Supporting Information

Lee et al. 10.1073/pnas.1114718109

SI Materials and Methods

Tumor Pathology. Whole brains within the skull were fixed in 10% formalin, decalcified with Formical-4 (Decal Chemical), and embedded in paraffin. Tissues were cut into 4-μm sections in the horizontal plane and H&E stained to generate computerized 3D renderings of the tumors. Sections were scanned by using a TissueFax (TissueGnostics) then stacked and aligned with the StackReg function of ImageJ, and 3D models were rendered with Imaris.

MRI and Chlorotoxin:Cy5.5 Imaging Analysis. MRI was performed by using a 3-T MRI system (Achieva; Philips) and a custom mouse head coil. Serial MRI scans were performed under halothane anesthesia with a 35-min coronal high-resolution, T2-weighted sequence [echo time (TE) = 110 ms, repetition time (TR) = 2,000 ms, bandwidth = 212, number of excitations (NEX) or signal averages = 2, pixels in-plane = 256 × 256, slice thickness = 320 μm, interslice gap = 160 μm]. Digital Imaging and Communications in Medicine (DICOM) images were exported from the MRI scanner to BioScribe and then imported into ITK-SNAP (http://www.itksnap.org). Chlorotoxin:Cy5.5 in vivo imaging was carried out as previously described (1).

Gene Amplification Analysis. *Gli2* amplification was assessed by real-time PCR using a SYBR Green PCR kit (Qiagen). Tumors were snap-frozen and analyzed for *Gli2* with three different primer sets and for Gapdh as normalization control. Genomic DNA from four mice of different genomic backgrounds was used as controls for each assay along with a no-template control. Four technical replicates were performed.

Western Blot Analysis. Tumor samples were homogenized in RIPA lysis buffer that included protease inhibitors. Equal amounts of proteins were loaded onto a 4–12% SDS/PAGE gel, transferred onto PVDF membrane, and probed with the antibody of interest: P-glycoprotein (Pgp; C219, 1:100; Covance).

Gene Expression Analysis. Total RNA was extracted with the RNeasy Kit (Qiagen). For quantitative RT-PCR analysis, cDNA synthesis was performed with the TaqMan Reverse Transcription kit (ABI) and TaqMan Master Mix and the 7300HT System (ABI). TaqMan primers for mouse *Gli1* and *Gapdh* were used. All conditions were run in triplicate and normalized to *Gapdh* controls.

For whole-genome expression analysis, RNA was hybridized to mouse-specific (MouseWG-6 v2.0) expression BeadChips (Illumina). Probe intensities were normalized with the Illumina-specific Lumi Bioconductor R package. Functional gene-interaction network analysis was performed by using Ingenuity Pathways Analysis (Ingenuity Systems).

Array-Comparative Genomic Hybridization. Genomic DNA was labeled and hybridized to Mouse Genome CGH Microarray 244K kit (Agilent) using WT littermate genomic DNA as a reference. Arrays were scanned with an Agilent microarray scanner, and image analysis was performed with Agilent Feature Extraction software.

Gli-Luciferase Reporter Assay. Gli-luciferase reporter assays were performed as previously described (2).

Pgp Functional Assay by Flow Cytometry. Pgp functionality was assessed by measuring fluorescent dye efflux of calcein-AM (Molecular Probes) with or without the Pgp inhibitor verapamil. First, 5×10^5 cerebellar *Patched1*-null ($Ptc^{C/C}$) tumor cells were incubated with or without 10 μM verapamil for 1 h at 37 °C, then with 1 μM calcein-AM for 30 min at 37 °C. Cells were washed and resuspended in PBS and analyzed for calcein fluorescence by flow cytometry (excitation: 495 nm, emission: 515 nm). Debris was eliminated by gating on forward versus side scatter. The function of Pgp is expressed as the mean percentage of fluorescence intensity of gated $FITC^+$ cells. The assays were performed in triplicate.

Statistical Considerations. Studies were designed to detect differences in event rates that approximately corresponded to a doubling of median improvement in survival of 3 wk with 90% power based on simulated power experiments. These estimates were made by using a two-sided log-rank test at $P < 0.05$ significance level. Survival analyses used animal death times and censoring times when animals were killed at ∼6 wk or as otherwise stated. Survival curves were plotted with the Kaplan–Meier method and compared by using a two-sided log-rank test. The Bonferroni test was used to correct multiple comparisons. Statistical analyses were performed in R statistical systems (http://www.r-project.org).

Immunohistochemical Staining. Tissues were cut into 4-μm sections and immunostained with the following antibodies: anti-Ki67 (1:1,000; Novocastra) and anti-Cleaved Caspase 3 (1:50; Biocare Medical). Sections were scanned with a TissueFax microscope (TissueGnostics). For Ki67 and Caspase 3 staining quantification, positive staining was quantified with National Institutes of Health image analysis software, Image J, and reported as percentage area of staining in the cerebellum.

1. Veiseh M, et al. (2007) Tumor paint: A chlorotoxin:Cy5.5 bioconjugate for intraoperative visualization of cancer foci. *Cancer Res* 67:6882–6888.

2. Yauch RL, et al. (2009) *Smoothened* mutation confers resistance to a Hedgehog pathway inhibitor in medulloblastoma. *Science* 326:572–574.

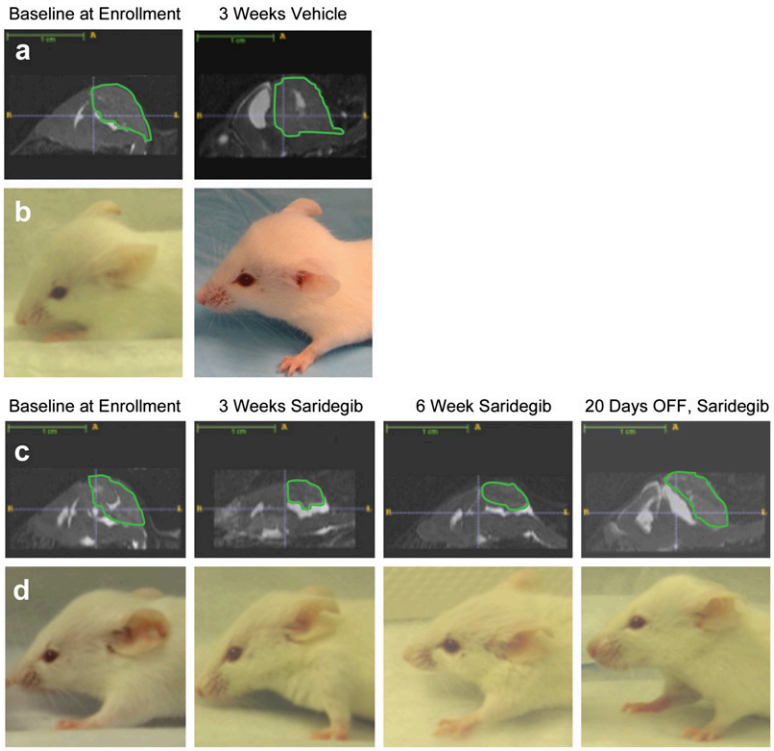

Fig. S1. Monitoring tumor response via MRI. (*A* and *C*) Representative MRI scans in the sagittal plane from vehicle-treated (*A*) or saridegib-treated (*C*) $Ptc^{C/C}$ mice are shown at enrollment, after 3 wk of daily saridegib treatment, after 6 wk of daily saridegib treatment, and after drug withdrawal. T2-weighted axial images were acquired at 3 T with a Philips MRI system and a custom mouse-head coil. Control mice were imaged at enrollment and after 3 wk on daily vehicle treatment, although no vehicle-treated mice survived until the 6-wk imaging time point. (*B* and *D*) Live animal images are shown in parallel with the MRI scans for vehicle-treated (*B*) and saridegib-treated (*D*) mice.

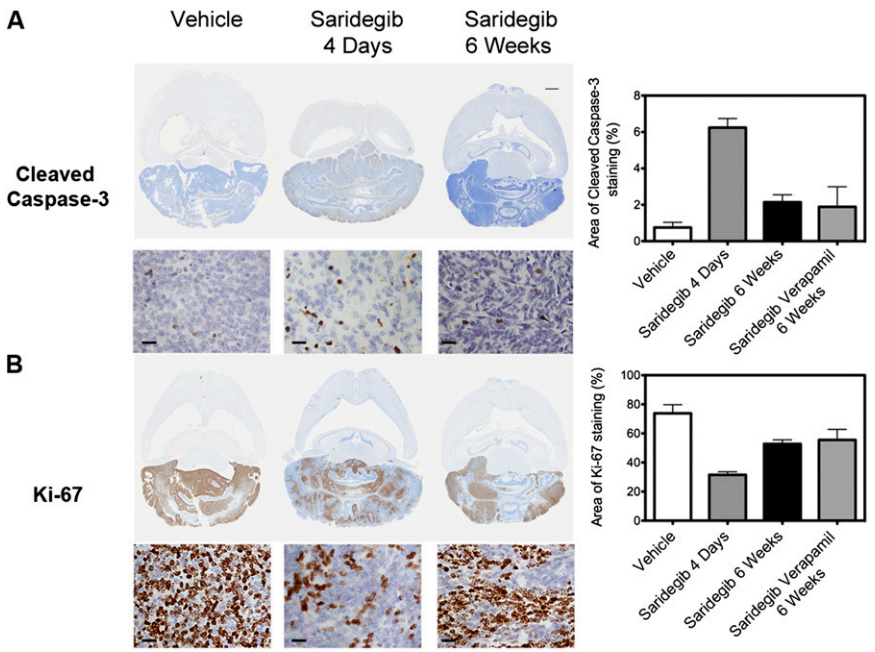

Fig. S2. Effect of saridegib on proliferation and apoptosis. Representative examples of immunohistochemical analysis of $Ptc^{C/C}$ tumors stained with Cleaved Caspase 3 (*A*) and Ki67 (*B*) antibodies are shown. Percentage area of active Caspase 3 and Ki67 were quantified with Image J. Compared with vehicle-treated controls, mice treated with the saridegib (20 mg/kg) show reduced expression of Ki67 and increased Cleaved Caspase 3 staining after 4 d of treatment. The effect of the drug on apoptosis and proliferation was reduced after 6 wk of daily therapy. Verapamil and saridegib combination therapy resulted in similar apoptotic and proliferative responses compared with saridegib alone. (Scale bars: *Upper*, 1 mm; *Lower*, 50 μm.) Individual data points represent the means ± SEM of three independent samples from each treatment.

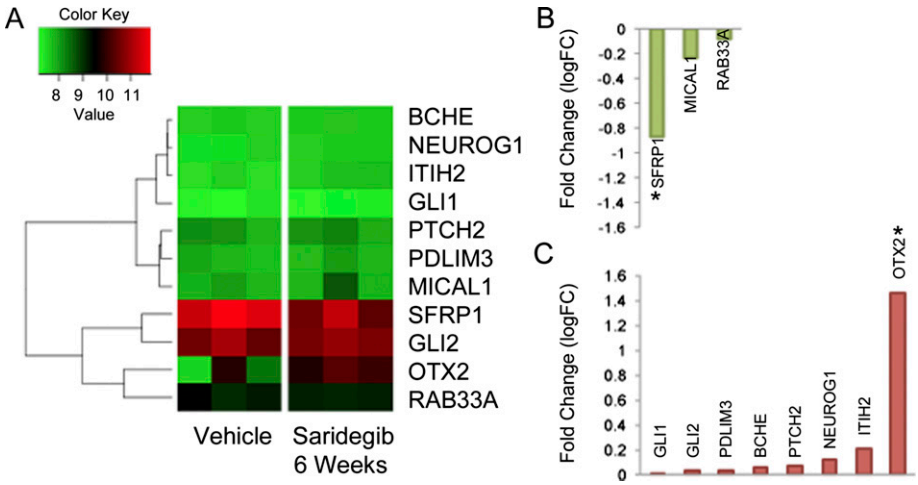

Fig. S3. Differential gene expression in vehicle- and saridegib-treated $Ptc^{C/C}$ samples. (*A*) Three independent vehicle- and saridegib-treated $Ptc^{C/C}$ samples were analyzed with the use of an Illumina expression array. The heat map depicts the expression of the Sonic Hedgehog (Shh) signature genes. (*B*) Differentially expressed Shh signature genes highly expressed in vehicle-treated compared with saridegib-treated $Ptc^{C/C}$ samples. (*C*) Differentially expressed Shh signature genes highly expressed in saridegib-treated compared with vehicle-treated $Ptc^{C/C}$ samples.

Table S1. Drug concentrations in plasma, brain, and tumor

			Tissue concentration		
Genotype	Treatment	Statistics	Cerebellum/tumor, ng/g	Forebrain, ng/g	Plasma, ng/mL
WT	None	Mean	0	0	0
		SD	0	0	0
	Saridegib for 4 d, daily, 20 mg/kg per dose	Mean	482	393	652
		SD	75	84	276
$Ptc^{C/C}$ intracranial tumors	None	Mean	0	0	0
		SD	0	0	0
	Saridegib for 4 d, daily, 20 mg/kg per dose	Mean	478	300	803
		SD	98	32	316
	Saridegib for 6 wk, daily, 20 mg/kg per dose	Mean	1,269	376	1,135
		SD	570	58	411
	Saridegib for 3 wk, daily, 20 mg/kg per dose; one dose at 70 mg/kg	Mean	5,956	1,659	1,830
		SD	2,302	421	349
	Saridegib for 3 wk, daily, 20 mg/kg per dose; 3 wk, one dose per week, 70 mg/kg	Mean	8,911	1,569	2,553
		SD	1,726	469	501
$Ptc^{C/C}$ flank allografts (WT recipients)	None	Mean	0	N/A	N/1
		SD	0	N/A	N/A
	Saridegib for 7 d, daily, 20 mg/kg per dose	Mean	47,320	N/A	N/A
		SD	27,887	N/A	N/A
	Saridegib, daily, 20 mg/kg per dose until tumor regrowth	Mean	22,053	N/A	N/A
		SD	3,834	N/A	N/A

N/A, not applicable.

Table S2. Top 10 networks and the associated functional categories of the genes significantly altered in saridegib-treated $Ptc^{C/C}$ tumors identified by Ingenuity Pathway Analysis

Category	*P*	No. of molecules	Genes up-regulated by more than twofold	Genes down-regulated by more than twofold
Cancer	2.19×10^{-27}–1.04×10^{-03}	173	MT1E, ANXA5, TIMP1, IFITM3, PLEKHB1, EFF1A2	CHRNA3, MTDH
Neurological disease	6.03×10^{-25}–8.09×10^{-04}	159	C3, SPP1, PDGDS, VSNL1, ENO2, PVALB, OTX2, CHI3L1, ITPR1, PCP2, LCN2, THY1, DCN, SLC24A2, C4B, MBP, IGFBP5, ATP2A3, HSPB8	CHRNA3, MTDH
Skeletal and muscular disorders	7.15×10^{-25}–1.24×10^{-03}	154	C3, SPP1, PDGDS, VSNL1, ENO2, PVALB, CHI3L1, ITPR1, LCN2, THY1, C4B, MBP, IGFBP5, ATP2A3, HSPB8	CHRNA3, MTDH
Cell death	1.27×10^{-19}–1.29×10^{-03}	140	LGALS3, C3, HBB, HBA1/HBA2, SPP1, PTGDS, CALB2, OTX2, CHI3L1, ITPR1, LCN2, Lyz1/Lyz2, THY1, DCN, MBP, IGFBP5, GADD45G, HSPB8, PCSK9, GFAP	MTDH
Cellular movement	9.06×10^{-15}–1.26×10^{-03}	96	LGALS3, C3, SPP1, VSNL1, CHI3L1, ITPR1, LCN2, Lyz1/Lyz2, THY1, DCN, C4B, IGFBP5, RASGRF1, GFAP	—
Cellular growth and proliferation	2.13×10^{-14}–1.08×10^{-03}	136	LGALS3, C3, SPP1, GPNMB, PTGDS, OTX2, ITPR1, LCN2, THY1, DCN, MBP, IGFBP5, GADD45G, HSPB8, RASGRF1, GAD1	—
Tissue development	1.18×10^{-13}–1.04×10^{-03}	126	LGALS3, C3, HBB, HBA1/HBA2, SPP1, PTGDS, VSNL1, OTX2, KNDC1, ITPR1, LCN2, ATP1B1, THY1, DCN, C4B, MBP, IGFBP5, GADD45G, RASGRP1,PCSK9, GFAP	CHRNA3
Nervous system development and function	2.55×10^{-11}–1.26×10^{-03}	96	C3, HBA1/HBA2, VSNL1, OTX2, CHI3L1, KNDC1, ITPR1, ATP1B1, THY1, SLC24A2, MBP, RASGRP1,PCSK9, GFAP	CHRNA3
Hematological system development and function	2.46×10^{-10}–1.24×10^{-03}	89	LGALS3, C3, HBB, HBA1/HBA2, SPP1, PTGDS, CHI3L1, LCN2, Lyz1/Lyz2, THY1, DCN, C4B, MBP, GADD45G, HSPB8, GFAP, GJA1	—
Immune cell trafficking	2.46×10^{-10}–1.24×10^{-03}	64	LGALS3, C3, SPP1, PTGDS, CHI3L1, LCN2, Lyz1/Lyz2, THY1, DCN, C4B, MBP, HSPB8, GFAP, ANXA5, NBL1, TIMP1	—

Genes from the dataset that met the log-ratio cutoff of $\geq|0.5|$ and $P < 0.05$ were considered for the analysis.

Table S3. Top genes differentially expressed in saridegib-treated $Ptc^{C/C}$ tumors

Symbol	Entrez gene name	Log ratio	*P*
Log ratio up-regulated			
LGALS3	Lectin, galactoside-binding, soluble, 3	2.232	1.56×10^{-16}
C3	Complement component 3	2.072	6.61×10^{-16}
HBB	Hemoglobin, β	2.000	6.62×10^{-09}
HBA1/HBA2	Hemoglobin, α1	1.741	2.07×10^{-08}
SPP1	Secreted phosphoprotein 1	1.736	3.12×10^{-10}
GPNMB	Glycoprotein (transmembrane) NMB	1.705	1.34×10^{-12}
PTGDS	Prostaglandin D2 synthase 21 kDa (brain)	1.687	6.73×10^{-13}
CALB2	Calbindin 2	1.612	7.28×10^{-13}
VSNL1	Visinin-like 1	1.535	1.98×10^{-13}
ITIH3	Inter-α-trypsin inhibitor heavy chain H3	1.529	4.69×10^{-15}
ENO2	Enolase 2 (γ, neuronal)	1.520	2.95×10^{-13}
PVALB	Parvalbumin	1.477	7.28×10^{-13}
OTX2	Orthodenticle homeobox 2	1.460	3.86×10^{-08}
Log ratio down-regulated			
RPRML	Reprimo-like	−2.173	5.42×10^{-19}
MAK16	MAK16 homolog (*Saccharomyces cerevisiae*)	−1.481	3.60×10^{-11}
THAP4	THAP domain-containing 4	−1.081	6.54×10^{-08}
CHRNA3	Cholinergic receptor, nicotinic, α3	−1.071	1.08×10^{-08}
MTDH	Metadherin	−1.055	1.39×10^{-07}
MAP2	Microtubule-associated protein 2	−0.953	8.11×10^{-06}
MCM6	Minichromosome maintenance complex component 6	−0.937	1.42×10^{-06}
GSTO1	GST ω1	−0.911	3.43×10^{-07}
SFRP1	Secreted frizzled-related protein 1	−0.868	1.16×10^{-05}
ISLR2	Ig superfamily containing leucine-rich repeat 2	−0.830	3.87×10^{-03}
OTX1	Orthodenticle homeobox 1	−0.820	9.15×10^{-05}

A cutoff of log ratio $\geq|0.5|$ and $P \leq 0.05$ were used to define focus genes in the network analysis.

Table S4. Primers used for sequencing

Exon	Forward primer, 5′ → 3′ (each prefixed with M13 forward)	Reverse primer, 5′ → 3′ (each prefixed with M13 reverse)
1	AAGCTGGCCCCAGACTTTC	CAACAGTTTGAGGCCTGAGC
2	GCATAAGGCAACCCTTAGCA	GCTTGACAACCATGCTCCAT
3	GCCCTATGAGGTAGGGGCTA	AGCCACAAAGGTGGCCTAAA
4	CACCAGGACATGCACAGCTA	GGACACAGGTCGGATTTGAA
5	AGCATTGCCCTGTTGTGTTC	CCAGCACGGTACCGATAGTTC
6	CTATGCCTTGATGGCTGGAG	GAACCTTGGTCATGGCTTTG
7	AGGCTCTGTCCCAGTTACCG	CCCCTTCTCAGAGGGAGTTG
8	TGTAGCCACCCTGGACTCAG	ACCTGCTCCTGTGCATTGAC
9	CCATGAGAATCACGCAGTGG	GGCTCCTGTGGCTCCTACTT
10	CTGTGAAGGCCTCAGCTCCT	CAGAGAAGAAGGAAGAGAGAGCAA
11	GCTCCAGGGTGGAATCTCTC	CACTGTCAGGGGGACAAAGA
12	ACCTGAAGGAGATGCCAAGG	CAGACACTTGGCCCACAGAC

PCR primers were designed with Primer3 and incorporated either M13 forward (5′-TGTAAAACGACGGCCAGT-3′) or M13 reverse (5′-CAGGAAACAGCTATGAC-3′) priming sites.