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Nmyc upregulation by sonic hedgehog signaling promotes proliferation in developing cerebellar granule neuron precursors

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SUMMARY

Hedgehog pathway activation is required for expansion of specific neuronal precursor populations during development and is etiologic in the human cerebellar tumor, medulloblastoma. We report that sonic hedgehog (Shh) signaling upregulates expression of the proto-oncogene *Nmyc* in cultured cerebellar granule neuron precursors (CGNPs) in the absence of new protein synthesis. The temporal-spatial expression pattern of *Nmyc*, but not other Myc family members, precisely coincides with regions of hedgehog proliferative activity in the developing cerebellum and is observed in medulloblastomas of *Patched* (*Ptch*) heterozygous mice. Overexpression of *Nmyc* promotes cell-autonomous G_1 cyclin upregulation and CGNP proliferation independent of Shh signaling. Furthermore, Myc antagonism in vitro significantly decreases proliferative effects of Shh in cultured CGNPs. Together, these findings identify *Nmyc* as a direct target of the Shh pathway that functions to regulate cell cycle progression in cerebellar granule neuron precursors.

Key words: Cerebellum, Sonic hedgehog, Proliferation, Cyclopamine, Medulloblastoma, Nmyc, Neural precursor, TRRAP, Mouse

INTRODUCTION

Signaling by the hedgehog family of secreted glycoproteins has been highly conserved through evolution (Ingham and McMahon, 2001). Hedgehog activity is required for fundamental processes during vertebrate development, including determination of the embryonic left-right axis and limb polarity as well as induction and patterning during organogenesis. Additionally, sonic hedgehog (Shh) signaling regulates proliferation of the developing lung (Bellusci et al., 1997), pancreas (Kim and Melton, 1998), retina (Jensen and Wallace, 1997) and central nervous system (CNS) (Dahmane and Ruiz-i-Altaba, 1999; Kalyani et al., 1998; Wallace, 1999; Wechsler-Reya and Scott, 1999). Proliferative effects of Shh have been especially well characterized in the external granule layer (EGL) of the cerebellum, where expansion of granule cell precursors is dependent upon Shh signaling during early postnatal development (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999).

Cerebellar granule neuron precursors (CGNPs) are generated in the rostral hindbrain during late embryonic development. They then migrate dorsally, where a second phase of postnatal proliferation takes place in the external granule layer of the cerebellum (EGL) (Altman and Bayer, 1997). Proliferating CGNPs express Math1, a bHLH transcription factor required for development (Ben-Arie et al., 1997; Helms and Johnson, 1998). Mature granule cells express other markers, including NeuN and the zinc finger transcription factor Zic (Aruga et al., 1994; Wechsler-Reya and Scott, 1999). Postmitotic granule precursors migrate to their final destination in the internal granule layer (IGL), where they undergo terminal differentiation (Altman and Bayer, 1997).

Shh pathway activation is implicated in the etiology of cerebellar tumors in humans as well as other types of cancer, and patched (*PTCH*), an inhibitory component of hedgehog signaling, has been identified as a tumor suppressor (Ruiz i Altaba, 1999). Individuals with Gorlin's syndrome, resulting from mutations of *PTCH*, have high rates of basal cell carcinoma and medulloblastoma (Hahn et al., 1996). Medulloblastoma, which is thought to derive from cerebellar granule cell precursors, is one of the most common solid tumors in children (Provias and Becker, 1996). Mutations of *PTCH* have also been found in 10-20% of sporadic medulloblastomas (Pietsch et al., 1997; Xie et al., 1997) and mice heterozygous for targeted mutations of *Ptch* develop cerebellar tumors (Goodrich et al., 1997). However, the intracellular mechanisms that underlie Shh effects on cell cycle progression during cerebellar development and tumorigenesis are poorly understood.

Shh is thought to bind to a receptor complex composed of the transmembrane proteins Ptch and smoothened (Smo). This in turn relieves Ptch-mediated inhibition of Smo activity (Ingham and McMahon, 2001). Smo, a member of the serpentine G-protein-coupled receptor family (van den Heuvel

and Ingham, 1996), is thought to activate an inhibitory G protein (DeCamp et al., 2000). Consequences of Shh signaling, in vivo and in vitro, can be inhibited experimentally by increasing cAMP levels or protein kinase A activity (Dahmane and Ruiz i Altaba, 1999; Fan et al., 1995; Hammerschmidt et al., 1996; Kenney and Rowitch, 2000; Wallace, 1999; Wechsler-Reya and Scott, 1999), or by treatment with the alkaloid, cyclopamine and related compounds (Berman et al., 2002; Dahmane et al., 2001; Incardona et al., 1998).

Smo signaling in *Drosophila* leads to post-translational activation of Cubitus interruptus protein, a member of the Gli family of zinc-finger transcription factors (Ruiz i Altaba, 1999). In vertebrates, Gli family members appear to be general targets of hedgehog signaling. Gli1 behaves as a transcriptional activator, whereas Gli3 functions as a repressor of hedgehog signaling (Dai et al., 1999; Sasaki et al., 1999). Although Gli2 appears to have distinct activator and repressor properties (Ruiz i Altaba, 1999; Sasaki et al., 1999), *Gli1* can fully compensate for *Gli2* function during CNS development (Bai and Joyner, 2001). Gli proteins are implicated in regulation of neural proliferation (Ruiz i Altaba, 1999), and *GLI1* was originally identified as a locus occasionally amplified in human gliomas (Kinzler et al., 1987). However, cerebellar proliferation is apparently normal in *Gli1* knockout mice (Park et al., 2000), indicating that additional factors must mediate proliferative effects of Shh.

Identification of Shh signaling intermediates that function in cell cycle regulation is key to understanding the role of this pathway in CNS proliferation during development and tumorigenesis. Shh treatment of CGNPs results in rapid upregulation of D-type cyclin genes, via a mechanism requiring protein synthesis (Kenney and Rowitch, 2000). Many mitogens signal to the cell cycle machinery by inducing expression of classical immediate-early genes (IEGs), such as members of the Fos, Jun and Myc families. Mitogen induction of IEGs occurs as a direct result of signaling pathway activation and does not require new protein synthesis (Sheng and Greenberg, 1990).

We report that Shh signaling in CGNPs induces the proto-oncogene Nmyc in a protein synthesis-independent manner. Of the Myc family members tested, only *Nmyc* was expressed in the proliferative zone of the cerebellum, suggesting a potential role for Nmyc in CGNP proliferation in vivo. *Nmyc* upregulation also occurred in proliferating neural precursor cells exposed to ectopic Shh in transgenic mice and in medulloblastomas of *Ptch* heterozygotes. *Nmyc* overexpression in vitro promoted CGNP proliferation and cyclin D1 protein upregulation in the presence of the Shh signaling inhibitor cyclopamine. Finally, antagonism of Nmyc activity significantly decreased CGNP proliferation induced by Shh. Together, our data provide compelling evidence that Nmyc functions downstream of Shh to promote cell cycle progression in cerebellar neuronal precursor cells during development and tumorigenesis.

MATERIALS AND METHODS

Cerebellar granule cell culture

Primary cultures of postnatal day (P) 4/5 mouse cerebellar granule cells were established precisely as described (Kenney and Rowitch, 2000), including an overnight adherence period in 10% fetal calf serum. To generate monolayer cultures enriched in CGNPs, cells were pre-plated twice for 30 minutes on 35 mm dishes coated with poly-D-lysine, then strained through a 70 μm nylon cell strainer (Falcon) before final resuspension and counting (Hatten, 1998). Quantitation of GFAP immunostaining revealed that glial cells accounted for less than 10% of cells in cultures enriched in this manner. Cells were resuspended at 3×10^6 cells/ml. One ml/well or 0.2 ml/coverslip was plated on poly-L-ornithine (Sigma)-treated glass cover-slips or six-well tissue culture plates (Falcon). For cycloheximide experiments, cells were 'rested' for 1 hour after the adherence period by incubation in DMEM-F12 with antibiotics alone to allow for downregulation of serum-stimulated intracellular signaling pathways. 'resting' medium was then exchanged for DMEM-F12/N2 supplement/25 mM KCl/antibiotics and Shh (3 μg/ml) or Shh vehicle±cycloheximide (10 μg/ml, Sigma). Shh was obtained from Biogen (Cambridge MA).

Cells destined for retroviral infection were prepared as above, except that Shh (1.5 μg/ml) was included during the adherence period. For 24 hours before infection, CGNPs were incubated in serum-free medium containing Shh (3 μg/ml) or Shh vehicle (indicated in figure legends). For infection, conditioned medium was removed and saved, and cells were exposed to freshly thawed retroviral supernatants for 2-3 hours, with intermittent rocking. Supernatants were removed and conditioned medium, with treatments added as indicated, was replaced. Alternatively, fresh medium was used with new Shh. Control cultures were treated with non-infectious, conditioned packaging cell medium, or UV-treated retroviral supernatants during the infection period. During analyses, no differences were observed between results obtained from cells cultured under these conditions. Cyclopamine, provided by William Gaffield (USDA, Albany, CA), was used at 1 μg/ml, as recommended.

Sample sizes and quantitation

All proliferation assessment experiments were repeated using pups from at least two separate litters. For immunochemistry experiments, two coverslips per treatment/infection were used, for each litter. Five fields of 100 cells per coverslip were analyzed by an unbiased observer, using DAPI staining to select homogeneously distributed cell fields. Thus, micrograph data shown are representative of a total n=20, 100-cell fields. Data are portrayed as fold change from Shh-treated GFP-infected cultures. GFP infected cultures did not differ from uninfected Shh-treated cultures in levels of proliferation (not shown). For flow cytometry (see below), experiments were performed using pups from three separate litters. With each separate litter, two wells of a six-well plate were used for each treatment/infection. Data represented in graphs are from n=6 experiments. Error bars represent standard error of the mean. Significance (P>0.01 in comparison with Shh-treated uninfected/GFP-infected CGNPs) was determined using the two-tailed t-test (Excel software). Sub-G_1-DNA quantitation was performed on two samples (two wells at 36 hours post-infection) for each experimental treatment. For northern blot and immunoblot analysis, two wells per treatment were pooled to maximize recovery of RNA and protein. Experiments for those analyses were repeated with three separate litters.

Retroviral constructs and production

Mouse *Nmyc* cassette (Wood et al., 2000), and *ΔMB2* cassette were prepared using standard methods for site-directed mutagenesis. *Mxi-SR* was a kind gift of Ron Depinho (Dana-Farber Cancer Institute) and *eGFP* was purchased from Clontech. All cassettes were cloned into the pWZL retroviral vector (Jay P. Morganstern, Millenium Pharmaceuticals, Cambridge, MA). pWZL IRES-*GFP* vector was provided by Steve Lessnick (Dana-Farber Cancer Institute). 293 EBNA (Invitrogen) packaging cells were co-transfected with retroviral constructs, gagpol, and vesicular stomatitis virus G

glycoprotein plasmids, using Fugene 6 transfection reagent (Roche). Packaging cells were re-fed 12 hours after transfection. Retroviral supernatants (4 ml) were harvested every 12 hours for 72 hours and kept at 4°C until they were pooled, filtered through 0.45 μm syringe filters, aliquoted and stored at –80°C until use.

Flow cytometry for cell cycle analysis

Flow cytometric analysis of propidium iodide (PI) staining was used to determine levels of proliferation and cell death as described (Darzynkiewicz et al., 1997; Kenney and Rowitch, 2000), using a FACScan (Becton Dickinson) and Cellquest software (Becton Dickinson) for data acquisition. Modfit software (Verity Software House) was used for quantifying sub-G_1 DNA levels.

Immunocytochemical labeling and analysis

For S-phase assessment, CGNPs plated on coverslips were pulsed with 25 μg/ml 8-bromo-deoxyuridine (BrdU) for 2 hours prior to fixation in 4% paraformaldehyde. Cell were washed in PBS and treated for 2 minutes with 2N HCl. Cells were then processed for immunocytochemistry using standard methods. Primary antibodies included mouse anti-BrdU (Becton-Dickinson), rabbit anti-GFP (Seedorf et al., 1999), mouse anti-GFAP (Sigma) and mouse-anti-NeuN (Chemicon). Fluorochrome-conjugated secondary antibodies were Cy-3 anti-rabbit, Cy-2 anti-mouse or FITC-conjugated anti-mouse (Jackson Immunoresearch Laboratories). Cells were co-stained with DAPI to label nuclei. Staining was visualized with a Nikon Eclipse E600 microscope. Images were captured using a SPOT 1 digital camera (Diagnostic Instruments) and processed using Adobe Photoshop 5.0 software.

RNA preparation and northern blot analysis

Total RNA was prepared by CsCl gradient centrifugation and ethanol precipitation. Ten μg of each sample was electrophoresed through a 1% agarose formaldehyde gel and transferred to a Hybond-N+ membrane (Amersham-Pharmacia). ^{32}P-labeled probes used for northern blotting were Gli1 (Hui et al., 1994), Jun (Tom Curran, St. Jude Children's Research Hospital, Memphis TN), Fos (Charles Stiles, DFCI, Boston MA), Mxi, Myc, Nmyc and Lmyc (all provided by Ron DePinho, DFCI, Boston, MA). Math1 cDNA was a gift of Jane Johnson (University of Texas Southwestern Medical Center, Dallas TX). Mouse GAPDH cDNA probe was PCR amplified from a mouse cDNA library.

Preparation of protein extracts and immunoblot analysis

Protein lysates were prepared and quantified as described (Kenney and Rowitch, 2000; Matsushime et al., 1994). Each sample (25 μg) was separated on 8% or 12.5% SDS-polyacrylamide gels, then transferred to Immobilon PVDF (Millipore) membranes. Primary antibodies used were anti-cyclin D1 and anti-Nmyc (sc-450 and sc-751, Santa Cruz), and anti-β-tubulin (T4026, Sigma). Peroxidase-conjugated secondary antibodies included donkey anti-mouse (Jackson Immunoresearch Laboratories) and goat anti-rabbit (Pierce). Blots were developed using enhanced chemiluminescence (ECL) (Amersham-Pharmacia), according to the manufacturer's instructions. Chemiluminescent immunoreactivity was detected using Kodak X-OMAT X-ray film.

Tissue preparation and in situ hybridization

Neonatal cerebella (PN1, PN7 and PN15) from 1% formalin/PBS perfused SWB mice and *Ptch* adult heterozygotes (Goodrich et al., 1997) or *Shh*-transgenic 12.5 dpc embryos (Rowitch et al., 1999) were drop-fixed in fresh 4% paraformaldehyde at 4°C. Samples were embedded in OCT and frozen 15-18 μm parasagittal sections were prepared on Superfrost plus slides. In situ hybridization on frozen sections was performed according to standard protocols (detailed protocol available upon request). Antisense digoxigenin-labeled riboprobes were generated from Myc, Nmyc and Lmyc constructs (provided by Ron DePinho, DFCI, Boston MA), *Gli1* (provided by Alex Joyner, Skirball Institute of Biomolecular Medicine, NY) or cyclin D1 (*Ccnd1*) (from Steve Elledge, Baylor College of Medicine, Houston TX) constructs.

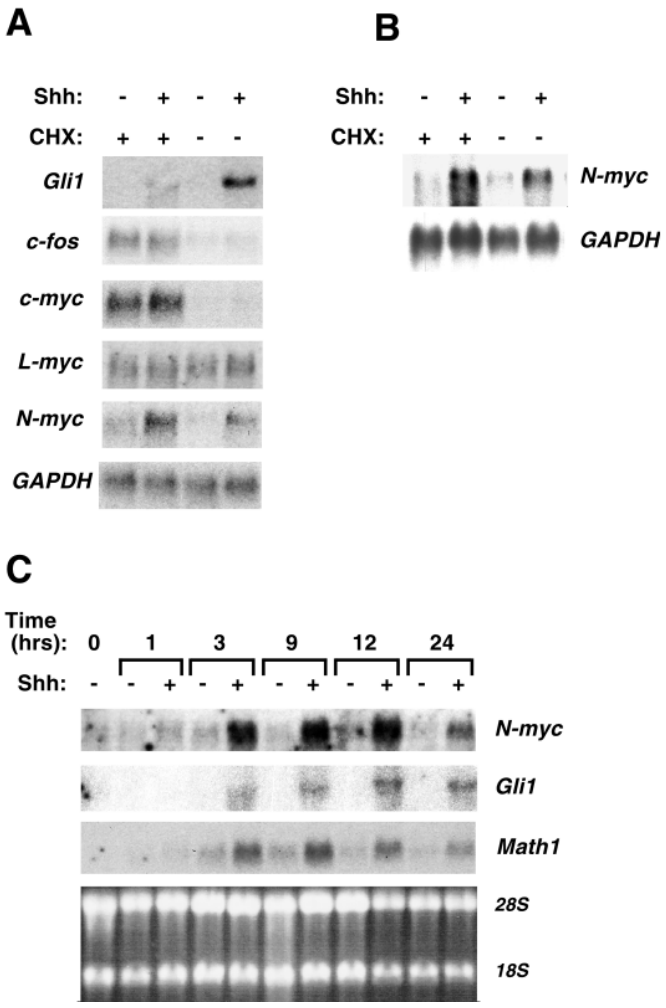

Fig. 1. *Nmyc* mRNA transcripts (*N-myc* in figure) are upregulated in Shh-treated CGNPs. (A) Total RNA was prepared from CGNPs (85% pure cultures) treated with Shh (3 μg/ml) for 3 hours±the protein synthesis inhibitor cycloheximide (10 μg/ml), as indicated above the lanes. Northern blot analysis was carried out using cDNA probes for candidate direct targets of Shh signaling. Representative autoradiographs for *Gli1*, *Myc* (*c-myc* in figure), *Lmyc* (*L-myc* in figure) and *Nmyc* are shown. *Gapdh* signal (bottom) shows equivalent loading. (B) Shh-mediated induction of *Nmyc* occurs in cultures enriched for CGNPs (>90% pure cultures). Cerebellar homogenates were enriched for CGNPs as described (Materials and Methods), then assayed as above for protein synthesis-independent up-regulation of Nmyc mRNA by Shh. A representative northern blot is shown (top). *Gapdh* signal (bottom) shows equivalent loading. (C) *Nmyc* expression is sustained for the duration of Shh exposure (top). The hedgehog signaling pathway is activated, as indicated by *Gli1* mRNA expression (middle). Shh signaling maintains a population of immature CGNPs, as indicated by *Math1* (*Atoh1* – Mouse Genome Informatics) expression (bottom). Representative northern analysis autoradiographs are shown. Ethidium bromide staining indicates equivalent lane loading.

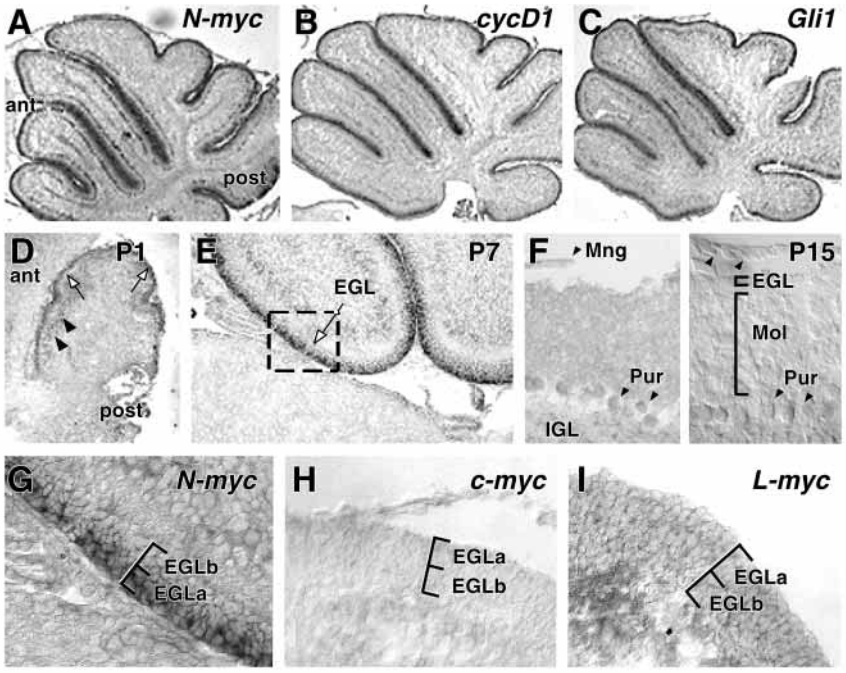

Fig. 2. *Nmyc* is expressed in proliferating precursors of the developing cerebellum. (A-C) In situ hybridization for *Nmyc*, (A) cyclin D1 (B) and *Gli1* (C) mRNA transcripts was performed on adjacent parasagittal frozen sections of PN 7 mouse cerebella. (A) *Nmyc* and (B) cyclin D1 expression co-localize to regions of Shh pathway activity and proliferation of CGNPs. (D-F) *Nmyc* expression is observed during the proliferative phase of cerebellar development at PN1 and PN7, but is undetectable in the PN15 or adult (not shown) mouse cerebellum. (D,E) Low power views of PN1 and PN7 cerebella shows the distribution of *Nmyc* mRNA transcripts primarily in the EGL region (white arrows). Additionally, a small *Nmyc*-expressing population is observed within deeper layers of the cerebellum at PN1 (arrowheads, D). (F) At PN15, *Nmyc* transcripts are undetectable in all cerebellar layers. DIC image at right allows discrimination of EGL and molecular layers. Arrowheads (top) indicate meninges that fail to express *Nmyc*. (G) At high magnification, *Nmyc* expression at PN7 clearly segregates to the EGLa, or proliferative compartment of the EGL. The EGLb comprises largely immature CGNPs that have recently left the cell cycle. By contrast, neither *Myc* (H) nor *Lmyc* (I) are expressed in the EGL at PN7. ant, anterior; post, posterior; EGL, external granule layer; Pur, Purkinje cells; Mol, molecular layer; Mng, meninges.

RESULTS

Shh stimulation directly induces *Nmyc* in granule neuron precursors

Upregulation of G_1 cyclin mRNA in CGNPs by Shh requires synthesis of unidentified protein intermediates (Kenney and Rowitch, 2000). Here, we asked whether known IEG transcription factors might link Shh signaling to cell cycle progression. IEGs are operationally defined as genes that are (1) induced by drugs that perturb protein synthesis and (2) 'superinduced' when protein synthesis is perturbed in the presence of growth factors, cytokines and other stimuli (Greenberg et al., 1986; Kelly et al., 1983; Lau and Nathans, 1985). The molecular basis of both the induction and super-induction responses of IEGs such as Fos and Myc family members has been well characterized (Greenberg et al., 1986; Lau and Nathans, 1987). The induction of IEG mRNA in the presence of protein synthesis inhibitors, including cycloheximide (CHX), occurs because IEG mRNA degradation depends upon IEG mRNA translation (Lau and Nathans, 1987). Constitutive low-level IEG transcription results in mRNA accumulation in the presence of protein synthesis inhibitors. The super-induction response results when factor-specific induction is combined with protein synthesis inhibition-mediated induction (Greenberg et al., 1986). To determine whether Shh signals to known classic IEGs in proliferating CGNPs, we examined the mRNA accumulation and super-induction responses of selected IEGs in CGNPs treated with Shh, in the presence/absence of the protein synthesis inhibitor cycloheximide (CHX).

As shown (Fig. 1A), 3 hours of CHX treatment alone resulted in increased levels of mRNA transcripts for *Myc* and *Fos* in CGNPs regardless of treatment, in keeping with original observations made in fibroblasts (Greenberg et al., 1986; Lau and Nathans, 1987). Cycloheximide-mediated accumulation of mRNAs for these genes indicates that they are transcribed in primary granule cell cultures, but it is evident from our analysis that their transcription is not specifically affected by Shh. Shh did not induce expression of *Lmyc* compared with vehicle-treated CGNPs (Fig. 1A), and Jun mRNA was undetectable in CGNP cultures (data not shown). Thus, *Myc*, *Fos*, *Lmyc* and *Jun* are unlikely to be direct targets of Shh in proliferating CGNPs.

Gli1 is a known transcriptional target of the Shh pathway in CGNPs (Weschler-Reya and Scott, 1999) and Gli proteins can be activated by hedgehog activity (Ruiz i Altaba, 1999). *Gli1* was strongly induced after 3 hours of treatment with Shh alone (Fig. 1A). CHX treatment alone did not induce *Gli1* mRNA, and sharply reduced the degree of *Gli1* upregulation in the presence of Shh. These results indicate that *Gli1* does not share the induction/super-induction response characteristic of IEGs. Newly synthesized Gli1 may regulate its own transcription (Dai et al., 1999). This could explain the antagonistic effect of CHX on *Gli1* mRNA induction by Shh.

Unlike other genes tested, *Nmyc* expression was strongly upregulated by Shh in the presence or absence of CHX (Fig. 1A). CHX treatment alone resulted in a low level of *Nmyc* mRNA elevation, in the manner of classically defined IEGs (Fig. 1A). These data suggest that Shh signaling pathway activation induces *Nmyc* by a mechanism that does not require new protein synthesis. Proliferation in CGNPs reaches high levels by 24 hours of Shh treatment (Kenney and Rowitch, 2000; Wechsler-Reya and Scott, 1999). Shh effects on the cell cycle machinery are first observed after 3 hours of treatment

(Kenney and Rowitch, 2000). We did not detect significant up-regulation of *Nmyc* mRNA until 3 hours of treatment with Shh+CHX (Fig. 1A,B).

Cerebellar homogenate cultures are an established system to characterize Shh proliferative effects on CGNPs (Kenney and Rowitch, 2000; Klein et al., 2001; Wechsler-Reya and Scott, 1999). These heterogeneous cultures comprise ~85% CGNPs, which express the bHLH transcription factor Math1 (Ben-Arie et al., 1997; Helms and Johnson, 1998; Kenney and Rowitch, 2000), as well as glia and a small percentage of other cell types (collectively defined as 'non-granule cell component'). Previous work indicates that CGNPs are the only cells in these cultures that proliferate in response to Shh (Dahmane and Ruiz-i-Altaba, 1999; Kenney and Rowitch, 2000; Wechsler-Reya and Scott, 1999). Nonetheless, to minimize any contribution of non-granule cells, we enriched for CGNPs in cerebellar homogenates (see Materials and Methods), then assayed cultures prepared in this manner for *Nmyc* upregulation by Shh. As shown in Fig. 1B, these enriched cultures exhibited protein-synthesis independent upregulation of *Nmyc* in response to Shh. These results confirm that Shh-induced *Nmyc* upregulation in cerebellar homogenate cultures is attributable to CGNPs, and rules out any significant contribution of the non-granule cell component.

Other neuronal cell types responding to extracellular stimuli typically upregulate IEG mRNA within minutes (Morgan and Curran, 1991; Sheng and Greenberg, 1990). By contrast, we observed a delay in *Nmyc* upregulation, which is most probably due to our culture conditions (see Materials and Methods) that include a 1 hour 'rest' period in factor-free medium before Shh treatment (Kenney and Rowitch, 2000). Cerebellar homogenates were initially maintained in serum-containing media to allow them to adhere and recover from harvest (Hatten, 1998). Although serum is not mitogenic for CGNPs, it is likely to contain factors that affect signaling pathways, which could antagonize or synergize with Shh activity. The resting period was thus used to minimize any potential contributions of serum-regulated pathways and reveal effects specific to Shh signaling. The timecourse we show for induction of *Nmyc* coincides with that of D-type cyclins in the absence of CHX (Kenney and Rowitch, 2000; Wechsler-Reya, 1999). Similarly, *Gli1* upregulation was first detectable after 3 hours of treatment in the absence of CHX (Fig. 1C), and lasted for the duration of this time course analysis. In summary, 3 hours is the earliest time point at which we have observed concerted activation of the hedgehog pathway and effects on cell cycle.

Proliferation in CGNPs reaches high levels by 24 hours of Shh treatment (Kenney and Rowitch, 2000; Wechsler-Reya and Scott, 1999). Cultures treated with Shh for 24 hours showed maintenance of *Math1* (*Atoh1* – Mouse Genome Informatics) expression (Fig. 1C), a CGNP-specific marker in the cerebellum required for granule cell development (Ben-Arie et al., 1997). Expression of *Math1* was used to confirm the presence of immature CGNPs in heterogeneous Shh-treated homogenate cultures (Kenney and Rowitch, 2000; Wechsler-Reya and Scott, 1999). Like *Gli1*, a classical Shh pathway target (Ruiz i Altaba, 1999), *Nmyc* was upregulated in CGNPs treated with Shh for at least 24 hours (Fig. 1C), indicating that the induction of *Nmyc* is not a transient response to Shh.

Nmyc is expressed in proliferating granule cell precursors in vivo

In mice, granule neuron precursor expansion in the EGL occurs primarily during the first 2 weeks of life (PN1-PN14) (Altman and Bayer, 1997). However, a role for Nmyc in this process has not been described, nor has *Nmyc* expression been characterized during expansion of granule neurons in vivo. We used in situ hybridization to determine expression of *Nmyc*, cyclin D1 and *Gli1*, an established Shh transcriptional target, in the PN7 cerebellum. As shown in Fig. 2A-C, we observed co-expression of *Nmyc*, cyclin D1 and *Gli1* in the EGL. To assess whether *Nmyc* is expressed in an appropriate developmental time course in vivo, we subjected cerebella from PN1-15 mice to analysis. *Nmyc* expression was readily detectable at PN1 and PN7 (Fig. 2D,E). At PN7, *Nmyc* expression was restricted to the proliferating outer zone of the EGL (EGLa) and was not detected in the inner granule layer (Fig. 2E,G), where differentiated granule cells lie. Furthermore, at PN15, when CGNP proliferation has ceased,

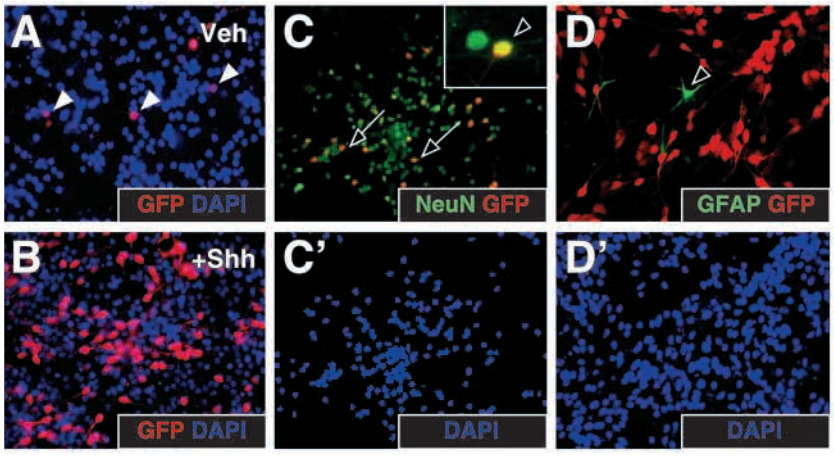

Fig. 3. Retroviral infection of heterogeneous primary cerebellar cultures is specific for proliferating CGNPs. (A,B) Cultures pretreated with Shh vehicle (Veh) alone prior to infection with GFP-carrying retroviruses showed only 5-10% infection levels (A), compared with ~70% infection levels in cells pre-treated with Shh (B). Merge of GFP-immunolabeled cells and DAPI-stained fields (blue) are shown. (A) Several infected cells are highlighted (arrowheads). (C,D) CGNPs infected with *GFP* retroviruses that subsequently exit the cell cycle co-express the neuronal marker, NeuN (FITC, green) (C), but not GFAP (FITC, green), an astroglial cell marker (D), indicating selective infection of neuronal precursors. After infection, cells were treated with vehicle alone for 48 hours prior to immunostaining. (C) Yellow pseudo-color indicates cells with overlapping expression (arrows); the inset shows infected cell with typical granule neuron morphology at higher power. (D) Arrowhead indicates a GFAP-positive cell. Note the paucity of GFAP-positive cells; we observed no astroglia that co-expressed GFP under our conditions of infection. (C′,D′) DAPI-staining (blue) of the fields shown in C,D.

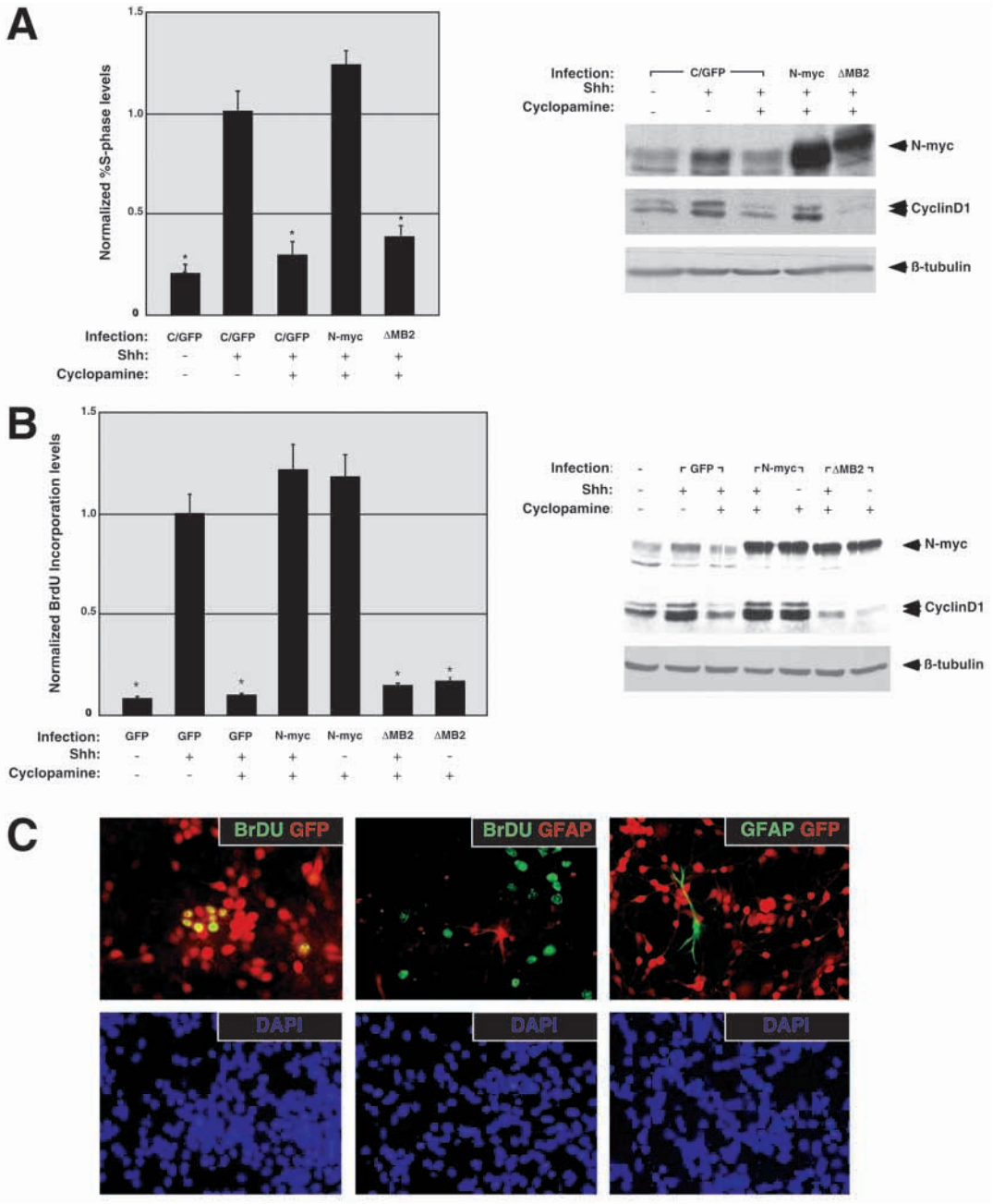

Fig. 4. Nmyc activity in cell cycle regulation lies downstream of Smo. Nmyc functions in a cell-autonomous manner. (A) 48 hours after infection and treatment as indicated below the graph (right), CGNPs (85% pure cultures) were harvested and processed for FACS analysis of cell cycle distribution. Percentages of cells in S-phase were normalized to levels in Shh-treated, uninfected/GFP infected samples. Bars represent average normalization from six samples per treatment. Error bars indicate standard error of the mean. **P*<0.01, in comparison with Shh-treated control CGNPs. A representative autoradiograph (left) shows western blot analysis of Nmyc (N-myc in figure) and cyclin D1 expression in CGNPs infected and treated with Shh/cyclopamine as indicated above the lanes. Tubulin levels demonstrate equivalent loading of the lanes. (B) Proliferation was assessed using BrdU immunostaining of CGNP cultures enriched for granule cells and treated as indicated below the graph. Numbers of BrdU immunopositive cells in Nmyc+GFP or ΔMB2+GFP-infected cells treated with Shh+cyclopamine or cyclopamine alone were normalized to numbers of BrdU-positive cells in GFP-infected, Shh-treated CGNP cultures. Twenty 100-cell fields were counted per treatment, using two separate litters of animals and two coverslips per treatment per litter. Error bars indicate standard error of the mean. **P*<0.01, in comparison with Shh-treated control CGNPs. (C) Phenotype of proliferating cells infected with Nmyc-carrying retrovirus. BrdU-GFP, BrdU-GFAP and GFAP-GFP immunostaining (as indicated) was carried out on Shh/cyclopamine treated cultures infected with *Nmyc-GFP* retrovirus. (Left) BrdU incorporation was exclusively associated with GFP expression (yellow pseudocolor indicates overlap). (Middle) BrdU incorporation was not observed in any GFAP-positive cells. (Right) No GFP-positive cells co-labeled with GFAP, indicating that glial cells were not infected. Note that yellow pseudocolor resulted in this image from a glial process underlying several neuronal cells. DAPI staining (bottom panels) shows distribution of cells.

Nmyc mRNA transcripts were no longer detected (Fig. 2F). Other myc family members tested (*Myc, Lmyc*) were not specifically expressed in the EGL (Fig. 2H,I) at any stage, consistent with results in Fig. 1. Thus, the temporal-spatial expression pattern of *Nmyc*, but not other myc family members, precisely coincides with regions of hedgehog proliferative activity in the developing cerebellum

Nmyc overexpression promotes cell-autonomous CGNP proliferation without smoothened activation

Gene over-expression has been employed to study hedgehog-stimulated proliferation in CNS precursors in cerebellar slice preparations (Solecki et al., 2001) and transgenic mice (Epstein et al., 1996; Rowitch et al., 1999). These methods, however, cannot distinguish primary versus secondary proliferative effects on surrounding cells. By contrast, monolayer cultures of CGNPs are suitable for the study of the cell-autonomous functions expected of transcription factors. Moreover, they minimize formation of aggregates, which can stimulate granule cell proliferation (Gao et al., 1991). Retroviral infection of monolayer cultures was the method we chose to investigate the role of Nmyc in Shh-regulated CGNP proliferation. Retroviruses exclusively infect proliferating cells (Roe et al., 1993), so we optimized conditions to enhance proliferation of CGNPs in vitro and minimize proliferation of non-CGNP cell types, such as glia and fibroblasts (see Materials and Methods). In particular, we observed that cultures grown in the presence of Shh for 24 hours before infection demonstrated high rates of infection with jellyfish green fluorescent protein (*GFP*)-carrying retroviruses (Fig. 3B) relative to cultures pre-treated with vehicle alone (Fig. 3A). To confirm selective infection of CGNPs under our conditions, primary CGNP cultures were infected with *GFP*-carrying retroviruses, then incubated in serum-free medium without Shh for 48 hours to ensure that infected cells exited the cell cycle and commenced differentiation. As shown in Fig. 3C,D, fluorescence immunocytochemistry indicated co-localization of GFP with NeuN, a marker of post-mitotic granule cells (Wechsler-Reya and Scott, 1999), but not cells expressing the astroglial marker, glial fibrillary acidic protein (GFAP).

For proliferation studies, CGNP cultures were infected with retroviruses expressing either GFP, wild-type mouse *Nmyc* or mutant *Nmyc* lacking the Myc box 2 domain (*ΔMB2*). The MB2 domain is required for myc interaction with TRRAP (McMahon et al., 1998), a co-factor essential for recruitment of histone acetyltransferase to target DNA. Cyclopamine, an alkaloid derived from the veratrum lily (Keeler, 1969), antagonizes hedgehog signaling during development in vivo (Incardona et al., 1998) because it prevents activation of Smo (Taipale et al., 2000). Cyclopamine also inhibits Shh-induced expansion of chick mid-brain precursor populations (Britto et al., 2002). Effects of cyclopamine on cycling CGNPs have not been previously investigated.

We used cyclopamine to assess whether (1) Smo inhibition blocks Shh-induced CGNP proliferation, (2) Nmyc lies downstream of Smo and (3) Nmyc can functionally rescue CGNP proliferation when Smo is inactivated. Proliferation was assessed 48 hours post-infection (~80 hours post-plating). Fresh medium with new Shh was provided after the infection period. It is known that GCNPs will proliferate at high levels with Shh treatment for up to 2 weeks and that proliferation is maximal at 3-4 days post-plating (Wechsler-Reya and Scott, 1999). Thus, our assay was performed within the time window during which Shh proliferative effects have been observed. Further, we confirmed upregulation of cyclin D1 proteins and ongoing effects of Shh on the molecular apparatus controlling cell cycle progression at these times (Fig. 4A,B). As shown in Fig. 4A, S-phase levels in cyclopamine/Shh-treated cells were significantly reduced in comparison with Shh-treated CGNPs. Western blot analysis confirmed that Shh-treated CGNPs expressed higher levels of Nmyc protein than vehicle-treated cells (Fig. 4A), and that cyclopamine treatment reduced levels of Nmyc protein in Shh-treated control/GFP-infected CGNPs.

Using flow cytometry, we measured levels of proliferation in CGNP cultures that were treated with cyclopamine/Shh and infected with Nmyc- or *ΔMB2*-carrying retroviruses. Retroviral protein expression was verified by western blotting (Fig. 4A). *ΔMB2*-infected, cyclopamine/Shh-treated CGNPs showed similar levels of cells in S-phase as GFP-infected Shh/cyclopamine-treated CGNPs (Fig. 4A). By contrast, *Nmyc*-infected cyclopamine/Shh treated cells maintained levels of proliferation similar to CGNPs exposed to Shh alone. These findings indicate that *Nmyc* expression is sufficient for CGNP proliferation in the absence of Smo-mediated hedgehog pathway activation. To confirm results of flow cytometry by an independent means, we measured proliferation in CGNP-enriched cultures by BrdU immunochemistry. As shown in Fig. 4B, this analysis yielded similar results. Cyclopamine alone had no effect on the ability of Nmyc to promote proliferation in CGNPs (Fig. 4B). CGNPs infected with *Nmyc+GFP* and subsequently treated with Shh vehicle alone also showed levels of proliferation similar to cells infected with GFP and treated with Shh (data not shown). Infection with *NmycΔMB2* and treatment with cyclopamine or Shh vehicle resulted in proliferation and cyclin D1 protein levels and similar to GFP-infected cells treated with Shh+cyclopamine (Fig. 4B and data not shown).

Nmyc might promote synthesis and release of secondary mitogens from infected cells. To determine whether proliferative effects of Nmyc on cell division were cell-autonomous, we used a retrovirus expressing both *Nmyc* and *GFP* (see Materials and Methods) to follow infected cells in Shh/cyclopamine-treated cultures. As shown in Fig. 4C, when Shh/cyclopamine-treated cultures were infected with *Nmyc+GFP* virus, we observed immunolabeling for BrdU exclusively in cells that co-expressed GFP. No BrdU-positive, GFP-negative cells were observed, out of twenty 100-cell fields examined. These results suggest that Nmyc acts within individual CGNPs to promote cell cycle progression.

Although our culture conditions were optimized for preferential infection of CGNPs, it was formally possible that the few glial cells present may have been infected with the *Nmyc* retrovirus. However, in *Nmyc+GFP* retrovirus-infected cultures treated with Shh/cyclopamine, we observed that both BrdU and GFP labeling consistently segregated away from cells expressing the astroglial marker, GFAP (Fig. 4C). Furthermore, the similar proliferative effects of Nmyc virus (Fig. 4B) despite the lack of glial cells in pre-plated, CGNP-enriched cultures indicates that these cells are not required to support CGNP proliferation in vitro. Together, these data rule out that CGNP proliferation in *Nmyc*-infected cultures reflects any significant contribution from glial cells.

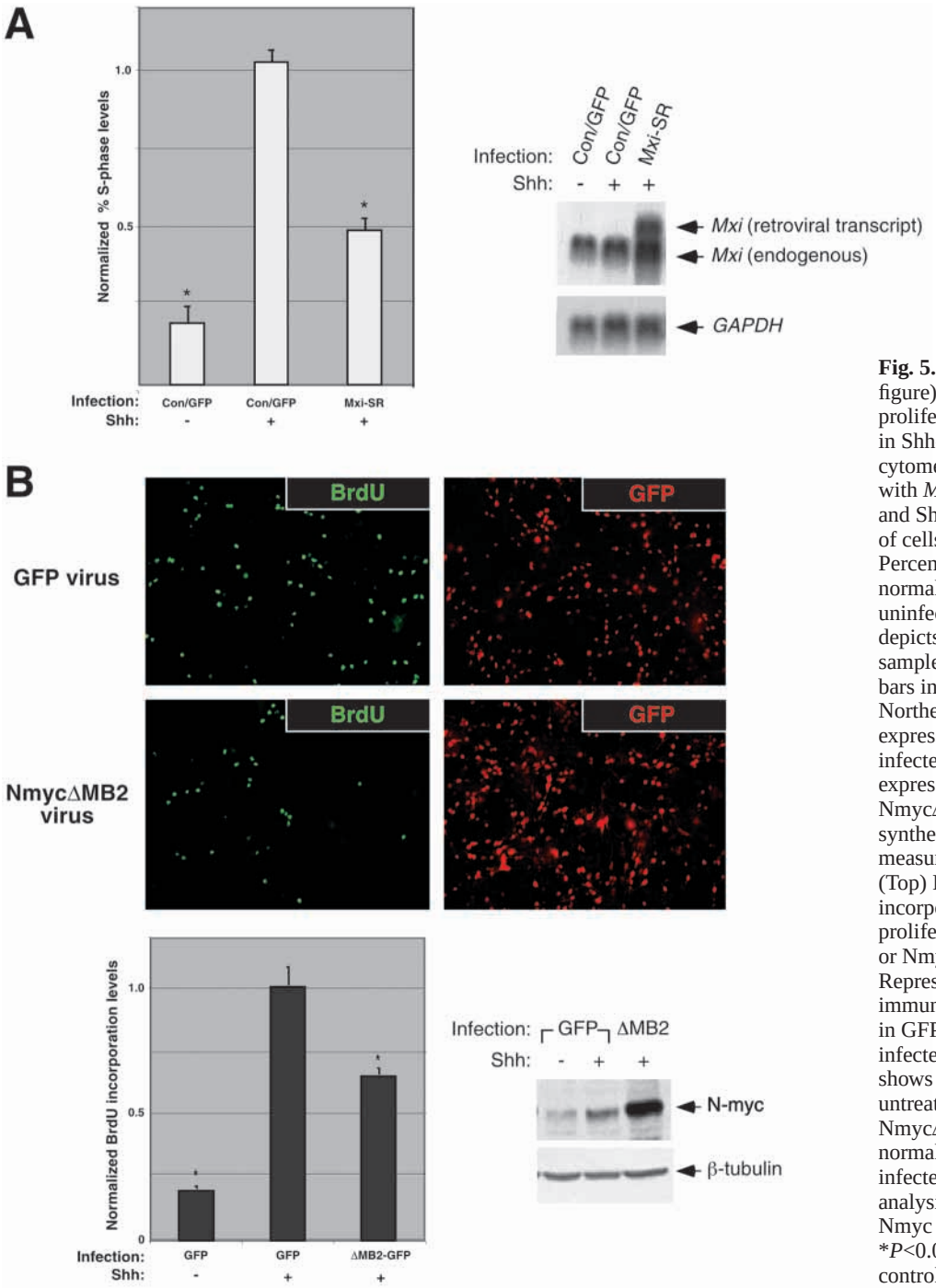

Fig. 5. Antagonism of Nmyc (N-myc in figure) activity reduces Shh-induced proliferation. (A) Mxi inhibits proliferation in Shh-treated cultures, as measured by flow cytometry. Thirty-six hours after infection with *Mxi-SR*-carrying retrovirus or controls and Shh-treatment, cycle phase distribution of cells was analyzed by flow cytometry. Percentages of cells in S phase were normalized to levels in Shh-treated, uninfected/GFP infected samples. The graph depicts average normalization from $n=6$ samples per treatment, per timepoint. Error bars indicate standard error of the mean. Northern blot analysis confirmed strong expression of *Mxi* (upper panel) in CGNPs infected as indicated. Endogenous *Mxi* expression can also be observed. (B) The NmycΔMB2 mutant inhibits DNA synthesis in Shh-treated cultures, as measured by BrdU immunolabling. (Top) Immunostaining to visualize BrdU incorporation was used to measure proliferation in CGNPs infected with GFP or NmycΔMB2+GFP retroviruses. Representative fields of BrdU immunostaining and GFP immunostaining in GFP-infected and NmycΔMB2+GFP-infected CGNPs are shown. (Bottom) Graph shows levels of BrdU incorporation in untreated, GFP-infected or Shh-treated NmycΔMB2+GFP-infected cells, normalized to levels in Shh-treated GFP-infected CGNP cultures. Western blot analysis (right) shows expression levels of Nmyc and NmycΔMB2 protein levels. *$P<0.01$, in comparison with Shh-treated control CGNPs.

A hallmark of the CGNP response to Shh is upregulation of D-type cyclin mRNA and protein (Kenney and Rowitch, 2000). Immunoblot analysis showed increased levels of cyclin D1 in Shh-treated cells, compared with vehicle treated controls (Fig. 4A,B). Cyclopamine co-treatment eliminated cyclin D1 protein expression in Shh-treated control cells and those infected with *GFP* or *ΔMB2* retroviruses. By contrast, *Nmyc*-infected CGNPs maintained cyclin D1 protein expression despite treatment with cyclopamine. These results suggest that a downstream consequence of Nmyc activity is upregulation of cyclin D1 protein expression.

Evidence that Nmyc activity is necessary for the full CGNP proliferative response to Shh

To further investigate potential roles for Nmyc, we asked whether Nmyc is required for Shh-mediated CGNP proliferation. A well-established means of blocking Myc function in vitro is by overexpression of Mad family members (Ayer and Eisenman, 1993; Lahoz et al., 1994; Lee and Ziff, 1999; Wu et al., 1996). Mad proteins, including Mad1, 3, 4 and Mxi, repress Myc target genes (Baudino and Cleveland, 2001) by binding to E-box sequences and recruiting histone deactelylase. Mxi overexpression blocks Myc- and *Nmyc*-

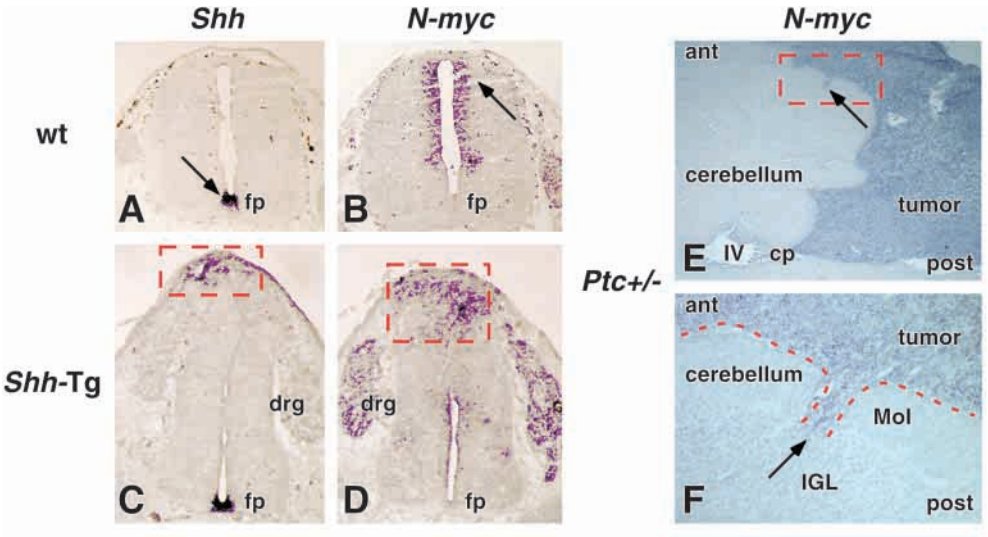

Fig. 6. *Nmyc* (*N-myc* in figure) is upregulated in proliferating neural tube precursors exposed to Shh and in medulloblastoma. (A-D) Hybridization for *Nmyc* and *Shh* mRNA transcripts was carried out in situ on adjacent transverse sections of spinal cord of 12.5 dpc wild-type (A) and *Shh* transgenic (Rowitch et al., 1999) embryos, which overexpress *Shh* in the dorsal neural tube (boxed) (C). (B,D) *Nmyc* is upregulated within proliferating neural precursors exposed to ectopic Shh (red box in D). Note that *Nmyc* is not a general Shh transcriptional target, because expression is absent in the floorplate (fp) where *Shh* is strongly expressed (compare A, arrow, with B). Additionally, *Nmyc* can be activated by mitogens other than Shh. *Nmyc* expression is observed in the dorsal spinal cord and dorsal root ganglion (drg) in the absence of adjacent *Shh* expression (compare B, arrow, with A; compare D with C). Thus, *Nmyc* is upregulated by Shh only in proliferating precursor cells. (E,F) *Nmyc* is upregulated in medulloblastomas of adult *Ptch* heterozygotes. Representative results of analysis of four animals are shown. (E) The normal adult cerebellum is devoid of *Nmyc* expression is at left in this image. By contrast, the tumor tissue expresses *Nmyc* strongly. (F) High-power view of boxed region in E shows possible area of continuity of the strongly *Nmyc*-expressing tumor cells (arrow) with the internal granule layer (IGL) and disruption of the molecular layer (Mol). The broken dashed line indicates the boundary between normal cerebellar tissue and medulloblastoma. ant, anterior; post, posterior; IV, fourth ventricle; cp, choroid plexus.

mediated, but not E1a-mediated transformation of fibroblasts (Lahoz et al., 1994).

As shown in Fig. 5A, endogenous *Mxi* is expressed in CGNPs in vitro. We infected Shh-treated CGNPs with a retrovirus carrying *Mxi-SR*, the strong repressor form of Mxi (Schreiber-Agus et al., 1995), then compared proliferation levels with control/*GFP*-infected CGNPs. Expression of *Mxi-Sr* is shown in Fig. 5A. Thirty-six hours after infection, proliferation in CGNPs was assayed by flow cytometry. *Mxi*-infected CGNPs treated with Shh showed significantly reduced proliferation compared with controls (Fig. 5A). To determine whether this decrease in proliferation reflected cell death, we quantified levels of fragmented 'sub-G_1' DNA, a hallmark of cell death that can be quantified by flow cytometry (Darzynkiewicz et al., 1997). The anti-proliferative effects of Mxi were not due to increased levels of cell death, as sub-G_1 DNA levels in Mxi-infected cells (5.3%±0.5, n=2) were similar to those in GFP-infected CGNPs (5.6%±0.7, n=2). These findings indicate that Mxi overexpression negatively regulates Shh-stimulated proliferation in CGNPs. Because Mxi represses Myc targets, and *Nmyc* is the only Myc family member expressed in CGNPs, our observations imply that Nmyc target gene activity is crucial for Shh to achieve its full mitogenic potential in CGNPs.

Although cell growth regulation targets substantially overlap between Myc and Mad (James and Eisenman, 2002), several targets for activation by Myc are not targets for Mad-mediated repression, and vice versa (O'Hagan et al., 2000). We wished to confirm our findings using a more specific means of blocking Nmyc activity. To this end, we infected CGNPs with a virus expressing *ΔMB2+GFP* and assessed levels of proliferation in these cells, compared with cells infected with GFP-carrying retroviruses alone. Proliferation was measured by quantitation of BrdU incorporation. These data indicated that ΔMB2 overexpression results in a significant decrease in Shh-induced CGNP proliferation (Fig. 5B). This suggests that overexpression of *NmycΔMB2* can compete with endogenous Nmyc in regulating growth-promoting target genes, resulting in reduced levels of proliferation. Together, the findings suggest that Nmyc activity is required to achieve the full proliferative response to Shh within CGNPs.

Hedgehog pathway activation causes *Nmyc* upregulation at ectopic locations in the neural tube and in medulloblastoma

Our findings indicate that Nmyc acts a link between hedgehog pathway activation and cell cycle progression within CGNPs. Shh has proliferative effects elsewhere in the CNS (Dahmane et al., 2001; Jensen and Wallace, 1997; Kalyani et al., 1998), and ectopic expression of *Shh* using the *Wnt1* regulatory element results in increased levels of neural precursor proliferation in the dorsal spinal cord of transgenic mice (Rowitch et al., 1999). We observed *Nmyc* upregulation in dorsal spinal cord of such transgenic embryos (Fig. 6D), suggesting that *Nmyc* is similarly upregulated in other CNS regions where Shh has mitogenic effects. These data further show that, in contrast to *Gli1*, *Nmyc* is not a general Shh transcriptional target. For example, *Nmyc* expression is absent in the floorplate, where *Shh* is strongly expressed (fp) (compare Fig. 6A,C with Fig. 6B,D). Second, it is clear that *Nmyc* can be activated by mitogens other than Shh. *Nmyc* expression is observed in proliferating progenitors in the dorsal half of wild-type spinal cord at 12.5 dpc (at a long distance from a source of Shh; Fig. 6B) and in the dorsal root ganglion (drg; Fig. 6B). Together with results shown in Figs 1, 2, these findings indicate that *Nmyc* can be recruited by Shh solely within immature, proliferating CNS precursor cells.

Mutation of the tumor suppressor *Ptch* is associated with

abnormal activation of hedgehog signaling and generation of medulloblastoma (Goodrich et al., 1997). We performed in situ hybridization on sections of medulloblastoma from *Ptch* adult heterozygotes. As shown in Fig. 6E, we observed dramatic induction of *Nmyc* in the tumors, in contrast to adjacent normal cerebellum. Indeed, the borders of the tumor appeared to be well demarcated by *Nmyc* expression, which highlighted an area of continuity between the IGL and tumor that largely occupied the space dorsal and posterior to the cerebellum (Fig. 6F). These findings suggest that *Nmyc* upregulation is a consequence of pathological hedgehog pathway activation as well as normal Shh signaling during development.

DISCUSSION

Nmyc: a target of hedgehog signaling in proliferating CGNPs

Sonic hedgehog signaling is required for expansion of neuronal precursor cells in the EGL of the cerebellum, and Shh is the most potent known mitogen for CGNPs in vitro. Activation of Shh signaling in cultured CGNPs rapidly results in increased levels of G_1 cyclin mRNA transcripts/proteins (Kenney and Rowitch, 2000), by a mechanism that requires new protein synthesis. We have shown that Shh activates expression of the proto-oncogene *Nmyc*, without requiring intermediate protein synthesis. The hedgehog signaling pathway is highly conserved and no genetic or biochemical interactions with Myc family members have yet been thoroughly explored, although recently published expression profiling studies using oligonucleotide microarrays have suggested that Nmyc may lie downstream of Shh signaling in the developing cerebellum (Zhao et al., 2002) and medulloblastoma (Pomeroy et al., 2002). These studies raise important questions regarding the role of Nmyc as a novel member of the hedgehog proliferative pathway.

Of numerous IEGs tested by northern blotting, only *Nmyc* expression was upregulated by Shh in the presence of cycloheximide in CGNPs, suggesting its transcription is directly regulated by the Shh signaling pathway. *Nmyc* overexpression was sufficient for CGNP proliferation, despite treatment with the hedgehog pathway antagonist cyclopamine, which interferes with Shh signaling by affecting the activation state of Smo (Taipale et al., 2000). Cyclopamine has been shown to interfere with the expansion of chick midbrain precursors, a Shh-mediated process (Britto et al., 2002). Interestingly, cyclopamine also reduces proliferation of certain brain tumor cells (Dahmane et al., 2001), which do not express Shh but do express Shh signaling targets. More recently, use of cyclopamine to inhibit the hedgehog signaling pathway was shown to block growth of mouse medulloblastomas in vitro and in vivo (Berman et al., 2002). Our data indicate that cyclopamine blocks Shh-induced proliferation and reduces levels of *Nmyc* protein in CGNPs. *Nmyc* overexpression sustained CGNP proliferation, despite cyclopamine treatment, indicating that Nmyc function lies downstream of Smo in the Shh signaling pathway.

It seems likely that *Nmyc* is not the only Smo target with a role in regulating cell cycle progression. Investigation of the proliferative targets of Shh has often focused on the Gli transcription factors, orthologs of the *Drosophila* hedgehog target *cubitus interruptus* (*ci*). Increased levels of *Gli1* mRNA transcripts are characteristic of Shh signaling pathway activation, and can be seen in CGNPs treated with Shh (Kenney and Rowitch, 2000; Wechsler-Reya and Scott, 1999). Our studies have not focused on a relationship between Gli activity and *Nmyc* regulation per se. Recently, a microarray analysis by Yoon and colleagues identified cyclin D2 as a Gli1 target in transformation assays (Yoon et al., 2002). *Nmyc* was not among the Gli1 targets described in that study.

Mutation of the DNA-binding domain of *Gli1* is not reported to result in cerebellar defects (Park et al., 2000), indicating the existence of *Gli1*-independent mechanisms for regulating cerebellar expansion. It is possible that Gli2 activity or antagonism of Gli3-mediated repression promotes Shh-stimulated Nmyc expression. The regulation of these factors is thought to involve post-translational processing (Ruiz i Altaba, 1999; Wang et al., 2000), a process that would enable target gene activation in the presence of protein synthesis inhibitors. However, this mechanism may not involve the induction/super-induction property of IEG mRNA regulation. An intriguing alternative is that *Nmyc* up-regulation by Shh occurs via a Gli-independent mechanism.

Shh-mediated regulation of *Nmyc* during cerebellar development and tumorigenesis

Nmyc is a member of the myc proto-oncogene family, which also includes *Myc* and *Lmyc*. Activity of Myc proteins is known to promote proliferation and/or transformation in many cell types (Henriksson and Luscher, 1996). Complete loss of Myc activity results in severely compromised cell cycle regulation (Mateyak et al., 1997). We found that *Nmyc* was highly expressed in the expansion zone of the EGL, where precursor proliferation is known to depend upon Shh activity. By contrast, we did not detect *Myc* or *Lmyc* expression in the developing EGL, nor were they upregulated by Shh in CGNP cultures. These findings suggest an important role for Nmyc in proliferation of CGNPs. We observed *Nmyc* upregulation in CGNP cultures and the dorsal embryonic spinal cord exposed to Shh, suggesting that signaling via *Nmyc* may be a general feature of Shh-induced proliferation in the CNS. However, in contrast to known general transcriptional targets of Shh (e.g. Gli genes), we note that Shh signaling effects on *Nmyc* regulation are unique to immature, proliferating precursor cells. Thus, cellular competence is evidently a critical determinant of the transcriptional response of *Nmyc* to Shh.

Increased levels of *MYCN* mRNA have been reported in certain cases of human medulloblastoma (Garson et al., 1989). More recently, *MYCN* upregulation has been observed in the desmoplastic form of medulloblastoma, the specific subtype of medulloblastoma associated with pathological activation of the hedgehog pathway (Pomeroy et al., 2002). By contrast, *MYCN* expression was not elevated in the majority of other (non-desmoplastic) medulloblastoma types analyzed. Consistent with findings in humans, we observed dramatic *Nmyc* upregulation in medulloblastomas from *Ptch* heterozygotes (Goodrich et al., 1997). Together, these findings are strong evidence that similar mechanisms operate downstream of hedgehog signaling during central nervous system development and tumorigenesis. Our results further suggest

that *MYCN* expression in desmoplastic medulloblastoma is a direct consequence of hedgehog pathway activation.

Evidence that Nmyc activity is required for the full Shh proliferative response in CGNPs

To establish a role for Nmyc in the Shh proliferative pathway in developing cerebellar precursors, it is necessary to examine cell cycle progression in the absence of *Nmyc* function. However, mice homozygous for *Nmyc* null or certain hypomorphic alleles mice die in utero or at birth (Charron et al., 1992; Moens et al., 1992; Sawai et al., 1991; Stanton et al., 1992), preventing assessment of *Nmyc* function during the proliferative postnatal phase of CGNP development. Recently, conditional targeting of murine *Nmyc* in the developing CNS has been reported to result in severe hypoplasia of cerebellum and defects in granule cell precursor proliferation (Knoepfler et al., 2002). These findings indicate that *Nmyc* function is necessary for cerebellar development. Yet, they leave unanswered the question of whether Nmyc activity is required downstream of Shh signaling during the postnatal phase of granule cell expansion.

In studies of Myc biology, overexpression of Mad family members is an established method for inhibiting Myc functions in proliferation, transformation and apoptosis (Ayer and Eisenman, 1993; Hurlin et al., 1995; Schreiber-Agus et al., 1995; Wu et al., 1996). These studies indicate that the repressive activity of Mad family members is specific, and Mad proteins are unlikely to behave as general transcriptional repressors; indeed, Mxi overexpression cannot inhibit transformation by E1a, another potent oncogene (Lahoz et al., 1994). The ability of *Mxi* overexpression to abrogate the mitogenic effects of Shh in vitro raises the possibility that Mad family members may regulate CGNP cell cycle exit during cerebellar development in vivo. In keeping with this, expression profiling studies indicate upregulation of several *Mad* family members at PN7, which coincides with the major wave of CGNP differentiation (Q. Zhao, A. Kho, I. Kohane and D. H. R., unpublished).

Our results imply that Mxi represses Nmyc targets, whose activity is crucial for Shh signaling to achieve its full proliferative capacity in CGNPs. To show that Nmyc activity is specifically involved in the proliferative response of CGNPs to Shh, we overexpressed a mutant *Nmyc* that lacks the Myc box 2 domain. This also resulted in a significant reduction in CGNP proliferation. Analogous mutants of Myc have dominant negative effects on transformation (Sawyers et al., 1992), and can attenuate entry into S-phase in fibroblasts (Conzen et al., 2000). The NmycΔMB2 mutant is incapable of interacting with TRRAP, a co-factor required for the transformation activity of Myc (McMahon et al., 1998). Although the NmycΔMB2 mutant lacks transforming activity, it can partially rescue cell cycle defects in Myc-null fibroblasts (Nikiforov et al., 2002). The activity of the NmycΔMB2 mutant, as well as requirements for TRRAP in neuronal precursors, have not been investigated. The inability of NmycΔMB2 to rescue proliferation in cyclopamine-treated CGNPs and its interference in Shh-induced CGNP proliferation raise the interesting possibility that neuronal precursors possess unique requirements for TRRAP activity in regulating proliferation-associated gene activation.

Although the levels of proliferation were reduced comparably with Mxi-SR or NmycΔMB2, in neither case did we observe complete cessation of proliferation. This is not surprising, as both of these methods allow for some residual Nmyc activity. When Myc activity is reduced, but not eliminated, Myc-dependent processes can still occur (Bazarov et al., 2001). Indeed, cultured fibroblasts that lack any Myc family member remain capable of proliferation albeit with an extended cell cycle length (Mateyak et al., 1997). Thus, abrogation of Nmyc activity might not necessarily lead to cell cycle exit within the time frame of our studies. Taken together, our findings of decreased CGNP proliferation with specific Nmyc antagonists in vitro, coupled with data that demonstrate essential functions for *Nmyc* during cerebellar development in vivo (Knoepfler et al., 2002), strongly suggest that *Nmyc* functions as an integral component of a Shh regulated pathway during proliferation of granule neuron precursors.

Mechanisms underlying cell cycle regulation by Nmyc in proliferating neuronal precursors

Myc proteins are characterized by specific functional domains, including a C terminus DNA-binding domain and a helix-loop-helix/leucine zipper domain, which mediates dimerization with the obligate partner of Myc, Max [Myn in the mouse (Prendergast et al., 1991)] (Henriksson and Luscher, 1996). Two highly conserved amino acid regions, Myc box 1 and Myc box 2, reside in the N-terminal transactivation domain. Myc box 2 associates with TRRAP protein (McMahon et al., 1998), which functions to recruit histone acetyltransferase to the transactivation domain of Myc (Park et al., 2001). This interaction is required for the transforming potential of Myc and *Nmyc* (McMahon et al., 1998). The Myc box 2 domain also appears to be required for the proliferative function of Nmyc in CGNPs, as overexpression of an *Nmyc* mutant lacking Myc box 2 was unable to rescue CGNP proliferation in the absence of Shh signal, and interfered with CGNP proliferation in the presence of Shh.

It is likely that Nmyc activates yet to be identified genes involved in cell cycle regulation. Recently, *Id2* was shown to be an Nmyc target in neuroblastoma (Lasorella et al., 2002). Some Myc targets include genes such as cyclin D2 (Bouchard et al., 1999) and *Cdc25* (Galaktionov et al., 1996), which control cell cycle progression. Metabolic regulators that enhance cell growth may also be targets of Myc (Dang, 1999). Finally, Myc has been implicated in repression of genes that promote cell cycle exit and differentiation (Claassen and Hann, 1999). Given the high level of relatedness between Nmyc and Myc protein structure, and the observation that Nmyc can rescue many of the defects in Myc null mutant mice (Malynn et al., 2000), it is possible that they have similar transcriptional targets.

We observed increased levels of cyclin D1 in *Nmyc*-infected cells despite the presence of cyclopamine. *Nmyc* is a direct transcriptional target of Shh signaling, whereas cyclin D1 mRNA upregulation was inhibited by cycloheximide (Kenney and Rowitch, 2000). Nmyc could be a candidate regulator of cyclin D1 expression. However, our present cyclin D1 protein measurements were taken 48 hours after infection and treatment, too long a length of time to assay for direct activation of Nmyc target genes. Nmyc may act indirectly, via activation of genes that otherwise promote cell growth, or by repressing genes that control the degradation of cyclin D1 message.

In summary, our results indicate that *Nmyc* is a direct downstream target of the canonical Shh signaling pathway in proliferating CGNPs. *Nmyc* expression was also found in medulloblastomas of *Ptch* mutant mice. Nmyc function is a key component of a Shh proliferative pathway essential for normal expansion of CGNP populations. These findings are the first to identify a direct connection between the Shh signaling pathway and a cell-intrinsic regulator of the cell cycle apparatus in primary neuronal cells. Proximal events in the progression from Smo activation to upregulation of *Nmyc* expression remain to be determined. Identification of Nmyc transcriptional targets will be crucial for understanding Shh-mediated cell cycle progression in CNS development. Moreover, because cerebellar granule cells are postulated to be the cell-of-origin for medulloblastoma, effective means to inhibit Nmyc activity might provide new approaches to control the growth of cerebellar tumors.

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REFERENCES

Altman, J. and Bayer, S. A. (1997). *Development of the Cerebellar System in Relation to its Evolution, Structure, and Functions*. Boca Raton, FL: CRC Press.

Aruga, J., Yokota, N., Hashimoto, M., Furuichi, T., Fukuda, M. and Mikoshiba, K. (1994). A novel zinc finger protein, zic, is involved in neurogenesis, especially in the cell lineage of cerebellar granule cells. *J. Neurochem.* **63**, 1880-1890.

Ayer, D. E. and Eisenman, R. N. (1993). A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. *Genes Dev.* **7**, 2110-2119.

Bai, C. B. and Joyner, A. L. (2001). Gli1 can rescue the in vivo function of Gli2. *Development* **128**, 5161-5172.

Baudino, T. A. and Cleveland, J. L. (2001). The Max network gone mad. *Mol. Cell. Biol.* **21**, 691-702.

Bazarov, A. V., Adachi, S., Li, S. F., Mateyak, M. K., Wei, S. and Sedivy, J. M. (2001). A modest reduction in c-myc expression has minimal effects on cell growth and apoptosis but dramatically reduces susceptibility to Ras and Raf transformation. *Cancer Res.* **61**, 1178-1186.

Bellusci, S., Furuta, Y., Rush, M. G., Henderson, R., Winnier, G. and Hogan, B. L. (1997). Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. *Development* **124**, 53-63.

Ben-Arie, N., Bellen, H. J., Armstrong, D. L., McCall, A. E., Gordadze, P. R., Guo, Q., Matzuk, M. M. and Zoghbi, H. Y. (1997). Math1 is essential for genesis of cerebellar granule neurons. *Nature* **390**, 169-172.

Berman, D. M., Karhadkar, S. S., Hallahan, A. R., Pritchard, J. I., Eberhart, C. G., Watkins, D. N., Chen, J. K., Cooper, M. K., Taipale, J., Olson, J. M. et al. (2002). Medulloblastoma growth inhibition by hedgehog pathway blockade. *Science* **297**, 1559-1561.

Bouchard, C., Thieke, K., Maier, A., Saffrich, R., Hanley-Hyde, J., Ansorge, W., Reed, S., Sicinski, P., Bartek, J. and Eilers, M. (1999). Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. *EMBO J.* **18**, 5321-5333.

Britto, J., Tannahill, D. and Keynes, R. (2002). A critical role for sonic hedgehog signaling in the early expansion of the developing brain. *Nat. Neurosci.* **5**, 103-110.

Brough, D. E., Hofmann, T. J., Ellwood, K. B., Townley, R. A. and Cole, M. D. (1995). An essential domain of the c-myc protein interacts with a nuclear factor that is also required for E1A-mediated transformation. *Mol. Cell. Biol.* **15**, 1536-1544.

Charron, J., Malynn, B. A., Fisher, P., Stewart, V., Jeannotte, L., Goff, S. P., Robertson, E. J. and Alt, F. W. (1992). Embryonic lethality in mice homozygous for a targeted disruption of the N-myc gene. *Genes Dev.* **6**, 2248-2257.

Claassen, G. F. and Hann, S. R. (1999). Myc-mediated transformation: the repression connection. *Oncogene* **18**, 2925-2933.

Conzen, S. D., Gottlob, K., Kandel, E. S., Khanduri, P., Wagner, A. J., O'Leary, M. and Hay, N. (2000). Induction of cell cycle progression and acceleration of apoptosis are two separable functions of c-Myc: transrepression correlates with acceleration of apoptosis. *Mol. Cell. Biol.* **20**, 6008-6018.

Dahmane, N. and Ruiz-i-Altaba, A. (1999). Sonic hedgehog regulates the growth and patterning of the cerebellum. *Development* **126**, 3089-3100.

Dahmane, N., Sanchez, P., Gitton, Y., Palma, V., Sun, T., Beyna, M., Weiner, H. and Ruiz i Altaba, A. (2001). The Sonic Hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis. *Development* **128**, 5201-5212.

Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M. and Ishii, S. (1999). Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. *J. Biol. Chem.* **274**, 8143-8152.

Dang, C. V. (1999). c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol. Cell. Biol.* **19**, 1-11.

Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T. and Traganos, F. (1997). Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry* **27**, 1-20.

DeCamp, D. L., Thompson, T. M., de Sauvage, F. J. and Lerner, M. R. (2000). Smoothened activates Galphai-mediated signaling in frog melanophores. *J. Biol. Chem.* **275**, 26322-26327.

Epstein, D. J., Marti, E., Scott, M. P. and McMahon, A. P. (1996). Antagonizing cAMP-dependent protein kinase A in the dorsal CNS activates a conserved Sonic hedgehog signaling pathway. *Development* **122**, 2885-2894.

Fan, C. M., Porter, J. A., Chiang, C., Chang, D. T., Beachy, P. A. and Tessier-Lavigne, M. (1995). Long-range sclerotome induction by sonic hedgehog: direct role of the amino-terminal cleavage product and modulation by the cyclic AMP signaling pathway. *Cell* **81**, 457-465.

Galaktionov, K., Chen, X. and Beach, D. (1996). Cdc25 cell-cycle phosphatase as a target of c-myc. *Nature* **382**, 511-517.

Gao, W. O., Heintz, N. and Hatten, M. E. (1991). Cerebellar granule cell neurogenesis is regulated by cell-cell interactions in vitro. *Neuron* **6**, 705-715.

Garson, J. A., Pemberton, L. F., Sheppard, P. W., Varndell, I. M., Coakham, H. B. and Kemshead, J. T. (1989). N-myc gene expression and oncoprotein characterisation in medulloblastoma. *Br. J. Cancer* **59**, 889-894.

Goodrich, L. V., Milenkovic, L., Higgins, K. M. and Scott, M. P. (1997). Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* **277**, 1109-1113.

Greenberg, M. E., Hermanowski, A. L. and Ziff, E. B. (1986). Effect of protein synthesis inhibitors on growth factor activation of c-fos, c-myc, and actin gene transcription. *Mol. Cell Biol.* **6**, 1050-1057.

Hahn, H., Wicking, C., Zaphiropoulous, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A. B., Gillies, S. et al. (1996). Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. *Cell* **85**, 841-851.

Hammerschmidt, M., Bitgood, M. J. and McMahon, A. P. (1996). Protein kinase A is a common negative regulator of Hedgehog signaling in the vertebrate embryo. *Genes Dev.* **10**, 647-658.

Hatten, M. E., Gao, W.-Q., Morrison, M. E. and Mason, C. A. (1998). The Cerebellum: purification and co-culture of identified cell populations. In *Culturing Nerve Cells* (ed. G. Bankeer and K. Goslin), pp. 419-459. Cambridge MA: MIT Press.

Helms, A. W. and Johnson, J. E. (1998). Progenitors of dorsal commissural interneurons are defined by MATH1 expression. *Development* **125**, 919-928.

Henriksson, M. and Luscher, B. (1996). Proteins of the Myc network:

essential regulators of cell growth and differentiation. *Adv. Cancer Res.* **68**, 109-182.

Hui, C. C., Slusarski, D., Platt, K. A., Holmgren, R. and Joyner, A. L. (1994). Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Dev. Biol.* **162**, 402-413.

Hurlin, P. J., Queva, C., Koskinen, P. J., Steingrimsson, E., Ayer, D. E., Copeland, N. G., Jenkins, N. A. and Eisenman, R. N. (1995). Mad3 and Mad4: novel Max-interacting transcriptional repressors that suppress c-myc dependent transformation and are expressed during neural and epidermal differentiation. *EMBO J.* **14**, 5646-5659.

Incardona, J. P., Gaffield, W., Kapur, R. P. and Roelink, H. (1998). The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. *Development* **125**, 3553-3562.

Ingham, P. W. (1998). Transducing Hedgehog: the story so far. *EMBO J.* **17**, 3505-3511.

Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.

James, L. and Eisenman, R. N. (2002). Myc and Mad bHLHZ domains possess identical DNA-binding specificities but only partially overlapping functions invivo. *Proc. Natl. Acad. Sci. USA* **99**, 10429-10434.

Jensen, A. M. and Wallace, V. A. (1997). Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. *Development* **124**, 363-371.

Kalyani, A. J., Piper, D., Mujtaba, T., Lucero, M. T. and Rao, M. S. (1998). Spinal cord neuronal precursors generate multiple neuronal phenotypes in culture. *J. Neurosci.* **18**, 7856-7868.

Keeler, R. F. (1969). Teratogenic compounds of Veratrum californicum (Durand). VII. The structure of the glycosidic alkaloid cycloposine. *Steroids* **13**, 579-588.

Kelly, K., Cochran, B. H., Stiles, C. D. and Leder, P. (1983). Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. *Cell* **35**, 603-610.

Kenney, A. M. and Rowitch, D. H. (2000). Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. *Mol. Cell. Biol.* **20**, 9055-9067.

Kim, S. K. and Melton, D. A. (1998). Pancreas development is promoted by cyclopamine, a hedgehog signaling inhibitor. *Proc. Natl. Acad. Sci. USA* **95**, 13036-13041.

Kinzler, K. W., Bigner, S. H., Bigner, D. D., Trent, J. M., Law, M. L., O'Brien, S. J., Wong, A. J. and Vogelstein, B. (1987). Identification of an amplified, highly expressed gene in a human glioma. *Science* **236**, 70-73.

Klein, R. S., Rubin, J. B., Gibson, H. D., DeHaan, E. N., Alvarez-Hernandez, X., Segal, R. A. and Luster, A. D. (2001). SDF-1 alpha induces chemotaxis and enhances Sonic hedgehog-induced proliferation of cerebellar granule cells. *Development* **128**, 1971-1981.

Knoepfler, P. S., Cheng, P. F. and Eisenman, R. N. (2002). N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. *Genes Dev.* (in press).

Lahoz, E. G., Xu, L., Schreiber-Agus, N. and DePinho, R. A. (1994). Suppression of Myc, but not E1a, transformation activity by Max- associated proteins, Mad and Mxi1. *Proc. Natl. Acad. Sci. USA* **91**, 5503-5507.

Lasorella, A., Boldrini, R., Dominici, C., Donfrancesco, A., Yokota, Y., Inserra, A. and Iavarone, A. (2002). Id2 is critical for cellular proliferation and is the oncogenic effector of N-myc in human neuroblastoma. *Cancer Res.* **62**, 301-306.

Lau, L. F. and Nathans, D. (1985). Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. *EMBO J.* **4**, 3145-3151.

Lau, L. F. and Nathans, D. (1987). Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc. *Proc. Natl. Acad. Sci. USA* **84**, 1182-1186.

Lee, T. C. and Ziff, E. B. (1999). Mxi1 is a repressor of the c-Myc promoter and reverses activation by USF. *J. Biol. Chem.* **274**, 595-606.

Malynn, B. A., de Alboran, I. M., O'Hagan, R. C., Bronson, R., Davidson, L., DePinho, R. A. and Alt, F. W. (2000). N-myc can functionally replace c-myc in murine development, cellular growth, and differentiation. *Genes Dev.* **14**, 1390-1399.

Mateyak, M. K., Obaya, A. J., Adachi, S. and Sedivy, J. M. (1997). Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. *Cell Growth Differ.* **8**, 1039-1048.

Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J. and Kato, J. Y. (1994). D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.* **14**, 2066-2076.

McMahon, S. B., van Buskirk, H. A., Dugan, K. A., Copeland, T. D. and Cole, M. D. (1998). The novel ATM-related protein TRRAP is an essential cofactor for the c- Myc and E2F oncoproteins. *Cell* **94**, 363-374.

Moens, C. B., Auerbach, A. B., Conlon, R. A., Joyner, A. L. and Rossant, J. (1992). A targeted mutation reveals a role for N-myc in branching morphogenesis in the embryonic mouse lung. *Genes Dev.* **6**, 691-704.

Morgan, J. I. and Curran, T. (1991). Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes fos and jun. *Annu. Rev. Neurosci.* **14**, 421-451.

Nikiforov, M. A., Chandriani, S., Park, J., Kotenko, I., Matheos, D., Johnsson, A., McMahon, S. B. and Cole, M. D. (2002). TRRAP-dependent and TRRAP-independent transcriptional activation by Myc family oncoproteins. *Mol. Cell Biol.* **22**, 5054-5063.

O'Hagan, R. C., Schreiber-Agus, N., Chen, K., David, G., Engelman, J. A., Schwab, R., Alland, L., Thomson, C., Ronning, D. R., Sacchettini, J. C. et al. (2000). Gene-target recognition among members of the myc superfamily and implications for oncogenesis. *Nat. Genet.* **24**, 113-119.

Park, H. L., Bai, C., Platt, K. A., Matise, M. P., Beeghly, A., Hui, C. C., Nakashima, M. and Joyner, A. L. (2000). Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. *Development* **127**, 1593-1605.

Park, J., Kunjibettu, S., McMahon, S. B. and Cole, M. D. (2001). The ATM-related domain of TRRAP is required for histone acetyltransferase recruitment and Myc-dependent oncogenesis. *Genes Dev.* **15**, 1619-1624.

Pietsch, T., Waha, A., Koch, A., Kraus, J., Albrecht, S., Tonn, J., Sorensen, N., Berthold, F., Henk, B., Schmandt, N. et al. (1997). Medulloblastomas of the desmoplastic variant carry mutations of the human homologue of Drosophila patched. *Cancer Res.* **57**, 2085-2088.

Pomeroy, S. L., Tamayo, P., Gaasenbeek, M., Sturla, L. M., Angelo, M., McLaughlin, M. E., Kim, J. Y., Goumnerova, L. C., Black, P. M., Lau, C. et al. (2002). Prediction of central nervous system embryonal tumour outcome based on gene expression. *Nature* **415**, 436-442.

Prendergast, G. C., Lawe, D. and Ziff, E. B. (1991). Association of Myn, the murine homolog of max, with c-Myc stimulates methylation-sensitive DNA binding and ras cotransformation. *Cell* **65**, 395-407.

Provias, J. P. and Becker, L. E. (1996). Cellular and molecular pathology of medulloblastoma. *J. Neurooncol.* **29**, 35-43.

Roe, T., Reynolds, T. C., Yu, G. and Brown, P. O. (1993). Integration of murine leukemia virus DNA depends on mitosis. *EMBO J.* **12**, 2099-2108.

Rowitch, D. H., St-Jacques, B., Lee, S. M., Flax, J. D., Snyder, E. Y. and McMahon, A. P. (1999). Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells. *J. Neurosci.* **19**, 8954-8965.

Ruiz i Altaba, A. (1999). Gli proteins and Hedgehog signaling. *Trends Genet.* **15**, 418-425.

Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M. and Kondoh, H. (1999). Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development* **126**, 3915-3924.

Sawai, S., Shimono, A., Hanaoka, K. and Kondoh, H. (1991). Embryonic lethality resulting from disruption of both N-myc alleles in mouse zygotes. *New Biol.* **3**, 861-869.

Sawyers, C. L., Callahan, W. and Witte, O. N. (1992). Dominant negative MYC blocks transformation by ABL oncogenes. *Cell* **70**, 901-910.

Schreiber-Agus, N., Chin, L., Chen, K., Torres, R., Rao, G., Guida, P., Skoultchi, A. I. and DePinho, R. A. (1995). An amino-terminal domain of Mxi1 mediates anti-Myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor SIN3. *Cell* **80**, 777-786.

Seedorf, M., Damelin, M., Kahana, J., Taura, T. and Silver, P. A. (1999). Interactions between a nuclear transporter and a subset of nuclear pore complex proteins depend on Ran GTPase. *Mol. Cell. Biol.* **19**, 1547-1557.

Sheng, M. and Greenberg, M. E. (1990). The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron* **4**, 477-485.

Solecki, D. J., Liu, X. L., Tomoda, T., Fang, Y. and Hatten, M. E. (2001). Activated Notch2 signaling inhibits differentiation of cerebellar granule neuron precursors by maintaining proliferation. *Neuron* **31**, 557-568.

Stanton, B. R., Perkins, A. S., Tessarollo, L., Sassoon, D. A. and Parada, L. F. (1992). Loss of N-myc function results in embryonic lethality and failure of the epithelial component of the embryo to develop. *Genes Dev.* **6**, 2235-2247.

Taipale, J., Chen, J. K., Cooper, M. K., Wang, B., Mann, R. K., Milenkovic, L., Scott, M. P. and Beachy, P. A. (2000). Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. *Nature* **406**, 1005-1009.

van den Heuvel, M. and Ingham, P. W. (1996). smoothened encodes a

receptor-like serpentine protein required for hedgehog signalling. *Nature* **382**, 547-551.

Wallace, V. A. (1999). Purkinje-cell-derived Sonic hedgehog regulates granule neuron precursor cell proliferation in the developing mouse cerebellum. *Curr. Biol.* **9**, 445-448.

Wang, B., Fallon, J. F. and Beachy, P. A. (2000). Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell* **100**, 423-434.

Wechsler-Reya, R. J. and Scott, M. P. (1999). Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron* **22**, 103-114.

Wood, M. A., McMahon, S. B. and Cole, M. D. (2000). An ATPase/helicase complex is an essential cofactor for oncogenic transformation by c-Myc. *Mol. Cell* **5**, 321-330.

Wu, M., Arsura, M., Bellas, R. E., FitzGerald, M. J., Lee, H., Schauer, S. L., Sherr, D. H. and Sonenshein, G. E. (1996). Inhibition of c-myc expression induces apoptosis of WEHI 231 murine B cells. *Mol. Cell. Biol.* **16**, 5015-5025.

Xie, J., Johnson, R. L., Zhang, X., Bare, J. W., Waldman, F. M., Cogen, P. H., Menon, A. G., Warren, R. S., Chen, L. C., Scott, M. P. et al. (1997). Mutations of the PATCHED gene in several types of sporadic extracutaneous tumors. *Cancer Res.* **57**, 2369-2372.

Yoon, J. W., Kita, Y., Frank, D. J., Majewski, R. R., Konicek, B. A., Nobrega, M. A., Jacob, H., Walterhouse, D. and Iannaccone, P. (2002). Gene expression profiling leads to identification of GLI1-binding elements in target genes and a role for multiple downstream pathways in GLI1-induced cell transformation. *J. Biol. Chem.* **277**, 5548-5555.

Zhao, Q., Kho, A., Kenney, A. M., Yuk Di, D. I., Kohane, I. and Rowitch, D. H. (2002). Identification of genes expressed with temporal-spatial restriction to developing cerebellar neuron precursors by a functional genomic approach. *Proc. Natl. Acad. Sci. USA* **99**, 5704-5709.