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Advances in Brief

Loss of *p53* but not *ARF* Accelerates Medulloblastoma in Mice Heterozygous for *patched*[1]

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Abstract

Brain malignancies represent the most common solid tumors in children, and they are responsible for significant mortality and morbidity. The molecular basis of the most common malignant pediatric brain tumor, medulloblastoma, is poorly understood. Mutations in several genes including the human homologue of the *Drosophila* segment polarity gene, *patched* (*PTCH*), the *adenomatous polyposis coli* gene (*APC*), *β-catenin*, and *p53* have been reported in subsets of hereditary and sporadic medulloblastoma. Inactivation of one *Ptc* allele in mice results in a 14% incidence of medulloblastoma. Here, we report a dramatic increase in the incidence (>95%) and accelerated development (prior to 12 weeks of age) of medulloblastoma in mice heterozygous for *Ptc* that lack *p53*. The acceleration of tumorigenesis in $Ptc^{+/-}$ mice is specific for loss of *p53*, because no change in tumor incidence was observed in $Ptc^{+/-}$ mice carrying a mutation in *APC* ($Min^{+/-}$) or in $Ptc^{+/-}$ mice deficient in $p19^{ARF}$. Thus, there is a specific interaction between *p53* loss and heterozygosity of *Ptc* that results in medulloblastoma. This may be a consequence of increased genomic instability associated with loss of *p53* function that may enhance the rate of acquisition of secondary mutations. $Ptc^{+/-}p53^{-/-}$ mice provide a useful model for investigation of the molecular bases of medulloblastoma and for evaluation of the efficacy of therapeutic intervention strategies in a spontaneously arising endogenous brain tumor.

Introduction

Medulloblastoma arises from the primitive neuroectoderm in the posterior fossa of children generally between the ages of 3 and 9. The tumors are derived from cerebellar granule precursor cells that undergo a dramatic proliferative expansion during the early phases of postnatal brain development. Several gene mutations have been described in medulloblastoma, although they occur in small subsets of tumors (1). A low frequency of mutations in *patched* (*PTCH;* Ref. 2), *p53* (3), the *adenomatous polyposis coli* (*APC*) gene (4), and *β-catenin* (5, 6) have been reported in subsets of sporadic medulloblastoma. In addition, brain tumors, including medulloblastoma, have been reported to occur more frequently in patients carrying germ-line mutations in *PTCH*, *APC*, or *p53* (7–9). In the case of heterozygous loss of *PTCH*, which is associated with Gorlin syndrome, also known as basal cell nevus syndrome (OMIM 109400), the incidence of medulloblastoma increases from 2 per million to 4 per hundred in children <18 years of age (10, 11). Thus, it is likely that there are several unknown prevalent mutations in these tumors that contribute to disease progression.

Progress in understanding the etiology of medulloblastoma has been hampered by the lack of an appropriate animal model. Recently, a mouse strain was generated in which *Ptc* was mutated by targeted disruption (12, 13). Homozygous deletion of *Ptc* results in embryonic lethality, whereas mice heterozygous for *Ptc* exhibit several features of Gorlin syndrome, including an increased propensity to develop tumors in the brain and soft tissues. Histological analysis of the brain tumors showed that they closely resemble human medulloblastoma (12, 14). However, only 14% of mice heterozygous for *Ptc* develop medulloblastoma over a period of 10 months, indicating that it is likely that additional genetic lesions are required for oncogenic transformation.

Ptc functions as a component of the receptor complex that transduces a signal from Hedgehog (Hh) through a complex pathway that was first described in *Drosophila* (7). The interaction of Shh, the mammalian orthologue of Hh, with Ptc relieves suppression of *smoothened* (*Smo*), resulting in increased transcription of *Gli1* and other target genes (7). During cerebellar development, Shh, produced by Purkinje cells, functions as a mitogen to stimulate proliferation of granule cell precursors (15). *Ptc* does not function as a classic tumor suppressor gene in medulloblastomas in $Ptc^{+/-}$ mice because the normal allele is not lost, and it continues to be expressed in tumors (14, 15).

p53 functions as a transcription factor that transduces signals elicited by physiological stress and DNA damage to regulate cell proliferation and apoptosis. Abrogation of p53 function attenuates both of these responses (17). The mouse tumor suppressor gene $p19^{ARF}$ ($p14^{ARF}$ in humans) is the product of an alternative reading frame encoded by the *INK4a-ARF* locus. *ARF* functions as a sensor of normal proliferative signals upstream of *p53* by interfering with *Mdm2*, a negative regulator of *p53* function (18). Thus, loss of $p19^{ARF}$ diminishes *p53* activity and promotes tumor formation (19). Mice deficient in *p53* do not develop brain tumors, although they are predisposed to develop tumors in several other tissues by 5 months of age (20, 21). Approximately 10% of *ARF*-null mice develop glial tumors by 6 months of age (19).

To address the possible involvement of tumor suppressor genes in medulloblastoma and to accelerate the incidence of these tumors, we crossed $Ptc^{+/-}$ mice with mice carrying mutations in other tumor suppressor genes. We selected *APC* because it regulates the levels of *β-catenin*, which functions in the *Wnt* signaling pathway (22). Humans with brain tumor-polyposis, or Turcot's syndrome, carry germline mutations in *APC*, and they have an increased incidence of tumors arising in colon and brain (8). In addition, mutations in *β-catenin* have been reported in spontaneous medulloblastoma, albeit at a low frequency (5, 6). We also crossed the $Ptc^{+/-}$ mice with mice carrying inactivating mutations in two major tumor suppressor genes that are defective in more than half of all human cancer, *p53* and *ARF* (18). These genes serve critical functions in the regulation of cell proliferation, apoptosis, and response to DNA damage (18, 23).

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Materials and Methods

Animals. The $Ptc^{+/-}$ mice used in this study were generated and maintained on a mixed C57Bl/6 × 129Sv background, as described previously (14), and crossed with mice carrying targeted disruptions in *p53*, *APC* (C57BL/6J-$Min^{+/-}$; Jackson Laboratories, Bar Harbor, ME), and *ARF* (19) to generate the following cohorts of mice: $Ptc^{+/-}p53^{+/+}$ ($n = 440$), $Ptc^{+/-}p53^{+/-}$ ($n = 68$), $Ptc^{+/-}p53^{-/-}$ mice ($n = 40$), $Ptc^{+/-}ARF^{-/-}$ ($n = 40$), and $Ptc^{+/-}Min^{+/-}$ ($n = 16$). Cohorts of mice were observed for tumor formation for a minimum of 6 months after birth. All mice were observed daily for signs of increased intracranial pressure and for evidence of enlarged occipital prominence three times weekly for at least 24 weeks. Animals were euthanized when they were moribund according to NIH-approved institutional guidelines or when they showed signs of increased intracranial pressure or when extracranial tumors were evident. Brains were removed from the surrounding calvarium, and tumor tissue was carefully separated from surrounding brain parenchyma under a dissecting microscope. In every mouse, the presence of tumor was confirmed by gross examination of the brain. If the mouse was not available for examination or if no tumor was detected, the cause of death was attributed to "unknown causes." 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 and processed for immunohistochemical analyses as described previously (14).

RNA Isolation and Northern Analysis. Total cellular RNA was isolated from 11 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, United Kingdom), 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 ^{32}P-labeled RNA probe. Filters were washed (twice × 20 min in 0.1 SSC, 0.1% SDS at 68°C) and exposed to MR film (Eastman Kodak) for 12–72 h at −80°C. Control and tumor tissues were analyzed by hybridization with ^{32}P-labeled RNA probes specific for mouse *Ptc* (12), *Gli1* (mouse EST clone 38654), and *mdm2*.

Immunoblot Analysis. Protein extracts were prepared by Dounce homogenization of 80–100 mg of snap-frozen tumor or normal tissue as described (14). Extracts were clarified by microcentrifugation at 14,000 rpm for 30 min. Protein lysates (200 μg) from medulloblastomas arising in $Ptc^{+/-}$ mice (tumor nos. 185, 199, 241, 448, 530, and 574), mouse leukemia cells known to express mutated (CR246) or wild-type P53 (CR205), medulloblastoma from a $Ptc^{+/-}p53^{-/-}$ mouse (1138), and normal adult mouse brain were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with anti-p53 antiserum (Ab7; Oncogene; 1:5000), followed by donkey antisheep IgG-horseradish peroxidase (Chemicon; 1:2500) diluted in 5% evaporated milk powder in 1% TBST [50 mM Tris-Cl, 0.15 M NaCl (pH 8.0) with 1% Tween]. The signal was detected by enhanced chemiluminescence. The membranes were stripped and incubated with antibodies directed against Ref-1 and β-tubulin to control for protein loading and transfer efficiency.

RT-PCR.[4] Two-step RT-PCR was 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). The reverse transcriptase first-strand cDNA synthesis reactions were carried out using 3 μg of total RNA prepared from adult C57BL/6 mouse cerebellum and from seven tumor samples (tumor nos. 185,199, 241, 448, 530, 574, and 646) according to the manufacturer's directions. Gene-specific oligonucleotides corresponding to sequences within the open reading frame of p53 were synthesized, and PCR amplification of overlapping regions was performed to generate templates for nucleotide sequencing. Sequence analysis of PCR products generated from both the sense and antisense strands of p53 were analyzed from two separate cDNA templates and from multiple PCR 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 complementary to regions covering the entire open reading frame of *p53*. Samples were electrophoresed, detected, and analyzed on PE/ABI model 373, model 37 Stretch, or model 377 DNA sequencers (Perkin-Elmer Applied Biosystems, Inc.). Sequence analysis was performed using Sequencher (Gene Codes Corp., Ann Arbor, MI) software.

Results and Discussion

The Loss of p53 Dramatically Accelerated the Age of Onset and the Incidence of Medulloblastoma in $Ptc^{+/-}$ Mice. Tumors were apparent as early as 4 weeks, and all of the $Ptc^{+/-}p53^{-/-}$ mice developed either brain tumors or died from unknown causes prior to 12 weeks of age (Fig. 1). Within this $Ptc^{+/-}p53^{-/-}$ cohort, 95% of mice were confirmed by gross and histological analysis to have tumors in the posterior fossa. In 5% of the mice, no brain tumor was apparent by gross examination at the time of death. A very low incidence of extracranial soft tissue sarcomas were noted to arise at similar frequencies in all cohorts of $Ptc^{+/-}$ mice. There was no acceleration in the incidence or time of onset of the extracranial tumors (soft tissue sarcomas, basal cell carcinomas, and lymphomas) described in $Ptc^{+/-}$ (7, 14) and $p53^{-/-}$ mice (21). This may reflect the fact that the mice became moribund with medulloblastoma prior to the time when they would develop these other tumors, which usually occur after 5 months of age (21). In contrast, no significant change in the age of onset or the incidence of tumors was observed in $Ptc^{+/-}p53^{+/-}$ mice (Fig. 1). Thus, complete loss of *p53* synergizes with haploinsufficiency of *Ptc* to produce a very high frequency of medulloblastoma in young mice.

The brain tumors in $Ptc^{+/-}p53^{-/-}$ mice arose in the same anatomical location in the posterior fossa, and they exhibited histology similar to the medulloblastomas found in $Ptc^{+/-}$ mice that did not carry *p53* mutations (14). Furthermore, the normal *Ptc* allele was expressed in all tumors examined, as described previously for tumors in $Ptc^{+/-}$ mice (14). *Gli1* mRNA was present at much higher levels in the tumors than in the control tissues (Fig. 2*A*). Transcription of

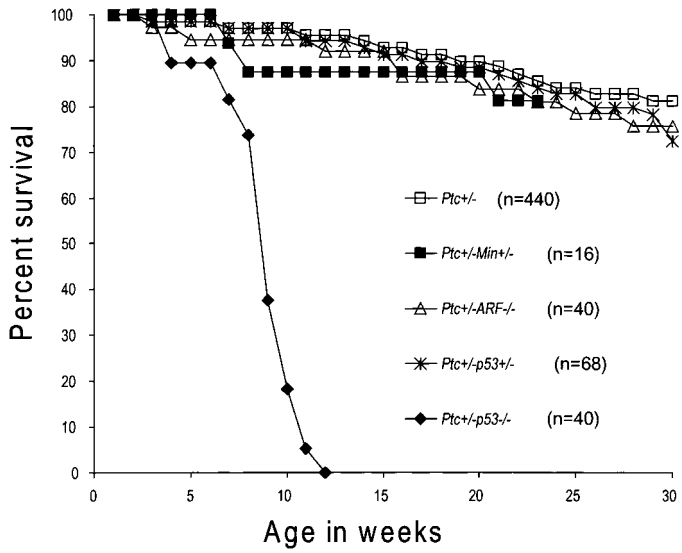

Fig. 1. Medulloblastoma incidence is increased and age of tumor onset is decreased in $Ptc^{+/-}$ mice lacking a normal *p53* gene. Greater than 95% of $Ptc^{+/-}p53^{-/-}$ mice spontaneously developed tumors in the posterior fossa between 10 and 12 weeks of age, compared with a 14% incidence of tumors in $Ptc^{+/-}$ mice by 10 months of age. A small percentage (3%) of the $Ptc^{+/-}p53^{-/-}$ mice died within the first 3–4 weeks of life, with no tumor detected by gross examination of the brain. All $Ptc^{+/-}p53^{-/-}$ mice died by 12 weeks of age. Brains were removed and examined for the presence of tumor. No significant acceleration in tumorigenesis was noted in $Ptc^{+/-}p53^{+/-}$, $Ptc^{+/-}ARF^{-/-}$, or in $Ptc^{+/-}Min^{+/-}$ mice.

[4] The abbreviation used is: RT-PCR, reversed transcription-PCR.

Gli1 is normally repressed by *Ptc*, and in adult, nonproliferating tissues, *Gli1* expression is not readily detected. However, during development, when cells are rapidly proliferating, repression of *Gli1* transcription is abrogated by the interaction of Shh with the Ptc/Smo receptor complex (7). When *Ptc* is mutated or absent, intrinsic signaling by *Smo* is not suppressed, resulting in increased transcription of *Gli1* and other downstream genes. A single copy of *Ptc* was sufficient to maintain repression of *Gli1* in the non-tumor containing $Ptc^{+/-}$ cerebellum, because no increases in *Gli1* levels were detected in control brain tissues compared with the dramatic increases in *Gli1* mRNA seen in the tumors (Fig. 2*A*). However, in medulloblastomas that arise in $Ptc^{+/-}$ mice, there is persistent expression of the wild-type *Ptc* allele, suggesting that derepression of *Gli1* expression does not require complete loss of *Ptc* (14). These data indicate that genes other than *Ptc* may influence *Gli1* expression in the cerebellum. Expression of *p53* mRNA was found to be elevated in all tumors (Fig. 2*B*). This is consistent with prior reports of elevated *p53* expression in populations of rapidly dividing cells during development and tumorigenesis (23, 24).

No Acceleration of Medulloblastoma Formation Was Observed in $Ptc^{+/-}Min^{+/-}$ Mice. Only 1 of 16 of these mice (6%) developed a posterior fossa tumor by 22 weeks of age. This tumor was phenotypically similar to the medulloblastomas found in $Ptc^{+/-}$ mice. These data suggest that genetic lesions in the *PTCH* and *APC* pathways found in subsets of sporadic human medulloblastoma do not act synergistically in mice.

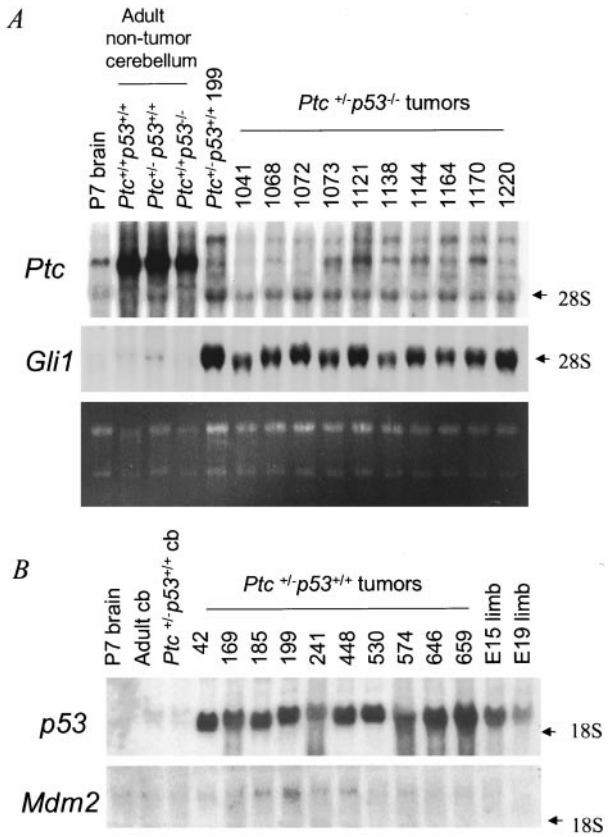

Fig. 2. Analysis of mRNA expression of medulloblastomas arising in $Ptc^{+/-}$ and $Ptc^{+/-}p53^{-/-}$ mice. *A*, persistent expression of *Ptc* and *Gli1* mRNA in tumors arising in $Ptc^{+/-}p53^{-/-}$ mice. Total RNA was prepared from the following tumors and control tissues: postnatal day 7 C57BL/6 mouse brain (P7 brain), C57BL/6 adult cerebellum ($Ptc^{+/+}p53^{+/+}$), non-tumor-bearing adult $Ptc^{+/-}$ cerebellum ($Ptc^{+/-}p53^{+/+}$), non-tumor-bearing adult $p53^{-/-}$ cerebellum ($Ptc^{+/+}p53^{-/-}$), tumor from one $Ptc^{+/-}$ mouse (*199*), and tumors from 10 $Ptc^{+/-}p53^{-/-}$ mice. Three transcripts (5, 8, and 12.5 kb) were detected with the *Ptc* probe. The major 8-kb *Ptc* transcript was decreased in all tumors examined compared with control tissues, whereas the 5- and 12.5-kb transcripts were expressed more robustly in tumors than in control tissues. A single *Gli1* mRNA transcript of 4 kb was detected in all tumors from $Ptc^{+/-}$ as well as from $Ptc^{+/-}p53^{+/+}$ mice at higher levels than those in control tissues. *B*, increased expression of wild-type *p53* mRNA in tumors from $Ptc^{+/-}p53^{+/+}$ mice. Northern analysis of total RNA prepared from medulloblastoma and control brain tissues revealed increased expression of *p53* mRNA and no increase in *mdm2* mRNA in tumor tissues.

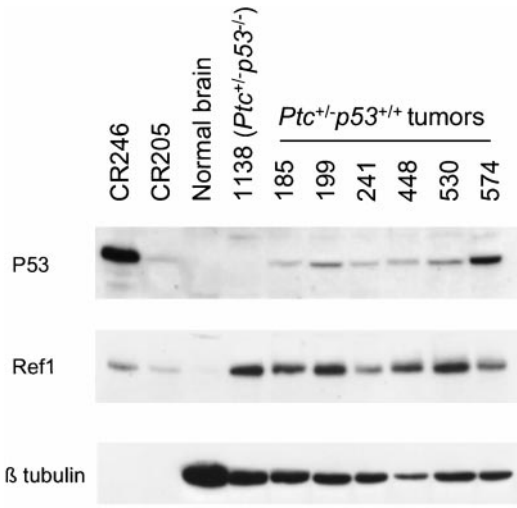

Fig. 3. p53 expression in medulloblastomas arising in $Ptc^{+/-}p53^{+/+}$ mice. Immunoblot analysis of protein extracts from medulloblastomas arising in $Ptc^{+/-}$ mice revealed very low levels of p53 expression in the medulloblastoma tissues compared with a murine B-cell leukemia (*CR246*) that expresses mutated p53 (E251G). In contrast, p53 expression was much lower in B-cell leukemia cells lacking p53 mutations (*CR205*). p53 protein was not detected in control tissues (normal brain and medulloblastoma arising in $Ptc^{+/-}p53^{-/-}$ mice). Sequence analysis of RT-PCR products amplified from these tumors revealed no mutations in p53.

***p53* Loss Is Not Required for Medulloblastoma Formation in $Ptc^{+/-}$ Mice.** The dramatic acceleration of medulloblastoma formation in $Ptc^{+/-}p53^{-/-}$ mice prompted us to investigate the status of *p53* in tumors arising in $Ptc^{+/-}$ mice in which there is no germ-line mutation of *p53*. Interestingly, these tumors contained high levels of *p53* mRNA compared with control tissues. In contrast, there was no consistent difference in *Mdm2* mRNA levels between normal and tumor tissues (Fig. 2*B*). The *Mdm2* gene product acts to repress *p53* activity, and amplification of *Mdm2* inactivates *p53* in a subset of astrocytomas (25, 26). Wild-type p53 protein has a short half-life, and it is not readily detected in populations of nonproliferating cells unless it has been stabilized by mutation (23). Therefore, we performed immunoblotting analysis to look for evidence of *p53* inactivation in tumors from $Ptc^{+/-}$ mice. As shown in Fig. 3, despite the increase in *p53* mRNA, p53 protein was present at significantly lower levels in medulloblastomas from $Ptc^{+/-}$ mice, compared with those observed in a mouse lymphoma with a E254G substitution mutation in *p53* (CR246). However, expression of p53 protein and mRNA were higher in the tumors than in control brain tissue, which contains relatively few proliferating cells (Figs. 2*A* and 3). Nucleotide sequence analysis of *p53* revealed no mutations in any of the seven tumor mRNAs examined. Thus, although germ-line loss of *p53* accelerates tumorigenesis in $Ptc^{+/-}$ mice, mutation of *p53* is not required for medulloblastoma formation. This contrasts with a report of increased medulloblastoma formation in mice carrying homozygous mutations in both the *retinoblastoma* (*Rb*) and *p53* genes. In this mouse model, brain tumors were not detected in mice in which only one of these genes was disrupted (27).

No Accelerated Tumor Formation Was Noted in $Ptc^{+/-}p53^{+/-}$ or in $Ptc^{+/-}ARF^{-/-}$ Mice. The lack of acceleration of medulloblastoma in $Ptc^{+/-}p53^{+/-}$ mice may be attributed to the very limited time window in which the presumed tumor precursor cells are proliferating. Granule cell precursors undergo rapid expansion in the external germinal layer of the cerebellum during the first 2 weeks of postnatal life.

In mice, these cells differentiate and migrate to their mature positions in the internal granular layer by the third week of postnatal development (28). In humans, this process is completed by the ninth postnatal month (29). Thus, $Ptc^{+/-}$ precursor cells have a limited number of cell divisions in which to acquire the additional mutation(s) that contribute to medulloblastoma formation.

Loss of *p53* leads to accumulation of cytogenetic abnormalities (23, 30). Indeed, we observed a much higher incidence of random chromosome loss in tumors from $Ptc^{+/-}p53^{-/-}$ mice compared with those from $Ptc^{+/-}p53^{+/+}$ mice. In $p53^{+/-}$ mice, there may be an insufficient number of cell generations to lose the remaining *p53* allele and to acquire other genetic changes. Additionally, no acceleration in tumorigenesis was noted in $Ptc^{+/-}ARF^{-/-}$ mice. This may be because *ARF* does not increase genomic instability, and therefore, the tumor precursor cells may be less prone to sustain DNA damage than cells deficient in *p53*. It is likely that the genomic instability associated with complete loss of *p53* function accelerates the mutation rate in granule cell precursors. This may synergize with the effects of reduced *Ptc* expression in these mice to increase the incidence of medulloblastoma.

Survivors of pediatric brain tumors have significant morbidity as a direct consequence of the therapy required to eradicate tumor cells from the developing brain of a child. Genetic mutations have been detected only in small subsets of medulloblastoma, and the molecular basis of the majority of these tumors remains to be elucidated. The high frequency and rapid onset of tumors in $Ptc^{+/-}p53^{-/-}$ mice provide a useful model to investigate other molecules that influence the balance between proliferation and cell death in the nervous system.

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