Supporting Information

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SI Materials and Methods

DNA Constructs. The N-terminally-tagged mouse Gli2 expression construct, pCEFL/3×HA-Gli2, was constructed by ligating three tandemly repeated PCR-amplified HA tags (3×HA), and mouse Gli2, into the EF1α promoter-containing pCEFL expression vector. pCEFL was provided by J. Silvio Gutkind (National Institutes of Health). pCEFL/mGFP-Gli2 was constructed by replacing the 3×HA tag in pCEFL/3×HA-Gli2 with monomeric GFP coding sequence, which was constructed by introducing the monomeric mutation, A206K, to EGFP. Motorless Kif3a dominant negative (Kif3a-DN = Kif3a-HL, Kif3a Head (Motor)-less) was provided by Trina Schroer (Johns Hopkins University). LMP/Dync2h1 shRNA was constructed by excising Dync2h1 shRNA fragment from pSM2/Dync2h1 (shRNA from Open Biosystems) and inserting into the LMP vector containing restriction sites for shRNA insertion and a PGK-puromycin resistance-IRES-GFP cassette.

Cells and Small Molecules. NIH 3T3 and NIH 3T3/HA-Gli2 cells were grown in DMEM supplemented with 10% bovine calf serum, 1% penicillin/streptomycin. The amphotropic retroviral packaging cell line Phoenix ampho was grown as described (1). NIH 3T3/HA-Gli2 cell lines were established by transfecting HA-tagged Gli2 construct in NIH 3T3 cells and selected with G418. Chemicals were used in the following concentrations unless otherwise indicated: cyclopamine, 3 μM; SAG1, 400 nM; SANT-1, 100 nM; purmorphamine, 10 μM; and vinblastine, 100 nM. Cyclopamine was a generous gift from Dale Gardner (USDA). SAG1, SANT-1, purmorphamine, and vinblastine were purchased from EMD/Calbiochem. Shh conditioned medium and ShhNp proteins were prepared as previously described (2).

Retroviral Infection. Stable NIH 3T3/HA-Gli2 cell lines with integrated LMP control vector or vector expressing shRNA against cytoplasmic dynein 2 heavy chain (Dync2h1 shRNA) were generated by retroviral infection and selection with 3.0 μg/mL of puromycin. The viral supernatant was harvested from LMP vector- or LMP/Dync2h1 shRNA-transfected Phoenix ampho packaging cells. NIH 3T3/HA-Gli2 cells were plated at a density of 10^5 cells/well of 24-well plates, and 24 h later, cells were infected with the viral supernatants using spin infection at 2,000 × g for 1 h at 30 °C with 6 μg/mL of polybrene (Sigma).

Antibodies. Polyclonal antibody against Smoothened was raised in rabbits using a synthetic peptide (CSRTNLMEAEILDADS, corresponding to Smo amino acids 777–791) conjugated to KLH, and affinity purified. Antibodies were used at the following concentrations: Rabbit anti-Smo antibody 1:1,000, mouse anti-HA antibodies 1:1,000 (clone 16B12, Covance), mouse anti-γ-tubulin antibodies 1:4,000 (Sigma). The primary cilium was stained with mouse monoclonal anti-acetylated tubulin antibodies (1:1,500, Sigma) or rabbit anti-detyrosinated tubulin (Glu Tubulin) antibodies (1:1,000, Millipore). Rabbit polyclonal antibodies against Gli2 that can detect endogenous Gli2 were provided by Baolin Wang (Weill Medical College of Cornell University).

Quantification of Microscopic Images. Spinning disc confocal and wide-field fluorescence images of about 30 Z-sections of 0.2 μm thickness for each field were obtained with a Leica spinning disc confocal microscope SD6000 equipped with an EM-CCD (electron multiplying-charge coupled device) camera and Z-galvanometer stage. Images were processed by LAS AF (Leica), Volocity (Improvision), or the open-source software ImageJ (National Institutes of Health). All wide field images were deconvoluted with LAS AF software through three or more blind iterative cycles in batches using a macro software (Workspace Macro Pro) before further quantification. The background subtracted from confocal or deconvoluted wide-field images was the mean fluorescence intensity in each color channel as measured in LAS AF software.

The amount of Smo in the primary cilium, and of Gli2 in the primary cilium and the nucleus were measured by three different methods. (*i*) Nuclear localization of Gli2 was measured using the colocalization module in Leica LAS AF software based on intensity correlation analysis. The colocalizing pixels of each color channel in an image above a threshold were measured in this module. This step was repeated throughout the stack of Z-sections, and combined data were analyzed. Nuclear Gli2 was determined by measuring colocalization between Gli2 and DAPI. Volocity has a similar colocalization module and analysis results were similar. (*ii*) Ciliary Smo and Gli2 were also measured by intensity correlation analysis and results largely agree with the measurements made by masking, described below. One complication encountered with intensity correlation analysis stems from the high degree of Gli2 concentration at the tip of the cilium, with Smo also distributed at the tip and along the shaft. Thus, although some of the Gli2 or Smo staining touches or overlaps acetylated or detyrosinated tubulin staining, it tends to extend beyond the modified tubulin staining at the ciliary tip. Measurements of colocalization by intensity correlation thus include part of Gli2 or Smo intensity but miss a considerable fraction of fluorescence from pixels at the ciliary tip. To more accurately determine fluorescence intensity of ciliary Smo and Gli2, we manually masked all of the cilia in an image using region of interest (ROI) marking tools in either LAS AF software or ImageJ, and then measured the intensity of each color channel in each ROI for each Z-section throughout the entire stack. The mean fluorescence intensities of ciliary Smo or Gli2 as presented in the figures were then calculated from the combined measurement values. (*iii*) We also used the following method to corroborate measurements obtained by the above method. An RGB image was split into three channel images. Working in the modified tubulin channel, all primary cilia in an image were masked in ImageJ according to the ImageJ manual. Briefly, a threshold value was selected for the acetylated or detyrosinated tubulin-stained image, and the image was converted into a binary image, and then all of the particles were counted and outlined. All outlined masked regions from this binary image were copied and pasted into the Gli2- or Smo-staining channels and fluorescence intensity for these channels was measured. This procedure was repeated for each section of the Z-series and summed results were analyzed.

Representative images presented in figures were identically treated for contrast enhancement in each series of panels.

Cell Extractions, Immunoprecipitation, and Immunoblotting. NIH 3T3/HA-Gli2 cells were lysed in a buffer containing 50 mM Hepes pH 7.4, 1% (vol/vol) Triton X-100, 150 mM NaCl, 2.5 mM EDTA, 10% (vol/vol) glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and EDTA-free complete mini protease inhibitor tablets (Roche). Nuclear and cytoplasmic extracts were isolated by subcellular fractionation using NE-PER kit (Pierce)

according to the manufacturer's instruction. Full length HA-Gli2 or repressor form of HA-Gli2 proteins were immunoprecipitated from 300 μg of protein extracts with rat anti-HA monoclonal antibody matrix (clone 3F10, Roche) followed by SDS/PAGE and Western blotting with a mouse anti-HA monoclonal antibody (clone 16B12, Covance). For direct immunoblotting experiments, 30 μg of total proteins was loaded in each lane.

1. Swift S, Lorens J, Achacoso P, Nolan GP (2001) Rapid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems. *Curr Protoc Immunol* Chapter 10:Unit 10 17C.
2. Taipale J, et al. (2000) Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. *Nature* 406:1005–1009.

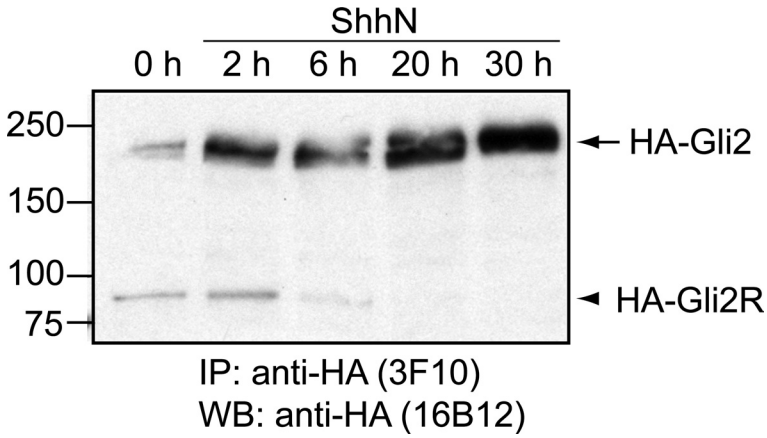

Fig. S1. Time course of Hh stimulation and quantification of Gli2 nuclear accumulation from biochemical fractionation. NIH 3T3/HA-Gli2 cells were incubated with ShhN for up to 30 h as indicated. Cells were lysed and analyzed by IP-Western with anti-HA antibodies.

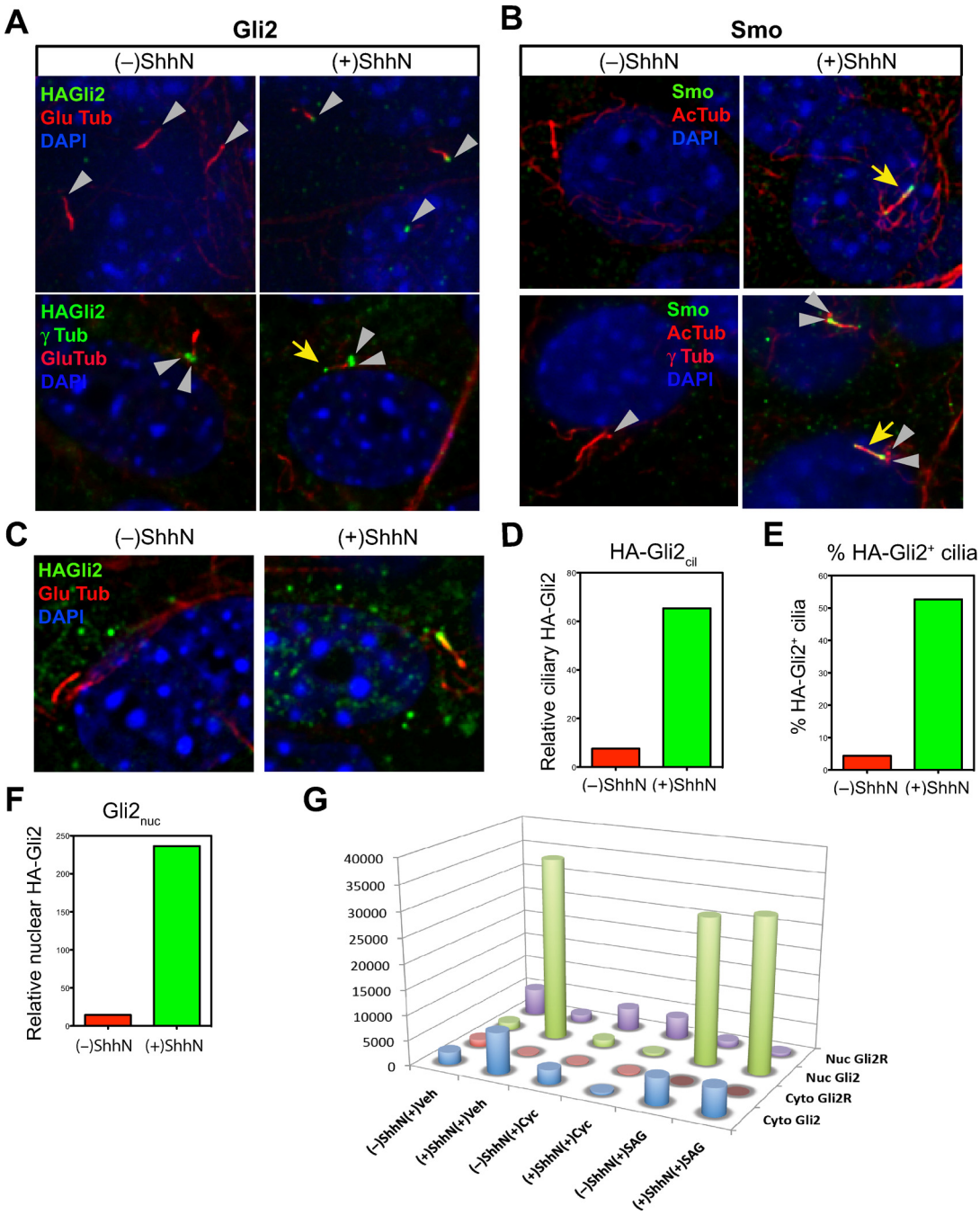

Fig. S2. Hh stimulation induces ciliary accumulation of Gli2 and Smo and nuclear accumulation of Gli2. (*A*) ShhN-induced accumulation of Gli2 at the primary cilium. NIH 3T3/HA-Gli2 cells were grown to confluency and incubated in the presence or absence of ShhN; cells were stained to visualize HA-Gli2, the primary cilium (Glu tubulin), and the nucleus (DAPI) in *Upper* panels, or HA-Gli2, the primary cilium (Glu tubulin), the centrosome (γ-tubulin), and the nucleus (DAPI) in *Lower* panels. In the *Lower Right* panel, the doublet of γ-tubulin staining (white arrowheads) marks the centrosome at the base of the primary cilium, thus indicating that HA-Gli2 (yellow arrow) accumulates at the tip. (*B*) ShhN-induced accumulation of Smo along the shaft of the primary cilium. NIH 3T3 cells were stained for Smo, acetylated tubulin, and DAPI (*Upper* panels), or Smo, acetylated tubulin, γ-tubulin, and DAPI (*Lower* panels). The doublet staining of γ-tubulin is located at the base (gray arrowheads) of the primary cilium. Yellow arrows indicate Smo staining. (*C*) NIH 3T3/HA-Gli2 cells incubated in the presence or absence of ShhN were stained to visualize HA-Gli2, the primary cilium (Glu tubulin), and the nucleus (DAPI). (*D*) The amounts of HA-Gli2 in cilia ($Gli2_{cil}$) in the presence or absence of ShhN were quantified from complete *Z*-series of immunofluorescence images. (*E*) The percentage of Gli2 positive cilia is shown. (*F*) The amounts of Gli2 in the nucleus ($Gli2_{nuc}$) in the presence or absence of ShhN were quantified from complete *Z*-series of immunofluorescence images. (*G*) The levels of full-length Gli2 (HA-Gli2) and its repressor form (HA-Gli2R) in cytoplasmic and nuclear fractions with various treatments as indicated were quantified from immunoblots (Fig. 2 *I* and *J*) using ImageJ software.

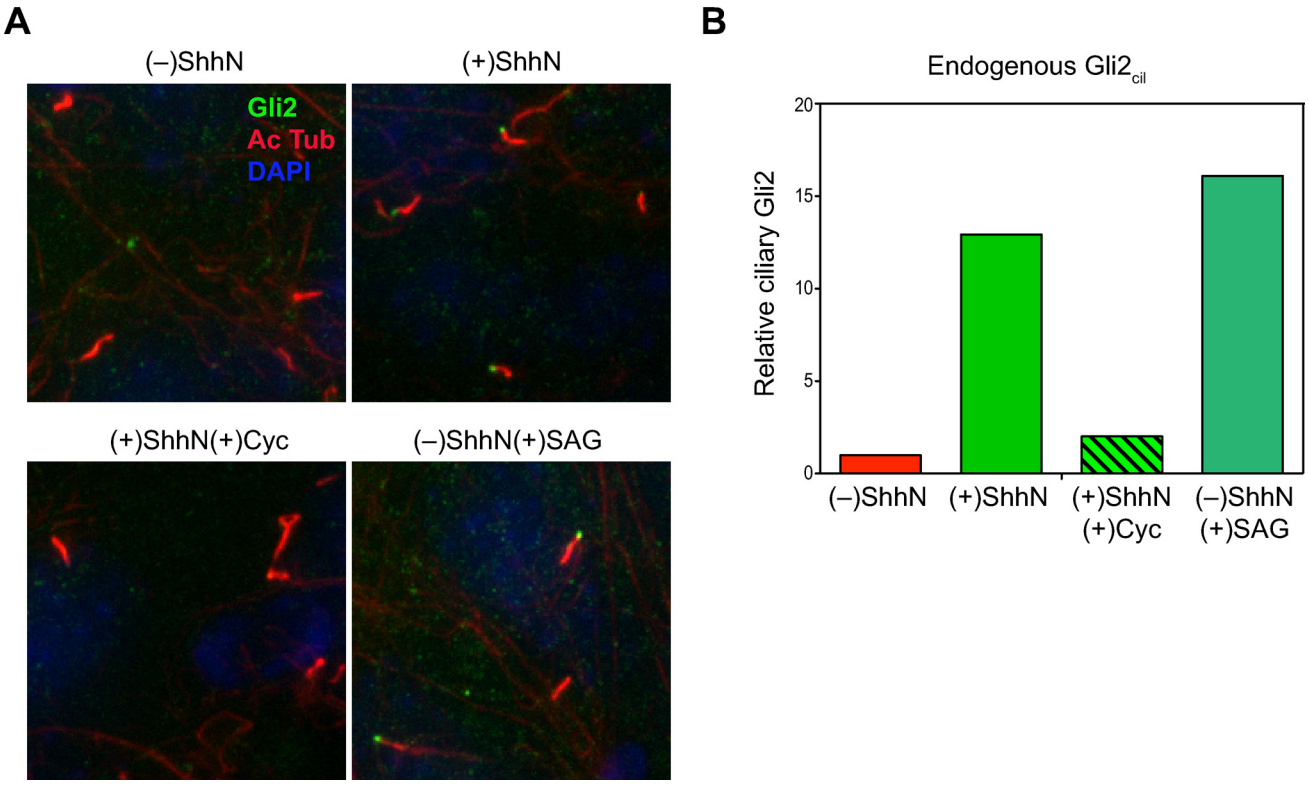

Fig. S3. Endogenous Gli2 accumulates at the primary cilium upon Hh stimulation. (*A*) NIH 3T3 cells were grown to confluency for 2 days and incubated in the presence or absence of ShhN, ShhN with cyclopamine, or SAG alone for 24 h. Cells were stained to visualize endogenous Gli2, acetylated tubulin, and the nucleus (DAPI). (*B*) The amounts of ciliary Gli2 in each condition were quantified from complete *Z*-series of immunofluorescence images.

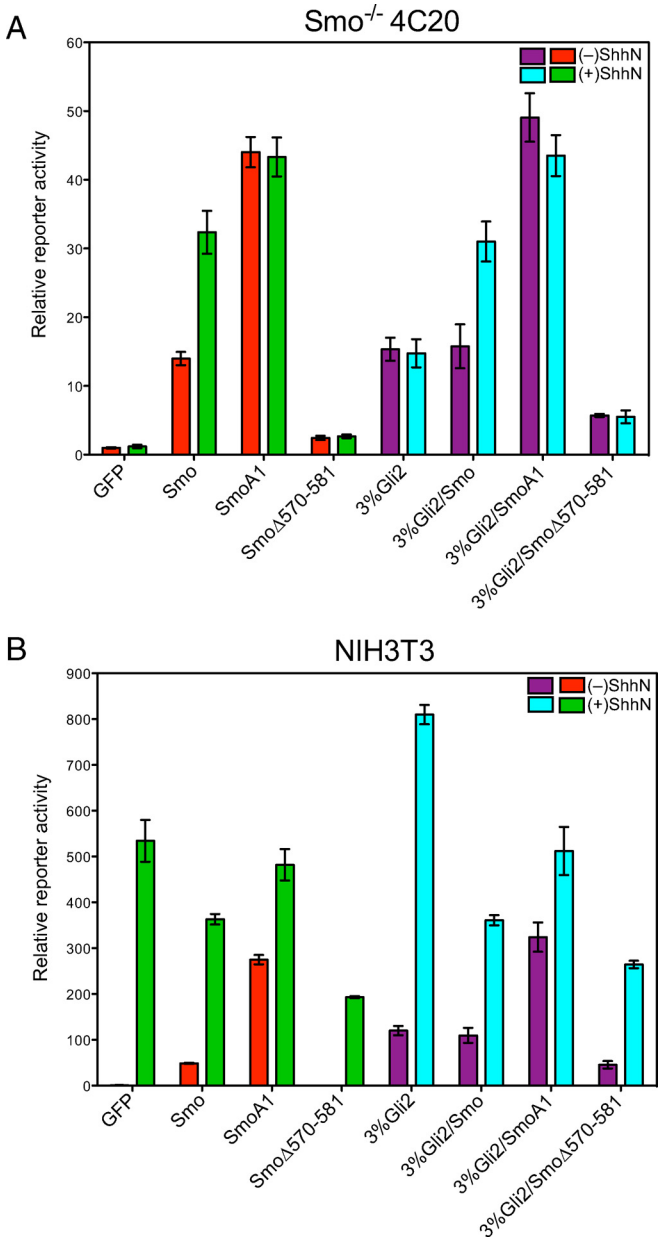

Fig. S4. A transdominant-negative form of Smo suppresses basal and Hh-induced pathway activity in the presence of Gli2. (*A*) Mouse embryonic fibroblast cells lacking Smo function (4C20) were transiently transfected with Gli-luc, SV40-Renilla luciferase control, and GFP or Gli2, either alone or in combination with either wild-type Smo, a constitutively activated Smo (SmoA1), or a dominant-negative form of Smo (SmoΔ570–581) that can suppress the basal pathway activity generated by transfection of Gli2. (*B*) NIH 3T3 cells were transfected as described in *A*. SmoΔ570–581 inhibits Hh-induced pathway activity in the control and also suppresses both the basal and Hh-induced activity in the presence of Gli2.

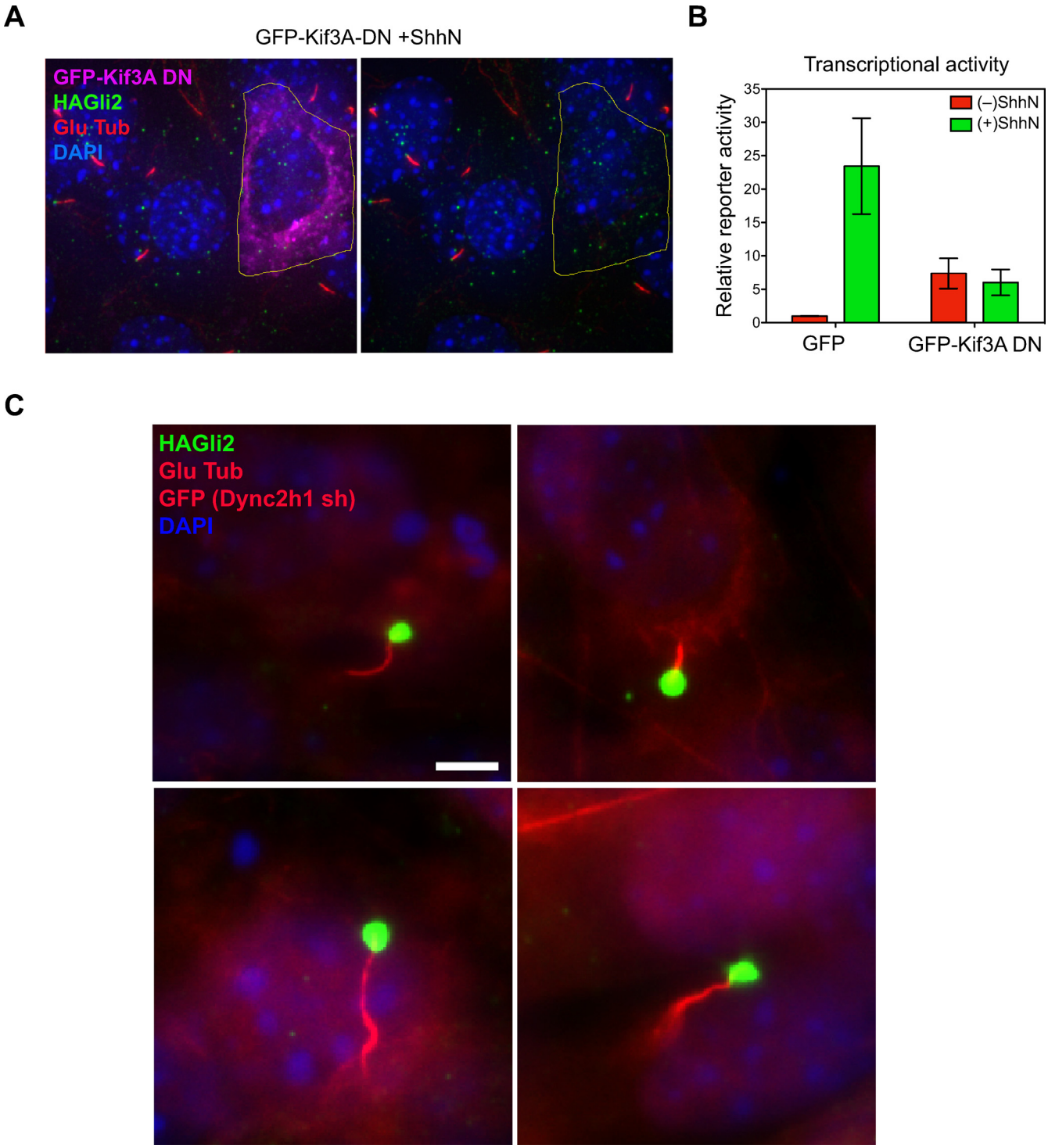

Fig. S5. The effects of inhibition of anterograde and retrograde transport on Gli2. (*A* and *B*) Dominant-negative Kif3a inhibits ciliogenesis and Hh pathway activation. NIH 3T3/HA-Gli2 cells transiently transfected with a Kif3a lacking its motor domain (GFP-Kif3a-DN) were incubated with ShhN. (*A*) A representative field is shown with GFP-Kif3a-DN staining (magenta) in the *Left* but not the *Right* panel to demonstrate that the transfected cell (yellow outline) contains no cilium. (*B*) Gli-luciferase reporter assays are shown. (*C*) Gli2 accumulates as ball-shaped structures at the tips of cilia in cells with disruption of cytoplasmic dynein 2. NIH 3T3/HA-Gli2 cells infected with a virus carrying dynein 2 heavy chain (Dync2h1) shRNA and selected for puromycin resistance were grown to confluency and left untreated for 24 h before immunofluorescence staining. Gli2 accumulation occurs at the tip in the absence of ShhN, sometimes forming ball-shaped structures ranging from 1 to 3 μm in size. Note that GFP from a PGK-puromycin-IRES-GFP cassette present in the Dync2 shRNA virus appears as a general red background. (Scale bar, 5 μm.)

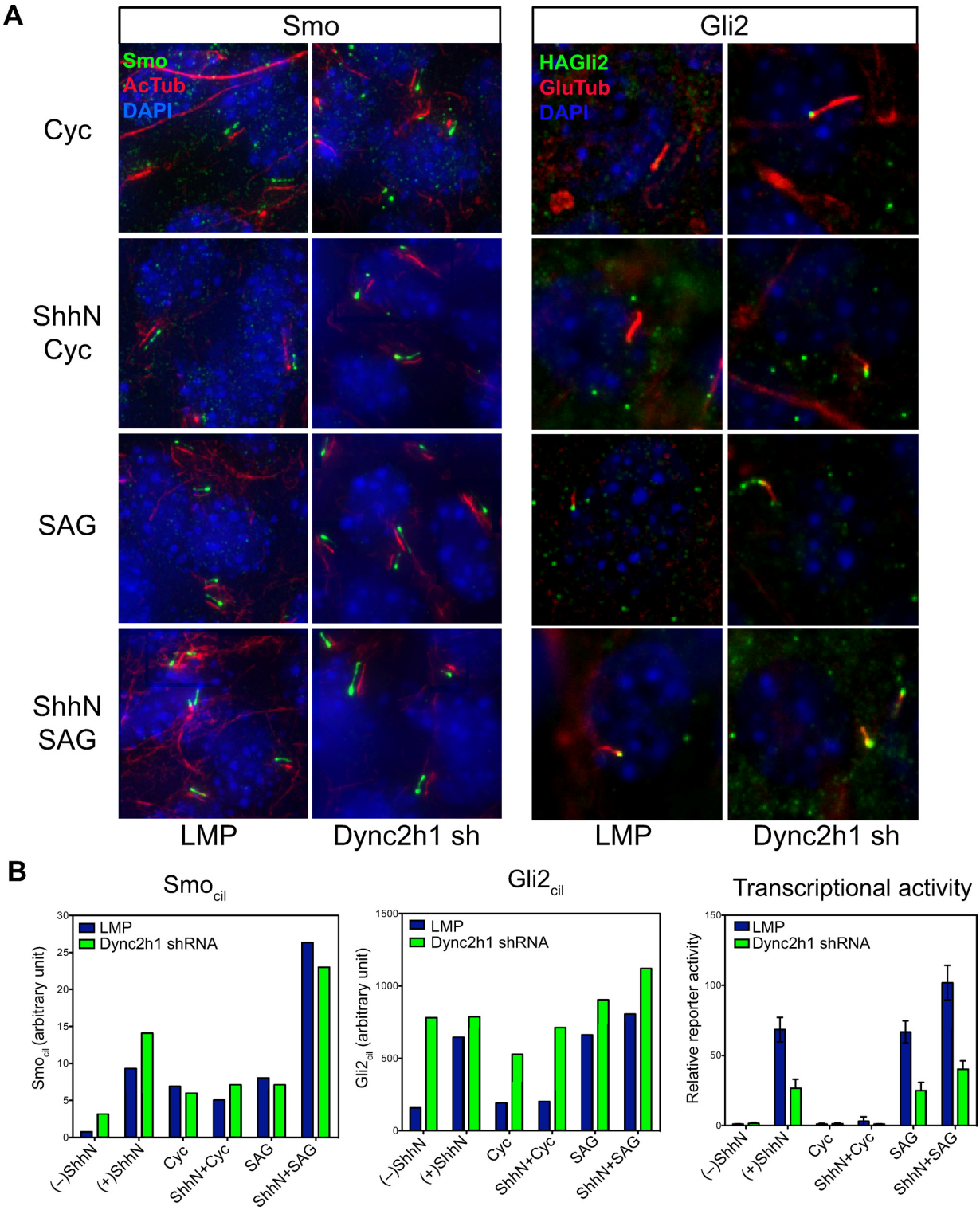

Fig. S6. Gli2 ciliary shuttling is not blocked by SAG1 or cyclopamine. (*A*) Effects of cyclopamine (Cyc) or SAG1 combined with shRNA-mediated knockdown of dynein 2 heavy chain on ciliary Smo and Gli2. NIH 3T3/HA-Gli2 cells infected with LMP vector or Dync2h1 shRNA viruses were selected with puromycin. Cells grown to confluency were incubated for 24 h with cyclopamine or SAG1 in the presence or absence of ShhN and stained for Smo or Gli2 as indicated. Smo staining (green) in the *Left* eight panels is shown as shifted overlays with staining for acetylated tubulin (red). (*B*) Smo and Gli2 levels in cilia (Smo$_{cil}$, *First* panel, Gli2$_{cil}$, *Middle* panel) were quantified from immunofluorescence images. The *Third* panel shows activity of the Gli-luciferase reporter assayed in parallel. This experiment was performed together with that in Fig. 4.

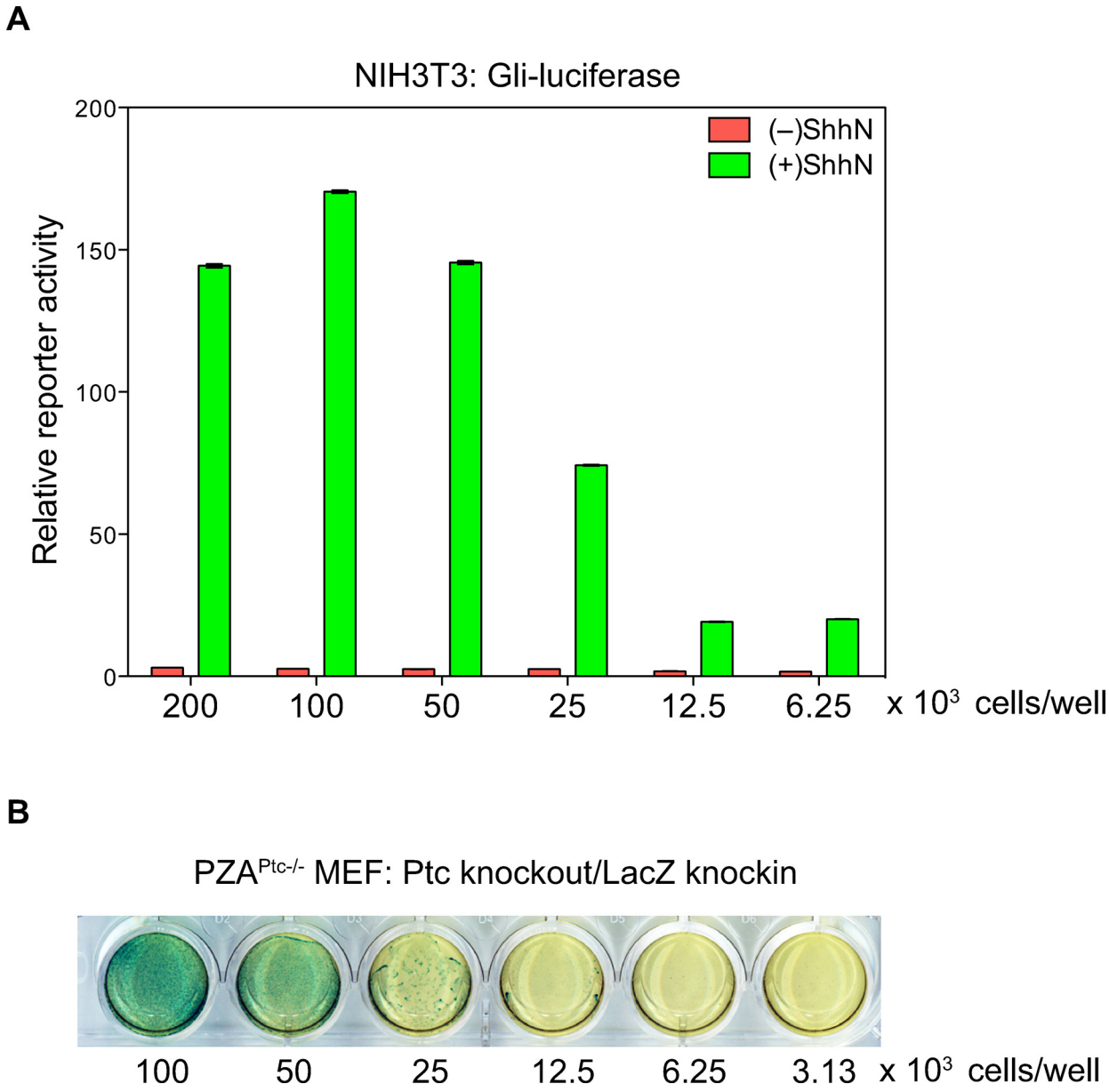

Fig. S7. The effect of cell density on Hh pathway activity in NIH 3T3 and PZA$^{Ptc-/-}$ MEF. (*A*) NIH 3T3 cells were plated in a series of decreasing densities in 24-well plates, and 2 days later, were incubated with ShhN in low serum medium for 30 h. Pathway activity was assessed by Gli-luciferase activity. (*B*) PZA$^{ptc-/-}$ MEF cells were plated in a series of decreasing densities in 24-well plates. Cells were incubated for 30 h in the absence of ShhN in low serum medium, and constitutive pathway activity was assayed by lacZ staining using X-gal.

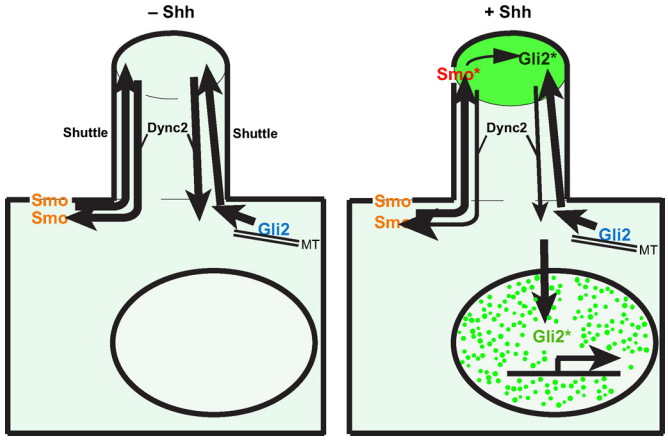

Fig. S8. Model of Gli2 trafficking as a dynamic monitor of Smo activity in the primary cilium. In the absence of Hh stimulation (*Left*), Smo and Gli2 shuttle through the primary cilium but do not accumulate because of export by the ciliary retrograde motor, cytoplasmic dynein 2 (Dync2). The entry of Gli2 but not Smo into the primary cilium requires cytoplasmic microtubules (MT). Upon Hh stimulation (*Right*), activated Smo accumulates in the primary cilium and acts there to trigger changes in Gli2 such that upon exit from the cilium it accumulates in the nucleus and activates transcription of target genes.

Table S1. Activity and localization of Smo and Gli2 under various conditions

Treatment	Presence of cilia	Activated Smo	Ciliary Smo	Ciliary Gli2	Transcriptional activation	Nuclear Gli2
No ShhN	Yes	No	−	−	−	−
No ShhN + SAG1	Yes	Yes	++	++	++	++
No ShhN + Pumorphamine	Yes	Yes	++	++	++	++
No ShhN + Cyc	Yes	No	+ or ++	−	−	−
No ShhN + SANT-1	Yes	No	−	−	−	ND
No ShhN + VNB	Yes	No	−	−	−	ND
No ShhN + Dync2 h1 shRNA	Yes	No	++	++*	−	ND
No ShhN + Dync2 h1 shRNA + Cyc	Yes	No	++	+*	−	ND
No ShhN + Kif3A DN	No	No	NA	NA	−	ND
No ShhN + Low density cells	No	No	NA	NA	−	ND
ShhN	Yes	Yes	++	++	++	++
ShhN + SAG1	Yes	Yes	+++	++	++	++
ShhN + Purmorphamine	Yes	Yes	+++	++	++	ND
ShhN + Cyc	Yes	No	++	−	−	−
ShhN + SANT-1	Yes	No?	−	−	−	ND
ShhN + VNB	Yes	Yes	++	−	−	ND
ShhN + Dync2 h1 shRNA	Yes	Yes	+++	+++*	+	ND
ShhN + Dync2 h1 shRNA + Cyc	Yes	No	+++	+*	−	ND
ShhN + Kif3A	No	Yes?	NA	NA	−	ND
ShhN + Low density cells	No	Yes?	NA	NA	−	ND

Definition of symbols: −, 0–10%; ±, 10–30%; +, 30–70%; ++, 70–150%; +++, >150% of the value produced by stimulation with ShhN alone. NA, not applicable; ND, not determined.
*Ball-shaped aggregate