

Corrigendum

Hedgehog and PI-3 kinase signaling converge on Nmyc1 to promote cell cycle progression in cerebellar neuronal precursors

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The authors wish to emphasise that the protein Nmyc1 referred to in this article is the same as N-myc.

Hedgehog and PI-3 kinase signaling converge on Nmyc1 to promote cell cycle progression in cerebellar neuronal precursors

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Summary

Neuronal precursor cells in the developing cerebellum require activity of the sonic hedgehog (Shh) and phosphoinositide-3-kinase (PI3K) pathways for growth and survival. Synergy between the Shh and PI3K signaling pathways are implicated in the cerebellar tumor medulloblastoma. Here, we describe a mechanism through which these disparate signaling pathways cooperate to promote proliferation of cerebellar granule neuron precursors. Shh signaling drives expression of mRNA encoding the Nmyc1 oncoprotein (previously N-myc), which is essential for expansion of cerebellar granule neuron precursors. The PI3K pathway stabilizes Nmyc1 protein via inhibition of GSK3-dependent Nmyc1

phosphorylation and degradation. The effects of PI3K activity on Nmyc1 stabilization are mimicked by insulin-like growth factor, a PI3K agonist with roles in central nervous system precursor growth and tumorigenesis. These findings indicate that Shh and PI3K signaling pathways converge on N-Myc to regulate neuronal precursor cell cycle progression. Furthermore, they provide a rationale for therapeutic targeting of PI3K signaling in medulloblastoma.

Key words: Cerebellum, Sonic hedgehog, Proliferation, GSK3, Medulloblastoma, Myc, Neural precursor, Mouse, Nmyc1 (N-myc)

Introduction

Developmental programs that coordinate neuronal precursor proliferation and cell cycle exit are poorly understood. Signaling by the secreted factor Sonic hedgehog (Shh) is essential for proliferation of neuronal precursors of the cerebellum, forebrain and midbrain (Ho and Scott, 2002). Shh signaling also promotes the proliferation of adult neural stem cells (Lai et al., 2003) and is implicated in the genesis of the cerebellar tumor medulloblastoma (Ho and Scott, 2002). Recent work has indicated an evolutionarily conserved role for Hedgehog (Hh) signaling in activation of G₁ cyclins (Duman-Scheel et al., 2002; Kenney and Rowitch, 2000). However, many aspects of Hedgehog signaling to the cell cycle machinery remain poorly understood. Indeed, the canonical Hh pathway, defined largely by genetic analysis, does not resemble classical mitogenic pathways.

Many well-characterized mitogens [e.g. insulin-like growth factor (IGF), platelet-derived growth factor] signal through receptor tyrosine kinases (RTKs) that lead to activation of the Ras-MAPK and phosphatidylinositol-3-kinase (PI3K) pathways (Marshall, 1995). By contrast, Shh binding to its receptor Patched (Ptc) relieves inhibition of the signaling pathway activated by the transmembrane protein Smoothed (Smo) (Ho and Scott, 2002), resulting in activation of Smo target genes including *Ptc* and the *Gli* family of transcription factors. However, several lines of evidence suggest that RTK signaling might be synergistic with Shh in its capacity to regulate proliferation of neuronal precursor cells. For example, although treatment of with CXCL12 (also called SDF1; the

cognate ligand of CXCR4) is not mitogenic in primary cerebellar granule neuron precursor (CGNP) cultures, it causes marked enhancement of Shh proliferative effects (Klein et al., 2001). Additionally, essential roles for IGF2 signaling have been described during Hedgehog-pathway-associated cerebellar tumorigenesis (Hahn et al., 2000).

The precursors for cerebellar granule neurons are generated in rhombomere 1 of the embryonic hindbrain and migrate dorsally to form the outer layer of the cerebellum, or external granule layer (EGL). Proliferation of granule cells in the EGL is largely postnatal in mammals (Altman and Bayer, 1997). Previous work has shown that Shh is required for granule cell precursor proliferation (Ho and Scott, 2002) and that Shh induces D-type cyclin expression (Kenney and Rowitch, 2000). In previous work, we surveyed a range of known immediate early factors and determined that proto-oncogene Nmyc1 was a direct target of Shh signaling in proliferating CGNPs (Kenney et al., 2003). Nmyc1 (previously known as N-myc) is a member of the Myc proto-oncogene family, which includes Myc and L-Myc. As heterodimeric complexes with the Max protein, Myc family members behave as transcriptional activators (Henriksson and Luscher, 1996) and also have gene repression capabilities (Wanzel et al., 2003). Nmyc1 activity is necessary for CGNP proliferation (Kenney et al., 2003; Knoepfler et al., 2002) and regulation of cyclinD1 and cyclinD2 expression during cerebellar development (Ciemerych et al., 2002). Gene activation by Myc family members involves interactions with chromatin remodeling machinery (Cole and McMahon, 1999) and this aspect of

Nmyc1 activity that is absolutely required for CGNP proliferation (Kenney et al., 2003; Oliver et al., 2003). These studies indicate that Nmyc1 is a crucial determinant of proliferation and growth arrest during central nervous system (CNS) development.

Identification of the intracellular events that integrate effects of divergent signaling pathways is crucial for a comprehensive understanding of growth control in both normal and neoplastic neuronal progenitors. To better understand interactions between the Shh and PI3K pathways during neuronal precursor proliferation, we have focused on determinants of Nmyc1 protein turnover and cell cycle progression in primary CGNP cultures. Here, we report that phosphorylation promotes Nmyc1 protein turnover and timely cell cycle exit in CGNP primary cultures. Nmyc1 phosphorylation in CGNPs requires GSK3 activity and is antagonized by PI3K signaling but is regulated independent of Shh activity per se. Our findings provide a mechanism by which complex regulation of a single intracellular target can integrate the effects divergent signaling pathways active in the developing brain.

Materials and methods

Cell culture and flow cytometry

Post-natal day 4/5 (PN 4/5) mouse CGNP cultures were prepared as described (Kenney and Rowitch, 2000), with the addition of two 30-minute pre-platings on poly-D-lysine, to enrich further for CGNPs (Hatten, 1998). Before the final plating on poly-ornithine, cell suspensions were passed through a 70 μ M nylon cell strainer (Falcon) to reduce the presence of aggregates. Cells were plated at a density of 3×10^5 cells cm^{-2} on poly-L-ornithine-coated six-well plastic plates or glass coverslips. For flow cytometry, CGNPs were fixed in ethanol, stained with propidium iodide and processed as described (Kenney et al., 2003). For CGNP treatments, Shh (19 kDa N-terminal fragment) was a kind gift from K. Williams (Biogen). Cyclopamine was provided by W. Gaffield (US Department of Agriculture). Cycloheximide, LiCl, LY294002 and wortmannin were purchased from Sigma. Lactacystin and GSK3 inhibitor II were from Calbiochem. Recombinant human IGF-1 was purchased from Gibco. SK-N-SH cells were maintained in RPMI (Gibco), supplemented with 10% fetal calf serum (Sigma) and penicillin-streptomycin (Gibco).

Mass spectrometry

HEK 293 cells were transiently transfected with Flag-tagged Nmyc1 in the pWZL retroviral vector using Fugene 6 transfection reagent (Roche). 36 hours after transfection, cells were lysed and Flag-Nmyc1 was immunoprecipitated with anti-Flag-antibody-bound agarose beads (Sigma, M2) at 4°C overnight. The protein was then eluted with 3 \times flag peptide (Sigma). The eluate was separated by SDS-PAGE, and the Coomassie-stained band corresponding to Nmyc1 was excised, trypsinized and subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis using Applied Biosystems DE-STR mass analysis instrumentation. Further fragmentation of the identified phosphorylated peptide at 1741.8544 Da was performed using tandem electrospray mass spectrometry with ion-trap technology (Thermal Finnegan LCQ-DECA).

Retroviral constructs

Flag-tagged wild-type Nmyc1 was cloned into the pWZL retroviral vector as described (Kenney et al., 2003). Nmyc1T50A, Nmyc1S54A, Nmyc1 T50E and Nmyc1 S54E were prepared using the QuikChange site-directed mutagenesis kit (Stratagene), with pWZL-Flag-Nmyc1 as template. Retroviral stock preparation and CGNP infection were carried out as previously described (Kenney et al., 2003).

Western blotting and immunohistochemistry

To detect proteins by immunoblotting, non-denaturing lysates were prepared from CGNPs as described (Kenney and Rowitch, 2000). Immunoblots were incubated overnight at 4°C in primary antibodies (Nmyc1, Santa Cruz sc-791; phospho-T58 Myc, CST 9401; Cyclin D1, Neomarkers Ab-3; Cyclin D2, Santa-Cruz M-20; β -tubulin, Sigma T4026; phospho-GSK3 β , CST 9331; phospho-S473 Akt, CST 9271), then developed using horseradish-peroxidase (HRP)-conjugated anti-rabbit (Pierce) or anti-mouse (Jackson) secondary antibodies and ECL reagents (Amersham). Immunohistochemical analysis of cryosections of PN 7, 15 and 21 mouse cerebella with polyclonal phospho-T58 Myc and monoclonal antibodies against Calbindin (Sigma) and PCNA (Dako) was carried out using standard protocols except that antigen retrieval was used. Immunofluorescence of anti-rabbit Cy3 and anti-mouse Cy2-conjugated secondary antibodies was visualized with a Nikon E600 microscope and documented with a SPOT digital camera.

Northern blotting

Northern blotting was carried out as described (Kenney et al., 2003). Prime-It II kits (Stratagene) were used to label cDNA probes with P³²-dCTP. Nmyc1 cDNA probe was a gift from R. DePinho (Dana-Farber Cancer Institute).

Transactivation assays

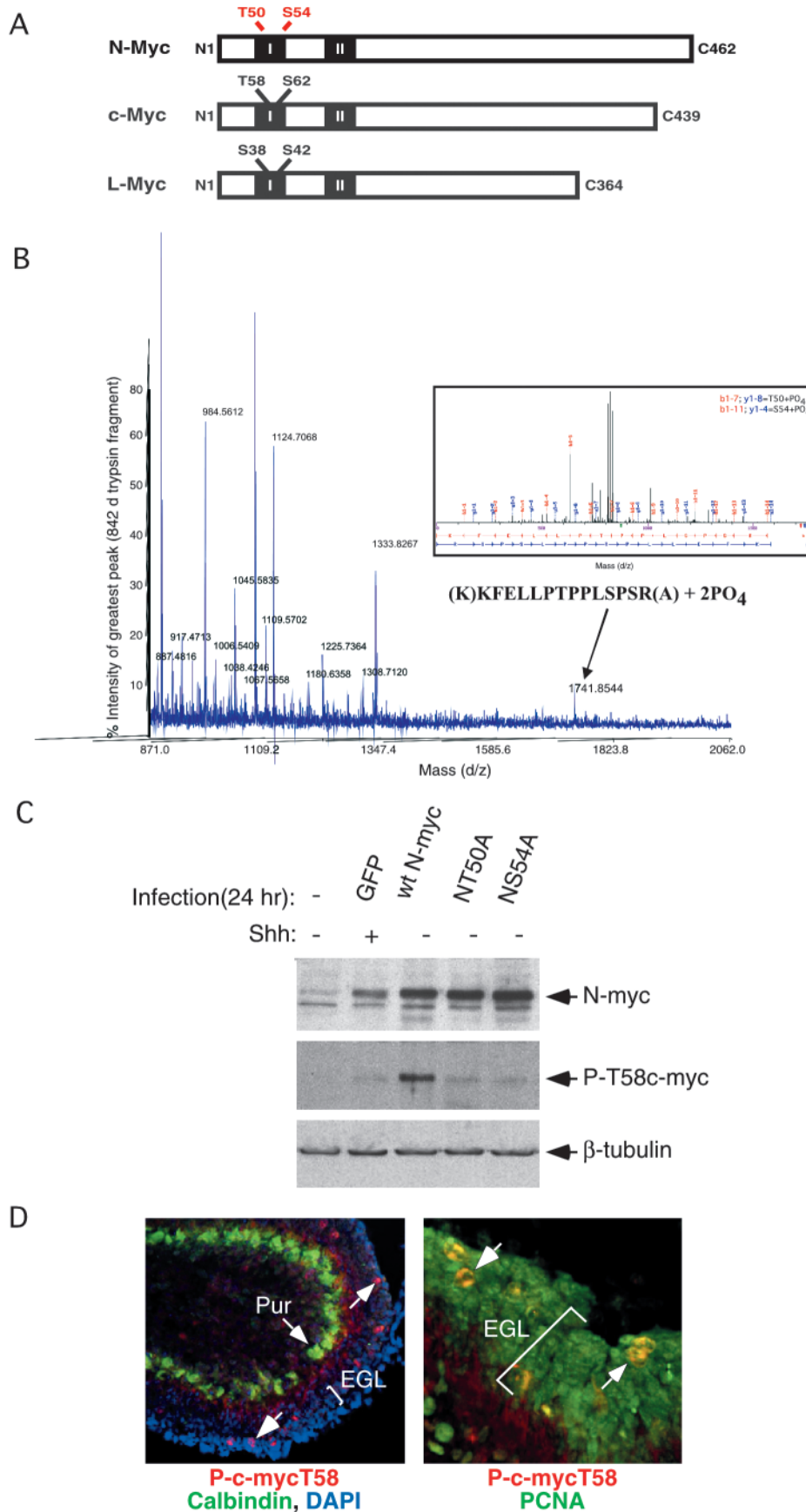
HeLa cells were seeded in 24-well plates at a density of 2×10^4 cells per well in 24-well format and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The following day, the cells were transfected with a mixture containing 20% pGL3-hCDK4 promoter, 10% renilla luciferase construct and 70% effector (pWZL-Nmyc/mutant constructs). Approximately 28 hours after transfection, the cells were harvested and analysed by the dual-luciferase assay system. The results are calculated as average fold activation relative to control pGL3-TK and using renilla luciferase activity values to correct for transfection efficiency. The experiments shown are averages from at least three independent experiments with standard deviation of the mean indicated.

Results

N-terminal phosphorylation of Nmyc1 in vitro and in vivo

Mutation of Myc's N-terminal phosphorylation sites in humans is associated with Burkitt's and other aggressive lymphomas (Albert et al., 1994; Henriksson et al., 1993), suggesting that disruption of the regulatory role played by these sites contributes to human disease. Previous analysis of Myc has implicated phosphorylation of N-terminal sites in Myc turnover (Niklinski et al., 2000; Sears et al., 2000) using rat embryonic fibroblasts (REFs), cell lines or non-mammalian cell systems (Salghetti et al., 1999). By contrast, Nmyc1 phosphorylation has never been established. Indeed, Myc phosphorylation has not been demonstrated in vivo and roles for Myc phosphorylation during normal development have yet to be identified.

Myc, L-Myc and Nmyc1 all feature residues in the highly conserved N-terminal Myc box 1 (MB1) domain, which could function as phosphate acceptor sites (Fig. 1A). We first asked whether Nmyc1 was phosphorylated in the N-terminal transactivation domain. We overexpressed Flag-tagged Nmyc1 in HEK 293 cells, then affinity purified Nmyc1 and subjected the protein to analysis by mass spectrometry, a precise and established means of determining peptide post-translational modifications (Littlepage et al., 2002). Analysis of peptides



derived from Flag-tagged Nmyc1 (Fig. 1B) demonstrated phosphorylation of the T50 and S54 residues of Nmyc1 Myc box 1.

We next sought to determine whether Nmyc1 phosphorylation occurs in neuronal precursors. Primary cultures derived from neonatal mouse cerebellum are a suitable and well-characterized system in which to model granule neuron precursor cell cycle regulation. Proliferative effects of Shh have been well characterized in such cultures (Ho and Scott, 2002). Nmyc1 is induced by Shh in CGNP cultures and it is the sole Myc family member expressed in the EGL *in vivo* (Kenney et al., 2003). To detect Nmyc1 phosphorylation in CGNPs, we tested a commercially available antibody against T-58-phosphorylated Myc (PT58-Myc), whose amino acid sequence in the Myc box 1 domain is highly conserved with Nmyc1 (Henriksson and Luscher, 1996). We infected Shh-treated CGNP cultures prepared from PN 4/5 mice with retroviruses expressing wild-type Nmyc1 or Nmyc1 mutated to non-phosphorylatable alanine residues at T50 or S54 (hereafter referred to as Nmyc1T50A and Nmyc1S54A, respectively). We prepared protein lysates and immunoblotted for total Nmyc1 or T50-phosphorylated Nmyc1 using PT58-Myc antibody. As shown (Fig. 1C), anti-PT58 Myc recognized wild-type Nmyc1 but not Nmyc1T50A or Nmyc1S54A. These results suggest that phosphorylation of T50 requires a priming phosphorylation event at S54, such that disabling S54 phosphorylation prevents T50 phosphorylation. To assess whether phosphorylated Nmyc1 is present *in vivo*, we used the anti-PT58 Myc reagent on histological sections of developing cerebellum. As shown (Fig. 1D), phosphorylated T58Nmyc1 protein was detected specifically in the PN 7 EGL and co-localized to cells expressing proliferating cell nuclear antigen (PCNA). Together, these data suggest that Nmyc1 phosphorylation is a feature of Nmyc1 post-translational modification within proliferating granule neuron precursor cells.

Phosphorylation of Nmyc1 is required for CGNP cell cycle exit

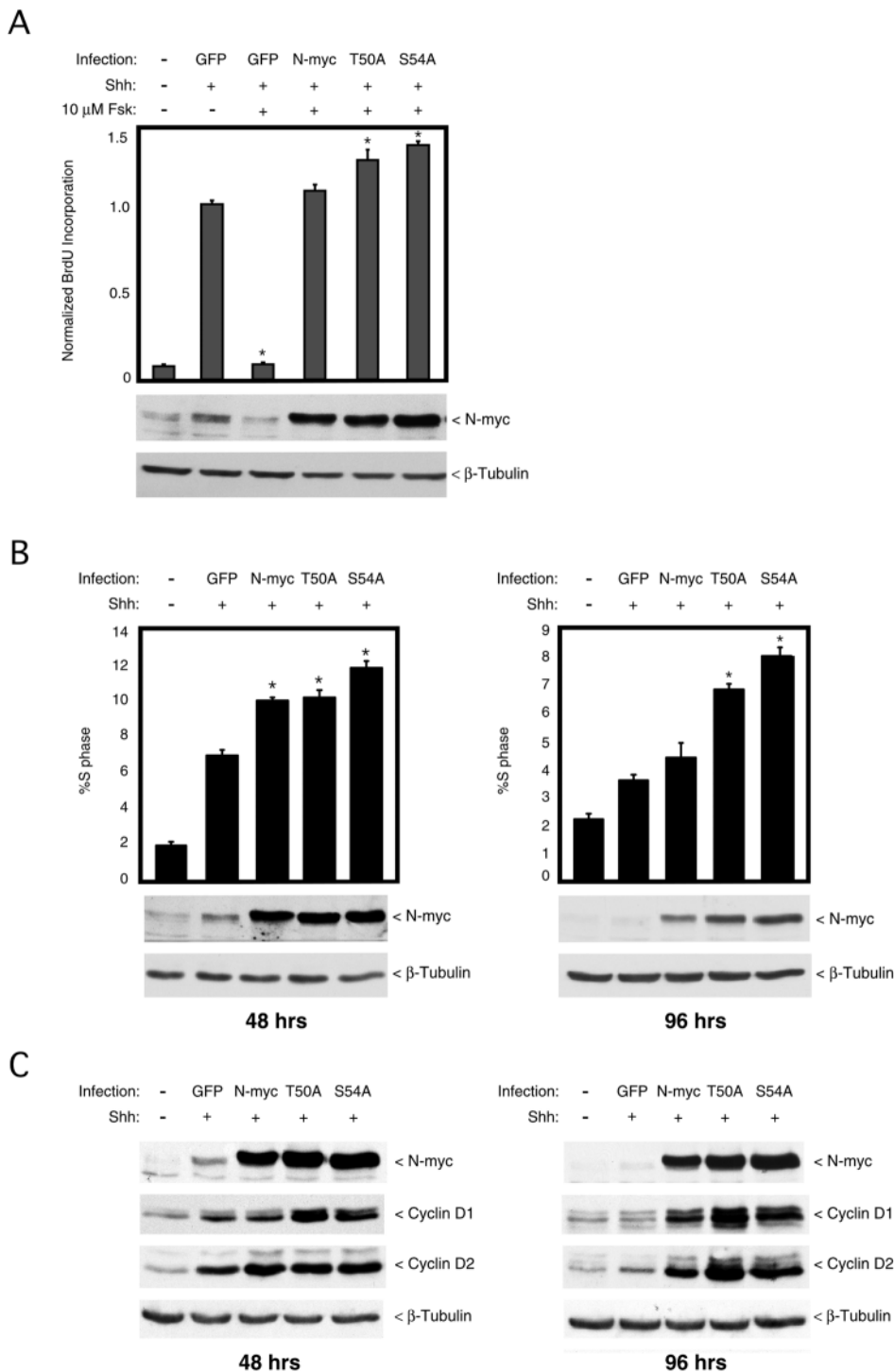
To assess the impact of Nmyc1 phosphorylation on neuronal precursor proliferation, we infected Shh-treated CGNP cultures with retroviruses expressing wild-type Nmyc1, Nmyc1T50A or Nmyc1S54A, and then compared the effects of these on cell cycle progression to green fluorescent protein (GFP)-virus-infected controls. To determine whether, like Nmyc1, Nmyc1T50A and Nmyc1S54A are sufficient for CGNP proliferation in the absence of Shh signaling, we treated infected CGNPs with the protein kinase A activator forskolin, an effective way of inhibiting Shh-induced CGNP proliferation (Kenney et al., 2003; Kenney and Rowitch, 2000; Wechsler-Reya and Scott, 1999). As previously shown (Kenney et al., 2003), ectopic expression of wild-type Nmyc1 maintains proliferation in CGNP cultures independent of Shh signaling (Fig. 2A). We found that Nmyc1 phosphorylation site mutants were similarly capable of maintaining proliferation in CGNP cultures despite treatment with forskolin (Fig. 2A). Indeed, levels of BrdU incorporation in Nmyc1T50A- and Nmyc1S54A-infected, but not wild-type Nmyc1-infected, cultures surpassed those with Shh treatment alone. These results suggest that prevention of Nmyc1 phosphorylation at either site does not result in defective

Nmyc1 protein and might even enhance Nmyc1 function in CGNPs.

The major phase of proliferation in the cerebellar EGL commences at birth and peaks at ~PN 7-8, diminishing thereafter as granule cells leave the cell cycle (Altman and Bayer, 1997). *In vitro*, CGNPs show a limited capacity for proliferation, with most cells eventually withdrawing from the cell cycle even in the presence of Shh (Miyazawa et al., 2000; Wechsler-Reya and Scott, 1999). Because both Nmyc1T50A and Nmyc1S54A showed an enhanced ability to rescue CGNP proliferation, we asked whether prevention of Nmyc1 phosphorylation might affect cell cycle exit. We maintained CGNP cultures in Shh for 2 or 4 days after infection with retroviruses expressing wild-type Nmyc1, Nmyc1T50A, Nmyc1S54A or GFP (control). We then used flow cytometry to assess cell cycle phase distribution. After 2 days of incubation, Shh maintained proliferation and endogenous Nmyc1 expression in control CGNP cultures. Infection with wild-type Nmyc1 or Nmyc1 phosphorylation site mutants resulted in robust levels of CGNP proliferation that were significantly higher than Shh treatment alone (Fig. 2B). However, 4 days after infection (~5.5 days after plating, equivalent to PN 9-10), proliferation levels in Shh-treated control CGNP cultures were greatly diminished. Similarly, S-phase levels in wild-type Nmyc1-infected CGNP cultures were greatly reduced (Fig. 2B) and not elevated significantly above Shh-treated controls. This contrasted with results in both Nmyc1T50A- and Nmyc1S54A-infected CGNP cultures, which showed ongoing proliferation at levels significantly higher than CGNP cultures treated with Shh alone. This enhancement was not due to variable levels of retroviral transcript expression (data not shown) or to differential effects on apoptosis, because flow cytometric analysis of cell cycle phase distribution revealed similar levels of fragmented 'sub-G₁' DNA in cells infected with either mutant or wild-type Nmyc1 retroviruses (data not shown). Together, these findings indicate that activity of non-phosphorylatable Nmyc1 is associated with enhanced CGNP proliferation and delayed cell cycle exit.

One possible explanation for these findings is that wild-type Nmyc1 – but not the phosphorylation mutants – undergoes progressive attenuation of its ability to transactivate target genes necessary for CGNP maintenance in the cell cycle. Recent biochemical and genetic evidence has implicated D-type cyclins as likely Nmyc1 targets in CGNPs *in vitro* and *in vivo* (Ciernerych et al., 2002; Kenney et al., 2003). We therefore assessed levels of cyclins D1 and D2 in Shh-treated CGNPs infected with viruses carrying wild-type Nmyc1 and the Nmyc1 mutants. As shown (Fig. 2C), at 96 hours after infection, all three Nmyc1 proteins tested promoted increased levels of cyclin D1 and D2 relative to untreated, uninfected controls. Notably, wild-type Nmyc1 retains its capacity to upregulate cyclin D1 and cyclin D2 4 days after infection despite the fact that levels of proliferation are not significantly elevated. Oliver et al. (Oliver et al., 2003) similarly found that ectopic expression of D-type cyclins is insufficient for CGNP proliferation. We conclude that the enhanced proliferative effects of Nmyc1 mutant T50A and S54A proteins reflect regulation of additional components of the cell cycle machinery beyond the D-type cyclins.

Fig. 2. Alanine replacement of Nmyc1 T50 or S54 residues results in enhanced proliferative effects and delayed CGNP cell cycle exit. (A) After 24 hours of Shh treatment alone (no serum), CGNP cultures were infected with the indicated viruses and forskolin (10 μ M) was added 24 hours later. After a further 24 hour incubation period, BrdU was added (2 hour pulse) and proliferation was assessed by BrdU immunostaining. The histogram shows levels of BrdU incorporation normalized to Shh-treated, control CGNP cultures as previously described (Kenney et al., 2003; Kenney and Rowitch, 2000). Wild-type Nmyc1 is sufficient for sustained proliferation in the presence of forskolin (fsk). Nmyc1T50A and Nmyc1S54A supported proliferation to a greater extent than wild-type Nmyc1, despite treatment with fsk. The western blot shows relative Nmyc1 protein levels. (B) Nmyc1T50A and Nmyc1S54A extend the phase of CGNP proliferation relative to Shh treatment alone. CGNP cultures were infected as indicated. Cell cycle phase distribution was assessed by propidium iodide flow cytometry at 48 hours (left) and 96 hours (right) after infection. The average percent of cells in S phase \pm s.e.m. derived from three independent experiments is shown in the histograms above representative western blots demonstrating relative Nmyc1 protein levels. Notice that, 96 hours after infection, the proportion of cells in S phase in Nmyc1T50A- and Nmyc1S54A-infected cultures, but not wild-type Nmyc1-infected cultures, remained significantly higher than Shh-treated controls. Western blots below the graphs show relative Nmyc1 protein levels. (C) Nmyc1-, Nmyc1T50A- and Nmyc1S54A-infected cells all show upregulation of cyclins D1 and D2 at 48 hours and 96 hours after infection. Representative western blot autoradiographs are shown. β -Tubulin immunoreactivity indicates equivalent loading of the lanes.



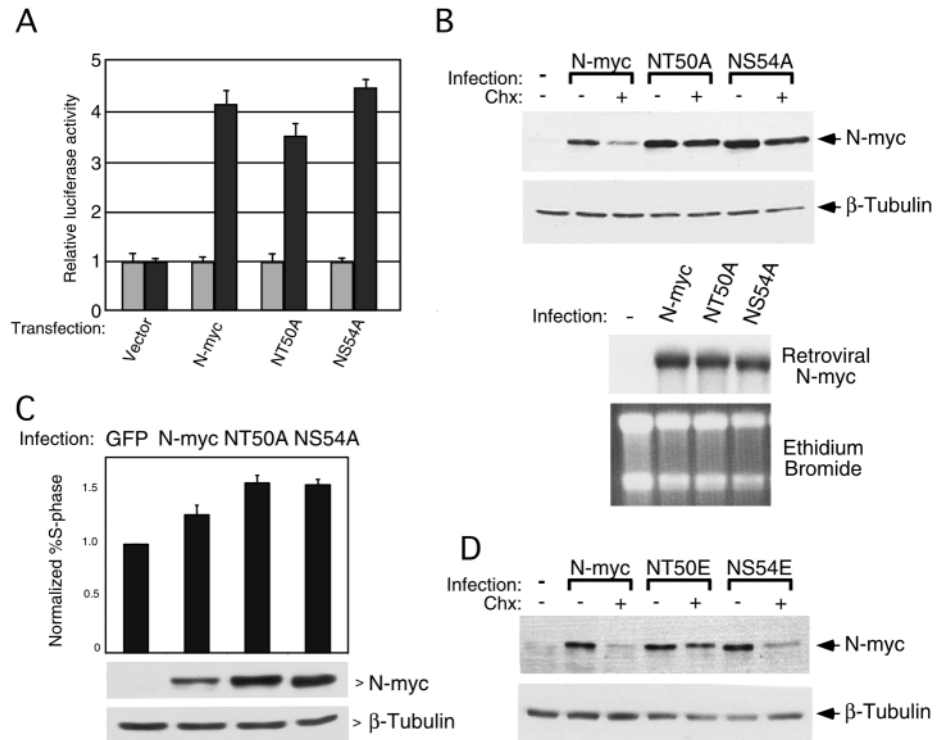
Phosphorylation accelerates Nmyc1 turnover in CGNPs

The mechanism that promotes CGNP proliferation by non-phosphorylatable Nmyc1 mutants could involve activation of additional gene targets. It is also possible that elevation of intracellular Nmyc1 protein levels per se and consequent protein-protein interactions could regulate cell cycle progression. To assess these possibilities further, we first asked whether mutation of N-terminal phosphorylation sites affected the ability of Nmyc1 to transactivate target genes.

Myc family members activate target genes by recognizing an E-box motif in the promoter. To determine effects of phosphorylation site mutation on Nmyc1's transactivation capacity, we transfected HeLa cells with a luciferase reporter under control of an E-box-containing promoter, then compared the ability of Nmyc1, Nmyc1T50A and Nmyc1S54A to transactivate this reporter. As shown in Fig. 3A, we did not observe differences between wild-type Nmyc1 and Nmyc1 phosphorylation mutants in terms of their ability to activate transcription of a luciferase reporter, suggesting

Fig. 3. Nmyc1T50A and Nmyc1S54A show transactivation capacity equivalent to wild-type Nmyc1 but have enhanced stability.

(A) Nmyc1, Nmyc1T50A and Nmyc1S54A were compared for their ability to activate target gene expression as determined by a luciferase reporter assay. The graph depicts relative luciferase activity in HeLa cells co-transfected with Nmyc1 or Nmyc1 phosphorylation mutant retroviral constructs, and a reporter plasmid containing luciferase driven by either the human *Cdk4* promoter (dark bars) or the same promoter lacking E boxes, Myc proteins' cognate DNA recognition sequence (light bars). Results shown are the average of three independent experiments \pm s.e.m. No significant differences in transactivation capacity between wild-type Nmyc1 or the phospho-mutants were detected. (B) Nmyc1T50A and Nmyc1S54A are more stable than wild-type Nmyc1. Primary CGNP cultures were infected with retroviruses expressing wild-type Nmyc1, Nmyc1T50A or Nmyc1S54A. After 24 hours, the cells were pulsed with cycloheximide for 30-90 minutes (three trials per time point) to prevent the synthesis of new protein and to allow an assessment of remaining previously synthesized protein. Protein lysates were prepared and blotted for Nmyc1. A representative western blot is shown from a 90 minute cycloheximide pulse. By 90 minutes, levels of retrovirally expressed wild-type Nmyc1 had substantially declined. Levels of Nmyc1T50A and Nmyc1S54A proteins were not strongly affected by cycloheximide treatment. Relative to retrovirally expressed wild type Nmyc1, increased levels of Nmyc1T50A and Nmyc1S54A protein levels can be seen (left), despite similar levels of retroviral transcript expression, as determined by northern blotting for Nmyc1 (right), consistent with an inability to be phosphorylated leading to protein stabilization and accumulation. (C) Nmyc1T50A and Nmyc1S54A enhance proliferation in human neuroblastoma cells, in comparison with ectopically expressed wild-type Nmyc1. SK-N-SH neuroblastoma cells were infected as indicated and proliferation was assessed after 48 hours using flow cytometry. The graph shows levels of cells in S phase, relative to GFP-infected cells. (D) Mutation of T50 or S54 to aspartic acid has different effects on Nmyc1 turnover. Cycloheximide chase assay was performed as described, and a representative western blot from a 90-minute cycloheximide chase is shown. Nmyc1S54E shows similar turnover characteristics to wild-type Nmyc1, whereas Nmyc1T50E is more stable than wild-type Nmyc1. These results indicate that the effect of phosphorylation at that site requires the presence of the phosphate group and does not depend merely upon the negative charge transferred by phosphorylation.



that phosphorylation does not regulate Nmyc1 transactivation capacity.

To determine whether Nmyc1 protein stability was affected by phosphorylation at T50 and S54, we infected CGNP cultures with wild-type Nmyc1, Nmyc1T50A or Nmyc1S54A retroviruses for 48 hours, and then treated cells with a cycloheximide pulse of up to 90 minutes to prevent new protein synthesis. Levels of wild-type Nmyc1 and the mutant proteins were compared by western blot. Cycloheximide chase is an established method for assaying turnover of cell cycle regulatory proteins (Schmidt et al., 2002), including Myc (Lehr et al., 2003). We chose this method because it permits assessment of relative stability and protein turnover despite the limiting quantities of cells in primary neuronal cell cultures (Monjaraz et al., 2000). As shown (Fig. 3B), we found that Nmyc1T50A and Nmyc1S54A proteins ectopically expressed in CGNP cultures showed little evidence of degradation, even after a 90-minute cycloheximide treatment. By contrast, ectopically expressed wild-type Nmyc1 protein was nearly undetectable. Moreover, we observed that Nmyc1T50A and Nmyc1S54A protein accumulated to higher levels than wild-type Nmyc1 during the 48-hour period after

infection. Increased levels of Nmyc1T50A and Nmyc1S54A were not a result of increased retroviral mRNA expression (Fig. 3B). Together, these findings indicate that prevention of Nmyc1 phosphorylation at T50 or S54 markedly enhances protein stability.

To determine whether the proliferative effects of Nmyc1 phosphorylation site mutations are restricted to CGNPs or might be observed in other cells of nervous system origin, we assessed the effects of Nmyc1, Nmyc1T50A and Nmyc1S54A expression on proliferation of human SK-N-SH neuroblastoma cells. As shown (Fig. 3C), the proportion of cells in S-phase in SK-N-SH cells infected with Nmyc1T50A or Nmyc1S54A was greater than that of cells infected with wild-type Nmyc1. Increased proliferation in cells infected with Nmyc1 phosphorylation mutants were also observed in Daoy cells, a poorly characterized medulloblastoma cell line (data not shown). Thus, inhibition of Nmyc1 phosphorylation at T50 or S54 increases the proliferative effect of Nmyc1 on several types of nervous-system-originating cells.

Transfer of a phosphate group to an amino acid residue results in the placement of a negative charge at that site, as well as affecting higher-order polypeptide structure. To

investigate whether the addition of a negatively charged amino acid would suffice to mimic Nmyc1 phosphorylation, we generated retroviruses expressing non-phosphorylatable Nmyc1 mutants with aspartic acid substitutions at positions 50 and 54 (Nmyc1T50E and Nmyc1S54E). We then infected CGNP cultures with retroviruses expressing these mutants and compared their stability with wild-type Nmyc1 using the cycloheximide pulse assay. We found that replacement of S54 with E resulted in a protein with limited cycloheximide resistance, similar to wild-type Nmyc1 (Fig. 3D), suggesting that the mechanistic consequences of phosphorylation at this site occur principally as a result of negative charge transfer. By contrast, Nmyc1T50E showed evidence of greater cycloheximide resistance than wild-type Nmyc1 (Fig. 3D). We conclude that the destabilizing effects of phosphorylation at Nmyc1 T50 occur in response to information provided by the phosphate group in addition to the negative charge it carries. Indeed, this has been previously reported for proteins whose half-life is regulated by phosphorylation (Lo et al., 2001).

Phosphorylation of Nmyc1 on T50 depends on GSK3

Several extracellular signals have been proposed to modulate proliferative effects of Shh in CGNP cultures, including those that activate RTK, Notch and chemokine receptors (Klein et al., 2001; Solecki et al., 2001; Wechsler-Reya and Scott, 1999). However, the precise intracellular mechanisms underlying such interactions are unclear. Because Nmyc1 phosphorylation and degradation are associated with CGNP cell cycle exit, we hypothesized that other signaling pathways might synergize with mitogenic effects of Shh via inhibition of Nmyc1 N-terminal phosphorylation. We first asked whether Shh signaling itself might, in addition to its roles in promoting Nmyc1 expression (Kenney et al., 2003), regulate Nmyc1 phosphorylation and turnover. We assayed for effects of Shh signaling on Nmyc1 phosphorylation on T50 by comparing the ratio of T50-phosphorylated Nmyc1 to total Nmyc1 in Shh-treated CGNPs with that of CGNPs when Shh signaling was blocked. To inhibit Shh signaling, we treated CGNP cultures with cyclopamine and submitted protein lysates to western blot analysis to determine the relative levels of wild-type and T50-phosphorylated Nmyc1 (Fig. 4A). Because Nmyc1 mRNA and protein levels diminish when Shh signaling is inhibited (Kenney et al., 2003), we used lactacystin [a proteasome inhibitor that blocks Nmyc1 degradation in neuroblastoma cells (Bonvini et al., 1998)] to preserve detectable levels of endogenous Nmyc1 protein. As shown in Fig. 4A, we observed that the level of T50-phosphorylated Nmyc1 relative to total Nmyc1 was similar in the presence or absence of Shh signaling. Because T50 phosphorylation requires phosphorylation of S54 (Fig. 1C), Shh activity at that site would be reflected in increased or decreased levels of T50 phosphorylation relative to total Nmyc1. Together, our results argue against a role for Shh in the regulation of Nmyc1 phosphorylation *in vitro*.

The sequence surrounding T50 includes a consensus GSK3 target sequence (Cohen and Frame, 2001). To determine whether GSK3 activity is necessary for phosphorylating endogenous Nmyc1 T50, we treated CGNPs with lithium (Li⁺), a classical antagonist of GSK activity (Cohen and Frame, 2001). As shown in Fig. 4B (left), Li⁺ resulted in a decrease in

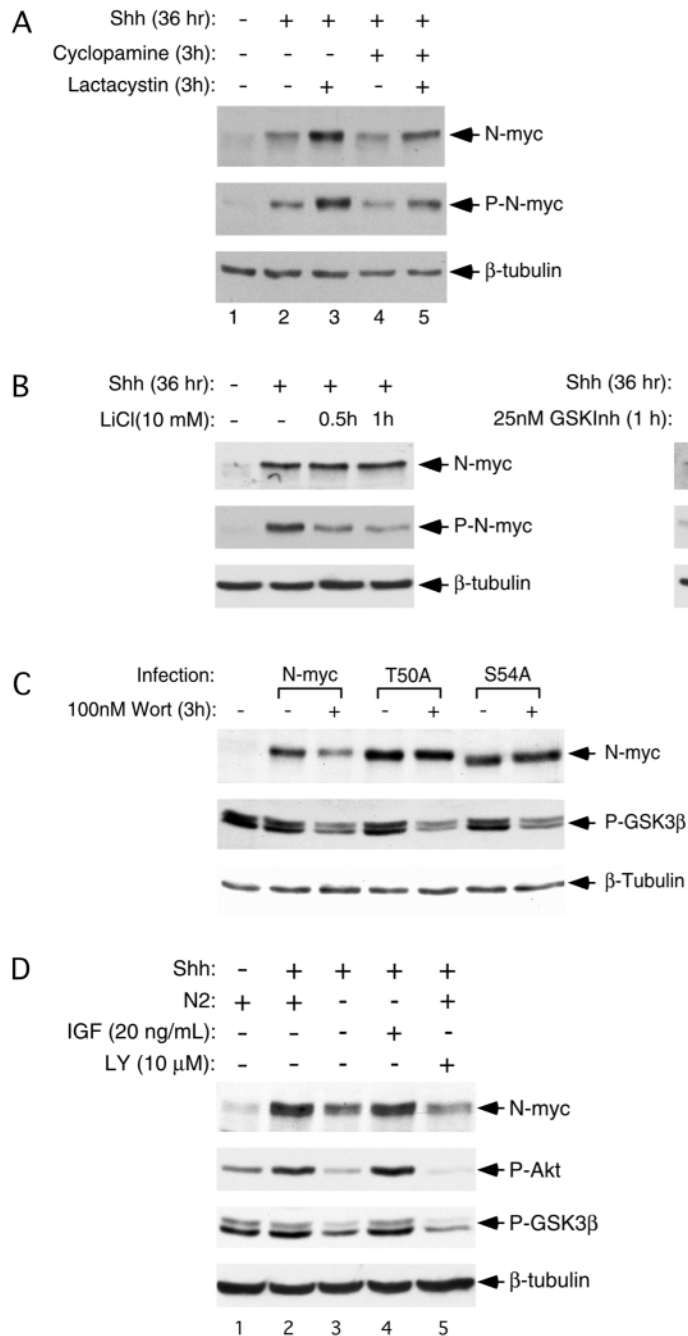
Nmyc1 T50 phosphorylation, detectable by 30 minutes and pronounced by 60 minutes. A commercially available GSK3 inhibitor had similar effects (Fig. 4B, right). These results indicate that GSK3 activity is necessary for phosphorylating Nmyc1 at T50.

Phosphorylation of Nmyc1 is regulated by PI3K signaling

If GSK3-mediated phosphorylation of Nmyc1 at T50 triggers its degradation, it follows that increased activity of GSK would destabilize wild-type Nmyc1. By contrast, based on our earlier observation (Fig. 3), we predicted that the stability of the non-phosphorylatable Nmyc1T50A and Nmyc1S54A would be unaffected by increasing GSK3 activity. Phosphorylation of GSK3 by PKB/Akt kinase causes GSK3 inactivation and PKB/Akt activity in CGNP cultures is positively regulated by PI3K (Dudek et al., 1997). We asked whether PI3K might stabilize Nmyc1 by modulating GSK3. We used wortmannin, a PI3K inhibitor, to promote increased GSK3 activity in CGNP cultures infected with vector carrying GFP, wild-type Nmyc1, Nmyc1T50A or Nmyc1S54A, and then used western blotting to assay the relative stability of the ectopically expressed Nmyc1 proteins. The effectiveness of wortmannin was demonstrated by reduced levels of phosphorylated (inactive) GSK3 in all of the treated samples (Fig. 4C). After 3 hours of wortmannin exposure, we found that levels of Shh-induced, endogenous Nmyc1 were substantially reduced (Fig. 4C, left). Likewise, retrovirally expressed wild-type Nmyc1 protein dropped in the presence of wortmannin (Fig. 4C, right). These findings suggest that increased GSK3 activity results in more rapid turnover of wild-type Nmyc1. Although it is possible that decreased levels of wild-type Nmyc1 in wortmannin-treated CGNP cultures could reflect reduced Nmyc1 protein synthesis (Brown and Schreiber, 1996), this is unlikely because wortmannin treatment had no effect on levels of retrovirally expressed Nmyc1T50A and Nmyc1S54A proteins (Fig. 4C, right). Indeed, these results show that the ability to be phosphorylated at T50 (and S54) is a requirement for Nmyc1 to be destabilized by PI3K pathway inhibition.

Previous work indicates that the PI3K pathway is required for granule cell survival and proliferation (Dudek et al., 1997). Activity of PI3K could regulate Nmyc1 turnover in response to multiple factors found in the developmental milieu. Indeed, several PI3K-activating pathways have been reported to enhance granule cell proliferation *in vivo* and *in vitro*. These include signaling through SDF-CXCR4, integrin receptors (which mediate CGNP cell-cell contacts) and IGF1 (Gao et al., 1991; Klein et al., 2001; Ye et al., 1996). In cultured CGNPs, signaling through the IGF receptor (IGFR) potently activates PI3K (Dudek et al., 1997).

CGNPs are normally cultured in N2 medium, which contains insulin at levels high enough to activate the IGFR (Dudek et al., 1997). To determine whether IGFR signaling stabilizes endogenous Nmyc1, we assessed the effects of insulin (N2) withdrawal on endogenous Nmyc1 protein levels. We cultured cells in N2 in the absence (Fig. 4D, lane 1) or presence (Fig. 4D, lanes 2-5) of Shh for 24 hours, at which time the medium was replaced as indicated (Fig. 4D). We observed a striking reduction in levels of Nmyc1 protein in CGNP cultures treated for 3 hours with Shh-supplemented medium lacking any N2 or IGF1 (Fig. 4D, lane 3). Reduced



IGFR stimulation resulted in decreased phosphorylation of Akt, a well-established downstream target of PI3K. We also observed decreased GSK3 phosphorylation, indicating increased GSK activity. We found similar reductions in Nmyc1 protein levels and evidence of decreased PI3K activity in cells treated with LY294002, a PI3K inhibitor (Fig. 4D, lane 5). Addition of IGF1 for 3 hours was sufficient to maintain Nmyc1 protein levels (Fig. 4D, lane 4), and IGF1- and N2-supplemented media were equally able to sustain phosphorylation of Akt and GSK3. These results indicate that, in the context of CGNP primary cultures, IGF1 stabilizes Nmyc1 proteins via activation of the PI3K pathway. They do not rule out the possibility that there are additional mechanisms to activate PI3K signaling in CGNPs in vivo or in vitro.

Fig. 4. Nmyc1 phosphorylation is regulated by GSK3 and the PI3K pathway in CGNPs, independent of Shh signaling. (A) CGNP cultures were treated with Shh, cyclopamine and/or the proteasome inhibitor lactacystin, which effectively blocks ongoing turnover of endogenous Nmyc1 (compare lanes 3 and 5 to lanes 2 and 4). Lysates were blotted to determine levels of T50-phosphorylated Nmyc1 relative to total Nmyc1 and results representative of three independent experiments are shown. Tubulin immunoreactivity indicates equivalent loading of the lanes. The level of T50-phosphorylated Nmyc1 relative to total Nmyc1 was comparable regardless of treatment with Shh or cyclopamine, indicating that Shh signaling has neither positive (destabilizing) nor negative (stabilizing) effects on endogenous Nmyc1 phosphorylation. (B) GSK3 activity is required for endogenous Nmyc1 phosphorylation. Shh-treated CGNP cultures were exposed to LiCl (left) or a commercially available GSK3 inhibitor (right) for the indicated length of time. Both methods for blocking endogenous GSK3 activity effectively reduced levels of Nmyc1 phosphorylation. (C) Activity of PI3K is required for stabilizing Nmyc1. CGNP cultures were infected as indicated.

After 24 hours, the cells were treated (3 hours) with wortmannin, a PI3K inhibitor. In the presence of wortmannin, levels of wild-type retroviral Nmyc1 were sharply reduced. By contrast, levels of Nmyc1T50A and Nmyc1S54A were unaffected, ruling out a general effect of wortmannin treatment on protein synthesis. These results also show that the non-phosphorylatable mutants do not depend on the PI3K pathway for stabilization. (D) IGF, a prominent activator of PI3K in CGNPs, stabilizes endogenous Nmyc1. CGNPs cultured in N2 (insulin)-containing medium were treated with (lanes 2-5) or without Shh (lane 1) for 24 hours. At this time, medium in lanes 2-4 was withdrawn and cells were washed extensively and treated with fresh medium containing Shh (lanes 2-5), N2 (lanes 2, 5), no N2 or IGF1 (lane 3), IGF1 (20 ng ml⁻¹, lane 4), or LY294002 (10 μM, lane 5). After 3 hours, protein lysates were prepared and western blotted for Nmyc1, phosphorylated Akt, and phosphorylated GSK3. Lack of N2 or IGF1 resulted in downregulation of the PI3K pathway, and decreased Nmyc1 protein. N2 and IGF1 were each capable of supporting PI3K pathway activity and Nmyc1 protein levels. LY294002 inhibited the PI3K pathway and promoted destabilization of endogenous Nmyc1.

Discussion

A crucial aspect of CNS organogenesis is the precise regulation of precursor population numbers. Shh signaling is essential for Nmyc1 expression and proliferation of developing cerebellar granule cell precursors, whereas activators of PI3K signaling (e.g. IGF, CXCR4) appear to regulate growth and survival as well as the magnitude of Hedgehog-induced proliferation. The intracellular targets that coordinate effects of these divergent signaling pathways, however, are poorly understood.

In the present study, we have investigated how post-translational modification of Nmyc1 affects its activity in neuronal precursors using the extensively characterized Shh-treated CGNP primary culture system. We found that Nmyc1 is phosphorylated on two highly conserved residues within Myc box 1. Our research has led to the implication of Nmyc1 phosphorylation in an important biological process, the regulation of cell cycle exit during CNS development. Indeed,

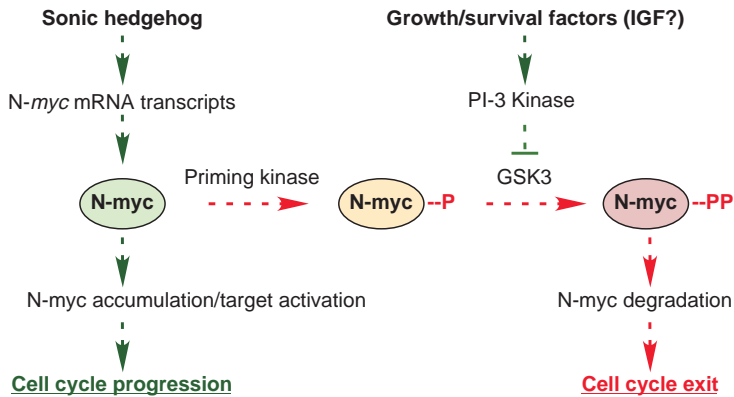


Fig. 5. Model for combined effects of Shh signaling and PI3K on cell cycle progression through Nmyc1 regulation in CGNP cultures. Previous work indicates that Shh signaling induces expression of the proto-oncogene transcription factor Nmyc1. Nmyc1 is necessary and sufficient for maintaining CGNP proliferation. The present findings indicate that maintenance of Nmyc1 protein levels in vitro is regulated in a Shh-independent manner. Our data support a role for IGF1 signaling in activation of the PI3K pathway, which inhibits GSK3-mediated phosphorylation of Nmyc1 T50 and its subsequent degradation. The kinase priming Nmyc1 for GSK3 phosphorylation, by acting at S54, remains to be identified. Other growth factors (e.g. SDF-1) or cell-cell interactions (e.g. via integrins) are candidates for PI3K regulation and inhibition of GSK3 activity in vivo. The combined effect of concerted Shh and PI3K activity is to promote G1 progression and precisely control the timing of cell cycle exit.

our studies are the first to demonstrate that phosphorylation is an important mechanism for regulation of Myc family protein levels during normal development. We found that phosphorylation of endogenous Nmyc1 can be prevented by inhibition of GSK3, and that endogenous Nmyc1 is stabilized by PI3K activation. These data, together with previous work (Kenney et al., 2003; Oliver et al., 2003), suggest that Shh mitogenic effects in the developing cerebellum result primarily from promoting increased levels of Nmyc1 expression and that, in turn, PI3K signaling inhibits GSK3-dependent phosphorylation and turnover of Nmyc1 protein. The cumulative result of Shh and PI3K signaling in CGNP cultures is to promote increased Nmyc1 levels and ongoing proliferation (Fig. 5).

The highly tissue-specific expression patterns of *Myc* genes and their antagonists, Mad family members (Chin et al., 1995; Dildrop et al., 1988; Hirvonen et al., 1990; Hurlin et al., 1995; Queva et al., 1998; Strieder and Lutz, 2002), suggest non-overlapping roles during development. Nmyc1 activity clearly plays a crucial role in the expansion of nervous system progenitor populations. Nmyc1-null mutants die between 10.5 days and 12.5 days of embryogenesis (Charron et al., 1992; Sawai et al., 1991; Stanton et al., 1992) with hypoplasia and structural defects in the developing nervous system. This occurs despite compensatory upregulation of *Myc* in some neuroepithelial tissues (Stanton et al., 1992). These observations suggest that Nmyc1 expression in the nervous system is uniquely responsive to specific developmental cues, rather than occurring as a response to general growth factor signaling, which might also upregulate *Myc*.

A requirement for Nmyc1 function in CNS precursors was

shown by Knoepfler et al. (Knoepfler et al., 2002), who followed a CNS-specific conditional gene ablation strategy. Their animals exhibited marked defects, including overall decreased brain size and evidence of premature neuronal differentiation. Additional abnormalities were found in the cerebellum, where granule neuron progenitor proliferation is known to depend on Shh signaling (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). In vitro, Shh treatment of CGNPs results in Nmyc1 upregulation but not induction of *Myc* or *L-Myc* in the presence or absence of new protein synthesis (Kenney et al., 2003). Thus, specificity for Nmyc1 function in CGNPs is probably due to precise upstream transcriptional regulation by the Hedgehog signaling pathway, a mechanism likely to be conserved in vivo, because Nmyc1 is the only *Myc* family member expressed in the proliferating EGL (Kenney et al., 2003).

IGF1-mediated activation of the PI3K pathway is crucial for the long-term survival of cultured CGNPs (Dudek et al., 1997) and, under our culture conditions, insulin is present at levels sufficient to activate the IGF receptor. We found that short-term withdrawal of insulin substantially destabilized Nmyc1 in cultured CGNPs, and that this destabilization could be prevented by substitution of insulin with IGF. IGF signaling is important for CNS development, and increased IGF activity results in cerebellar hyperplasia (de Pablo and de la Rosa, 1995; Ye et al., 1996). In addition to enhancing growth by promoting survival, IGF-mediated activation of PI3K could have positive effects on the cell cycle regulatory apparatus. Indeed, PI3K negatively regulates Forkhead transcription factors, which can promote cell cycle exit by repressing cyclin D (Schmidt et al., 2002). The finding that IGF-stimulated PI3K activity can stabilize Nmyc1 provides additional insight as to the molecular mechanisms underlying the pro-proliferative effects of PI3K signaling in neuronal precursors.

Our data indicate that Nmyc1 T50 phosphorylation in CGNP cultures depends on GSK3, suggesting that Nmyc1 turnover is regulated by PI3K signaling. In addition to IGF, PI3K can be activated by other factors present in the milieu of the developing cerebellum, including CXCL12-CXCR4 and integrin signaling. Additionally, Wnt signaling is a powerful antagonist of GSK3 activity, although Wnt-mediated GSK3 regulation is distinct from the PI3K pathway (Cohen and Frame, 2001). We observed that inhibition of Shh signaling did not affect Nmyc1 phosphorylation in CGNP cultures. However, an indirect role for Shh in regulating Nmyc1 phosphorylation cannot be ruled out. Indeed, several lines of evidence suggest that IGF pathway components are transcriptional targets of Shh in medulloblastomas (Hahn et al., 2000), CGNPs (Oliver et al., 2003; Zhao et al., 2002) and a Shh-responsive cell line (Ingram et al., 2002). These findings suggest that Hedgehog and PI3K signaling interactions could be regulated during cerebellar development in vivo by local Shh induction of upstream PI3K activators (e.g. IGF2). We have focused on granule neuron precursors of the cerebellum but the ability of Shh and IGF to promote proliferation in neural stem cells (Arsenijevic et al., 2001; Lai et al., 2003) suggests that a synergy between PI3K and Shh signaling might apply to additional CNS precursor

populations, including those of the adult brain. In keeping with this, we note that Nmyc1 is expressed in the post-natal (P10) mouse hippocampus (A.M.K. and D.H.R., unpublished). Further work is required to establish precise roles for Nmyc1 phosphorylation in neural progenitors in vivo.

We observed that increasing GSK3 activity resulted in enhanced Nmyc1 turnover, probably through T50 phosphorylation. Conversely, preventing the phosphorylation by mutating T50 or S54 to alanine resulted in Nmyc1 accumulation. GSK3 requires a priming phosphorylation event in order to recognize its target site (Cohen and Frame, 2001). A probable Nmyc1 GSK3 priming site is S54, which is analogous to Myc S62. Our findings indicate that S54 phosphorylation is required for phosphorylation of Nmyc1 T50, consistent with regulation by GSK3-dependent kinase activity. Further, it is clear that the inability to be phosphorylated at Nmyc1 S54 stabilizes the protein. In contrast, some studies have suggested that MAPK activity at S62 transiently stabilizes Myc before T58 phosphorylation by GSK3 in vitro (Sears et al., 2000). Others indicate that MAPK might not phosphorylate Myc (Lutterbach and Hann, 1999), and we have shown that MAPK is dispensable for Shh proliferative effects in CGNP cultures (Kenney and Rowitch, 2000). We conclude that MAPK is unlikely to prime S54 phosphorylation of Nmyc1 in CGNPs and that the identity of the priming kinase remains to be determined.

Our observation of Nmyc1S54A mutant protein stabilization and enhanced proliferative effects is in contrast to some Myc studies, which have suggested that mutation of S62 might cripple Myc function in fibroblasts or cell lines (Chang et al., 2000; Sears et al., 2000). Others have shown that mutation of Myc S62 enhances its ability to transform REFs (Henriksson et al., 1993). Although these findings might highlight general differences in the regulation of Myc and Nmyc1 activity, they might also reflect features of cell-type-specific regulation in neuronal cells, as opposed to the non-neuronal cells used in the Myc studies. In any case, our studies demonstrate that N-terminal phosphorylation of Nmyc1 in CGNPs promotes protein turnover and cell cycle exit rather than affecting the ability of Nmyc1 to regulate target gene transcription. The mechanism through which stabilized Nmyc1 enhances CGNP proliferation remains to be determined and might involve other Myc functions such as transcriptional repression (Wanzel et al., 2003) or direct antagonistic interactions with proteins that promote cellular differentiation (Wechsler-Reya et al., 1998).

Although we were unable to establish culture conditions of sufficient duration to determine the effects of GSK3 inhibition on endogenous Nmyc1 accumulation, it has been reported that inhibition of GSK activity enhances CGNP proliferation (Cui et al., 1998). We observed that accumulation of phosphorylation-mutant proteins took place over a relatively long time course (48-96 hours), whereas GSK3-mediated degradation could be measured after only a few hours. These findings suggest that additional mechanisms for Nmyc1 degradation exist in CGNPs. For example, ubiquitination of Myc via association Myc box 2 domain with ubiquitin ligases results in protein turnover (Kim et al., 2003; Lehr et al., 2003). In *Drosophila*, GSK3 phosphorylation of the canonical Hedgehog signaling target cubitus interruptus leads to its proteolysis (Jia et al., 2002; Price and Kalderon, 2002), raising

the intriguing possibility that GSK3-mediated antagonism might be a general feature of Hedgehog signaling.

Abnormally prolonged or increased activity of the Myc family of basic helix-loop-helix leucine zipper transcription factors is associated with many types of cancer (Nesbit et al., 1999). Nmyc1 function, in particular, is implicated in the generation and/or maintenance of neuroblastomas, gliomas and Hedgehog-associated cases of medulloblastoma in humans and mice (Herms et al., 1999; Kenney et al., 2003; Nesbit et al., 1999; Oliver et al., 2003; Pomeroy et al., 2002). We observed that Nmyc1 phosphorylation takes place in the developing cerebellum in vivo, and that preventing Nmyc1 phosphorylation enhances CGNP proliferation. Furthermore, we show that IGF-mediated PI3K activation is an important regulator of Nmyc1 stability in primary cerebellar cultures. Interestingly, evidence of increased IGF pathway activity and IGF2 expression has been found in human and animal models of medulloblastomas (Del Valle et al., 2002; Pomeroy et al., 2002). Our data suggest that increased Nmyc1 stabilization caused by PI3K pathway activation could occur as a result of enhanced IGF pathway activity in tumors, thereby contributing to tumor growth. Manipulation of signaling pathways to promote GSK3-dependent phosphorylation of Nmyc1 protein might therefore prove an effective adjuvant anti-tumor strategy in such cases of cancer in humans.

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