N-\textit{myc} Is an Essential Downstream Effector of Shh Signaling during both Normal and Neoplastic Cerebellar Growth

Beryl A. Hatton, Paul S. Knoepfler, Anna Marie Kenney, David H. Rowitch, Ignacio Moreno de Alborán, James M. Olson, and Robert N. Eisenman

Abstract

We examined the genetic requirements for the Myc family of oncogenes in normal Sonic hedgehog (Shh)–mediated cerebellar granule neuronal precursor (GNP) expansion and in Shh pathway–induced medulloblastoma formation. In GNP-enriched cultures derived from N-\textit{myc\\textsuperscript{F/F}} and c-\textit{myc\\textsuperscript{F/F}} mice, disruption of N-\textit{myc}, but not c-\textit{myc}, inhibited the proliferative response to Shh. Conditional deletion of c-\textit{myc} revealed that, although it is necessary for the general regulation of brain growth, it is less important for cerebellar development and GNP expansion than N-\textit{myc}. In \textit{vivo} analysis of compound mutants carrying the conditional N-\textit{myc} null and the activated \textit{Smoothened} (ND2:SmoA1) alleles showed, that although granule cells expressing the ND2:SmoA1 transgene are present in the N-\textit{myc} null cerebellum, no hyperproliferation or tumor formation was detected. Taken together, these findings provide \textit{vivo} evidence that N-\textit{myc} acts downstream of Shh/Smo signaling during GNP proliferation and that N-\textit{myc} is required for medulloblastoma genesis even in the presence of constitutively active signaling from the Shh pathway.

Introduction

Medulloblastoma is the most common childhood malignancy of the central nervous system and, despite aggressive multimodal therapy with surgery, radiation, and chemotherapy, 5-year survival rates have only approached recently >60% (1). Medulloblastomas are thought to derive from granule precursor cells of the cerebellum and comprise several histologic and prognostic subgroups. The desmoplastic subtype is distinguished from “classic” medulloblastoma by the presence of relatively acellular nodules within the dense sheets of malignant cells (2).

The mitogenic activity of the Sonic hedgehog (Shh) pathway is well characterized in the development of the cerebellum, where Shh drives the proliferation of granule neuronal precursors (GNP) in the external granular layer (EGL; refs. 3, 4). GPNs originate from the rhombic lip during embryonic development and then migrate out to cover the outer surface of the developing cerebellum during both late embryogenesis and the postnatal period, where they undergo significant expansion to form the EGL (5). Shh is secreted from Purkinje cells and binds to the Patched (PTCH) receptor on the GPNs, which relieves inhibition of the G-coupled transmembrane receptor Smoothened (Smo). Smo then activates the Gli family of zinc finger transcription factors (6–8) and the proto-oncogene N-\textit{myc} (9), as well as expression of \textit{cyclin D} and \textit{cyclin E} (10), which together are thought to promote the proliferation of GPNs in the EGL (11).

Numerous studies have shown that deregulated Shh signaling is critical to the genesis and survival of both classic and desmoplastic subtypes of sporadic medulloblastoma (12–16). Genetic alterations associated with medulloblastoma include activating mutations in \textit{Smo} and inactivating mutations in negative regulators of Shh signaling, such as \textit{Ptc1} and \textit{suppressor of fused} (\textit{SUFU}). Thus, Shh signaling regulates normal cerebellar development, and deregulation of this signaling plays a critical role in the formation of medulloblastomas.

In addition to mutations in the \textit{Ptc1}, \textit{SUFU}, and \textit{Smo} genes, amplifications of the proto-oncogenes N-\textit{myc} and c-\textit{myc} have also been observed in 5% to 8% of medulloblastoma cases (17). The Myc family of basic helix-loop-helix leucine zipper proteins act as transcriptional regulators that direct cell growth and proliferation, and Myc family members play a key role in promoting the progression of the cell cycle in proliferating cells (for reviews, see refs. 18, 19). \textit{In vitro} studies with cultures enriched for GPNs have shown that treatment with soluble Shh protein up-regulates N-\textit{myc} expression and that N-\textit{myc} overexpression can promote GNP proliferation even in the absence of Shh treatment (9). Overexpression of c-\textit{myc} in cultures of nestin-positive neural progenitors promoted their proliferation (20), but treatment of GNP-enriched cultures with Shh did not induce c-\textit{myc} expression (9), suggesting that c-\textit{myc} may not be transcriptionally regulated by Shh. Furthermore, expression of dominant-negative mutants of myc block the proliferative response induced by Shh (9, 21), and conditional knockout of N-\textit{myc} within the brain severely disrupts the expansion of GPNs within the cerebellum (11), linking N-\textit{myc} to normal granule cell development.

We reported previously a transgenic mouse model, in which the expression of a constitutively activated form of \textit{Smoothened} (SmoA1) was driven in cerebellar GPNs through the use of the 1-kb \textit{neuroD2} promoter (ND2:SmoA1; ref. 16), whose expression begins during embryonic development. The SmoA1 mutation was originally identified in cases of sporadic basal cell carcinoma, and the mutated Smo protein was shown to transform rat embryonic fibroblasts in culture (22). Mutations that activate \textit{Smo} have also been associated with sporadic brain tumors (23–25). The ND2:SmoA1 mice exhibit a high penetrance (48%) of cerebellar...
tumors that histologically resemble human medulloblastoma with a median tumor onset of 6 months (16).

Whereas the role of Shh in driving GNPs expansion within the developing cerebellum and the link between deregulated Shh signaling and medulloblastoma formation have been clearly established, the requirement for Myc family function has not been as well defined in Shh-induced tumorigenesis. In particular, previous studies have not examined the role of N-myc by genetic loss-of-function analysis in the context of medulloblastoma tumorigenesis. Furthermore, potential roles for the other major myc family member, c-myc, in GNP expansion are unknown. To address these important questions, we ablated N-myc or c-myc using Cre/lox conditional targeting both in vitro with cultured GNPs and in vivo with conditional knockout mice.

Materials and Methods

Cerebellar GNP cultures. GNPs were isolated from wild-type (WT) N-myc<sup>Fl/Fl</sup> and c-myc<sup>Fl/Fl</sup> mice at postnatal day 5 (P5) and cultured overnight in the presence of Shh (3 µg/ml) and serum and then with Shh and without serum for 3 to 5 days as described previously (11, 26). For infection, Shh-conditioned medium was removed and cells were incubated with retroviral supernatants for 2 hours. Viral supernatants were then removed and Shh-conditioned medium was replaced. GNP cultures were infected on three sequential days leading to an infection rate of >90% as verified by green fluorescent protein (GFP) status. The biologically active, modified GFP was labeled with a monoclonal nestin antibody and 3,3′-diaminobenzidine reagent. For analysis of ND2:SmoA1 expression, sections were processed with standard immunohistochemical methods. A monoclonal anti-PentaHis antibody (Molecular Probes, Eugene, OR) was used with a FITC-conjugated anti-mouse IgG antibody (Jackson Immunoresearch Laboratories) and a DAPI costain was used to label cell nuclei.

Results and Discussion

N-myc is required for the proliferative response of cultured GNPs to Shh. GNPs were isolated from N-myc<sup>Fl/Fl</sup> and WT control mice at the height of their postnatal expansion (P5), cultured as described previously (26), and infected with retroviruses encoding bicistronic Cre recombinase and GFP. A retrovirus expressing GFP alone was used as a control. GNP cultures were harvested and concentrated to 30 million GNP/ml and serum and then with Shh and without serum for 3 to 5 days as described previously (11, 26). For infection, Shh-conditioned medium was removed and cells were incubated with retroviral supernatants for 2 hours. Viral supernatants were then removed and Shh-conditioned medium was replaced. GNP cultures were infected on three sequential days leading to an infection rate of >90% as verified by green fluorescent protein (GFP) status. The biologically active, modified GFP was labeled with a monoclonal nestin antibody and 3,3′-diaminobenzidine reagent. For analysis of ND2:SmoA1 expression, sections were processed with standard immunohistochemical methods. A monoclonal anti-PentaHis antibody (Molecular Probes, Eugene, OR) was used with a FITC-conjugated anti-mouse IgG antibody (Jackson Immunoresearch Laboratories) and a DAPI costain was used to label cell nuclei.

Table 1. Histologic analysis of combined conditional N-myc knockout × ND2:SmoA1 mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Time point, mo (n)</th>
<th>Tumor formation</th>
<th>Hyperplasia</th>
<th>Persistent EGL</th>
<th>Diminished cerebella</th>
<th>Reduced foliation</th>
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<td>WT N-myc, SmoA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3 (20)</td>
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<td>11</td>
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<td>19</td>
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<tr>
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experiment. At 5 days after infection, GFP-Cre-infected cells were verified to be N-myc deficient by immunofluorescent staining (Fig. 1C). We have found previously that such N-myc null GNPs display no change in apoptosis or in specification of granule cell identity as determined by cleaved caspase-3 and Zic1 immunofluorescence, respectively (30). At 3 and 5 days following infection, control and null GNPs were pulsed for 2 hours with BrdUrd to label G1 and S phase cells and then fixed and stained to examine BrdUrd incorporation. In both WT and GFP-infected control cultures by day 5, ~80% of cells incorporated BrdUrd, indicating a highly proliferative population. In contrast, only 10% of the N-myc-deficient cells incorporated BrdUrd, an 8-fold reduction (Fig. 1A and B). A similar level of reduced proliferation is observed in knockout GNPs derived from N-myc<sup>F5/Fl</sup> nestin-Cre P5 pups.8

Figure 1. Acute knockout of N-myc strongly blocks progenitor proliferation in response to Shh. A, GNP cultures isolated from P5 N-myc<sup>F5/Fl</sup> mice and WT control mice were infected with retroviruses encoding GFP-Cre recombinase or GFP alone as a control. GNPs were cultured in the presence of Shh and pulsed with BrdUrd 2 hours before fixation in 4% paraformaldehyde, stained with anti-BrdUrd (red) and costained with DAPI to label cell nuclei (blue), and examined using fluorescent microscopy. B, quantification of BrdUrd incorporation in GNPs isolated from c-myc<sup>F5/Fl</sup>, N-myc<sup>F5/Fl</sup>, and WT mice at 3 and 5 days after infection with GFP-Cre or the GFP control. Bars, SE. C, control GFP and GFP-Cre-infected GNP cultures were stained with fluorescent-tagged anti-N-myc (red) and DAPI (blue) to verify N-myc deficiency induced by the Cre recombinase.

Figure 2. Loss of c-myc does not disrupt cerebellar development in vivo but retards overall brain growth. A, c-myc<sup>F5/Fl</sup> nestin-Cre mice were crossed to c-myc<sup>WT/WT</sup> mice to generate mice null for c-myc. The conditional N-myc knockout mice were generated as described previously (11). Whole brains from 2- to 3-month-old mice are shown with their cerebella outlined. Note that, although the overall size of the c-myc null brain is moderately decreased compared with the control brains lacking nestin-Cre expression, the development of the c-myc null cerebellum was not specifically impaired in contrast to the conditional N-myc knockout mouse brain. B, mice lacking c-myc displayed an 18% reduction in total brain mass compared with WT, WT Cre-positive, and c-myc<sup>F5/Fl</sup> Cre-negative controls. The data on N-myc null brain mass were reported previously (11). Bars, SE. C, sagittal sections of mouse cerebella from control, c-myc null, and N-myc null mice at 2 to 3 months of age were stained with H&E after fixation in 4% paraformaldehyde.

* P.S. Knoepfler and Robert N. Eisenman, unpublished data.
The markedly reduced levels of proliferation in N-myc null cells were similar to those seen in GNPs cultured in the absence of Shh (Fig. 1B), suggesting that N-myc is an essential downstream effector of Shh signaling.

**c-myc function is generally required for normal brain growth but has no specific roles in cerebellar development.** N-myc is expressed in the proliferative region of the EGL, where Shh activity is known to drive GNP proliferation (4, 9, 31). These studies indicated that N-myc is the only myc family member in the cerebellum that is regulated by Shh and raised the possibility that N-myc may have a unique role in the proliferative expansion of GNPs, a notion supported by studies showing that the conditional knockout of N-myc within the developing nervous system prevented the full expansion of GNPs (11). Nonetheless, c-myc is also expressed in the developing brain (32) and low levels or transient expression of c-myc could potentially contribute to GNP growth.

To further address the role of c-myc in the development of the cerebellum, we conditionally knocked out c-myc in neural stem and progenitor cells in vivo using nestin-Cre. As shown in Fig. 2A, mice conditionally null for c-myc did not display a cerebellum-specific phenotype as was seen in the conditional N-myc knockout mice (11). Although the overall size of the c-myc null brain was diminished (18% reduction; Fig. 2B) compared with that of control mice, the expansion of GNPs and the development of the cerebellum were not specifically impaired by the loss of c-myc (Fig. 2A). The cerebella of animals lacking c-myc resembled those of control animals in the thickness of the inner granular layer (IGL) as well as in the extent of cerebellar foliation (Fig. 2C). In contrast, animals lacking N-myc in neural stem and progenitor cells displayed a reduction in total brain mass (Fig. 2B) and a failure in full expansion of the GNPs (Fig. 2C) as was shown previously (11).

To examine whether the acute loss of c-myc would have a similar effect on the proliferative response of GNPs to Shh in vitro, Cre/lox conditional targeting was used to knockout c-myc in primary GNP cultures. Cultures were infected with the GFP-Cre retrovirus or the control GFP virus, pulsed with BrdUrd 3 days after infection, and fixed and stained to examine BrdUrd incorporation. The infection of c-mycFl/Fl GNP cultures with the GFP-Cre or GFP control retroviruses did not affect their proliferative response to Shh in either case. Approximately equal fractions of both control and GFP-Cre infected cells incorporated BrdUrd (Fig. 1B), indicating that c-myc is dispensable for the proliferative response of GNP cultures to Shh. The levels of BrdUrd incorporation were lower in the control c-mycFl/Fl cultures infected with GFP alone compared with the control GFP-infected N-mycFl/Fl or WT cultures due to experimental variability in the preparation of the cultures. Note that N-mycFl/Fl cultures infected with GFP-Cre had incorporation levels below both c-mycFl/Fl and WT cultures infected with either GFP-Cre or GFP alone. Thus, it seems that both c-myc and N-myc have important roles in the overall growth of the brain but that N-myc has a more critical role in cerebellar development and specifically in the expansion of GNPs. Because the function of N-myc was clearly critical to the proliferation of GNP cultures in response to Shh, we chose to examine the requirement for N-myc in the context of ND2:SmoA1-driven cerebellar hyperplasia and tumor formation.

**N-myc functions downstream of Smo and is necessary for the hyperproliferation induced by constitutive activation of the Shh pathway.** In addition to the formation of medulloblastomas, 55% of mice carrying the ND2:SmoA1 transgene display hyperproliferation of the granular layer by 3 months of age (Table 1). Expanded pockets of granule cells are present in the IGL of these mice, indicative of the excess proliferation of these cells. Furthermore, 80% of ND2:SmoA1 mice have a persistent EGL (Table 1), which is normally absent by postnatal day 20 in WT mice (17). These populations of cells also express the nuclear antigen Ki-67 (Fig. 3A and B), which marks proliferative cells, and the neural stem cell marker nestin (Fig. 3E and F), indicating that the presence of the

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**Figure 3.** Regions of hyperplasia and persistent EGL (PEGL) express Ki-67 and nestin in N-mycWT/WT, SmoA1+ cerebella. Cerebellar sections from 3-month-old N-mycWT/WT, SmoA1+ mice displaying immunoreactivity for the mitotic marker Ki-67 (A) and the neural stem cell marker nestin (E) in regions of granule cell hyperplasia. Cerebellar sections from N-mycFl/Fl, SmoA1 mice displaying immunoreactivity for Ki-67 (B) and nestin (F) in regions with a persistent EGL, whereas cells in the IGL express neither marker. Immunohistochemical staining with antibodies against Ki-67 (C) and nestin (G) in cerebellar sections from 3-month-old WT mice (N-mycWT/WT, SmoA1+ ) shows that neither Ki-67 nor nestin is expressed. Similarly, no Ki-67 (D) or nestin (H) expression was observed in the cerebella of mice lacking N-myc and positive for the ND2:SmoA1 transgene (N-mycFl/Fl, SmoA1+ ). Brown, positive staining; light blue, a nuclear haematoxylin counterstain. Magnification, ×40 magnification.
ND2:SmoA1 transgene may sustain granule neural precursors in a progenitor state. Ki-67 and nestin expression was not seen in WT controls (Fig. 3C and G) or in ND2:SmoA1 cerebella lacking hyperplastic or persistent EGL phenotypes. This granule cell hyperplasia and the persistent EGL observed at 3 months may thus represent early stages in the formation of cerebellar tumors in the ND2:SmoA1 mice. Real-time PCR analysis showed that N-myc expression is elevated in cerebellar tumors generated in the ND2:SmoA1 medulloblastoma model (16), suggesting that the overexpression of N-myc may be involved in the initiation of hyperplasia and progression to tumor formation in SmoA1-induced medulloblastoma.

To determine whether N-myc is necessary for the hyperproliferation of GNPs seen in the ND2:SmoA1 transgenic mice, we crossed the ND2:SmoA1 mice with conditional (Nestin-Cre) N-myc knockout mice (11) to generate mice that possess persistently elevated Shh pathway activity in the cerebellum while lacking N-myc in the same region. These crosses produced four general groups of mice that we subsequently analyzed (Table 1). The number of mice that expressed ND2:SmoA1 and was null for N-myc in the cerebellum matched the number predicted by Mendelian inheritance, showing that this combination was not embryonic lethal.

The ND2:SmoA1 × N-myc conditional knockout mice were initially analyzed at 3 months of age. From birth onward, mice nullizygous for N-myc displayed moderate growth retardation and, by 3 weeks of age, began to exhibit tremors, an ataxic gait and raised tails, regardless of the presence or absence of the ND2:SmoA1 transgene. These phenotypes are also characteristic of the N-myc knockout mice (11). Forty-one mice were sacrificed at 3 months of age and it was noted that, compared with WT controls (Fig. 4A), nullizygous N-myc mice had significantly diminished cerebella in both the presence (Fig. 4C) and absence (Fig. 4D) of the ND2:SmoA1 transgene. Expression of the ND2:SmoA1 transgene was confirmed by immunofluorescent staining against the His tag present on the Smo protein encoded by the ND2:SmoA1 transgene. Staining with an anti-His antibody verified the expression of the ND2:SmoA1 transgene within granule cells of the N-myc null, ND2:SmoA1+ (N-myc−, SmoA1+) cerebella (Fig. 5A) as was seen in N-mycWT/WT, ND2:SmoA1+ controls (Fig. 5C). Therefore, ND2:SmoA1 expression was not disrupted by the loss of N-myc, and expression occurs throughout the remaining GNP population.

Histologic examination revealed a strong reduction in cerebellar foliation in mice lacking N-myc, regardless of the presence (Fig. 4G) or absence (Fig. 4H) of the ND2:SmoA1 transgene. Note that the granular, molecular, and Purkinje cell layers are present. Additionally, the expression of Ki-67 and nestin was not observed in cerebella lacking N-myc (Fig. 3D and H), suggesting that N-myc may be required for maintaining a proliferative, precursor-like

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**Figure 4.** N-myc is essential for the hyperplasia and tumorigenesis induced by deregulated Shh signaling. A and E, representative brain and H&E-stained cerebellar section from an N-mycWT/WT, SmoA1− mouse (WT control) at 6 months of age. B and F, example of a tumor in an N-mycWT/WT, ND2:SmoA1+ mouse. Sheets of small round blue cells disrupt the overall cerebellar architecture, with normal granule cell organization remaining only in small areas of the cerebellum (F). Representative brain and H&E-stained cerebellar section from an N-myc−, SmoA1− mouse (C and G) and from a mouse lacking both N-myc and the ND2:SmoA1 transgene (N-myc−, SmoA1−; D and H). Mice lacking N-myc exhibit an overall reduction in size of cerebellum, diminished thickness of granule cell layer, and incomplete foliation in both the presence (G) and absence (H) of the ND2:SmoA1 transgene. Additionally, no hyperplasia, persistent EGL, or tumor formation is evident in N-myc−, SmoA1− mice (see Table 1). Whole brains in (A–D) were photographed at 6 months of age and the cerebellal have been outlined in yellow; note that the relative differences in cerebellar size were also found in brains from 3-month-old mice. H&E-stained cerebellar sections taken at 6 months are shown at ×5 magnification in (E–H). Note that relative differences in cerebellar histology were also found in brains from 3-month-old mice.
population of cells during Shh-induced tumorigenesis. That constitutive activation of Smo fails to reverse the nullizygous N-myc overall brain and cerebellar-specific phenotypes suggests that N-myc is a critical downstream effector of Smo in the Shh signaling pathway. These findings also showed the requirement for N-myc in Shh-induced hyperplasia and in the persistent EGL observed in the majority of mice carrying the ND2:SmoA1 transgene.

**N-myc is required for tumorigenesis induced by deregulated Shh signaling.** Further analysis was carried out in mice at 6 months of age to examine the requirement for N-myc in ND2:SmoA1-driven medulloblastoma formation. Fifty-five mice from our study were sacrificed at 6 months. Mice nullizygous for N-myc and positive for the ND2:SmoA1 transgene phenotypically resembled the N-myc conditional knockout mice with significantly diminished cerebella (Fig. 4C). Histologic analysis at this time point revealed that mice lacking N-myc (Fig. 4G and H) have defects in cerebellar foliation compared with that of WT controls (Fig. 4E), with a reduction of the granular layer despite the presence of the ND2:SmoA1 transgene. No evidence of hyperproliferation or tumor formation was detected in mice lacking N-myc or in the WT controls (Table 1). Mice WT for N-myc and positive for the ND2:SmoA1 transgene developed hyperplasia and tumors (Fig. 4B and F) with an incidence similar to that seen in the study of the ND2:SmoA1 model (Table 1). These results indicate that N-myc is necessary for the hyperproliferation and tumor formation induced by the ND2:SmoA1 transgene. Furthermore, the hyperactivity of the Shh signaling pathway induced by the ND2:SmoA1 transgene is unable to compensate for the loss of N-myc in both normal GNP expansion as well as in hyperproliferation and tumor formation. Taken together, these findings provide *in vivo* evidence that N-myc acts downstream of Shh and Smo signaling during GNP proliferation and that N-myc is required for medulloblastoma genesis even in the presence of constitutively active signaling from the Shh pathway.

Although the loss of N-myc limits the expansion of the GNP population during normal cerebellar development, its loss does not ablate these cells entirely. GNPs are still specified and the granular layer does not fail to form. Thus, the cells thought to be the originators of tumor formation are still present within cerebella lacking N-myc. The phenotype of the N-myc null cerebellum was attributed previously to the inhibition of GNP proliferation. In addition, precocious differentiation of precursor cells in the cerebral cortex in the absence of N-myc was hypothesized to contribute to the failure of overall brain growth (11), indicating that N-myc may be functioning to maintain cells in a progenitor-like state. This role is consistent with the function of the Shh signaling pathway, which has been shown to prevent the terminal differentiation of GNPs (3, 4, 26), and whose deregulation might contribute to maintaining a subpopulation of GNPs in a progenitor-like state during tumorigenesis.

The results of this study also suggest that other components of the Shh signaling pathway are unable or insufficient to activate the proliferative machinery of cell in the absence of N-myc despite the constitutive activation of the Smoothened signal beginning in early embryonic development, showing that N-myc plays a central role in Shh-mediated proliferation and tumorigenesis.

The requirement for N-myc during Shh-induced hyperplasia and tumorigenesis makes it an appealing target for therapeutic intervention in the treatment of medulloblastoma. Studies using GNP cultures have shown that the overexpression of N-myc can rescue the inhibition induced by treatment of these cultures with the Shh/Smo antagonist cyclopamine (21). Taken together with the results of our current study, these findings suggest that the prevention of overactive Shh signaling through the inhibition of N-myc activity may be beneficial. Our study shows that N-myc is a necessary downstream effector of Shh/Smo signals *in vitro* and *in vivo* and that N-myc may be an efficient target for the prevention of tumor growth because it may act as an integrator of multiple signaling pathways that act to drive GNP proliferation, in addition to being a key effector of the Shh signaling pathway. N-myc levels in GNPs are regulated in part by glycogen synthase kinase-3β phosphorylation, which targets N-myc for proteasome degradation (33). Therefore, antagonism of N-myc activity by phosphatidylinositol 3-kinase blockage or other targeted therapies could lead to a clinically relevant reduction in the pool of proliferating cells that progress to malignant tumors.

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References
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