Supporting Information

Hyman et al. 10.1073/pnas.0907134106

SI Materials and Methods

Shh-LIGHT2 Assay for Hh Pathway Activation and Library Screening Conditions. Shh-N-conditioned medium was prepared as previously described (1). Shh-LIGHT2 cells (2), an NIH 3T3-derived line stably transfected with Gli-dependent firefly luciferase (8×GliBS-FL; ref. 3) and constitutive *Renilla* luciferase (pRLTK, Promega) reporters, were cultured in DMEM (Invitrogen) containing 10% calf serum (CS, HyClone), 400 μg/mL geneticin, 200 μg/mL zeocin, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. For 96-well plate assays, Shh-LIGHT2 cells were seeded into each plate (10,000 cells/well) and cultured to confluency. The Shh-LIGHT2 cells were then grown in DMEM containing 0.5% CS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, either 5% Shh-N-conditioned medium or 500 nM SAG, and various concentrations of the HPIs, cyclopamine, forskolin, or GANT-61. After the cells were cultured for another 30 h, the resulting firefly and *Renilla* luciferase activities were measured using a Dual Luciferase Reporter kit (Promega) and a Veritas microplate luminometer (Turner Biosystems).

To screen 122,755 compounds from the Stanford High-Throughput Bioscience Center collection, the Shh-LIGHT2 assay was modified as follows. Shh-LIGHT2 cells were cultured in ten 10-cm tissue culture dishes using DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. When the cells reached >90% confluency, they were removed by trypsinization and diluted to a final volume of 50 mL culture medium. This cell suspension was then plated into clear-bottom, white-walled 384-well plates using a Titertek Multidrop dispenser (50 μL/well) and the cells were cultured until they reached confluency (3–4 days). The culture medium was then manually removed from each plate, and DMEM containing 0.5% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin was added using the Multidrop dispenser (40 μL/well). The plates were then placed into an automated Staccato system (Caliper Life Sciences), which includes an automated CO_2 incubator. An automated protocol was then run, in which a Multidrop dispenser added 10 μL of a SAG solution in 0.5% CS medium (final SAG concentration of 500 nM) to columns 1–22 and 100 nL of each compound solution was added to the plates using a V&P Scientific pin tool, resulting in a final compound screening concentration of 10 μM. The SAG-free wells were used as negative controls. After 28–32 h, the assay medium was manually removed and the plates were frozen. To assay the firefly luciferase activities, the plates were thawed and placed into a Twister II stacker in the Staccato system. Bright-Glo luciferase substrate (Promega) was added by the Multidrop dispenser and the plates were analyzed on an Analyst GT microplate reader (2 min in the dark, 0.2 s integration/well; Molecular Devices). Hits were defined as compounds that reduce the firefly luciferase signal by at least 50% compared to inhibitor-free controls.

Primary hits were then re-tested in duplicate in an eight-point, 1:1 serially diluted dose-response curve with a starting concentration of 20 μM. In these experiments, the median inhibitory concentration (IC_{50}) for both firefly and *Renilla* luciferase were determined using a Dual-Glo kit (Promega). Compounds that exhibited IC_{50}s <10 μM and did not inhibit *Renilla* activity were selected for further study, including HPI-1 through HPI-4.

HPI Acquisition and Synthesis. Upon the completion of the primary screen and dose-response studies, additional quantities of HPI-1 through HPI-4 were purchased from ChemDiv, Specs, Chembridge, or Abminter and their chemical compositions were verified by mass spectrometry. HPI-1 and HPI-2 were also re-synthesized through the following procedures.

General Synthetic Procedures. All chemicals used for organic synthesis were purchased from Sigma-Aldrich, Acros, or TCI and used without further purification. Anhydrous conditions were maintained under N_2 using standard Schlenk line techniques and oven-dried glassware. Compounds were purified by flash chromatography using SiO_2 (EM Science) as the stationary phase. ^1H and ^{13}C NMR spectra were taken on Varian Inova 400 and 500 MHz spectrometers in $CDCl_3$, and chemical shifts are reported as parts per million (ppm) downfield of the internal control trimethylsilane (TMS). High-resolution mass spectrometry (HRMS) data were obtained on a Micromass Q-TOF hybrid quadrupole liquid chromatography-mass spectrometer at the Stanford University Mass Spectrometry Facility. Purity of final compounds was assessed using a Waters 2795 HPLC system equipped with a dual wavelength UV detector, a reverse-phase (C_{18}) 2.1 × 30 mm Agilent Zorbax HPLC column containing a 3.5-μm Stablebond stationary phase, and a mobile phase of water and acetonitrile, each containing 0.1% formic acid.

E)-4-(2-methoxyphenyl)but-3-en-2-one (1). To a solution of *o*-anisaldehyde (3.00 g, 22.0 mmol) in acetone and water was added 2.0 mL of 50% NaOH, and the resulting mixture was allowed to stir for 3 days at room temperature. The reaction mixture was then extracted twice with dichloromethane, and the organic layers were pooled, dried with $MgSO_4$, and concentrated in vacuo to yield **1** as a white solid (4.12 g, 100%). Compound **1** was carried on to the next reaction without further purification.

5-(2-methoxyphenyl)cyclohexane-1,3-dione (2). To a solution of sodium ethoxide (0.53 g Na dissolved in 1.37 mL of ethanol, 23.4 mmol) in 15 mL anhydrous ethanol was added diethyl malonate (3.60 mL, 23.4 mmol) followed by **1** (4.12 g, 23.4 mmol). The reaction mixture was refluxed for 16 h, after which it was cooled and extracted with chloroform/water. The aqueous layer was collected and distilled, leaving a residue, which was re-dissolved in 15 mL of 2 N NaOH and refluxed for 4 h. After the solution was cooled to room temperature, 15 mL of 5 N H_2SO_4 was added and the mixture was refluxed for an additional 2 h. Cooling of the reaction mixture then yielded **2** as a pale yellow precipitate, which was isolated by filtration and washed with water (5.00 g, 100%)

HPI-1. To a dry round-bottom flask was added 3-hydroxybenzaldehyde (1.12 g, 9.16 mmol), dione **2** (2.00 g, 9.16 mmol), methoxyethyl acetoacetate (1.47 g, 9.16 mmol), ammonium acetate (1.10 g, 9.16 mmol), and the ionic liquid N,N-methylbutylimidazolium tetrafluoroborate (0.21 mL). The solution was stirred for 10 min at 90 °C. The reaction mixture was applied directly to SiO_2 column, and HPI-1 was purified by flash chromatography (ethyl acetate/hexanes, 6:4) to yield HPI-1 as pale yellow crystalline solid (2.69 g, 64%). ^1H NMR (400 MHz, $CDCl_3/CD_3OD$): δ 7.18–6.61 (m, 8H), 5.06 (s, 1H), 5.01 (s, 1H), 4.12 (m, 2H), 3.81 (s, 3H), 3.55 (m, 2H), 3.33 (s, 2H), 3.32 (s, 3H), 2.64–2.49 (m, 4H), 2.36 (s, 3H). ^{13}C (500 MHz, $CDCl_3$): δ 196.6, 167.7, 157.1, 156.1, 151.0, 148.4, 144.6, 130.3, 129.1, 127.9, 127.1, 120.6, 119.8, 115.0, 113.3, 112.3, 110.6, 105.3, 70.4, 62.8, 58.7, 55.1, 42.3, 36.2, 33.1, 32.5, 19.2. HRMS (*m/z*): $[M]^+$ calc. for $C_{27}H_{29}NO_6Na$, 486.1893; found, 486.1891. HPLC (water/

acetonitrile, 0.1% formic acid, 0–95%, 25 min): retention time, 10.3 min; 96% pure.

Methyl-4,7-dimethoxy-1H-indole-2-carboxylate (3). A dry round-bottom flask was charged with NaN_3 (0.900 g, 13.7 mmol) and 5 mL DMF. Methyl bromoacetate (1.20 mL, 13.1 mmol) was added dropwise to this solution, and the mixture was stirred for 2.5 h at room temperature. A white precipitate formed, and an equivalent volume of H_2O was added. The resulting slurry was extracted three times with diethyl ether, and the pooled organic layer was washed six times with water, dried over $MgSO_4$, and concentrated in vacuo to give methyl azidoacetate (1.5 g, 50%). This crude material was combined with 2,5-dimethoxybenzaldehyde (0.640 g, 3.83 mmol) in 15 mL methanol (prechilled in an acetone/water dry ice bath), and the mixture was added at −10 °C to a solution of NaOMe (0.650 g, 11.5 mmol) in methanol (6 mL). The yellow slurry was then stirred for 45 min at −10 °C and then overnight at 4 °C. Ice water was added to the reaction, and the resulting precipitate was collected, dissolved in dichloromethane, dried over $MgSO_4$, and concentrated in vacuo to give the azidocinnamate intermediate (0.65 g, 66%). The yellow solid was refluxed in 12 mL xylene for 30 min, after which N_2 evolution ceased, and then refluxed for an additional 15 min. A yellow precipitate formed upon cooling of the reaction mixture, which was collected by filtration and washed with petroleum ether to give indole **3** as a yellow solid (0.46 g, 79%).

4,7-Dimethoxy-1-(2-oxopropyl)-1H-indole-2-carboxylic acid (4). α-Bromoacetone was prepared by combining 225 mL dichloromethane/methanol (7:3) and acetone (1.00 mL, 13.6 mmol) with tetrabutylammonium tribromide (6.20 g, 12.9 mmol). After 1 h, the red solution became colorless, and the dichloromethane/methanol was removed by distillation. The remaining solution was diluted with 25 mL diethyl ether and washed three times with water to remove tetrabutylammonium bromide. The solution was than dried over $MgSO_4$ and concentrated to give a 50% α-bromoacetone solution in diethyl ether.

The N-alkyl indole was then prepared under anhydrous conditions by combining **3** (0.100 g, 0.425 mmol), α-bromoacetone (0.180 g, 1.28 mmol), anhydrous K_2CO_3 (0.170 g, 1.28 mmol), 18-crown-6 (16 mg, 0.064 mmol), and 0.5 mL DMF in a round-bottom flask fitted with a reflux condenser. This solution was allowed to stir at 80 °C for 3 h. The indole was then purified by SiO_2 flash chromatography (ethyl acetate/hexanes, 7:3) and saponified with 2 N NaOH (aq) in methanol at 40 °C to obtain the carboxylic acid **4** (0.10 g, 83%).

HPI-2. Cyclooctylamine (3.80 mL, 26.8 mmol) and ethyl formate (2.10 mL, 25.5 mmol) were stirred together for 3 h. Water (50 mL) was added to the reaction, and the mixture was extracted with ethyl acetate. The combined organic layers were dried over $MgSO_4$, and concentrated in vacuo to yield crude N-cyclooctylformamide (3.21 g, 80%). The formylated product was then dehydrated by phosphorus oxychloride (1.20 mL, 12.4 mmol) in 10 mL petroleum ether/pyridine (3:5). Purification by SiO_2 flash chromatography using hexanes afforded the isocyanide product (2.1 g, 75%).

An oven-dried and N_2-purged sealed vial was charged with carboxylic acid **4** (122 mg, 0.44 mmol), N,N-dimethylethane-1,2-diamine (48.2 μl, 0.44 mmol), cyclooctyl isocyanide (60.6 mg, 0.440 mmol) and 300 μL MeOH. After 16 h, the reaction mixture was purified directly by SiO_2 flash chromatography (ethyl acetate/hexanes, 1:9) to yield HPI-2 as a tan crystalline solid (90 mg, 42%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.62 (s broad, 1H), 7.31 (d, J = 4.0 Hz, 1H), 6.58 (d, J = 8 Hz, 1H), 6.35 (d, J = 8 Hz, 1H), 5.59 (d, J = 12 Hz, 1H), 4.21 (m, 1H), 4.08 (d, J = 12 Hz, 1H), 3.92 (s, 6H), 3.72 (m, 2H), 3.57 (m, 2H), 2.76 (m, 2H), 2.48 (s, 6H), 1.75 (s, 3H), 1.64–1.24 (m, 14H). ^{13}C (500 MHz, $CDCl_3$): δ 178.46, 161.04, 148.50, 142.24, 127.59, 127.21, 120.35, 104.99, 104.96, 98.97, 65.19, 57.65, 55.89, 55.58, 52.82, 50.28, 44.86, 39.37, 32.13, 31.87, 26.88, 26.77, 25.26, 23.97, 23.20, 21.04. HRMS (m/z): $[M]^+$ calc. for $C_{27}H_{40}N_4O_4Na$, 507.2947; found, 507.2948. HPLC (water/acetonitrile, 0.1% formic acid, 0−–95%, 25 min): retention time, 9.35 min; 99% pure.

Confirmation of HPI Activity by Quantitative RT-PCR. Shh-LIGHT2 cells were seeded into 96-well plates (10,000 cells/well) and cultured to confluency in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were cultured further in DMEM containing 0.5% CS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 5% Shh-N-conditioned medium, and various concentrations of the HPIs. After 30 h, the Shh-LIGHT2 cell lysates were used to prepare cDNA using a Cells-to-CT kit (Ambion) according to the manufacturer's protocols. The cDNA was then quantified with *Ptch1* and *GAPDH* Taqman probes (Mm1306905_m1 and Mm99999914_g1, Applied Biosystems) on a Roche Lightcycler 480, using the 2nd derivative/maximum method to obtain Ct values. Biological triplicates were analyzed using the ΔΔCt method to determine fold-changes for each HPI condition relative to the maximum pathway activation associated with Shh-treatment alone.

BODIPY-Cyclopamine/Smo Binding Assay. Smo-binding assays were conducted with BODIPY-cyclopamine and Smo-overexpressing HEK 293T cells as previously described (2, 4), using a CMV-promoter-based SV40 origin-containing expression construct for Smo-Myc_3 (murine Smo containing three consecutive Myc epitopes at the C terminus). HEK 293T cells were seeded into eight-well chambered coverslips (Labtek) (80,000 cells/well) and cultured in DMEM containing 10% FBS (Invitrogen), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were cultured until they reached 55 to 65% confluency (14–18 h), after which they were transfected with the Smo-Myc_3 expression construct and Transit-LT1 (Muris Bio) according to the manufacturer's protocols. Twenty-four hours after transfection, the cells were washed with PBS and cultured in DMEM containing 0.5% FBS, 5 nM BODIPY-cyclopamine, and various concentrations of either cyclopamine or individual HPIs. After 30 min, 10 μM Hoescht 33342 was added to each well, and the HPIs were incubated with the cells for an additional 30 min. The cells were then washed two times with PBS buffer, once with phenol red-free DMEM containing 0.5% FBS, and immediately imaged using a DMI6000B compound microscope (Leica). Images were background-substracted using ImageJ software with a rolling ball size of 75 pixels, and BODIPY-cyclopamine intensity was then determined using Metamorph software. Circular regions with a diameter of 300 pixels were placed over regions containing uniformly confluent cells, and the pixel intensities of approximately 20 regions from four independent images was used to determine the average BODIPY-cyclopamine levels for each experimental condition.

Shh-EGFP Assay for Hh Pathway Activation. A Gli-dependent enhanced green fluorescent protein reporter (Shh-EGFP) was generated by excising firefly luciferase cDNA from the 8XGliBs vector (4) using *Nco*I/*Hpa*I, and ligating in EGFP cDNA excised from the pEGFP-C1 vector (Clontech) by *Nco*I/*Afl*II digestion. NIH 3T3 cells were seeded into a six-well plate (150,000 cells/well), cultured in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin for 24 h, and then transfected with 1 μg/well of Shh-EGFP plasmid, 50 ng/well of the zeocin resistance-conveying vector pVgRXR (Invitrogen), and FuGENE 6 according to the manufacturer's protocols. The cells were grown to confluency and treated with DMEM containing 0.5% CS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 5% Shh-N-conditioned medium for 30 h. Following Shh-N treat-

ment, the adherent cultures were dissociated into single cells with 0.05% trypsin-EDTA (300 μL/well; Invitrogen) for 5 min. The cells were then suspended in 1 mL PBS containing 1% CS and sorted on a BD FACSAria (excitation: 488 nm; emission: 530/30 nm) to enrich for cells expressing EGFP in a Shh-N-responsive manner. Clonal populations were then cultured from single cells in DMEM containing 10% CS, 400 μg/mL zeocin, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

To identify Shh-EGFP clones with maximum Shh-N-responsiveness, individual lines were seeded into 24-well plates (45,000 cells/well) and cultured for 48 h. The cells were then treated with DMEM containing 0.5% CS, 100 U/mL pencillin, 0.1 mg/mL streptomycin, or the culture medium plus 5% Shh-N-conditioned medium. After 30 h, the adherent cultures were treated with 0.05% trypsin-EDTA (150 μL/well; Invitrogen) for 5 min to dissociate them into single cells. The cells were then resuspended in 500 μL PBS containing 1% CS and analyzed on a BD FACSCalibur (excitation: 488 nm; emission: 530/30 nm). 10,000 cells were used to assess EGFP expression levels as a measure of Hh pathway activation, and the data were analyzed using FlowJo software (Tree Star). Clones with low basal EGFP levels and maximum Shh-N-induced EGFP expression were selected for further use.

To assess the inhibitory activities of the HPIs in the Shh-EGFP cells, a clonal line with maximum Shh-responsiveness was cultured in 24-well plates as described above. During treatment with Shh-N-conditioned medium, the cells were incubated either DMSO or individual HPIs (each at a concentration 10-fold greater than its IC_{50} in the Shh-LIGHT2 assay or 30 μM, whichever was lower: 15 μM HPI-1, 20 μM HPI-2, 30 μM HPI-3, and 30 μM HPI-4). The resulting EGFP levels were then evaluated by FACS and quantified using the FlowJo software.

C3H10T(1/2) Assay for Hh Pathway Activation. C3H10T(1/2) cells (ATCC) were plated into 96-well plates using DMEM containing 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 5% Shh-N-conditioned medium, and various concentrations of the Hh pathway inhibitors. After approximately 40 h, the cells were washed with PBS and lysed in 50 μL buffer containing 50 mM Tris-HCl, pH 9.5, 150 mM NaCl, 50 mM $MgCl_2$, and 1% Triton X-100. Alkaline phosphatase activities in the cell lysates were quantified by adding 10 μL of the lysate to 50 μL of CDP-Star chemiluminescence reagent (Perkin–Elmer) and measuring the resulting chemiluminescence on a Veritas microplate luminometer.

***Ptch1*$^{-/-}$ Fibroblast Assay for Hh Pathway Activation.** Hh pathway activation in *Ptch1*$^{-/-}$ fibroblasts was assayed as previously described (2), using the knocked-in β-galactosidase gene as a reporter for Hh target gene expression. *Ptch1*$^{-/-}$ fibroblasts were grown to 70–80% confluence in a 15-cm dish, trypsinized, and then resuspended in 40 mL DMEM containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. This cell suspension was aliquoted into 96-well plates (150 μL/well), cultured overnight, and then treated with DMSO or various concentrations of the HPIs for 28 h. Cell viability was measured with CellTiter 96 AQ (Promega) according to the manufacturer's protocols. The cells were then lysed in Tropix lysis solution (30 μL/well; Applied Biosystems) and β-galactosidase levels were quantified by a Tropix Galacto-Star kit (Applied Biosystems) on a Veritas microplate luminometer.

SmoM2-LIGHT Assay for Hh Pathway Activation. SmoM2-LIGHT cells (previously named SmoA1-LIGHT cells; ref. 2) were grown to 70–80% confluence in a 15-cm dish, trypsinized, and then resuspended in 40 mL DMEM containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. This cell suspension was aliquoted into 96-well plates (150 μL/well), cultured overnight, and then treated with DMSO or various concentrations of the HPIs for 28 h. Cells were then washed once with PBS then treated with 50 μL of passive lysis buffer (Promega). Hh pathway-dependent firefly luciferase activity in the lysates was quantified using Bright-Glo reagent and a Veritas microplate luminometer. Constitutive β-galactosidase activity was measured using the Tropix Galacto-Star kit.

Wnt-LIGHT Assay for Wnt Pathway Activation. Wnt3a-conditioned medium was prepared by culturing L cells stably expressing Wnt3a (ATCC) in DMEM containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. After the cells reached 70% confluency, they were cultured in fresh medium, and the resulting Wnt3a-condition medium was collected 30 h later. To generate a Wnt pathway-reporter cell line (Wnt-LIGHT cells), L cells were seeded into a six-well plate and cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin until they reached 50% confluency. The cells were then transfected with 1 μg/well of SuperTopFlash reporter (5), which contains contains seven TCF/LEF enhancer sites upstream of a basal promoter and firefly luciferase cDNA, 50 ng/well of constitutive *Renilla* reporter pRLSV40 (Promega), 50 ng/well of the geneticin resistance-conveying vector pcDNA3 (Invitrogen), and FuGENE 6 according to the manufacturer's protocols. Clonal populations were obtained by culturing the transfected cells in DMEM containing 10% FBS, 1 mg/mL geneticin, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Individual clones were isolated by ring-cloning.

To quantitatively assess Wnt pathway activation in the Wnt-LIGHT cells clones, the cells were then cultured in 48-well plates using DMEM containing 10% FBS, 400 μg/mL geneticin, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. After the cells reached confluency, they were grown for another 24 h with either fresh culture medium, Wnt3a-conditioned medium, or medium containing 30 mM LiCl. Through this process, a clone exhibiting maximum Wnt3a responsiveness and minimum basal pathway activation was identified. Firefly luciferase activity in this clone typically increases over 200-fold upon stimulation with Wnt3a-conditioned medium.

To determine whether the Hh pathway inhibitors affect Wnt signaling in the Wnt-LIGHT cells, the cells were cultured to 70–80% confluence in a 15-cm dish, trypsinized, and then resuspended in 40 mL of DMEM containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. This cell suspension was then aliquoted into 96-well plates (150 μL/well) and cultured overnight. The following day, the growth medium was replaced with Wnt3a-conditioned medium containing either DMSO or various concentrations of the HPIs. The cells were cultured further for 24–28 h, after which their firefly and *Renilla* luciferase activities were measured using a dual luciferase kit and Veritas microplate luminometer. To confirm that firefly luciferase activity is indicative of Wnt target gene expression, the Wnt-LIGHT cells were also stimulated with Wnt3a-conditioned medium and various concentrations of the Axin-stabilizer IWR-1. The cells were cultured for 48 h to achieve maximum IWR-1-dependent Axin stabilization, and the resulting firefly and *Renilla* luciferase activities were measured as before.

FRET Assay for Smo Multimerization. To construct cyan and yellow fluorescent protein-tagged forms of murine Smo (Smo-CFP and Smo-YFP), CFP or FYP was fused in frame to the Smo C terminus, using the *Nhe*I/*Sal*I sites in the pGE-Smo vector and the following primer sequences: 5′-GTA CGC TAG CAT GGT GAG CAA GGG CGA GCT G-3′, and 5′-GTA CGT CGA CTC ACT TGT ACA GCT CGT CCA TG-3′.

For FRET analysis of cultured cells, NIH 3T3 cells were seeded into 6-well plates (150,000 cells/well) and cultured in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL

streptomycin for 10–16 h. The cells were then transfected with 1 μg/well of the Smo-CFP and Smo-YFP expression constructs (1:1 mixture) and FuGENE 6, cultured for another 24 h, and treated with either 10 μM cyclopamine, 500 nM SAG, or individual HPIs (10 μM) in the absence or presence of 10% Shh-N-conditioned medium for 5 h. The cells were washed with PBS buffer, fixed with 4% paraformaldehyde for 20 min, and mounted on slides in 80% glycerol. Fluorescence signals were acquired with the 100× objective of a Zeiss LSM510 confocal microscope with the following conditions: CFP was excited by 458-nm light and the emission was collected through a BP 480–520-nm filter. YFP was excited by 514-nm light and the emission was collected through a BP 535–590-nm filter. The CFP signal was obtained before and after photobleaching YFP (CFP_{BP} and CFP_{AP}, respectively) using the full power of the 514-nm laser line for 1–2 min at the top half of each cell, leaving the bottom half unbleached as an internal control. The CFP fluorescence intensity was analyzed using Metamorph software (Universal Imaging Corp.), and the energy transfer efficiency was calculated using the formula: $FRET\% = ((CFP_{AP}\text{-}CFP_{BP})/CFP_{AP}) \times 100$. Photobleached areas in at least ten cells were analyzed for each experimental condition.

Smo Trafficking Assay. NIH 3T3 cells were seeded into 24-well plates (40,000 cell/well) containing polyD-lysine-coated 12-mm glass coverslips and cultured in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin until they reached 85–90% confluency. The medium was changed to DMEM containing 0.5% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin and the cells were cultured for another 12 h. The cells were then treated with either DMSO, 3 μM cyclopamine, 15 μM HPI-1, 20 μM HPI-2, 30 μM HPI-3, or 30 μM HPI-4. Shh-N-conditioned medium was added to appropriate wells at a final concentration of 5%. After 12 h, the cells were fixed in 4% paraformaldehyde for 10 min at room temperature, washed three times with PBS, permeabilized for 1 min with PBS containing 0.1% Triton X-100, washed again three times with PBS, and then blocked with PBS containing 1% normal goat serum for 3 h. The coverslips were then treated with mouse anti-N-acetylated-α-tubulin (clone 6–11B-1, Sigma; 1:1,000 in blocking buffer) and rabbit anti-Smo antibody (6) (1:2,000 dilution in blocking buffer) for 2 h at room temperature and washed 3 × 5 min with PBS. The coverslips were incubated next with Alexa Fluor 594-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated donkey anti-rabbit IgG antibodies (Invitrogen; 1:1,000 dilutions in blocking buffer) for 1 h at room temperature. After washes with PBS and a 5-min incubation with 4,6-diamidino-2-phenylindole (DAPI), the samples were mounted using Prolong Gold (Invitrogen) and imaged with an inverted Leica DMIRE2 laser scaning confocal microscope.

Ciliary Smo levels were quantified by designating ciliary regions according to N-acetylated-α-tubulin staining intensity using Metamorph software. Thresholded areas were further filtered for size and shape to remove non-ciliary regions, and additional ciliary regions were included manually. The ciliary regions were then transferred to the corresponding images of Smo antibody staining, and the average pixel intensity was recorded. Between 10–40 cilia were analyzed for each condition.

***Su(fu)*$^{-/-}$ Fibroblasts Assay for Hh Pathway Activation.** *Su(fu)*$^{-/-}$ fibroblasts were cultured in a 15-cm dish with DMEM containing 10% FBS containing 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 μg/mL gentamicin. The cells were trypsinized and transfected in batch with 8xGliBS-FL (285 ng), constitutive *Renilla* luciferase reporter (15 ng; phRLCMV, Promega), and pEGFP-C1 (100 ng as a carrier; Clontech). The transfected cells were then seeded into 24-well plates (25,000 cells/well) and cultured further in the same medium. After 24 h, the cells were then cultured in DMEM containing 0.5% FBS and either DMSO or various concentrations of the HPIs, cyclopamine, or GANT-61 for another 40 h. The resulting firefly and *Renilla* luciferase activities were measured using a dual luciferase kit and Veritas microplate luminometer.

Hh Pathway Activation Mediated by the Overexpression of Gli, Gli2, Gli2ΔN, Gli2ΔGSK, or Gli2ΔPKA. Gli1, Gli2, Gli2ΔGSK, or Gli2ΔPKA pcDNA-derived vectors for expressing N-terminally Xpress-tagged proteins were used as previously described (7). To generate a Gli2ΔN expression construct with the same vector background, the pcDNA Gli2 construct was PCR amplified using the following primer sequences: 5′-GCC TTC ACT TTT CCC CAC CCC ATC-3′, and 5′-CCA CCA CAC TGG ATC CTT AGG TAC C-3′. Ligation of the linear PCR product then provided the pcDNA Gli2ΔN vector. NIH 3T3 cells were seeded into 24-well plates (35,000 cells/well) and cultured in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were co-transfected the following day with 220 ng/well pcDNA-derived Gli1, Gli2, Gli2ΔN, or Gli2 phospho-site mutant expression vectors and 80 ng/well of a 1:15 mixture of pRLTK (Promega) and 8XGliBS-FL. After transfection, cells were grown to confluence (≈48 h). The cell were then incubated for an additional 28–32 h in DMEM containing 0.5% CS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and either DMSO, 50 μM forskolin, 50 μM LY294002, or various concentrations of the HPIs, cyclopamine, or GANT-61. Firefly and *Renilla* luciferase activities were measured using a dual luciferase kit and a Veritas microplate luminometer.

To confirm the stability of the wildtype and mutant Gli2 proteins, their expression levels were analyzed by immunoblotting. HEK 293T cells were grown in DMEM containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were cultured in 10-cm dishes and then transfected at 50% confluency in the following manner: 61 μL of 2 M $CaCl_2$, and 10 μg of the appropriate pcDNA Gli2 vector was diluted to a final volume of 500 μL in H_2O and added slowly to 500 μL of 2× HBS buffer (50 mM HEPES, pH 7.1, 280 mM NaCl, and 1.5 mM Na_2HPO_4). After a 1-min incubation at room temperature, each transfection mixture was added to a 10-cm dish of HEK 293T cells, which were cultured an additional 40 h and then lysed in buffer [1 mL/plate; 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 250 mM NaCl, 2 mM EDTA, 20 mM NaF, 2 mM Na_3VO_4, 1 mM 2-mercaptoethanol, and EDTA-free protease inhibitor mixture (Roche)]. This suspension was transferred to a 1.5-mL centrifuge tube and incubated on ice for 30 min, with vortexing every 5 min. The cell lysates were then centrifuged at 20,000 × *g* at 4 °C for 20 min. Supernatants were removed, and a BCA protein assay was performed to quantify protein levels. Twenty micrograms of total protein was resolved on 4–12% Bis-Tris Criterion XT gel and transferred to a PVDF membrane. The membrane was dehydrated with methanol, and then probed with rabbit anti-Gli2 antibody [SCBT H-300; 1:2000 dilution in blocking buffer (PBS containing 3% nonfat dried milk and 0.01% Tween 20)] for 90 min. Blots were washed 4 × 5 min in PBS containing 0.01% Tween 20 and then probed with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (GE Healthcare; 1:10,000 dilution in blocking buffer) for 1 h. Blots were washed 4 × 3 min in PBS, and the immunoreactive bands were detected with a SuperSignal West Dura kit and a ChemiDoc XRS system. To assess protein loading, the blots were rinsed with PBS, incubated for 1 min in methanol, washed 3 × 5 min in PBS, and then re-blocked for 1 h in PBS containing 5% nonfat dried milk and 0.1% Tween 20. The membranes were then incubated for 1 h with rabbit anti-importin β1 antibody (sc-11367, Santa Cruz Biotechnology; 1:500 dilution in blocking buffer), washed 3 × 10 min with PBS containing 0.1% Tween 20, incubated with donkey anti-rabbit IgG antibody (GE Healthcare; 1:10,000 dilution in

PBS containing 0.1% Tween 20), washed 3 × 10 min with PBS containing 0.1% Tween 20, and then analyzed by chemiluminescence as described above.

PKA-Mediated CREB Phosphorylation Assay. NIH 3T3 cells were seeded into 12-well plates and grown to 80% confluency in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were then cultured overnight in DMEM containing 0.5% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The serum-starved cells were treated with DMSO or 10 μM H89 for 30 min, after which additional DMSO, 50 μM forskolin, 15 μM HPI-1, 20 μM HPI-2, 30 μM HPI-3, or 30 μM HPI-4 were added to the wells. The cells were cultured for an additional 2 h and then lysed in hot 2× SDS/PAGE sample buffer (100 mM Tris, pH 6.8, 200 mM DTT, 0.02% bromophenol blue, 20 mM NaF, 2 mM sodium orthovanadate, 4% SDS, and 20% glycerol). After the samples were boiled for 5 min, they were resolved on 4–12% Criterion XT gels (Bio-Rad) and transferred to PVDF membranes (Millipore). The membranes were blocked overnight in TBST buffer (Tris-buffered saline with 0.1% Tween 20) containing 5% BSA and then probed with either anti-phosphorylated CREB (87G3, Cell Signaling Technology; 1:1,000 dilution) or CREB (48H2, Cell Signaling Technology, 1:1,000 dilution) antibodies. After extensive washing in TBST, the membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (GE Healthcare, 1:10,0000 dilution), and the immunoblotted proteins were visualized using a SuperSignal West Dura Extended Duration kit (Pierce) and a ChemiDoc XRS system (Bio-Rad).

PI3K and MAPK Signaling Assays. NIH 3T3 cells were seeded into 6-well plates and grown to 80% confluency in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells were then serum-starved for 8 h in DMEM containing 0.5% CS and either DMSO, 15 μM HPI-1, 20 μM HPI-2, 30 μM HPI-3, or 30 μM HPI-4. Fifty micromolar LY294002 and 10 μM U0126 were used as positive controls. After serum starvation, cells were stimulated with 10 ng/mL PDGF BB for 30 min, washed in PBS, and lysed in buffer containing 50 mM Tris·HCl pH 7.4, 250 mM NaCl, 20 mM NaF, 2 mM sodium orthovanadate, 2 mM EDTA, 2 mM PMSF, 1% Triton X-100, and EDTA-free protease inhibitor mixture (Roche). Cell lysates were clarified by centrifugation at 20,000 × *g* and total protein concentrations were determined by the BCA assay (Pierce). Fifteen micrograms of protein from each lysate was mixed with 6× SDS/PAGE sample buffer (300 mM Tris-HCl, pH 6.8 containing 60% glycerol, 12% SDS, 600 mM DTT, and 0.05% bromophenol blue), boiled for 5 min and then resolved on 4–12% Bis-Tris Criterion XT gels. The electrophoresed samples were transferred to PVDF membranes and blocked overnight in TBST buffer containing 5% BSA. The membranes were then probed with anti-phosphorylated Akt (193H12, Cell Signaling Technology; 1:1,000 dilution), Akt (9727, Cell Signaling Technology, 1:1,000 dilution), phosphorylated p44/p42 MAPK (20G11, Cell Signaling Technology), or p44/p42 MAPK (137F5, Cell Signaling Technology) antibodies. After extensive washing in TBST, the membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (GE Healthcare, 1:10,000 dilution), and the immunoblotted proteins were visualized using a SuperSignal West Dura Extended Duration kit (Pierce) and a ChemiDoc XRS system.

Generation of FLAG-Gli1 and FLAG-Gli2 Retroviruses. Gli1 and Gli2 were amplified from a mouse oligo dT primed cDNA library with Phusion DNA polymerase (NEB), and subcloned into pCR-Blunt II−TOPO (Invitrogen) using the following PCR (PCR) primers (Gli1: 5′-GCG CCT CTC CCA CAT ACT AGA AAT CT-3′, 5-TAG GAA ATA CCA TCT GCT TGG GGT TC-3′) and (Gli2: 5′-CAC CTG CAT GCT AGA GGC AAA CTT TT-3′, 5′-TCA GGC CTA GTT AAC ACT TTG GGA CA-3′). The resulting vectors were used as templates for amplification of Gli1 and Gli2 with primers containing *Not*I and *Bgl*II restriction sites: (Gli1: 5′-GAA TGC GGC CGC GTT CAA TCC AAT GAC TCC AC-3′, 5′-GAA GAT CTT TAG GCA CTA GAG TTG AGG-3′) and (Gli2: 5′-GAA TGC GGC CGC GGA GAC TTC TGC CCC AGC CC-3′, 5′-GAA GAT CTT AGG TCA TCA TGT TTA AAA AC-3′). The PCR products were digested with *Not*I and *Bgl*II and ligated into the pCMV 3×FLAG 26 vector (Sigma). This plasmid construct was then used as a template for generating FLAG-tagged Gli1 and Gli2 cDNAs with flanking Gateway recombination sites using the common forward primer for FLAG 5′-AAA AAG CAG CCT CAG CCA CCA TGG ACT ACA AAG ACC ATG ACG GTG-3′ and the reverse primers 5′-AGA AAG CTG GGT CTT AGG CAC TAG AGT TGA GGA ATT G-3′ and 5′-AGA AAG CTG GGT CTT AGG TCA TCA TGT TTA AAA AC-3′ for Gli1 and Gli2, respectively. These products were re-amplified with Gateway flanking primers (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC A-3′ and 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC-3′) to add complete *att*B1 and *att*B2 sites and then recombined into the Gateway entry vector pDNR207 (Invitrogen). Entry vectors were sequence verified and recombined into the pBMN-IRES-tdTomato-DEST or pBMN-IRES-hcRed-DEST vectors to generate FLAG-Gli1-tdTomato and FLAG-Gli2-hcRed retroviral constructs. The pBMN-IRES-tdTomato-DEST and pBMN-IRES-hcRed-DEST vectors contain a Gateway cassette inserted into the polylinker region, followed by an internal ribosome entry sequence (IRES) for expression of the tdTomato (8) and hcRed (9) fluorescent proteins, respectively, as a reporter of infection efficiency.

To generate retroviruses for FLAG-Gli1 or FLAG-Gli2 expression, HEK 293T cells (ATCC) were grown in DMEM containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were cultured in 10-cm dishes and then transfected at 30% confluency in the following manner: 61 μL of 2 M $CaCl_2$, 6 μg of pBMN vector (pBMN-Gli1-IRES-TdTomato or pBMN-Gli2-IRES-hcRed), and 3 μg of pCL-ECO retrovirus packaging vector (Imgenex) were diluted into 500 μL of nuclease-free H_2O (Invitrogen) and added slowly to 500 μL of 2× HBS buffer (50 mM HEPES, pH 7.1, 280 mM NaCl, and 1.5 mM Na_2HPO_4). Following a 1-min incubation at room temperature, the mixture was gradually added to a single 10-cm plate. Retroviral supernatants were collected 24 h later with a full medium replacement and then collected again after an additional 24 h. The combined supernatants were passed through a 0.45-μm filter and stored at −80 °C. TdTomato or hcRed expression was used to confirm that a greater than 90% infection rate was achieved.

Generation of Cell Lines Stably Expressing FLAG-Gli1 or FLAG-Gli2. To generate a FLAG-Gli1-expressing cell line, Shh-LIGHT2 cells were seeded into a 24-well plate (40,000 cells/well) and cultured in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin for 18 h. The cells were then infected with FLAG-Gli1-TdTomato retrovirus, and after 30 h, TdTomato-expressing cells were isolated on a Vantage SE/FACS DiVa cell sorter (excitation: 598 nm; emission: 575/26 nm). Clonal cell lines were cultured in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, and individual clones that exhibited FLAG-Gli1 expression by immunofluorescence microscopy were isolated and expanded.

To generate a FLAG-Gli2-expressing cell line, Shh-EGFP cells was added to a 24-well plate (40,000 cells/well) and cultured in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin for 18 h. The cells were then infected with FLAG-Gli2-hcRed retrovirus, and after 30 h, hcRed-expressing

cells were isolated on a Vantage SE/FACS DiVa cell sorter (excitation: 598 nm; emission: 620/20 nm). Clonal cell lines were cultured in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, and individual clones that exhibited FLAG-Gli2 expression by immunofluorescence microscopy were isolated and expanded.

Analysis of Gli2 Processing and Shh-Dependent Gli2 Stabilization. To analyze Gli2 processing, a Shh-EGFP clone expressing low levels of FLAG-Gli2 was cultured in 60-mm plates and grown to confluency in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The culture medium was then replaced with DMEM containing 10% CS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and either DMSO, 3 μM cyclopamine, 15 μM HPI-1, 20 μM HPI-2, 30 μM HPI-3, or 30 μM HPI-4. Each compound incubation was also done in the absence and presence of 5% Shh-N-conditioned medium, and the cells were maintained under these conditions for 24 h. The cells were subsequently solubilized in lysis buffer [100 μL/well; 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 250 mM NaCl, 2 mM EDTA, 20 mM NaF, 2 mM Na_3VO_4, 2 mM 2-mercaptoethanol, and EDTA-free protease inhibitor mixture (Roche)]. This suspension was transferred to a 1.5-mL centrifuge tube and incubated on ice for 30 min, with vortexing every 5 min. The cell lysates were then centrifuged at 20,000 $\times$ g at 4 °C for 20 min. Supernatants were removed, and a BCA protein assay was performed to quantify protein levels.

Mouse anti-FLAG M2 agarose slurry (20 μL, Sigma) was pelleted by centrifugation and incubated with 800 μg protein from each condition for 90 min at 4 °C and then washed three times with 1 mL of wash buffer (75 mM Tris-HCl buffer, pH 8.0 containing 225 mM NaCl, 0.5% Triton X-100) and centrifuged at 2,300 $\times$ g at 4 °C. The agarose beads were suspended in 30 μL of 1$\times$ SDS/PAGE sample buffer (50 mM Tris-HCl, pH 6.8 containing 10% glycerol, 2% SDS, 50 mM DTT, 200 mM 2-mercaptoethanol, and 0.001% bromophenol blue), and the mixture was boiled for 5 min. The samples were then resolved on a 4–12% Bis-Tris Criterion XT gel and transferred to a PVDF membrane. The membrane was dehydrated with methanol, and then probed with rabbit anti-FLAG antibody [Sigma; 1:1,000 dilution in blocking buffer (PBS containing 3% nonfat dried milk and 0.01% Tween 20)] for 90 min. Blots were washed 4 $\times$ 5 min in PBS containing 0.01% Tween 20 and then probed with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (GE Healthcare; 1:10,000 dilution in blocking buffer) for 1 h. Blots were washed 4 $\times$ 3 min in PBS, and the immunoreactive bands were detected with a SuperSignal West Dura kit and a ChemiDoc XRS system.

Protein bands were quantified using Quantity One software (Bio-Rad). For each experiment, boxes of equal size were drawn around the FLAG-Gli2 and FLAG-Gli2R bands. Band intensities were then normalized to the average intensity for all bands in a given experiment. The results from all experiments were averaged and normalized relative to the basal level of FLAG-Gli2 (DMSO treatment without Shh-N-conditioned medium).

Analysis of Gli1 Stability. To analyze Gli1 expression levels, FLAG-Gli1-expressing Shh-LIGHT2 cells were cultured in 12-well plates and grown to confluency in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The culture medium was then replaced with DMEM containing 10% CS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and either DMSO, 15 μM HPI-1, 20 μM HPI-2, 30 μM HPI-3, or 30 μM HPI-4. The cells were maintained under these conditions for 24 h. The cells were subsequently solubilized in lysis buffer [100 μL/well; 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 250 mM NaCl, 2 mM EDTA, 20 mM NaF, 2 mM Na_3VO_4, 2 mM 2-mercaptoethanol, and EDTA-free protease inhibitor mixture (Roche)]. This suspension was transferred to a 1.5-mL centrifuge tube and incubated on ice for 30 min, with vortexing every 5 min. The cell lysates were then centrifuged at 20,000 $\times$ g at 4 °C for 20 min. Supernatants were removed, and a BCA assay was performed to quantify protein levels. Fifteen micrograms of protein from each sample was solubilized in 1$\times$ SDS/PAGE sample buffer (50 mM Tris-HCl, pH 6.8 containing 10% glycerol, 2% SDS, 50 mM DTT, 200 mM 2-mercaptoethanol, and 0.001% bromophenol blue) and boiled for 5 min. The samples were then resolved on a 4–12% Bis-Tris Criterion XT gel and transferred to a PVDF membrane. The membrane was dehydrated with methanol, and then probed with mouse anti-FLAG M2 antibody [Sigma; 1:1,000 dilution in blocking buffer (PBS containing 3% nonfat dried milk and 0.01% Tween 20)] for 90 min. The blots were washed 4 $\times$ 5 min in PBS containing 0.01% Tween 20 and then probed with horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (GE Healthcare; 1:10,000 dilution in blocking buffer) for 1 h. The membranes were washed again 4 $\times$ 3 min in PBS, and immunoreactive bands were detected with a SuperSignal West Dura kit and a ChemiDoc XRS system. To probe for loading controls, the blots were rinsed with PBS, incubated for 1 min in methanol, washed 3 $\times$ 5 min in PBS, and then re-blocked for 1 h in PBS containing 5% nonfat dried milk and 0.1% Tween 20. The membranes were then incubated for 1 h with rabbit anti-importin β1 antibody (sc-11367, Santa Cruz Biotechnology; 1:1,000 dilution in blocking buffer), washed 3 $\times$ 10 min with PBS containing 0.1% Tween 20, incubated with donkey anti-rabbit IgG antibody (GE Healthcare; 1:10,000 dilution in PBS containing 0.1% Tween 20), washed 3 $\times$ 10 min with PBS containing 0.1% Tween 20, and then analyzed by chemiluminescence as described above.

Protein bands were quantified using Quantity One software (Bio-Rad). FLAG-Gli1 and importin β1 bands were normalized to the average band intensities for each experiment. The ratio of these two values was then used to determine the relative amount of FLAG-Gli1 per condition. Results from three independent experiments were then averaged, and normalized to FLAG-Gli1 levels in DMSO-treated cells.

Immunostaining of FLAG-Gli Lines and NIH 3T3 Cells. The FLAG-Gli1-expressing or FLAG-Gli2-expressing clonal cell lines were seeded into 24-well plates (80,000 cells/well) containing polyD-lysine-coated 12-mm glass coverslips. The cells were grown to confluency in DMEM containing 10% CS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin and then cultured for an additional 24 h in DMEM containing 0.5% CS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and either DMSO, 20 μM nocodazole, 15 μM HPI-1, 20 μM HPI-2, 30 μM HPI-3, or 30 μM HPI-4. The cells were subsequently fixed in 4% paraformaldehyde for 10 min at room temperature followed by treatment with methanol at −20 °C for 2 to 5 min. The cells were washed with PBS, permeabilized with 0.2% Triton X-100 for 2 to 5 min, and then blocked with PBS containing 2% BSA for 3 h at room temperature. The coverslips were then treated with mouse anti-FLAG M2 antibody (Sigma; 1:1,000 dilution in blocking buffer) and rabbit anti-Arl13b (10) (1:1,000 dilution in blocking buffer) for 3 h at room temperature and washed 3 $\times$ 5 min with PBS. The coverslips were incubated next with Alexa Fluor 594-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated donkey anti-rabbit IgG antibodies (Invitrogen; 1:1,000 dilutions in blocking buffer) for 2 h at room temperature. After washes with PBS and a 5-min incubation with 4,6-diamidino-2-phenylindole (DAPI), the samples were mounted using Prolong Gold (Invitrogen) and imaged with an upright Leica DM4500B compound microscope. To quantify ciliary Gli2 localization, a circular region with diameter equal to the average width of cilia as determined by the anti-Arl13b antibody staining was manually placed at the distal end of each cilium. Regions were transferred

from the anti-Arl13b antibody image to the anti-FLAG antibody image, and FLAG staining intensities within those areas were assessed. Between 40 and 80 cilia were analyzed for each condition. To normalize ciliary FLAG-Gli2 levels with respect to compound-dependent changes in total FLAG-Gli2 levels, the absolute average intensities for each condition were divided by the fold-change in FLAG-Gli2 levels, as determined by the quantitative immunoblotting described above. Quantification of ciliary FLAG-Gli1 levels was performed in an analogous manner. Since HPI-4 perturbs primary cilia formation, only Arl13b-positive structures that could be clearly identified as cilia were used for Gli protein quanitification in HPI-4-treated cells.

To assess cytoplasmic microtubule structures in cells treated with the HPIs, NIH 3T3 cells were cultured on polyD-lysine-coated 12-mm glass coverslip, treated with the HPIs, and fixed with 4% paraformalehyde as described above. In place of the methanol treatment, the cells were permeabilized with PBS containing 0.1% Triton X-100 for 1 min. Washing, blocking, and antibody incubation steps were then conducted as before, using mouse anti-α-tubulin (DM1-A, Sigma, 1:2,000 in blocking buffer). Secondary antibody treatments, DAPI staining, mounting, and imaging were then conducted as described above.

Analysis of *Math1-cre:SmoM2* CGNP Proliferation. To generate *Math1-cre:SmoM2* CGNPs (11), a *Math1-cre* driver was used to conditionally express an activated *Smo (SmoM2)* allele. The *Gt(ROSA)26Sortm1 (Smo/EYFP)Amc*/J) mouse line was obtained from Jackson Laboratory. Generation and characterization of *Math1-cre* transgenic animals that carry bacteriophage P1 cre recombinase under control of a 1.4 kb upstream *Math1* enhancer element has been described previously (12). Primary cultures of *Math1-cre:SmoM2* CGNPs were established by triturating cerebellar tissue from P7 mice and plating the dissociated cells onto polyD-ornithine-coated plates in DMEM-F-12 with N2 supplement, 25 mM KCl, and antibiotics. After culturing the cells for 24 h, they were treated with individual HPIs (20 μM), cyclopamine (5 μM), or a DMSO control for 24 h. Cell proliferation was then quantified by immunocytochemistry, and the expression of cyclin D1, Gli1, Gli2, and N-Myc were assessed by immunoblotting or RT-PCR.

For immunocytochemistry, the *Math1-cre:SmoM2* CGNP cultures were fixed in 4% paraformaldehyde for 30 min at room temperature. The cells were then incubated in PBS containing 5% normal goat serum, 0.1% BSA, and 0.3% Triton X-100 for 1 h and with anti-phosphorylated histone H3 antibody (9706, Cell Signaling Technology; 1:500 dilution) overnight at 4 °C. Each sample was then washed with PBS and incubated with Alexa Fluor 555-conjugated anti-rabbit IgG antibody (Molecular Probes; 1:1,000 dilution) and embedded. Immunofluorescently stained cultures were imaged using a Nikon Eclipse E600 microscope equipped with a 20× objective. Approximately 30,000 cells were analyzed for each experimental condition.

To detect proteins by immunoblotting, *Math1-cre:SmoM2* CGNP cultures were dissociated in lysate buffer (50 mM HEPES, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Triton X-100, 10% glycerol, and 1 mM DTT), resolved on a 10% SDS/PAGE gel, and transferred onto a PVDF membrane. The membrane was incubated overnight at 4 °C with anti-cyclin D1 (Ab-3, Neomarkers; 1:1,000 dilution) or anti-β-tubulin (T4026, Sigma; 1:5,000 dilution) antibodies. After extensive washing in 10 mM Tris-HCl, pH 8.0 containing 0.1% Triton X-100, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce; 1:10,000 dilution) or donkey anti-mouse IgG (Jackson Laboratories, 1:10,000 dilution) antibodies, and the immunoblotted proteins were visualized with an ECL kit (Amersham) and a Konica SRX-101A film processor.

For RT-PCR analyses, total RNA from *Math1-cre:SmoM2* CGNP cultures was extracted with TRIzol (Invitrogen) according to the manufacturer's protocol. The RNA was purified further with an RNeasy kit (Qiagen), and then reverse-transcribed with an Advantage RT-for-PCR Kit (Clontech) and random hexamer primers. PCR was performed using a Stratagene RoboCycler Gradient 96 temperature cycler with a Hot Top Assembly, the Titanium TaqDNA polymerase (Clontech), and the following primers: *Gli1*: 5′-ACA GCG GGG GCA GAA GTC G-3′, 5′-CCT CAG CCC CAG TAT CCC CAG TCG-3′; *Gli2*: 5′-GGC GCC CTG ACC CCC TTA TTC TG-3′, 5′-CCT GCG GTG GCC TTG TAG C-3′; *N-Myc*: 5′-GGG GGC TCA GGC TCT TCG CTT TTG-3′, CCC GCC GTG GTC TTC CCC TTC C-3′, *β-actin*: the Clontech Mouse β-Actin Control Amplimer Set. Typical PCR conditions were as follows: an initial denaturation at 95 °C for 1 min followed by 28 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, extension at 70 °C for 2 min, and a final extension at 72 °C for 7 min. The resulting PCR products were analyzed on 2% agarose gels.

1. Chen JK, Taipale J, Young KE, Maiti T, Beachy PA (2002) Small molecule modulation of Smoothened activity. *Proc Natl Acad Sci USA* 99:14071–14076.
2. Taipale J, et al. (2000) Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. *Nature* 406:1005–1009.
3. Sasaki H, Hui C, Nakafuku M, Kondoh H (1997) A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* 124:1313–1322.
4. Sinha S, Chen JK (2006) Purmorphamine activates the Hedgehog pathway by targeting Smoothened. *Nat Chem Biol* 2:29–30.
5. Kaykas A, Yang-Snyder J, Heroux M, Shah KV, Bouvier M, Moon RT (2004) Mutant Frizzled 4 associated with vitreoretinopathy traps wild-type Frizzled in the endoplasmic reticulum by oligomerization. *Nat Cell Biol* 6:52–58.
6. Rohatgi R, Milenkovic L, Scott MP (2007) Patched1 regulates hedgehog signaling at the primary cilium. *Science* 317:372–376.
7. Riobo NA, Lu K, Ai X, Haines GM, Emerson CP, Jr (2006) Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling. *Proc Natl Acad Sci USA* 103, 4505–4510.
8. Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY (2004) Improved monomeric red, orange, and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nat Biotechnol* 22:1567–1572.
9. Gurskaya NG, et al. (2001) GFP-like chromoproteins as a source of far-red fluorescent proteins. *FEBS Lett* 507:16–20.
10. Caspary T, Larkins CE, Anderson KV (2007) The graded response to Sonic Hedgehog depends on cilia architecture. *Dev Cell* 12:767–778.
11. Schuller U, et al. (2008) Acquisition of granule neuron precursor identity is a critical determinant of progenitor cell competence to form Shh-induced medulloblastoma. *Cancer Cell* 14:123–134.
12. Schuller U, et al. (2007) Forkhead transcription factor FoxM1 regulates mitotic entry and prevents spindle defects in cerebellar granule neuron precursors. *Mol Cell Biol* 27:8259–8270.

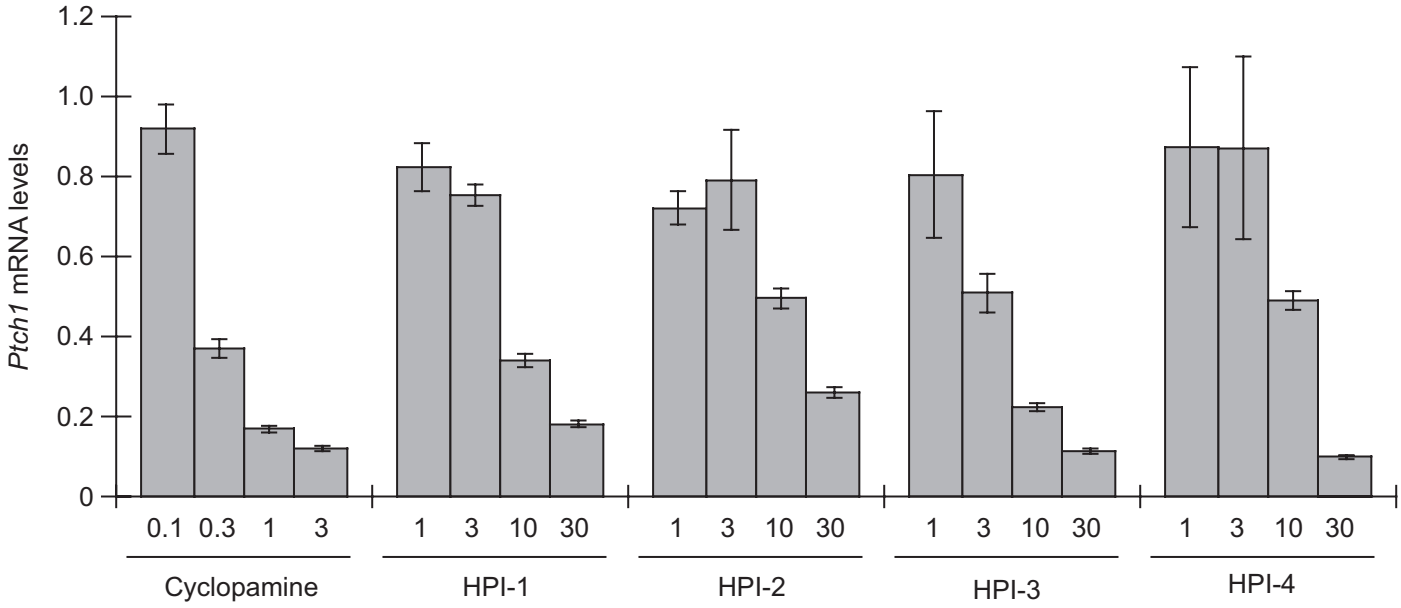

Fig. S1. Inhibition of endogenous Hh target gene expression by the HPIs. Cyclopamine and the individual HPIs inhibit endogenous *Ptch1* expression in Shh-LIGHT2 cells treated with Shh-conditioned medium, as determined by quantitative RT-PCR. Data for each experimental condition are the average *Ptch1* transcript levels of triplicate samples normalized with respect to *GAPDH* and expressed as a fraction of that observed in Shh-stimulated Shh-LIGHT2 cells ± SD.

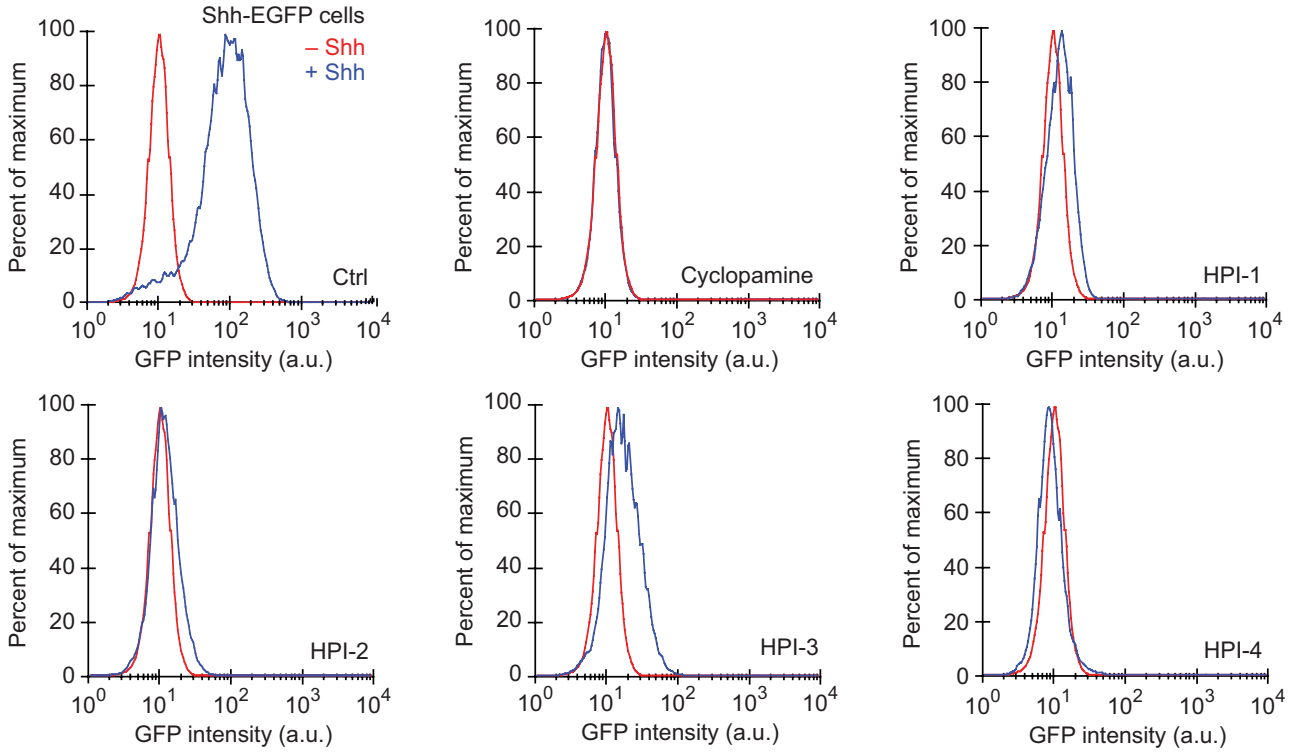

Fig. S2. Inhibition of Hh pathway activity in Shh-EGFP cells by the HPIs. Hh pathway activity in Shh-EGFP cells can be monitored by fluorescence-activated cell sorting (FACS), as demonstrated by the difference in fluorescence intensity observed between untreated cells (red) and those stimulated with Shh-conditioned medium (blue). Shh-EGFP cells treated simultaneously with Shh and either cyclopamine or individual HPIs exhibit fluorescence intensities similar to those of untreated cells. The compounds were used at concentrations 10-fold greater than their IC50s in the Shh-LIGHT2 assay or 30 μM, whichever was lower (3 μM cyclopamine, 15 μM HPI-1, 20 μM HPI-2, 30 μM HPI-3, and 30 μM HPI-4).

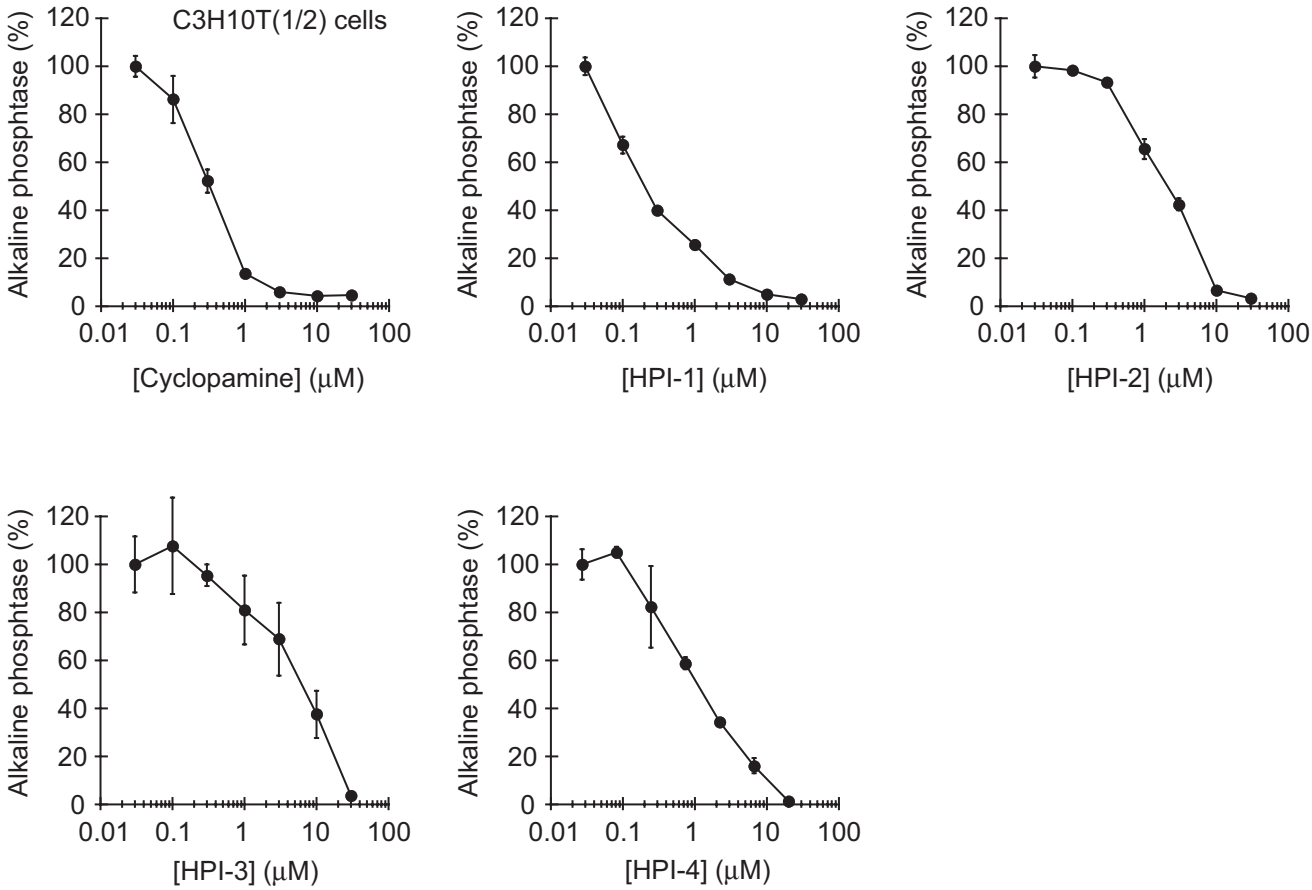

Fig. S3. Inhibition of Shh-dependent osteogenesis by the HPIs. Cyclopamine and the individual HPIs block the Shh-induced differentiation of C3H10T(1/2) cells into alkaline phosphatase-expressing osteoblasts. Data are the average of triplicate samples ± SD.

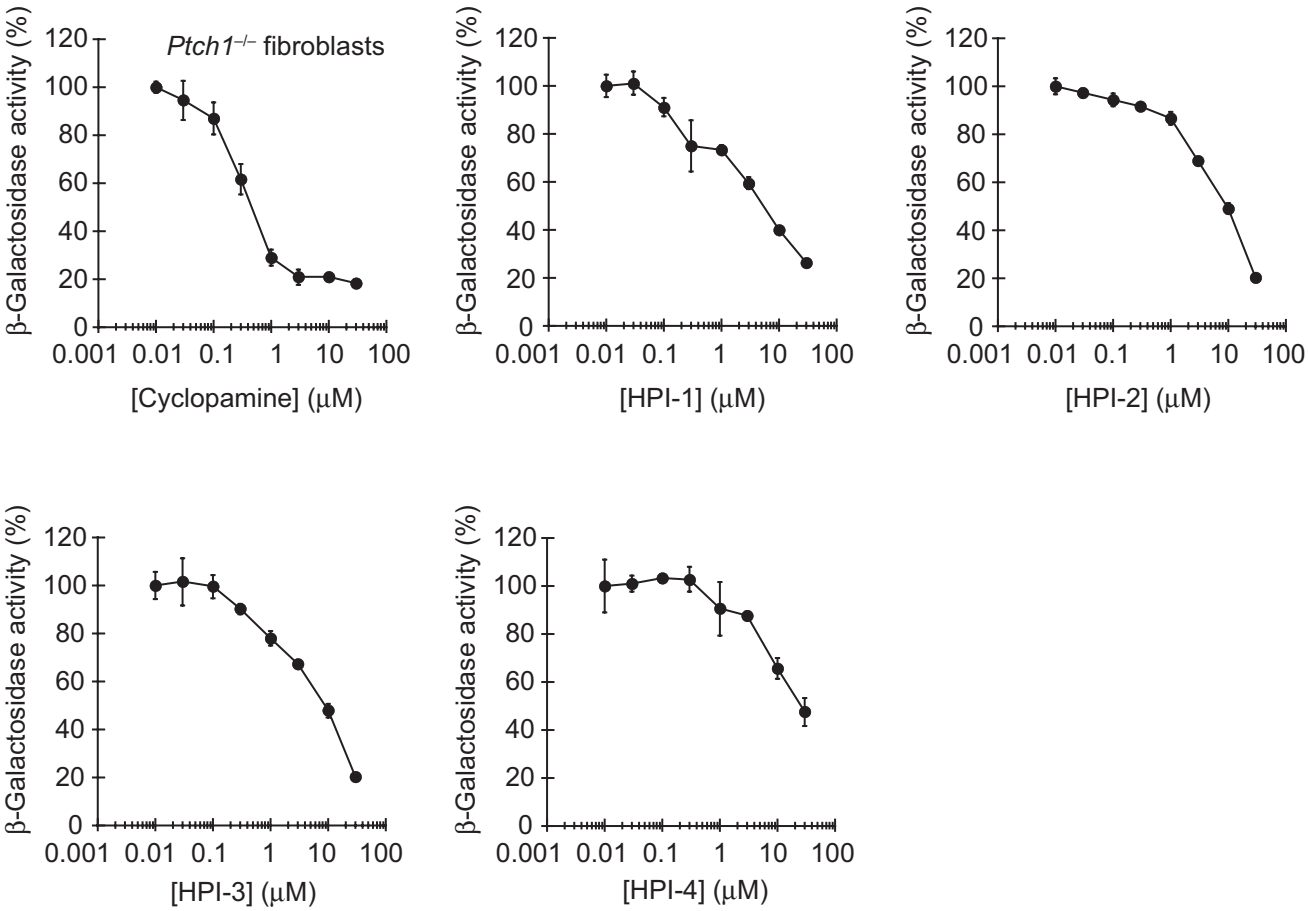

Fig. S4. Inhibition of constitutive Hh pathway activity in *Ptch1*$^{-/-}$ fibroblasts by the HPIs. Cyclopamine and the individual HPIs inhibit Hh pathway activity in *Ptch1*$^{-/-}$ fibroblasts as determined by cellular levels of the β-galactosidase reporter knocked into the *Ptch1* coding region. Data are the average of triplicate samples ± SD.

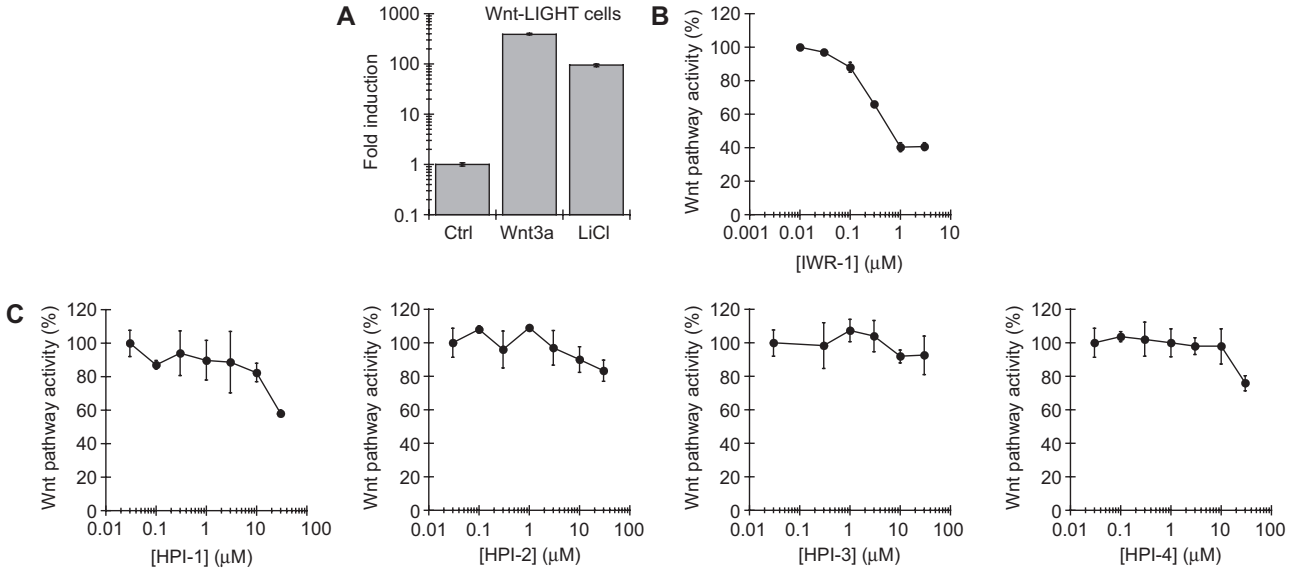

Fig. S5. The HPIs do not inhibit Wnt signaling. (*A*) Activation of the TCF/LEF-dependent firefly luciferase reporter in Wnt-LIGHT cells upon stimulation with either Wnt3a-conditioned medium or 30 mM LiCl. (*B*) Inhibition of Wnt3a signaling in this cell line by Axin-stabilizing compound IWR-1. (*C*) None of the HPIs significantly inhibit Wnt3a-induced pathway activation in Wnt-LIGHT cells at concentrations associated with Hh pathway blockade. Data are the average of triplicate samples ± SD.

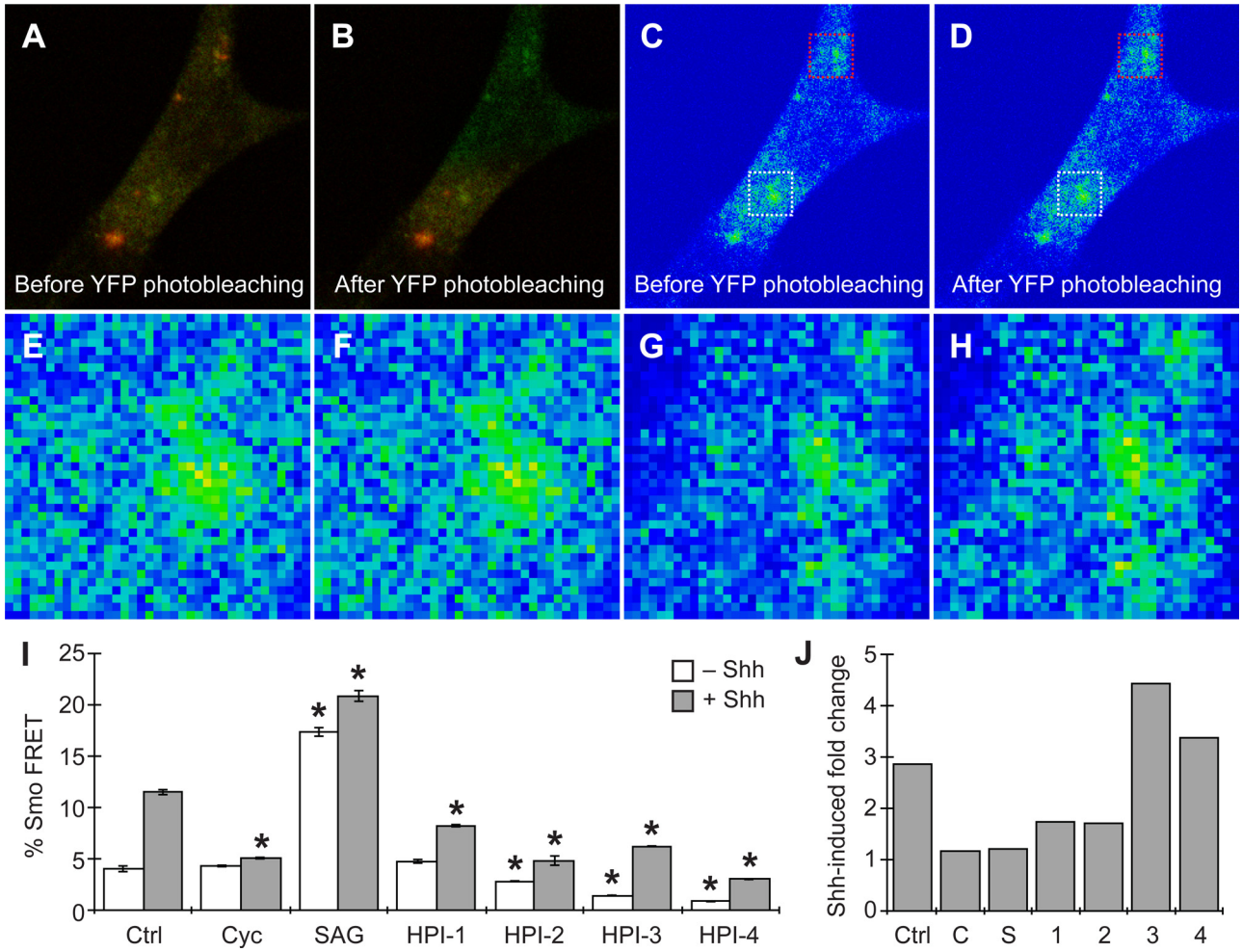

Fig. S6. Modulation of basal and Shh-induced Smo-CFP/Smo-YFP FRET by the HPIs. (*A* and *B*) Merge of Smo-CFP (green) and Smo-YFP (red) fluorescence in transfected NIH 3T3 cells before and after the top half of the cell is subjected to YFP photobleaching. (*C* and *D*) False color images of Smo-CFP fluorescence intensities before and after YFP photobleaching, with selected regions within the non-photobleached and photobleached halves indicated by the white and red squares, respectively. (*E*) Close-up view of the white-bordered region in panel *C*. (*F*) Close-up view of the white-bordered region in panel *D*. (*G*) Close-up view of the red-bordered region in panel *C*. (*H*) Close-up view of the red-bordered region in panel *D*. (*I*) Percentage of Smo-CFP/Smo-YFP FRET associated with 10 μM cyclopamine, 0.5 μM SAG, or 10 μM doses of the HPIs. Data are the average of two independent experiments $\pm$ SD., with at least 10 individual cells analyzed in total for each compound treatment. Asterisks indicate $P < 0.001$ for Smo-CFP/Smo-YFP FRET levels associated with compound treatment vs. the DMSO control. (*J*) Shh-induced fold change in Smo-CFP/Smo-YFP FRET for each condition. Note that Shh treatment causes an increase in Smo-CFP/Smo-YFP FRET, which is blocked by cyclopamine. Smo-CFP/Smo-YFP FRET is also Shh-insensitive in cells treated with SAG, since the compound alone can fully activate Smo. The HPIs exhibit partial effects on the Shh-induced fold-change in Smo-CFP/Smo-YFP FRET and/or basal FRET levels.

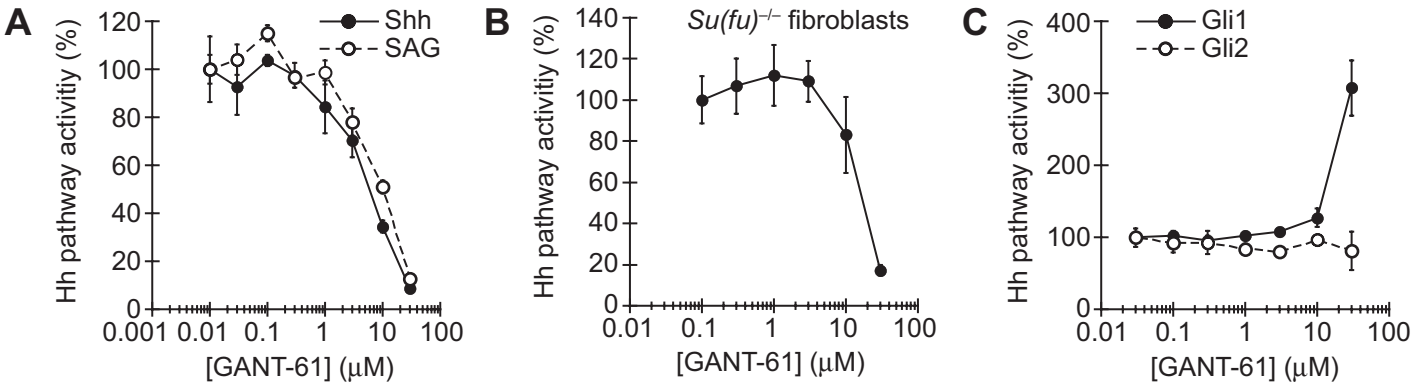

Fig. S7. Epistatic mapping of GANT-61 activity relative to Shh, Smo, Su(fu), Gli1, and Gli2. (*A*) GANT-61 inhibits Hh pathway activity in Shh-LIGHT2 cells induced by Shh-conditioned medium or 0.5 μM SAG with similar potencies. Data are the average of triplicate samples ± SD. (*B* and *C*) GANT-61 blocks the constitutive Hh pathway activity in *Su(fu)*$^{-/-}$ fibroblasts but is ineffective against Hh pathway activation in NIH 3T3 cells induced by Gli1 or Gli2 overexpression. Hh pathway activity was measured with a transfected Gli-dependent firefly luciferase reporter and a constitutive *Renilla* luciferase expression construct, and data are the average of triplicate samples ± SD. The apparent increase in Gli1-dependent luciferase activity at high GANT-61 concentrations is due to a decrease in *Renilla* luciferase levels that may be indicative of compound cytotoxicity.

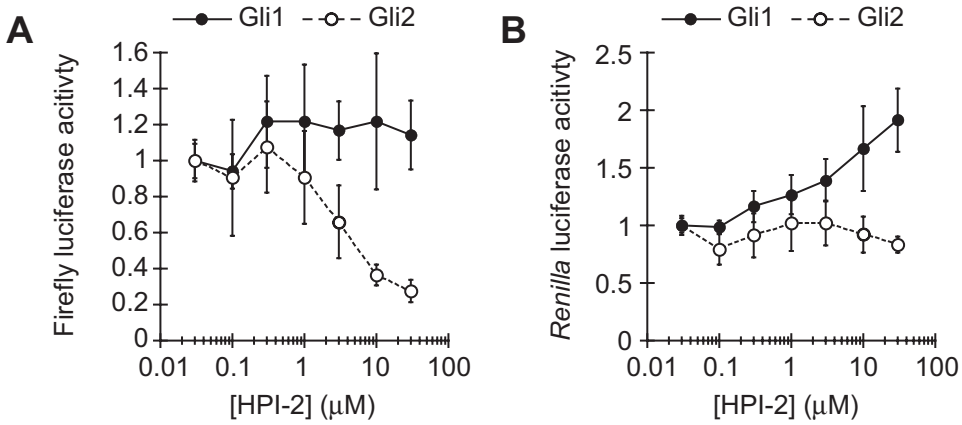

Fig. S8. HPI-2 selectively inhibits Gli2. (*A*) Relative firefly luciferase levels in Shh-LIGHT2 cells transfected for Gli1 or Gli2 overexpression and treated with various doses of HPI-2. (*B*) Corresponding *Renilla* luciferase levels under the same experimental conditions. Note that the effect of HPI-2 on the relative luciferase activity in Gli1-expressing cells is primarily due to an increase in *Renilla* luciferase levels. Data are the average of triplicate samples ± SD.

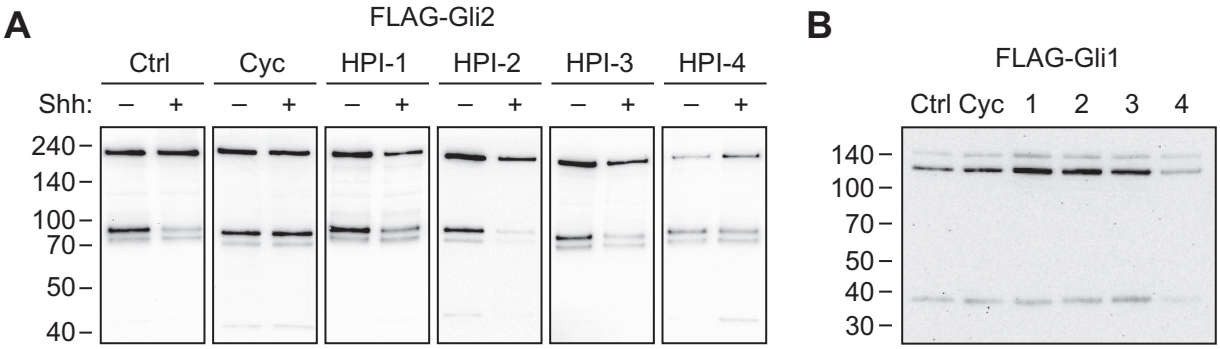

Fig. S9. Effects of the HPIs on Gli processing and stability. (*A*) Whole-gel view of the western data shown in Fig. 4*A*, demonstrating that full-length and repressor forms of FLAG-Gli2 are the predominant isoforms. (*B*) Whole-gel view of the western data shown in Fig. 4*B*. In addition to the full-length FLAG-Gli1 protein, a 38-kDa fragment presumably resulting from protein degradation was also observed.

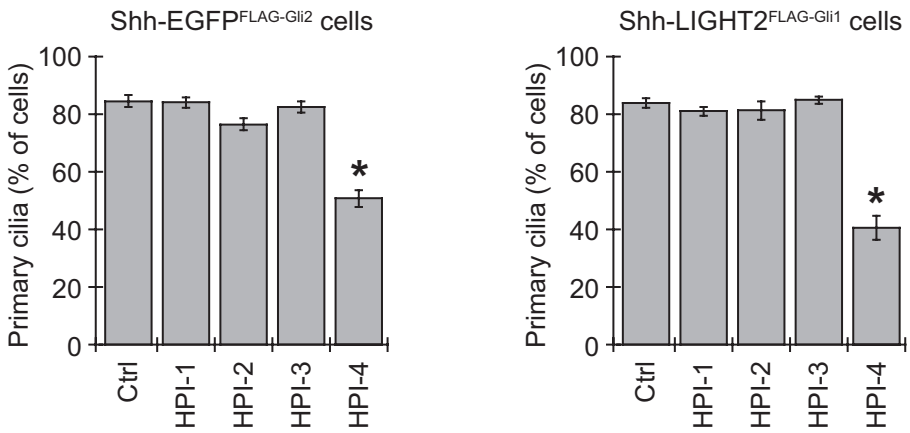

Fig. S10. Effects of the HPIs on ciliogenesis. HPI-1, HPI-2, and HPI-3 do not affect ciliogenesis in either Shh-EGFP$^{FLAG-Gli2}$ or Shh-LIGHT2$^{FLAG-Gli1}$ cells. However, HPI-4 disrupts primary cilia formation in both cell lines. Data for each experimental condition are the average of at least 12 independent images ± SEM., each containing approximately 30 cells. Asterisks indicate $P < 0.001$ for the percentage of cells with primary cilia associated with compound treatment vs. the DMSO control.

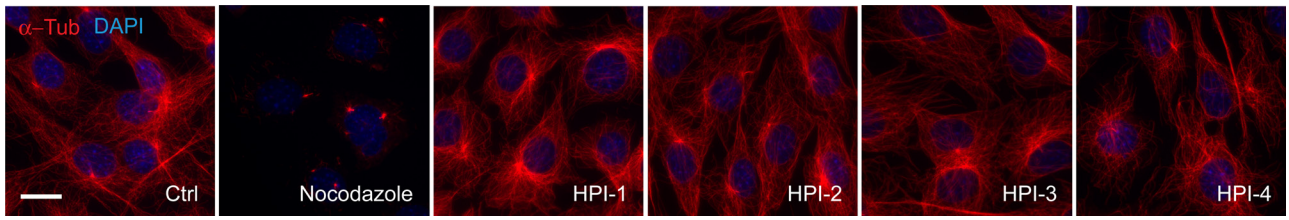

Fig. S11. The HPIs do not perceptibly alter the general microtubule cytoskeleton. NIH 3T3 cells were treated with the individual HPIs and stained with an antibody against α-tubulin. Doses of 15 μM HPI-1, 20 μM HPI-2, 30 μM HPI-3, and 30 μM HPI-4 were tested, and DMSO and 20 μM nocodazole were used as negative and positive controls, respectively. (Scale bar, 10 μm.)

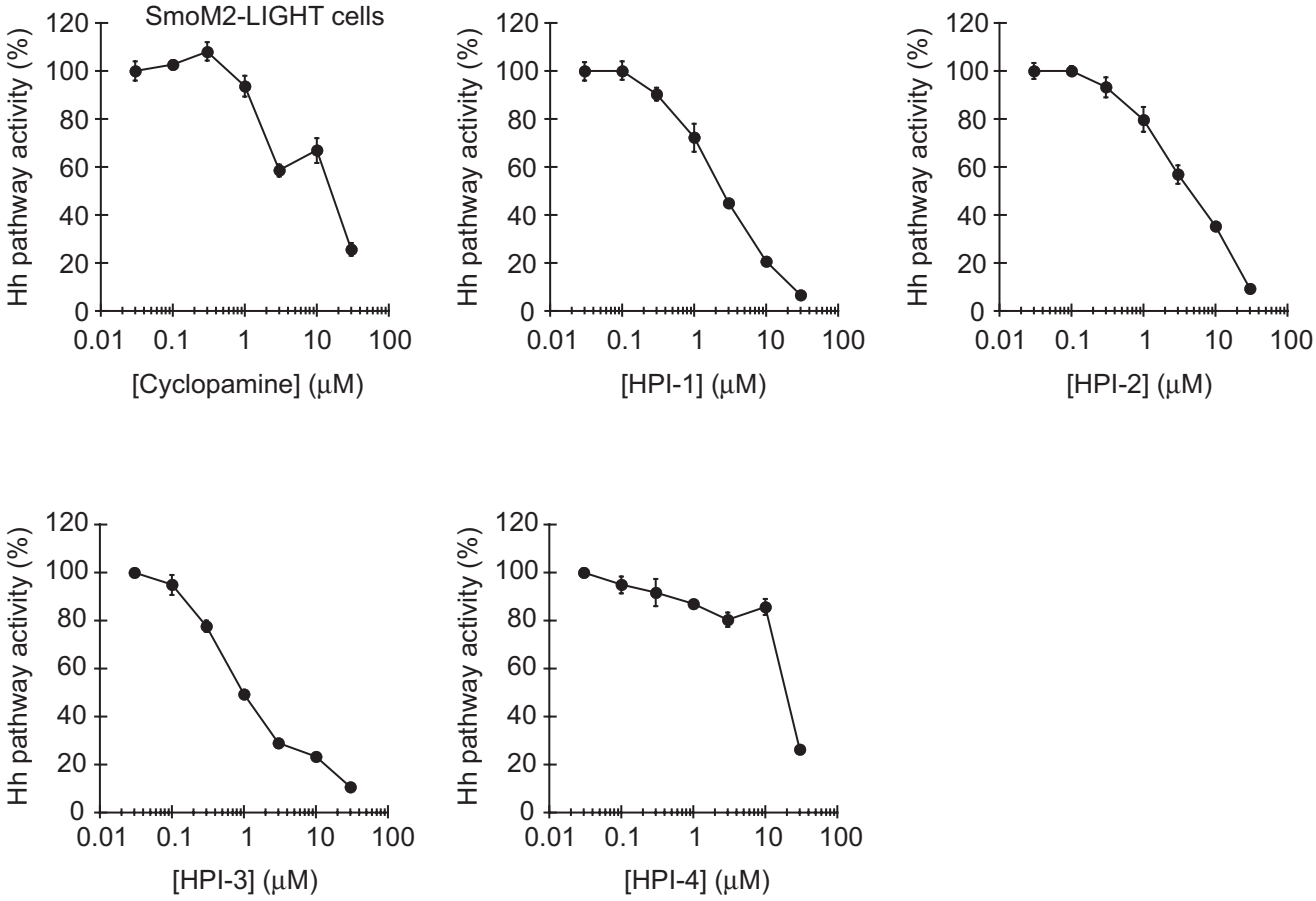

Fig. S12. Inhibition of Hh pathway activity in SmoM2-LIGHT cells by the HPIs. The constitutive Hh pathway activity in SmoM2-LIGHT2 cells is inhibited by the HPIs with IC_{50}s similar to those observed in Shh-stimulated Shh-LIGHT2 cells (see Fig. 1*C* and Table 1). In contrast, cyclopamine is significantly less potent against SmoM2-dependent pathway activity (see Fig. 1*A*). Data are the average of triplicate samples ± SD.