Oligodendrocyte Specification in Zebrafish Requires Notch-Regulated Cyclin-Dependent Kinase Inhibitor Function

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Cyclin-dependent kinase inhibitors (Cdks) influence both cell-cycle progression and differentiation of neural cells. However, the precise roles of Cdks in coordinating formation of neurons and glia and the mechanisms that regulate expression of genes that encode Cdks in the vertebrate CNS remain unknown. Here, we report that, in zebrafish, expression of the Cdk gene cyclin-dependent kinase inhibitor 1c (cdkn1c), a p57 homolog, is negatively regulated by Delta–Notch signaling and that Cdkn1c function is required for neural plate cells to stop dividing and differentiate as neurons on schedule, even in the absence of Notch signaling activity. Furthermore, Cdkn1c function is required for specification of oligodendrocytes from ventral spinal cord precursors. We propose that levels of cdkn1c expression are an important factor in regulating neural development: high levels of Cdkn1c promote cell-cycle exit and neuronal development, whereas, during late embryogenesis, neural cells that have low but functional levels of Cdkn1c, regulated by Notch activity, are specified for oligodendrocyte fate.

Key words: oligodendrocyte; cell cycle; cell fate; neural precursor; neurogenesis; spinal cord

Introduction

During vertebrate neural development, proliferative precursor cells produce different types of postmitotic neurons at different times, revealing a correlation between the time at which a precursor stops dividing and neuronal identity. A particularly good illustration of this was provided by experiments in which cerebral cortex precursors were transplanted from young donors to older hosts (McConnell and Kaznowski, 1991). Remarkably, precursors that remained proliferative at the time of transplantation adopted fates appropriate for the host, whereas those that stopped dividing before transplantation retained donor identity. Thus, specification of multipotent cortical precursors for particular neuronal identities apparently occurred at about the time of cell-cycle exit. Cell-cycle control, therefore, might be an essential feature of mechanisms that specify neuronal fate.

Timing also seems to be important for glial development because, in general, glia are produced after neurons. However, for oligodendrocytes, the myelinating cell type of the CNS, the relationship between the proliferative status of a neural precursor and specification for oligodendrocyte fate is not clear. In particular, cells fated to give rise to oligodendrocytes do not stop dividing but exist as proliferative oligodendrocyte progenitor cells (OPCs), which have characteristic behaviors, morphologies, and gene expression profiles (Baumann and Pham-Dinh, 2001; Miller, 2002). Eventually, many OPCs stop dividing and differentiate as myelinating oligodendrocytes, whereas others apparently persist into adulthood (Levison et al., 1999; Horner et al., 2000). Here, cell-cycle control influences the balance of immature OPCs and myelinating oligodendrocytes.

Molecules that likely play widespread roles in the timing of neuronal and glial development include cyclin-dependent kinase inhibitors (Cdks). Cdks negatively regulate complexes composed of cyclins and cyclin-dependent kinases (Cdks), which promote progression through the cell cycle (Cunningham and Roussel, 2001). For instance, cyclin E assembles with cdk2 to drive the G1 to S phase transition. Cdki proteins of the Cip/Kip class, which include p21Cip1, p27Kip1, and p57Kip2, bind to cyclin E-cdk2 in a 1:1 stoichiometry and prevent entry into S phase. Although various experimental strategies provided evidence that Cdki functions promote formation of postmitotic neurons and regulate proliferation and differentiation of OPCs (Casaccia-Bonnefil et al., 1997, 1999; Durand et al., 1998; Zezula et al., 2001; Carruthers et al., 2003; Vernon et al., 2003), the mechanisms that control Cdki expression in the developing CNS and whether Cdki function is necessary to specify neural precursors for oligodendrocyte fate have not been fully explored.

This report describes a series of loss- and gain-of-function experiments designed to investigate regulation of cdki gene expression and how cdki function influences the timely formation of neurons and oligodendrocytes. Our results show that, in zebrafish, Notch signaling limits the number of neural cells that express cyclin-dependent kinase inhibitor 1c (cdkn1c), and cdkn1c function is necessary for production of early born neurons at the appropriate time. Surprisingly, we also learned that cdkn1c func-
tion is necessary for OPC specification from neural precursors, thereby expanding the previously described roles of Cdks in oligodendrocyte development.

Materials and Methods

Fish breeding and maintenance. Embryos were produced by pairwise matings, raised at 28.5°C in egg water or embryo medium (EM) (Westerfield, 2000), and staged according to hours postfertilization (hpf), days postfertilization (dpf), and morphological criteria (Kimmel et al., 1995). Tg(ollg2:egfp)XdnSu(H)myc, pCS2hsp70 at the 3′ created a reading frame fusion to sequence encoding the Myc epitope tag. We mated germ-line-transformed founders to wild-type fish, raised the anti-Myc immunocytochemistry. To establish stable lines, we again heat shocked embryos by incubating them at 40°C for 30 min and, after a recovery period, fixed them and performed anti-Myc immunocytochemistry. To confirm expression in one-fourth of the embryos should inherit both transgenes, which we confirmed by anti-Myc immunocytochemistry (data not shown). To induce expression of Cdkn1cMyc or a Myc-tagged dominant-negative DNA-binding mutant form of frog Suppressor of Hairless (dnSu(H)Myc), we intercrossed Tg(hsp70:cdkn1c-myc) or Tg(hsp70: XdnSu(H)Myc) adults, respectively, raised embryos at 28.5°C, transferred them to EM at 40°C for 30 min, and continued incubation at 28.5°C until appropriate stages for fixing.

Morpholino injections. An antisense morpholino oligonucleotide (MO) having the sequence 5′-TGCCATATGTCTAAAGTGCTA-TGC-3′ (Gene Tools, LLC, Philomath, OR) was designed to target the cdkn1c 5′-untranslated region sequence and translation start site. The morpholino oligonucleotide was dissolved in 1× Danieau solution at a concentration of 20 μg/μl and further diluted with distilled water. Two nanograms of morpholino oligonucleotide were injected into one- to two-cell-stage embryos. For control experiments, a similar amount of a standard control MO having the sequence 5′-CCCTTCTACCTGAT- TACAATTTATA-3′ was injected. To test the ability of cdkn1c MO to block Cdkn1c expression, we injected MO into embryos produced by Tg(hsp70:cdkn1c-myc) adults and heat shocked embryos at 7.5 hpf by incubating them at 38°C for 30 min, fixed them at 11 hpf, and processed them for anti-Myc immunocytochemistry.

Results

Identification of zebrafish cdkn1c

We identified zebrafish expressed sequence tags (ESTs) with sequence similarity to p27 and p57 genes of other vertebrate species using basic local alignment search tool (Altschul et al., 1990). These ESTs were derived from genes now known as cdkn1b and cdkn1c, respectively (Sprague et al., 2001). We did not find ESTs with homology to p21. Whole-mount in situ RNA hybridization detected no cdkn1b expression during early stages of neural development (data not shown, but see ZFIN, http://zfin.org/cgi-bin/webdriver?Mival = aa-XPatview.apg&OID = ZDB-XPAT-030521-5). In contrast, neural plate and spinal cord cells expressed cdkn1c (Figs. 1, 2A). Notably, the neural plate expression of cdkn1c is like that of p27Xic1 of Xenopus laevis and Xenopus tropicalis (Hardcastle and Papalopulu, 2000; Carruthers et al., 2003; Vernon et al., 2003). Thus, Cdkn1c may regulate early neural development in zebrafish similarly to p27Xic1 in frogs.

Notch signaling regulates cdkn1c expression

We showed previously that Notch signaling is required to limit the formation of early born primary neurons and maintain proliferative neural precursors (Appel et al., 2001; Park and Appel, 2003). Because p27Xic1 promotes formation of neurons within the neural plate of frogs (Carruthers et al., 2003; Vernon et al., 2003), we speculated that maintenance of neural precursors by Notch is mediated by transcriptional repression of cdkn genes. Therefore, we compared the distribution of cdkn1c transcripts between embryos that had different levels of Notch activity. In wild-type embryos, neural plate cells expressed cdkn1c RNA in a mosaic pattern, consistent with the pattern of the earliest born primary neurons, and adaxial mesoderm cells expressed cdkn1c uniformly at high level (Fig. 1A, D). Cells that expressed cdkn1c did not incorporate the thymidine analog BrdU, a marker of S-phase cells, which supports the idea that nonproliferative cells express cdkn1c (Fig. 3G). In both dla+/−/ddl/− embryos, which lack functions of the DeltaA and DeltaD ligands (Appel et al., 1999; Holley et al., 2000), and mb−/− embryos, which are deficient for Heat-induced gene expression. To induce expression of constitutively active Notch1a, embryos were collected from matings of Tg(hsp70: GAL4) and Tg(UAS:Notch1a−/−-myc) adults and raised at 28.5°C. At 7.5 hpf, embryos were transferred to EM at 39.0°C for 30 min and then returned to 28.5°C until the three-somite stage (11 hpf). Approximately one-fourth of the embryos should inherit both transgenes, which we confirmed by anti-Myc immunocytochemistry (data not shown). To induce expression of Cdkn1cMyc or a Myc-tagged dominant-negative DNA-binding mutant form of frog Suppressor of Hairless (dnSu(H)Myc), we intercrossed Tg(hsp70:cdkn1c-myc) or Tg(hsp70: XdnSu(H)Myc) adults, respectively, raised embryos at 28.5°C, transferred them to EM at 40°C for 30 min, and continued incubation at 28.5°C until appropriate stages for fixing.
an E3 ubiquitin ligase necessary for efficient Notch signaling activity (Itoh et al., 2003), excess neural plate cells expressed cdkn1c RNA, but adaxial expression appeared normal (Fig. 1B,C,E). This is consistent with formation of excess primary neurons in these mutant embryos (Jiang et al., 1996; Schier et al., 1996; Appel et al., 2001; Itoh et al., 2003). In contrast, forced expression of a constitutively active form of Notch1a (Scheer and Campos-Ortega, 1999; Scheer et al., 2001, 2002) just before the beginning of neurogenesis concomitantly inhibited neural plate cdkn1c expression (Fig. 1F) and formation of primary neurons (Fig. 1G,H) but did not affect adaxial cell cdkn1c expression (Fig. 1F). Thus, Notch signaling regulates cdkn1c expression in neural plate but not adaxial mesoderm cells.

We next asked whether Notch activity continuously limits cdkn1c expression and neurogenesis by using the transgenic line Tg(hsp70:XdShSu(H)mwc) (Latimer et al., 2005) to express dnSu(H)MyC (Wettstein et al., 1997) under transcriptional control of the zebrafish hsp70 promoter (Shoji et al., 1998). At 36 hpf, control embryos expressed cdkn1c RNA in columns of spinal cord cells that were just outside the proliferative cells that occupy the medial spinal cord (Fig. 2A). Similarly staged embryos heat shocked at 24 hpf to induce dnSu(H)MyC expressed cdkn1c at high level throughout the spinal cord (Fig. 2B), consistent with our observations that Notch signaling inhibits cdkn1c expression in neural plate cells. To examine the effect of this treatment on formation of neurons and oligodendrocytes, we repeated the experiment using Tg(olg2:egfp);Tg(hsp70:XdShSu(H)mwc) embryos, in which olg2:EGFP expression marks ventral spinal cord precursors that give rise to motor neurons, interneurons, and oligodendrocytes (Shin et al., 2003; Park et al., 2004). All olg2:EGFP+ cells developed as neurons, with a concomitant loss of medial spinal cord (Fig. 2D) and no OPCs formed (Fig. 2F). Thus, Notch signaling is required throughout the period of motor neuron production to inhibit cdkn1c expression and maintain a pool of precursors that can give rise to OPCs. These data establish that, in zebrafish, Notch activity negatively regulates cdkn1c expression and support the possibility that Notch inhibition of cell-cycle exit and primary neurogenesis and maintenance of spinal cord precursors occurs, at least in part, via inhibition of cdkn1c.

**Loss of cdkn1c function increases neural cell proliferation and delays primary neuron production**

To investigate cdkn1c function, we designed an antisense MO to block translation of cdkn1c mRNA. We tested the effectiveness of our cdkn1c MO by injecting it into Tg(hsp70:cdkn1c-myc) embryos, which express Myc epitope-tagged Cdkn1c under control of the heat shock 70 promoter (Shoji et al., 1998). The cdkn1c MO has sequence complementary to the transcript expressed from the transgene and should inhibit its translation. Indeed, cdkn1c MO-injected embryos expressed Cdkn1cMyc at much lower levels than noninjected embryos (Fig. 3A,B).

If Cdkn1c promotes cell-cycle exit and primary neurogenesis, embryos that lack Cdkn1c function should have a deficiency of primary neurons. At neural plate stage, 80.2% (325 of 405) of cdkn1c MO-injected embryos had few primary neurons (compare Figs. 3C, 1G). This is consistent with the possibility that Notch inhibition of cdkn1c expression prevents formation of neurons in the zebrafish neural plate. To further establish the relationship between Notch signaling and Cdkn1c function, we injected embryos produced by intercrosses of mib+/− adults with control or cdkn1c MOs. Twenty-six percent (43 of 165) of embryos injected with control MO produced excess primary neurons, close to the expected frequency of 25% homozygous mutant embryos (data not shown). In contrast, 12.6% (20 of 159) of embryos injected with cdkn1c MO formed groups of densely spaced neurons evident of a mib mutant phenotype, although, overall, these embryos had deficits of primary neurons (Fig. 3D). Most remaining embryos had few neurons, similar to wild-type embryos injected with cdkn1c MO (data not shown). Thus, loss of cdkn1c function suppressed the excess primary neuron phenotype of mib+/− embryos, suggesting that Notch-signaling-deficient embryos produce excess primary neurons because they fail to limit cdkn1c expression to subsets of neural plate cells.

In mice, flies, and nematodes, cells that were deficient for Cdk1 activity underwent additional divisions but ultimately exited the cell cycle and differentiated (de Nooij et al., 1996; Lane et al., 1996; Hong et al., 1998; Lowenheim et al., 1999; Levine et al., 2000; Miyazawa et al., 2000; Fukuyama et al., 2003). Frog em-
bryos injected with MOs made to block p27<sup>Xic1</sup> translation were not examined at later stages of development to determine whether neural cells eventually exited the cell cycle (Carruthers et al., 2003; Vernon et al., 2003). To learn whether zebrafish embryos that lack <i>cdkn1c</i> function undergo a delayed primary neurogenesis, we examined MO-injected wild-type embryos at 20 hpf using RNA probe to detect p27<sup>Xic1</sup> expression, which marks Rohon-Beard (RB) sensory neurons and CaP and VaP motor neurons (Appel et al., 1995). Both RB and CaP and VaP neurons were evident at 20 hpf (<i>n</i> = 128 embryos) (Fig. 3F), indicating that loss of <i>cdkn1c</i> function delayed but did not prevent primary neurogenesis. Notably, <i>cdkn1c</i> MO-injected embryos had a small but statistically significant increase in the number of <i>isl2</i><sup>+</sup> primary motor neurons. Whereas each ventral hemisegment of control embryos had one (CaP) or two (CaP and VaP) <i>isl2</i><sup>+</sup> cells (Fig. 3E), 36.7% of the <i>cdkn1c</i> MO-injected embryos had one to three <i>isl2</i><sup>+</sup> cells per hemisegment (Fig. 3F). Cells counted from 12 hemisegments each of five control and five <i>cdkn1c</i> MO-injected embryos with excess primary motor neurons revealed an average of 1.5 and 2.4 <i>isl2</i><sup>+</sup> cells, respectively (<i>p</i> < 0.001). To test the possibility that excess primary motor neurons resulted from excess cell proliferation, we incubated embryos with the thymidine analog BrdU to label S-phase cells. In wild-type embryos, neural
plate cells that expressed cdkn1c did not incorporate BrdU (Fig. 3G). In contrast, many neural plate cells of embryos injected with cdkn1c MO were both cdkn1c positive and BrdU positive (Fig. 3H). These data indicate that cdkn1c function is necessary for some neural plate cells to stop dividing and differentiate as primary motor neurons on schedule.

**cdkn1c function is required for oligodendrocyte specification in zebrafish**

The above data are consistent with the following model. During early stages of neural development, cells that have low levels of Notch activity express cdkn1c, exit the cell cycle, and develop as primary neurons, whereas those that have high levels of Notch activity do not express cdkn1c and remain proliferative. Subsequently, some of these precursors escape from Notch inhibition, express cdkn1c, and develop as secondary neurons. This model raises the question as to whether, after the period of neuron production, cdkn1c function is necessary for glial cell specification.

To investigate a possible role in oligodendrocyte specification, we first examined cdkn1c expression by in situ RNA hybridization to transverse sections obtained from Tg(olig2:egfp) embryos (Shin et al., 2003; Park et al., 2004). At 3 dfp, many spinal cord cells expressed cdkn1c, including olig2:EGFP" OPCs and differentiating oligodendrocytes (Fig. 4A, B). Second, we injected cdkn1c MO into Tg(olig2:egfp) embryos. At 3 dfp, when dorsally migrated OPCs were evident in control embryos, similarly staged cdkn1c MO-injected embryos had few dorsal OPCs (data not shown) (Fig. 5H). OPCs were still absent from dorsal spinal cord at 4 dfp (Fig. 5B) and 5 dfp (data not shown), suggesting that OPCs were not simply delayed in their development. Because motor neuron expression of EGFP might obscure OPCs that failed to migrate dorsally in cdkn1c MO-injected embryos, we tested plp/dm20 expression, which marks the myelinating subpopulation of oligodendrocyte lineage cells (Brosamle and Halpenny, 2002; Park et al., 2002). Whereas 4 dfp wild-type embryos had numerous plp/dm20" cells in dorsal and ventral spinal cord (Fig. 5C), 83 of 96 similarly staged cdkn1c MO-injected embryos had very few plp/dm20" spinal cord cells (Fig. 5D). Additionally, we used immunocytochemistry to examine expression of Sox10, which is the earliest known marker of OPCs in zebrafish (Park et al., 2002). Relative to wild type (Fig. 5E), cdkn1c MO-injected embryos had few Sox10" OPCs (Fig. 5F).

Notch-signaling-deficient embryos also have excess primary neurons and a deficit of oligodendrocytes (Appel et al., 2001; Park and Appel, 2003). In these embryos, all spinal cord precursors stop dividing too soon and differentiate as neurons, and this is revealed as an excess of Hu" neurons and absence of Hu" cells at 1 dfp. To determine whether cdkn1c-deficient embryos similarly lack oligodendrocytes because they fail to maintain the precursors that give rise to them, we labeled cdkn1c MO-injected Tg(olig2:egfp) embryos with anti-Hu antibody. At 3 dfp, wild-type embryos had dorsal and ventral EGFP", Hu" OPCs, ventral EGFP", Hu" neurons, and EGFP", Hu" cells near the ventral ventricle (arrows), and EGFP", Hu" ventral neurons (bracket). H, cdkn1c MO-injected embryo had EGFP", Hu" precursors (arrows) and EGFP", Hu" ventral neurons (bracket) but no OPCs. Scale bar: (in H) A–D, 40 μm; E–F, 20 μm; and G, H, 15 μm. wt, Wild type.
function is necessary to specify oligodendrocyte lineage cells from olig2:EGFP+ spinal cord precursors.

Previously, we showed that embryos in which a constitutively active form of Notch1a was expressed beginning at 24 hpf had about a twofold excess of OPCs (Park and Appel, 2003). To determine whether formation of excess OPCs is dependent on cdkn1c function, we injected cdkn1c MO into cleavage stage Tg(hsp70:GAL4); Tg(UAS:Notch1a-Myc); Tg(olig2:egfp) embryos. 

A. Control embryo in which heat shock at 24 hpf produced excess EGFP+ Sox10+ OPCs (arrowheads). 
B. Embryo injected with cdkn1c MO and heat shocked at 24 hpf lacked OPCs. Anti-Myc labeling (blue) reveals Notch1a-Myc expression that persisted after heat-shock induction. The arrows mark Sox10+ Schwann cells. Schwann cells are distributed intermittently along the anteroposterior axis, which accounts for their absence in the transverse section shown in A. Scale bar: (in B) 20 μm.

Figure 6. Formation of excess OPCs by Notch activity is dependent on cdkn1c function. 
Panels show transverse sections, dorsal up, of Tg(hsp70:GAL4); Tg(UAS:Notch1a-Myc); Tg(olig2:egfp) embryos. 

A. Control embryo in which heat shock at 24 hpf produced excess EGFP+ Sox10+ OPCs (arrowheads). 
B. Embryo injected with cdkn1c MO and heat shocked at 24 hpf lacked OPCs. Anti-Myc labeling (blue) reveals Notch1a-Myc expression that persisted after heat-shock induction. The arrows mark Sox10+ Schwann cells. Schwann cells are distributed intermittently along the anteroposterior axis, which accounts for their absence in the transverse section shown in A. Scale bar: (in B) 20 μm.

Figure 7. Time-dependent expression of cdkn1c rescues the OPC deficit produced by cdkn1c MO. All images are of transverse sections of Tg(hsp70:cdkn1c-myc); Tg(olig2:egfp) embryos, dorsal up. The dashed circle outlines the spinal cord, and the arrows mark Sox10+ Schwann cells. 

A. B. Induction of Cdkn1cMyc expression at 24 hpf did not promote premature formation of Sox10+, olig2:EGFP+ OPCs at 32 hpf. C, D. Induction of Cdkn1cMyc expression at 28 hpf did not promote formation of excess or ectopic Sox10+, olig2:EGFP+ OPCs at 48 hpf. E, F. Induction of Cdkn1cMyc expression at 28 hpf produced OPCs (arrowheads) in cdkn1c MO-injected embryo at 48 hpf.

Our data are paradoxical, in that we showed that Notch inhibits cdkn1c expression and can promote formation of excess OPCs but that cdkn1c function is required for OPC specification. We speculated that ventral neural precursors that begin to escape Notch inhibition, even after transiently induced expression of constitutively active Notch, and express low levels of cdkn1c after neurons are produced are specified as OPCs. One test of this idea would be to block Cdkn1c function just before OPC specification. However, we lack a method to do this. Instead, we heat shocked Tg(hsp70:cdkn1c-myc) embryos to induce Cdkn1cMyc expression at 24 hpf. At 48 hpf, noninjected embryos had excess Sox10+, olig2:EGFP+ OPCs (Fig. 6A), consistent with our previous results. In contrast, embryos injected with cdkn1c MO had a deficit of OPCs (Fig. 6B), showing that cdkn1c function is required to mediate OPC specification by Notch signaling.

becomes depleted at later stages of development. Indeed, anti-Myc immunocytochemistry revealed substantial Cdkn1cMyc expression when transgenic embryos were heat shocked at 28 hpf (Fig. 7F). These embryos produced, on average, 2.30 OPCs per section (66 sections from eight embryos) (Fig. 7E), in contrast to nontransgenic, cdkn1c MO-injected embryos, which produced an average of 0.86 OPCs per section (66 sections from eight embryos; p < 0.00001) (images not shown but compare with Fig. 5B, D, F, H). Thus, cdkn1c expression induced after most neurons are produced but before oligodendrocyte specification rescues the oligodendrocyte deficit caused by cdkn1c MO. We conclude that specification of oligodendrocyte fate from olig2+ spinal cord precursors requires cdkn1c function.

Discussion
Notch signaling coordinates neurogenesis by regulating cdkn1c expression

One critical mechanism for coordinating neural cell proliferation, specification, and differentiation is Notch signaling. Typically, membrane-bound ligands, such as Delta proteins, interact with transmembrane Notch receptors, causing transduction of a signal to the nucleus of Notch-expressing cells that influences
gene transcription (Kadesch, 2004). Among the consequences of Delta–Notch signaling activity in the CNS is differential expression of proneural class genes, such as neurogenin1, which encode basic helix-loop-helix (bHLH) transcription factors that promote cell-cycle exit and neuronal development (Kageyama and Ohtsuka, 1999). Cells that have relatively high Notch activity inhibit proneural gene expression and remain in the cell cycle, whereas those with low Notch activity express proneural genes, stop dividing, and develop as neurons. Functional tests of the Notch pathway reveal that signaling is important to diversify neural cell fate in vertebrate embryos. For example, zebrafish embryos deficient for Notch activity failed to maintain proliferative precursors and produced excess early born neurons at the expense of later-forming neurons and glia (Appel and Eisen, 1998; Appel et al., 2001; Park and Appel, 2003), and expression of constitutively active Notch blocked neuronal development and routed precursors into glial developmental pathways (Furukawa et al., 2000; Scheer et al., 2001; Park and Appel, 2003). Evidence also points to a role for Notch activity in regulating development of oligodendrocyte lineage cells. In culture, Notch activity inhibited differentiation of OPCs (Wang et al., 1998).

Previously published data raised the possibility that genes regulated by Notch activity might include those that encode Cdkis. For example, flies deficient for the Cdki Decapo underwent additional rounds of cell division, whereas overexpression of Decapo caused cells to stop dividing prematurely (Lane et al., 1996). Similarly, mice and frogs that lacked p27Kip1 and p27Xic3 function, respectively, had increased numbers of proliferating cells (Kiyokawa et al., 1996; Carruthers et al., 2003; Vernon et al., 2003), and overexpression of p27Xic3 in frog embryos reduced proliferation and caused neural plate cells to form excess primary neurons (Hardcastle and Papalopulu, 2000; Vernon et al., 2003). Thus, Notch activity could prevent cell-cycle exit and neuronal differentiation by inhibiting cdki gene expression. In principle, this could occur because Notch activity directly inhibits cdki gene transcription or Notch activity inhibits expression of genes that encode activators of cdki transcription. Consistent with the latter possibility, P19 embryonal carcinoma cells transfected with constructs that expressed proneural factors expressed elevated levels of p27Kip1 and p27Xic3 and withdrew from the cell cycle (Farah et al., 2000). Genomic DNA near the p21Cip1 transcription start site contains E-box sequences, which bind bHLH transcription factors, and these mediate enhancement of p21Cip1 transcription by bHLH proteins in cell-culture assays (Pagliuca et al., 2000). In flies, decapo expression is positively regulated by a sense, which encodes a proneural bHLH factor (Wallace et al., 2000).

Our data establish that Notch signaling regulates cdkn1c expression within the CNS of zebrafish embryos. Nonproliferative neural plate cells expressed cdkn1c in a mosaic pattern consistent with the pattern of primary neuron formation. Excess neural plate cells expressed cdkn1c and developed as primary neurons in mutant embryos that were deficient for Notch signaling, whereas embryos that expressed a constitutively active Notch had few cdkn1c+ neural plate cells and deficits of primary neurons. At late stages of neurogenesis a conditional block of Notch signaling activity also caused excess spinal cord cells to express cdkn1c, stop proliferating, and develop as neurons. Thus, Notch signaling may regulate the orderly formation of neurons over time by regulating transcription of cdkn1c.

cdkn1c function is required for timely formation of neurons

Previous work using frogs showed that p27Xic1 function is required to limit neural cell proliferation and promote formation of primary neurons from neural plate cells (Carruthers et al., 2003; Vernon et al., 2003). We found a similar effect for loss of cdkn1c function in zebrafish embryos. However, we also learned that these embryos eventually produced primary motor neurons, sometimes in excess number. This result is remarkably similar to the consequences of disrupted Cdki activity in nematodes, wherein vulva precursor cells underwent only one additional round of cell division to produce excess ventral hypodermal cells in the absence of cki-1 function (Hong et al., 1998). Thus, the simplest interpretation of our data is that, in the absence of cdkn1c activity, neural plate cells proceed through one extra division but remain competent to respond to signals that specify primary motor neuron fate.

This interpretation has an interesting implication. We have analyzed clones made by marking single cells within a precursor domain that gives rise to motor neurons, interneurons, and oligodendrocytes (Park et al., 2004). The size and cell-type composition of the clones was highly variable, suggesting that specification of cell fate results from integration of extrinsic signals that change with time. Consistent with this, we found that time-dependent manipulation of Notch and Hedgehog signaling alters the number of cells specified for motor neuron, interneuron, and oligodendrocyte fates (Park and Appel, 2003; Park et al., 2004). If an obligate relationship exists between neural cell-cycle exit and fate specification, we would predict that, in the absence of cdkn1c function, medial neural plate precursors that normally give rise to primary motor neurons would instead produce cells that develop after primary motor neurons. Because this, apparently, is not the case, our results raise the possibility that precursors can be specified for primary motor neuron fate and retain this identity through an additional round of cell division. Thus, cdkn1c function probably is only a part of a mechanism that coordinates neural cell cycle exit and neuronal specification.

cdkn1c function is required for oligodendrocyte specification

Cdkis also play important roles in regulating proliferation and differentiation of OPCs, proliferative cells committed to the oligodendrocyte lineage. For example, relatively undifferentiated OPCs expressed low levels of p27Kip1 protein, whereas progressively more fully differentiated cells expressed higher levels (Durand et al., 1997). Cultured OPCs derived from p27Kip1 mutant mice proliferated more than OPCs from wild-type mice (Casaccia-Bonnefil et al., 1997; Durand et al., 1998; Zeeula et al., 2001) and had impaired ability to develop the multiple branching processes characteristic of differentiated oligodendrocytes (Casaccia-Bonnefil et al., 1997; Zeeula et al., 2001). p27Kip1 mutant mice had more proliferative OPCs than wild-type OPCs, they had the same proliferative characteristics as wild-type OPCs, they did not fully differentiate (Zeeula et al., 2001). One caveat of the mouse loss-of-function tests is that mice express multiple cdki genes in overlapping patterns within the nervous system (Zhu and Skoulitchi, 2001). Thus, mouse Cdkis might have significant functional redundancy. In contrast to the mouse p21 and p27 mutants, in which OPCs are formed but do not proliferate or differentiate properly, we found that zebrafish embryos that had reduced function of cdkn1c, a p57 homolog, did not produce spinal cord OPCs. Instead, the olig2:EGFP+ precursors that normally give rise to OPCs appeared to remain in an undifferentiated state. Spinal cord cells of zebrafish embryos express high levels of cdkn1c but not cdkn1b, a p27 homolog, and we did not find a p21 homolog. This raises the possibility that, in zebrafish, a single
gene provides most or all of Cdki function necessary to regulate oligodendrocyte development. Thus, the absence of spinal cord OPCs in cdkn1c-deficient zebrafish embryos reveals a new role for Cdki function in specification of OPCs from proliferative neural precursors. In frogs, retinal cells lipofected with antisense RNA formed fewer Müller glia than controls (Ohnuma et al., 1999). These data raise the possibility that Cdkis have widespread roles in glial specification.

We also found that expression of cdkn1c from a transgene before OPC specification restored OPC development in embryos injected with cdkn1c MO. This provides strong evidence for our conclusion that cdkn1c function is necessary for OPC specification. However, transgenic cdkn1c expression was not sufficient to induce formation of OPCs prematurely or in excess number at the normal time of OPC specification. This is in contrast to over-expression of p27(Xic1) in frog retina, which promoted formation of excess Mu¨ller glia and a concomitant reduction in the number of bipolar neurons (Ohnuma et al., 1999). Previously, we showed that oligodendrocyte specification is temporally and spatially constrained (Park and Appel, 2003; Park et al., 2004). Thus, cdkn1c does not function as a determination factor but as a necessary trigger for cells that are competent to respond to other signals that instruct oligodendrocyte specification.

Rising Cdki protein levels may regulate OPC cell-cycle exit and differentiation. We showed that Notch inhibition of cdkn1c expression limits formation of neurons and maintains a population of neural precursors, yet that cdkn1c function is also necessary for precursors to give rise to OPCs. We propose that, after formation of most neurons, remaining ventral spinal cord precursors that escape Notch-mediated inhibition begin to express cdkn1c and that the initial low level of Cdkn1c protein triggers OPC specification without causing cells to stop dividing. Subsequent accumulation of Cdkn proteins might then drive OPCs from the cell cycle and promote oligodendrocyte differentiation.

References