Small Molecule Intervention in a PKC-Gli Axis

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Materials. Compounds were purchased from the following companies: Cayman Chemical Company: PMA, Ing, saGly, oaGly, Cer, Gö6976, PSI, and PKC9; Santa Cruz Biotechnology: SAG, SC9, and Pro; Fisher Scientific: PDBu; BOC Sciences: TPPB; Adooq Bioscience: ILV, GANT, and IngMb; Toronto Research Chemicals: S10. Recombinant human Sonic Hedgehog protein (hSHH-N) was purchased from R&D Systems (Cat. No. 8908-SH-005). All other reagents were of molecular biology grade and purchased from Sigma. Unless noted otherwise, tissue culture media and additives were from Invitrogen. The Dual Luciferase Reporter Assay System, Bright-Glo reagent, and CellTiter 96 AQueous One Solution Cell Proliferation Assay System were from Promega.

Cell Culture. Shh-LIGHT2 and NIH-3T3 cells were grown in DMEM containing 1% sodium pyruvate, 1% penicillin/streptomycin, and 10% calf serum. Sufu-KO-LIGHT cells were grown in DMEM containing 1% sodium pyruvate, 1% penicillin/streptomycin, 10% fetal bovine serum, 150 μg/mL zeocin, and 400 μg/mL geneticin (39). C3H10T1/2 cells were grown in DMEM containing 1% sodium pyruvate, 1% penicillin/streptomycin, and 10% fetal bovine serum. TM3-Gli-Luc cells were grown in F12 Ham's/DMEM (1:1) containing 1% sodium pyruvate, 1% penicillin/streptomycin, 5% horse serum, 2.5% fetal bovine serum, and 15 mM HEPES, pH 7.3. ASZ001 cells were grown in M154CF containing 1% penicillin/streptomycin, 2% chelated fetal bovine serum, human keratinocyte growth supplement (Invitrogen), and 0.05 mM $CaCl_2$. All cell lines were grown at 37 °C with 5% CO_2. Serum starvation was performed with media containing 0.5% fetal bovine serum for Sufu-KO-LIGHT, C3H10T1/2, and ASZ001 cells, 0.5% calf serum for Shh-LIGHT2 and NIH-3T3 cells, and 0.4% horse serum and 0.1% fetal bovine serum for TM3-Gli-Luc cells.

RNA-Seq Analysis. NIH-3T3 cells were grown to 100% confluence in a 24-well plate and treated with DMSO or 200 nM SAG in starvation media. After 30 h, the medium was aspirated and the cells were rinsed with PBS. Cells were lysed using the Qiagen QIAshredder homogenizer kit. Total RNA was extracted using the Qiagen RNeasy Mini kit, and RNA quality was assessed on an Agilent 2100 Bioanalyzer prior to library preparation. Samples were processed using the Illumina TruSeq Stranded mRNA Sample Preparation Kit, using a 3-cycle PCR for final amplification. Samples were analyzed on a HiSeq 4000 with a 76 paired end read length and read depth of 4×10^7 counts per sample. Differential expression was analyzed using DESeq2 with false discovery rate (FDR)-corrected p-value of 0.1. Biological triplicates were analyzed for each condition.

Shh-LIGHT2 Assays. Shh-LIGHT2 cells were seeded in 96-well plates at a density of 3.5 x 10^4

cells/well. After 24 h, medium was changed to starvation media containing compounds at the concentration specified for 30 h. After 30 h, media was removed from all wells. Cells were washed with PBS and then treated with Passive Lysis Buffer (20 μL/well, Promega Dual Luciferase Assay kit). After 15 min of rocking, 10 μL lysate was transferred to a white bottom assay plate, and Bright-Glo and Stop & Glo reagents were used to measure Firefly and Renilla luciferase signal on a GloMax luminometer. Gli activity was calculated as the ratio of Firefly/Renilla luciferase signal. For each compound, percent Gli inhibition or activation was assessed versus Gli-driven luciferase activity induced by 200 nM SAG as a control (100%). All dose-response curves were generated using GraphPad Prism software.

Sufu-KO-LIGHT Assays. Sufu-KO-LIGHT cells were seeded in 96-well plates at a density of 3.5 x 10^4 cells/well. After 24 h, medium was changed to starvation media containing compounds at the concentration specified. After 30 h, cells were incubated with 20 μL/well CellTiter AQueous One at 37 °C for 30 min. Cell viability was measured at 490 nm on a Molecular Devices Spectramax M2 spectrophotometer. Cells were then washed with PBS and incubated with 100 μL/well Bright-Glo reagent for 5 minutes at room temperature. Firefly luciferase signal from total cell lysate was measured on a GloMax luminometer. Gli activity was calculated as the ratio of Firefly/CellTiter signal. For each compound, percent Gli inhibition or activation was assessed Gli-driven luciferase activity in the presence of DMSO as a control (100%). All dose-response curves were generated using GraphPad Prism software.

qPCR Assays. Shh-LIGHT2 or Sufu-KO-LIGHT cells were seeded in 96-well plates at a density of 3.5 x 10^4 cells/well. After 24 h, medium was changed to starvation media containing TPPB (**1**) at the concentration specified for 30 h. After 30 h, media was removed from all wells, cells were washed with 100 μL PBS, snap frozen in liquid N_2, and stored at –80 °C. Upon thawing, cells were homogenized using TRIzol (ThermoFisher) and RNA was extracted using the PureLink RNA Mini Kit (ThermoFisher). cDNA was prepared from total RNA using the QuantiSure First Strand cDNA Synthesis Kit (Gene Scientific). qPCR analysis was performed in triplicate for each sample using the AccuAmp™ Probe qPCR Master Mix/ Low ROX (Gene Scientific) on an Agilent MX3005P QPCR System. The ΔΔCt method was used for relative quantification of gene expression using B2M as a reference gene.

qPCR Primers/Probes:

B2M

Forward Primer	GGG TGG AAC TGT GTT ACG TAG
Reverse Primer	TGG TCT TTC TGG TGC TTG TC
Probe	/56-FAM/CCG GAG AAT /ZEN/GGG AAG CCG AAC ATA C/3IABkFQ/
Gli1	
Forward Primer	TTG GAT TGA ACA TGG CGT CT
Reverse Primer	CCT TTC TTG AGG TTG GGA TGA
Probe	/56-FAM/AGC TGG AGG /ZEN/TCT GCG TGG TAG A/3IABkFQ/

NIH-3T3 Luciferase Assays. NIH-3T3 cells were seeded 10 cm plates at a density of 2.5 x 10^5 cells/plate to 80% confluence over 36 h. Cells were transfected with the pGL4.23-GW plasmid (Addgene plasmid #60323) using Lipofectamine 3000 (Invitrogen) according to manufacturer's protocol. After 24 h cells were dissociated and reseeded into 96-well plates at a density of 3.5 x 10^4 cells/well. After 24 h, media was changed to starvation media containing compounds at the concentration specified. After 30 h, cells were incubated with either 20 µL CellTiter AQueous One at 37 °C for 30 min or 100 µL Bright-Glo reagent at room temperature for 5 min. Cell viability was measured as absorbance at 490 nm on a Tecan Spark 10M. Firefly luciferase signal from total cell lysate was measured on a Tecan Spark 10M. Luciferase activity was calculated as the ratio of Firefly/CellTiter relative to DMSO (100%). All dose-response curves were generated using GraphPad Prism software.

C3H10T1/2 Assays. C3H10T1/2 cells were seeded in 96-well plates at a density of 1x10^4 cells/well. After 24 h, medium was changed to starvation media containing compounds at the concentration specified. After 48 h, cells were incubated with either 20 µL CellTiter AQueous One at 37 °C for 1 h or lysed in 50 µL lysis buffer (100 mM Tris pH 9.5, 250 mM NaCl, 25 mM $MgCl_2$, 1% Triton X-100) for 45 min at room temperature with rocking. Cell viability was measured as absorbance at 490 nm on a Tecan Spark 10M. Alkaline phosphatase activity was measured by transferring 10 µL lysate to a 96-well white bottom assay plate and adding 50 µL of CDP-Star chemiluminescence reagent (Perkin-Elmer) and measuring the resulting chemiluminescence on a Tecan Spark 10M. Gli activity was calculated as the ratio of chemiluminescence/absorbance relative to 200 nM SAG (100%). All dose-response curves were generated using GraphPad Prism software.

mBCC Assays. ASZ001 cells were seeded in 96-well plates at a density of 1x10^4 cells/well. After 24 h, medium was changed to starvation media containing compounds at the concentration specified. After

48 h, cells were incubated with 20 µL CellTiter AQueous One at 37 °C for 1 h. Cell viability was measured as absorbance at 490 nm on a Tecan Spark 10M and calculated relative to DMSO (100%). All dose-response curves were generated using GraphPad Prism software.

TM3-Gli-Luc Assays. TM3-Gli-Luc cells were seeded in 96-well plates at a density of $3.5x10^4$ cells/well. After 24 h, medium was changed to starvation media containing compounds at the concentration specified. After 30 h, cells were incubated with either 20 µL CellTiter AQueous One at 37 °C for 30 min or 100 µL Bright-Glo reagent at room temperature for 5 min. Cell viability was measured as absorbance at 490 nm on a Tecan Spark 10M. Firefly luciferase signal from total cell lysate was measured on a Tecan Spark 10M. Gli activity was calculated as the ratio of Firefly/CellTiter relative to 200 nM SAG (100%). All dose-response curves were generated using GraphPad Prism software.

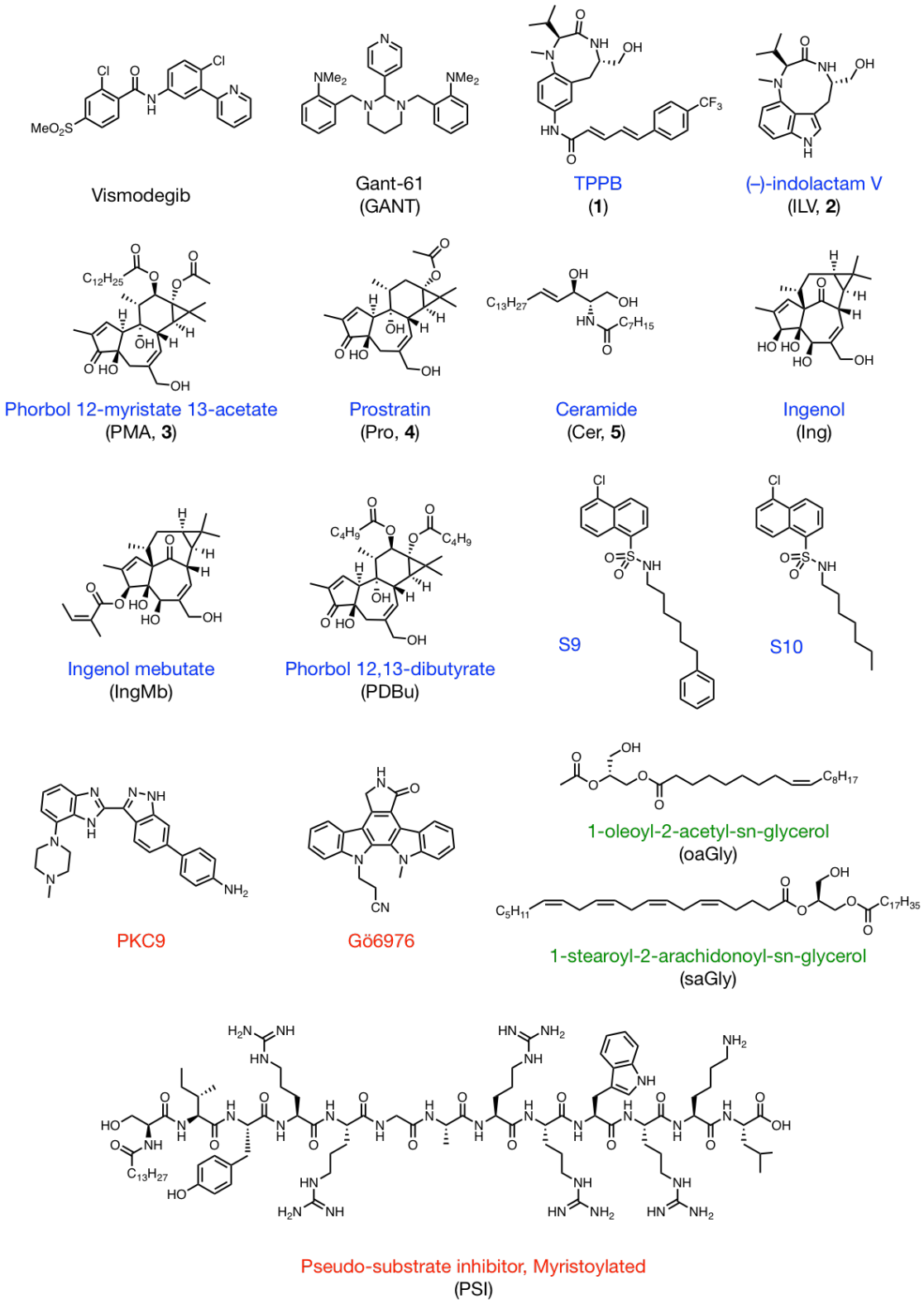

Fig. S1. Structures of compounds tested in Shh-LIGHT2 and Sufu-KO-LIGHT cells. Blue: regulatory domain C1-binding site activators; green: DAG-based lipid activators; red: catalytic domain inhibitors.

Shh-LIGHT2 Cell Line					
	Nanomolar Potency				
	Compound	**Gli Luciferase (% control)**	**Error**	**IC50 (nM)**	**Error**
	Vismodegib	30	5	37	2.5
	TPPB	33	4	20	7
	ILV	43	4	33	7.8
	PMA	52	13	53	18
	Cer	74	1	65	15
	Micromolar Potency				
	Compound	**Gli Luciferase (% control)**	**Error**	**EC50 or IC50 (μM)**	**Error**
	Ing	90	1	3.2	0.6
	IngMb	51	6	0.61	0.34
	Pro	55	5	0.13	0.06
	PDBu	52	12	8.9	3.3
	S9	92	7	8.3	1.3
	S10	67	9	2.9	0.6
	Go6976	95	9	2.7	2.3
	PKC9	99	7	2.2	0.5
	PSI	100	13	0.36	0.12
	saGly	104	3	0.24	0.01
	oaGly	80	9	1.1	0.4

Table S1. Evaluation of PKC effectors in Shh-LIGHT2 cells treated with SAG (200 nM). Relative Gli luciferase activity at 1.5 μM dose of each compound relative to SAG alone (200 nM) as 100%. Half maximal inhibitory (IC_{50}) concentration was determined for each test compound. All values are the mean of $n \geq 3$ biological replicates ± s.d.

Sufu-KO-LIGHT Cell Line					
	Nanomolar Potency				
	Compound	**Gli Luciferase (% control)**	**Error**	**IC50 (nM)**	**Error**
	TPPB	26	4	67	29
	ILV	31	3	13	6
	PMA	53	9	18	10
	Pro	27	5	98	52
	Micromolar Potency				
	Compound	**Gli Luciferase (% control)**	**Error**	**IC50 (μM)**	**Error**
	Vismodegib	78	4	>5	--
	GANT	4	17	2.2	0.2
	Cer	101	--	>5	--

Table S2. Evaluation of PKC effectors in the Sufu-KO-LIGHT cells. Relative Gli luciferase activity at 1.5 μM dose of each compound relative to DMSO (100%). Half maximal inhibitory (IC_{50}) concentration was determined for each compound. All values are the mean of $n \geq 3$ biological replicates ± s.d.

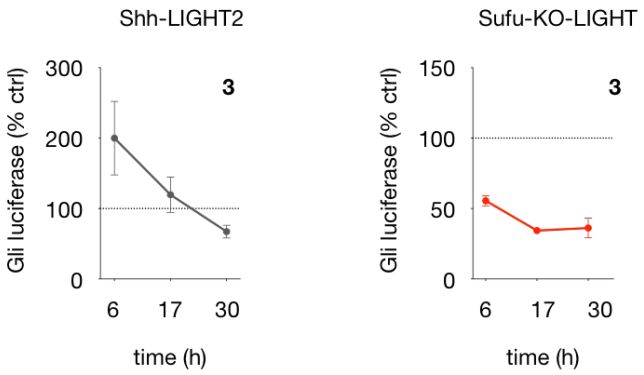

Fig. S2. Evaluation of PMA (**3**) at 6, 17, and 30 h in Shh-LIGHT2 treated with SAG (200 nM) or Sufu-KO-LIGHT cells. Relative Gli luciferase activity at 1.5 μM dose of **3** in Shh-LIGHT2 cells treated with 200 nM SAG and Sufu-KO-LIGHT cells. Control is Shh-LIGHT2 cells treated with 200 nM SAG (100%). Values are the mean of $n \geq 3$ biological replicates ± s.d.

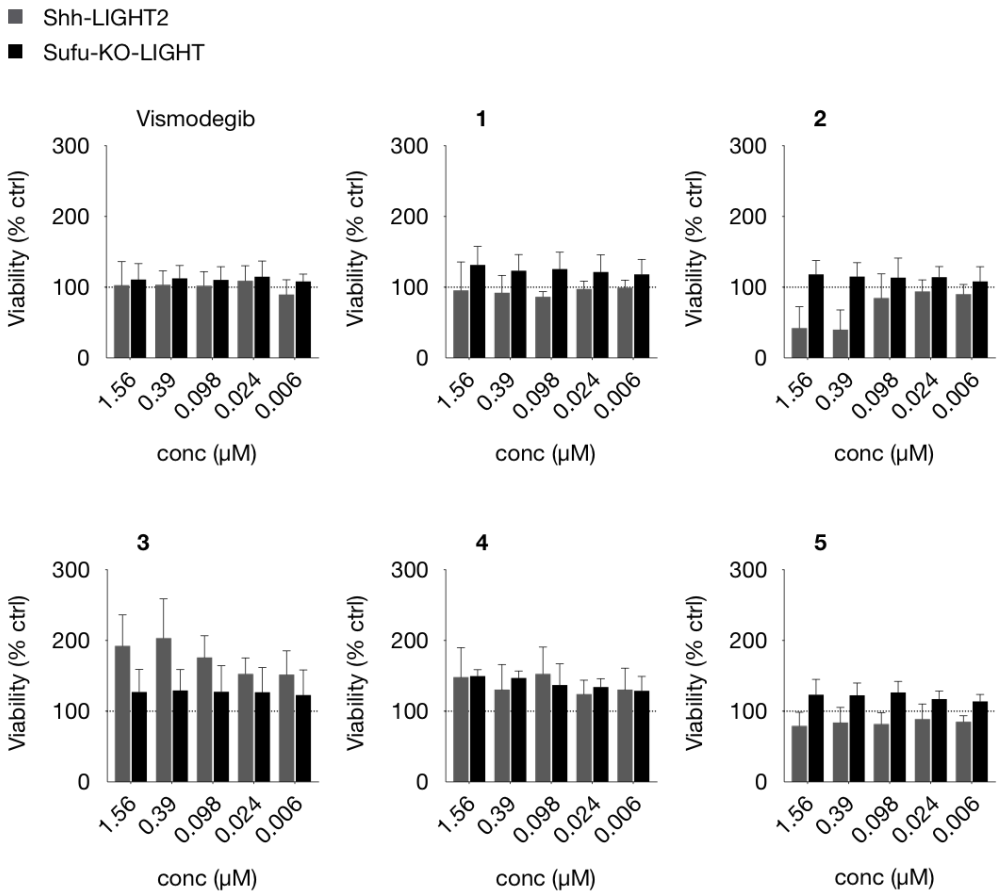

Fig. S3. Percent viability in Shh-LIGHT2 cells (TK-driven Renilla luciferase) and Sufu-KO-LIGHT cells (absorbance, CellTiter) relative to control for Vismodegib, TPPB (**1**), ILV (**2**), PMA (**3**), Pro (**4**), and Cer (**5**) at sub-micromolar concentrations in Shh-LIGHT2 cells relative to DMSO (100%). Concentrations displayed on graph are in micromolar. All values are the mean of n $\geq$ 3 biological replicates ± s.d.

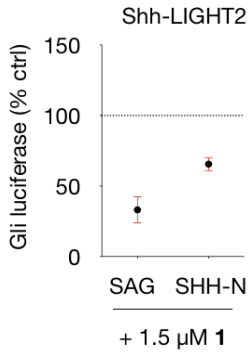

Fig. S4. Evaluation of TPPB (**1**) in Shh-LIGHT2 cells treated with SAG (200 nM) or recombinant human Sonic Hedgehog protein hSHH-N (10 ng/mL). Relative Gli luciferase activity at 1.5 μM dose of **1** in Shh-LIGHT2 cells. Control is Shh-LIGHT2 cells treated with 200 nM SAG or 10 ng/mL hSHH-N (100%). Values are the mean of $n \geq 3$ biological replicates ± s.d.

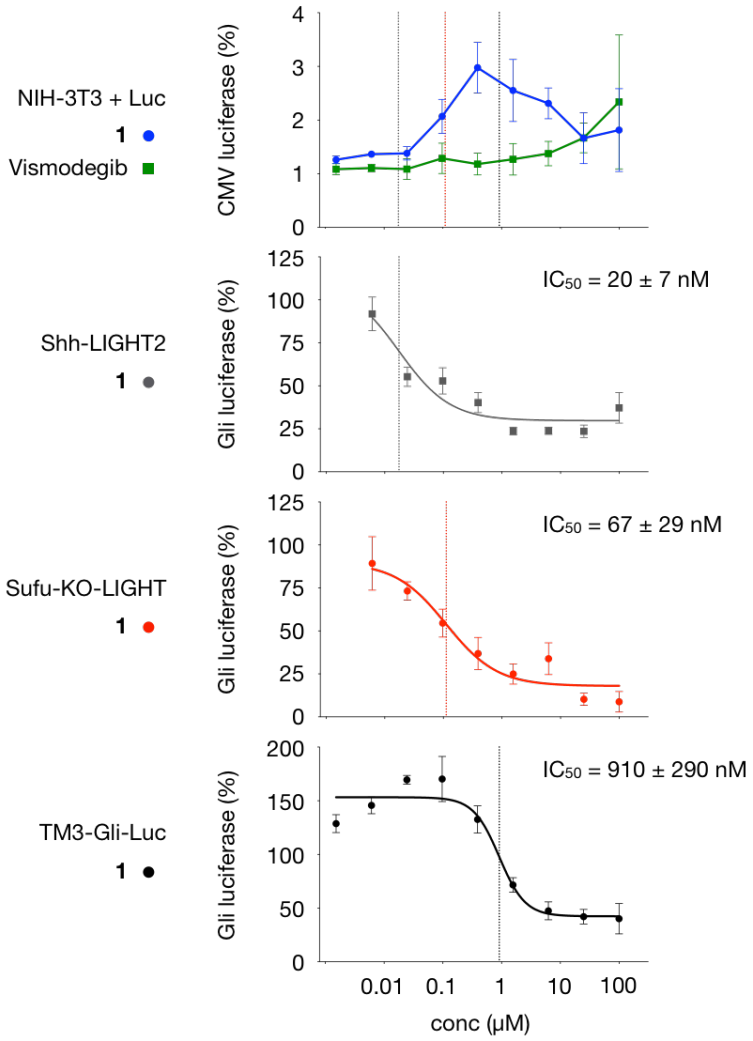

Fig. S5. Top panel: activation of luciferase enzyme by **1** or Vismodegib in NIH-3T3 cells transiently transfected with CMV-driven luciferase, normalized to CellTiter and calculated relative to DMSO (100%). Lower panels: inhibition of Gli-driven luciferase expression in Shh-LIGHT2 cells, Sufu-KO-LIGHT cells, and TM3-Gli-Luc cells[1] stimulated with 200 nM SAG, normalized to TK-driven Renilla luciferase (Shh-LIGHT2) or CellTiter (Sufu-KO-LIGHT, TM3-Gli-Luc) and calculated relative to 200 nM SAG (100%). Vertical lines indicate IC50 values for **1** in each cell line, demonstrating that inhibition of Gli-driven luciferase expression overcomes activation of luciferase enzyme at relevant concentrations.

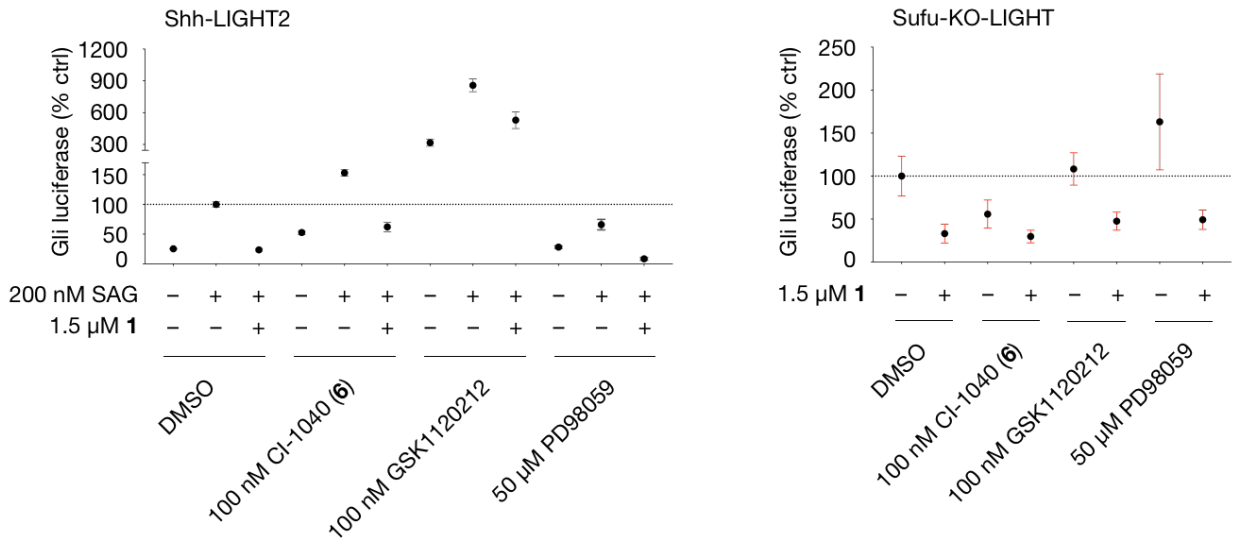

Fig. S6. Inhibition of Gli-driven luciferase activity by 1.5 μM 1 in the presence of MEK inhibitors. Relative Gli-driven luciferase inhibition in Shh-LIGHT2 cells by **1** (1.5 μM) in the presence of SAG (200 nM) and MEK inhibitors CI-1040 (**6**, 100 nM), GSK112012 (100 nM),[S2] and PD98059 (50 μM).[S3] Relative Gli-driven luciferase inhibition in Sufu-KO-LIGHT cells by **1** (1.5 μM) in the presence of MEK inhibitor **6** (100 nM). For all values, Gli luciferase is calculated relative to Gli-driven luciferase activity induced by 200 nM SAG in Shh-LIGHT2 cells (100%) or DMSO in Sufu-KO-LIGHT cells (100%). All values are the mean of $n \geq 3$ biological replicates ± s.d.

SUPPLEMENTARY REFERENCES

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