

Supplemental Data

Article

Medulloblastoma Can Be Initiated

by Deletion of *Patched*

in Lineage-Restricted Progenitors or Stem Cells

Zeng-Jie Yang, Tammy Ellis, Shirley L. Markant, Tracy-Ann Read, Jessica D. Kessler, Melissa Bourboulas, Ulrich Schüller, Robert Machold, Gord Fishell, David H. Rowitch, Brandon J. Wainwright, and Robert J. Wechsler-Reya

Supplemental Experimental Procedures

Antibodies

Primary antibodies used for immunostaining included NeuN (1:100), chicken anti-GFP (1:100), mouse anti-Cre (1:200), rabbit anti-GABRA6 (1:100) and mouse anti-O4 (1:200) from Chemicon/Millipore (Temecula, CA); mouse anti-Ki67 (1:100), rabbit anti-BLBP (1:500), mouse-anti-BrdU (clone 3D4) and mouse anti-Nestin (1:100) from BD Biosciences (San Jose, CA), rabbit anti-GFP (1:100) and rabbit anti-Pax2 (1:200) from Molecular Probes/Invitrogen, (Carlsbad, CA), mouse anti-Calbindin-D28K (1:250) and mouse anti-S100 (1:1000) from Sigma; rabbit anti- β -Tubulin (TuJ1, 1:3000) from Covance (Berkeley, CA), and rabbit anti-Sox2 (1:3000, gift from Dr. Larysa Pevny, UNC-Chapel Hill). Anti-Group B1 Sox antibodies (1:2000) were a gift from Hisato Kondoh, Osaka University.

Secondary antibodies included Alexa Fluor 568 anti-mouse IgM (1:200), Alexa Fluor 594 anti-rabbit IgG (1:200) and Alexa Fluor-488 anti-mouse IgG (1:200) from Invitrogen and FITC-anti-rabbit IgG (1:200, Jackson ImmunoResearch, West Grove, PA).

Primers

Gli1 (used in Figure 1J)

Forward: ATGGAGAGAGCCCGCTTCTTT

Reverse: TTATGGAGCAGCCAGAGAGACCAG

N-myc (used in Figure 1J)

Forward: AACAAAGGCGGTAACCACTTTCAC

Reverse: TGCTGCTGATGGATGGGAAC

Cyclin D1 (used in Figure 1J)

Forward: ACCCTGACACCAATCTCCTCAAC

Reverse: TGGATGGCACAATCTCCTTCTG

Patched (used in Figure 1I and Figure 3I to detect expression in *Ptc*^{C/C} mice)

Forward: GGCAAGTTTTTGGTTGTGGGTC

Reverse: CCTCTTCTCCTATCTTCTGACGGG

Patched exon 3 (used in Figure S3)

Forward: ACCCGTCAGAAGATAGGAGAAGA

Reverse: GCACACGACTGGCCTGGA

Patched exon 14 (used in Figure S3)

Forward: GCCTCACAGTAACACCCGGTA

Reverse: GCTCGGCGGTGGTGTAGTAC

In Situ Hybridization

Tumor-bearing mice were perfused with 4% PFA and brains were embedded in OCT and cut into 12- μ M sections. Sections were fixed in 4% PFA, acetylated and incubated for 1h at room temperature in pre-hybridization buffer (50% formamide, 5X SSC, 1X Denhardt's, 250 μ g/ml yeast tRNA, 500 μ g/ml 1 herring sperm DNA). Sections were hybridized overnight at 65°C in hybridization buffer (50% deionized formamide, 1X Denhardt's, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 10 mM Na₂HPO₄, pH 7.4, 10% dextran sulfate, 0.5 mg/ml yeast tRNA) containing digoxigenin (DIG)-UTP-labeled probes for *Math1*. Probes were synthesized using a DIG labeling kit (Roche). After hybridization, sections were incubated overnight at 4°C with alkaline phosphatase-conjugated anti-DIG antibodies (Roche). Bound probe was visualized by incubating slides in NBT/BCIP overnight in the dark. Coverslips were mounted with Aqua-Polymount (Polysciences, Warrington, PA).

Virus Infection

To generate Cre retrovirus, *cre* cDNA was cloned into *MSCV-IRES-GFP* (from Irv Weissman, Stanford University). The plasmid was transfected into 293T cells with helper plasmids encoding gag-pol and vesicular stomatitis virus envelope glycoprotein. Virus-containing supernatant was harvested and concentrated by centrifugation. For infection, supernatant was added to cells at the beginning of culture. After 48 h, cells were harvested and infected (GFP⁺) cells were sorted by using a FACSVantage SE flow cytometer (BD Bioscience).

Table S1. Tumor Induction in *Math1-creER/Ptc^{C/C}* Mice Treated with Tamoxifen at Various Postnatal Ages

Age at Tamoxifen Treatment	Number of Animals	Tumor Incidence	Median Age of Tumor Onset (Weeks)
E10.5	8	0	—
E14.5	7	100%	10
P4	21	100%	13
P8	17	100%	15.5
P10	7	28.6%	19
P12	16	0	—

Mice were exposed to tamoxifen at the indicated ages and monitored for symptoms of tumor growth. Animals that developed symptoms were sacrificed and cerebella were subjected to histological analysis to confirm the presence of tumors. Asymptomatic mice were sacrificed after 8 months and analyzed for the presence of ectopic proliferating cells; no such cells were found in any of these mice.

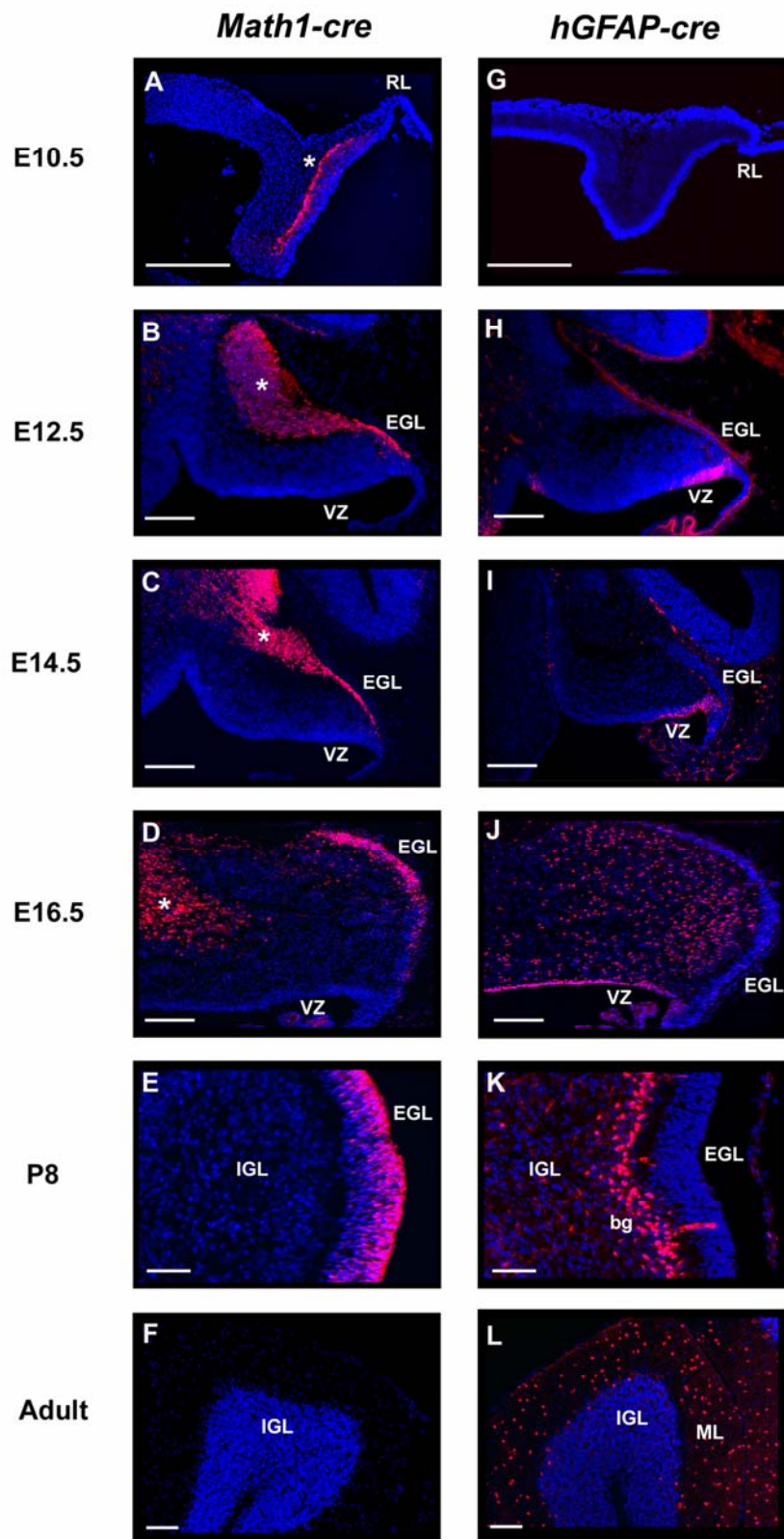


Figure S1.

Figure S1. Expression of Cre Protein in *Math1-cre* and *hGFAP-cre* Cerebellum

Cerebellar sections from *Math1-cre* (A-F) and *hGFAP-cre* (G-L) transgenic mice at various developmental stages (E10.5 - adult) were stained with anti-cre antibody (red) and counterstained with DAPI (blue). In the *Math1-cre* cerebellum, Cre expression is first seen at E10.5 (A) in rhombic lip (RL)-derived cells destined for the deep cerebellar nuclei and brainstem. These cells migrate around the circumference of the cerebellum during embryonic development (* in A-D) and leave the cerebellar cortex before birth. Between E12.5 and E14.5, the RL begins to produce GNPs. GNPs populate the EGL and maintain Cre expression until 2-3 weeks after birth (B-E). By adulthood (F), all GNPs have differentiated into mature granule neurons, migrated into the IGL, and lost expression of Cre. In the *hGFAP-cre* cerebellum, no Cre expression is detected at E10.5 (G). Cre-expressing cells are first seen in the caudal part of the ventricular zone (VZ) at E12.5 (H) (Staining of meninges and choroid plexus in this section is also seen with no primary antibody, and is therefore considered non-specific). Between E14.5 and E16.5 (I-J), Cre expression expands rostrally through the VZ, and is also seen in scattered astroglial cells throughout the cerebellar parenchyma. After birth (K), prominent Cre expression is seen in Bergmann glia (bg) and in astrocytes in the molecular layer (ML) and IGL. This expression pattern is maintained into adulthood (L). Note that no Cre is detected in the VZ of *Math1-cre* cerebella, or in the EGL of *hGFAP-cre* cerebella. Scale bars represent 25 μ m.

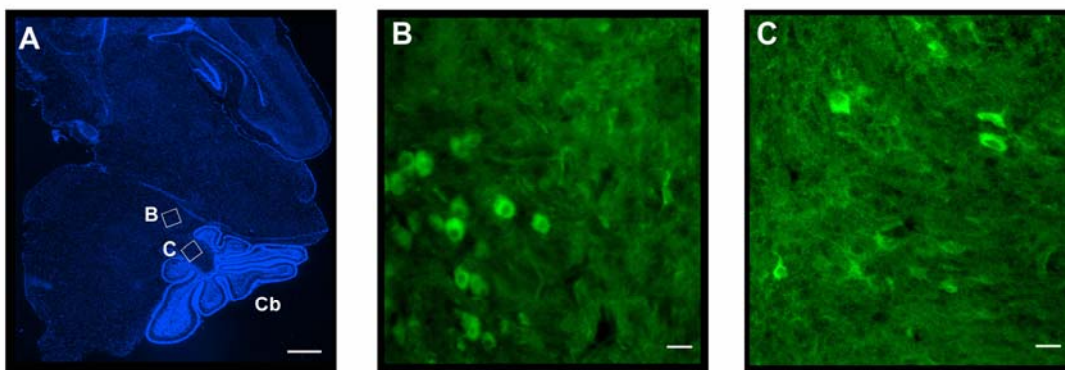


Figure S2. *Math1-cre*-Expressing Cells Include Progenitors that Seed the Brainstem and Deep Cerebellar Nuclei

Sagittal sections from P8 *Math1-cre/R26R-GFP* mice were stained with DAPI (A) and with anti-GFP antibodies (B and C). In addition to the GFP expression detected in the EGL (see Figure 1), less intense GFP was detected in cells scattered throughout the brainstem (B) and deep nuclei (C). Boxes in A indicate regions shown in B and C. Cb, cerebellum. Scale bars: A, 1mm; B and C, 40 μ m.

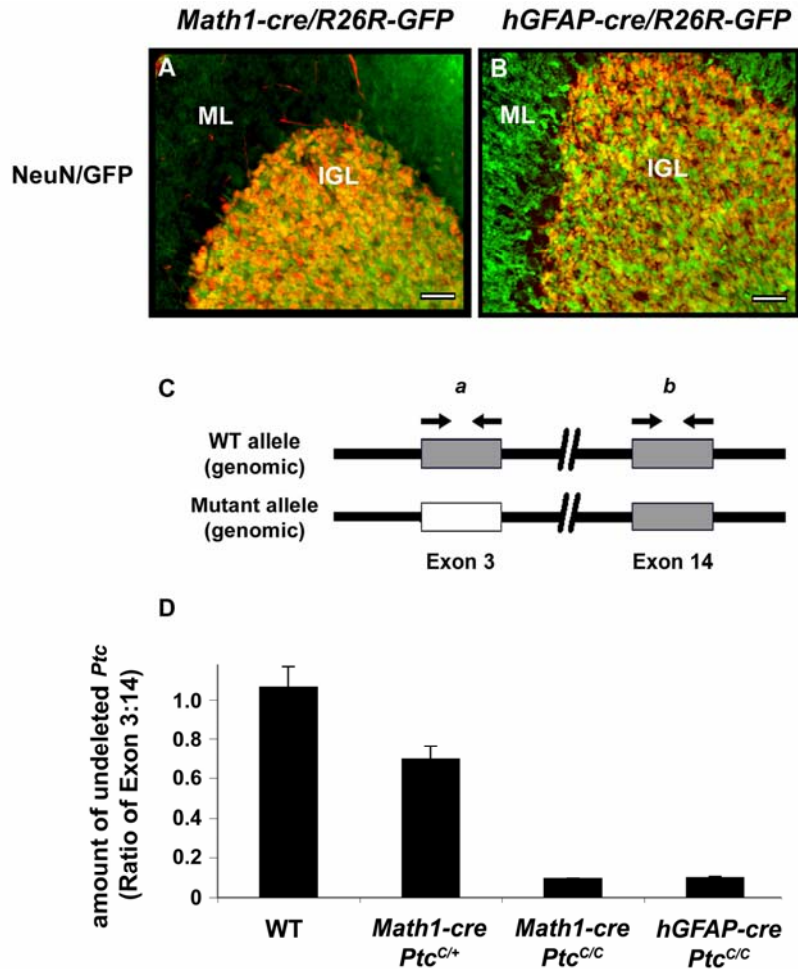


Figure S3. Efficiency of Cre-Mediated Recombination in *Math1-cre* and *hGFAP-cre* Mice

(A and B) Induction of GFP in *R26R-GFP* reporter mice. Cerebellar sections from P21 *Math1-cre/R26R-GFP* (A) and *hGFAP-cre/R26R-GFP* (B) animals were stained with anti-GFP (green) to label cells that had expressed Cre during development and anti-NeuN (red) antibodies to label postmitotic granule neurons. Note that in both strains, >90% of NeuN⁺ cells coexpressed GFP (orange/yellow staining). Scale bars represent 25 μ m. C, D. Deletion of *Ptc* in *Ptc^{C/C}* mice.

(C) Two different primer sets were designed to analyze *Ptc* genomic DNA. Primer set “a” amplifies part of exon 3, which is deleted in the mutant allele; primer set “b” is specific for exon 14, which is present in both wild type and mutant alleles.

(D) Cells were isolated from the EGL of wild type (WT), *Math1-cre/Ptc^{C/+}*, *Math1-cre/Ptc^{C/C}*, or *hGFAP-cre/Ptc^{C/C}* mice by laser capture microdissection. Genomic DNA extracted from captured cells was used for quantitative real-time PCR using primer sets a and b. The amount of undeleted *Ptc* in each sample was calculated by dividing the level of exon 3 product by the total amount of *Ptc* DNA (represented by exon 14 product). As shown in D, for wild type EGL, the ratio of exon 3 to exon 14 (E3:E14) was close to 1, consistent with the fact that wild type EGL has not undergone exon 3 deletion. In contrast, 90% of genomic *Ptc* has been deleted in the EGL of *Math1-cre/Ptc^{C/C}* and *hGFAP-cre/Ptc^{C/C}* mice by P8; similar results were seen at P1. Data represent means of triplicate samples \pm SEM.

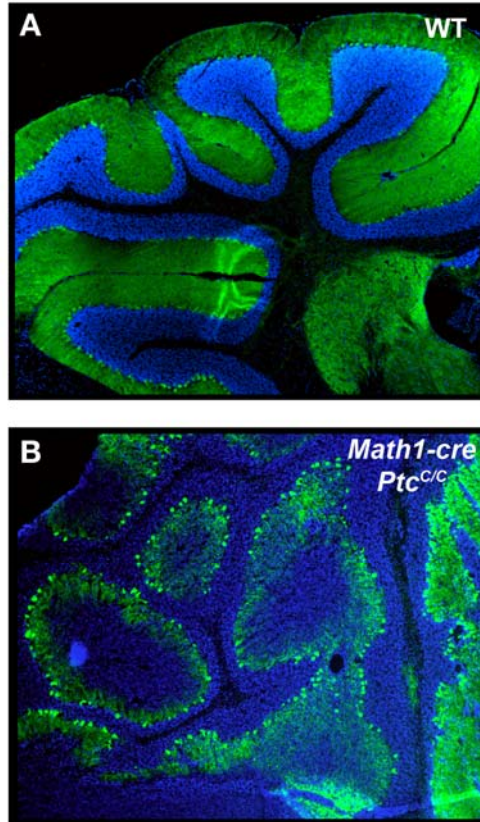


Figure S4. Purkinje Cell Differentiation in *Math1-cre/Ptc^{C/C}* Mice

Cerebellar sections from wild type (A) and *Math1-cre/Ptc^{C/C}* (B) mice were stained with anti-calbindin (green) and counterstained with DAPI (blue). In the mutant cerebellum (B), Purkinje cells surround regions of proliferating GNPs, and are surrounded by postmitotic granule neurons, as shown in Figure 3E-F. Scale bars represent 300 μ m.

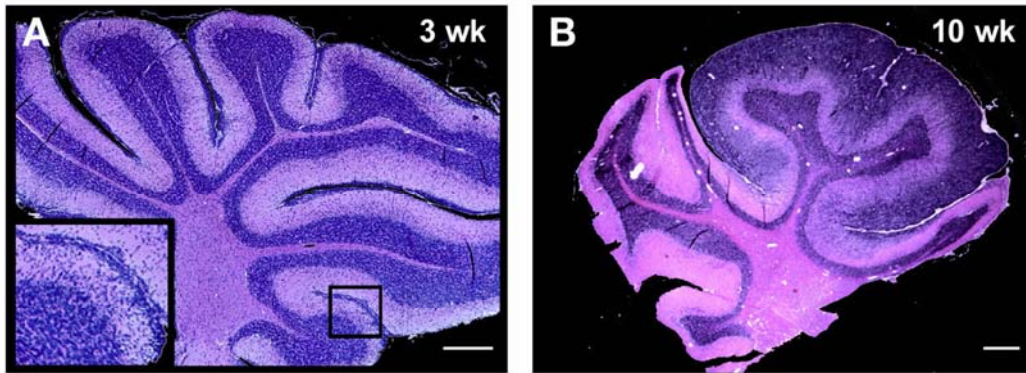


Figure S5. Postnatal Deletion of *Ptc* in GNPs Results in Hyperplasia

Math1-creER/Ptc^{C/C} mice were treated with tamoxifen at P4 and sacrificed at 3 weeks (A) or 10 weeks (B) of age. Cerebella were sectioned and stained with H&E. At 3 weeks (A), all mutant animals have persistent GNP-like cells covering the surface of the cerebellum (boxed region is expanded in inset). By 10 weeks of age (B), most ectopic cells have moved away from the surface and differentiated, but some lobes still contain hyperplastic regions on the surface. Tumors develop in all animals between 10-19 weeks of age (see Figure 4). Scale bars represent 300 μm .

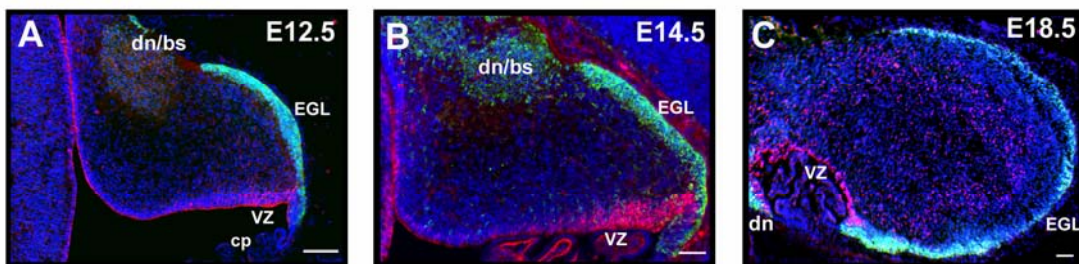


Figure S6. *Math1* and *hGFAP* Are Expressed in Discrete Populations of Cells

Cerebellar sections from *Math1-GFP/hGFAP-cre* embryos at E12.5 (A), E14.5 (B) and E18.5 (C) were double-stained with anti-GFP (green) and anti-Cre (red) and counterstained with DAPI (blue). At each stage, *hGFAP-cre* is highly expressed in the ventricular zone (VZ) and in parenchymal astrocytes and excluded from the EGL, whereas *Math1-GFP* is expressed in the EGL and progenitors of the deep nuclei and brainstem (dn/bs) and excluded from the VZ. Scale bars represent 20 μm .

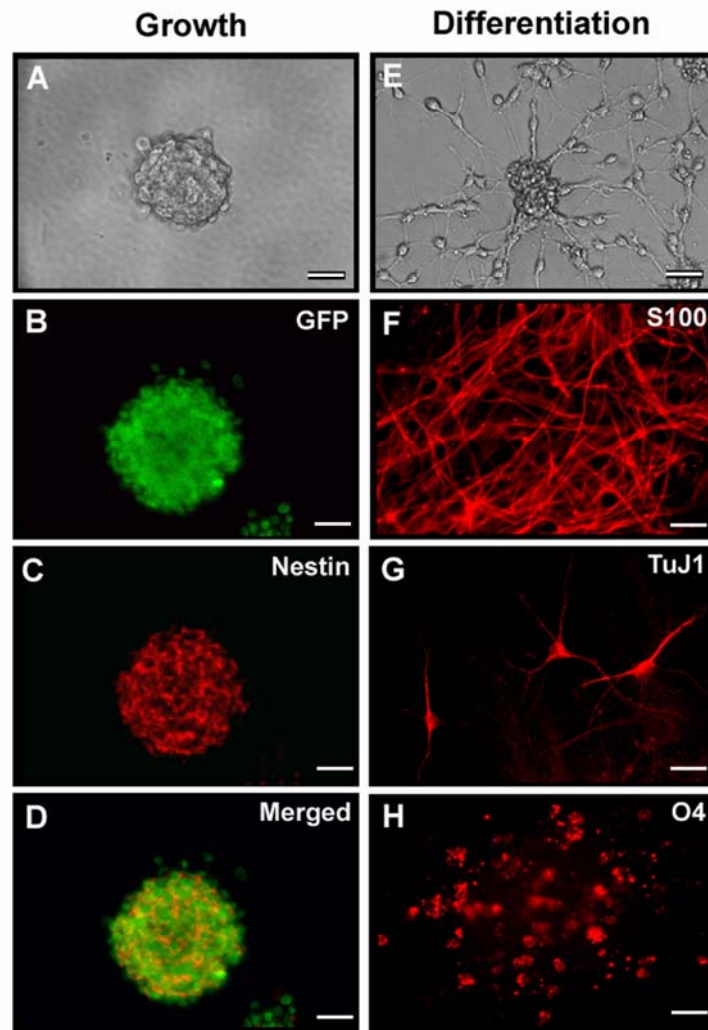


Figure S7. *hGFAP*-Expressing Cells from the Embryonic Cerebellum Include Multipotent, Neurosphere-Forming Cells

GFP⁺ cells were FACS-sorted from E14.5 *hGFAP-GFP* embryos and cultured in NSC proliferation medium with 10 ng/ml FGF and 20 ng/ml EGF.

(A-D) Neurospheres observed after 7 days in culture were photographed using bright-field microscopy (A) or stained with anti-GFP (green, B, D) and anti-Nestin (red, C, D). Most cells in neurospheres were found to coexpress GFP and Nestin (D).

(E-H) Neurospheres were also dissociated and plated in NSC differentiation medium. After 7 days in culture, many cells adhered to the dish and extended processes (E). Differentiation cultures were photographed with bright-field (E) or stained with antibodies to detect differentiated astrocytes (S100, F), neurons (β 3-tubulin, G) or oligodendrocytes (O4, H). Scale bars represent 15 μ m.

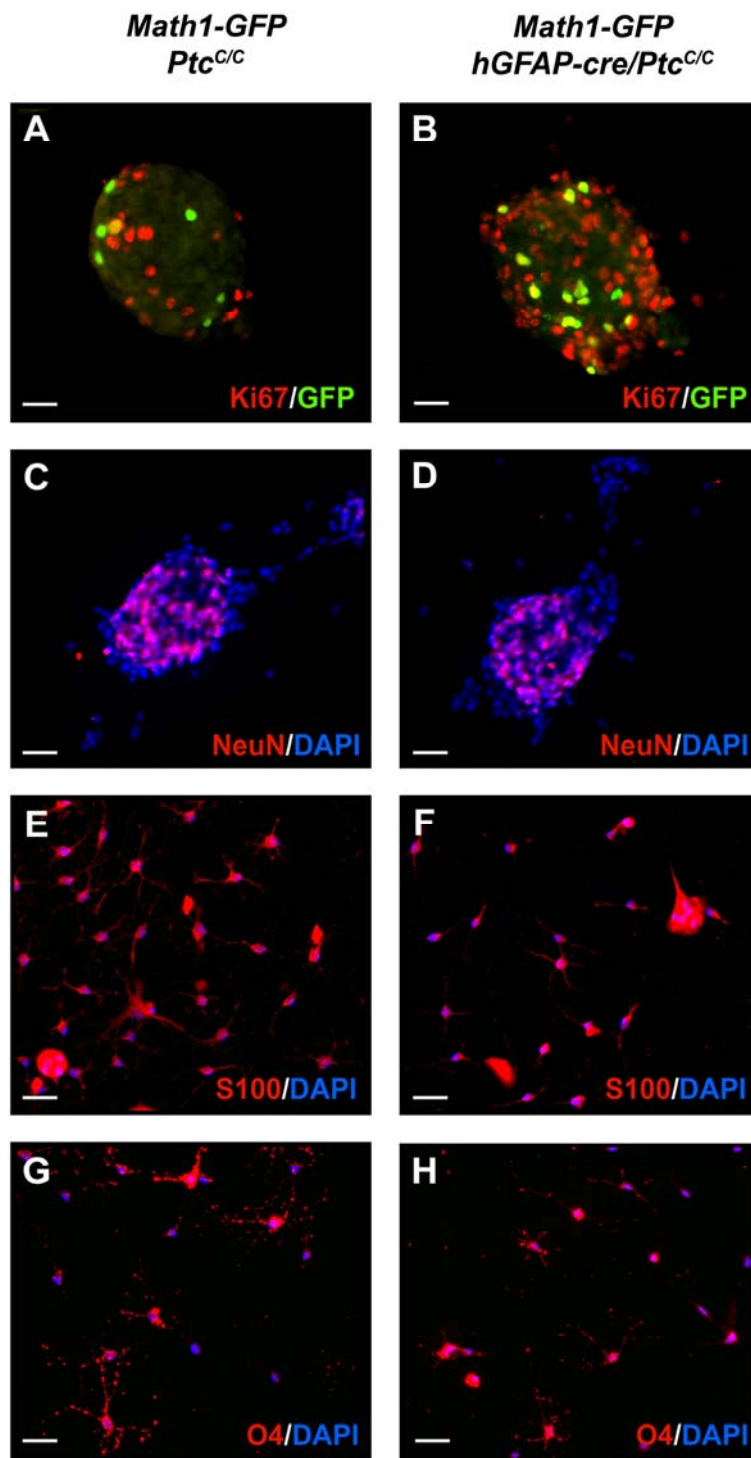


Figure S8.

Figure S8. Differentiation of Stem Cells after Deletion of *Ptc*

To determine the lineage potential of neural stem cells after deletion of *Ptc*, we analyzed differentiation of neurospheres from *Ptc*^{C/C} (A, C, E, G) or *hGFAP-cre/Ptc*^{C/C} (B, D, F, H) embryos at E14.5. To enrich for stem cells (and deplete cells that had already committed to the granule lineage), we did these experiments using animals carrying a *Math1-GFP* transgene and FACS-sorted *GFP*⁻ cells. Cells were plated at clonal density in neurosphere growth media for 5 days, and then transferred to differentiation media and cultured for an additional 3-5 days.

(A–H) Granule lineage commitment was monitored based on expression of the *Math1-GFP* protein (green), and proliferation was detected by staining for *Ki67* (red) after 3 days in differentiation media (A and B). Neuronal and glial differentiation was analyzed after 5 days by staining with antibodies specific for neurons (*NeuN*, red in C-D), astrocytes (*S100*, red in E-F) and oligodendrocytes (*O4*, red in G-H); all cultures were counterstained with *DAPI* (blue) to detect nuclei. Neurospheres from both control (*Ptc*^{C/C}) and mutant (*hGFAP-cre/Ptc*^{C/C}) mice were able to differentiate into all three lineages. The increased number of *Math1-GFP*⁺ cells (B) and *NeuN*⁺ cells (D) in mutant neurospheres could represent either skewed commitment of *Ptc*-deficient stem cells to the granule lineage or increased proliferation of cells once they have committed to this lineage. Scale bars represent 15 μ m.