SUPPLEMENTAL INFORMATION

Materials and methods

Cell culture. The human hepatoblastoma cell line HepG2 and the human pancreatic carcinoma cell line PANC-1 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100,000 units/liter penicillin, 100mg/liter streptomycin and 100 mg/liter gentamycin. The normal rat cholangiocyte cell line was a generous gift from Dr. Nicholas F. LaRusso, Mayo Clinic, Rochester, MN.

Stable transfection of SMO hairpin RNA expression construct. To obtain stably lenti virus transduced cells, cells were transduced with lentiviral particles containing pLenti6/TR one day before transduction with the inducible lentiviral SMO-shRNA constructs following the manufacturer's protocol (Invitrogen, Carlsbad, CA). Twenty-four hours after transduction with lentiviral particles containing SMO-shRNA constructs, fresh medium containing 10 µg/mL Blasticidin (Invitrogen) was added and the cells were incubated at 37^{o}C for an additional 24 hours. Twenty-four hours after incubation, cells were trypsinized and replated in fresh media containig 10 µg/mL Blasticidin and 500 µg/mL Zeocin^{TM} (Invitrogen). Surviving clones were separated using cloning rings and individually cultured.

Human tissue samples and RNA isolation. This minimal risk study was approved by the Mayo Clinic Institutional Review Board. Tumor and matching benign hepatic tissue of 45 patients with intra- and extrahepatic cholangiocarcinoma was obtained during surgical

resection. Tissue was immediately shock frozen and stored at -80^0 C. At the time of analysis, tissue was thawed on ice and mRNA isolated using RNeasy isolation kit (QIAGEN, Valencia, CA) according to the manufacturer's recommendations. Nucleic acid purity and quality was confirmed by spectrophotometry ($OD_{260/280,\ 230/260}$) and Agilent analysis. Only samples with $OD_{260/280,\ 230/260} \geq 1.8$ and RNI score ≥ 5 were used for real time-PCR.

Realtime RT-PCR. Transcriptional analysis of Hedgehog signaling pathway-associated genes in human samples were analyzed by real time-PCR. 5μg human mRNA was reverse transcribed using Moloney Leukemia Virus-reverse transcriptase (Invitrogen, Carlsbad, CA). Real-Time PCR was performed using SYBR Green I Master kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. PCR primers are depicted in **Supplemental Table 1**. As an internal control, 18S ribosomal RNA expression levels were used and Hedgehog-related gene expression was calculated using the standard curve method.

Statistical Analysis. All data represent at least three independent experiments are expressed as mean ± SE. Differences between groups were compared using two-tailed Student's *t*-tests.

Materials. Reagents were purchased from the following suppliers: DAPI was from Sigma; recombinant human TRAIL was from R&D Systems; cyclopamine was from LC Laboratories (Woburn, MA); TRAIL-R1/DR4 agonist, HGS-ETR1, and TRAIL-R1/DR5

agonist, HGS-ETR2, were kindly provided from Dr. R. Humphreys (Human Genome Sciences Inc., Rockville, MD); recombinant human Sonic hedgehog ligand was from R&D Systems.

Supplemental Table 1

Realtime RT-PCR primers

Gene	Primer sequences for realtime RT-PCR
SHh	5’-GATGTCTGCTGCTAGTCCTCG-3’
	5’-CACCTCTGAGTCATCAGCCTG-3’
IHh	5’-TGGCATGCATTGGTACTCTC-3’
	5’-GCTTGCAGCTCTATGACTAC-3’
DHh	5’-GAGACTCTTTCACAGCTTGG-3’
	5’-TATCACCTCCTCTCAGTACG-3’
GLI1	5’-GGGATGATCCCACATCCTCAGTC-3’
	5’-CTGGAGCAGCCCCCCCAGT-3’
GLI2	5’-TGGCCGCTTCAGATGACAGATGTTG-3’
	5’-CGTTAGCCGAATGTCAGCCGTGAAG-3’
GLI3	5’-GGCCATCCACATGGAATATC-3’
	5’-TGAAGAGCTGCTACGGGAAT-3’
Smoothened	5’-GTTCTCCATCAAGAGCAACCAC-3’
	5’-CGATTCTTGATCTCACAGTCAGG-3’
Patched-1	5’-CCACAGAAGCGCTCCTACA-3’
	5’-CTGTAATTTCGCCCCTTCC-3’
DR4	5’-CAGAACGTCCTGGAGCCTGTAAC-3’
	5’-ATGTCCATTGCCTGATTCTTTGTG-3’
DR5	5’-GGGAAGAAGATTCTCCTGAGATGTG-3’
	5’-ACATTGTCCTCAGCCCCAGGTCG-3’

Supplemental Table 2

Sequences inserted into pLenti4/BLOCK-iT-DEST

shRNA	Sequences for the inserted oligos
shSMO (1)	sense: CAC C**TG CAC AGC TAC ATC GCG GC**T TCA AGA GA**G CCG CGA TGT AGC TGT GCA** antisense: AAA A**TG CAC AGC TAC ATC GCG GC**T CTC TTG AA**G CCG CGA TGT AGC TGT GCA**
shSMO (2)	sense: CAC C**AC CCC AAA CCC ATC TTT TG**T TCA AGA GA**C AAA AGA TGG GTT TGG GGT** antisense: AAA A**AC CCC AAA CCC ATC TTT TG**T CTC TTG AA**C AAA AGA TGG GTT TGG GGT**
Scramble	sense: CAC C**TA TTA ATG TTA ATA TGT TT**T TCA AGA GA**A AAC ATA TTA ACA TTA ATA** antisense: AAA A**TA TTA ATG TTA ATA TGT TT**T CTC TTG AA**A AAC ATA TTA ACA TTA ATA**

Figure legend

Supplemental Figure 1. Cyclopamine sensitizes KMCH cells to TRAIL cytotoxicity in a concentration- and time-dependent manner. Cells were pretreated with cyclopamine for 24 hours. After cyclopamine pretreatment, human recombinant TRAIL (5 ng/ml) was added and the cells were incubated for the indicated time periods. Cells were then stained by DAPI and cells with apoptotic morphology were quantified as a percent of the total cells.. Data are mean ± SEM.

Supplemental Figure 2. Cyclopamine does not sensitize HepG2 cells or normal rat cholangiocytes to TRAIL cytotoxicity. Cells were pretreated with cyclopamine (5μM) for 24 hours. After cyclopamine pretreatment, human recombinant TRAIL (500 ng/ml) was added and the cells were incubated for an additional 6 hours. Cells were then stained by DAPI and cells with apoptotic morphology were quantified as a percent of the total cells. Data are mean ± SEM.

Supplemental Figure 3. SMO is highly expressed in KMCH cells. PANC-1 cells were used as a positive control and tetracycline induced shSMO-KMCH cells were used as a negative control. Whole cell lysates from PANC-1, KMCH, and shSMO-KMCH cells with and without tetracycline induction were probed using a SMO antibody. shSMO-KMCH cells were treated with tetracycline (1μg/mL) for 48 hours before isolating whole cell lysates. Actin was used as a loading control.

Supplemental Figure 4. Hedgehog pathway components were expressed in human cholangiocarcinoma tissues. Total cellular RNA was extracted from human cholangiocarcinoma tissues. mRNA expression of Hedgehog-related genes was quantified by realtime RT-PCR. The relative expression of mRNA was expressed as a ratio of target gene/18S (internal control) copies/μL.

Supplemental Figure 5. GLI3 over-expression inhibits 8x-GLI reporter activities in KMCH cells. A pGL3 empty construct and a reporter construct containing 8x GLI-binding sites (8x-GLI) were used to evaluate the promoter activity upon GLI3 overexpression. GLI3-overexpressed KMCH cells were co-transfected with 25 ng of pRL-CMV and 0.5 μg of indicated pGL3-based DR4 promoter reporter plasmids or 8x-GLI reporter plasmids. Both firefly and Renilla luciferase activities were quantified. Data (firefly/Renilla luciferase activity) are expressed as fold changes over pGL3 empty vector. Mean ±SEM, * $p<0.01$