Supporting Information

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SI Materials and Methods

Antibodies. Rabbit anti-Smo (a gift from Helene Faure, Institut de Neurobiologie Alfred Fessard, Gif-sur-Yvette, France), mouse anti-Smo (Santa Cruz Biotechnology) for Western blot analysis, mouse anti-acetylated tubulin (Sigma-Aldrich), mouse anti–α-tubulin (Sigma-Aldrich), rabbit anti–γ-tubulin (Sigma-Aldrich), chicken anti-GFP (Aves Labs), and mouse anti-BrdU (Neo-Markers) were used for immunofluorescence analyses.

Cell Lines. NIH 3T3 and HEK293T cells were obtained from American Type Culture Control. *Ptch1*$^{-/-}$ fibroblasts, *Sufu*$^{-/-}$ fibroblasts, and Shh-LIGHT2 cells were described previously (1–3). *Smo*$^{-/-}$ MEFs were a gift from J. Taipale (Karolinska Institutet, Stockholm, Sweden). SNAP-Smo cells were a gift from R. Rohatgi (Stanford University, Stanford, CA). A SmoM2 overexpression construct was transfected into *Smo*$^{-/-}$ MEFs to assess the ability of SAs to inhibit oncogenic Hh pathway activity. IMCD3 Smo-YFP cells were generated by transfecting IMCD3 cells with a Smo-YFP reporter construct and selecting stable clones. ASZ1 cells (a gift from E. Epstein, Children's Hospital, Oakland, CA) were used to examine the localization of endogenous ciliary Smo and expression of downstream Hh pathway mediators. BATgal MEFs were used for Wnt activity assays as described previously (4).

Library Screening Conditions Using IMCD3 Smoothened-YFP Cells. For the screen, IMCD3 cells stably expressing YFP-tagged Smoothened (Smo) were cultured in DMEM/F12 containing 10% (vol/vol) FBS, 15 mM Hepes buffer, 400 μg of geneticin (G418), and 1× antibiotic/antimycotic. To screen a total of 12,250 compounds, cells were plated onto 188-μm-thick, 384-well black microtiter imaging plates (Aurora Biotechnologies) at 4×10^4 cells/well in 50 μL of media. Cells were dispensed using a Matrix WellMate bulk dispenser (Thermo Scientific). The cells were incubated at 37 °C for 24 h. After 24 h, compounds were added as follows: 50 nL of compound (10 mM) in 100% DMSO was transferred to cells using a 384 floating pin tool fitted with 50 nL hydrophobic-coated slot pins (V&P Scientific) attached to a Biomek FX^P workstation (Beckman Coulter). Positive and negative controls were plated in an identical manner. Compounds were dispersed into the culture media by dipping pins into the assay plate and shaking on a Variomag Teleshake 1536 (Thermo Scientific) at 2,000 rpm. The final concentration of compound after transfer was 10 μM in 0.1% DMSO. Cells were then incubated at 37 °C for another 16–20 h.

After 16–20 h, cells were live-imaged for the presence of ciliary Smo-YFP. Before imaging, 10 μL/mL of Hoescht 34442 (Sigma-Aldrich), a live cell nuclear dye, was added using the Matrix WellMate bulk dispenser for a final concentration of 5 μg/mL in 60 μL. Cells were incubated with Hoescht 34442 at 37 °C for 30 min, followed by washing with 50 μL of 1× PBS using a Biotek EL406 cell washer. The cells were then imaged in 1× PBS to reduce autofluorescence from the media, which vastly improved the signal-to-noise ratio.

Cells were imaged with an IN Cell 2000 automated multimodal fluorescence microscope (GE Healthcare). The entire well was captured as a single field using the 10× objective in combination with the large-format 4-megapixel CCD sensors. Images were captured at a rate of 3 plates per hour.

Image Analysis. The image stack of each plate was segmented and quantified using IN Cell Developer 1.7 (GE Healthcare). The nuclei were segmented using object segmentation (kernel 9, sensitivity, 95%) and postprocessed using erosion (kernel 7) and sieved to keep objects ≥1.32 mm^2. The nuclei target set was used to "clump break" a second nuclei target set, which was segmented using object segmentation (kernel 9; sensitivity, 95%), postprocessed using dilation (kernel 5), followed by a clump break and then a final postprocess erosion (kernel 5).

Cilia were segmented using object segmentation (kernel 3; sensitivity, 1%). One-to-one target linking was used to identify primary cilium entirely enclosed within the nuclear border of the clump-broken nuclei target set. If more than one cilia-like object was detected within the border of the nucleus, then the larger object was selected as the best match. From these target sets, the total number of nuclei and linked cilia were measured to produce a normalized fraction of cells ciliated, defined as

$$\text{Fraction of Cells Ciliated} = \text{Total Number of Linked Cilia}/\text{Total Number of Cells}$$

This assay had a Z-factor of 0.6 ± 0.09, using 0.1% DMSO as the maximum signal and 10 μM of SMDC 172294 (SA3) as the minimum signal.

ChemDiv Identification of Smo Antagonists (SAs), Ciliogenesis Antagonists (CAs), and Negative Controls (NCs).

SA1: C561-0772
SA2: C561-0769
SA3: 8012–8916
SA4: C614-1058
SA5: C614-0211
SA6: C696-0083
SA7: C736-0402
SA8: C741-0238
SA9: C741-0580
SA10: C548-3499
CA1: C618-0518
CA2: C614-5661
NC3: C548-3627
NC4: C548-3631
NC5: C548-3546

Generation of IMCD3 Smo-YFP Cell Line. Full-length mouse Smo cDNA was cloned into the Clontech Living Color vector EYFP, conferring kanamycin and G418 resistance to the construct. The Smo-EYFP construct was transfected into IMCD3 cells that were 70% confluent using JetPei transfection reagent (Polyplus) according to the manufacturer's instructions, and then cultured for 24 h in DMEM, 10% (vol/vol) FBS, and 1× antibiotic/antimycotic. At 24 h after transfection, the standard DMEM media was replaced with DMEM containing G418 at three different concentrations: 400 μg/mL, 500 μg/mL, and 600 μg/mL. Cells were placed under selection for 2 wk with daily media changes. Most cells treated with 500 μg/mL and 600 μg/mL of G418 died during the selection process, and then clones were selected from cells placed under 400 μg/mL G418 selection. After 2 wk, 20 stable clones were chosen and allowed to grow to confluence. Of the 20 clones, clones 6 and 7 had the best Smo-YFP localization to the primary cilium, with clone 7 exhibiting the best overall ciliary Smo-YFP signal. All subsequent screening was done with IMCD3-SmoYFP cells derived from clone 7.

SSTR3 Inhibition by SA Assay. ASZ1 cells were transiently transfected with 1 μg of a construct expressing SSTR3-GFP. Cells were transfected using jetPRIME (Polyplus) according to the manufacturer's instructions. At 24 h after transfection, cells were fixed with 4% (wt/vol) PFA for 10 min and blocked in 2.5% (wt/vol) BSA plus 0.1% Triton-X for 1 h. Anti-chicken GFP primary antibody (Aves Labs) and anti-mouse acetylated tubulin (Sigma-Aldrich) were used at a dilution of 1:1,000. Cells were incubated in primary antibodies overnight at 4 °C. After overnight incubation, cells were washed three times for 10 min each, followed by addition of secondary antibodies Alexa Fluor 488 donkey anti-chicken and Alexa Fluor 555 donkey anti-mouse (both 1:400; Molecular Probes). Cells were incubated in secondary antibodies for 1 h and then washed in 1× PBS three times, after which the coverslips were mounted and imaged. Cells were imaged with a Nikon C1si Spectral Imaging confocal microscope with a 100× objective. Images were analyzed using ImageJ software.

SA Treatment Apoptosis Assay. ASZ1, NIH 3T3, and IMCD3 Smo-YFP cells were plated onto coverslips in 24-well plates at 60% confluence. Cells were treated with 25 μM SA1–10 in full media for 5 d, with the addition of new full-serum media plus 25 μM SAs every other day for 5 d. After the 5-d incubation, cells were fixed, and the number of cells undergoing apoptosis was quantitated using the Roche In Situ Cell Death Detection Kit, Fluorescein, in accordance with the manufacturer's instructions.

Immunostaining in ASZ Cells and Quantification of Cilia. ASZ1 cells were plated onto coverslips in 24-well plates (25,000 cells/well) and cultured to confluence in 154-CF medium (Cascade Biologicals), 2% (vol/vol) chelexed FBS, 1× antibiotic/antimycotic, and 0.5 mM calcium chloride. Chelexed FBS was prepared as described previously (5). After confluence, cells were treated with 25 μM SA compounds and LDE225 or DMSO and cultured for another 24 h in Opti-MEM reduced-serum media. After 24 h, cells were fixed in 100% methanol for 7 min and washed with 1× PBS three times for 5 min each and blocked in 2.5% (wt/vol) BSA plus 0.1% Triton-X for 1 h. Anti-rabbit Smo primary antibody (a gift from Helene Faure, Institut de Neurobiologie Alfred Fessard, Gif-sur-Yvette, France) was used at a dilution of 1:200, and anti-mouse acetylated tubulin (Sigma-Aldrich) was used at 1:1,000. Cells were incubated in primary antibodies overnight at 4 °C. After the overnight incubation, cells were washed three times for 10 min each, followed by the addition of secondary antibodies Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 555 donkey anti-mouse (both at 1:400; Molecular Probes). Cells were incubated in the secondary antibodies for 1 h and washed three times in 1× PBS, after which the coverslips were mounted and imaged. Cells were imaged using a Nikon C1si Spectral Imaging confocal microscope with a 100× objective, and images were processed with Nikon Elements software. Ciliary Smo levels were quantified by designating ciliary regions according to acetylated tubulin staining and obtaining the pixel intensity of the corresponding region of Smo antibody staining. Between 30 and 50 cilia were analyzed for each compound, and pixel intensities were averaged.

Localization of Endogenous Smo to the Ciliary Membrane in NIH 3T3 Cells. NIH 3T3 cells were plated onto coverslips in 24-well plates and grown until confluence. Cells were treated with 25 μM SA8 and SA9 and 500 nM Smoothened agonist (SAG; EMD Bioscience) for 7 h in DMEM containing 10% (vol/vol) FCS and 1× antibiotic/antimycotic. After 7 h, cells were fixed in 100% methanol for 7 min, washed three times with 1× PBS for 5 min each, and blocked in 2.5% (wt/vol) BSA plus 0.1% Triton-X for 1 h. Anti-rabbit Smo primary antibody (a gift from Helen Faure, Institut de Neurobiologie Alfred Fessard, Gif-sur-Yvette, France) was used at a dilution of 1:200, and anti-mouse acetylated tubulin (Sigma-Aldrich) was used at 1:1,000. Cells were incubated in primary antibodies overnight at 4 °C. After the overnight incubation, cells were washed three times for 10 min each, after which secondary antibodies Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 555 donkey anti-mouse (both at 1:400; Molecular Probes) were added. Cells were incubated in the secondary antibodies for 1 h, washed three times in 1× PBS, and the coverslips were mounted and imaged. Cells were imaged using a Nikon C1si Spectral Imaging confocal microscope with a 100× objective.

Smo Trafficking Assay. SNAP-Smo–expressing fibroblasts were plated in 24-well plates in duplicate and grown until confluence. To determine whether SA treatment affected the lateral trafficking of Smo, live SNAP-Smo cells were labeled with SNAP-Surface 488 (1:200; New England Biolabs) for 30 min at 37 °C. Cells were washed three times for 10 min with full media to remove the SNAP-Surface 488, then incubated with 25 μM SA1–10 plus 500 nM SAG for 1 h. After 1 h, the cells were washed with PBS and fixed in 4% (wt/vol) PFA for 10 min.

To determine whether SA treatment inhibited intracellular Smo trafficking, Surface SNAP-Smo was first blocked using SNAP-Surface Block (1:200; New England Biolabs) for 30 min in DMEM plus 20% (vol/vol) FBS. After blocking, cells were rinsed twice in full media and then washed three times for 15 min each in full media. The cells were then incubated overnight in 500 nM SAG plus 25 μM SA1–10 in DMEM plus 0.5% FBS. The next day, cells were washed two times in full media and incubated with SNAP-Cell TMR Star (1:400; New England Biolabs), a cell-permeable, red fluorescent SNAP substrate, in DMEM plus 20% (vol/vol) FBS for 30 min. After labeling with SNAP-Cell TMR Star, cells were washed two times for 15 min in full media plus 500 nM SAG and SAs. The SAs were added to the wash to prevent the possibility of any newly synthesized, untreated Smo from becoming labeled by any SNAP-Cell TMR remaining after the wash and localizing to the cilium. After the incubation, cells were washed in full media and PBS and fixed for 10 min with 4% (wt/vol) PFA. For both assays, after PFA fixation, the samples were washed three times with 1× PBS for 5 min each and blocked in 2.5% (wt/vol) BSA plus 0.1% Triton-X for 1 h. Anti-mouse acetylated tubulin (Sigma-Aldrich) was used at a dilution of 1:1,000. Cells were incubated in primary antibody overnight at 4 °C. After the overnight incubation, cells were washed three times for 10 min each, followed by the addition of secondary antibodies Alexa Fluor 555 donkey anti-mouse for the lateral trafficking assay and Alexa Fluor 488 donkey anti-mouse for the intracellular trafficking assay (both at 1:400; Molecular Probes). Cells were incubated in secondary antibodies for 1 h and then washed three times in 1× PBS, after which the coverslips were mounted and imaged. Cells were imaged using a Nikon C1si Spectral Imaging confocal microscope with a 100× objective.

BODIPY-Cyclopamine/Smo Binding Assay. HEK293T cells were plated in 48-well plates (Thermo Scientific) treated with 5% poly-D-lysine (Sigma-Aldrich; 80,000 cells/well) in DMEM containing 10% (vol/vol) FBS and 1× antibiotic/antimycotic. Cells were cultured for 14 h until they reached ∼60% confluence, then transfected with a Smo-myc$_3$ expression construct under the CMV promoter using jetPRIME (Polyplus) according to the manufacturer's instructions. At 24 h after transfection, the cells were washed with 1× PBS and incubated with phenol-red free DMEM containing 0.5% FBS and varying concentrations of SAs, CAs, cyclopamine, or DMSO for 30 min. After 30 min, 10 μM Hoescht 33342 was added, and the cells were incubated for another 30 min. The cells were then washed with 1× PBS and phenol-red free DMEM and immediately imaged using a Zeiss Axio Observer D1 compound microscope at a 40× objective. Images were background-subtracted using ImageJ with a rolling

ball size of 75 pixels, and BODPIY-cyclopamine intensity was assessed using Nikon Elements software. Circular regions with an area of 21 pixels were placed randomly over areas of evenly confluent cells, and pixel intensities from ~20 individual regions from eight independent images were averaged and used to determine the average BODIPY-cyclopamine levels for each experimental condition.

Assay for SA and CA1 Activity by Quantitative RT-PCR. ASZ1 cells or *Ptch1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were plated into 48-well plates (200,000 cells/well) and cultured to confluence in 154-CF medium, 2% (vol/vol) chelexed FBS, and 0.5 mM calcium chloride for ASZ1 cells, and in DMEM and 10% (vol/vol) FBS and 1× antibiotic/antimycotic for *Ptch1*$^{-/-}$ MEFs. After confluence, ASZ1 and *Ptch1*$^{-/-}$ MEFs were treated with 25 μM SA1-10, CA1, LDE225, and Vismodegib or DMSO in Opti-MEM reduced-serum media for an additional 24 h. After 24 h, RNA was isolated from the cells using the RNAeasy RNA Extraction Kit (Qiagen) according to the manufacturer's instructions. cDNA was prepared from 100 ng of total RNA using the Maxima First-Strand cDNA Synthesis Kit (Fermentas). cDNA was quantified with *Gli1*, *Ptch1*, and *β-actin* primers on a LightCycler system (PerkinElmer/Roche) using the second derivative/maximum method to determine C_t values. Biological quadruplicates were analyzed to determine the percent change in each SA and CA relative to the 100% pathway activation as determined by the DMSO control. Primer sequences were as follows: for *Gli1*, 5′ GGT GCT GCC TAT AGC CAG TGT CCT C 3′ and 5′ GTG CCA ATC CGG TGG AGT CAG ACC C 3′; for *Ptch1*, 5′ TGT CTG GAG TCC GGA TGG A 3′ and 5′ TGA TTG TGG AAG CCA CAG AAA A 3′; for *β-actin*, 5′ GGC CCA GAG CAA GAG AGG TAT CC 3′ and 5′ ACG CAC GAT TTC CCT CTC AGC 3′.

In addition, to determine whether any of the SA compounds inhibited transcription of *Smo*, ASZ1 cells were plated and treated as above and treated with 25 μM SAs, Vismodegib, and cyclopamine or DMSO. After 24 h, RNA was isolated from the cells as before, and *Smo* expression was quantified as above using the *Smo* primers 5′ CCA GTA ACC CTG TCC TGC AT 3′ and 5′ AAG GGA AAA CCA CAC AGC AC 3′.

To determine whether the structural analogues NC1 and NC2 could inhibit Hedgehog (Hh) pathway activity in ASZ1 cells, ASZ1 cells were plated and treated as above and treated with 25 μM SA7-9 and NC1 and NC2 for 24 h. After 24 h, RNA was isolated from the cells as before, and *Gli1* expression was quantified as above.

Gli Luciferase Assay for Hh Pathway Activity. Shh-LIGHT2 cells were plated into 96-well plates (2,000 cells/well) and cultured to confluence in DMEM containing 10% (vol/vol) FCS, 1× antibiotic/antimycotic, 0.4 mg/mL of G418, and 0.15 mg/mL of zeocin. On confluence, cells were treated with various concentrations of the SAs, CAs, and cyclopamine or DMSO, plus 500 nM SAG in Opti-MEM reduced-serum media for 24 h. After 24 h, firefly and *Renilla* luciferase activity were measured using the Promega Dual Luciferase Kit and a Veritas microplate luminometer (Promega).

To determine whether the SA10 analogues NC3–5 could inhibit Hh pathway activity, Shh-LIGHT2 cells were cultured as above and treated with 25 μM SA10, NC3, NC4, NC5, and LDE225 or DMSO plus 500 nM SAG in Opti-MEM for 24 h. After 24 h, firefly and *Renilla* luciferase activity were measured using the dual luciferase kit and Veritas microplate luminometer (Promega).

Wnt Pathway Activity Assay. β-galactosidase reporter under the control of canonical Wnt signals (BATgal) MEFs were plated into 96-well plates (1,100 cells/well) and cultured to confluence in DMEM containing 10% (vol/vol) FBS and 1× antibiotic/antimycotic. On confluence, the BATgal MEFs were treated with 10 ng of recombinant mouse Wnt3a (R&D Systems), 25 μM SA compounds, and 10 μM Wnt pathway inhibitor Dkk-1 (R&D Systems) or DMSO. The BATgal MEFs were incubated for 24 h, after which cellular β-galactosidase levels were assessed using the Galato-Light β-Galactosidase Reporter Gene Assay System (Applied Biosystems) according to the manufacturer's instructions.

SmoM2-Induced Hh Pathway Activity Assay. *Smo*$^{-/-}$ MEFs were plated into 96-well plates (1,000 cells/well) and transfected with pGEN-SmoM2-YFP, a SmoM2 overexpression construct, Gli luciferase, and *Renilla* reporters using jetPRIME according to the manufacturer's instructions and cultured for 24 h in DMEM, 10% (vol/vol) FBS, and 1× antibiotic/antimycotic. After 24 h, transfected cells were treated with 25 μM SA1-10, Vismodegib, LDE225, and cyclopamine or DMSO for an additional 24 h, after which firefly and *Renilla* luciferase activity were measured using the dual luciferase kit and Veritas microplate luminometer (Promega).

***Sufu*$^{-/-}$ Activity Assay.** *Sufu*$^{-/-}$ MEFs were plated in 48-well plates (250,000 cells/well) and cultured in DMEM containing 10% (vol/vol) FBS and 1× antibiotic/antimycotic until confluent. On confluence, cells were cultured in Opti-MEM reduced serum media with 25 μM SA1–10, LDE225, and cyclopamine or DMSO for an additional 24 h. After 24 h, RNA was isolated and cDNA was prepared as described above. *Gli1* mRNA expression levels were analyzed using qRT-PCR primers for *Gli1* as described above.

Smo Immunoblot Analysis. IMCD3 Smo-YFP cells were grown in 10-cm plates until confluent. On confluence, the cells were treated with 25 μM SA1–10 for 24 h, then washed with PBS and lysed with 300 μL of RIPA buffer plus protease inhibitor (1:200; Roche). Cell lysate was collected, and 40 μg of total protein was used for Western blot analyses. Smo protein was detected using the mouse anti-Smo primary antibody (1:500; Santa Cruz Biotechnology).

Analysis of ASZ1 Cell Proliferation. ASZ1 cells were plated onto coverslips in a 24-well plate (150,000 cells/well) and cultured for 24 h in Opti-MEM low-serum medium with 25 μM SA1–10 or DMSO for 24 h. After 24 h, standard full-serum ASZ1 mediuim, 154-CF medium, 2% (vol/vol) chelexed FBS, and 0.5 mM calcium chloride were added. Then 25 μM SA1–10, cyclopamine or DMSO, and 10 μM bromodeoxyuridine (BrdU; Sigma-Aldrich) were added to the cells for another 24 h. On the addition of complete medium, cells were released from the cell cycle arrest induced by starvation, and cell division resumed. After 24 h, cells were fixed in 4% (wt/vol) PFA for 15 min and then denatured with 4N HCl for 10 min. The HCl was quenched by the addition of 0.1 M sodium borohyride (Sigma-Aldrich) and three washings with 1× PBS. Cells were blocked in 2.5% (wt/vol) BSA plus 0.1% Triton-X for 1 h. Cells were incubated in primary antibody (mouse anti-BrdU antibody, 1:100; NeoMarkers) overnight at 4 °C. After primary incubation, cells were washed three times in 1× PBS for 10 min each, and the secondary antibody (Alexa Fluor 488 donkey anti-mouse; 1:400), was added. Cells were incubated in secondary antibody for 1 h at room temperature, following by three washings in 1× PBS and coverslip mounting. Cells were imaged with a Zeiss Axio Observer D1 compound microscope with a 63× objective. The total numbers of BrdU-positive and BrdU-negative cells were counted using ImageJ software.

Flow Cytometry Analysis of SA-Treated ASZ1 Cells. ASZ1 cells were plated onto 10-cm plates and grown to 60% confluence, then treated with 25 μM SA1–10 for 24 h. After 24 h, cells were treated with 10 μg/mL Hoechst 33342 for 30 min, washed three times with PBS, and harvested. After harvesting, cells were resuspended in 500 μL of FACS buffer with 10 μg/mL of propidium iodide (Invitrogen). Cells were collected with a FACSAria cell sorter (BD Biosciences).

Immunostaining and Analysis of α-Tubulin Microtubule Cytoskeleton and γ-Tubulin Centrosomes. ASZ1 and IMCD3 Smo-YFP cells were plated onto coverslips in a 24-well plate (500,000 cells/well), and cultured until confluence in 154-CF medium, 2% (vol/vol) chelexed FBS, 1× antibiotic/antimycotic, and 0.5 mM calcium chloride for ASZ1 cells and in DMEM/F12 containing 10% (vol/vol) FBS, 15 mM Hepes buffer, 400 μg of G418, and 1× antibiotic/antimycotic for IMCD3 Smo-YFP cells. After confluence, ASZ1 cells were treated with 10 μM CA2 in Opti-MEM reduced-serum medium for 24 h, and IMCD3 Smo-YFP cells were treated with 10 μM CA1 in Opti-MEM reduced-serum medium for 24 h. After 24 h, cells were washed with 1× PBS and fixed in 100% methanol for 2 min. Cells were washed three times with 1× PBS for 5 min each and then blocked in 2.5% (wt/vol) BSA with 0.1% Triton-X for 1 h. Mouse anti–α-tubulin primary antibody (Santa Cruz Biotechnology) and rabbit anti–γ-tubulin primary antibody (Sigma-Aldrich) were used at a 1:1,000 dilution. Cells were incubated in primary antibodies for 2 h at room temperature in blocking solution, followed by the addition of secondary antibodies Alexa Fluor 488 donkey anti-mouse and Alexa Fluor 555 donkey anti-rabbit (both at 1:400; Molecular Probes). Cells were incubated in secondary antibodies for 1 h, then washed three times in 1× PBS and coverslip-mounted. Cells were imaged using a Nikon C1si Spectral Imaging confocal microscope with a 100× objective.

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5. So PL, et al. (2006) Long-term establishment, characterization and manipulation of cell lines from mouse basal cell carcinoma tumors. *Exp Dermatol* 15:742–750.

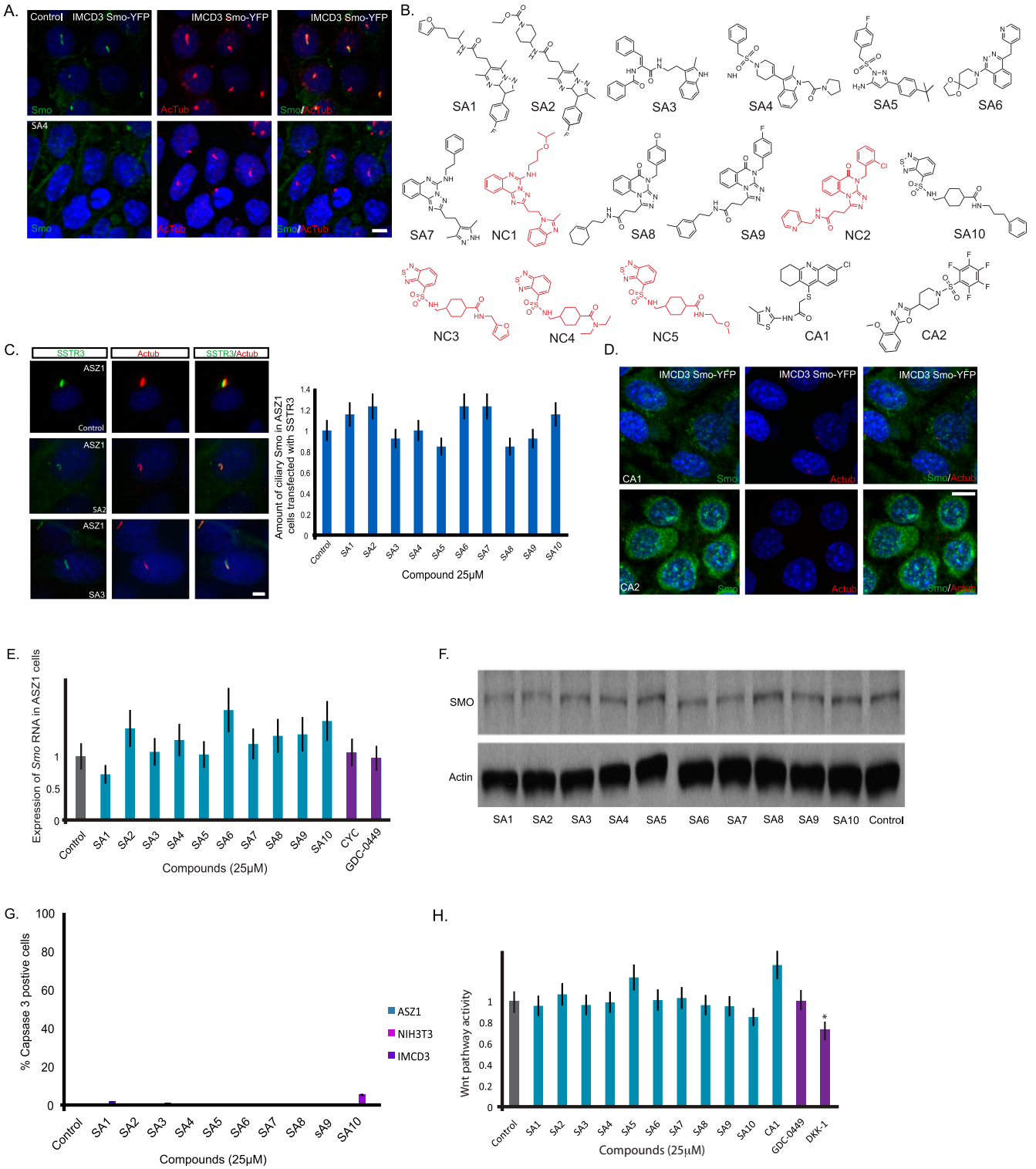

Fig. S1. (*A*) IMCD3 Smo-YFP cells treated with 25 μM SA4 display abrogated ciliary Smo-YFP localization; all SAs behave similarly. Acetylated tubulin is not affected in treated cells. (*B*) Depictions of the chemical structures of SAs (SA1–10), CAs (CA1 and CA2), and NCs (NC1–5). (*C*) The SA compounds do not prevent the ciliary localization of SSTR3. ASZ1 cells transiently transfected with SSTR3-GFP and treated with SA1–10 show no loss of SSTR3-GFP localization to the cilia. (*D*) IMCD3 Smo-YFP cells treated with 10 μM CA1 or CA2 display abrogated acetylated tubulin. (*E*) Quantitation in arbitrary units of *Smo* expression in ASZ1 cells treated with 25 μM SA compound, as measured by qRT-PCR. SA compounds do not inhibit *Smo* transcription. (*F*) SA1–10 treatment of IMCD3 Smo-YFP cells does not change the amount of total Smo protein in the cell, as assessed by immunoblot. (*G*) SA1–10 treatment of ASZ1, IMCD3 Smo-YFP, and NIH 3T3 cells for 5 d does not cause increased apoptosis in the cell lines. (*H*) Quantitation of β-galactosidase activity in BATgal MEFs treated with 25 μM compound. Wnt3a-induced β-galactosidase activity is inhibited by 10 μM Dkk-1, but not by SA1–10 or CA1. (Scale bars: 20 μm.) Data for *A*, *B*, *C*, and *F* are the average of duplicated measurements; data for *D* are the average of quadruplicate measurements; data for *G* are the average of triplicate measurements ± SD.

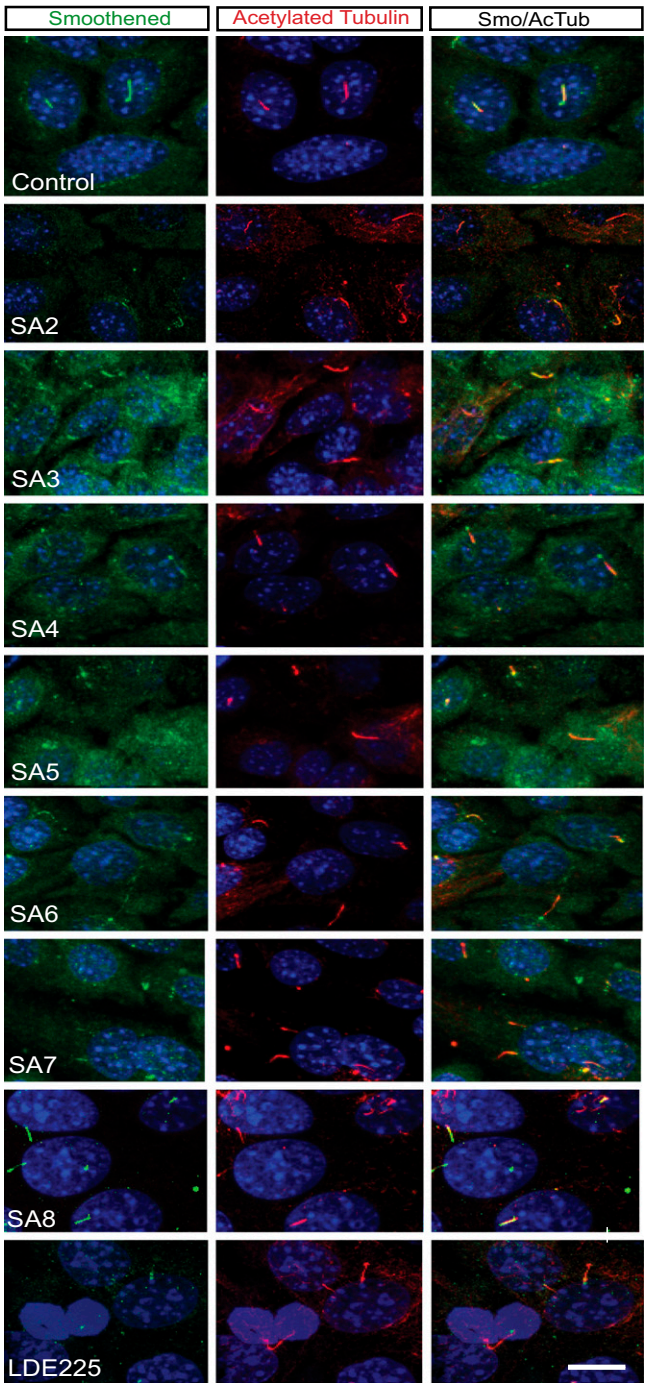

Fig. S2. SA inhibition of endogenous ciliary Smo in BCC-like ASZ1 cells. ASZ1 cells are stained for nuclei (DAPI; blue), Smo (green), and cilia (acetylated tubulin; red). SA2-6 and LDE225 but not SA8 decrease the ciliary localization of Smo in ASZ1 cells. (Scale bar: 20 μm.)

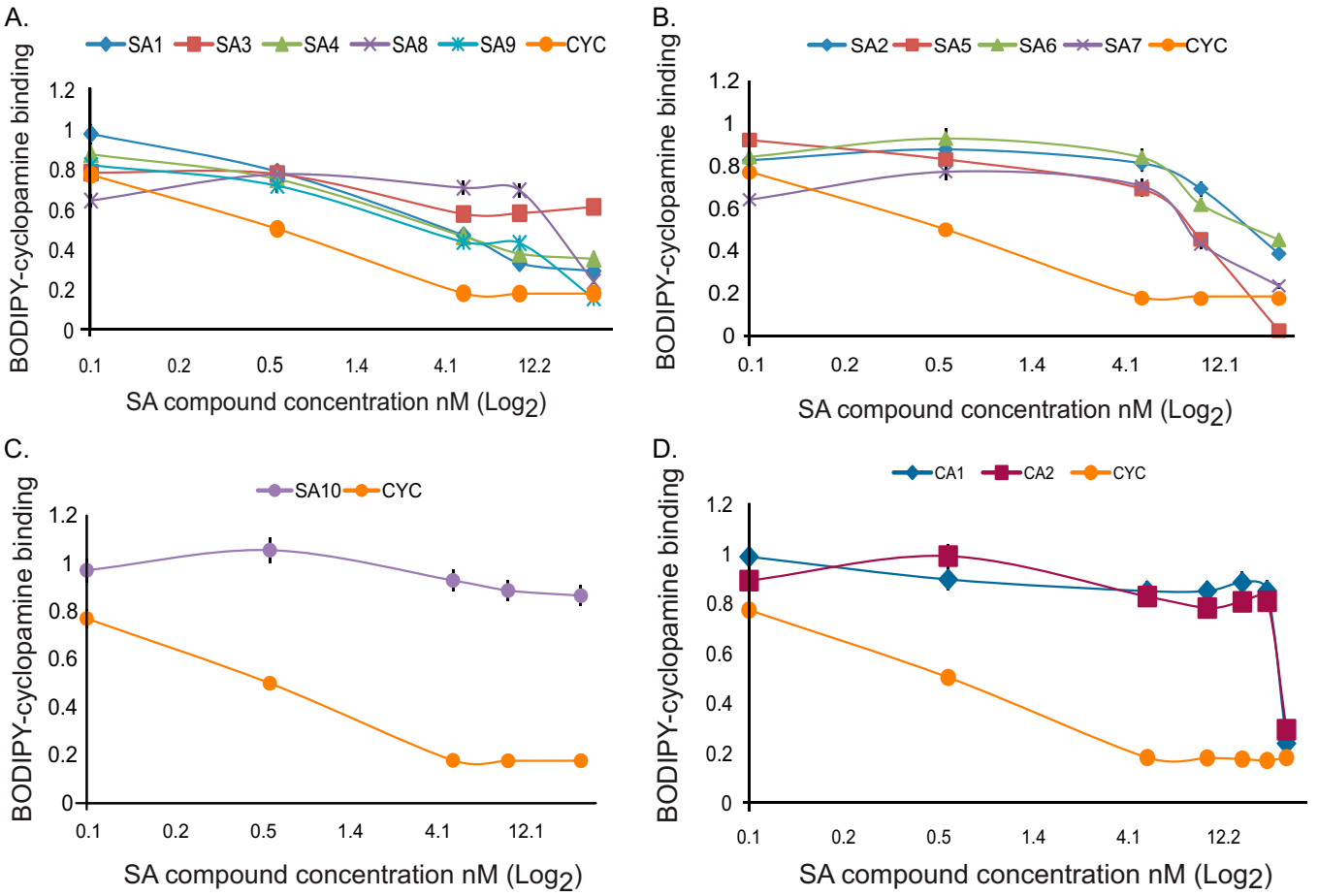

Fig. S3. (*A*) Average relative BODIPY-cyclopamine fluorescence intensity from eight independent micrographs of Smo-transfected HEK293T cells treated with SA1, 3, 4, 8, and 9. Unlabeled cyclopamine was used as a positive control. (*B*) Similar quantitation of BODIPY-cyclopamine intensity of Smo-transfected HEK293T cells treated with SA2, 5, 6, and 7. (*C*) Quantitation of BODIPY-cyclopamine intensity of Smo-transfected HEK293T cells treated with SA10. Unlike the other SA compounds, SA10 does not inhibit the binding of BODIPY-cyclopamine to Smo. (*D*) CA compounds do not affect BODIPY-cyclopamine binding. Average BODIPY-cyclopamine intensity of eight independent images of HEK293T cells treated with CA compounds and compared with unlabeled cyclopamine ±SD.

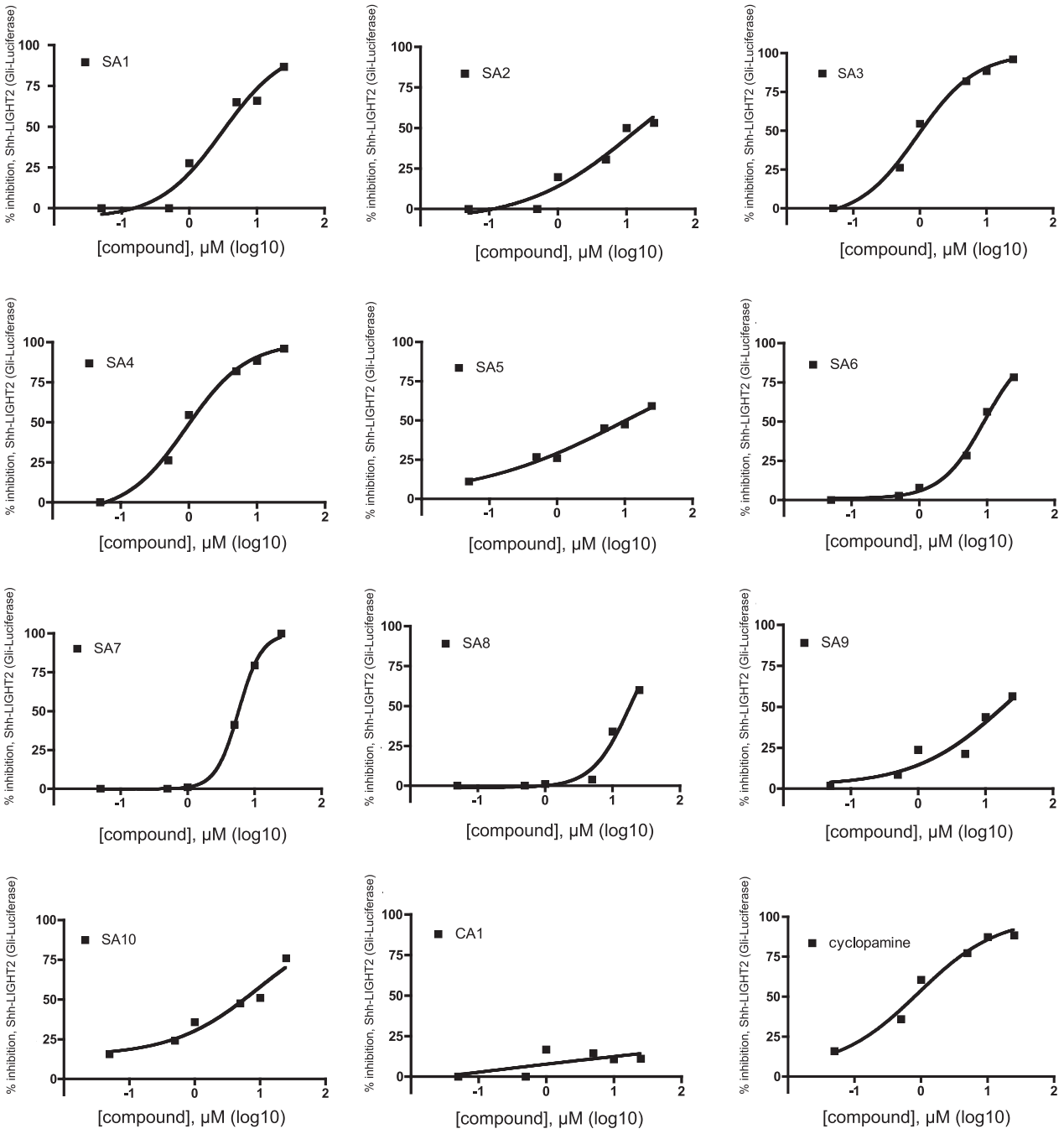

Fig. S4. SA1–10 and cyclopamine can inhibit Hh pathway activity, as measured by the Shh-LIGHT2 activity assay. CA1 does not inhibit Hh pathway activity. Data are the average of triplicate measurements.

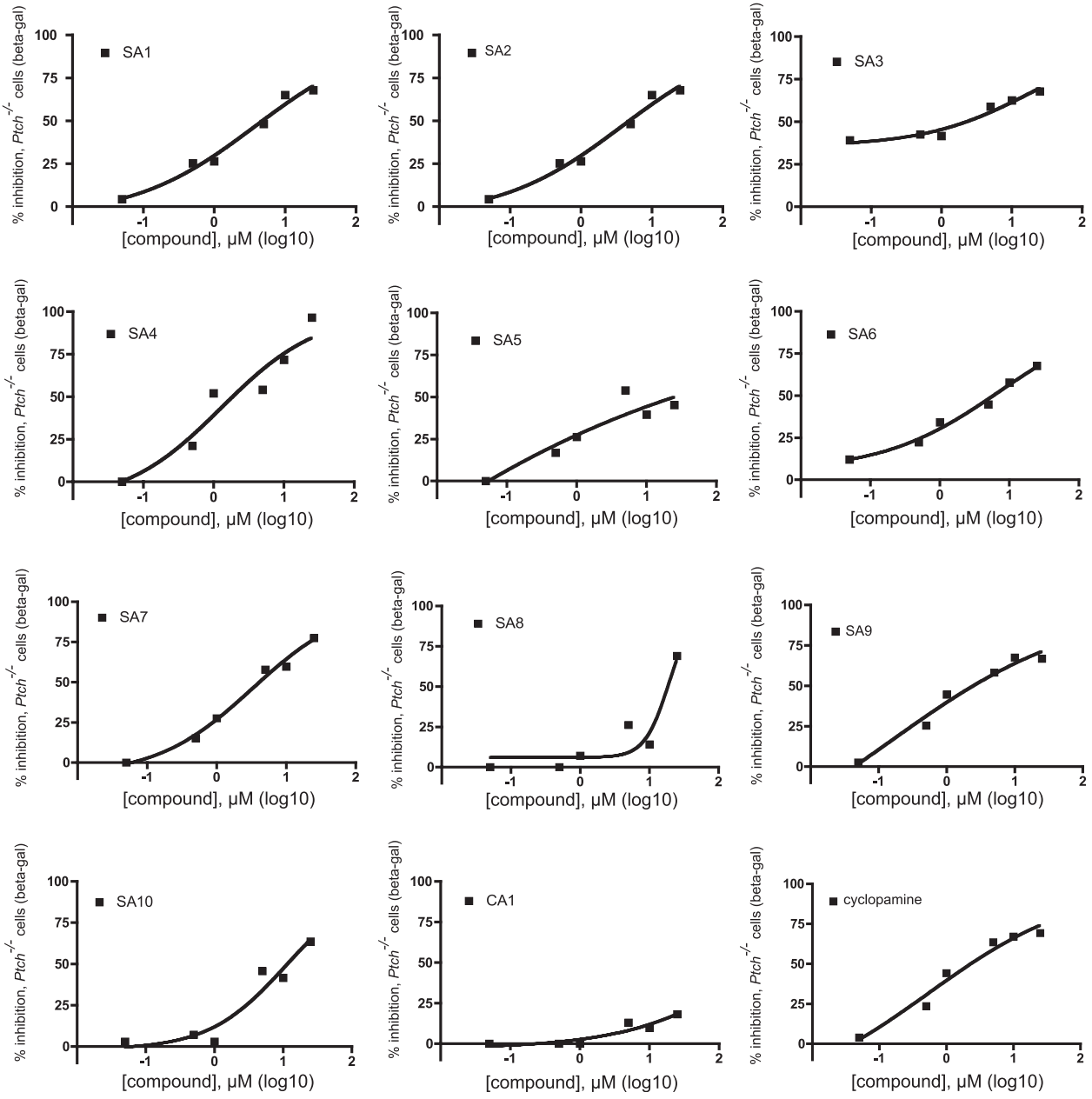

Fig. S5. Inhibition of Hh pathway activity in *Ptch1*$^{-/-}$ MEFs by SA1–10, CA1, and cyclopamine. SA1–10 and cyclopamine inhibit Hh pathway activity, as measured by the levels of β-galatosidase under the control of Ptch1 promoter. CA1 does not reduce cellular levels of β-galatosidase and thus does not inhibit Hh pathway activity. Data are the average of triplicate measurements.