Article Title: Proteoglycan interactions with Sonic Hedgehog specify mitogenic responses
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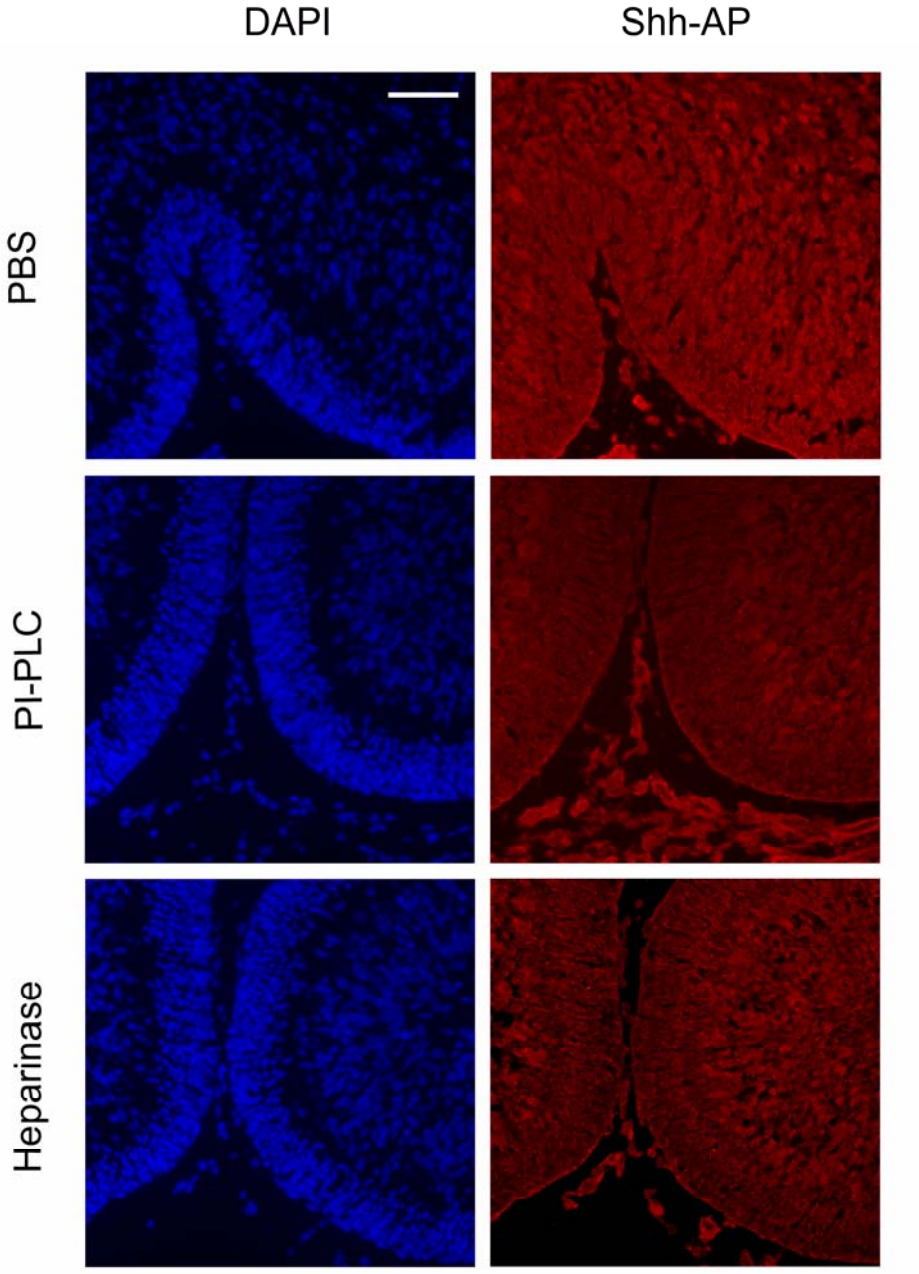
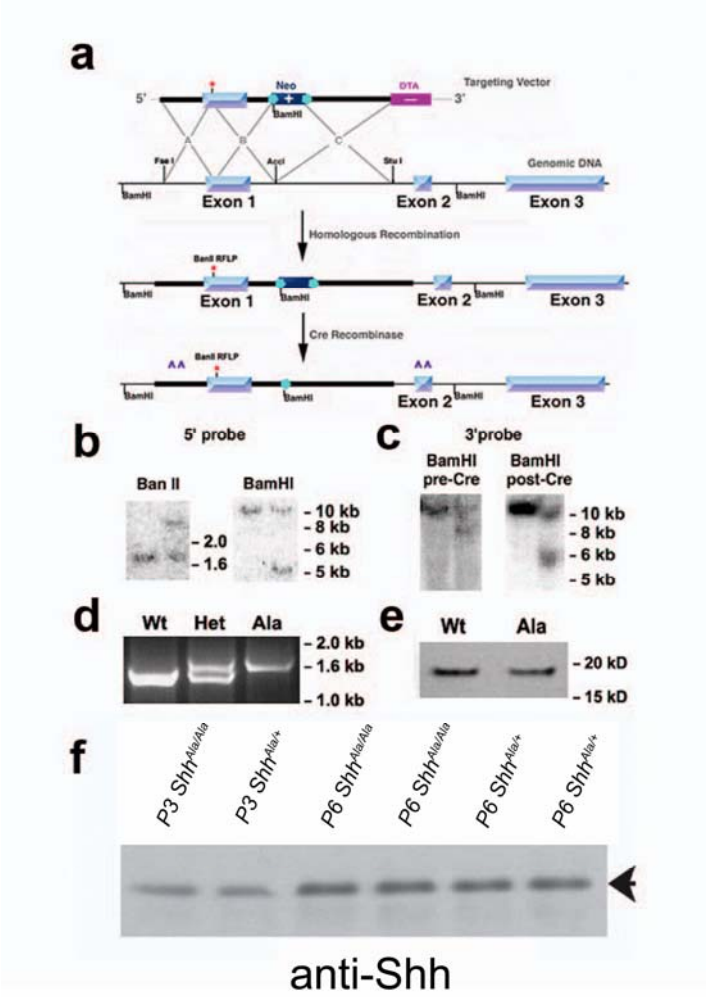
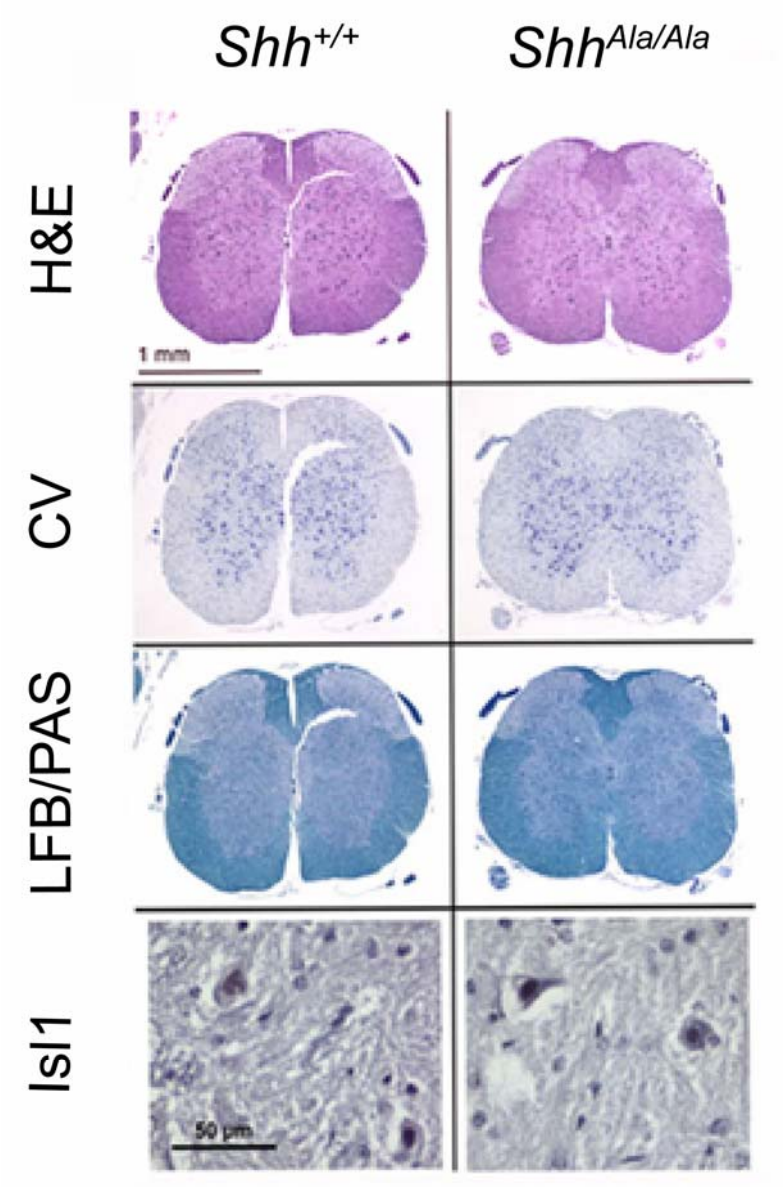
SUPPLEMENTARY DATA



Supplementary Figure 1. High magnification images demonstrate a reduction of Shh-AP binding in the EGL after GPI-linked proteins or the sulfated glycans of heparin are cleaved. DAPI is in blue, anti-alkaline phosphatase in red. Scale bar, 100 μm

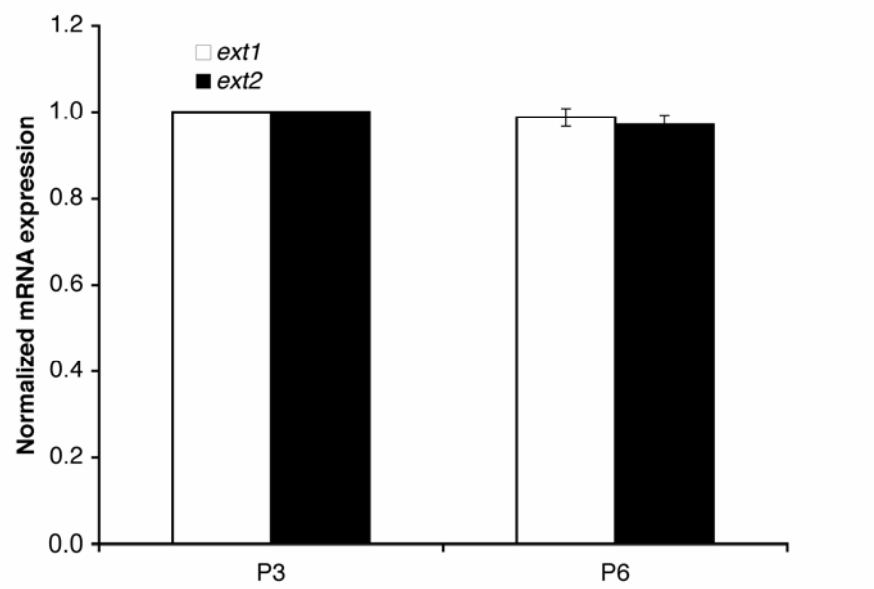


Supplementary Figure 2. Construction of *Shh*^{Ala} and *Shh*^{Ctl} mice
(a) Targeting strategy. Targeting vector included exon 1 with the *Shh*^{Ala} mutations (*), a neo cassette in the intron between exon 1 and 2 flanked by loxP sites (green pentagons), and a diphtheria toxin A gene at the 3' terminus. The 5' arm extends 4.6 kb and the 3' arm extends 2.9 kb. G418-resistant clones were screened for homologous recombination by PCR. Up arrowheads indicate sites of 5' and 3' probes used for confirmatory Southern blots. Transfected Cre recombinase was used to excise the neo cassette from ES clones.
(b),(c). Southern blots of DNA from positive clones digested with BanII and BamH1, both before and after Cre-mediated excision. Left lanes are wild type clones; right lanes are correctly targeted ES clones. Blots were probed with the 5' probe (b) or 3' probe (c) as indicated; bands of the correct size were seen.
(d) Identification of *Shh*$^{+/+}$, *Shh*$^{Ala/+}$ and *Shh*$^{Ala/Ala}$ mice by PCR and BanII digest.
(e) Equivalent Shh protein expression in *Shh*$^{+/+}$ and *Shh*$^{Ala/Ala}$ E10.5 embryos.
(f) Shh^{Ala} expression is normal. Proteins from *Shh*$^{Ala/+}$ and *Shh*$^{Ala/Ala}$ cerebella were analyzed by immunoblot with anti-Shh following immunoprecipitation.



Supplementary Figure 3. *Shh*$^{Ala/Ala}$ mice exhibit normal spinal cord patterning.

Differentiation of spinal cord proceeds normally into adulthood. Wild type or *Shh*$^{Ala/Ala}$ adult mice were fixed, and spinal cords were stained by hematoxylin and eosin, cresyl violet, luxol fast blue/PAS (to visualize myelination), or Isl1 (to visualize motor neurons). The spinal cords of *Shh*$^{Ala/Ala}$ mice are smaller but no patterning defects are seen.



Supplementary Figure 4. Expression levels of *ext1* and *ext2* in P3 and P6 cerebella are equivalent in C57BL/6 mice.
Expression levels of *ext1* (open bars) and *ext2* (black bars) in C57BL/6 were quantified by qRT-PCR. No age-dependent changes in expression levels were observed.

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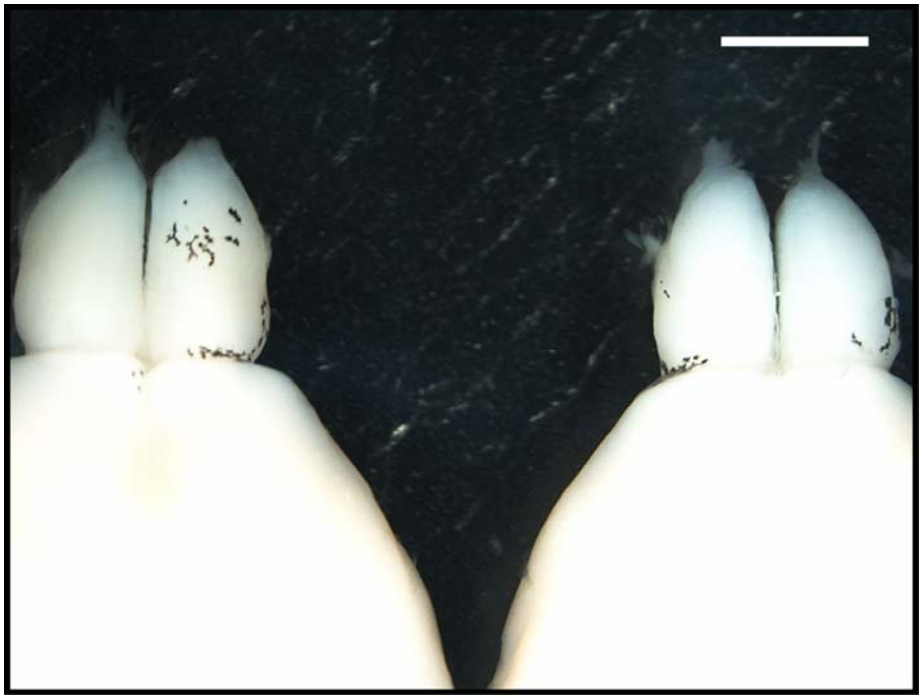

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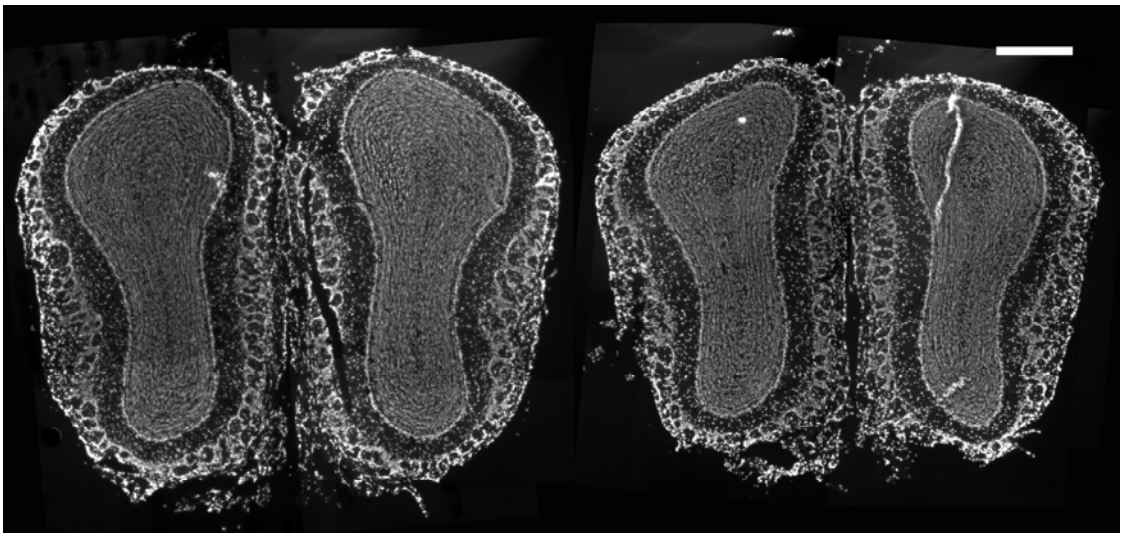
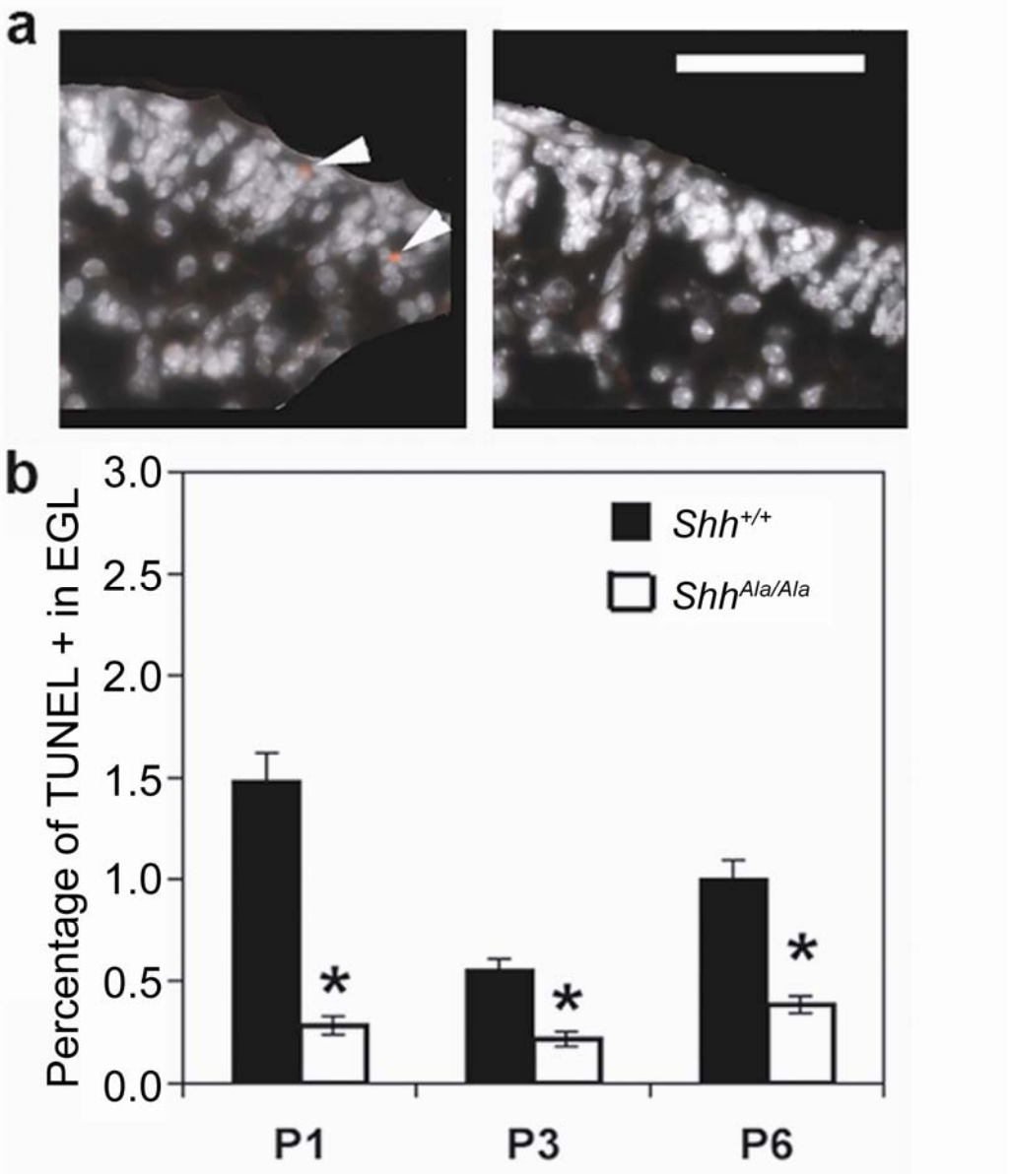
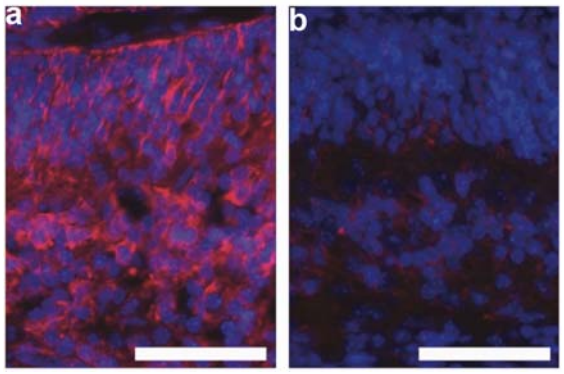


Supplementary Figure 5. High magnification images of adult *Shh*$^{+/+}$ and *Shh*$^{Ala/Ala}$ olfactory bulbs demonstrate that mutants are reduced in size. (a) Gross images of the olfactory bulbs of a *Shh*$^{+/+}$ (left) and a *Shh*$^{Ala/Ala}$ (right) adult male. Scale Bar, 2 mm. (b) 12 μm coronal sections through the olfactory bulb of a *Shh*$^{+/+}$ (left) and a *Shh*$^{Ala/Ala}$ (right) adult male. Each section is 65% of the distance from the tip of the olfactory bulb to the flexure. Scale bar, 500 μm



Supplementary Figure 6. Apoptosis is decreased in *Shh*$^{Ala/Ala}$ mice.
(a) Cerebellar sections from *Shh*$^{Ala/Ala}$ and wild type littermates were processed for TUNEL staining (shown in red). Positive cells are indicated by arrows. Scale bar, 50 μm
(b) During early postnatal development, the percentage of TUNEL-positive EGL cells in mutants is less than that of wild type animals (*p<0.001).



Supplementary Figure 7. Shh immunostaining is specific.
Wild type P6 cerebellar sections were immunostained with antibody to Shh (a), or with the same concentration of antibody following preincubation with recombinant Shh-alkaline phosphatase (b). Scale bar, 50 μm

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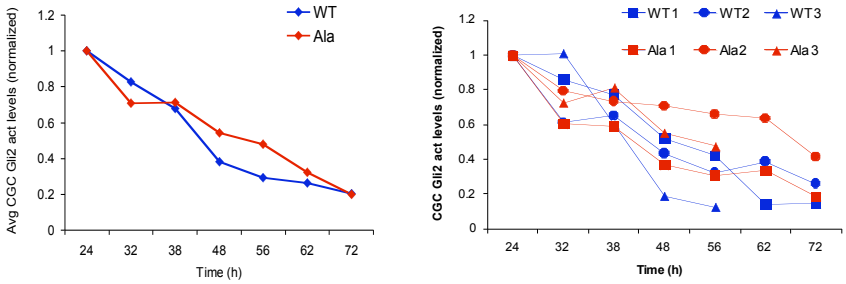

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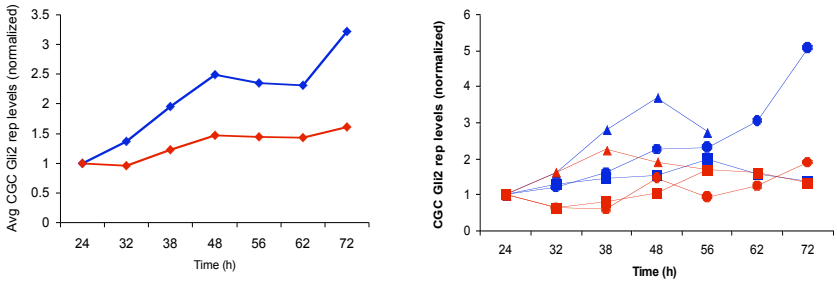

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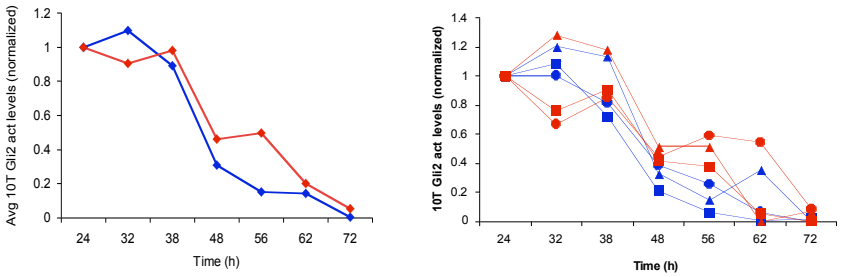

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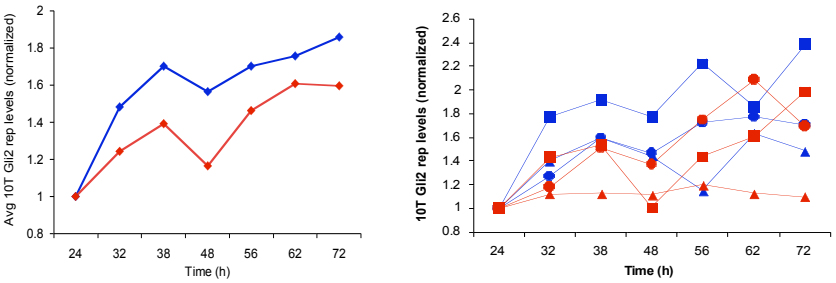

Supplementary Figure 8. Quantitation of Gli2^{Act} and Gli2^{Rep} over time. Values are normalized to loading control and relative to the value at 24 hours. Three independent experiments using cerebellar granule cells (**(a)** and **(b)**) and three independent experiments using C3H10T1/2 cells (**(c)** and **(d)**) were performed. Graphs at left are averages, while graphs at right are of each of the individual experiments. Within the graphs representing individual experiments, data obtained in the same experiment are represented by the same symbol (circle, square or triangle).

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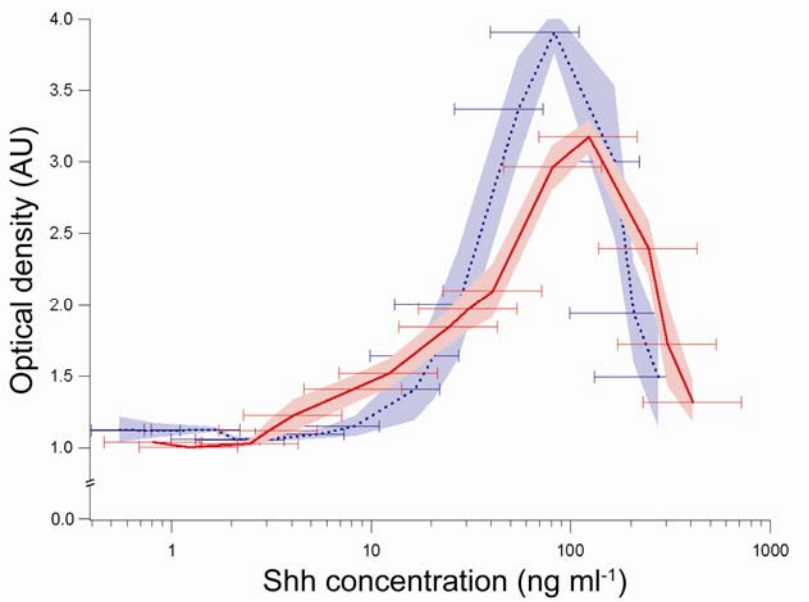

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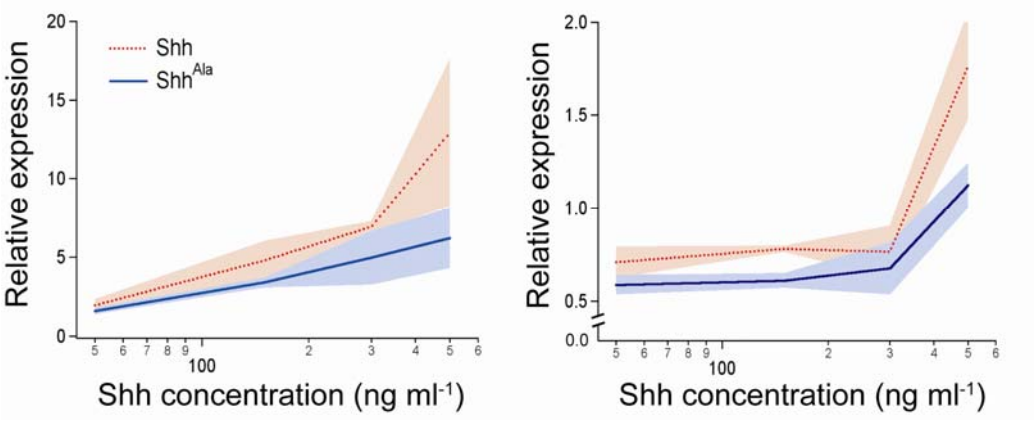
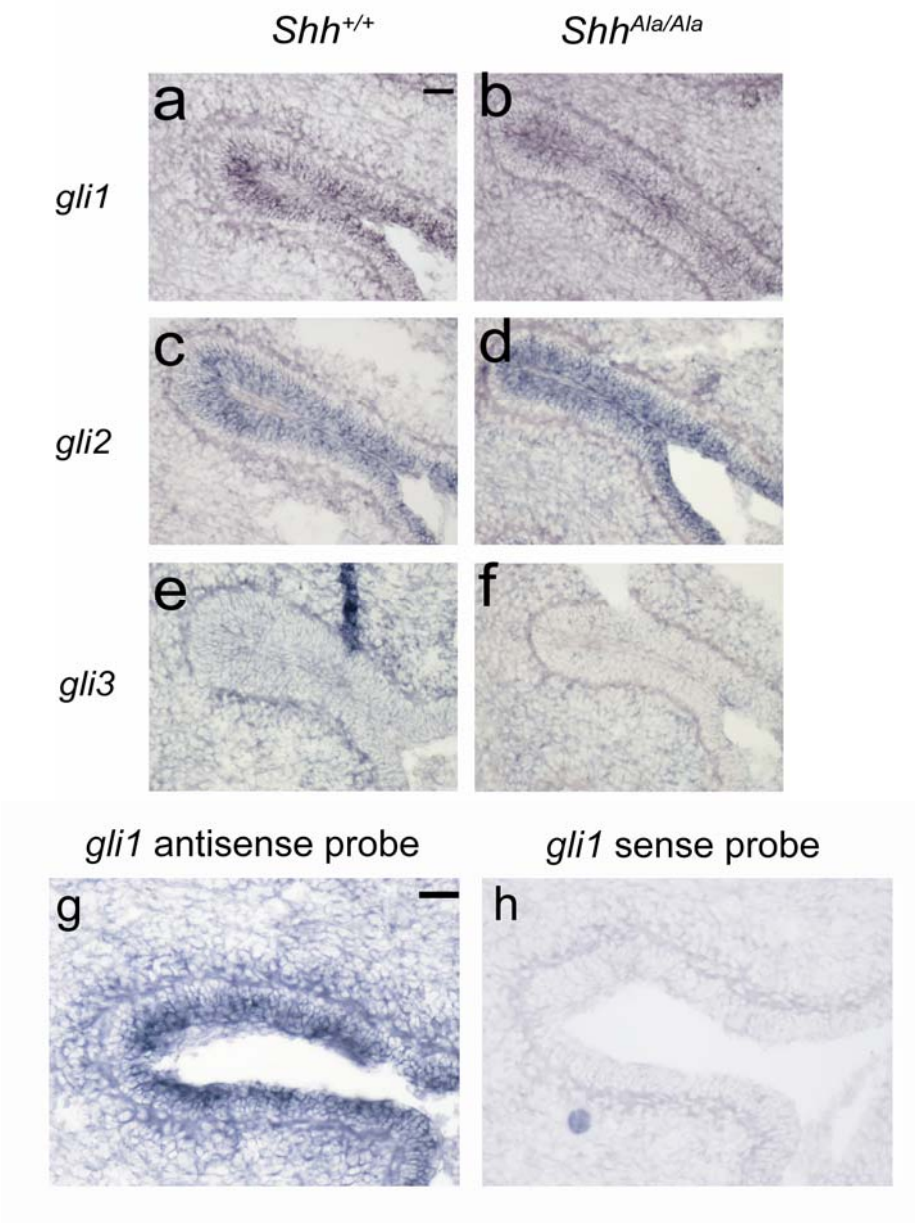


Supplementary Figure 9. Dose response curves demonstrate Shh^{Ala} is as efficacious as Shh and differential induction of gene subsets is not a function of dose.

(a) C3H10T1/2 cells were stimulated with equivalent amounts of wild type or Shh^{Ala}, and alkaline phosphatase activity was measured. Shh^{Ala} (blue) is as efficacious as wild type protein (red) in inducing differentiation of C3H10T1/2 cells. Shading represents y standard error; range of Shh or Shh^{Ala} concentrations at each point is represented by the horizontal bars. (b) *gli1* expression is induced to similar levels throughout a range of equivalent doses of Shh (red curve) and Shh^{Ala} (blue curve) (left), while *Cyclin D2* mRNA levels are induced to a lesser extent by Shh^{Ala} throughout this same range (right).



Supplementary Figure 10. Distribution of *gli* expressing cells is not changed in *Shh*$^{Ala/Ala}$ mice.

(a-f) Shh target genes *gli1* (a-b), *gli2* (c-d) and *gli3* (e-f) in P3 *Shh*$^{Ala/Ala}$ and *Shh*$^{+/+}$ cerebella do not show a change in the pattern of expression (*in situ* hybridization). Scale bar, 20μm

(g,h) Sections of P3 *Shh*$^{+/+}$ cerebella were hybridized with antisense (g) and sense probes (h) for *gli1*. No background hybridization was observed with sense probes. Scale bar, 20μm

SUPPLEMENTARY METHODS

Mutagenesis

Plasmid containing wild type full length *Shh* was a generous gift of P. Chuang. Mutations to generate the Shh^{Ala} allele were introduced by Quikchange (Stratagene). Primer sequences were designed to introduce the desired amino acid changes (Arg34Ala and Lys38Ala) as well as delete a Ban II restriction site to allow for clone selection. Sense and antisense mutagenesis primer sequences are as follows:
5'-GGCCTGGCAGAGGGTTTGGAAAGGCGCGCCACCCCGCAAAGCTGAC-3'
and 5'-GTCAGCTTTGCGGGGTGGCGCGCCTTTCCAAACCCTCTGCCAGGCC-3'. Mutants were confirmed by direct sequencing. Plasmids containing sequences for alkaline-phosphatase tagged N-terminal wild type or Shh^{Ala} were described previously[1].

Transient transfection

Plasmids containing full length *Shh* and Shh^{Ala} were transiently transfected into HEK cells and plasmids containing *N-Shh:AP* and *N-*Shh^{Ala}*:AP* were transiently transfected into COS7 cells, all using Lipofectamine2000 (Invitrogen). 24 hour conditioned media were collected 60 hours post-transfection. Conditioned media from HEK cells transfected with full length *Shh* or Shh^{Ala} were collected and concentrated 10-fold using AmiconUltra concentration devices with a molecular weight cutoff of 10K (Millipore). Samples were analyzed by Western blot with anti-Shh (SHH N-19; Santa Cruz, sc-1194) to quantify protein concentration. Conditioned media from AP-tagged constructs were also assayed for alkaline phosphatase activity[1].

Animals

Mice expressing eGFP under the control of chicken beta-actin promoter and cytomegalovirus enhancer were from The Jackson Laboratory (C57BL/6-Tg(ACTB-EGFP)1Osb/J, #003291). BALB/c breeders were purchased from Charles River Laboratories. Shh^{Ala} animals and Shh^{Ctl} were generated as detailed below and maintained on a mixed 129sv/J and C57BL/6J background as heterozygous breeding pairs, or bred to C57BL/6J. All experimental animal procedures were performed in accordance with the National Institutes of Health guidelines and were approved by the Dana-Farber Cancer Institutional Animal Care and Use Committee.

Generation of mutant animals

Genomic DNA containing *Shh* was obtained from the RPCI-22 129/Sv mouse genomic BAC clone number 63F20. A 4.6 kb FseI/AccI fragment inclusive of exon 1 and a 2.9 kb AccI/StuI fragment in intron 1 were used as 5' and 3' flanking arms, respectively, and inserted into a targeting vector backbone (pPGK-NEO/DTA; from D Paul) containing a floxed Neomycin resistance gene (Neo) for positive selection and a diphtheria toxin subunit A (DTA) sequence for negative selection. The 5' loxP sequence also introduced a BamHI restriction

site. The *Shh*^{Ala} mutation was introduced into exon 1 using Quikchange as described above.

The *Shh*^{Ala} allele was targeted to the endogenous *Shh* gene by homologous recombination in ES cells. G418-resistant ES clones were screened by PCR for correct targeting using a sense primer external to the 5' targeting arm and an antisense primer within the neo cassette. Clones identified as positive by PCR were subjected to Southern blot of BamHI-digested genomic DNA with 5' and 3' internal and external probes to confirm site-specific integration. A second round of Southern blots using the 5' probe on Ban II-digested genomic DNA allowed distinction between 5' crossover events that had incorporated the mutation in exon 1 and crossover events that were downstream of the mutation site (contain wild type *Shh* exon 1). Clones with the former events were used for generation of chimeric *Shh*^{Ala} animals while those with the latter events were used for generation of chimeric *Shh*^{Ctl} animals. Cre-mediated excision of the Neo cassette was performed prior to using the cells in blastocyst injections by transfection with a pOG231 expression plasmid with a CMV promoter regulating Cre with a nuclear localization signal (from Stephen O'Gorman). Excision of the Neo cassette was confirmed by repeat Southern blot. Chimeric males with germline transmission were bred to C57BL/6J wild type mice to generate *Shh*$^{Ala/+}$ and *Shh*$^{Ctl/+}$ heterozygotes.

Shh$^{Ala/Ala}$ animals have the Cardin-Weintraub motif mutation in exon 1 and a 32-bp residual loxP site within the adjacent intron. *Shh*$^{Ctl/Ctl}$ animals have wild type exon 1 with the residual intron 1 loxP site and provide an ideal control for the knock-in mutations. These lines were later interbred yielding animals with the intronic loxP site and either the *Shh*^{Ala} or wild type *Shh* allele. *Shh*$^{Ala/+}$ mice were bred to *Shh*$^{+/-}$ animals (from D Rowitch) to generate *Shh*$^{Ala/-}$ animals.

Genotyping

For analysis of the *Shh*^{Ala} mutation, PCR was performed on genomic DNA with primers 5'-CCAGCCCTGCACTTTGCTA-3' and 5'-GGTCTCTACCTGAGTCATCAGC-3', followed by digestion with Ban II restriction enzyme (New England BioLabs). Products were run on a 1.2% agarose gel. The wild type allele was identified by bands at 1.4 kb + 0.2 kb. The *Shh*^{Ala} allele was identified by a band at 1.6 kb. For analysis of the loxP site in the *Shh* intron, PCR was performed on genomic DNA with primers 5'-ATCTGCTCCCGACCACCTTAAATC-3' and 5'-GGGTTGGGAATCAGAAAGGCTACT-3', followed by digestion with BamHI. Wild type intron 1 was identified by a single band at approximately 0.9 kb whereas the presence of the residual loxP site with BamHI digestion yielded bands at 0.4 kb + 0.6 kb.

Heparin binding assay

Heparin-coated plates were purchased from Lifespan Technologies. All reagents used were purchased from Sigma unless otherwise specified. First, the wells were washed for 5 minutes with 100μl/well of binding buffer (20mM Tris-HCl pH 7.4, 150mM sodium chloride, 2mM calcium chloride, 2mM magnesium

chloride , 0.01% Tween-20) and blocked with 100μl/well of 1% Bovine Serum Albumin (BSA) in binding buffer for 1 hour at room temperature. Shh-AP, Shh^{Ala}-AP or AP were added to each well at a concentration of 3.2 μg/μl in the absence or presence of various concentrations of heparin (heparin sulfate sodium salt from bovine kidney; Sigma, H7640) and incubated for 1 hour at room temperature. Wells were washed with binding buffer and with alkaline phosphatase assay buffer and then developed with substrate p-nitrophenyl phosphate disodium salt hexahydrate (PNPP). A microplate spectrophotometer was used to read absorbance at 405nm.

Section binding assay

In situ proteoglycan binding was evaluated by the methods of Friedl[1, 2]. 8 μm sagittal cryosections from P6 BALB/c pups were treated with PBS, vehicle control, 500 mU/ml of heparinases (Sigma) in PBS, or 500 mU/ml of PI-PLC in PBS, first for 1 hour at 37°C then overnight at 4°C. Autofluorescence was decreased by treating with 0.05% sodium borohydride for 10 minutes at room temperature, followed by 0.1 M glycine at 4°C overnight. Non-specific binding was blocked with 1% BSA in PBS for 1 hour at room temperature. Equimolar Shh:AP or Shh^{Ala}:AP were added for 1 hour at room temperature. Sections were washed with PBS containing 0.5 M NaCl then stained with rabbit anti-human alkaline phosphatase (Biomeda) and visualized with Alexa 546-conjugated goat anti-rabbit IgG (Invitrogen).

Immunohistochemistry

The following antibodies were used for immunohistochemistry: phospho-Histone H3 (clone 6G3, Cell Signaling; 1:500 dilution), BrdU (BD, Cat # 555627; 1:100), Shh (Santa Cruz N-19, sc-1194; 1:50). Non-specific antibody binding was blocked by incubating slides in 5% BSA, 3% NGS, and 0.1% Triton X-100 in PBS, for 1 hour. Primary antibodies in block were applied for 1 hour at room temperature or overnight at 4°C. Staining was visualized with Alexa Fluor-546 or -488 labeled secondary antibodies (Invitrogen) and DAPI counterstain.

Specialized staining protocols were carried out as follows: For phospho-Histone H3 staining, sections were pre-treated with 1mM Tris pH 8.0 and 0.5M EDTA in PBS heated to 99°C for 15 minutes. For BrdU staining, sections were permeabilized with 0.4% Triton X-100 and 5% NGS in PBS for 30 minutes, then treated with 2N HCl for 20 minutes at 37°C, followed by 0.1M sodium borate for 10 minutes. For Shh immunostaining, sections were blocked in PBS containing 5% horse serum without detergent for 1 hour (block), then incubated overnight with anti-Shh in block at 4°C. For competition studies to assess specificity, anti-Shh was incubated for 1 hour at 4°C with either supernatant from HEK cells expressing Shh-AP or control supernatant from HEK cells expressing AP alone before use. Shh immunostaining was quantified using Image J software (NIH).

Apoptotic cells were detected by TdT-mediated dUTP nick-end labeling (TUNEL). Sections were permeabilized, using 0.2% Triton X-100 and 5% NGS in PBS, for 1 hour at room temperature. Slides were equilibrated with TUNEL Dilution Buffer (Roche) for 10 minutes, then incubated at 37°C for 1 hour with

reaction mixture (25nM biotin UTP, 0.5mM dGTP, 0.5 mM dATP, 0.5 mM dCTP in TUNEL Dilution Buffer) containing 200 U/ml terminal deoxytransferase (TdT) TUNEL Enzyme (Roche). The TdT reaction was terminated with 2X SSC. Biotin-UTP labeling was visualized with Cy3-conjugated Streptavidin (Jackson Immuno). Nuclei were counterstained with DAPI.

Skeletal staining

P5 animals were sacrificed, skinned, and eviscerated to remove all soft tissues. Bones and cartilage were fixed in 95% ethanol for 1 week then transferred to acetone for 2 days. Skeletons were stained for 1 day at 37°C and 2 days at room temperature in a solution of 0.005% Alizarin Red S, 0.015% Alcian Blue 8GS, 5% acetic acid, 70% ethanol. Skeletons were rinsed in water and cleared in 1% potassium hydroxide for 24 hours. Additional clearing and transfer into 100% glycerol was performed by a series of incubations in graded KOH/glycerol solutions for a period of 1 week each.

BrdU labeling

Mice at P1, P3, P6, and P12 were injected ip with BrdU (100μg/g animal weight), sacrificed after 4 hours, and fixed in 4% PFA. Brains were cryoprotected in 30% sucrose. Matched 8 μm cryosections from *Shh*$^{Ala/Ala}$ pups and littermate controls were mounted on the same slide. Adult animals 5–6 months of age were injected ip with BrdU (200 μg/g animal weight). After 2 hours, animals were perfused with PBS then 4% PFA, and 30μm serial coronal cryosections were cut and mounted on glass slides.

***In situ* hybridization**

E10.5 embryos or P3 cerebella were fixed overnight with 4% PFA in PBS, then cryoprotected in sucrose. 14 μm serial sections were cut and mounted on glass slides. For hybridization in spinal cord, axial sections at the level of the heart of embryos were used. *Shh* probe was from D Rowitch; all others were from Q Ma. Probe synthesis/labeling and *in situ* hybridization were performed as described[3].

Size measurements

6 month old animals were weighed then sacrificed. Brains were dissected and total brain weight (including olfactory bulbs, cerebrum, cerebellum, and brainstem to the level of the cervicomedullary junction) was recorded. Cerebella were removed and weighed separately. Height and width of olfactory bulbs (OB) were measured from 10 μm thick coronal cryosections. OB length was assessed after serial cryosectioning by counting the number of coronal sections from the tip of the bulb to the flexure where the olfactory tract meets the frontal cortex. OB volume was calculated using the equation for the volume of a solid elliptical tube: Volume = π*A*B*C, where A is (OB height / 2), B is (OB width / 2), and C is OB length. Significance was calculated by 2-tailed Student's t-test.

In vivo proliferation and apoptosis

Analyses of p-Histone H3 staining, BrdU labeling and TUNEL were performed on cerebella from 3–5 pairs of matched wild type and mutant littermates at ages P1, P3, P6, P9, and P12. For each pair, 4–6 sagittal vermal cerebellar sections were stained for analysis. Images were taken with a Spot Camera and Nikon Eclipse E800 microscope using a 40X objective at identical sites in the EGL for comparison between wild type and mutant animals. For P1 sections four images were taken on each side of the primary and secondary fissures, at a point midway along the depth of those fissures. For P3, P6, P9, and P12 sections, 8 images were taken, on either side of the pre-culminate fissure, primary fissure, secondary fissure, and posterolateral fissure, at a point midway along the depth of those fissures. For each image, the number of phospho-Histone H3, BrdU or TUNEL positive nuclei was counted, and the proliferative or apoptotic index was calculated by dividing the number of positive cells by the number of DAPI stained cells in the EGL. Images were quantified by a reviewer blinded to genotype. Reviewers also recorded the cerebellar location of positive cells as anterior (in the vermis midway in the fissure between folia IV and V), middle (midway in the fissure between folia V and VI) and posterior (midway in the fissure between folia VIII and IX). Statistical significance was calculated by 2-tailed Student's t-test.

For analyses of staining in coronal sections of adult animals, every 9th section through the extent of the SGL or SVZ was stained for phospho-Histone H3 or BrdU. Analyses were performed on at least 3 pairs of adult *Shh*$^{Ala/Ala}$ animals and age-matched, sex-matched controls. All positively stained cells in the appropriate locations were counted while continuously focusing up and down through the thickness of the section. For quantification, the SGL was defined as the inner half of the thickness of the dentate gyrus, extending two cell widths into the hilus; only labeled cells within this zone were counted. Final counts for SGL and SVZ were multiplied by 9 to obtain a value for the total counts through the entire extent of the structures. Images were quantified by a reviewer blinded to genotype. Data are shown as mean +/– s.e.m. Significance was calculated by 2-tailed Student's t-test by pairwise comparison.

Analyses of p-Histone H3 staining (and DAPI counterstain) were also performed on spinal cords from 2 pairs of E10.5 *Shh* or *Shh*$^{Ala/Ala}$ animals using the staining protocol listed above. Images were taken of axial sections that were at the same level relative to the heart. All p-H3+ cells within the spinal cord were counted by a blinded reviewer. Data are shown as mean +/– s.e.m. Significance was calculated by 2-tailed Student's t-test. Also, the total spinal cord area was determined by thresholding the DAPI image and defining a region equivalent to the spinal cord (minus the canal), using MetaMorph (Molecular Devices). The ratio of p-H3+ cells per unit area spinal cord was then determined.

Primary GCP cultures

Cerebella were dissected, meninges were removed, and tissue was incubated in 1 mg/ml trypsin with 125 U/ml DNase (Sigma) for 20 minutes at 37◦C. Trypsinization was quenched with DMEM with 10% FCS. Cells were

washed and dissociated by serial trituration in cold Hank's buffered saline. After panning, dissociated cells were passed through a 70 μm nylon mesh strainer and plated in GCP media (1x N2, 15 mM HEPES, 24 mM KCl, 6 mg/ml Glucose, 100 U/ml Penicillin, 100 μg/ml Streptomycin in DMEM/F-12 (1:1)) in 6 well plates (density of 4 x 10^6 cells/well) or 12 well plates (density of 2 x 10^6 cells/well). 1 hour after plating, 100 μl of concentrated conditioned media prepared from wild type *Shh*-transfected, Shh^{Ala}-transfected, or mock-transfected HEK cells were added to each well. Cells were harvested for RNA at 18 hours after stimulation.

In vitro proliferation and apoptosis assays

6 x 10^5 dissociated P6 cerebellar granule cells were plated onto poly-L-ornithine-coated (Sigma, P4957) 12 mm glass coverslips (Bellco). For the apoptosis assay, at 18 hours, cells were fixed with 4% PFA for 30 minutes (4◦C), permeabilized for 15 minutes in 0.1% Triton X-100 (Sigma) in PBS (RT) and blocked for 1 hour in a blocking solution consisting of 10% NGS, 1% BSA and 0.1% Triton X-100/PBS (RT). Anti-activated caspase 3 (Abcam, ab13847) was diluted (1:500) in blocking solution and applied to the cells overnight (4◦C). Cells were washed 3X in 0.1% Triton X-100 in PBS. A 1:500 dilution of Alexa-Fluor 546 goat anti-rabbit (Invitrogen, A11035) in 0.1% Triton X-100/PBS was applied (1 hour, RT), then cells were extensively washed (0.1% Triton X-100/PBS). Coverslips were mounted (ProLong Gold antifade reagent with DAPI, Invitrogen, P36936) and imaged. To assess proliferation, 12 hours post-plating, cultures were treated with 3 μg/ml of BrdU. 36–40 hours post-plating, cultures were stained for BrdU as described before with the exception of treating with 2N HCl for 10 minutes instead of 30 minutes. The percentage of cells that were caspase-positive or BrdU-positive was determined by use of the Cell Scoring Module of MetaMorph (Molecular Devices).

Quantitative RT-PCR

RNA was purified from GCP cultures using RNAqueous-4-PCR (Ambion). RNA was purified from whole cerebella dissected from P1–P2 pups using Trizol (Invitrogen). RNA samples were treated with DNAseI (Ambion). cDNA was generated using the high capacity cDNA Archive Kit (Applied Biosystems). Taqman quantitative PCR was performed on an Applied Biosystems 7700 quantitative PCR instrument with Sequence Detector Software. The following primer and probe mixes from Applied Biosystems were used: *GAPDH, Shh, Ptc1, Gli1, Gli3, N-Myc, Cyclin D1, Cyclin D2*, and *Bmi1*. Primers and probe for *Cyclin E* were as follows:
5'-ATGCCTCAGTACCCACAGCA-3', 5'-ACGGAACCATCCATTTGACA-3', and 5'-6FAM-CCTTATGGTGTCCTCGCTGCT-MGBNFQ-3'.
Primers and probe for *Gli2* were as follows: 5'–GGACCTGAGGAGAGTGTGGA -3', 5'–CATGCCACTGTCATTGTTGG–3'; and 5'– 6FAM AGAGCTCCGGGCTTTGTCAGTCCAG-MGBNFQ–3'.

Starting target transcript levels (S) and GAPDH transcript levels (G) were calculated from their respective C_t values by the formulas $S = 2^{-Ct}$ and $G = 2^{-Ct}$. GAPDH-normalized transcript levels (N) were then calculated by the formula N =

S/G. When showing gene expression data from wild type versus *Shh*$^{Ala/Ala}$ animals *in vivo*, or cultured granule cells stimulated with wild type versus Shh^{Ala} protein, the GAPDH-normalized transcript levels in wild type animals was assigned a value of 1, and the corresponding GAPDH-normalized transcript level in *Shh*$^{Ala/Ala}$ mutant animals is expressed as a number relative to 1. For each experiment, samples were run in triplicate, and the mean of the triplicate values was taken as the transcript level. Each figure represents the mean of 3–8 experiments. Significance was calculated by 2-tailed z-test.

Perdurance assays

C3H10T1/2s (1.75 x 10e5 cells per 12 well) were seeded in growth media (10% Serum Supreme (BioWhittaker), 2 mM L-glutamine (GIBCO 25030), 100 U/ml Penicillin, 100 μg/ml Streptomycin (Invitrogen Cat# 15140-122), 100 μM β-mercaptoethanol, 0.5 μg/ml $ZnS0_4$ in D-MEM (Invitrogen Cat# 11995-065)). 24 hours later, cells were stimulated with 150 ng/ml of Shh or Shh^{Ala} in assay media (1x N2 supplement (GIBCO # 17502-048), 2 mM L-glutamine, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 100 μM β-mercaptoethanol, 0.5 μg/ml $ZnS0_4$ in D-MEM). After another 24 hours, cells were rinsed 3x with PBS. Cells were either scraped into ice-cold lysis buffer (50 mM Na-Tris, pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, 1 mM DTT, 10 mM NaF, 1 mM activated sodium orthovanadate, 1 mM PMSF, Protease Inhibitor Cocktail (Roche)) or were fed with fresh assay media. Lysates were collected at 24, 32, 38, 48, 56, 62 and 72 hours after stimulation. Samples were solubilized with agitation for 1 hour at 4◦C, then centrifuged for 1 hour at 16,100 x g. Supernatants were placed in fresh tubes and frozen (–20◦C) or processed further for immunoblotting. Sample buffer (final: 5% glycerol, 1.5% SDS, 0.01% bromphenol blue, 2.5% β-mercaptoethanol, 62.5 mM Na-Tris) was added to lysates, which were incubated at RT for 15 minutes. The procedure for granule cell precursors is identical, except that GCPs were seeded the day of stimulation at 2 x 10e6/poly-L-ornithine-coated 12-well. After 1 hour, cells were refed with GCP media with 150 ng/ml of Shh or Shh^{Ala}.

Immunoblotting

For analyses of protein levels in P3 *Shh*$^{+/+}$ and *Shh*$^{Ala/Ala}$ cerebella, cerebella were lysed in lysis buffer (as listed above). Protein concentration was determined by the Bradford method. 50 μg of total protein were loaded per well. For western analyses of C3H10T1/2s, GCP and cerebellar lysates, blots were probed with anti-Shh (Santa Cruz N-19, sc-1194; 1:250), anti-Gli1 (Cell Signaling, V812, cat # 2534), anti-Gli2 (Aviva Systems Biology, cat # ARP31885_T100, 1.25 μg/ml) or anti-Gli3 (Santa Cruz, cat # sc-20688) after blocking for 1 hour in 5% nonfat dry milk in 1x TBS-T. Anti-goat (Santa Cruz, sc-2354 or sc-2020) or anti-rabbit (BioRad, cat# 170-6515) HRP-conjugated secondaries (1:2500) were used. All antibodies were diluted in block. Blots were developed using Amersham ECL per manufacturer's directions. Blots were quantitated using Quantity One 1-D Analysis software (BioRad).

C3H10T1/2 Differentiation Assay

To assess the relative activities of Shh and Shh^{Ala}, equivalent amounts were used to induce osteogenic differentiation of C3H10T1/2s (ATCC, CCL-226) as previously described[4], with a few exceptions. C3H10T1/2s were maintained in growth media (as detailed above). 72 hours prior to assay, C3H10T1/2s were seeded at 1 x 10e6 in 96 well plates. The day of assay, cells were refed with assay media (also detailed above) and samples to be assayed were added for a consistent final volume. Cells were returned to the incubator. 4–5 days after sample addition, cells were rinsed with 1x HBSS. 50 μl of lysis buffer (0.2% NP-40, 1 mM $MgCl_2$) were added to each well. Samples went through 2 freeze-thaw cycles (–80◦C (15 minutes), then 37◦C (15 minutes), twice). 50 μl of a 2x Alkaline Phosphatase Substrate solution (120 mM PNPP, 50 mM $MgCl_2$, 0.5 μg/ml BSA, 2.21 M Diethanolamine) were then added. The reaction was allowed to proceed for 1–3 hours at 37◦C. Absorbance was then read at 405 nm.

Organotypic slice culture overlay assay

Cerebella were dissected from P6 C57BL/6, $Shh^{+/+}$ or $Shh^{Ala/Ala}$ littermates and washed in HBSS containing 36 mM glucose and 15 mM HEPES, pH 7.4. Cerebella were embedded in 2% low melting point agarose in HBSS. 250 μm thick midsagittal slices were cut with a vibratome and transferred to porous membrane inserts (Millipore) bathed in GCP medium. Slices were maintained in a 37◦C, 7.5% CO_2 humidified incubator. For introduction of dissociated GFP+ cells into the cerebellar slice cultures, GFP+ granule cell precursors were isolated from P6 actin-GFP+ pups as described[5]. 10,000 dissociated GFP+ GCPs (100 μl of a single-cell suspension of 1 x 10^5 cells/ml) were overlaid on each cerebellar slice. The effect of exogenous heparan sulfate proteoglycans was evaluated using slices from postnatal day 6 littermates of the GFP transgenic animals. Slices were incubated for 18 hours, with or without heparan sulfate salts (Sigma, 100μg/ml). BrdU (30 μg/ml) was present during the final 6 hours of culture. Slices were fixed in 4% PFA. Double-labeling immunofluorescence staining was performed on the slices as previously described[5] using antibodies against BrdU (BD; 1:75) and GFP (gift of Dr. P. Silver, Dana-Farber Cancer Institute, 1:6,000). All cells positive for BrdU and GFP were counted in the slice and the locations of the labeled cells (EGL, ML, IGL, white matter) were also recorded. All cells double positive for GFP and BrdU were confirmed by 1 μm optical sections using Delta Vision Deconvolution software. The proliferative index in each location was calculated by dividing the number of double positive cells by the number of GFP-positive cells in that layer. Each figure represents the mean of 3–4 experiments. Significance was calculated by 2-tailed Student's t-test.

Palmitoylation assay

HEK cells at 70% confluence were mock-transfected or transfected with *Shh* constructs (*Shh* , Shh^{Ala}, Shh^{C24S}) using Lipofectamine2000. 36 hours post-transfection, cells were cultured in labeling medium containing 500 μCi/ml [9,10-^{3}H] palmitate for 4 hours. Dishes were placed on ice and the cells were washed

twice with cold PBS before harvesting in lysis buffer. Lysates were cleared by centrifugation at 20,000 x g for 30 minutes at 4°C, then immunoprecipitated with Shh 5E1 antibody (ATCC) and Protein A/G Ultralink beads (Pierce). Immunoprecipitates were separated by 12% Bis-Tris SDS-PAGE and transferred onto Immobilon PVDF membrane. Membranes were sprayed with Enhance solution (Perkin Elmer) and exposed to Kodak Biomax film for 14 days at –80°C, then washed with TBS-T to remove Enhance solution, and then probed with goat polyclonal anti-Shh (Shh N-19; Santa Cruz) followed by donkey anti-goat IgG-HRP (Santa Cruz). Bands were visualized by ECL (Pierce SuperSignal).

Statistical Analysis

Comparable conditions were evaluated for significance by performing a 2-tailed Student's t-test. Comparison of normalized data was done by performing a 2-tailed z-test.

References for Supplementary Materials:

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