anti-mouse and ECL detection (GE Healthcare). Blots were exposed to BioMax light film (Kodak).

**Immunofluorescence microscopy.**

S12 cells were plated on 8-well slides at 3x10^4 cells/well and reverse transfeeted with siRNAs as above. After 72 hours knockdown, with the final 16-24 hours in serum-free media ± 200ng/ml octyl-Shh (Genentech), cells were fixed with 3% PFA for 20 minutes at room temperature and quenched for 10 minutes in 50 mM NH₄Cl. Cells were then penneabilized with 0.1% Triton-X-100 (Sigma) in PBS for 10 minutes, blocked in 1% BSA for 30 minutes and subsequently incubated with the following primary antibodies: rabbit anti-Smo 1μg/ml, mouse anti-TPRA40 (1:50), in conjunction with 1:3000 mouse anti-acetylated tubulin and/or 1:2300 mouse anti-gamma tubulin or rabbit anti-Aril 3b (1:400). After 3x 10 minute washes, Cy3 or FITC-conjugated secondary anti-rabbit or anti-mouse antibodies (Jackson Immunoresearch) were applied for at least 20 minutes at room temperature, washed 3x and coverslips were mounted using ProLong Gold with DAPI (Invitrogen). Slides were imaged using Axio Imager M2 microscope with a 60X objective and SlideBook 5.5 software. The percentage of cilia (as
identified by acetylated tubulin or Aril 3b staining) that stained for TPRA40 or Smo in the presence or absence of Hh stimulation was counted manually.

293 CRE-luciferase assay for cyclic AMP.

HEK293 cells were plated at 10,000 cells/well in 96-well white walled plates. Cells were reverse transfected with 50ng Cre-luciferase, 10 ng Renilla Luciferase (Proniega) and 90 ng of murine TPRA40 with an N-terminal HA tag (pRK-nHA-TPRA40) with 0.45µl of Fugene 6. After 36 hours, different amounts of Forskolin (Cell Signaling) were added for 4 hours and the Firefly and Renilla luciferase were measured using Dual Glow Luciferase Substrate (Promega) and a TopCount or EnVision himinometer.

Statistics. Comparison of TPRA40-depleted versus non-depleted cells was performed using the student's unpaired t-test (ww.graphpad.com). *, p < 0.05; **, p < 0.01; ***, p < 0.001

Sequence Information
SEQ ID NO: 1- Human TPRA40 amino acid sequence (GenBank Accession No. AAH5071 1.1)
MDTLEEVTWANGSTALPPPLAPNISVPHRCLLLLYEDIGTSRVRYWDLPLLIPNLFLIF
LLWKLPSARAKIRITSSPIFITFYILVFWALVGIALRASMTSNAATVADKILWEI
TRFFLLAJELSVIILGLAFGLESKSSKRSKVLAIETTVLSLAYSVTQGTELILYPDHAHSA
EDFNIYGHGGRQFWLVSFFFLVYSLVYLPSRKTLKISLPSRSSFYVVAGILALLNL
LQGLGSVLLCDIIEGLCCVDATTFLYFSFAPIYVAFLRGGSGEPKILFSYKCQVDE
TEEPDVHLPQPYAVARREGLEAAGAGASASYSSTQFDSAGGVAYLDDIASMPCHTG

SEQ ID NO: 2- Mouse TPRA40 amino acid sequence (GenBank Accession No. NP_036036.2)
MASLQEAJNGSTAWPPTASNISEPHQLLLLLLYEIDGSSRVRVYWDLLLLIPNVLFFIIIFLWK
LPLARKIRWSPFITFYILVWVALVGIARAVSMTVSAADATVADKILWEITRFLL
AIELSVIILGLAFGLESKSSKRSKVLATTVLSLAYSVTQGTELILYPDLSHLSAEDFNIYGHG
GRQFWLVSFFFLVYSLWILPKTLKERVSLSRSSFYVVAGILATNTLLQGLGSALLC
ANUVGLCCVDATTFLYFSFFAPLIYVAFLRGFFGSEPKJLFSYKCQVDEAEEPDMHLQP
YAVARREGIESAGPACASAANYSTQFDSAGVAYLDDIASMPCHTGSINSTDSERVKA1
NA

SEQ ID NO: 3- Zebrafish TPRA40 amino acid sequence (GenBank Accession No.
NP_001025422.1)
MLETVTDVASFVHYGNTSVFPTADNSSEIIIPDGESNISKPRCHLQVLYDDIGTSRVRYWD
VMLLIPNVAFLVFLMWKPSARAKIRLTSSPIFVAFYILVFVAAVGITRAIVSMSMTVSTS
AATLIDKVLWEITRFLLAIELSVIIGLAFHGLESKSSIKRVLAITAVLSAYSITQGTELIR
FPDKHLSAKDFNIYGHGGRHFMLASSCCFFLVY SLIVILPKTPIRERISLPKRSFYVYGIL
ALLNLQGLGSALLCADIIEGLCCvOWTFLWSWAPLIVvTFLKGFFGSEPKnLFSYK
SEQ ID NO: 4- exemplary murine Glil 5’ primer
GCA GTG GGT AAC ATG AGT GTC T

SEQ ID NO: 5- exemplary murine Glil 3’ primer
AGG CAC TAG AGT TGA GGA ATT GT

SEQ ID NO: 6- exemplary murine Glil probe
CTC TCC AGG CAG AGA CCC GAG C

SEQ ID NO: 7- exemplary human Glil 5’ primer
CGC TGC GAA AAC ATG TCA AG

SEQ ID NO: 8- exemplary human Glil 3’ primer
CCA CGG TGC CGT TTG GT

SEQ ID NO: 9- exemplary human Glil probe
CAG TGC ATG GTC CTG ACG CCC A
SEQ ID NO: 10- exemplary murine TPRA40 5' primer
TGC AGG AGG CCA ATG GAA

SEQ ID NO: 11- exemplary murine TPRA40 3' primer
GGG CTC ACT GAT ATT GGA TGC T

SEQ ID NO: 12- exemplary murine TPRA40 probe
ACA GCG TGG CCA CCG CCC

SEQ ID NO: 13- exemplary human TPRA40 5' primer
CCT GGT CTA CTC TCT GGT GGT CAT

SEQ ID NO: 14- exemplary human TPRA40 3' primer
CCG AGA AGG CAG GGA GAT G

SEQ ID NO: 15- exemplary human TPRA40 probe
CCC AAG ACC CCG CTG AAG GAG C

SEQ ID NO: 16- Mouse TPRA40 siRNA #9
GGAGGAGUUUCUACGUGUA

SEQ ID NO: 17- Mouse TPRA40 siRNA #10
CGGCAGUUCUAGCUGUCA

SEQ ID NO: 18- Mouse TPRA40 siRNA #11
CGACAGUUGCUGACAAGAU

SEQ ID NO: 19- Mouse TPRA40 siRNA #12
GCCAUUGAGCUGAGUGUGA

30
SEQ ID NO: 20- Human TPRA40 siRNA #6
GGUCAGCUCCJGCUUCUUC

SEQ ID NO: 21- Human TPRA40 siRNA #7
5 GAGAGUAAGUCCAGCAUCA

SEQ ID NO: 22- Human TPRA40 siRNA #8
GGAGGAGCUUCUACGUGUA

SEQ ID NO: 23- Human TPRA40 siRNA #9
10 CCACAACCUUCCUGUACUU

SEQ ID NO: 24- Mouse Galpha(i) siRNA #1
GAAUAGCACAGCCAAAUUA

SEQ ID NO: 25- Mouse Galpha(i)l siRNA #2
15 GGAUGAUGCUCGCCAACUU

SEQ ID NO: 26- Mouse Galpha(i)l siRNA #3
20 UAACAGACGUCAUAAAAA

SEQ ID NO: 27- Mouse Galpha(i)l siRNA #4
GAAGAGGAGUGUAAGCAGU

SEQ ID NO: 28- Mouse Smo siRNA #1
25 CAAUUGGCCUGGUGCUUAU

SEQ ID NO: 29- Mouse Smo siRNA #2
GAGCGUAGCUUCCGGGACU

SEQ ID NO: 30- Mouse Smo siRNA #3
30
GGAGUAGUCUGGUUCGUGG

SEQ ID NO: 31- Mouse Snio siRNA #4
GCUACAAGAACUAUCGGUA

5

SEQ ID NO: 32- Mouse Ift88 siRNA#5
GUAGCUAGCUGCUUUAGAA

SEQ ID NO: 33- Mouse Ift88 siRNA#6
10 CGUCAGCUCUCACUAAUAA

SEQ ID NO: 34- Mouse Ift88 siRNA#7
GCUUGGAGCUUAUUAACAUU

15 SEQ ID NO: 35- Mouse Ift88 siRNA#8
CGGAGAAUGUUGAAUUGUU

SEQ ID NO: 36- Mouse Ssnal siRNA #1
GAACCUGACUAAAGCCACA

20 SEQ ID NO: 37- Mouse Ssnal siRNA #2
GCAACGAGUUUUGACCCGAC

SEQ ID NO: 38- Mouse Ssnal siRNA #3
25 GGAGUUGUGUCAGAAGCGA

SEQ ID NO: 39- Mouse Ssnal siRNA #4
ACGAGUUGGUCAGAAGCGA

30 SEQ ID NO: 40- TPRA40 Sense Morpholino
ACATCGGTCACCGTCTCCAGCATTC
SEQ ID NO: 41- TPRA40 Antisense Morpholino
ACAACCGTGACCCCTCTCACCATTCC

SEQ ID NO: 42- human Smoothened amino acid sequence (GenBank Accession No. NP_005622.1)
MAAAARPARGPEPLLLGLLLLLLGLDPGRGAASSGNATGPGPRSAAGGSARRSAAVTGPPP
PLSHCGRAAPCEPLRYNVCLGSLPYGATSTLLAGDSDSQEEAHKLVLWSGLRNAPR
CWAIVQPLLCAVYMPKCDNDRVELPSRTLQATRGPACIVERERGWPDFLRTCPDPE
GCTNENQNKFNSSGQCEVPVLRSTDNPKSWYEVIEGCGIQCNPLFTEAEIHQDMHSYA1A
AFGAVTGIXTLFTL1ATFVARDWRSNRYPAVILFYWACFFVGSIGWL.AQFMDGAAEEIV
CRADGTMRLGEPTSNETLSCVIIFVTVYV5ALA4GW WFW I.TYACHTSF5KLGTYQPI,
SGKTSYFinXTWSLPFVT_TVAIIAVAQVDGSVSGICFVGKYKNRYRAGFXAPIGLVXI
VGGYFLIRGVMTLSIFKSNHGPLLSEEKAINETMLRLGIFGFLAFGFIJFTSFCHYDFD
NQAEVERSFREDYVLQANXV IGLPTKQIPDPCEIKNRPSLLVX KINLFAMFTGTGJAMSTW
VTKATLILLWRRTWCRGLTQSDDEPKRKKSKMIKAASKRHELLOQPQGELSFSMI-IT
VSHDGVPVAGLAFDLNEPSADVSSAWAQHVTKMWARRGAILPQDISVTPVATPVPEEQA
NLWLEEAISELQKRKLGRKKKRKKKKEVCPLAPPHELHPAPAPSTIPRLPQLPRQKC
LVAAGAWGAGDSRQCGAWTLVSNFCPEPSPPQDPFLPSAPAVAWAHGRQGLPIH
SRTNLMDEC3LMDA3DSDF

SEQ ID NO: 43- human Suppressor of Fused (SuFu) amino acid sequence (GenBank Accession No. NM_016169.2)
MAELRPSGAPGPTAPPAPAPAFASLFPPGLHAIYGECRRLYPDQPNPLQVTAVKY
WLGDPDLDYVSMYVRGSPSANIPEHWHYISFGLSDLYGDNRVHEFTGTDGPSFGFEGE
LTFRKLKRTGESAPPTWPAMQGLARYVQFQSENTTCSGHSVSHSLDNEISRIQHML
LTEDPQMQPETYTFVTLQIVGVCTEELHSAQQWNGQGILELLRTPVIAGGPGWLTID
MRGETIFEDPHLQERVDKGIEITGDSNLGVSAKACAWDDLSPPEDEDEDSRSICGTQPR
RLSGKDWGRTLRRGLESNKPVLPINPQNRQNGLAHDRAPSRKDSLES3DSSTAIPHEL
RTRQLESVHLETSIQSEGALIPLCLRGRLHHRHFTYSITGDMAITFVSTGVEGAFATEE
HPYAAHGPVVLQILTEEFTKMLEDLEDLTSPEEFKXPKEYSWPEKKLVSILIDVFDFSD
PLH

SEQ ID NO: 44- human Suppressor of Fused (SuFu) cDNA sequence (GenBank Accession No. NM_016169.2)

CGCCGCTGCAGCGCGAGCTAGACCTCGGCAGCCCCCATCGCCTCGGGGAGTC
CTCACACCACGGAGTCCGCCCGCTGGCCCGTCAGTGCTCTCCCCGCTGTTTGC
CACCTTTCCAGCAGCCTGCCCTACCGACCCGATGGCGGAGCTGCGGCCTAGCGGCG
CCCCCGCCACCGCGCCCCCCCTGGCCTGGCCCGACTGCCCCCCCGGCCTTCGCTT
CGCTCTTTTCCCCGGGACTGCACGCCCATCTACGGGAGAGTGCCGCGCCCGCCTT
AACCCCTGGACACTAGCATGCCACATGTGACAGATGCATGACACATCGCACACTCGGCGGAGTGGAAGGCGCCTTTGCCACTGAGGAGCATCCTT
ACGATCGTGTGGTGTGGTGGTGGTGGAAGGCGCCTTTGCCACTGAGGAGCATCCTT
GGGAGCAGGAGTTGGACCTCCATGGAGCCACTAGGCCTGGCCTCCTCTACACATCCC
CAGGGCTATCTGGTTAATTCCATCAAGCTCAGAGTTAAAAGGCATATCAGCCTGG
AGTATTTGGGAGAGACTGCTGCAGATCCCCGCCAGCAAGATGCAGCAGCCTCTC
CTGAACTCTCTCTTGGCTGGGACCTGCTGACGAGCTCCCTGCTGAGTTACAGTCTGG
5
CTTCCCATCTGCTGGGTCCTCCTCATTGTTGGGTTGAGGATTGGGACATTTTCTGAG
CTAAGCTTTGGTCAATTAGTTTGTGAAGCACCTGGTCAGCAACCTGCCCCAGACCTGGA
GGGTCTTTGGACTGAAGGTAGACACCAGACAGATGGTGCCCTTGGCCCTGCTGCTG
AGTATTGGGAGAGACTGGCTGCAGATCCCCGCCAGCCAAGATGCAAGCCACTCGG
GACCTGAGTGTGAGCAGCTGCTCTACTGCGCCCTGAGGACTTACCAGAGGAGGCCC
10
CTTCCCATCTGCTGGGTCCTCCTCATTGTTGGGTTGAGGATTGGGACATTTTCTGAG
CTAAGCTTTGGTCAATTAGTTTGTGAAGCACCTGGTCAGCAACCTGCCCCAGACCTGGA
GGGTCTTTGGACTGAAGGTAGACACCAGACAGATGGTGCCCTTGGCCCTGCTGCTG
AGTATTGGGAGAGACTGGCTGCAGATCCCCGCCAGCCAAGATGCAAGCCACTCGG
GACCTGAGTGTGAGCAGCTGCTCTACTGCGCCCTGAGGACTTACCAGAGGAGGCCC
15
CTTCCCATCTGCTGGGTCCTCCTCATTGTTGGGTTGAGGATTGGGACATTTTCTGAG
CTAAGCTTTGGTCAATTAGTTTGTGAAGCACCTGGTCAGCAACCTGCCCCAGACCTGGA
GGGTCTTTGGACTGAAGGTAGACACCAGACAGATGGTGCCCTTGGCCCTGCTGCTG
AGTATTGGGAGAGACTGGCTGCAGATCCCCGCCAGCCAAGATGCAAGCCACTCGG
GACCTGAGTGTGAGCAGCTGCTCTACTGCGCCCTGAGGACTTACCAGAGGAGGCCC
20
CTTCCCATCTGCTGGGTCCTCCTCATTGTTGGGTTGAGGATTGGGACATTTTCTGAG
CTAAGCTTTGGTCAATTAGTTTGTGAAGCACCTGGTCAGCAACCTGCCCCAGACCTGGA
GGGTCTTTGGACTGAAGGTAGACACCAGACAGATGGTGCCCTTGGCCCTGCTGCTG
AGTATTGGGAGAGACTGGCTGCAGATCCCCGCCAGCCAAGATGCAAGCCACTCGG
GACCTGAGTGTGAGCAGCTGCTCTACTGCGCCCTGAGGACTTACCAGAGGAGGCCC
25
CTTCCCATCTGCTGGGTCCTCCTCATTGTTGGGTTGAGGATTGGGACATTTTCTGAG
CTAAGCTTTGGTCAATTAGTTTGTGAAGCACCTGGTCAGCAACCTGCCCCAGACCTGGA
GGGTCTTTGGACTGAAGGTAGACACCAGACAGATGGTGCCCTTGGCCCTGCTGCTG
AGTATTGGGAGAGACTGGCTGCAGATCCCCGCCAGCCAAGATGCAAGCCACTCGG
GACCTGAGTGTGAGCAGCTGCTCTACTGCGCCCTGAGGACTTACCAGAGGAGGCCC
30
TCACCTAGATGTCGTTTCTTCTTGGCCCCCTCTCTCTCTCTCTTAATCTAAGTGCATTAA
ACATCTTTGCAG
SEQ ID NO: 45 - Mouse Gli3 siRNA #5
GAACAACCCUAGUCAAGGA

SEQ ID NO: 46 - Mouse Gli3 siRNA #6
GCUCUACCGUGCAGAAUUA

SEQ ID NO: 47 - Mouse Gli3 siRNA #7
GCUUAGUGCUGCAAAAUUA

SEQ ID NO: 47 - Mouse Gli3 siRNA #8
CCUCACAUAUCAACACUCA

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.
We Claim:

1. A method of screening for a TPRA40 antagonist, wherein the method comprises:
   a) contacting a cell that expresses TPRA40, adenylyl cyclase and a reporter construct indicative of adenylyl cyclase activity with an activator of adenylyl cyclase and an agent:
   b) determining, as compared to an untreated control, whether the agent rescues adenylyl cyclase activity suppressed by TPRA40 expression, wherein if the agent increases adenylyl cyclase activity relative to the untreated control, which untreated control comprises TPRA40 expressing cells that are contacted with the adenylyl cyclase activator but are not contacted with the agent, then the agent is identified as a TPRA40 antagonist.

2. A method of identifying a TRPA40 antagonist, comprising:
   a) providing a cell that expresses TRPA40 and that expresses a reporter gene capable of indicating adenylyl cyclase activity;
   b) contacting the cell with an activator of adenylyl cyclase and with an agent, wherein the cells are contacted with the activator and the agent simultaneously, concurrently, or consecutively; and
   c) determining, as compared to a control, whether the agent rescues adenylyl cyclase activity induced by the activator, wherein if the agent increases the adenylyl cyclase activity relative to the control, then the agent is identified as a TPRA40 antagonist.

3. A method of screening for an agent for inhibiting the proliferation, growth or survival of a cancer cell, wherein said method comprises:
   a) screening for an agent that binds to TPRA40 protein, reduces expression of TPRA40, inhibits transport of TPRA40 protein to the plasma membrane or to primary cilia, prevents activation of TPRA40 or uncouples TPRA40 from God;
   b) contacting a cancer cell with an amount of the agent identified in step a), wherein said cancer cell is responsive to hedgehog protein or comprises one or more mutations in a hedgehog signaling pathway gene, wherein said one or more mutations results in increased hedgehog signaling and/or activation of the hedgehog signaling pathway in the absence of ligand, and
c) determining, as compared to a control, whether said agent inhibits the proliferation or growth of said cancer cell, wherein if said agent inhibits cell proliferation or growth relative to the control, then an agent that inhibits the proliferation or growth of said cancer cell is identified.

5. A method of screening for an agent for inhibiting hedgehog signaling in a cell, wherein said method comprises:
   a) screening for an agent that binds to TPRA40 protein, reduces expression of TPRA40, inhibits transport of TPRA40 protein to the plasma membrane or to primary cilia, prevents activation of TPRA40 or uncouples TPRA40 from G
d) contacting a cell with an amount of the agent identified in step a), wherein said cell is responsive to hedgehog protein or comprises one or more mutations in a hedgehog signaling pathway gene, wherein said one or more mutations results in increased hedgehog signaling and/or activation of the hedgehog signaling pathway in the absence of ligand, and
c) determining, as compared to a control, whether said agent inhibits hedgehog signaling in said cell, wherein if said agent inhibits hedgehog signaling in said cell relative to the control, then an agent that inhibits hedgehog signaling is identified.

5. A method of identifying a TPRA40 antagonist, wherein said method comprises:
   a) screening for an agent that binds to TPRA40 protein;
   b) contacting a cell with an amount of the agent identified in step a), wherein said cell is responsive to hedgehog protein or comprises one or more mutations in a hedgehog signaling pathway gene, wherein said one or more mutations results in increased hedgehog signaling and/or activation of the hedgehog signaling pathway in the absence of ligand, and
c) determining, as compared to a control, whether said agent that binds to TPRA40 protein also inhibits hedgehog signaling in said cell, wherein if said agent inhibits hedgehog signaling in said cell relative to the control, then the agent is identified as a TPRA40 antagonist.

6. The method of any of claims 1-5, wherein said cell is in culture.

7. The method of any of claims 1-5, wherein said cell is in an animal.
8. The method of any of claims 1-7, wherein said agent is a small molecule.

9. The method of any of claims 1-7, wherein said agent is a polypeptide or an antibody.

10. The method of any of claims 1-7, wherein said agent is an siRNA or shRNA that decreases TPRA40 translation.

11. The method of any of claims 1-9, wherein said agent binds TPRA40 protein.

12. The method of any of claims 3-11, wherein said agent is identified in step a) using a yeast two-hybrid screen.

13. The method of any of claims 1-11, wherein said agent is identified in step a) using a high throughput binding or activity screen of a small molecule library.

14. The method of any of claims 1-13, wherein said agent inhibits transport of the TPRA40 protein to the plasma membrane or to primary cilia.

15. The method of any of claims 3-14, wherein said agent is identified in step a) by a method comprising the steps of:
   i) contacting a cell expressing TPRA40 with an agent,
   ii) determining the localization of TPRA40 in said first cell expressing TPRA40 using immunofluorescence.

16. The method of any of claims 3-14, wherein said agent is identified in step a) by a method comprising the steps of:
   i) contacting a cell expressing TPRA40 with an agent; and
   ii) determining the levels of TPRA40 in a plasma membrane or ciliary membrane fraction.
17. The method of any of claims 1-16, wherein said agent reduces expression of TPRA40 protein or RNA.

18. The method of any of claims 3-17, wherein said agent is identified in step a) by a method comprising:
   i) contacting a cell expressing TPRA40 with an agent; and
   ii) determining activity of TPRA40 in said cell using a G/i-luciferase reporter or adenylyl cyclase reporter assay.

19. The method of any of claims 3-17, wherein said agent is identified in step a) by a method comprising:
   i) contacting a cell expressing TPRA40 with an agent; and
   ii) determining the expression of Giri in said cell by RT-PCR or Western Blotting.

20. The method of any of claims 3-17, wherein said agent is identified in step a) by a method comprising:
   i) contacting a cell expressing TPRA40 with an agent; and
   ii) determining the expression of TPRA40 in said cell using RT-PCR or Northern Blot analysis of TPRA40 RNA or Western Blot, flow cytometry, immunofluorescence or immunohistochemistry analysis of TPRA40 protein.

21. The method of any of claims 1-20, wherein said cell used in step (b) comprises one or more mutations in a hedgehog signaling pathway gene.

22. The method of any of claims 1-21, wherein said one or more mutations are in smoothened, and the cell has a smoothened gain-of-function.

23. The method of any of claims 1-22, wherein said gain-of-function smoothened mutation results in a constitutively active smoothened protein.
24. The method of any of claims 1-23, wherein said one or more mutations are in *patched*, and the cell has a patched loss-of-function.

25. The method of any of claims 1-24, wherein said tumor overexpresses a hedgehog protein.

26. The method of any of claims 1-25, wherein said one or more mutations are in *suppressor-of-fused*, and the cell has suppressor-of-fused loss-of-function.

27. The method of any of claims 1-26, wherein a reporter gene is used in order to determine whether adenylyl cyclase activity has been rescued by an agent.

28. The method of claim 27, wherein the reporter gene is a luciferase gene controlled by a cAMP response element.

29. The method of any of claims 1-28, wherein the activator of adenylyl cyclase is forskolin.

30. The method of any of claims 1-28, wherein the activator of adenylyl cyclase activity is 8-bromo-cAMP.

31. The method of any of claims 1-30, wherein the agent is further assessed in an assay for hedgehog signaling.

32. The method of claim 31, wherein the assay for hedgehog signaling comprises the steps of:

i. contacting a cell with an amount of the agent, wherein said cell is responsive to hedgehog protein or comprises one or more mutations in a hedgehog signaling pathway gene, wherein said one or more mutations results in increased hedgehog signaling and/or activation of the hedgehog signaling pathway in the absence of ligand, and
ii. determining, as compared to a control, whether said agent inhibits hedgehog signaling in said cell, wherein if said agent inhibits hedgehog signaling in said cell relative to the control, then an agent that inhibits hedgehog signaling is identified.

33. The method of any one of claims 1-32, wherein the TPRA40 is exogenously expressed in a cell.

34. The method of claim 33, wherein the TPRA40 is stably expressed in the cell.

35. The method of claim 33, wherein the TPRA40 is transiently expressed in the cell.

36. The method of claim 33, wherein the cell is transformed with a vector expressing TPRA40 protein.

37. The method of any one of claims 1-36, wherein the cell is an S12 cell.

38. The method of any one of claims 1-36, wherein the cell is a 293T cell.

39. The method of any one of claims 1-38, wherein the reporter gene is a luciferase gene controlled by a cAMP response element.

40. The method of any one of claims 1-39, wherein the activator is a combination of one or more of forskolin, 8-bromo-cAMP or dibutyryl-cAMP.

41. A method of screening for a TPRA40 agonist, wherein the method comprises:
   a) contacting a cell that expresses TPRA40, adenylyl cyclase and a reporter construct with an activator of adenylyl cyclase and an agent;
   b) determining, as compared to an untreated control, whether the agent suppresses adenylyl cyclase activity, wherein if the agent suppresses adenylyl cyclase activity relative to the untreated control, which untreated control comprises TPRA40 expressing cells that are contacted
with the adenvyl cyclase activator but are not contacted with the agent, then the agent is identified as a TPRA40 agonist.

42. A method of identifying a TRPA40 agonist, comprising:

a) providing a cell that expresses TRPA40 and that expresses a reporter gene capable of indicating adenvyl cyclase activity;

b) contacting the cell with an activator of adenvyl cyclase and with an agent, wherein the cells are contacted with the activator and the agent simultaneously, concurrently, or consecutively; and

c) determining, as compared to a control, whether the agent suppresses adenvyl cyclase activity induced by the activator, wherein if the agent suppresses the adenvyl cyclase activity relative to the control, then the agent is identified as a TPRA40 agonist.

43. A method of screening for an agent for inducing hedgehog signaling in a cell, wherein said method comprises:

a) screening for an agent that binds to TPRA40 protein, induces expression of TPRA40, facilitates transport of TPRA40 protein to the plasma membrane or to primary cilia, induces activation of TPRA40 or promotes its coupling with God;

b) contacting a cell with an amount of the agent identified in step a), and

c) determining, as compared to a control, whether said agent induces hedgehog signaling in said cell, wherein if said agent induces hedgehog signaling in said cell relative to the control, then an agent that induces hedgehog signaling is identified.

44. A method of identifying a TPRA40 agonist, wherein said method comprises:

a) screening for an agent that binds to TPRA40 protein;

b) contacting a cell with an amount of the agent identified in step a), and

c) determining, as compared to a control, whether said agent that binds to TPRA40 protein also induces hedgehog signaling in said cell, wherein if said agent induces hedgehog signaling in said cell relative to the control, then the agent is identified as a TPRA40 agonist.

45. The method of any of claims 45-44, wherein the agent is a small molecule.
46. The method of any of claims 41-44, wherein the agent is a polypeptide.

47. The method of any of claims 41-44, wherein the agent is a polynucleotide.

48. The method of any of claims 41-47, wherein said cell is in culture.

49. The method of any of claims 41-47, wherein said cell is in an animal.

50. The method of any of claims 41-47, wherein said agent hinds TPRA40 protein.

51. The method of any of claims 41-50, wherein said agent is identified in step a) using a yeast two-hybrid screen.

52. The method of any of claims 41-51, wherein said agent is identified in step a) using a high throughput binding or activity screen of a small molecule library.

53. The method of any of claims 45-52, wherein said agent facilitates transport of the TPRA40 protein to the plasma membrane or to primary cilia.

54. The method of any of claims 43-53, wherein said agent is identified in step a) by a method comprising the steps of:
   i) contacting a cell expressing TPRA40 with an agent,
   ii) determining the localization of TPRA40 in said first cell expressing TPRA40 using immunofluorescence.

55. The method of any of claims 41-54, wherein said agent is identified in step a) by a method comprising the steps of:
   i) contacting a cell expressing TPRA40 with an agent; and
   ii) determining the levels of TPRA40 in a plasma membrane or ciliary membrane fraction.
56. The method of any of claims 41-55, wherein said agent increases expression of TPRA40 protein or RNA.

57. The method of any of claims 41-56, wherein said agent is identified in step a) by a method comprising:
   i) contacting a cell expressing TPRA40 with an agent; and
   ii) determining activity of TPRA40 in said cell using a G//-luciferase reporter or adenylyl cyclase reporter assay.

58. The method of any of claims 41-56, wherein said agent is identified in step a) by a method comprising:
   i) contacting a cell expressing TPRA40 with an agent; and
   ii) determining the expression of TPRA40 in said cell by RT-PCR or using Northern Blot analysis of TPRA40 RNA.

59. The method of any of claims 41-56, wherein said agent is identified in step a) by a method comprising:
   i) contacting a cell expressing TPRA40 with an agent; and
   ii) determining the expression of TPRA40 in said cell by Western Blot, flow cytometry, immunofluorescence or immunohistochemistry analysis of TPRA40 protein.

60. The method of claim 59, wherein a reporter gene is used in order to determine whether adenylyl cyclase activity has been suppressed by an agent.

61. The method of claim 60, wherein the reporter gene is a luciferase gene controlled by a cAMP response element.

62. The method of any of claims 41-61, wherein the activator of adenylyl cyclase activity is forskolin.
63. The method of any of claims 41-61, wherein the activator of adenylyl cyclase is 8-bromo-cAMP or dibutyryl-cAMP.

64. The method of any of claims 41-63, wherein the agent is further assessed in an assay for hedgehog signaling.

65. The method of any one of claims 41-64, wherein the TPRA40 is exogenously expressed in a cell.

66. The method of claim 65, wherein the TPRA40 is stably expressed in the cell.

67. The method of claim 65, wherein the TPRA40 is transiently expressed in the cell.

68. The method of claim 65, wherein the cell is transformed with a vector expressing TPRA40 protein.

69. The method of any of claims 41-68, wherein the cell is an S12 cell.

70. The method of any of claims 41-68, wherein the cell is a 293T cell.

71. The method of claim 41-70, wherein the reporter gene is a luciferase gene controlled by a cAMP response element.

72. A method of reducing hedgehog signaling in a cell, wherein said cell is responsive to hedgehog protein or comprises one or more mutations in a hedgehog signaling pathway gene, wherein said one or more mutations results in increased hedgehog signaling and/or activation of the hedgehog signaling pathway in the absence of ligand, wherein said method comprises the step of contacting said cell with an effective amount of a TPRA40 antagonist.

73. A method of inhibiting unwanted growth, proliferation or survival of a cell, wherein said cell is responsive to hedgehog protein or comprises one or more mutations in a
hedgehog signaling pathway gene, wherein said one or more mutations results in increased hedgehog signaling and/or activation of the hedgehog signaling pathway in the absence of ligand, wherein said method comprises the step of contacting said cell with an effective amount of a TPRA40 antagonist.

74. A method of inhibiting growth, proliferation or survival of a tumor cell, wherein said cell is responsive to hedgehog protein or comprises one or more mutations in a hedgehog signaling pathway gene, wherein said one or more mutations results in increased hedgehog signaling and/or activation of the hedgehog signaling pathway in the absence of ligand, wherein said method comprises the step of contacting said cell with an effective amount of a TPRA40 antagonist.

75. The method of any of claims 72-74, wherein the TRPA40 antagonist comprises an agent that inhibits the expression and/or activity and/or localization of TPRA40 by: (i) binding to TPRA40 DNA, RNA, or protein.

76. The method of any of claims 72-75, wherein hedgehog signaling is hyperactive in the cell.

77. The method of any one of claims 72-76, wherein said cell comprises one or more mutations in a hedgehog signaling pathway gene.

78. The method of claim 77, wherein said one or more mutations are in smoothened, and the cell has a smoothened gain-of-function.

79. The method of claim 78, wherein gain-of-function smoothened mutation results in a constitutively active smoothened protein.

80. The method of any of claims 76-79, wherein said one or more mutations are in patched and the cell has a patched loss-of-function.
81. The method of any of claims 72-76, wherein said cell or an adjacent cell overexpresses a hedgehog protein.

82. The method of claim 81, wherein said overexpressed hedgehog protein is Sonic hedgehog protein.

83. The method of claim 81, wherein said overexpressed hedgehog protein is Indian hedgehog protein.

84. The method of claim 81, wherein said overexpressed hedgehog protein is Desert hedgehog protein.

85. The method of claim 77, wherein said one or more mutations are in suppressor-of-fused, and the cell has suppressor-of-fused loss-of-function.

86. The method of any of claims 76-85, wherein prior to contacting said cell with said TPRA40 antagonist, said cell is determined to have one or more mutations in a hedgehog signaling pathway gene or otherwise determined to exhibit hedgehog signaling activity.

87. The method of claim 86, wherein said cell is determined to exhibit hedgehog signaling activity by measuring GUI or Patched1 levels.

88. The method of any of claims 72-87, wherein said cell is a cell in culture.

89. The method of claim 88, wherein the method comprises contacting a culture comprising a plurality of cells.

90. The method of any of claims 72-89, wherein said cell is in a vertebrate, and contacting said cell comprises administering the TPRA40 antagonist to the vertebrate.

91. The method of claim 90, wherein said vertebrate is a human subject.
92. The method of claims 90 or 91, wherein said cell is a cancer cell and/or said vertebrate is a vertebrate diagnosed with cancer.

93. The method of claim 92, wherein said 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, meningioma, glioma, chondrosarcoma, gall bladder, sarcoma, rhabdomyosarcoma, basal cell carcinoma, gastric, ovarian, esophageal, pancreatic, leukemia, lymphoma, multiple myeloma or testicular cancer cell.

94. A method of inhibiting unwanted growth, proliferation or survival of a cell, wherein said cell comprises one or more mutations in suppressor-of-fused, in which one or more mutations result in the cell having suppressor-of-fused loss-of-function, wherein said one or more mutations results in increased hedgehog signaling and/or activation of the hedgehog signaling pathway in the absence of ligand, wherein said method comprises the step of contacting said cell with an effective amount of a TPRA40 antagonist.

95. A method of inhibiting growth, proliferation or survival of a tumor cell, wherein said cell comprises one or more mutations in suppressor-of-fused resulting in the cell having suppressor-of-fused loss-of-function, wherein said one or more mutations results in increased hedgehog signaling and/or activation of the hedgehog signaling pathway in the absence of ligand, wherein said method comprises the step of contacting said cell with an effective amount of a TPRA40 antagonist.

96. The method of any of claims 94 or 95, wherein said cell is a cell in culture.

97. The method of claim 96, wherein the method comprises contacting a culture comprising a plurality of cells.

98. The method of any of claims 94-97, wherein said cell is in a vertebrate, and contacting said cell comprises administering the TPRA40 antagonist to the vertebrate.
99. The method of claim 98, wherein said vertebrate is a human subject.

100. The method of any of claims 98 or 99, wherein said cell is a cancer cell and/or said vertebrate is a vertebrate diagnosed with cancer.

101. The method of claim 100, wherein said cancer cell is a cancer cell selected from the group consisting of: a medulloblastoma, meningioma, adenoid cystic carcinoma, basal cell carcinoma and rhabdomyosarcoma cancer cell.

102. The method of any of claims 72-101, wherein said TPRA40 antagonist comprises a polynucleotide molecule that inhibits the expression of TPRA40.

103. The method of claim 102, wherein the polynucleotide molecule is an antisense oligonucleotide that hybridizes to a TPRA40 transcript to inhibit expression of TPRA40.

104. The method of any of claims 72-103, wherein said TPRA40 antagonist comprises an RNAi that targets the TPRA40 mRNA transcript.

105. The method of claim 104, wherein the RNAi comprises an siRNA.

106. The method of claim 105, wherein said siRNA is 19-23 nucleotides in length.

107. The method of claim 105 or 106, wherein said siRNA is double stranded, and includes short overhang(s) at one or both ends.

108. The method of claim 104, wherein the RNAi comprises an shRNA.

109. The method of claim 105 or 108, wherein said siRNA or shRNA targets TPRA40 niRNA transcript.
110. The method of claim 104 or 109, wherein said siRNA comprises one or more of the nucleotide sequences selected from: SEQ ID NOs: 16-23.

111. The method of any of claims 72-101, wherein said TPRA40 antagonist is a small molecule that binds to TPRA40.

112. The method of any of claims 72-101, wherein said TPRA40 antagonist comprises an antibody that binds to TPRA40 protein.

113. The method of claim 112, wherein said antibody is a monoclonal antibody.

114. The method of any of claims 72-101, wherein said TPRA4G antagonist comprises a polypeptide antagonist.

115. The method of any one of claims 72-114, wherein said cell is also contacted with an additional antagonist of the hedgehog signaling pathway.

116. The method of claim 115, wherein said additional antagonist of the hedgehog signaling pathway is a veratrum-type steroidal alkaloid.

117. The method of claim 116, wherein said veratrum-type steroidal alkaloid is cyclopamine or KAAD-cyclopamine or a derivative thereof.

118. The method of claim 116, wherein said veratrum-type steroidal alkaloid is jervine, IPI-269609 or IPI-926.

119. The method of claim 115, wherein said antagonist is a non-veratrum-type synthetic small molecule inhibitor of Smoothened.

120. The method of claim 119, wherein said antagonist is Erivedge (vismodegib), BMS-833923 (XLS 19), LDE225 (Erismodegib), PF-04449913, NVP-LDE225, NVP-LEQ506,
TAK-441, XL-319, LY-2940680, SEN450, Itraconazole, MRT-10, MRT-83, or PF-04449913.

121. The method of claim 115, wherein said additional antagonist of the hedgehog signaling pathway is an antibody.

122. The method of claim 121, wherein said antibody is an antibody that binds Sonic, Indian and/or Desert hedgehog protein.

123. The method of claim 115, wherein said additional antagonist of the hedgehog pathway is a small molecule hedgehog inhibitor.

124. The method of claim 123, wherein said hedgehog inhibitor is robotkinin.

125. The method of claim 115, wherein said additional antagonist of the hedgehog signaling pathway is selected from the group consisting of: vismodegib, sonidegib, BMS-833923, PF-04449913, and LY2940680.

126. The method of claim 115, wherein said additional antagonist of the hedgehog signaling pathway is an RNAi antagonist.
Figure 1

n=4; * p < 0.05, ** p < 0.01

A

Gli-luc (% siTNC + Hh)

siNTC | silFT88 | siSna1 | siTPRA40

-Bh | +Hh

B

Gli-luc (% HhAg)

DMSO | HhAg1.2

siNTC | silFT88 | siSna1 | siTPRA40

% knockdown (mRNA): n/a | 72% | 75% | 80%
**Figure 3 (continued)**

### mGli1 expression in S12 Cells

- **Expression relative to NTC**

  - NTC
  - siTPRA Pool
  - siTPRA #9
  - siTPRA #10
  - siTPRA #11
  - siTPRA #12

  - No Hh
  - Hh

### mTPRA expression in S12 cells

- **Expression relative to NTC**

  - NTC
  - siTPRA40 Pool
  - siTPRA40 #9
  - siTPRA40 #10
  - siTPRA40 #11
  - siTPRA40 #12

  - No Hh
  - Hh
Figure 4

Human *Gli1* expression

![Bar chart showing human *Gli1* expression levels with NTC and siTPRA40 treatments at different concentrations of cycloamine compared to DMSO.](chart.png)
Figure 6

Gli-Luciferase normalized to DMSO NTC

- siNTC
- siTPRA40

DMSO
PKA Inhibitor (14-22 amide)
Figure 10

n=4 (293 cell transfection)

CRE-luc + GFP

CRE-luc + TPRA40

Relative CRE-luc activity

µM Forskolin

0.5 0.75 1 10 20

0 20 40 60 80 100 120

TPRA40

αS

αβ

cAMP

CRE

Luciferase

293 cells
Figure 11A

Gli-Luciferase normalized to NTC + Hh

siNTC  siTPRA40  siGa (i)  siTPRA40+ siGa (i)
## Alignment of human, mouse and zebrafish TPRA40 homologs

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<th>Alignment</th>
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<th>moTPRA40</th>
<th>zfTPRA1</th>
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### TM1
- huTPRA40: ILYFVVAALVGIARAVSMTSTSNAATVAKILWTRFFLLAILSVII
- moTPRA40: ILYFVVAALVGIARAVSMTSTSDAAATVAKILWTRFFLLAILSVII
- zfTPRA1: ILYFVVAALVGIARAVSMTSTSSAALVKLWTRFFLLAILSVII

### TM2
- huTPRA40: ILVDFVVAALVGIARAVSMTSTSNAATVAKILWTRFFLLAILSVII
- moTPRA40: ILVDFVVAALVGIARAVSMTSTSNAATVAKILWTRFFLLAILSVII
- zfTPRA1: ILVDFVVAALVGIARAVSMTSTSNAATVAKILWTRFFLLAILSVII

### TM3
- huTPRA40: LGLAFGHLLSKSIRKRLTAAIVSLASSVTSQGTLLYPAHLSAKFNF
- moTPRA40: LGLAFGHLLSKSIRKRLTAAIVSLASSVTSQGTLLYPAHLSAKFNF
- zfTPRA1: LGLAFGHLLSKSIRKRLTAAIVSLASSVTSQGTLLYPAHLSAKFNF

### TM4
- huTPRA40: IYGHGGRQFWLVSSECFEEFLVYSLVLPKTPRKLIRSLPSRSSFVYYAGI
- moTPRA40: IYGHGGRQFWLVSSECFEEFLVYSLVLPKTPRKLIRSLPSRSSFVYYAGI
- zfTPRA1: IYGHGGRQFWLVSSECFEEFLVYSLVLPKTPRKLIRSLPSRSSFVYYAGI

### TM5
- huTPRA40: LALLNLQGLGSLVCGLCCVAATTFLYFSSFAPLIVYVAFLRGFF
- moTPRA40: LALLNLQGLGSLVCGLCCVAATTFLYFSSFAPLIVYVAFLRGFF
- zfTPRA1: LALLNLQGLGSLVCGLCCVAATTFLYFSSFAPLIVYVAFLRGFF

### TM6
- huTPRA40: GSPKLFPSYKCQVAPVHPQPYAVARRGGLAAGAAGASASAXYS
- moTPRA40: GSPKLFPSYKCQVAPVHPQPYAVARRGGLAAGAAGASASAXYS
- zfTPRA1: GSPKLFPSYKCQVAPVHPQPYAVARRGGLAAGAAGASASAXYS

### TM7
- huTPRA40: STQFSAAGVAYLIAASPCHTGSIINSTSSRWKAINA373
- moTPRA40: STQFSAAGVAYLIAASPCHTGSIINSTSSRWKAINA369
- zfTPRA1: STQFSAAGVAYLIAASPCHTGSIINSTSSRWKAINA369
### A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/566
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)
G01N

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

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
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<td>par [0664], par [0743] et seq., par [0773] et seq., par [0830]–[0834]</td>
<td>1,2, 6–14, 17, 21–42, 45–53, 55–71</td>
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</table>

* 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) on which is cited to establish the publication date of another citation or other special reason (as specified)
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*AT* document member of the same patent family

Date of the actual completion of the international search
10 April 2015

Date of mailing of the international search report
03/07/2015

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

Authorized officer
Rosin, Oliver
INTERNATIONAL SEARCH REPORT

<table>
<thead>
<tr>
<th>Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)</th>
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<td>This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:</td>
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<tr>
<td>1. □ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:</td>
</tr>
<tr>
<td>2. □ Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:</td>
</tr>
<tr>
<td>3. □ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).</td>
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<th>Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)</th>
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<td>This International Searching Authority found multiple inventions in this international application, as follows:</td>
</tr>
<tr>
<td>see additional sheet</td>
</tr>
<tr>
<td>1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.</td>
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<tr>
<td>2. □ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.</td>
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<tr>
<td>3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:</td>
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<tr>
<td>4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:</td>
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<td>1, 2, 41, 42 (completely) ; 6-14, 17, 21-40, 45-53, 55-71 (partially)</td>
</tr>
</tbody>
</table>

Remark on Protest
- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.
This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 2, 41, 42 (completely); 6-14, 17, 21-40, 45-53, 55-71 (partially)
   Screening methods encompassing TPRA40 signaling using an adenylyl cyclase readout system.

2. claims: 3-5, 15, 16, 18-20, 43, 44, 54 (completely); 6-14, 17, 21-40, 45-53, 55-71 (partially)
   Screening methods encompassing TPRA40 and hedgehog signaling.

3. claims: 72-126
   Methods of reducing hedgehog signaling and reducing cell growth.
<table>
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<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
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<td>US 2011214189 A1</td>
<td>01-09-2011</td>
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