The Normal patched Allele Is Expressed in Medulloblastomas from Mice with Heterozygous Germ-Line Mutation of patched

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ABSTRACT

Defects in a developmental signaling pathway involving mammalian homologues of the Drosophila segment polarity gene, patched (ptc) and its ligand, sonic hedgehog (shh), contribute to tumor formation in several tissues. Recently, a subset of medulloblastoma, the most common malignant brain tumor in children, was found to contain somatic mutations in the human ptc gene. In addition, basal cell nevus syndrome (BCNS), or Gorlin syndrome, which is characterized by developmental anomalies and a predisposition to skin and nervous system malignancies, is associated with germ-line mutation of ptc. Targeted disruption of both alleles of ptc in mice results in embryonic lethality. However, ptc+/− mice survive and develop spontaneous cerebellar brain tumors, suggesting that ptc may function as a tumor suppressor gene. Therefore, we investigated ptc+/− mice as a model for human medulloblastoma. We report that 14% of ptc+/− mice develop central nervous system tumors in the posterior fossa by 10 months of age, with peak tumor incidence occurring between 16 and 24 weeks of age. The tumors exhibited several characteristics of human medulloblastoma, including expression of intermediate filament proteins specific for neurons and glia. Full-length ptc mRNA was present in all tumors analyzed, indicating that there was no loss of heterozygosity at the ptc locus. Nucleotide sequence of ptc mRNA from four tumors failed to identify any mutations. However, a comparison of the normal ptc sequence from C57BL/6 and 129Sv mice did reveal several polymorphisms. High levels of gli1 mRNA and protein were detected in the tumors, suggesting that the shh/ptc pathway was activated despite the persistence of ptc expression. These data indicate that haploinsufficiency of ptc is sufficient to promote oncogenesis in the central nervous system.

INTRODUCTION

Medulloblastoma, a primitive neuroectodermal tumor that arises in the cerebellum, is the most common malignant brain tumor in children. Peak incidence occurs at 5 years of age, and most tumors appear during the first decade of life (1). Although comparative genome hybridization identified several candidate chromosomal rearrangements in medulloblastoma, no specific fusion gene products were identified (2, 3). However, a subset of medulloblastoma has been identified with allelic loss of chromosome 9q22 (4–8), a region that contains ptc (9, 10). In contrast to numerous reports of p53 mutation in adult brain tumors, mutations in p53 have only rarely been demonstrated in pediatric medulloblastoma (11). The discovery that the human homologue of the Drosophila segment polarity gene, ptc, is mutated in a subset of spontaneous (4–8) and basal cell nevus syndrome-associated medulloblastoma (12) provided the first genetic clue to the etiology of medulloblastoma. ptc mutations have been detected in both desmoplastic and classic medulloblastoma, and they cannot be correlated with a specific tumor subtype (7).

The ptc gene was identified during a genetic screen for embryonic lethal mutations in Drosophila. Alterations in ptc are associated with aberrant body segmentation of the larva and early lethality (13). ptc encodes a 12-pass transmembrane protein (14) that functions as part of the receptor complex for hh (15). During development, ptc represses constitutive signaling by smo, another component of the hh receptor complex. This results in reduced transcription of several target genes in the pathway including members of the transforming growth factor β family, wnt genes, ci, and ptc itself (16). Binding of hh to the ptc/smo receptor complex relieves this ptc-mediated repression and results in increased activity of the transcription factor ci, which elevates expression of target genes (17).

In vertebrates, mutations in several members of the hh/ptc pathway have been linked to developmental defects in the nervous system and to malignancies in several tissues. Mutations in shh, a vertebrate homologue of hh, are associated with neural tube defects in humans, including spina bifida and holoprosencephaly (18, 19). In human tumors, inactivation of ptc abrogates its repressor function, increasing the level of expression of gli1, a vertebrate transcription factor related to ci, that was originally identified as an amplified gene in a glioblastoma (20). Although it is present in glioblastomas and sarcomas, gli1 is not readily detected in adult brain RNA (21). Overexpression of gli1 in the epidermis of frogs causes lesions resembling human BCC, similar to those induced in mice by overexpression of shh (19). The hh/ptc pathway is thought to play a critical role in regulating cell proliferation and differentiation during morphogenesis.

Germ-line mutations in ptc are responsible for BCNS (also known as Gorlin Syndrome; OMIM 109400), an autosomal dominant disease (22) characterized by a larger body size, developmental and skeletal anomalies, fibromas of soft tissues, radiation sensitivity, basal cell carcinoma, and medulloblastoma (19, 23). Although only a small proportion of medulloblastoma is associated with BCNS (10% of children diagnosed with medulloblastoma at age 2 years or under; Ref. 12), these patients are very sensitive to radiation, which is often used in the treatment of medulloblastoma. In BCNS patients, ptc appears to function as a classic tumor suppressor gene because the second allele is lost in the majority of BCCs (12). Mice heterozygous for ptc resemble BCNS patients in that they exhibit a larger body size, skeletal abnormalities, cerebellar tumors (24), radiation sensitivity (25), and skin lesions similar to BCC after radiation (26). In mice, homozgyous loss of ptc results in embryonic lethality at 9.5–10.5 days after fertilization (24).

We investigated tumorigenesis in ptc+/− mice to assess the utility of this strain as a model for pediatric medulloblastoma. In our colony, we found that 14% of ptc+/− mice develop posterior fossa tumors by 10 months of age. The histological appearance, site of origin, and presence of intermediate filament proteins indicated that the mouse tumors were very similar to human medulloblastoma. In contrast to the situation with BCC in mice and humans, we found that the normal ptc allele was retained and expressed in mouse medulloblastomas. Thus, haploinsufficiency of ptc promotes medulloblastoma formation.
in mice by a mechanism that does not require complete loss of ptc expression.

MATERIALS AND METHODS

Animals. A colony of ptc+/- mice was established using two pairs of mice, obtained from Matthew Scott, Stanford University. These mice were either crossed with C57BL/6 or 129Sv (currently designated by The Jackson Laboratory as 129 x 1/Sv) mice. Mice were maintained in the animal facility at SICRH on a 12-h light/dark cycle. Animals were genotyped by PCR amplification (24) of genomic DNA extracted from tail biopsies. Approximately 1000 mice (200 129Sv and 800 C57BL/6) were generated and genotyped to obtain 499 ptc+/- mice for the present study. Heterozygous mice were maintained and observed up to 10 months of age. Animals were sacrificed when they showed signs of increased intracranial pressure (ataxia, decreased movement, paresis of hind limbs, enlarged occipital prominence, hunched back, and/or poor grooming).

Dissection of Brains and Tumor Tissues. Brains were removed from the surrounding calvarium, and tumor tissue was carefully separated from surrounding brain parenchyma under a dissecting microscope. Generally, there was no capsule surrounding tumors, and malignant tissue was separated from unraveled brain by blunt dissection based on the difference in tissue consistency and the anatomy of the cerebellum. Fresh tissue was snap frozen and stored at -80°C for later extraction of RNA, DNA, and protein. For histochemical analyses, animals were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 M PBS.

Histochemistry. After perfusion, tissues were postfixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 2–6 h at room temperature. Subsequently, they were immersed in 20% sucrose for cryoprotection and stored at 4°C. Tissue was embedded in freezing medium, frozen, sectioned at a thickness of 12 µm on a cryostat, and mounted onto Superfrost + slides (Fisher Scientific, Pittsburgh, PA).

Frozen sections of tumor tissue were treated with antibodies to neurofilament-H (NF; 1:200; Chemicon International, Temecula, CA), GFAP (1:200; Dako A/S, Copenhagen, Denmark), vimentin (1:200; Zymed Laboratories, San Francisco, CA), Nestin (1:50; Rat401; Iowa Hybridoma Tissue Bank), and synaptophysin (1:200; Chemicon International). Primary antibodies were diluted in 0.1 M PBS with 0.1% Triton X-100 and applied to tissue overnight at 4°C. Secondary antibodies were applied according to Vectastain Elite ABC instructions (Vector Laboratories, Burlingame, CA), and detection was carried out with 3,3′-diaminobenzidine reagent (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

RNA Isolation and Northern Analysis. Total cellular RNA was isolated from 18 mouse medulloblastomas using Trizol (Ambion, Inc., Austin, TX) according to the manufacturer’s directions. Five to 10 µg of total RNA were electrophoresed on a 0.8% agarose-formaldehyde gel, transferred to a nitrocellulose filter (Hybond N+; Amersham Pharmacia, Buckinghamshire, England), and hybridized under stringent conditions (18 h at 68°C in 5% SSPE, 50% formamide, 5% Denhardt’s solution, 1% SDS, and 0.1 mg/ml denatured salmon sperm DNA) with a 32P-labeled RNA probe. Filters were washed twice for 20 min each in 0.1× SSC, 0.1% SDS at 68°C and exposed to MR film (Kodak) for 12–72 h at -80°C. Control and ptc+/- tissues were analyzed by hybridization with 32P-labeled DNA probes specific for mouse ptc (24), gil1 (Mouse EST clone 38654), gl2 (27), reelin (28), trkC (29), and random primed DNA probes specific for lacZ and β-actin (RediPrime; Amersham Pharmacia Biotech UK).

Generation of Gli1 Antisera. Antibodies were generated in rabbits against COOH region peptides corresponding to amino acids 803–818 of mouse Gli1. Peptides were synthesized as multiple antigen peptides (Research Genetics, Huntsville, AL) to avoid generating antibodies to a carrier protein. Rabbits were immunized with 1 mg of peptide in Freund’s Complete adjuvant and boosted approximately every 2 weeks with 0.5–0.25 mg of peptide in incomplete adjuvant, and blood was collected via the central ear artery at 10 days after immunization. Crude sera were collected after allowing the blood to clot overnight at 4°C and used at stated dilutions without further purification. Sera were screened in immunoblot, immunofluorescence, and immunoprecipitation assays and selected for further evaluation.

Transfection and Immunoblot Analysis. COS-7 cells or 293T cells were transfected with an expression plasmid encoding human gil1 cDNA (30) using FuGene6 (Boehringer Mannheim) according to the manufacturer’s directions.

Forty-eight h after transfection, the cells were lysed in 2× SDS sample buffer (100 µl per 35-mm dish) for immunoblot analysis. As a positive control for native Gli1, we analyzed protein extracts from the rhabdomyosarcoma cell line Rh30, which expresses high levels of gil1 (31).

Protein extracts were prepared by Dounce homogenization of 80–100 mg of snap-frozen tumor or normal tissue in 300 µl of ice-cold lysis buffer [1% Triton X-100, 30 mM HEPES (pH 7.5), 10% glycerol, 150 mM NaCl, 5 mM MgCl2, 1 mM EGTA, 0.1% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml trypsin inhibitor, and 1 µg/ml pepstatin]. Extracts were cleared by microcentrifugation at 14,000 rpm for 30 min. One hundred µg of the protein extracts or 6 µl of 293T cell lysates were loaded per lane on an 8% polyacrylamide gel. After separation on a polyacrylamide gel, proteins were electrotransferred onto nitrocellulose membranes, incubated with an antibody directed against mouse Gli1 amino acids 803–818 (1:5000), followed by incubation with a rabbit IgG conjugated to horseradish peroxidase at 40 milliunits/ml, and products were visualized by enhanced chemiluminescence (Boehringer Mannheim). Membranes were stripped and incubated with an antibody against Ref-1 (32) to control for protein loading and transfer efficiency.

Reverse Transcription-PCR. Two-step reverse transcription-PCRs were carried out to maximize uniformity of PCR templates for all reactions. cDNA was derived in 20 µl volumes with random hexamers, oligo dT, and gene-specific priming using SuperScript reverse transcriptase (Life Technologies, Inc., Rockville, MD). Reverse transcriptase first-strand cDNA synthesis reactions were carried out using 3 µg of total RNA prepared from adult C57BL/6 and 129Sv mouse cerebellum, C57BL/6 E15 limbs, and four tumor samples (tumor numbers 199, 241, 448, 646), according to the manufacturer’s directions. PCR products were analyzed from two separate cDNA synthesis reactions.

Nucleotide Sequencing. Sequencing reactions were performed by the Hartwell Center for Biotechnology at St. Jude Children’s Research Hospital on template DNA using rhodamine or dRhodamine dye-terminator cycle sequencing ready reaction kits with AmpliTaq DNA polymerase FS (Perkin-Elmer, Applied Biosystems, Inc., Foster City, CA), and synthetic oligonucleotides. Samples were electrophoresed, detected, and analyzed on Perkin-Elmer Applied Biosystems model 373, model 37 Stretch, or model 377 DNA sequencers. Sequence analysis was performed using Sequencher (Genes Codes Corp., Ann Arbor, MI) software.

Restriction Enzyme Digest of PCR Products. The nucleotide polymorphism at position 4015 of ptc in C57BL/6 and 129Sv mouse DNA disrupts the recognition site for the restriction enzyme, MaeII (ACGGT; Boehringer Mannheim). To differentiate between the polymorphic alleles, PCR fragments amplified from tail DNA from 129Sv mice, C57BL/6 mice, and tail DNA from four ptc+/- mice (nos. 199, 241, 448, and 646) that developed tumors were plated from 129Sv mice, C57BL/6 mice, and tail DNA from four ptc+/- mice (nos. 199, 241, 448, and 646) that developed tumors were

![Fig. 1. Histogram of medulloblastoma incidence in ptc+/- mice. All brain tumors arose in the posterior fossa and were detected by physical examination of the mice for signs of increased intracranial pressure. Brains were dissected and examined to confirm the presence of tumors. Mouse age at time of tumor detection was calculated to the nearest week at time of death.](Image 308x96 to 560x292)
treated with MaeII. The PCR reaction generated a 524-bp fragment from the 3' end of the ptc open reading frame (primers ptcF8 corresponding to bases 3781–3804 and ptcR8 complementary to bases 4283–4305). MaeII digestion of 0.5 µg of each PCR product was performed with buffer supplied by manufacturer. The presence of the C57BL/6 and 129Sv alleles was determined by comparison of untreated (−) and MaeII-treated PCR products by agarose gel electrophoresis in the presence of ethidium bromide.

RESULTS

Mice Heterozygous for ptc Develop Brain Tumors in the Posterior Fossa. Fourteen % of ptc+− mice between the ages of 8 and 48 weeks of age developed spontaneous intracranial tumors resembling medulloblastoma that became large enough to cause a change in normal behavior. There was no significant difference in tumor inci-
dence between the two mouse strains (C57BL/6, 14%; 129Sv, 12%). The earliest tumors were detected at 8–10 weeks of age (Fig. 1). Peak incidence occurred between 16 and 24 weeks, although some tumors arose in mice older than 28 weeks of age (Fig. 1). The tumors were readily apparent on gross examination of the brains (Fig. 2C), and they arose consistently in the posterior fossa, generally displacing normal cerebellar tissue (Fig. 2D). The surface of the tumor was smooth (Fig. 2, C and D) and lacked cerebellar foliations (Fig. 2, A and B). Five % of the ptc+/− mice developed an enlarged cranial vault with a thin membrane bridging the area in which calvarial sutures would form in normal mice. These mice had significantly compressed cortical layers with enlarged ventricles, consistent with hydrocephalus (Table 1). Four % of the mice exhibited skeletal anomalies (polydactyly) or soft tissue tumors arising in skeletal muscle and connective tissues (Table 1). Brains from ~30 asymptomatic adult heterozygous mice were examined grossly under a ×2.5 dissecting microscope. No tumors were detected, although small regions of focal thickening of the cerebellar folia were noted in three mice.

**Histological Analysis of Tumors.** Histological analysis revealed that the tumors, which lacked a distinct capsule, were comprised of densely packed cells with prominent nuclei and scant cytoplasm. In the majority of cases, the tumors displaced and compressed the normal cerebellar architecture (Fig. 2E). Tumor cells did not infiltrate normal areas of the cerebellum, and they did not migrate along white matter tracts. The tumors were well circumcursed by a border of compressed cells that expressed intermediate filament proteins of neuronal lineage, including NF (Fig. 2G) and synaptophysin (Fig. 2H). GFAP immunoreactivity was noted in patches of cells throughout the tumor mass (Fig. 2F). It was not possible to determine whether a single cell expressed both NF and GFAP, although there were significant areas of overlapping GFAP and NF immunoreactivity. A few scattered areas of vimentin immunoreactivity were detected in three of the five tumors analyzed. No grossly apparent lesions were noted elsewhere in the neuroaxis. On the basis of the histological appearance, location, and expression of markers, the tumors in ptc+/− mice closely resemble human medulloblastoma.

**Persistence of Normal ptc mRNA in Tumors.** Northern analysis of mRNA extracted from medulloblastomas arising in ptc+/− mice indicated that the normal ptc allele was expressed in all tumors examined (Fig. 3). Two major ptc transcripts were detected in both tumor and control tissues that migrated slower than 28S rRNA, at approximately 8 and 12.5 kb (Fig. 3). In 5 of the 10 tumor samples, a smaller transcript (~5 kb) was also detected (tumors of mice nos. 42, 185, 199, 530, and 646) and in E15 limb. The levels of ptc mRNA detected in tumors varied, but it was always less than that observed in the adult mouse cerebellum (Fig. 3). Northern analysis was also carried out using a lacZ probe to detect mRNAs derived from the targeted allele. The lacZ probe identified a distinct but less abundant RNA species in tumor samples that was larger than the normal ptc mRNAs and was not present in RNA extracted from normal tissues.

**gli1 Is Expressed in Tumors.** Transcription of gli1, a member of the gli family of transcription factors, is normally repressed by ptc signaling (19). gli1 expression is increased after the interaction of Shh with the Ptc/Smo receptor complex, which relieves the repression of Ptc, or after inactivation of ptc by mutation. Therefore, we compared the expression of gli1 in ptc+/+ brain tumors and in control tissues to determine whether the ptc pathway was activated. A single gli1 mRNA transcript of ~4 kb was present at low levels in normal and ptc+/− brain tissues; however, this mRNA species was present at much higher levels in all tumors examined (Fig. 3). The increased expression of gli1 mRNA observed in tumors compared with adult control tissues implies that gli1 transcription is derepressed in ptc+/− tumors. In contrast, there was no expression of gli3, another gli family member, in the tumor tissues. However, gli3 mRNA expression was detected in E15 and E19 limbs.

**Nucleotide Sequence Analysis of ptc mRNA and Detection of Polymorphisms.** The second ptc allele is mutated frequently in skin tumors from individuals with a heterozygous germ-line mutation in ptc (9, 10). To determine whether the normal allele of ptc was mutated in medulloblastomas from ptc+/− mice, we compared the nucleotide sequence of ptc from four tumors, C57BL/6 cerebellum and C57BL/6 normal cerebellar tissue in medulloblastomas from ptc+/− mice. No grossly apparent lesions were noted elsewhere in the neuroaxis. On the basis of the histological appearance, location, and expression of markers, the tumors in ptc+/− mice closely resemble human medulloblastoma.
embryonic limbs. To ensure sequence fidelity, we carried out two complete sequencing passes of the ptc open reading frame using two different PCR reactions for both sense and antisense strands of ptc cDNA. Each PCR reaction was repeated with two independent preparations of cDNA from each sample. No mutations were detected in any of the four tumors examined. However, several sequence discrepancies were uncovered between the tumors and the normal ptc sequence from C57BL/6. Sequence analysis of selected regions of tail DNA from the two mouse strains, C57BL/6 and 129Sv, used in this study demonstrated that the sequence discrepancies corresponded to five polymorphisms (Table 2 and Figs. 5 and 6). One of the five polymorphisms (C or T at nucleic acid residue 4015) resulted in a codon change from ACG (threonine in C57BL/6 mice) to ATG (methionine in 129SvJ mice) at amino acid residue 1339 (Table 2). In fact, both of these alternatives were detected in the sequence chromatogram of the PCR products obtained from genomic DNA extracted from ptc1/2 mouse 448. The presence of both alleles generated two peaks on the chromatogram at position 4015 (Fig. 5). This polymorphism generates a MaeII restriction site in the C57BL/6 ptc sequence that is not present in the 129Sv ptc sequence (Fig. 6). Treatment of the PCR product amplified from the C57Bl/6 allele (but not the 129Sv allele) resulted in two fragments of 235 and 289 bases that migrated as a close doublet on the 1% agarose gel (Fig. 6). The disrupted ptc allele, which would be expected to be present in all heterozygous mice, is derived from 129Sv ES cells; therefore, it is not cleaved at this site by MaeII. Mice crossed onto the 129Sv background (e.g., mouse 241) contain a normal 129Sv ptc allele as well as the targeted 129Sv allele, neither of which is digested by MaeII (Fig. 6). In contrast, the three other ptc1/2 mice examined, which were crossed with C57BL/6 mice (i.e., mice nos. 199, 448, and 646), have a normal ptc sequence derived from C57BL/6 containing an intact MaeII restriction site (Fig. 6). Because these are heterozygous mice, they should also carry the targeted 129Sv allele, which does not

<table>
<thead>
<tr>
<th>Base</th>
<th>C57BL/6/J codon</th>
<th>129Sv codon</th>
<th>Result in amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>3180 (T or C)</td>
<td>ATT</td>
<td>ATC</td>
<td>No change</td>
</tr>
<tr>
<td>3318b</td>
<td>GAG</td>
<td>GAG</td>
<td>No change</td>
</tr>
<tr>
<td>3438 (C or T)</td>
<td>ACC</td>
<td>ACT</td>
<td>No change</td>
</tr>
<tr>
<td>3498 (G or A)</td>
<td>CCG</td>
<td>CCA</td>
<td>No change</td>
</tr>
<tr>
<td>3561 (T or C)</td>
<td>CCT</td>
<td>CCC</td>
<td>No change</td>
</tr>
<tr>
<td>4015 (C or T)</td>
<td>ACG</td>
<td>ATG</td>
<td>Thr or Met at 1339</td>
</tr>
</tbody>
</table>

- Polymorphisms in ptc were identified by nucleotide sequence analysis ptc from C57BL/6 and 129Sv mouse tail DNA. Discrepancies in nucleic acid sequence between the two strains and comparison of our sequencing with the mouse ptc sequence in GenBank are highlighted in bold typeface in the codon columns.
- One nucleic acid discrepancy was identified at nucleotide 3318 between the mouse ptc sequence registered in GenBank (accession no. U46155) and the ptc sequence obtained from both mouse strains in the present study. This discrepancy does not result in an amino acid change.

Fig. 4. Immunoblot analysis of Gli1 protein expression in mouse tumors. Protein lysates from tumors (mouse nos. 42, 169, 185, 199, 241, 448, 530, 574, 646, and 659), normal cerebellum, normal brain excluding cerebellum, ptc1/2 cerebellum, normal mouse embryonic day 15 limb (E15 Limb), E15 head, 293 cells transfected with vector alone or with hGli1, and the rhabdomyosarcoma tumor cell line Rh30, which is known to express high levels of gli1 mRNA (31), were separated on 8% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with anti-Gli1 serum, followed by antirabbit IgG-horseradish peroxidase, and the signal was detected by enhanced chemiluminescence. The membranes were stripped and incubated with an antibody directed against Ref-1 as a control for protein loading and transfer efficiency.

Fig. 5. Chromatogram of nucleic acid sequence analysis of a PCR fragment amplified from tail DNA extracted from mouse 448. The PCR reaction amplified regions from both C57BL/6- and 129Sv-derived ptc alleles, creating a mixed template for sequencing. Note that there are two peaks at position 4015 representing the mixed template, indicating both A and G (corresponding to T and C in the sense strand of ptc).
In this proliferative phase, granule cells exit the cell cycle and migrate superficially to the external germinal layer of the cerebellum during development. After proliferation of early granule cell precursors in the germinal layer, the normal cerebellum is comprised of a proliferation of inwardly directed radial fibers to assume their mature position in the internal granule layer (37). Medulloblastoma is thought to arise from a primitive neuroectodermal cell, perhaps a precursor of cerebellar granule cells (38). Recent studies have demonstrated that mutations in components of shh/ptc pathway can result in medulloblastoma formation and other tumors. Loss of ptc expression, as a consequence of germ-line mutation or somatic mutation in sporadic tumors, has been linked to medulloblastoma. Furthermore, overexpression or activating mutations in shh also lead to abrogation of ptc function and subsequent tumorigenesis (39–41).

Mice heterozygous for ptc, as a consequence of the targeted disruption of one allele, develop brain tumors and provide a unique opportunity to investigate an animal model for medulloblastoma (24, 25). Medulloblastomas that arise in ptc<sup>−/−</sup> mice express several of the intermediate filament proteins found previously to be present in human medulloblastoma cells. This is consistent with the proposed primitive neuroectodermal cell of origin of the tumors. Tumor cells retain the potential to differentiate along multiple pathways including neuronal, glial, and ependymal cell lineages (38). Expression of NF, synaptophysin, trkC, and reelin as well as GFAP within the tumor suggests that both neuronal and glial differentiation occurs. Here we show that the normal ptc allele is retained and expressed in medulloblastomas arising in ptc<sup>−/−</sup> mice. We demonstrate at least two major ptc transcripts (approximately 8 and 12.5 kb) in all control and tumor samples and 5 of 10 tumors also expressed a third, smaller transcript of ~5 kb. At least five ptc mRNA transcripts have been detected in human tissues (9), and the presence of the two larger ptc transcripts in control as well as tumor tissues in the present study suggests that several ptc transcripts are also expressed by mouse tissues. The levels of ptc expression varied among the tumors, regardless of mouse strain, age at tumor detection, or anatomical location within the cerebellum. This demonstrates that complete loss of ptc expression is not a prerequisite for tumorigenesis. Thus, ptc is not acting as a classic tumor suppressor gene in this mouse model.

Consistently, gli1, a candidate target gene thought to be activated by the hh/ptc pathway, was found to be expressed at high levels in all of the mouse medulloblastomas analyzed (Figs. 3 and 4). This is an indication that there is increased shh signaling or reduced ptc activity in the tumor cells (19). The expression levels of gli1 were too low in the normal tissues examined to determine whether loss of one allele of ptc caused up-regulation of expression. In contrast to the patterns of gli1 expression, variable levels of ptc mRNA were observed among the tumors, and some showed only very low levels of expression. Previously, Ptc was reported to repress its own transcription, and reduced levels of Ptc activity were believed to increase transcription of ptc (42–44). This does not appear to be the case in mouse medulloblastoma cells. In addition, there was approximately twice as much ptc mRNA in cerebellar extracts from normal mice compared with ptc<sup>−/−</sup> mice (Fig. 3), indicating that Ptc does not repress its own expression in mouse cerebellum. The regulatory pathway involving ptc is rather complex, and it is possible that there are additional defects in the tumor cells. Furthermore, the expression patterns of the genes we examined may differ in the proposed granule cell precursor population from which the tumors are believed to arise, compared with the postmitotic granule neurons present in the adult cerebellum.

Medulloblastomas in ptc<sup>−/−</sup> mice seem to arise from a focal lesion, and it is clear that many areas of the cerebellum have a relatively normal histological appearance (Fig. 2D). Thus, ptc haploinsufficiency alone is not sufficient to induce tumor formation, and it is likely that the tumors contain additional genetic lesions. Here we demonstrate that the additional mutations do not involve the second allele of ptc; however, it is still possible that other components of the pathway are mutated in the mouse tumors. The majority of human...
In the 129Sv strain, one of the polymorphisms results in a methionine at amino acid residue 1339 compared with a threonine in C57BL/6 mice. This residue is located in the terminal 100 amino acids of the Ptc protein in a region of Ptc that interacts with Smo (47, 48).

Complete inactivation of ptc appears to be an important step in the development of adult basal cell carcinoma and in a subset of human medulloblastomas (5–8). However, our data demonstrate that brain tumors of similar phenotype and anatomical location arise in mice with no loss of heterozygosity of the ptc locus. Similarly, in medulloblastomas from individuals with BCNS, the second ptc allele was reported to be mutated in only one of three cases examined (12). In approximately one-half of the sporadic medulloblastomas analyzed in which 9q loss or mutation of ptc was demonstrated, no mutations were found in the second ptc allele (4–8). Persistence of the wild-type ptc allele and variable levels of ptc mRNA expression were detected in many tumors (4, 7). This raises the question: what is the functional consequence of ptc haploinsufficiency that leads to medulloblastoma formation? One possibility is that the reduced level of Ptc expression, caused by loss of one allele, results in increased proliferation of granule cell precursors. The increase in cell proliferation may be balanced by elevated levels of cell death such that the total number of granule neurons does not change. In fact, during normal brain development there is an overproduction of neurons and a subsequent loss of excess cells by apoptosis. The increased turnover of granule cell precursors would enhance the possibility that additional mutations would arise in these cells. These mutations could either promote continued cell division or escape from cell death, resulting in tumorigenesis. Hopefully, continued analysis of ptc+/− mice will elucidate the molecular and cellular mechanisms underlying formation of medulloblastoma in mice and humans.

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