Two Tumor Suppressors, p27\(^{Kip1}\) and Patched-1, Collaborate to Prevent Medulloblastoma

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Abstract

Two cyclin-dependent kinase inhibitors, p18\(^{Ink4c}\) and p27\(^{Kip1}\), are required for proper cerebellar development. Loss of either of these proteins conferred a proliferative advantage to granule neuron progenitors, although inactivation of Kip1 exerted a greater effect. Mice heterozygous for Patched-1 (Ptc1+/−) that are either heterozygous or nullizygous for Kip1 developed medulloblastoma rapidly and with high penetrance. All tumors from Ptc1+/−/Kip1+/− or Ptc1+/−/Kip1+/− mice failed to express the wild-type Ptc1 allele, consistent with its role as a canonical “two-hit” tumor suppressor. In contrast, expression of the wild-type p27\(^{Kip1}\) protein was invariably maintained in medulloblastomas arising in Ptc1+/−/Kip1+/− mice, indicating that Kip1 is haploinsufficient for tumor suppression. Although medulloblastomas occurring in Ptc1+/− mice were histopathologically heterogeneous and contained intermixed regions of both rapidly proliferating and nondividing more differentiated cells, tumors that also lacked Kip1 were uniformly less differentiated, more highly proliferative, and invasive. Molecular analysis showed that the latter medulloblastomas exhibited constitutive activation of the Sonic hedgehog signaling pathway without loss of functional p53. Apart from gains or losses of single chromosomes, with gain of chromosome 6 being the most frequent, no other chromosomal anomalies were identified by spectral karyotyping, and half of the medulloblastomas so examined retained a normal karyotype. In this respect, this mouse medulloblastoma model recapitulates the vast majority of human medulloblastomas that do not sustain TP53 mutations and are not aneuploid. (Mol Cancer Res 2009;7(1):33–40)

Introduction

Normal cerebellar development requires the correct balance between the early proliferation of granule neuron progenitors (GNP) and their subsequent postmitotic differentiation and migration to their final location within the mature organ. In the mouse, GNPs arise in the rhombic lip during embryonic days E9.5 and E11.5 and migrate outward to form the external germinal layer (EGL) of the developing cerebellum (1). At birth (postnatal day 0; P0), the EGL is composed of a single layer of GNPs overlaying Purkinje cells that secrete the mitogen Sonic hedgehog (Shh). GNPs expressing the Shh receptor Patched-1 (Ptc1) are stimulated to undergo cell division, resulting in the rapid expansion of the EGL with maximum proliferation occurring at P5. GNPs then exit the cell cycle, differentiate, and migrate through the Purkinje cell layer to reside as postmitotic neurons within the internal granule layer (IGL). As they migrate, granule neurons extend retrograde axons that synapse with Purkinje neuron dendrites in the vacated EGL, thereby generating the outer molecular layer of the adult cerebellum, which is largely devoid of neuronal cell bodies. The cerebellum is completely formed within the first 3 to 4 weeks of life in the mouse and within 12 to 16 months in humans (1, 2).

Cell cycle progression of GNPs is regulated by the cyclin-dependent kinases, Cdk4 and Cdk6 that, in complexes with their allosteric regulators, cyclins D1 and D2, are required for proper development of the cerebellum (3). Opposing the action of cyclin-dependent kinases are two Cdk inhibitors, p18\(^{Ink4c}\) and p27\(^{Kip1}\), that enforce cell cycle exit and help to maintain the neuronal postmitotic state (4). Whereas p18\(^{Ink4c}\) is a specific inhibitor of Cdk4 and Cdk6, p27\(^{Kip1}\) preferentially targets cyclin E-dependent and cyclin A-dependent Cdk2, which acts later in G1 phase to help guide the G1 to S phase transition (5). \(^{Ink4c}\) mRNA is transiently expressed just as proliferating GNPs enter mitosis, and \(^{Kip1}\) mRNA is completely extinguished by P14. Nevertheless, p18\(^{Ink4c}\) and p27\(^{Kip1}\) expression is restricted to postmitotic neurons in the outer EGL and is maintained in granule neurons within the IGL throughout the life of the animal (6-8).

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Received 8/5/08; revised 9/25/08; accepted 9/30/08.

Grant support: NCI grant P01-CA-096832 (M.F. Roussel and C.J. Sherr) and an NCI Cancer Center Core grant CA-21765 to St. Jude Children’s Research Hospital, La Fondation pour la Recherche Medicale and a Gephardt Endowed Fellowship (O. Ayrault), and the American Lebanese-Syrian Associated Charities of St. Jude Children’s Research Hospital. C.J. Sherr is an Investigator of the Howard Hughes Medical Institute.

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doi:10.1158/1541-7786.MCR-08-0369
Medulloblastomas, the most common malignant pediatric brain tumors, are thought to arise, at least in part, from GNPs that fail to exit the cell cycle, migrate, and properly differentiate (9). Genetic anomalies in the SHH signaling pathway including mutations in PATCHED and SUFU (a negative regulator of the SHH-stimulated transcription factor GLI1) have been detected in ~10% of human medulloblastomas (1). Methylation of the CDKN2C/INK4C promoter was shown in ~10% of medulloblastomas from 43 patients admitted to St. Jude Children’s Research Hospital, and a somewhat higher percentage (22% of 73 medulloblastomas) expressed no detectable p18<sup>INK4c</sup> protein (6). Although mice heterozygous for Ptc1 develop medulloblastoma at a relatively low frequency (10-12), the time of onset and incidence of tumor development increases dramatically in an Ink4c nullizygous or heterozygous background (6). Low levels of p27<sup>Kip1</sup> have been associated with poor outcome in many forms of human cancer, including brain tumors such as astrocytoma and glioblastoma (13). However, the role of Kip1 in medulloblastoma has not previously been evaluated. We now provide evidence that genetic disruption of either one or two alleles of Kip1 in Ptc1<sup>+/-</sup> mice greatly increases the incidence of medulloblastomas. All such medulloblastomas lose expression of the wild-type Ptc1 allele but retain functional p53, thereby providing a highly penetrant mouse medulloblastoma model that closely mimics a subset of the human disease (1).

Results
Expression of Cell Cycle Regulatory Proteins during Cerebellar Development

To compare the expression of key cell cycle regulators with that of p27<sup>Kip1</sup> and p18<sup>Ink4c</sup> during postnatal cerebellar development, we analyzed their protein levels in normal cerebellar tissues between P1 and P30 (Fig. 1A). Cyclin D1, cyclin D2, Cdk4, and Cdk2 protein levels peaked at P5, corresponding to the time of maximum GNP proliferation, and slowly decreased thereafter. Cdk2 levels were undetectable by P17, whereas cyclin D1, cyclin D2, and Cdk4 levels were maintained at low levels until P30. Cdk6 levels remained relatively constant between P1 and P20. P27<sup>Kip1</sup> was detected from P1 through P30; its level was relatively constant between P1 and P7 but increased at P10, as more cells exited the cell division cycle (6) and remained elevated thereafter. In contrast, p18<sup>Ink4c</sup> was barely detectable at P1. Its expression reached a peak at P10 after which it progressively declined and was no longer detectable by P20. Two other Cip/Kip proteins, p21<sup>Cip1</sup> and p57<sup>Kip2</sup>, were not expressed at significant levels (data not shown), consistent with earlier reports (7, 8). Together, these data suggest that p18<sup>Ink4c</sup> and p27<sup>Kip1</sup> coordinate cell cycle exit, whereas the continuing expression of p27<sup>Kip1</sup> presumably helps to maintain neurons in their postmitotic state.

We next evaluated the effect of Kip1 inactivation on the proliferation of cultured GNPs and compared the results with those obtained with GNPs lacking Ink4c, the loss of which was previously revealed to endow cells with an enhanced proliferative capacity (6). GNPs purified from the cerebella of wild-type, Ink4c-null, and Kip1-null mice were cultured in the absence of Shh for 1 day (Fig. 1B, a) or for 3 days (Fig. 1B, b), or in the complete absence of Shh for 1 day followed by a 2-day restimulation with Shh (Fig. 1B, c). Cultures were exposed to bromodeoxyuridine (BrdUrd) for 24 hours either throughout the duration of the 24-hour experiment (a) or during the final 24-hour intervals (b and c) before fixing the cells and determining the percentage that had incorporated the precursor. When deprived of Shh for 1 day, GNPs from Kip1-null, Ink4c-null, and wild-type mice displayed comparable levels of DNA synthesis with at least 30% of the cells incorporating BrdUrd during the labeling period (Fig. 1B, a). However, after 2 days of Shh starvation, virtually no GNPs from wild-type mice incorporated BrdUrd in the ensuing 24 hours; in contrast, a significant fraction of GNPs explanted from Ink4c-null and,
to a greater extent, Kip1-null mice could still incorporate BrdUrd (Fig. 1B, b). When GNPs were grown in the absence of Shh for 1 day followed by a 2-day restimulation with Shh, an even more significant proliferative advantage was documented for cells lacking either Ink4c or Kip1 (Fig. 1B, c). These data are consistent with previous reports demonstrating enhanced in vitro proliferation of GNPs from Kip1-null mice compared with those from wild-type animals (7, 8). Therefore, loss of Kip1 can delay cell cycle exit and maintain GNP proliferation even in the absence of Shh.

FIGURE 2. Kip1 loss increases the incidence of medulloblastomas in Ptc1 heterozygous mice. A. Illustration of survival curves for Ptc1+/− mice retaining two wild-type Kip1 alleles (+/+, black line; n = 70), or lacking one (−/+, gray dotted line; n = 144) or two (−/−, gray line; n = 30) Kip1 alleles. Mice showing signs of disease without any obvious brain tumors developed hydrocephaly, hemangiosarcomas, intestinal tumors, rhabdomyosarcomas, or lymphomas. Not all mice succumbed to tumors. B. Survival curves of Ptc1+/− mice of different Kip1 genotypes with confirmed medulloblastoma development. Line designations are the same as for A: 30 mice in each cohort for Kip1+/+, 86 for Kip1+−, and 20 for Kip1−− genotypes. C. Detection by immunoblotting of the p27Kip1 protein in GNPs purified from a P7 wild-type cerebellum and in purified tumor cells from medulloblastomas arising in Ptc1+/−;Kip1+/+ and Ptc1+/−;Kip1−− mice (top). Actin was used as a loading control. D. Expression of p27Kip1 determined by immunohistochemistry (brown) in cerebellar sections from Ptc1+/− mice of different Kip1 genotypes. All sections were counterstained with hematoxylin (blue). a, b, and c, sections taken from normal cerebellar tissues from Ptc1+/−;Kip1+/+, Ptc1+/−;Kip1−−, and Ptc1+/−;Kip1−− mice, respectively. d, e, and f, p27Kip1 expression in medulloblastomas arising in Kip1+/+, Kip1−−, and Kip1+/+ mice, respectively. Magnification, ×10 (b) and ×40 (a, c-f). The unstained region represents the external molecular layer of the adult cerebellum which is practically devoid of cell bodies. a and c, cells within the p27Kip1-positive IGL.

Increased Medulloblastoma Incidence in Ptc1+/−; Kip1+−− and Ptc1+/−;Kip1−− Mice

To assess whether loss of Kip1 might accelerate medulloblastoma formation in tumor-prone Ptc1 heterozygous mice, Kip1-null mice were interbred with Ptc1+/− animals to derive Ptc1+/−;Kip1−− offspring and later-generation Ptc1+/−;Kip1−− cohorts that were prospectively followed for tumor formation. The overall survival of Ptc1+/− mice of the three possible Kip1 genotypes revealed detrimental effects of Kip1 loss of function on life span, with biallelic Kip1 deletion exerting the most drastic effect (Fig. 2A). Although the majority of morbidity in these cohorts was due to medulloblastoma development, a fraction of the mice in each group developed other tumor types (hemangiosarcomas, intestinal tumors, rhabdomyosarcomas, and lymphomas) as well as hydrocephalus unrelated to tumor development (Table 1). The latter abnormalities collectively accounted for medulloblastoma-independent deaths as the animals were further aged (10, 12, 14).

Ptc1+/− mice were previously reported to develop medulloblastoma with a relatively low incidence (12-22%) and a mean time of tumor occurrence of 5 to 6 months (10, 12),
A greater incidence of medulloblastomas (42%) in Ptc1+/−;Kip1+/− mice from our colony (Fig. 2A) was most likely due to strain-specific genetic differences (14, 15). Loss of one or two alleles of Kip1 further increased the overall incidence of medulloblastoma in Ptc1+/− mice to ~60% and ~67%, respectively (Table 1). Although the inactivation of one Kip1 allele did not increase the incidence of other tumor types observed in Ptc1+/− mice (12-14% overall), 30% of Ptc1+/−; Kip1−/− mice developed tumors other than medulloblastomas (Table 1). No grossly overt pituitary tumors, a hallmark of Kip1-null mice (16-18), were observed in Ptc1+/−;Kip1−/− mice with medulloblastoma, reflecting the more rapid deaths of the compound-deficient animals. Indeed, when we limited our focus to those mice that developed medulloblastoma only (Fig. 2B), their median times of survival were shorter than those of their overall cohorts, documenting that medulloblastomas arose earlier than other tumor types (compare Fig. 2B to Fig. 2A). Among the animals with medulloblastoma only, the Ptc1+/−;Kip1−/− mice died somewhat more rapidly (median survival, 114 days) than the Ptc1+/−;Kip1+/− (127 days) or Ptc1+/−;Kip1+/+ mice (158 days). Differences in survival between Ptc1+/−;Kip1+/− versus Ptc1+/−;Kip1−/− mice were not significant (P = 0.13), but inactivation of both Kip1 alleles significantly accelerated medulloblastoma formation (Ptc1+/−;Kip1+/− versus Ptc1+/−;Kip1+/+; P = 0.016; Ptc1+/−;Kip1−/− versus Ptc1+/−;Kip1+/−; P = 0.034).

Therefore, Kip1 collaborates with Ptc1 to prevent the development of medulloblastoma and other tumor types.

We confirmed by immunoblotting that p27Kip1 expression was maintained in tumor cells purified from medulloblastomas resected from six of six Ptc1+/−;Kip1+/− mice (Fig. 2C, lanes 4-9). The levels of p27Kip1 expressed in these tumors were approximately half of those detected in medulloblastomas arising in Ptc1+/−;Kip1+/− mice (Fig. 2C, lanes 2 and 3), consistent with the concept that Kip1 is haploinsufficient for tumor suppression (19). Immunohistochemical staining of sections of normal cerebellar tissue from 1-month-old Ptc1+/− animals confirmed that p27Kip1 was localized within the nuclei of mature postmitotic neurons residing in the IGL (Fig. 2D, a). As expected, no staining was detected in mature granule neurons in the IGL of Ptc1+/−;Kip1−/− mice (Fig. 2D, b), whereas p27Kip1 was present in their Kip1+/− counterparts (Fig. 2D, c). P27Kip1 was also found in the nuclei of medulloblastomas that arose spontaneously in Ptc1+/−; Kip1+/− mice (Fig. 2D, d), whereas as expected, it was not detected in medulloblastomas from Ptc1+/−;Kip1+/− mice (Fig. 2D, e). Notably, p27Kip1 expression was retained in the nuclei of four of four Ptc1+/−;Kip1+/− medulloblastomas (Fig. 2D, f shows representative data). Because nucleotide sequencing of PCR-amplified Kip1 cDNAs from the latter medulloblastomas revealed no mutations, the collective findings confirmed that Kip1 is haploinsufficient for medulloblastoma suppression, as it is in other tumor types (19).

**Table 1. The Incidence of Medulloblastomas and Other Tumors Arising in These Cohorts**

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Medulloblastoma</th>
<th>Other tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ptc1+/−;Kip1+/+</td>
<td>30/70 (42.8%)</td>
<td>10/70 (14.3%)</td>
</tr>
<tr>
<td>Ptc1+/−;Kip1−/−</td>
<td>86/144 (59.7%)</td>
<td>17/144 (11.8%)</td>
</tr>
<tr>
<td>Ptc1+/−;Kip1+/−</td>
<td>20/30 (66.7%)</td>
<td>9/30 (30%)</td>
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**Molecular Characterization of Medulloblastomas from Ptc1+/−;Kip1−/− Mice**

Medulloblastomas from Ptc1+/−;Kip1−/− mice lose the expression of the wild-type Ptc1 allele (22). We therefore assessed expression of Ptc1 in purified tumor cells from medulloblastomas occurring in Ptc1+/−;Kip1−/− and Ptc1+/−;Kip1+/− mice. By quantitative real-time PCR, we found that Ptc1 mRNA could not be detected in all such tumors (data not
shown), implying that the Shh signaling pathway was constitutively activated. To confirm this, tumor cells purified from the cerebella of Ptc1+/−;Kip1−/− animals were cultured in the presence of cyclopamine, a specific inhibitor of Shh signaling (23). These cells completely ceased proliferating after 3 days of treatment (data not shown), underscoring their dependence on the Shh pathway.

The transcription factor, Math1/Atoh1, is expressed in proliferating GNPs, but its expression is extinguished once GNPs in the EGL exit the cell cycle and begin to differentiate and start migrating toward the IGL (24). Down-regulation of Math1/Atoh1 in response to signaling by bone morphogenetic proteins (BMP) or transcriptional repression mediated by Hic1 induces the neuronal differentiation of GNPs and of Ptc1−/− medulloblastoma tumor cells derived from them (25, 26). Suggesting that Math1/Atoh1 expression is required to prevent the differentiation of GNPs. Math1/Atoh1 expression is also a hallmark of human medulloblastomas harboring a SHH signaling pathway expression signature (25). Immunoblotting of protein lysates revealed that Math1/Atoh1 was expressed in Ptc1−/− tumor cells purified from mice of all three Kip1 genotypes (Fig. 4A), confirming that the Shh signaling pathway was constitutively activated in these medulloblastomas. The levels of Math1/Atoh1 were elevated in medulloblastomas from Ptc1+/−;Kip1−/− mice, consistent with the enhanced proliferative capacity and more aggressive nature of these tumors.

BMPs inhibit the proliferation of tumor cells from Ptc1+/−;Ink4c−/− mice and induce their differentiation by rapid posttranscriptional down-regulation of Math1/Atoh1 (25). Despite their relatively elevated levels of Math1/Atoh1 expression, tumor cells from Ptc1+/−;Kip1−/− mice remained sensitive to BMP treatment (S phase: 18% without treatment, 5% after BMP treatment; n = 2) suggesting that BMP-dependent cell cycle arrest and differentiation is independent of both Kip1 and Ink4c.

P21<sup>Cip1</sup> and p27<sup>Kip1</sup> are necessary for the assembly and stability of D-type cyclins into complexes with Cdk4 and Cdk6 (5, 27). In turn, mouse embryo fibroblasts, hepatocytes, and thymic lymphoid cells lacking p27<sup>Kip1</sup> and p21<sup>Cip1</sup> each exhibited reduced levels of D-type cyclins (27). Although the levels of cyclin D1 in purified tumor cells from medulloblastomas from Ptc1+/−;Kip1+/+ and Ptc1+/−;Kip1+/− mice differed between individual tumors, they were higher or equivalent to those observed in P7 wild-type GNPs (Fig. 4A). In the same tumors, cyclin D2 levels were somewhat less variable and were similar to those observed in P7 wild-type GNPs. However, reduced overall levels of cyclins D1 and D2 were observed in purified tumor cells from medulloblastomas arising in Ptc1+/−;Kip1−/− animals, consistent with previous observations that D-type cyclins are more rapidly degraded when p27<sup>Kip1</sup> is absent (27, 28). The fact that medulloblastomas lacking Kip1 arise more rapidly implies that, in the absence of p27<sup>Kip1</sup>, unfettered “downstream” Cdk5 drive the cell cycle even when cyclin D levels are reduced (28).

To assess their p53 status, tumor cells were purified from medulloblastomas of Ptc1+/−;Kip1−/− mice, cultured for 1 day, and then subjected to 15 Gy of ionizing radiation. P53 and p21<sup>Cip1</sup> protein levels, detected by immunoblotting 2 and 4 hours after irradiation, accumulated in response to ionizing radiation (Fig. 4B), confirming that p53 and a canonical p53-responsive gene product could be activated by DNA damage in these tumors. Spectral karyotyping analysis of medulloblastomas from Ptc1+/−;Kip1+/− and Ptc1+/−;Kip1−/− mice revealed that they had not sustained chromosomal translocations but exhibited recurrent gains of chromosomes 6 and 8 (Table 2). The most frequent event was trisomy 5 in 50% of cases (5 of 10), as previously reported in several mouse medulloblastoma models (29-31). The overall lack of aneuploidy and presence of a normal karyotype in half of the analyzed tumors is consistent with the fact that retention of p53 counters the widespread genomic instability generally associated with its loss of function.
cycle and migrate into the IGL. P27Kip1, which accumulated to cycle exit in GNPs. During the early postnatal period, the EGL
their overlapping but differential functions in regulating cell
postnatal development of the cerebellum are consistent with
B.

Accumulation of p53 and p21Cip1 and p4hafter
Ptc1+/-;Kip1+/- mice. Zn2+-treated MT-Arf cells

Discussion

Several lines of evidence suggest that Ink4 and Cip/Kip1 proteins collaborate to induce G1 phase arrest (5). In cycling
cells, Ink4 gene expression is limited, and most Cip/Kip proteins are assembled into complexes with cyclin D-Cdks. Induction of p18^INK4C and its binding to Cdk4 and Cdk6 not only disrupts cyclin D-Cdk complexes and accelerates cyclin D turnover but mobilizes p27^KIP1 into complexes with cyclin E/A-
Cdk2, thereby inhibiting all G1 cyclin-dependent kinase activity and inducing exit from the cell cycle. These interrelationships are consistent with observations that inactivation of either Ink4c
and Kip1 in mice leads to similar phenotypes, including organomegaly and predisposition to certain tumor types (16-18, 32, 33). Moreover, both Ink4c and Kip1 can act as haploinsufficient tumor suppressors (6, 19, 34). However, the fact that the simultaneous disruption of both genes widens the tumor spectrum and further accelerates tumor formation argues that the two Cdk inhibitors not only functionally collaborate but act synergistically (35).

The patterns of expression of p27^KIP1 and p18^INK4C during postnatal development of the cerebellum are consistent with their overlapping but differential functions in regulating cell cycle exit in GNPs. During the early postnatal period, the EGL rapidly expands, and maximal overall GNP proliferative rates are manifested at P5 to P7, after which the cells exit the division cycle and migrate into the IGL. P27^KIP1, which accumulated to maximal levels by P10, is detected exclusively in postmitotic GNPs, whether present in the inner part of the EGL during its expansion or later in the IGL; its expression in mature granule neurons then persists throughout the lifetime of the mice (6, 7). However, Ink4c mRNA expression in GNPs is transient, peaking at P7 but becoming undetectable by P14 (6). Because the p18^INK4C protein is relatively stable (half-life, ~12 hours), its expression lags behind that of its mRNA. Peak levels of p18^INK4C were detected at P10 and declined slowly thereafter, vanishing by P17 to P20. Thus, p18^INK4C expression persists even in the absence of new synthesis throughout the period when most postmitotic cells migrate into the IGL.

Constitutive activation of the Shh signaling pathway induced medulloblastomas whose formation was accelerated when either one or two alleles of Kip1 were co-inactivated. Although co-inactivation of Ptc1 and Kip1 alleles in the mouse germ line predisposed to several tumor types, medulloblastomas were by far the most frequent in the younger animals. Medulloblastomas from Ptc1+/-;Kip1+/- mice were less differentiated and more invasive than medulloblastomas from their Ptc1+/-;Kip1+/+ counterparts, correlating with the greater incidence and more rapid course of medulloblastomas in animals lacking p27^KIP1 function. Medulloblastomas lacking Kip1 expressed high levels of Math1/Atoh1, a basic helix-loop-helix transcription factor expressed exclusively in that subset of GNPs that are actively proliferating. This underscores the idea that p27^KIP1 helps to trigger the exit of GNPs from the cell cycle and enforces their differentiation. In tumors arising in Ptc1+/-;Kip1+/- mice, the wild-type p27^KIP1 protein was persistently expressed in purified tumor cells, consistent with previously documented Kip1 haploinsufficiency in other tumor types (19). In contrast, the wild-type Ptc1 allele was not expressed in tumor cells and functioned as a canonical tumor suppressor gene.

Although many mouse medulloblastoma models rely on the loss of functional p53 for high tumor penetrance, medulloblastomas from Ptc1+/-;Kip1+/- or Ptc1+/-;Kip1+/- mice, like those arising in Ptc1+/-;Ink4c+/- or Ptc1+/-;Ink4c+/- mice, retained functional wild-type p53 and were not aneuploid, similar to the vast majority of human medulloblastomas (1). This raises the possibility that inactivation of either Kip1 or Ink4c might limit the p53 response to oncogenic signaling through the altered Patched pathway, thereby bypassing any
need to mutate p53 in this setting. Indeed, Carneiro and coworkers suggested that the absence of p27kip1 might curb the p19ARF-induced p53 response during pituitary tumorigenesis (36); however, the inactivation of ARF does not accelerate medulloblastoma formation in Ptc1+/− mice (12), effectively ruling out such a mechanism. Neurons lacking Ptc1 exhibit an increased sensitivity to DNA-damaging agents (37), so the retention of a functional p53 checkpoint in this subset of tumor cells was done as described (30).

Histopathology, Immunohistochemistry, and Spectral Karyotyping

Histopathology and immunohistochemistry were done as previously described (30) using the following antibodies: anti-NeuN (clone A60, 1:50, Chemicon), anti-Ki67 (1:1,000; Vector Laboratories), and anti-p27kip1 (1:100, C19, Santa Cruz Biotechnology). Spectral karyotyping was done as previously described (30).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Materials and Methods

Animal Husbandry

Kip1-null male mice (18) were bred to Ptc1+/− female mice (10) to obtain Ptc1+/−;Kip1+/− F1 animals. By intercrossing these double heterozygotes, we obtained Ptc1+/−;Kip1+/− females and Ptc1+/−;Kip1+/− males which were bred to one another to generate other genotypes. Mice were observed twice weekly for a period of 10 months. All mice were maintained on a mixed C57BL/6 × 129Sv background. The generation of Ptc1+/−;Ink4c−/− mice was previously described (6). Mice were housed in an American Association of Laboratory Animal Care–accredited facility and maintained in accordance with NIH guidelines. The Animal Care and Use Committee at St. Jude Children’s Research Hospital approved all procedures. Survival curves and median survival were plotted and calculated using GraphPad Prism 4.

Purification and Culture of Primary GNPs and GNP-Like Tumor Cells

Purification of GNPs from the cerebella of mice with different genotypes and of GNP-like tumor cells from medulloblastomas were done as described (6, 25). Proliferation was determined by BrdUrd incorporation assay done with 6 × 10^5 GNPs per milliliter plated in poly-D lysine and Matrigel precoated eight-well Lab-Tek glass dishes (6). We counted >500 cells per well and recorded the number of BrdUrd-positive cells detected by fluorescence within the total population enumerated by counterstaining with 4′,6-diamidino-2-phenylindole (Sigma). Each experiment was repeated at least thrice. Purified tumor cells were grown in the absence of Shh and treated with cycloamine or BMP, as previously described (25). Zn^2+/-inducible MT-Arf cells were used as positive controls for p53 and p21cip1 induction as previously described (39).

Protein Analysis by Immunoblotting and Quantitative Real-time PCR

Proteins extracted from cells were electrophoretically separated on polyacrylamide denaturing gels, transferred onto nitrocellulose membranes, and immunoblotted with the design-


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