Supplementary Information

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Table S1. *In silico* alanine scanning for residues of ZF4 and ZF5 most involved in Gli1-ZF binding to DNA.

Zinc finger	Gli1 mutant	ΔΔG binding (MM-PBSA)[a]	ΔΔG binding (MM-GBSA)[a]
ZF4	S346A	1.75 ± 1.02	0.85 ± 0.99
ZF4	R354A[*]	14.87 ± 1.04	7.01 ± 1.06
ZF4	K350A	15.21 ± 0.97	6.70 ± 0.98
ZF4	R348A	9.63 ± 1.04	1.07 ± 1.00
ZF4	K340A	10.19 ± 1.01	8.82 ± 1.02
ZF5	S377A	3.31 ± 1.05	1.48 ± 1.00
ZF5	S378A	-1.19 ± 1.03	3.82 ± 1.01
ZF5	R380A	14.72 ± 1.06	8.58 ± 0.99
ZF5	K381A[*]	11.82 ± 1.03	6.77 ± 0.98
ZF5	K360A	13.48 ± 1.05	10.11 ± 0.97
ZF5	K371A	10.91 ± 1.00	6.89 ± 0.96

[a] values are in kcal/mol. Deviation is the Standard Error of the Mean (SEM)

[*] Energy contribution was calculated by considering explicit bridging water molecules.

Movie S1. Molecular Dynamics trajectory of Gli1ZF/DNA. The movie shows the conformational behavior of the Gli1ZF/DNA complex in explicit water (TIP3P type, removed for clarity of presentation) simulated by classic Molecular Dynamics with Amber11. Trajectory was visualized with VMD. The movie describes the Gli1ZF/DNA system in its geometrical and energetic convergence status. Gli1 is showed as colored surface (different colors corresponds to different

atom types) while DNA is showed as orange backbone and colored sticks. Non polar H atoms were omitted.

Supplementary Material and Methods

Cell culture, **Tranfection, Reagents.** HEK293T, MEF, Ptch1$^{-/-}$ MEF, Smo$^{-/-}$ MEF, SuFu$^{-/-}$ MEF, HepG2 cells were cultured in DMEM plus 10% FBS; Jurkat T cells were cultured in RPMI 1640 (Gibco-BRL, Grand Island, NY) plus 10% FCS; Daoy cells were cultured as previously described (Mazzà *et al*, 2013); Ptch1$^{+/-}$ MB-SCs were cultured as previously described (Po *et al*, 2010). All media contained L-glutamine and antibiotics. ASZ001 BCC cells were cultured in 154CF medium (Gibco-BRL) plus 2% FBS chelated with Chelex 100® sodium form (Sigma Aldrich, St. Louis, MO,USA), calcium chloride 0.05 mM (Gibco-BRL, Grand Island, NY) and antibiotics. Transient transfections were performed using Lipofectamine 2000 or Lipofectamine Plus (Invitrogen, Eugene, Or, USA). For RNA interference, Daoy cells were transfected with non-targeting siRNA as control or with double-stranded siRNAs targeting human Gli1 and Gli2 (On target-Plus SMARTpool reagents; Dharmacon, Lafayette,CO). Transfections were performed with HiPerFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cerebellar GCPs (from 4-days old mice) were isolated and cultured as previously described (Di Marcotullio *et al*, 2011). Murine MBs were isolated from Ptch1$^{+/-}$ mice (EMMA, Monterotondo, Italy). Tissues were collected as previously described (Po *et al*, 2010) and immediately prepared cell suspensions were used for short-term cultures to keep Hh-sensitivity *in vitro* (Sasai *et al*, 2006; Berman *et al*, 2002; Kool *et al*, 2014). Cells were treated with ShhN (3 µg/ml, R&D Systems Inc, MN, USA) or GANT61 (Enzo Life Science, UK).

Site-directed mutagenesis

To mutagenize Flag-Gli1 construct, the QuickChange XL Site-Directed Mutagenesis Kit and the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) were used. The reaction was carried out according to the manufacturer's protocol.

Preparation of the virtual library. The *in house* unique library was composed of 816 natural products. Two-dimensional molecular sketches were first converted into SMILES strings and then transformed in the three-dimensional SDF format. The LigPrep application of the Schrodinger Maestro suite (LigPrep, 2011) was used for ionizing compounds at pH = 7.5 ± 1, for generating tautomers and for energy minimization with the OPLS2005 force field (Jorgensen *et al*, 1996). Ionization states endowed with a normalized probability higher than 0.6 were retained in the library. After this step the library was composed of 1111 individual entries. QikProp was used for calculating chemical and chemico-physical features of all compounds showing that more than 90%

of molecules are in accordance with the Lipinski rule of five and more than 93% are within the limits set by the 95% of drugs in the market (Figure S3) (QikProp, 2011; Lipinski *et al*, 2001).

Docking-based Virtual Screening. The GOLD docking program (version 5.0.1) (Verdonk *et al*, 2003) was used to dock the *in house* library of natural products toward the putative binding site of Gli1 located within ZF4 and ZF5 of Gli1. A representative Gli1ZF structure was extracted from MD trajectories and used as rigid receptor in docking simulations. The GoldScore function was used, which is generally recommended for binding sites particularly solvent-exposed or accounting for several H-bonding in ligand-protein interaction, such as the ZF4 and ZF5. GA efficacy was set to 150% and 50 runs for each ligands were performed; other parameters were kept at their default values. The binding site for docking simulations was centered on the middle point within ZF4 and ZF5, in correspondence of the side chain of T374, and had a radius of 18 Å in order to scan almost all accessible surface of Gli1ZF. Ligand docking poses within Gli1ZF were clustered based on a RMS tolerance of 1.5 Å. After visual inspection, the most representative pose of each ligand (top ranking pose of the most populated cluster) was selected for the subsequent rescoring procedure, which was carried out with the Molecular Mechanics Generalized Born Surface Area (MM-GBSA) method implemented in Amber11 (Case *et al*, 2010). Only the few compounds able to interact at least with the side chain of a key residue highlighted by the mutagenisis study were further considered. The final selection of virtual hits to be progressed to *in vitro* tests was driven by the combination of binding pose and ligand efficiency (LE = ΔG_{MMGBSA}/#heavy atoms). The ff99bsc0 and General Amber (GAFF) force fields were used for parameterizing protein and ligands, respectively. Zinc ions were treated following a bound approach, using force field parameters adapted from previous study on a similar Zn-coordination system (Mori *et al*, 2010).

Characterization of GlaB

GlaB characterization data: mp:102-104 °C; TLC (Hexane:Ethylacetate, 60 :40 v/v): Rf = 0.26.
^{1}H NMR (400 MHz, Acetone-δ_6): δ 7.94 (s, 1H, H-8), 7.16 (d, *J*=1.6 Hz, 1H, H-15), 6.99 (dd, *J*=8.0 Hz and 1.6 Hz, 1H, H-11), 6.89 (d, *J* = 8.0 Hz, 1H, H-12), 6.50 (d, *J* = 2.0 Hz, 1H, H-1), 6.42 (d, *J* = 2.0 Hz, 1H, H-3), 5.43 (m, 2H, 2 x =C*H*), 4.51 (d, *J*=6.8 Hz, 4H, OCH_2), 3.86 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 1.70 (s, 6H, 2 CH_3), 1.67 (s, 6H, 2 CH_3). ^{13}C NMR (400 MHz, $CDCl_3$): δ 175.59 (s, C=O), 164.02 (s, C-2), 161.23 (s, C-4), 159.67 (s, C-9), 150.20 (d, C-8), 148.89 (s, C-14), 148.51(s, C-13), 136.85 (s, 2 x C=), 126.50 (s, C-7), 125.00 (s, C-10), 121.44 (d, C-11), 120.65 (d, 2 x =*C*H), 115.50 (d, C-15), 110.00 (s, C-5), 114.00 (d, C-12), 96.47 (d, C-3), 92.60 (d, C-1), 65.95 (t, 2 x O*C*H_2), 56.44 (q, O*C*H_3), 55.93 (q, O*C*H_3), 25.62 (q, 2 x *C*H_3), 18.13 (q, 2 x *C*H_3). IR (KBr): IR (KBr): 3078, 1646, 1607, 1457, 1257 cm^{-1}; ESI-MS $C_{27}H_{30}O_6$ (m/z): 451.1 (M^++H); HRMS: 451.211500 (MH^+); found: 451.211495 (MH^+).

mRNA expression analysis. Total RNA was isolated with Trizol (Invitrogen, Eugene, Or, USA)

and reverse transcribed with Superscript II reverse transcriptase and random hexamers (Invitrogen, Eugene, Or, USA). Quantitative real-time PCR (qRT-PCR) analysis of *Gli1, Gli2, Ptch1, Ptch2, Nanog, Oct-4, Cyclin D1, Cyclin D2, NMyc, HIP1, Sfrp1, Bmp2, IGF2, PCNA*, *Pfkfb3, β-2 microglobulin, HPRT, GAPDH,* mRNA expression was performed on each cDNA sample using the ABI Prism 7900HT Sequence Detection System employing Assay-on-Demand Reagents (Applied Biosystems, Foster City, CA, USA). A reaction mixture containing cDNA template, TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA, USA) and primer probe mixture was amplified using standard qRT-PCR thermal cycler parameters. Each amplification reaction was performed in triplicate and the average of the three threshold cycles was used to calculate the amount of transcript in the sample (using SDS version 2.3 software). mRNA quantification was expressed, in arbitrary units, as the ratio of the sample quantity to the quantity of the calibrator. All values were normalized with two endogenous controls, *β-2 microglobulin, HPRT*, *GAPDH*, which yielded similar results.

Chromatin immunoprecipitation (ChIP). MEF cells transfected with Flag-tagged Gli1 plasmid or pcDNA 3.1 were crosslinked and chromatin immunoprecipitation was carried out with (1:200) mouse anti Flag-M2 antibody (Sigma Aldrich, St. Louis, MO, USA). Eluted DNA was analyzed by qRT-PCR. Oligonucleotides used for PCR amplification were: *Ptch1* promoter, forward 5´-GAGCATTCCTTAATGGAAG-3´, reverse 5´-CTGCAACGCGATTGGCTCT-3´; *GAPDH* Control: forward 5´-GACGGCCGCATCTTCTTGT-3´, reverse 5´-CCTGGTGACCAGGCGC-3´ (as described in Canettieri *et al*, 2010; Coni *et al*, 2013b).

Luciferase, Cell proliferation and MB-SCs neurosphere forming assay. Luciferase assay was performed in HEK293T or MEF cells transfected with GliBS-luciferase reporter, pRL-TK *Renilla* (normalization control) and other DNA constructs as indicated. Luciferase and renilla activity were assayed with a dual-luciferase assay system (Promega, Madison, WI, USA). Results are expressed as luciferase/renilla ratios and represent the mean ± SD of at least three experiments, each performed in triplicate. Cell proliferation was evaluated by BrdU detection (Roche, Welwyn Garden City, UK) accordingly to the manufacturer's instructions and as previously described (De Smaele *et al*, 2011). Nuclei were counterstained with Hoechst reagent. For the neurosphere forming assay, cells were plated at clonal density (1–2 cells/mm^2) into 96-well plates and cultured in selective medium as previously described (Po *et al*, 2010).

Kinase assay: 27 kinases were selected from Cerep Comprehensive Kinase Profile (http://www.cerep.fr) and subjected to kinase assay testing GlaB at 10 μM concentration (2 replicates for every assay).

Western Blot Analysis. Cells were lysed in standard radioimmunoprecipitation assay buffer plus

protease inhibitors (Roche, Welwyn Garden City, UK). Lysates were separated on SDS-PAGE and immunoblotted using standard procedures. Mouse anti-Gli1 and rabbit anti-Caspase3 antibodies were from Cell Signaling (Beverly, MA, USA); anti-rabbit PCNA and anti-rabbit Oct-4 antibodies were from Abcam (Cambridge, UK); anti-Flag-HRP was from Sigma Aldrich; mouse anti-NMyc, rabbit anti-Cyclin D1, goat anti-Actin and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All antibodies were used at different dilutions followed by enhanced chemiluminescence (ECL, Amersham Pharmacia).

Immunohistochemistry and TUNEL assays. For immunohistochemical staining tissues were formalin fixed and paraffin embedded. Sections were incubated with (1:200) rabbit polyclonal-Gli1 H300 antibody (Santa Cruz Biotecnology) or (1:100) rabbit monoclonal-Ki67 antibody (Thermo Fisher Scientific, MA, USA) or (1:200) mouse monoclonal-Ki67 antibody (Leica Microsystems) diluted in PBS. Detection was carried out with the Mouse-to-Mouse HRP (DAB) staining system (ScyTek Laboratories, Logan, UT, USA) accordingly to the manufacturer's instructions. Cell death on paraffin embebbed sections was evaluated by in situ cell death detection kit, POD (Roche, Welwyn Garden City, UK), whereas cell death on Ptch1$^{+/-}$ MB-SCs was permormed using the in situ cell death detection kit, TMR red (Roche), both accordingly to the manufacturer's instructions.

Case DA, Darden TA, Kollman PA (2010) AMBER 11. University of California, San Francisco, California, USA

De Smaele E, Di Marcotullio L, Moretti M, Pelloni M, Occhione MA, Infante P, Cucchi D, Greco A, Pietrosanti L, Todorovic J, Coni S, Canettieri G, Ferretti E, Bei R, Maroder M, Screpanti I, Gulino A (2011) Identification and characterization of KCASH2 and KCASH3, 2 novel Cullin3 adaptors suppressing histone deacetylase and Hedgehog activity in medulloblastoma. *Neoplasia* 13: 374-385

Jorgensen WL, Maxwell DS, Tirado-Rives J (1996) Development and Testing of the OPLS All-Atom Force Field on Conformational Energetics and Properties of Organic Liquids. *J Am Chem Soc* 118: 11225-11236

LigPrep, version 2.5, Schrodinger, LLC, New York, NY, 2011

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews* 46: 3-26

QikProp, version 3.4, Schrödinger, LLC, New York, NY (2011) QikProp, version 3.4, Schrödinger, LLC, New York, NY