Supplemental Materials and methods

Generation of the BDEneu cell line with dominant-negative Ptch1

The pKT2C-Ptch1ΔL2-EGFP is a construct containing the coding sequence for dominant-negative Ptch1ΔL2 which lacks the Shh-binding domain in its second extracellular loop (deleted coding sequence for amino acids 738-939). Expression of the Ptch1ΔL2 protein in rat cells functions as a dominant-negative constitutive repressor of Smo activation [1]. The bicistronic template construct pCIG-AscI-Ptch1ΔL2-IRES-EGFP was a generous gift from Dr. Yimin Zou from University of California San Diego [1]. This construct was cloned into a transposon pKT2C-eG in order to transfect the BDEneu cells. Amplification of the Ptch1ΔL2 sequence and incorporation of MfeI sites were carried out using the following primers:

forward 5'-AACAATTGACTATGGCCTCGGCTGGT-3',

reverse 5'-TTCAATTGTCAGAAAATAAGTTTTTGT-3'.

The amplified product was cloned into the pCR-BluntII vector (Invitrogen, Carlsbad, CA, USA) and sequenced. The Ptch1ΔL2 sequence of the construct was excised using MfeI and cloned into the EcoRI site of the pKT2C-eG vector. Correct orientation of ligation products was verified by restriction digest and sequencing. The BDEneu cell line with stable transfection of Ptch1ΔL2 ($BDE^{\Delta Loop2}$) was generated using the *Sleeping Beauty* transposon transfection system [2]. Cells were co-transfected using FuGENE HD (Promega, Madison, WI, USA) with the following constructs: the pKT2C-Ptch1ΔL2-EGFP, pKT2P-PTK carrying a puromycin resistance gene, and pKC-SB100X carrying the transposase encoding sequence (obtained from the Genetic Core of the NIDDK funded Center for Cell Signaling in Gastroenterology, P30DK084567). Cells were selected with puromycin (14 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) and screened by examining for EGFP expression using a fluorescence microscope (Nikon Eclipse TE200,

Melville, NY, USA) equipped with 488 nm and 509 nm excitation and emission filters, respectively. Incorporation of the *Ptch1*$^{\Delta L2}$ was confirmed by a polymerase chain reaction (PCR). Briefly, the genomic DNA was extracted from BDEneu and BDE^{Loop2} cells using the Genomic DNA Miniprep Kit (Sigma-Aldrich) and analyzed by agarose gel electrophoresis after an amplification of the *Ptch1*Δ*L2* (206 bp) fragment using Hot-Start *Taq* (Denville Scientific, South Plainfield, NJ, USA) with the following primers: forward 5'-GCTCCTTTCCTCCTGAAACC; reverse 5'- GTCATCAGAGCCAGGACCAT.

Quantitative real time PCR (qRT-PCR)

To study expression of the canonical Hh pathway marker Gli1, cells at 80% confluency were incubated in a serum-free medium overnight and then collected or treated with either vehicle or purmorphamine (2 μM, x 72 hours; Calbiochem, Billerica, MA, USA). Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and the reverse transcription polymerase chain reaction (qRT-PCR) using Moloney leukemia virus reverse transcriptase and random primers (Invitrogen, Carlsbad, CA, USA) was performed for reverse transcription into the complementary DNA. Quantitation of mRNA was accomplished with the SYBR Green-based detection technology (Molecular Probes; LightCycler; Roche Molecular Biochemicals, Mannheim, Germany). Primers are listed in Supplemental Table 1.

Cell proliferation assay

Cells were cultured into a flat-bottomed 96-well plate at 10^3 density per well. The premixed WST-1 cell proliferation reagent (Clonthech, Mountain Veiw, CA, USA) was added to the wells.

Absorbance per well was quantified with a multiwell plate reader (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Cell surface protein biotinylation assay

Cells were cultured in 100-mm dishes and treated with either vehicle or purmorphamine (2 μM, Calbiochem) with and without PTX (200 μg/ml, x 16 hours; Sigma-Aldrich). Surface proteins were biotinylated, cells lysed, and labeled surface proteins were affinity purified using the Cell Surface Protein Isolation Kit according to manufacturer's instructions (Pierce, Thermo Scientific). Precipitated proteins were immunoblotted for Smo on TGX 10% precast gel (Criterion, Bio-Rad, Hercules, CA, USA). Antibodies used are listed in Supplemental Table 2.

Animal experiments

All protocols involving animal studies were approved by the Institutional Animal Care and Use Committee, Mayo Clinic, Rochester, MN, USA. Adult male Fisher 344 rats (Harlan, Indianapolis, IN, USA) were anesthetized with 2% Isoflurane in oxygen, a midline incision was performed below the sternum, and the left hepatic lobe bile duct was identified and ligated with a 6-0 silk suture material to promote cholestasis. To study the effect of genetic interruption of the Hh signaling pathway, $4x10^6$ of the BDEneu or $BDE^{\Delta Loop2}$ cells were suspended in 0.05 ml of sterile PBS and implanted into the left hepatic lobe. The laparatomy wound was closed with an absorbable 6-0 vicryl suturing material, and analgesia was provided in an immediate postoperative period with buprenorphine 0.05 mg/kg subcutaneously. Animals were euthanized at desired time intervals and analyzed for the presence of the primary tumor and metastases as previously described [3]. Metastases burden was quantified for each examined site (omentum,

peritoneum, and diaphragm) on a scale from 0 to 4 based on the metastases size. In studies employing pharmacological inhibition of the Hh signaling pathway, animals were treated intraperitoneally daily over a selected time periods with either vehicle or the small molecule inhibitor of Smo, vismodegib (25 mg/kg; GDC-0449; Active Biochemicals, Wan Chai, Hong Kong and LC Laboratories, Woburn, CA, USA).

Statistical analysis

At least three replicates were obtained for each condition in experiments with cell cultures. In animal experiments, at least 9 animals were included in each experimental group. For quantitative continuous data differences between two groups, data were analyzed by Student's t-test with a threshold for significance defined at $p < 0.05$. When multiple comparisons were possible, ANOVA coupled with Bonferroni post hoc correction was applied.

References

[1] Parra LM, Zou Y. Sonic hedgehog induces response of commissural axons to Semaphorin repulsion during midline crossing. Nature neuroscience 2010;13(1): 29-35.

[2] Hackett PB, Ekker SC, Largaespada DA, McIvor RS. Sleeping beauty transposon-mediated gene therapy for prolonged expression. Advances in genetics 2005;54: 189-232.

[3] Sirica AE, Zhang Z, Lai GH, Asano T, Shen XN, Ward DJ, et al. A novel "patient-like" model of cholangiocarcinoma progression based on bile duct inoculation of tumorigenic rat cholangiocyte cell lines. Hepatology 2008;47(4): 1178-1190.

Supplementary Fig. legend

Supplemental Fig. 1. CCA cells lacking cilia have variable Gli1 expression and do not display canonical Hh signaling with translocation of the transcriptional factor Gli2 to the cell nuclei. (A) Total RNA from the non-malignant (BDE1) and malignant (KMCH, HuCC-T1, Mz-ChA-1, BDEneu, and BDE$^{\Delta Loop2}$) cells was isolated and subjected to qRT-PCR for quantitation of basal expression of Gli1 mRNA. 18S mRNA expression was used as an internal control. Relative expression was determined (delta-delta CT compared to 18S) and results are presented as basal expression of Gli1 mRNA in the non-malignant and malignant cells. (B) The non-malignant (BDE1) and malignant (HuCC-T1, Mz-ChA-1, and BDEneu) cells were cultured in 4-well chambers and treated with either vehicle (Veh) or purmorphamine (Purm; 2 μM) for 8 hours. Cells were examined with fluorescence microscopy for Gli2 (green) translocation to the cell nuclei (blue). Results are shown as representative images of the cells at resolution of x630.