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(54) **Title:** IN VIVO SCREEN FOR HEDGEHOG (HH) PATHWAY ANTAGONISTS

(57) **Abstract:** A fish from the family of Cyprinidae comprising an inhibited Kif7 gene and wherein the fish is homozygous for the inhibited Kif7 gene.



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IN VIVO SCREEN FOR HEDGEHOG (Hh) PATHWAY ANTAGONISTS**Technical Field**

5 The present invention generally relates to a fish from the family of Cyprinidae comprising an inhibited *Kif7* gene. The present invention also relates to methods of screening for Hedgehog (Hh) pathway antagonists using a fish of the present invention.

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Background

Hedgehog (Hh) signaling plays a crucial role in animal development, controlling cell type specification, proliferation and survival in a variety of contexts through a pathway that has been highly conserved during evolution. Hh signaling also functions post-embryonically to control tissue homeostasis and aberrant activity of the pathway has been implicated in numerous types of cancer.

20 Activation of the Hh pathway has been implicated in 25% of all human cancers, making it an important target for the development of anti-cancer therapeutics. To date, known anti-Hh therapeutics in clinical trials target the GPCR-like protein, Smoothened (Smo); this is an obligate components of the Hh pathway that acts just downstream of the receptor, Patched (Ptc).

30 Although some drugs have shown impressive efficacy, there are reports of rapid acquisition of resistance through mutation of Smoothened, underlining the need for the development of drugs against multiple targets in the pathway.

 Mutations in Hh pathway genes acting downstream of Smoothened have also been reported to be associated with tumours, emphasizing the need for drugs targeting downstream pathway components. Thus, there is a need to provide an *in vivo* model of Hh pathway activation downstream of Smoothened.

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 The *in vivo* model of the present invention advantageously provides for an alternative screening method that is robust, non-quantitative and cost effective.

Summary of Invention

According to a first aspect, there is provided a fish from
5 the family of Cyprinidae comprising an inhibited *Kif7* gene and
wherein the fish is homozygous for the inhibited *Kif7* gene.

In a second aspect, there is provided a zebrafish
comprising an inactivated *Kif7* gene and a reporter gene.

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In a third aspect, there is provided a *Danio rerio*
comprising two inactivated copies of the *Kif7* gene and a
reporter gene, wherein the *Kif7* gene is inactivated using zinc
finger nuclease-induced mutagenesis and the reporter gene
15 encodes green fluorescent protein.

In a fourth aspect, there is provided a method of
identifying a compound capable of inhibiting the Hedgehog cell
signalling pathway comprising the following steps:

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(a) contacting the compound with a fish of any one of the
preceding claims and measuring the expression level of the
reporter gene;

(b) contacting a control compound with a control group of
the fish and measuring the expression level of the
25 reporter gene;

(c) comparing the reporter gene expression levels obtained
from steps (a) and (b);

wherein a decreased expression of the report gene relative
to the control group indicated that the compound is capable of
30 inhibiting the Hedgehog cell signalling pathway.

Brief Description of Drawings

The accompanying drawings illustrate a disclosed
35 embodiment and serves to explain the principles of the disclosed
embodiment. It is to be understood, however, that the drawings
are designed for purposes of illustration only, and not as a
definition of the limits of the invention.

Figure 1 is a schematic diagram showing what happens when there is no Hh binding to the Ptc receptor of a normal cell. This results in Ptc repressing the activity of smoothened and thereby the activation of the downstream signaling pathway. In the absence of Smo activity, *Kif7* inhibits Gli transcriptional activity and hence there is no or reduced expression of the reporter protein.

Figure 2 is a schematic diagram showing what happens when Hh binds to the Ptc receptor in a normal cell. When Hh binds to Ptc, this removes the inhibitory effect of Ptc on Smo, which in turn results in Smo inhibiting the activity of *Kif7*. This allows Gli to activate the *Hh* reporter gene resulting in the expression of the reporter protein.

Figure 3 is a schematic diagram showing what happens when a normal cell is exposed to Hh and an inhibitor of Smo, such as Cyclopamine. Binding of Cyclopamine to Smo results in inactivation of the latter, even in the absence of Ptc mediated inhibition; this in turn removes the inhibitory effect of Smo upon *Kif7*. This allows *Kif7* to inhibit the activity of Gli, resulting in no or reduced expression of the reporter protein.

Figure 4 is a schematic diagram showing what happens in a *Kif7* homozygous mutant cell. Since *Kif7* is inactivated, the activity of Gli is not repressed and this results in high expression levels of the reporter protein.

Figure 5 is a schematic diagram showing what happens in a *Kif7* homozygous mutant cell when the cell is exposed to Cyclopamine. The binding of Cyclopamine to Smo results in the removal of the inhibitory effect of Smo upon *Kif7*. However, in contrast to a normal cell (see Figure 3), because *Kif7* is

inactivated, Gli activity is not affected and hence the reporter protein is expressed at high levels.

Figure 6 is a schematic diagram showing what happens when a *Kif7* homozygous mutant cell is exposed to a novel chemical inhibitor which affects the activity of Gli, or of factors required for its activation, stability or nuclear import. In this case, although *Kif7* is inactivated and therefore unable to regulate the activity of Gli, because the novel inhibitor acts directly upon Gli or positive modulators of its activity, this results in no or reduced expression of the reporter protein.

Figure 7A is schematic diagram of the generation of mutant alleles of zebrafish *Kif7*. Nucleotide sequence of the zebrafish *Kif7* gene chosen for targeting and amino acid sequences of the Zinc Finger Proteins selected to recognize this sequence. Each of the latter was fused to a subunit of the FokI nuclease as shown. The upper section shows the identifiers of Sangoma two-finger library from which fingers recognizing each triplet motif were selected. The central block of sequences represent clones picked randomly from each pool prior to selection. The lower block of sequences derives from clones picked following selection. Asterisks indicate the sequences chosen for use as the mutagenic Left and Right Zinc Finger Proteins (ZFP) as shown in (A).

Figure 7B is a collection of images of *in situ* hybridization and immunofluorescence characterizing the mutant phenotype of zebrafish *Kif7*.

Figure 8 is a collection of images of immunofluorescence and western blot results that shows *Kif7* controls processing, trafficking and activity of Gli2a..

Figure 9 is a collection of images of *in situ* hybridization and immunofluorescence showing differential activities of *Kif7* in the neural tube versus the mesoderm.

Figure 10 is a collection of images of immunofluorescence showing cilia length of *Kif7* mutants.

Definitions

As used herein, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a gene" includes a plurality of genes.

As used herein, "copies" of genes are also known in the art as "alleles". This latter term signifies the naturally-occurring copy of a given gene in a cell. Usually there are two copies of a gene in a diploid cell. In some situations (e.g., trisomy 21) there are three copies, but four or more copies of one or more entire chromosomes, or extra copies of genes or chromosomal fragments are also encountered naturally. Cells may also be experimentally altered to change the expression of specific genes. In one example, the fish may have one copy of the *Kif7* gene inactivated. In another example, the fish may have two copies of the *Kif7* genes inactivated.

As used herein, "homozygous" refers to a cell in which all copies (typically two in most eukaryotic cells) of a given gene are mutated.

As used herein, a nucleotide sequence is "operably linked" to another nucleotide sequence when it is placed in a functional relationship with another nucleotide sequence. For example, if a reporter gene is operably linked to a regulatory region, this generally means that the reporter gene may only be expressed if the regulatory region is activated. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary join two protein coding regions, contiguous and in reading frame. For example, the reporter gene as disclosed herein may be operatively linked to a regulatory region of one or more genes of the Hedgehog pathway. In another example, the gene of the Hedgehog pathway is the *Eng2a* gene and/or the *slow myosin heavy chain 1* gene. In another example, the regulatory region is a cis-regulatory region.

As used herein, "inhibiting, inactivating and/or reducing the expression and/or activity" of a gene, mRNA and/or promoter used herein refers to the prevention, reduction, inactivation

and/or inhibition of the expression and/or activity and/or function of a particular gene, transcript, mRNA and/or promoter. It is understood that the phrase is relative, and does not require absolute suppression of the transcript. Thus, in some examples, reducing, inactivating and/or inhibiting the expression of *Kif7* gene and/or transcript, may cause the *Kif7* gene to be inhibited completely or partially. In particular preventing, reducing and/or inhibiting the expression of *Kif7* gene and/or transcript, requires that, following application of the *Kif7* gene and/or transcript is expressed at least 5 % less than wild type, such as at least 10 % less, at least 15 % less, at least 20 % less, at least 25 % less, or even more reduced. Thus, in some particular examples, the reduction and/or inhibition of expression of the *Kif7* gene is at least by about 30 %, about 40 %, about 50 %, about 60 %, or more of the wild type expression. In specific examples, the reduction, inactivation and/or inhibition of expression of the *Kif7* gene is at least by 70 %, 85 %, 85 %, 90 %, 95 %, or even more. Furthermore, in some examples, preventing, reducing, inactivating and/or inhibiting the expression of *Kif7* gene and/or transcript, may cause the *Kif7* gene to be inhibited temporarily or permanently.

As used herein, the phrase inhibited "temporarily" refers to inhibition of a gene for a limited period of time wherein the gene may reverted back to normal expression after a period of time. The reversal of inhibition may be performed deliberately as in the reversal of morpholino temporary inhibition by mRNA rescue experiments. For example, temporary inhibition of a gene as disclosed herein may use, but not limited to, of any one of the following, short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA) and morpholino oligonucleotides.

As used herein, the phrase inhibited "permanently" refers to inhibition of a gene wherein reversal of inhibition is not possible. Permanent inactivation of a gene of the present disclosure may be conducted by for example, but not limited to,

the introduction of one or more mutations into the *Kif7* gene or any sequence controlling expression of the *Kif7* gene which results in a loss of function or lack of expression of the *Kif7* gene. The mutations may be introduced by, for example, but not limited to one or more of the following methods: site-directed mutagenesis, insertional mutagenesis, PCR site-directed mutagenesis, plasmid mutagenesis, cassette mutagenesis and zinc finger nuclease-induced mutagenesis.

Detailed Bibliographic references mentioned in the present specification are for convenience listed in the form of a list of references and added at the end of the examples. The whole content of such bibliographic references is herein incorporated by reference.

Additionally, unless otherwise defined, any terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies described in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY and Ausubel et al., *Current Protocols in Molecular Biology*, Wiley Interscience Publishers, (1995) as well as text describing the culture of fish such as Westerfield, M. (2000), *"The Zebrafish Book"*, A guide for the laboratory use of zebrafish (*Danio rerio*). 4th ed., Univ. of Oregon Press, Eugene and related texts such as Marie A. Di Berardino *"Genomic Potential of Differentiated Cells"* (1997) Columbia University Press. As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with

manufacturer defined protocols and/or parameters unless otherwise noted.

Detailed description of the invention

An exemplary non-limiting embodiments of a fish comprising
5 an inhibited *Kif7* gene will now be disclosed.

In one embodiment the fish may be homozygous for the inhibited *Kif7* gene.

In one embodiment, zinc finger nuclease mediated targeted mutagenesis was used to generate mutant alleles of genes in the
10 zebrafish.

In another embodiment, the fish may be selected from the group consisting of freshwater, brackish water, and saltwater (marine water) species of fish.

In another embodiment, the fish may be selected from a
15 fish of the *Oryzias* species and the *Danio* species.

In another embodiment, the fish may be from the Adrianichthyidae family and include, for example, *Oryzias melastigma* (alternative name *Oryzias dancena*) (Marine or brackish medaka), *Oryzias latipes* (Japanese medaka), *Oryzias*
20 *celebensis*, *Oryzias marmoratus*, *Oryzias matanensis*, *Oryzias nigrimas* (black buntingi), *Oryzias orthognathus* (buntingi), and *Oryzias profundicola*.

In another embodiment, the fish may be from the family of Cyprinidae and include, for example, *Danio rerio*
25 (Zebrafish), *Danio aesculapii*, *Danio albolineatus*, *Danio choprae*, *Danio dangila*, *Danio erythromicron*, *Danio feegradei*, *Danio jaintianensis*, *Danio kerri*, *Danio kyathit*, *Danio margaritatus*, *Danio meghalayensis*, *Danio muongthanhensis*, *Danio nigrofasciatus*, *Danio quagga*, *Danio quangbinhensis*, *Danio*
30 *roseus*, *Danio tinwini* and *Danio trangi*.

Zebrafish (*Danio rerio*), in particular, may be utilized in the present disclosure. For example, zebrafish are amenable to genetic screens, modifier screens, and chemical screens; develop rapidly ex-utero; are transparent for much of their life cycle
35 and produce large clutches of offspring weekly. Advantageously, Zebrafish may be raised in relatively small facilities (housing

up to about 54 adult fish in a single 9 liter tank), and can reliably produce offspring in large quantities, with each mature female typically laying between 100 to 300 eggs per week. These eggs are fertilized externally, and the embryos are transparent
5 allowing the early development of hematopoietic tissues and other organ and tissue systems to be directly observed using only a dissecting microscope. Embryonic development is extremely rapid with most organ systems including blood cell formation being fully developed by 5 days post fertilization. Full
10 reproductive maturation is reached by about 3 months. In one embodiment, the fish as used herein may be an embryo, a larva or an adult.

In one embodiment, the fish may further comprise a reporter gene. Thus, in another embodiment, there is provided a
15 zebrafish comprising an inactivated *Kif7* gene and a reporter gene.

The reporter gene as disclosed herein may include, but not limited to luciferase, galactosidase, chloramphenicol, acetyltransferase, b-glucuronidase, and alkaline phosphatase.
20 The reporter gene may encode a visually identifiable protein. The protein may include, but not limited to fluorescent protein, luminescent protein or beta-galactosidase. The fluorescent protein may include, but not limited to green fluorescent protein (GFP), reef coral fluorescent protein (RFP), Blue
25 fluorescent protein (BFP), Yellow Fluorescent Protein (YFP), and a red fluorescent protein designed for high solubility and low aggregation (dsRED2). In one embodiment, the fluorescent protein may be GFP. The reporter gene may comprise a transcription stop-site.

30 In one embodiment, the reporter gene may be operatively linked to a regulatory region of one or more genes of the Hedgehog pathway.

In one embodiment, there is provided a *Danio rerio* that may include two inactivated copies of the *Kif7* gene and a
35 reporter gene, wherein the *Kif7* gene may be inactivated using zinc finger nuclease-induced mutagenesis and the reporter gene may encode green fluorescent protein.

Also disclosed is a method of identifying a compound capable of inhibiting the Hedgehog cell signalling pathway that may include the following steps: (a) contacting the compound with a fish as disclosed herein and measuring the expression level of the reporter gene; (b) contacting a control compound with a control group of the fish and measuring the expression level of the reporter gene; (c) comparing the reporter gene expression levels obtained from steps (a) and (b); wherein a decreased expression of the report gene relative to the control group indicates that the compound may be capable of inhibiting the Hedgehog cell signalling pathway.

In one embodiment, the fish may be contacted with the compound by injecting the compound into the fish, wherein the injection into the fish may be in conjunction with a carrier. As disclosed herein, the carrier may be any carriers that may be injected into a fish, for example, but not limited to solvent, lipid or peptide. In another embodiment, the method may be capable of identifying a compound capable of inhibiting the Hedgehog cell signaling pathway downstream of *Kif7* protein. In particular, the expression level of the reporter gene may be measured by light or fluorescent microscopy.

Also disclosed is a zebrafish homozygous for loss of function mutations in the *Kif7* gene that also carry a GFP-based reporter of Hh pathway activity. *Kif7* acts downstream of Smoothened to negatively regulate the activity of the Gli transcription factors, which mediate the response of cells to Hh signals.

The zygotic inactivation of *Kif7* in zebrafish is found not to be lethal: however, homozygous mutant animals produce homozygous mutant progeny that show strong upregulation of the Hh GFP reporter gene. It is found that this effect is insensitive to the Smoothened inhibitor, cyclopamine, confirming that the pathway is upregulated downstream of Smoothened.

As the loss of *Kif7* derepresses the Hh pathway downstream of Smo, the embryos provide the basis for a screen for a highly specific class of Hh inhibitors. *Kif7* mutant mouse embryonic

fibroblasts (MEFs) have previously been reported but their use in screening for specific inhibitors has not been proposed.

Examples

5 Non-limiting examples of the invention will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention.

10

MATERIALS AND METHODS

Zebrafish strains and husbandry

Adult fish were maintained on a 14 hour light/10 hour dark cycle at 28°C in the AVA (Singapore) certificated IMCB Zebrafish Facility. Previously described zebrafish strains used were: *ptc1*^{hu1602}, *ptc2*^{tj222} (Koudijs et al., 2008); *igu*^{ts294} (Wolff et al., 2004); *Tg(eng2a:eGFP)*ⁱ²³³ (Maurya et al., 2011) and *Tg(fli::GFP)* (Lawson and Weinstein, 2002); *gli1* (dtr) *gli2a* (yot).

20 Generation, selection and genotyping of *Kif7* mutant alleles

Plasmids encoding Zinc-finger nucleases (ZFN) specific for the Zebrafish *Kif7* (set 3) gene were purchased from Sigma. The Zinc Finger Nuclease Binding Site is shown below (ZF-
25 nucleotide recognition site in bold italics).

Set 3:

24631 tcATCCAAATTCCATTTTGTGGAC**CCTGGCAGGATCAGAG**cg (SEQ ID NO: 1)

24630 ag**TAGGTTTAAGGTAAAA**CACCTGGACCGTCCTAGTCTCgc (SEQ ID NO: 2)

Capped polyadenylated RNA from each plasmid was produced
30 by in vitro transcription and a range of doses was injected into one-cell stage zebrafish embryos. Embryos were injected with approximately 100pg had around 30% rate of deformity at 24 hpf (hours post-fertilisation). Genomic DNA prepared from non-deformed 24hpf embryos injected with this dose was used as a PCR
35 template to analyse potential somatic mutations.

Roche Titanium 454 amplicon sequencing showed that 6.3% of the amplicon molecules had insertions or deletions at the target site. G0 adults derived from embryos injected with ZFN capped RNA were in-crossed and their progenies (G1) individually genotyped by PCR using the forward primer (*Kif7* exF2:CGAGGTGCTGAGTCTCTTAGAGT) (SEQ ID NO: 3) and reverse primer (*Kif7* ex R1:TGAATCCCTGTATGGGATATGGGT) (SEQ ID NO: 4) followed by Sanger sequencing.

Founders transmitting two different alleles (*Kif7* Δ 8, an 8bp del: GGAACCTGG) and *Kif7* Δ 7, a 7bp del: TTGTGGA) were selected and used to establish stable mutant lines. The full nucleic acid sequence of each allele is shown below.

Underline + Highlighting = *Kif7*-delta8 mutant

Bold italics + Underline = *Kif7*-delta7 mutant

>zf-*Kif7*-cDNA (mutants marked) (SEQ ID NO: 5)

ATGTCTCCAAAAGGAGTCGGCCACAGTAAGGTGGAAGAAAGTGCTGTACAGGTGGCCGTCCGCGTGCGTC
CGTTTGCTTCCCAAAGAGATTTTGCACAGCCATGAGAGCTGCATCACTTCAGACCCAGAGGAGCGGAGGGT
CACCTTGGGCAATGACCGCCACTTCCACTGTGACTTTGTGTTTTGAGGACGGCTCTACCCAGGAGGAGGTG
TACACTAACTGCGTTCAACCACTTATCGAGGCCTTCTTTTCATGGATTTAATGCTACCGTGTCTGCCTATG
GGCAGACAGGCTCAGGCAAAACCTACACCATTTGGGGAAGCCAGCATTTCCGCTTTTAGGGATGATGAACA
GGGCATCATTCCCAGAGCTGTGGCCGAGATCTTCAAACCTGTTGGACGAGAATGACCTCATTGACTTCTCG
GTCCGGGTGTCTTACATGGAGGTTTACAAAGAGGTCTTCAGAGACCTTTTGGAAAGTTGAGACGGCCAGTA
AAGACATACATATCCGAGAGGACGAAAGAGGGAATGTAGTGCTTTGTGGTGTAAGGAATGTGAAGTGG
GGGACTTGACGAGGTGCTGAGTCTCTTAGAGTCAGGAAAGACAGCCCGTCACACAGGGGCCACCCAGATG
AATCCCACTCAAGTCGCTCTCACACCATCTTCACTGTGCTGATGGAGCAGCGGCGTGGAGGCTCTCGAG
CAGCGAATGGCTCTGTGCAGATCCTCTCATCCAAATTCCATT***TTGTGGACCTGG***CAGGATCAGAGCGCAT
CCTTAAACCGGCAACACCGGCGAACGGCTCAAGGAGAGCATTGAGATCAACAGTGGACTTCTTGTCTT
GGAAATGTCATTGGAGCGCTTGGGGACCCCAAAGAAAAGGCACCCATATCCCATACAGGGATTCAAAAA
TCACCAGGATCTTAAAGACTCTCTTGGAGGCAACGCCAAACCTGATGATTGCCTGCATTAGCCCTTC
GTCCTCCGACTTTGATGAGAGCCTAAACACACTCAACTACGCCAAGCGTGCTCGTAACATACAAAACCGG
GCTACAGTCAACTGCCGGGGGGAACAGATCGCATTGAGGGCCTTGAACCTCAGATCAAAGCGCTGAGAC
GAGCCCTTGAACCGCCAGCGCTCTGAACCCGCATTATTGCACGATCTGACCCTGAGAAGAGACTTCG
GCCGTTTGAAGTGGATGTAAGGAAGCTGCAAGCTGAGAGCGCCCACTATAGGACGTGCACCGACTCTGCC
TACAGGCTTTTAAACAGAGCTGCAGGGCGAAGGAACCTTAATGCGGGGCAAATCCTGCGGGTTAAAGAGT
GGCTGTGTGGTGTGAGGAGGAACGCAGCGGTTTGACCTCAGCGTCAGGACTCGACAGCGGCATTGAAAG
CAGCTCTACTGAGGACAGCACTGCGCTCAAAGGAGACAAGCCGTGCTGAACAACAGGATCTTGTGAAG
GAGGATTGGAGAGGAGAGCGGGAGGACTACACTTCCCAGCTGCAAGCCCAGATTGAGCAACTCGAGCAAG
AAAACACTGACTTTTTAGTCGCCTTGAAGATGCCATGGAACAGTACAAACAACAGAGCGATAAGCTACA
GGAGCAGCAGGATCTAATAGCGGAGCTTACAGTCTGTAGCTCAGCCTGGAGGAGCGGGGTTTCTCCAC
CTGAAACAGAGACCTCACACTGCTCCTATCAACTCACTACTGCAGACTCCAGACCGCCTCACTCCTCCCT
GCGACTCTGATGTGGGCAGAGCCTCGCCAGACAGCTTGACGTCGGTGCATCAGTAGACAGCATGTCATA
TTCAGAACAGACCCAGTGGGACGGCACACATGGAACACGCACTGTGAAAGCTCTAGGAACTGAACAGA
GATGAAGATGGCCACATGCAAAACCACTAGAGACAAACGCAAATCCATCAATGTAACCTGGACAAAAAAG
ACATTGCAATTCCCCAAGGACCTTTTGGTGGGACCAGGACAGCCCTTCTCAGACTCTAGGACTCTGTCA
TCCTCTGGGCATGCAATTCAACAGACGCACGTCTAACAGCAGCATTGGCGAGAGCTCGGTGTGGGAGAGC
GTGAGAGGTTTTGGAGGGGAGTTTTGCTCCGACCGAGGACTTCTCCAGGCTCAGCAGAAGATCAGAGAGC
TCTCCATCACCATTGCGATGAAGGAGGAGCTGATAAAAGAGCTGGTCAAGACAGGGAAAGACGCTCAGGC
CATGAACAGGCAGTACAGTCGTAAGATCTCAGAGCTGGAGGCCGAGGCAGAGCAGGCCCGAGTGGAGCTC
ACAGAGGCACAAAAGCAGCTGCAGGAGCTGGAGGTTTCAAGGCGGCCGTGATGCCGTTGACCGCTCTAAAG
CTCAGGAGTGCAGGAGGAAATCGCAGCTGCGCAGAGCAAAGTTTCAAGGTGTTGAAGCAGAAGCAGCGTGA

CACTGCGCAGCTGGCCTCTCTGTCTGCTCAGAGCGAACGGCGCGTGCAGGAAGTGGAGAGAAACGTGCAG
 AACATGAAGCAGCAGCAGGACCTTCTGCAGCGCCGCTGCGTGAAGAGAGTGCAGAGAAGAGACGGCTGG
 AGACCGAGATGCAGAAAGGAAAGCACAGAGTCAAGGAGCTGGAGATAAAGAACGAACAACAGCAGAAAAT
 TCTGAGGATTAAGACGGAGGAAATCGCAGCCTTCCAGCGGCAAAGGAGAAGTGGAAAGTAACGGCTCGGTC
 5 GTATCACTAGAGGAACAGCAGAAGATAGAGGAGCAGAAACGCTGGCTTGATGAGGAAATGGAGAAGGTTT
 TGGATCAACGACGTGGCCTTGAAGACCTGGAAGGAGAGCTGACCAAGAGGGAAGAGATCTTAGCCAAAA
 AGAAGCTTTACTCTGGGAGCGCAGTGGCCTGGAATCCAAAAAGCTTCGCTCAAGCCAGGCTCTTAGTCAA
 GACCTGTAACTTTGTGCGAGCCGGATTGAGTCTCTGGAACGGGAGCTCACTGAGAGGAACGGTCTTCTGC
 GGAGTGGCAGTGCTCAGGACTCCAGCAGATCAGACAGGAGATCTCCAACCTTCGGCAGGAGAAGGAGCT
 10 GCTGCTTAAACAACGAGTGGAGCTGGATGACAACTGAGACAGGGCAACTTGCTCTCTCTGAGGAAGAA
 CGAACACTTTTCCAGTTGGACGAGGCTATCGAGGCTTTGGATGCTGCTATTGAGTACAAAAACGAGGCGA
 TCAACCCAGAGACAGAGGCAGCTGCGGGCGTCTGGAAGCATGTTGACCCAATGGGAGATGAACCTGATGGC
 CAACTCACCTACCTGTCTGTCATCAGAAACAGAGCTCTGCTTTGCAAATACTTTGATAAGGTTGTGTCT
 CTTCTGTGAGGAGGAGCGCAGGCTGCAGATGGCTCTCGCTGAGCTGGAAGTGGAGTTGAGGAACAGCAGA
 15 ACTTGGTTCGGGTGGTTGGAGGCAGCTCTGGAGAGACAGCAGCTCGAGGCAGATCGCAGACTCACGCAGCA
 GCAGAAAGAGCACGAGAGGAACATTGAGTACTGCTGCAGCAGTGCAGAGAGCAGATGGATGAGGGTCTG
 GCGGGGAGACTGCGGCAGTACGAGGGCTTGATCCACAACCTCAGCAAAGAGCTGAATTTTTGCAAATAG
 CTAATCAGGAGCTGAACATCAAACTCAGGGAAATGTGTGGCCCTGTAAATCTCACTGGAGAACAATGCAA
 AGGTCTGAATTGTGACAGTCTGCTTTTAGCTGGAGCTCAGAGCAGAGTGGCTGAAGATGTAAAGCCTATC
 20 ATTGACGCAGAGAGAGTTTCAAGTCAAGAGAAGAGATGCGAGAGCCGTTAAATGCTCCTTTACCGGCCA

Delta-8 protein (8 aa after mutation) (SEQ ID NO: 6)

25 atgtctccaaaaggagtcggccacagtaaggtggaagaaagtgctgtacaggtggccgtc
 M S P K G V G H S K V E E S A V Q V A V
 cgcgtgcgtccgttgccttcccaaagagattttgcacagccatgagagctgcatcacttca
 R V R P L L P K E I L H S H E S C I T S
 gacccagaggagcggagggtcaccctgggcaatgaccgccacttccactgtgactttgtg
 30 D P E E R R V T L G N D R H F H C D F V
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 L P A

45

Alternative generation, selection and genotyping of *Kif7* mutant alleles

Plasmids encoding Zinc-finger nucleases (ZFN) specific for
 the zebrafish *Kif7* (set 3) gene were purchased from Sigma.
 50 Capped polyadenylated RNA from each plasmid was produced by in
 vitro transcription and a range of doses was injected into one-
 cell stage zebrafish embryos. Embryos injected with
 approximately 600 pg had around 30% rate of deformity at 24 hpf

(hours post-fertilisation). Genomic DNA prepared from non-deformed 24hpf embryos injected with this dose was used as a PCR template to analyse potential somatic mutations.

Roche Titanium 454 amplicon sequencing showed that 2.5% (6.5% is by 8 of 127 colony PCR and sequencing) of the amplicon molecules had insertions or deletions at the target site. G0 adults derived from embryos injected with ZFN capped RNA were in-crossed and their progenies (G1) individually genotyped by PCR using the forward primer (*Kif7* exF2: 5' CGAGGTGCTGAGTCTCTTAGAGT) (SEQ ID NO: 8) and reverse primer (*Kif7* exR1: 5' TGAATCCCTGTATGGGATATGGGT) (SEQ ID NO: 9) followed by Sanger sequencing.

Founders transmitting two different alleles (*Kif7* Δ 8, an 8-bp del: GGACCTGG, *Kif7* Δ 7, a 7-bp del: TTGTGGA) were selected and used to establish stable mutant lines. The full nucleic acid sequences of mutated alleles are shown in the accompanying file (Table 1).

***In situ* hybridization and immunofluorescence**

Standard *in situ* hybridization (ISH) was performed with anti-Dig alkaline phosphatase and chromogenic substrate NBT/BCIP as previously described (Oxtoby and Jowett, 1993). RNA probe used for *in situ* hybridization was synthesised from the full length template and sheared into 500 bp fragments with 60 mM Na₂CO₃/40mM NaHCO₃ (any long RNA probe generated by Ashish). RNA probes for other genes were prepared from templates as previously described: *ptc2* (formerly *ptc1*) (Barth and Wilson, 1995; Concordet et al., 1996), *nkx2.2* (Barth and Wilson, 1995; Concordet et al., 1996).

Whole-mount antibody staining was performed as previously described at the following dilutions: mAb 4D9 (anti-Engrailed; DHSB) at 1:50-1:200; rabbit anti-Prox1 (1:5000) (Elworthy et al., 2008); rabbit anti- γ -tubulin (1:500; Sigma); mouse anti- γ -tubulin (1:500; Sigma); mouse anti-acetylated α -tubulin (1:800; Sigma); rabbit anti-*Kif7* (1:1000). The secondary antibodies were: Alexa488-conjugated goat anti-mouse or rabbit, Alexa546-conjugated goat anti-mouse and Alexa568-conjugated goat anti-

rabbit, Alexa633-conjugated goat anti-secondary antibodies (1:1000, Invitrogen). Bright field microscopy images were acquired with an AxioCam HRc mounted on a Zeiss AXIO Imager M2, Olympus DP70 on MVX10 or Leica DFC300 FX mounted on MZ16FA. Fluorescent specimens were imaged using the 60X or 100x oil immersion objective on an Olympus Fluoview 1000 confocal microscope. Images were acquired using Olympus FV10-ASW software.

10 Synthetic RNA, DNA and morpholino for injection

The plasmids, pCS2-GFP-Kif7 (Tay et al., 2005), pCS2-GFP-Dzip1 (Kim et al., 2010) were linearized with NotI. Capped synthetic mRNAs were transcribed *in vitro* with SP6 mMessage mMachine Kit (Ambion) and injected into fertilized eggs. BAC Gli2a-GFP was used as previously reported (Kim et al., 2010).

SuFu morpholino (Wolff et.al., 2003)

Cyclopamine treatment of embryos

20 Cyclopamine treatment followed a standard method with immersion in 40 μ M cyclopamine (Toronto Research Chemicals) from 50% epiboly as previously described (Wolff et al., 2003).

Generation of antibodies

25 SuFu

Kif7: The peptide, L₁₂₃₁TGEQCKGLNCDSL₁₁LAGAQS₁RVAEDVKPIIDAERVQKSREEMREPVNAPLPATWRRSSLP TEDQYTMEELRQRAACELPNNRIVQPGMNSTHWSGSTSL₁₃₃₀ (SEQ ID NO: 10) was injected into the rabbit to generate one anti-Kif7 polyclonal antibody (Sdix, USA).

DZIP1: The peptide, V₇₀₈TTLSDSDWTGDGSEMEEINLSQLHKHTDQNGNLKNVTHSNVKALGKSLEKQLAARGPKKPAG GVNTFLEKPTDVRNTRQNAKKELKYSDDD₇₉₈ (SEQ ID NO: 11) was injected into rabbits to generate two anti-Dzip1 polyclonal antibodies (Sdix, USA).

Western blot analysis

5 Embryos were de-chorionated, deyolked, and homogenized manually in ice cold PBS without Ca^{2+} and Mg^{2+} in the presence of complete protease inhibitor cocktail (Roche). The embryo pellet was lysed in RIPA buffer (50mM Tris.HCl, pH8.0 /150mM NaCl /1%NP-40/0.5% Na.Deoxycholate /0.1%SDS/ protease inhibitor
10 cocktail/1mM PMSF). Samples were microcentrifuged for 10 min at 4°C, loading buffer (62.6 mM Tris HCl, pH 6.8; 2% SDS; 0.01% bromophenol blue; 10% glycerol; 100 mM DTT) was added to the supernatant and the equivalent of 30 embryos run on each lane of a 7.5% acrylamide denaturing gel at 30 mA for 120 mins, and
15 electroblotted onto Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore). PVDF strips were blocked in 5% milk powder PBS 0.1%Tween20 for 1 hr, and incubated with rabbit anti-zebrafish Gli2a (1:5000) (Maurya et al., 2011), rabbit anti-zebrafish Kif7 (1:5000), rabbit A anti-zebrafish Dzip1 (1:7500),
20 rabbit B anti-zebrafish Dzip1 (1:5000), mouse anti-Sufu (1:100) for 1 hr at room temperature. After washing, primary antibody was detected with ECL HRP-conjugated anti-rabbit IgG (1: 50,000) and anti-mouse IgG (1:50,000). Chemiluminescent Substrate was SuperSignal West Femto (Pierce). The loading amount of protein
25 extract among specimens was evaluated by gamma-Tubulin level with either rabbit or mouse anti-gamma Tubulin (1:5000; Sigma). Signal quantification was performed using Adobe Photoshop software.

Immunoprecipitation

30 Embryos were de-chorionated using pronase (2mg/ml, Sigma) and rinsed with egg water and then PBS with 2x protease inhibitor (PI; Roche). Embryos were de-yolked by yellow tip on ice and rinsed in ice-cold PBS/2x PI. The embryos were
35 centrifuged at 200xg for 5 mins. The pellet was stored at -80°C degree. For 1000x embryos at 18-somite stage, 1 ml of triton buffer (20mM Tris-HCl, pH8.0/137mM NaCl/10% Glycerol/1%Triton

X100/2mM EDTA)/2xPI/10mM PMSF is added to extract the proteins. Tubes were horizontally put on ice for 30 mins or longer. The lysates were spinned at 10,000xg for 15 mins. The suspension was transferred into a new 2 ml eppendorf. Forty μ l of lysate was
5 taken out as the input control. In 1ml of lysate, 150 μ l of magnetic Dyna-bead (Invitrogen) was added to pre-clear for 3 hours at 4°C degree. After removing the pre-clear beads, 10 μ g of rabbit primary Ab was added into lysate. The affinity binding between rAb and the specific protein was facilitated by shaking
10 at 4°C degree overnight. The 120 μ l of dynabead protein A was added in 1 ml of lysate and incubated at 4°C degree for no more than 1 hour to pull down the complex of rAb-target protein. The beads were washed with 1.5ml of lysis buffer/2xPI per tube 4 to 6 times. During the last time of washing, the beads were
15 transferred to a new tube. Elution was performed by adding 40 μ l of 2x SDS NuPage LDS/100 mM DTT (Invitrogen) per tube and by heating at 95°C for 5 mins.

**Example 1 - Kif7 controls the response of cells to Hh in the
20 zebrafish by regulating Gli2 trafficking, processing and
interaction with SuFu**

Using zinc finger nuclease (ZFN) mediated targeted mutagenesis (Doyon et al., 2008; Foley et al., 2009; Meng et
25 al., 2008), stable germ line transmissible mutant alleles of the zebrafish *Kif7* gene was generated. Three fish were identified that transmit deletion mutations; two of these alleles cause frame-shifts resulting in premature termination codons that are predicted to yield proteins truncated in the motor domain of the
30 *Kif7* protein, similar to those found in human Joubert's syndrome patients (Figure 7). Surprisingly, animals homozygous or trans-heterozygous for these mutant alleles completed embryogenesis and showed no obvious manifestations of aberrant Hh pathway activity at 24 hpf; in particular, the specification of slow
35 twitch muscle fibres, a sensitive read-out of Hh activity in the zebrafish embryo, appeared normal. By 2.5 dpf, however, some mutant larvae displayed ectopic expression of the *eng2a:eGFP*

reporter gene in the myotome (Figure 7). Nevertheless, the homozygous and trans-heterozygous fish were fully viable and grew into phenotypically normal adults. This situation contrasts markedly with the peri-natal lethality of mice homozygous for *Kif7* loss of function alleles, but mirrors the finding that some Joubert syndrome patients are homozygous for mutated *KIF7* alleles.

The lack of significant disruption of Hh pathway activity in the absence of zygotic *Kif7* function may be due to maternally derived *Kif7* mRNA present in newly fertilized eggs. Consistent with this, *Kif7* protein was readily detectable in the primary cilia of *Kif7* homozygous embryos at 24hpf. To test this inference further, *Kif7* homozygous females were crossed with *Kif7* homozygous males. The resulting MZ (maternal + zygotic) *Kif7* mutant embryos showed no detectable *Kif7* protein in their primary cilia and exhibited a dramatic up-regulation of the *eng2a:eGFP* reporter gene in the myotome at 30 hpf (Figure 8). In contrast to the effects of full activation of the Hh pathway that causes a complete switch of muscle progenitors to the slow twitch fibre lineage (Wolff et al., 2003), the expansion of *eng2a:eGFP* expression was largely restricted to fast-twitch muscle fibres (Figure 8), indicative of an increased specification of medial fast fibres that are normally induced in response to Hh activity after the initial Hh-dependent specification of slow twitch fibre type has occurred (Wolff et al 2003). It follows that Hh pathway activity can be partially restrained in muscle progenitors, even in the complete absence of *Kif7* activity. In line with these findings, transcription of *ptc2*, a direct target of the Hh pathway, was ectopically expressed throughout the fast-twitch muscle domain of the somites at 24hpf (Figure 8).

Consistent with *Kif7* acting downstream of Smo to negatively regulate transcriptional activation of Hh targets by the Gli transcription factors, genetic removal of Smo activity from MZ*Kif7* embryos or their treatment with the Smo antagonist cyclopamine had little effect on the ectopic expression of *ptc2* or *eng2a:eGFP* (data not shown and Figure 7); in both cases,

however, there was a reduction/loss of MPs, indicating that removal of *Kif7* is not sufficient to fully activate the pathway in the absence *Smo* (Figure 9). Notably, the ectopic activation of *eng2a:eGFP* in *MZKif7* was completely suppressed by heterozygosity for the semi-dominant *yot* allele that encodes a truncated repressor form of the *Gli2a* protein; this demonstrates that loss of *Kif7* causes a shift in the balance between the repressor and activator forms of the *Gli* proteins.

To investigate this more directly, *Gli2a* processing in wild type was compared to *MZKif7* mutant embryos by Western blot analysis. A previous study has shown that in 28hpf zebrafish embryos, the ratio of full length (FL) to the truncated repressor (R) form of *Gli2a* is approximately 1:1 (Ben et al 2011). Consistent with the processing of *Gli2a* being regulated by Hh signaling, this ratio is markedly increased in *ptc1;ptc2* double mutant embryos and substantially decreased in embryos treated with cyclopamine (Ben et al, 2011). In *MZKif7* embryos, the *Gli2a* FL:R ratio was found to be similarly perturbed, though much less markedly than in *ptc1;ptc2* double mutants. Interestingly, a shift in mobility of the full-length form of *Gli2a* both in *MZKif7* and wild-type embryos between the 21hpf and 26 somites stages (Figure 9) was observed. These findings show that loss of *Kif7* causes a subtle imbalance in the levels of the activator (or full-length) and repressor forms of *Gli2a*, an effect consistent with the partial de-repression of pathway activity seen in the myotome of *MZKif7* embryos.

Kif7 activity has also been implicated in regulating the dissociation of *Gli* proteins from *SuFu*, a key step in their maximal activation. *Gli2a* was immunoprecipitated from wild-type and *MZKif7* mutant embryos. Western blot was subsequently used to analyse *Gli2a* interaction with *SuFu* and *Kif7*. As expected, both *SuFu* and *Kif7* proteins co-precipitates with *Gli2a* from wild type embryos, whereas only *SuFu* could be detected in the immunoprecipitates from *MZKif7* embryos. Notably, the relative levels of *SuFu* were significantly increased in the *MZKif7* embryos, consistent with decreased dissociation of the two proteins in the absence of *Kif7*.

In the present disclosure, the GFP-Gli2a fusion protein was found to localise at the tip of the primary cilium in wild type embryos in response to Hh activity (Kim et al 2010). It was also consistently restricted to the base of primary cilia in
5 MZKif7 mutant embryos.

Taken together these data demonstrates a dual role for Kif7, both to promote the processing of Gli2a to its repressor form and also to promote the activation of full length Gli2a through its transport to the tip of primary cilium and
10 dissociation from the SuFu protein. In this view, the effect of the modest increase in FL-Gli2a relative to the Repressor form is partly mitigated by the attenuation of its dissociation from SuFu. To test this, an antisense morpholino mediated oligonucleotides (which caused an 80% decrease in SuFu levels as
15 judged by Western blot analysis) was used to knock-down SuFu activity in MZKif7 mutant embryos. Such embryos showed a dramatic increase in the number of slow twitch fibres in the myotome, indicative of significant enhancement of pathway activation, a much stronger effect than seen in the absence of
20 either SuFu or Kif7 alone. Gli2a is largely dispensable for muscle development in the zebrafish myotome, its absence being compensated for by Gli1 activity (Wolff et al. 2003). Thus any reduction in Gli2a activation caused by loss of Kif7 would be masked by this compensatory function of Gli1. To confirm this,
25 the detour (dtr) mutation was used to remove Gli1 activity from MZKif7 mutant embryos. Homozygous dtr embryos show normal specification of Hh dependent muscle cell types (Wolff et al. 2003); however, removal of Gli1 activity caused a complete suppression of ectopic Eng expression in MZKif7 embryos as well
30 as the loss of MPs, which require the highest level of Hh pathway activity for their specification (Figure 9). This finding demonstrates that Kif7 is required for full activation of the Gli2a protein.

35 **Example 2 - Distinct effects of Kif7 loss in the ectoderm vs. the mesoderm**

Shh plays a major role in patterning the neural tube and the de-repression of the pathway caused by *ptc* mutations results in the ectopic expression of Hh target genes both in mouse and fish embryos (Figure 9). In mouse embryos homozygous for loss of function *Kif7* alleles, there is a modest expansion of the expression domains of patched 1 and of the ventral neural tube markers *Nkx2.2* and *olig2*, effects that are consistent with a partial de-repression of the Hh pathway. In zebrafish *MZKif7* embryos, by contrast, no changes in the neural tube expression domains of either *nkx2.2a* or *olig2* could be detected. Notably, however, *olig2* expression was found to be partially uncoupled from its dependence on Smo activity by complete elimination of *Kif7* activity. In addition, the domain of *ptc2* expression is found to be slightly expanded in the neural tube of *MZKif7* embryos. Taken together, these findings demonstrates a subtle role for *Kif7* in modulating Hh pathway activity in the neural tube in zebrafish. Even the simultaneous removal of SuFu function from *MZKif7* embryos had no effect on the expression domains of either *nkx2.2a* or *olig2* in the spinal cord, although, ectopic expression of *nkx2.2a* was consistently seen in the hindbrain (Figure 11). This contrasts with the dramatic ventralisation of the neural tube seen in mouse SuFu loss of function mutants.

Loss of *Kif7* activity results in increase in length of primary cilia independent of Hh pathway activity

MEFs derived from Joubert Syndrome patients display longer primary cilia. What remains unclear is if this is due to a direct involvement of *Kif7* in ciliary biogenesis or an indirect role mediated by Hh signaling or through its control of PKA activity. To analyze the role of *Kif7* in cilia formation further, *MZKif7* embryos were stained with acetylated tubulin. *MZKif7* embryos were found to have a significantly longer cilia length as compared to wild type embryos. It has previously been shown that upregulation of Hh signaling by removing both *ptc1* and *ptc2* results in longer primary cilia. However, *Kif7* mutants

as disclosed herein show primary cilia that are even longer than in *ptc1*, *ptc2* double mutants (Figure 12). To test whether longer primary cilia in MZKif7 are a result of ectopic Hh signaling, cilia length in *yot*, MZKif7 double mutant embryos was assayed.

5 These mutants show a complete suppression of Hh target genes, similar to that seen in *yot*^{-/-} embryos. The double mutants were found to display longer cilia, similar to MZKif7 mutants, whereas *yot*^{-/-} and wild type embryos have shorter primary cilia. Similar effects of *Kif7* on cilia length in MZKif7, *dtr* double
10 mutants were also observed (data not shown). This demonstrates that *Kif7* controls primary cilia length independent of its effects on Hh signaling. In addition MZKif7 embryos were also found to display larger basal bodies as compared to wild type, when assayed with anti γ -tubulin antibodies.

15

Applications

The present invention may be used to screen for novel
5 inhibitors of the Hh pathway acting downstream of Smoothened.

Advantageously, the method of screening is robust, non-quantitative, cheap and based on an *in vivo* assay.

10 Advantageously, a large number of homozygous *Kif7* mutant fish embryos can be easily produced, allowing very rapid visual screening of thousands of compounds in a matter of weeks.

It will be apparent that various other modifications and
15 adaptations of the invention will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention and it is intended that all such modifications and adaptations come within the scope of the appended claims.

20

Claims

1. A fish from the family of Cyprinidae comprising an inhibited *Kif7* gene and wherein the fish is homozygous for the inhibited *Kif7* gene.

5

2. The fish of claim 1, further comprising a reporter gene.

10

3. The fish of claim 1 or 2, wherein the fish is of the Danioninae sub-family.

15

4. The fish of claim 3, wherein the fish is of the *Danio* or *Devario* genus or which is selected from any one of the following species: *Danio rerio* (Zebrafish), *Danio aesculapii*, *Danio albolineatus*, *Danio choprae*, *Danio dangila*, *Danio erythromicron*, *Danio feegradei*, *Danio jaintianensis*, *Danio kerri*, *Danio kyathit*, *Danio margaritatus*, *Danio meghalayensis*, *Danio muongthanhensis*, *Danio nigrofasciatus*, *Danio quagga*, *Danio quangbinhensis*, *Danio roseus*, *Danio tinwini* and *Danio trangi*.

20

5. A zebrafish comprising an inactivated *Kif7* gene and a reporter gene.

25

6. The fish of any one of the preceding claims, wherein the fish is an embryo, or a larva or adult.

7. The fish of any of the preceding claims wherein the *Kif7* gene is inhibited completely or partially.

30

8. The fish of claim 7, wherein the *Kif7* gene is inhibited temporarily or permanently.

35

9. The fish of claim 8, wherein the *Kif7* gene is inhibited temporarily using one or more of the following: short interfering nucleic acid (siNA), short interfering

RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA) and morpholino oligonucleotides.

5 10. The fish of claim 8 wherein the *Kif7* gene is inactivated permanently by introducing one or more mutations into the *Kif7* gene or any sequence controlling expression of the *Kif7* gene which results in a loss of function or lack of expression of the *Kif7* gene.

10

11. The fish of claim 10, wherein the mutations are introduced by one or more of the following methods: site-directed mutagenesis, insertional mutagenesis, PCR site-directed mutagenesis, plasmid mutagenesis, cassette
15 mutagenesis and zinc finger nuclease-induced mutagenesis.

12. The fish of claim 11, wherein both copies of the *Kif7* gene are inactivated.

20

13. The fish of any of claims 2 to 12, wherein the reporter gene encodes a visually identifiable protein.

25

14. The fish of claim 13, wherein the protein is a fluorescent protein, luminescent protein, or beta-galactosidase.

30

15. The fish of claim 14, wherein the fluorescent protein is green fluorescent protein, red fluorescent protein or reef coral fluorescent protein.

35

16. The fish of any one of claims 2 to 15, wherein the reporter gene is operatively linked to a regulatory region of one or more genes of the Hedgehog pathway.

17. The fish of claim 16, wherein the gene of the Hedgehog pathway is the *Eng2a* gene and/or the *slow myosin heavy chain 1* gene.

18. The fish of claim 16 or 17, wherein the regulatory region is a cis-regulatory region.

5 19. A *Danio rerio* comprising two inactivated copies of the *Kif7* gene and a reporter gene, wherein the *Kif7* gene is inactivated using zinc finger nuclease-induced mutagenesis and the reporter gene encodes green fluorescent protein.

10 20. A method of identifying a compound capable of inhibiting the Hedgehog cell signalling pathway comprising the following steps:

15 (a) contacting the compound with a fish of any one of the preceding claims and measuring the expression level of the reporter gene;

(b) contacting a control compound with a control group of the fish and measuring the expression level of the reporter gene;

20 (c) comparing the reporter gene expression levels obtained from steps (a) and (b);

25 wherein a decreased expression of the report gene relative to the control group indicates that the compound is capable of inhibiting the Hedgehog cell signalling pathway.

30 21. The method of claim 20, wherein the fish is contacted with the compound by injecting the compound into the fish.

22. The method of claim 21, wherein the compound is injected into the fish in conjunction with a carrier.

35 23. The method of claim 22, wherein the carrier is a solvent, lipid or peptide.

24. The method of any one of claims 20 to 23, wherein the method refers to identifying a compound capable of inhibiting the Hedgehog cell signaling pathway downstream of *Kif7* protein.

5

25. The method of any one of claims 20 to 24, wherein the expression level of the reporter gene is measured by light or fluorescent microscopy.

10

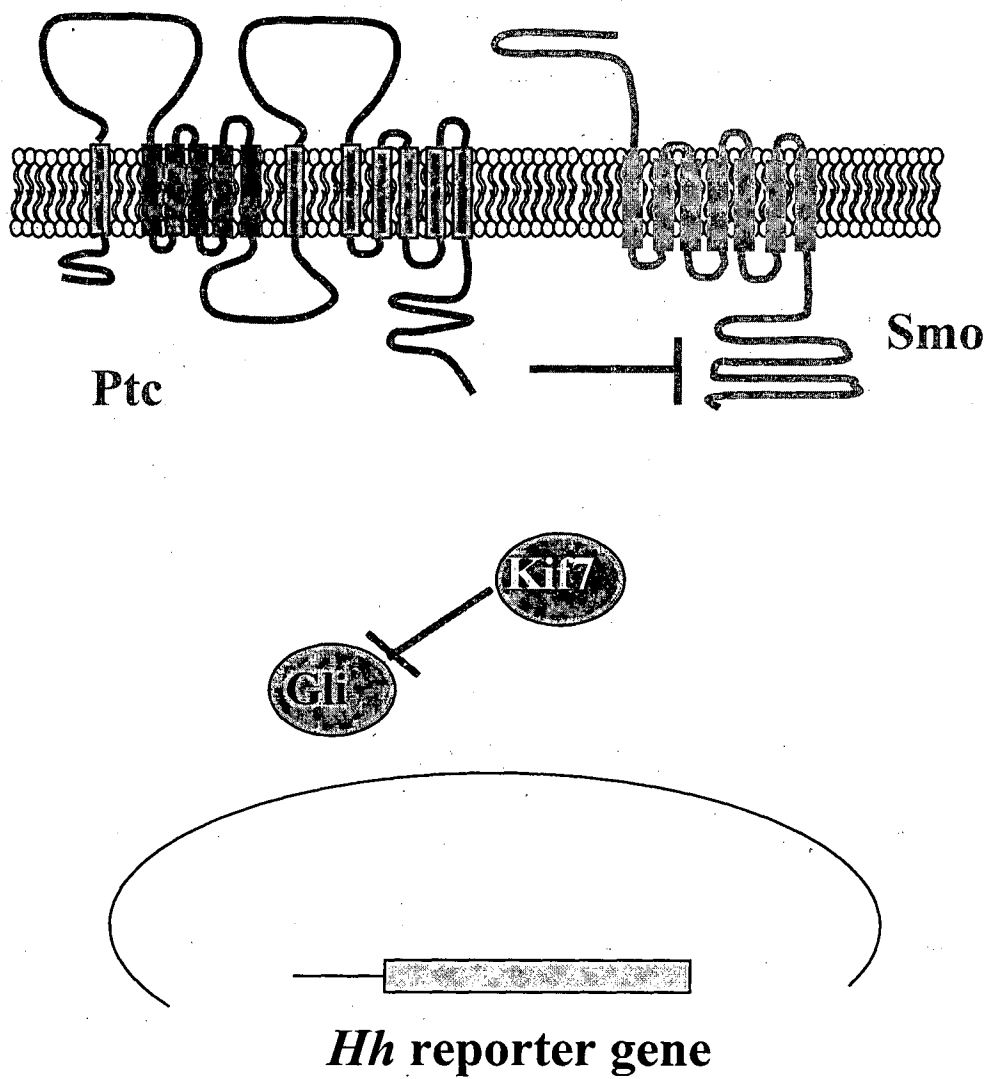


Figure 1

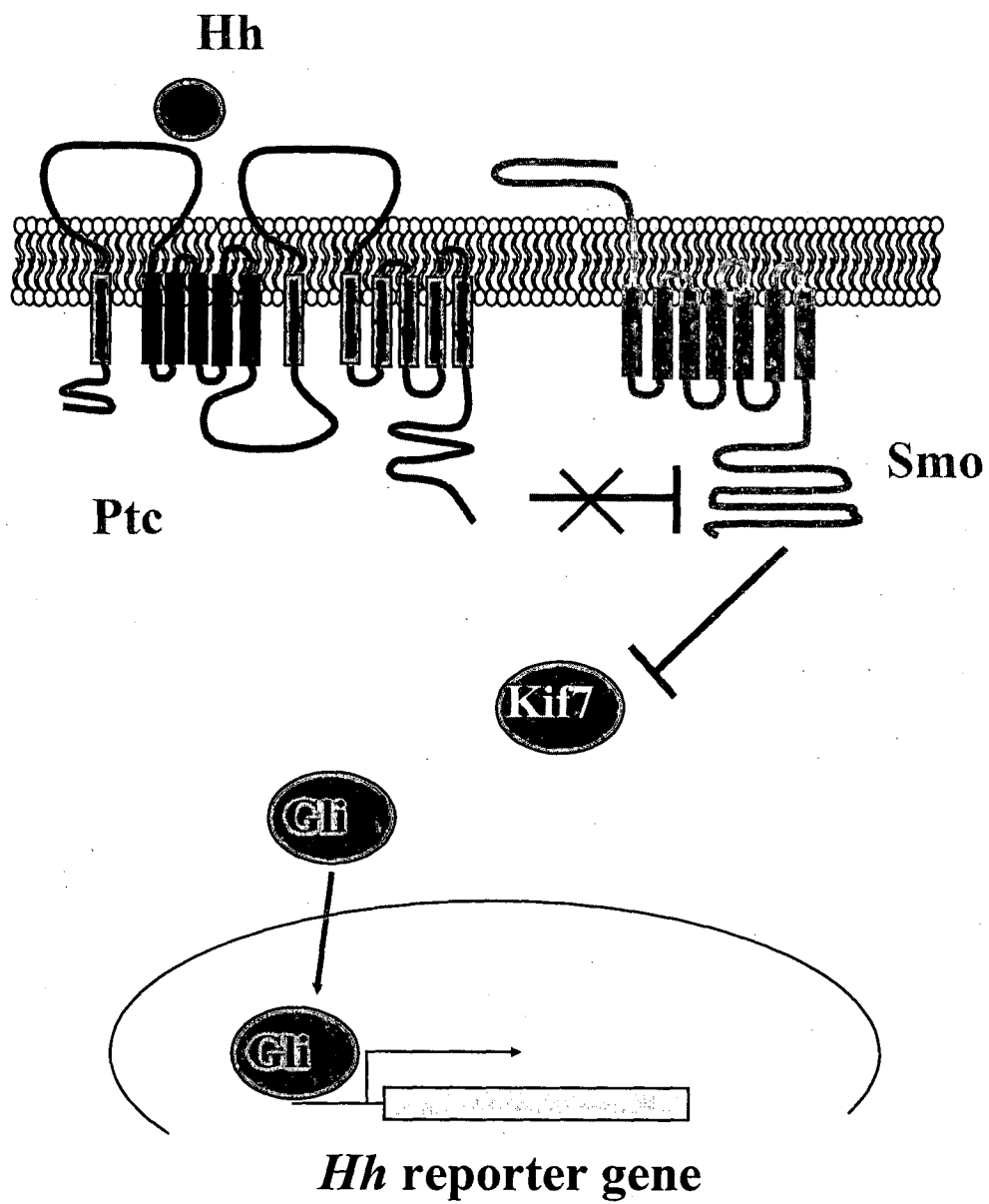


Figure 2

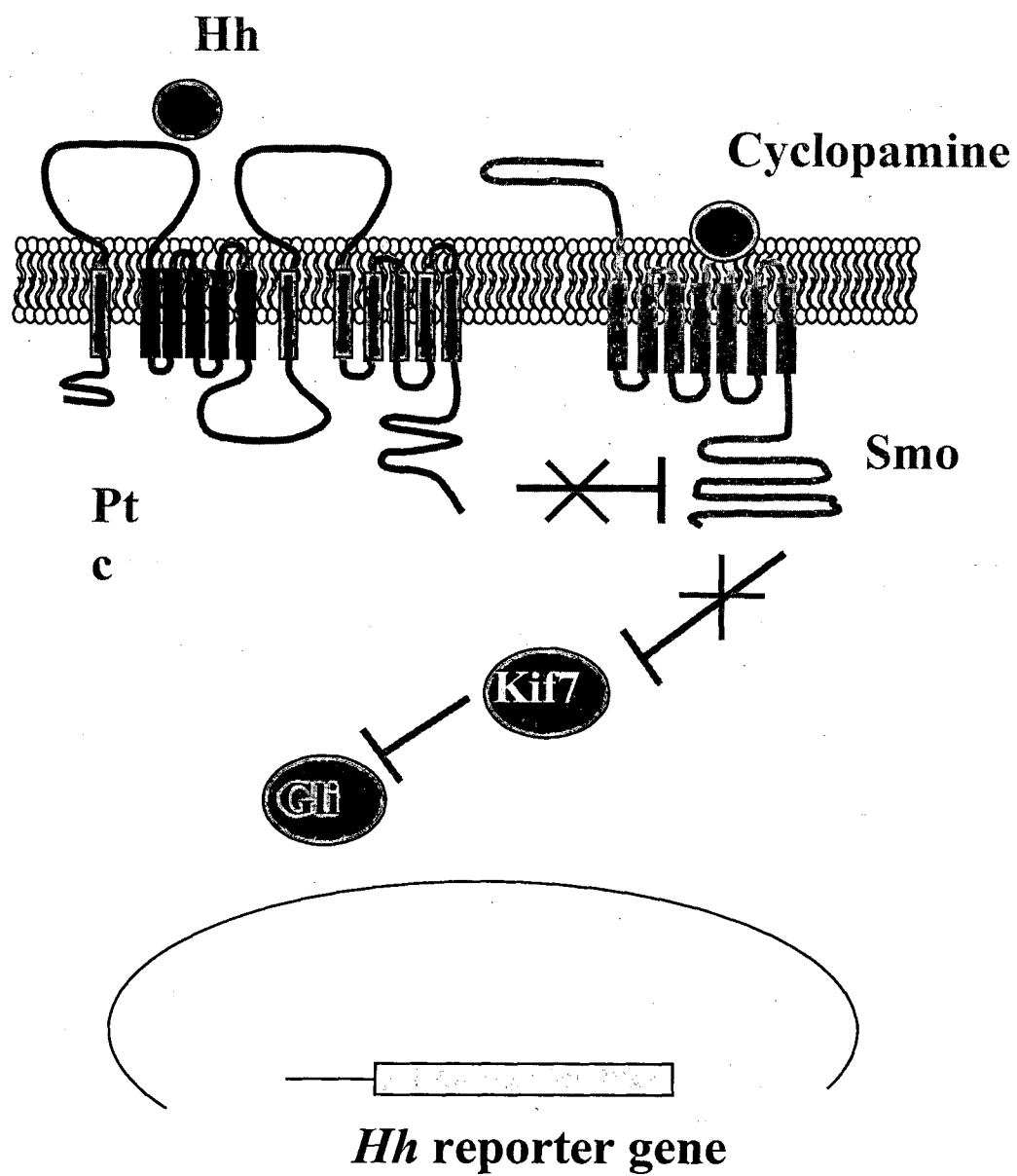


Figure 3

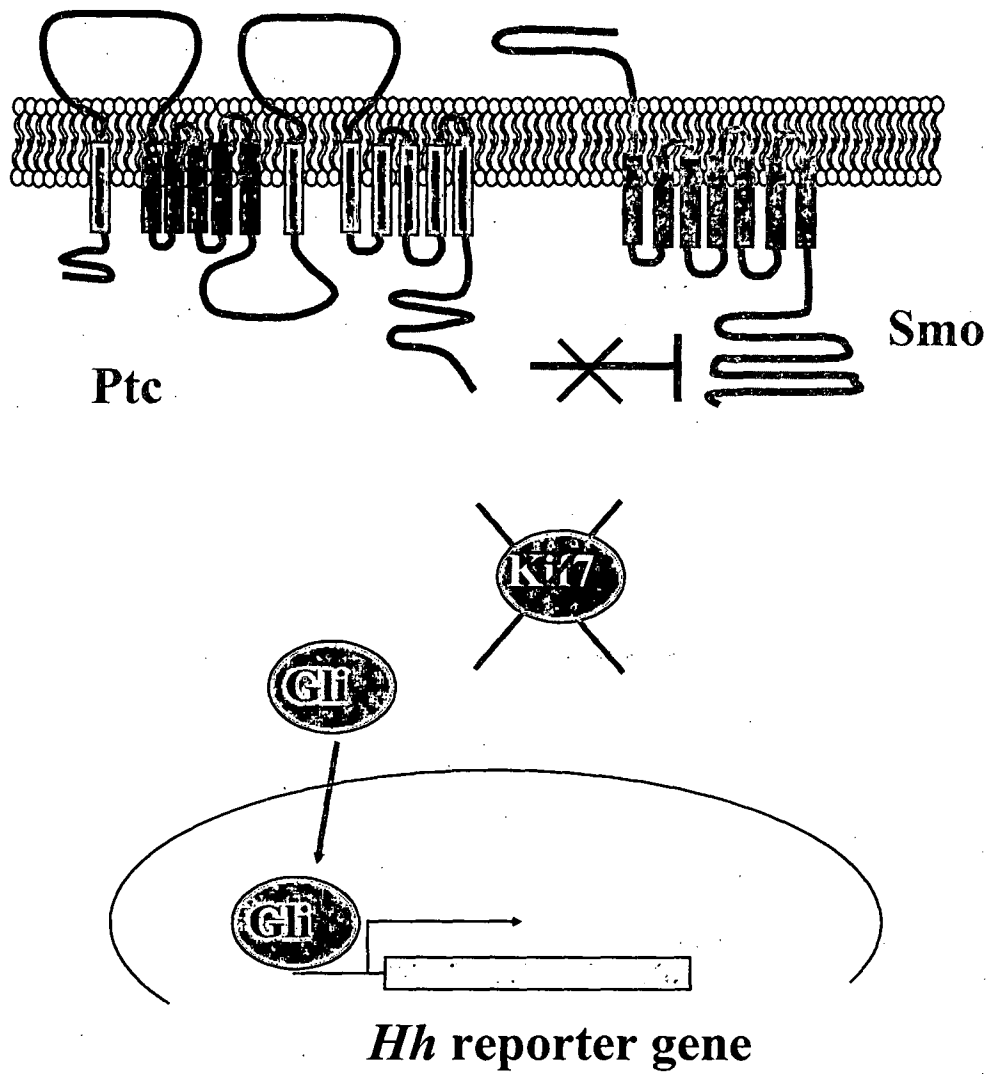


Figure 4

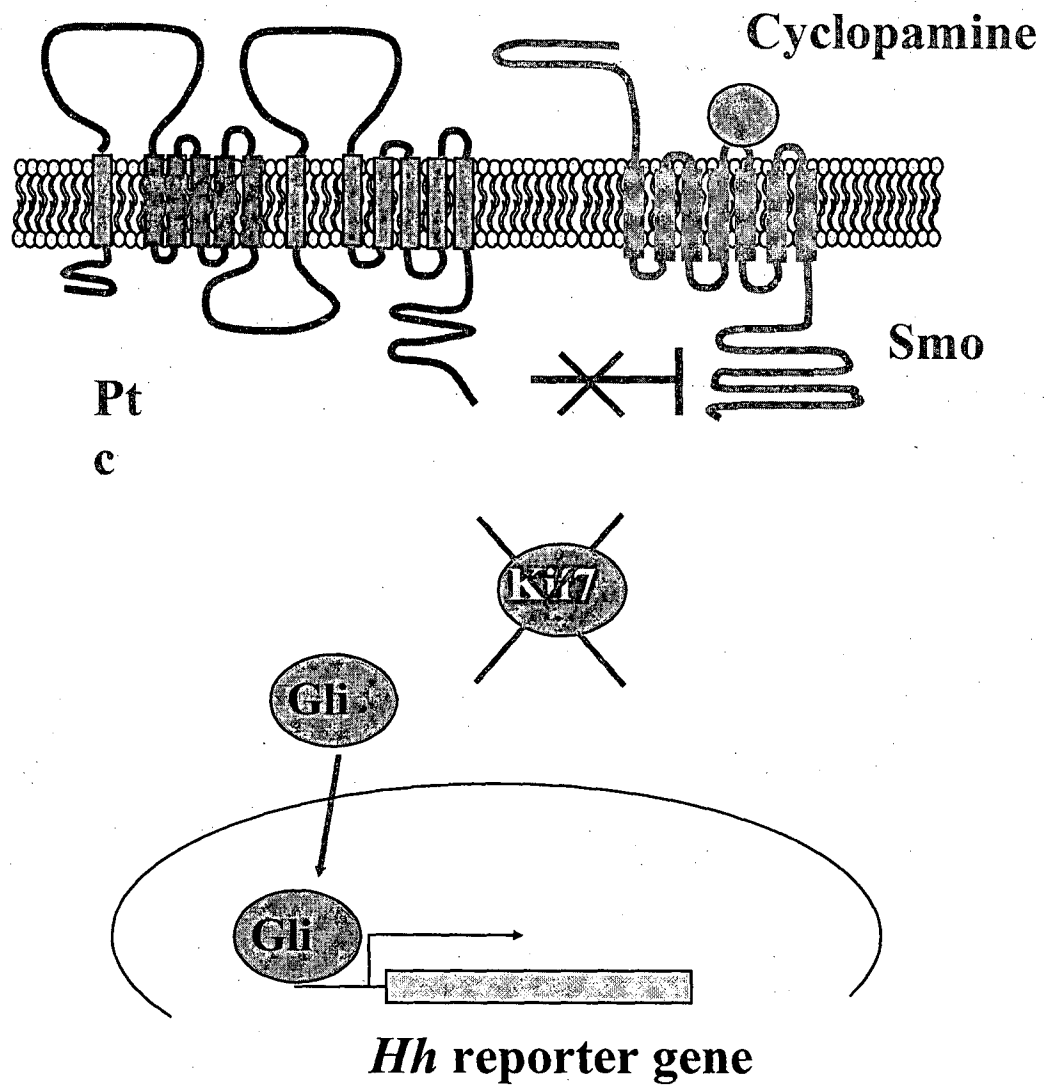


Figure 5

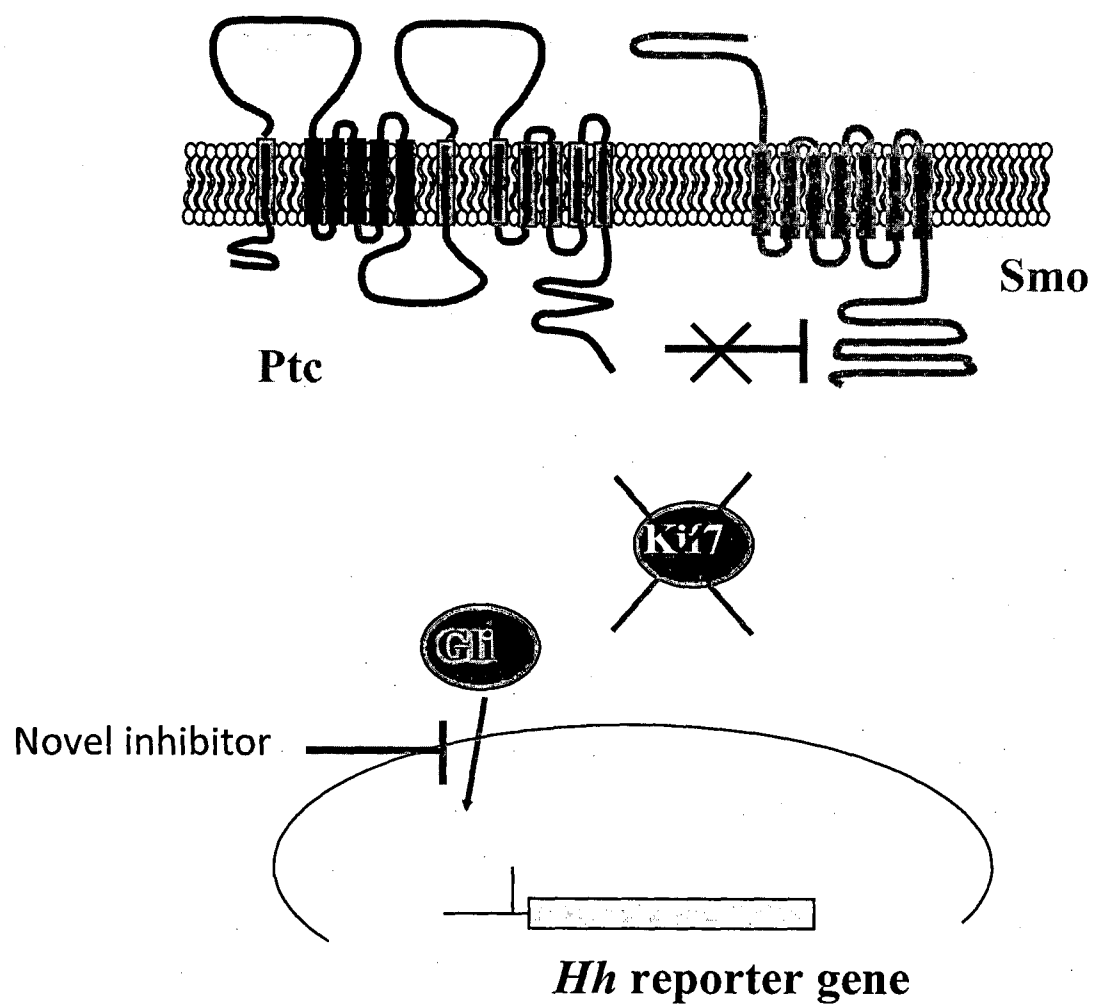


Figure 6

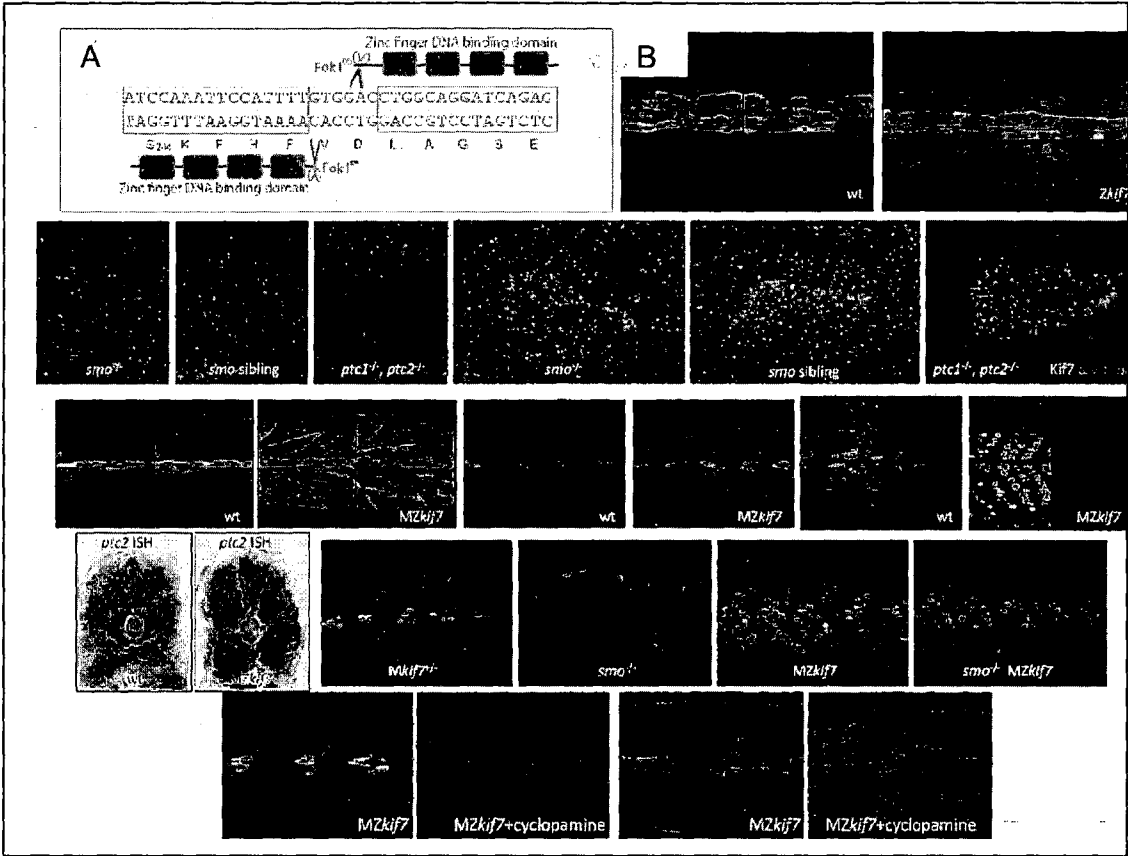


Figure 7

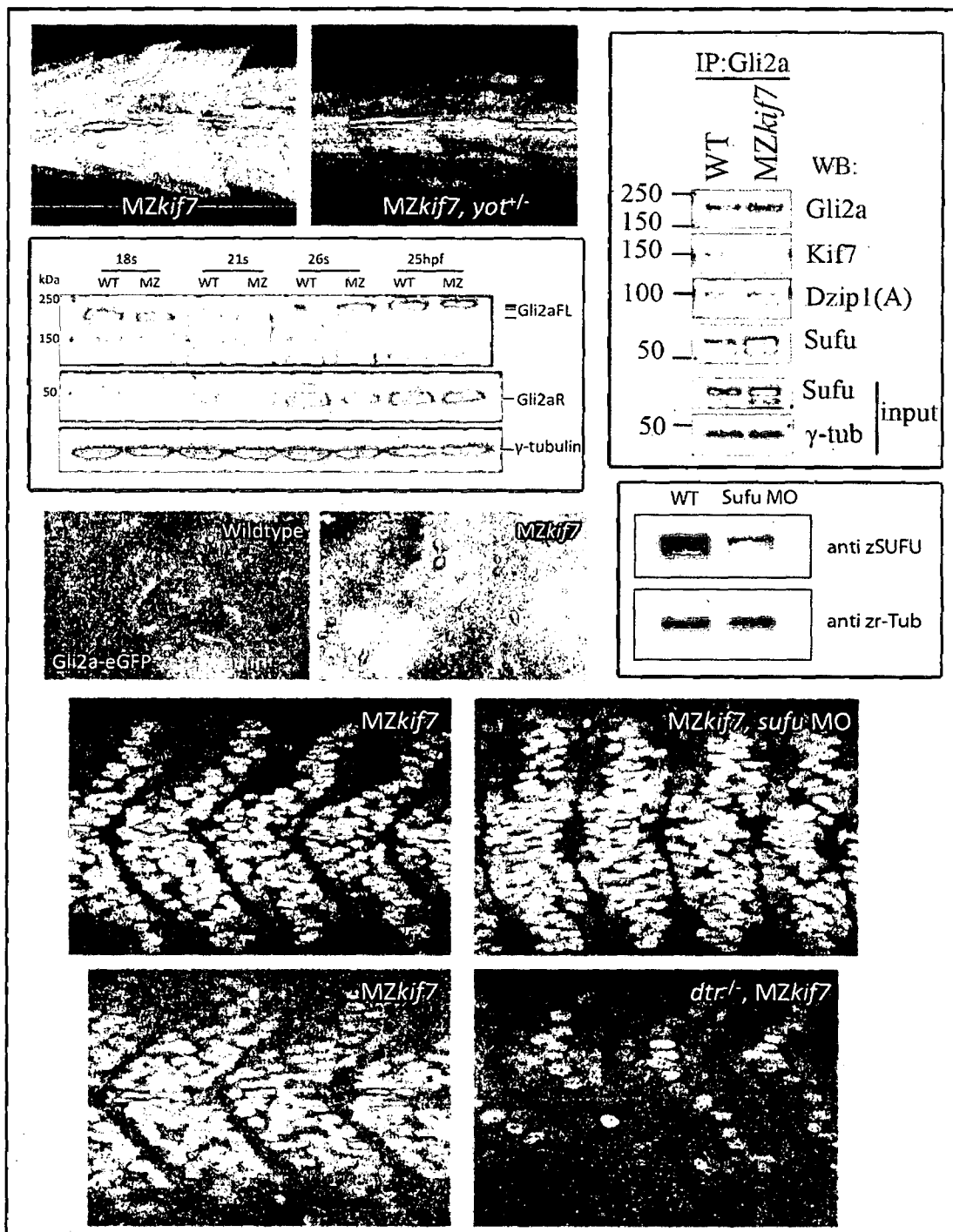


Figure 8

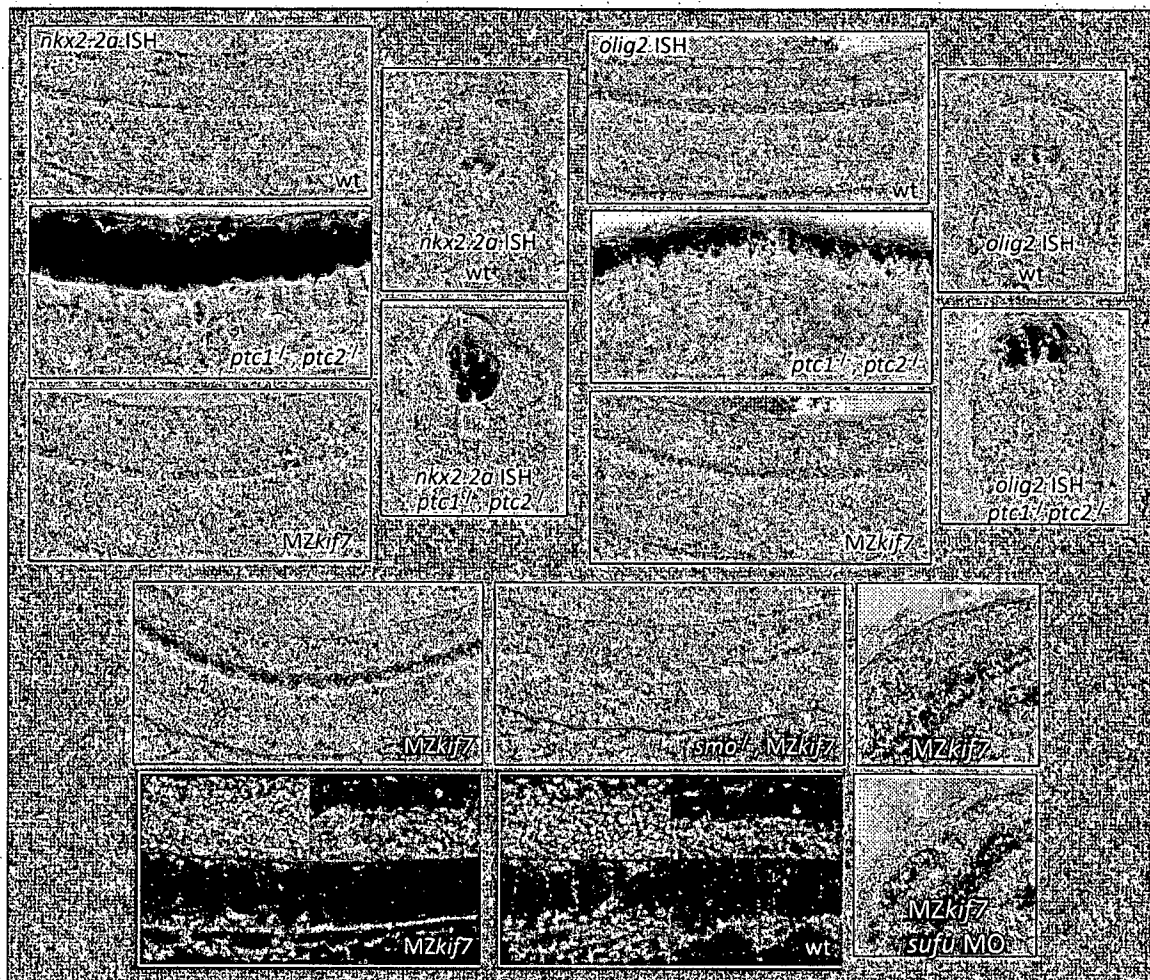
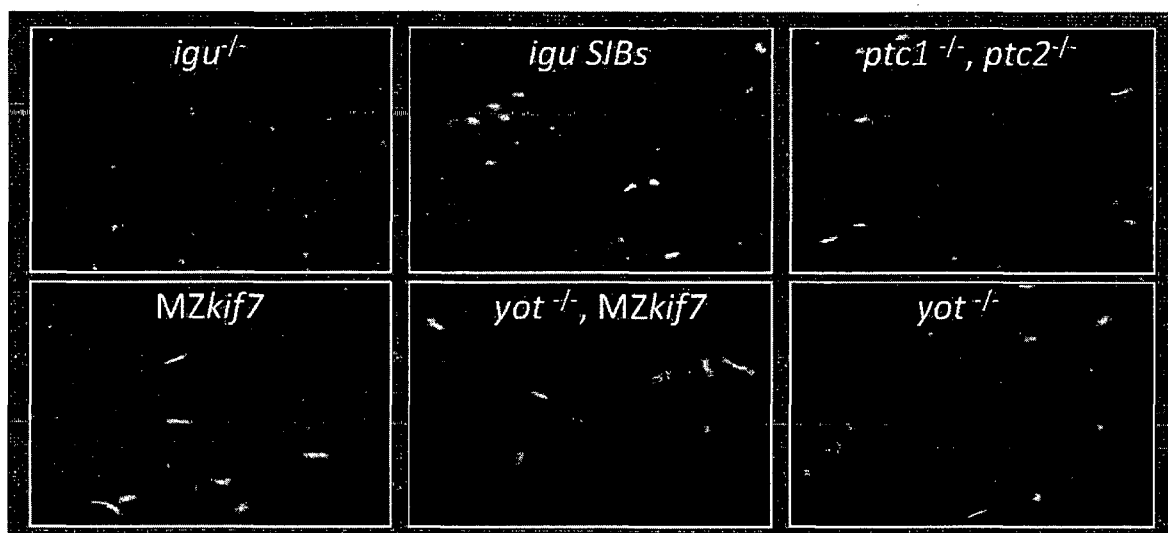


Figure 9

**Figure 10**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2012/000434**A. CLASSIFICATION OF SUBJECT MATTER****A01K 67/027 (2006.01) C12Q 1/68 (2006.01)**

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)

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)

WPI, EPODOC, MEDLINE, HCA, BIOSIS, EMBASE: zebrafish, cyprinidae, oryzias, danio, adrianchthyidae, minnow, carp, tinca, rutilus, danio, davario, brachydanio, oryzias, kif7, kinesin family member 7, cos2, costal 2 and like terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

☒ Further documents are listed in the continuation of Box C
 ☐ See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

 Date of the actual completion of the international search
 13 December 2012

 Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2012/000434
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAY, S.Y. et al. 'A homologue of the Drosophila kinesin-like protein Costal2 regulates Hedgehog signal transduction in the vertebrate embryo' Development, 2005, vol. 132, pages 625-634 page 626, page 629, column 1, paragraph 2, figures 4 and 5, page 630, column 1, fig. 4	1-19
X	WILSON, C.W. et al. 'Fused has evolved divergent roles in vertebrate Hedgehog signalling and motile ciliogenesis' Nature, 2009, vol. 459, pages 98-102 & Online supplemental methods retrieved from the internet on 12 December 2012 <URL: http://www.nature.com/nature/journal/v459/n7243/full/nature07883.html > Methods column 1, paragraph 3, fig. 2	1, 3, 4 and 7-9
X	PUTOUX, A. et al. 'KIF7 mutations cause fetal hydroletharus and acrocallosal syndromes' Nature Genetics, June 2011, vol. 43, pages 601-606 & Online supplemental methods retrieved from the internet on 12 December 2012 <URL: http://www.nature.com/ng/journal/v43/n6/full/ng.826.html > Online methods column 1, paragraph 6, fig. 2	1, 3, 4 and 7-9
A	BILL, B.R. et al. 'A Primer for Morpholino Use in Zebrafish' Zebrafish, 2009, vol. 6, pages 69-77 page 76, column 1	10-12 and 19