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Delta-Notch signaling regulates oligodendrocyte specification

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SUMMARY

Oligodendrocytes, the myelinating cell type of the central nervous system, arise from a ventral population of precursors that also produces motoneurons. Although the mechanisms that specify motoneuron development are well described, the mechanisms that generate oligodendrocytes from the same precursor population are largely unknown. By analysing mutant zebrafish embryos, we found that Delta-Notch signaling is required for spinal cord oligodendrocyte specification. Using a transgenic, conditional expression system, we also learned that constitutive Notch activity could promote formation of excess oligodendrocyte progenitor cells (OPCs). However, excess OPCs are induced only in ventral spinal cord at the time that OPCs normally develop. Our data provide evidence that Notch signaling maintains subsets of ventral spinal cord precursors during neuronal birth and, acting with other temporally and spatially restricted factors, specifies them for oligodendrocyte fate.

Key words: Delta, Notch, Oligodendrocytes, Motoneurons, Neural precursors, CNS, Spinal cord, Zebrafish

INTRODUCTION

The Delta-Notch signaling pathway regulates crucial aspects of neural cell fate specification and differentiation in both invertebrate and vertebrate embryos. For example, in *Drosophila melanogaster*, mutations that impair function of the Delta ligand, Notch receptor or other components of the signaling pathway resulted in the formation of excess neurons at the expense of epidermis (Campos-Ortega, 1995). Reduction of Notch signaling in vertebrate embryos also produced excess neurons at early stages of central nervous system (CNS) development, which correlated with loss of proliferative neural precursor cell populations and absence of some late-forming neurons (Chitnis et al., 1995; Henrique et al., 1997; Appel et al., 2001). Conversely, forced expression of Delta or constitutively active, intracellular forms of Notch ($Notch^{ac}$) blocked neuronal differentiation (Coffman et al., 1993; Chitnis et al., 1995; Henrique et al., 1997). These observations helped form the view that modulation of Delta-Notch signaling regulates the transition of proliferative neural precursors to post-mitotic differentiated cells. Thus, downregulation of Notch activity in subsets of precursors at different times might allow them to respond to other, temporally regulated instructive factors that specify them for different neuronal and glial fates (Bertrand et al., 2002). In this model, Notch signaling plays a permissive, rather than instructive, role in neural cell fate specification.

Several lines of evidence have now raised the possibility that Notch signaling directly promotes development of some types of glial cells. First, formation of Müller glia in the retina was promoted by forced expression of $Notch^{ac}$ and by overexpression of Hes1 and Hes5, which are downstream components of the Notch signaling pathway (Furukawa et al., 2000; Hojo et al., 2000; Scheer et al., 2001). Second, in utero retroviral infection of the telencephelon of mouse embryos showed that $Notch^{ac}$ could promote the formation of radial glia, which gave rise, postnatally, to astrocytes (Gaiano et al., 2000; Chambers et al., 2001). Third, in cells of the peripheral nervous system, activation of Notch signaling induced the formation of Schwann cells from purified neural crest stem cells (Morrison et al., 2000) and permitted glial development while blocking neurogenesis, in cultured quail neural crest cells (Wakamatsu et al., 2000). The manner in which Notch promotes glial development is unknown, although, in dorsal root ganglia, the asymmetric distribution of Numb proteins might influence Delta-Notch mediated decisions for neuronal or glial fate (Wakamatsu et al., 2000).

Until recently, most work investigating the role of Notch signaling in development of oligodendrocytes, the myelinating glial cell type of the CNS, focused on control of oligodendrocyte differentiation. When immature oligodendrocyte progenitor cells (OPCs) purified from rat optic nerve were exposed to Notch ligands, they did not differentiate, in contrast to control experiments (Wang et al., 1998). Consistent with this observation, selective inactivation of Notch1 in OPCs in vivo caused their premature differentiation (Genoud et al., 2002) and oligodendrocytes became myelinated too soon in mice heterozygous for a *Notch1* mutation (Givogri et al., 2002). Additionally, astrocytes in demyelinating lesions of human multiple sclerosis patients upregulated Jagged1, a Notch ligand, and Jagged1 blocked maturation of purified human OPCs (John et al., 2002). These observations provide strong evidence that Notch signaling inhibits differentiation of cells once they enter the oligodendrocyte developmental

pathway. Less clear is whether Notch signaling also promotes specification of OPCs from neural precursors. Neurospheres from *delta-like 1* mutant mice produced a deficit of OPCs and, conversely, addition of a soluble Notch ligand to wild-type neurospheres enhanced their formation (Grandbarbe et al., 2003). However, multipotent adult hippocampus-derived progenitors produced fewer oligodendrocytes when they were transfected with Notch^{ac} (Tanigaki et al., 2001), although the markers used in this study did not distinguish between the possibilities that Notch activity blocked specification or subsequent differentiation. When electroporated into chick spinal cords, Notch^{ac} could promote formation of OPCs only when co-expressed with Olig2, a basic helix-loop-helix (bHLH) transcription factor necessary for oligodendrocyte development (Zhou et al., 2001).

Here, we provide in vivo, genetic evidence that Delta-Notch signaling is required for spinal cord oligodendrocyte specification. Spinal cord precursors of mutant zebrafish embryos that had reduced Notch signaling activity stopped dividing prematurely and developed as early-born neurons at the expense of later-born oligodendrocytes. By contrast, transgenic embryos in which Notch^{ac} was driven by a conditional expression system had a deficit of neurons and concomitant increase of OPCs. However, formation of OPCs in response to Notch^{ac} was restricted to their normal time and place of development. We also show that, subsequent to OPC specification, Notch^{ac} blocked oligodendrocyte differentiation, consistent with previous in vitro data. Our data indicate that Notch signaling is important throughout development of oligodendrocytes by promoting specification of OPCs from neural precursors and regulating their subsequent differentiation into mature oligodendrocytes.

MATERIALS AND METHODS

Wild-type, mutant and transgenic zebrafish

Embryos were produced by pair matings of fish raised in the Vanderbilt University Zebrafish Facility. Except for heat-shock experiments, embryos were raised at 28.5°C and staged according to hours post-fertilization (hpf) and morphological criteria. We used the following mutant alleles: *dla^{dx2}* (Appel et al., 1999); *dld/aei^{AR33}* (Holley et al., 2000); and *mib^{ta52b}* (Jiang et al., 1996). For some *dla;dld* double mutant experiments, we used a new hypomorphic *dld* allele that we will describe elsewhere. We produced double mutant embryos by intercrossing *dla$^{+/-}$;dld$^{+/-}$* adults. Double mutant embryos were identified by phenotype, which included a somite defect characteristic of *dld$^{-/-}$* embryos, an irregularly shaped brain, an abnormally curved body and absence of trunk pigmentation. The double mutant combination was fully penetrant, producing phenotype in approximately 1/16 of progeny from matings of doubly heterozygous adults. *TG[hsp:GAL4]* and *TG[UAS:Notch1a^{ac}]* transgenic lines were described previously (Scheer, 1999). To produce *TG[hsp:GAL4];TG[UAS:Notch1a^{ac}]* embryos, we crossed carriers that were either homozygous or heterozygous for each transgene.

Labeling methods and photomicroscopy

Manually dechorionated embryos were labeled with BrdU by incubating them for 20 minutes on ice in a solution of 10 mM BrdU and 15% DMSO in embryo medium (15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM NH_2PO_4, 0.7 mM $NaHCO_3$). The BrdU solution was replaced with embryo medium and embryos were incubated for 20 minutes at 28.5°C. The embryos were then anesthetized using 3-aminobenzoic acid ethyl ester, fixed in 4% paraformaldehyde, embedded in 1.5% agar/5% sucrose and frozen in 2-methyl-butane chilled by immersion in liquid nitrogen. Sections (10 μm) were obtained by using a cryostat microtome. Sections were treated with 2 N HCl for 1 hout, washed with PBS, blocked in PBS plus 2% sheep serum and 2 mg ml^{-1} bovine serum albumen (BSA) and then incubated with anti-BrdU antibody.

Previously described RNA probes included *sox10* (Dutton et al., 2001), *ngn1* (Blader et al., 1997), *tlxa* (Andermann and Weinberg, 2001), *olig2* and *plp1/dm20* (Park et al., 2002). In situ RNA hybridization was performed as described previously (Hauptmann and Gerster, 2000). Embryos for sectioning were treated as described above. Flat mounted embryos were dissected from the yolk and mounted in 75% glycerol.

For immunohistochemistry, we used the following primary antibodies: mouse anti-BrdU [G3G4, 1:1000, Developmental Studies Hybridoma Bank (DSHB), Iowa City, Iowa, USA], mouse anti-HuC/D (1:20, Molecular Probes, Eugene, Oregon, USA), rabbit anti-phospho-histone-H3 (1:1000, Upstate Biotechnology, Charlottesville, Virginia, USA). For fluorescent detection of antibody labeling, we used Alexa Fluor 568 goat anti-mouse conjugate (1:200, Molecular Probes) and Alexa Fluor 488 goat anti-rabbit conjugate (1:200, Molecular Probes). Hoechst labeling was performed by incubating sections in Hoechst for 10 minutes following immunolabeling.

In situ hybridization and Hoechst/immunofluorescence images were obtained using a Spot digital camera mounted on a compound microscope. All other fluorescence images were obtained using a Zeiss LSM510 Meta laser scanning confocal microscope. All images were imported into Adobe Photoshop. Image manipulation was limited to levels, curves, hue and saturation adjustments.

Heat shock experiments

Heat shock was applied to transgenic and control embryos in embryo medium for 30 minutes at 40°C to induce Notch1a^{ac} expression. Following heat shock, embryos were incubated at 28.5°C. Heat-shocked, transgenic embryos were identified by phenotype, which consisted of touch insensitivity, abnormal brain morphology and indistinct somites. We confirmed our identification by labeling a subset of embryos with anti-Myc antibody, which detects the Myc epitope fused to Notch1a^{ac} (Scheer et al., 2001). To analyse the number of spinal cord *olig2^{+}* and *sox10^{+}* cells, we counted cells in 20 transverse sections from each of five nontransgenic and five transgenic embryos. Statistical significance was determined using Student's *t* test.

RESULTS

Delta-Notch signaling is required for spinal cord oligodendrocyte formation

We showed previously that zebrafish embryos mutant for *deltaA* (*dla*), which encodes one of four known zebrafish Delta-like ligands, had reduced numbers of proliferative spinal cord cells and excess neurons (Appel et al., 2001). We extend those data here to show that, at 24 hpf, spinal cords of double mutant embryos lacking function of *dla* and the related *deltaD* (*dld*) gene (*dla$^{-/-}$;dld$^{-/-}$* embryos) had few S-phase cells as revealed by BrdU incorporation (Fig. 1B). Similarly, spinal cord cells of embryos homozygous mutant for *mind bomb* (*mib*), which encodes a ubiquitin ligase necessary for efficient Notch signaling (Itoh et al., 2003), did not incorporate BrdU (Fig. 1C). Immunocytochemical labeling of phosphorylated histone H3, which marks M-phase cells, and Hu proteins, which reveals mostly post-mitotic neurons and some neural cells in S-phase (Marusich et al., 1994), showed that 24 hpf wild-type

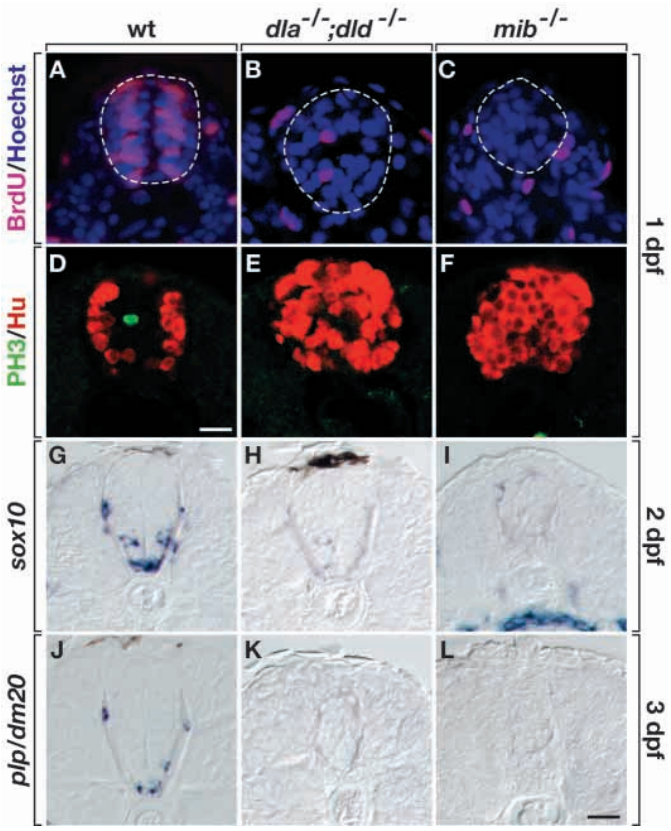

Fig. 1. Delta-Notch is required to maintain the spinal cord precursor pool and for OPC specification. All images show transverse sections, dorsal to the top. (A-C) 1 dpf embryos treated with BrdU and anti-BrdU antibody (pink) to mark S-phase cells and Hoechst (blue) to reveal nuclei. *dla*$^{-/-}$;*dld*$^{-/-}$ (B) and *mib*$^{-/-}$ (C) embryos had fewer S-phase spinal cord cells than wild type (A). Spinal cords are outlined. (D-F) 1 dpf embryos labeled with anti-phospho-histone-H3 antibody to mark M-phase cells (green) and pan neuronal anti-Hu antibody (red). *dla*$^{-/-}$;*dld*$^{-/-}$ (E) and *mib*$^{-/-}$ (C) embryos had excess neurons and a deficit of M-phase cells relative to wild type (D). (G-I) 2 dpf embryos probed for *sox10* expression, which marks OPCs, by in situ RNA hybridization. Spinal cord cells of *dla*$^{-/-}$;*dld*$^{-/-}$ (H) and *mib*$^{-/-}$ (I) embryos did not express *sox10*. (J-L) 3 dpf embryos probed for *plp1/dm20* RNA expression, which marks premyelinating oligodendrocytes. *dla*$^{-/-}$;*dld*$^{-/-}$ (K) and *mib*$^{-/-}$ (L) embryos did not express *plp1/dm20*. Scale bars: 20 μm.

embryos had a small number of M-phase cells located near the ventricle and neurons distributed along the pial surface of the spinal cord (Fig. 1D). By contrast, *dla*$^{-/-}$;*dld*$^{-/-}$ and *mib*$^{-/-}$ embryos had few, if any, M-phase cells and large excesses of neurons (Fig. 1E,F). In mutant embryos, neurons appeared in positions normally occupied by proliferative cells. In fact, nearly every spinal cord cell was Hu^{+}. Thus, Delta-Notch signaling limits formation of spinal cord neurons and maintains a population of proliferative neural precursors.

A ventral spinal cord precursor domain, called pMN, produces first motoneurons and then oligodendrocytes (Richardson et al., 2000; Zhou and Anderson, 2002). Previous work showed that reduction of Notch activity in zebrafish embryos resulted in formation of excess primary motoneurons (Dornseifer et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998; Appel et al., 2001). As production of excess neurons occurred at the expense of proliferative precursors in *dla*$^{-/-}$;*dld*$^{-/-}$ and *mib*$^{-/-}$ embryos, we predicted that these embryos would lack oligodendrocytes. In zebrafish, the earliest gene expression apparently specific to oligodendrocyte lineage cells is that of *sox10*, which, at 48 hpf, marks OPCs (Park et al., 2002) (Fig. 1G). Neither *dla*$^{-/-}$;*dld*$^{-/-}$ nor *mib*$^{-/-}$ embryos expressed *sox10* in spinal cord (Fig. 1H,I). To confirm the absence of OPCs from mutant embryos, we tested expression of *plp1/dm20*, which marks premyelinating oligodendrocytes (Timsit et al., 1995; Park et al., 2002). By contrast to 3 days post-fertilization (dpf) wild-type embryos (Fig. 1J), neither *dla*$^{-/-}$;*dld*$^{-/-}$ nor *mib*$^{-/-}$ embryos expressed *plp1/dm20* (Fig. 1K,L). These observations are consistent with the possibility that, within the pMN domain, Delta-Notch signaling limits formation of early-born primary motoneurons and promotes formation of later-born OPCs at least in part by maintaining a subset of proliferative precursors.

Delta-Notch signaling regulates *olig2* and *ngn1* expression differently

A combinatorial code of bHLH proteins might specify pMN precursors for motoneuron or oligodendrocyte fates, whereby cells that express Olig2 and Ngns develop as motoneurons and those that express only Olig2 develop as oligodendrocytes (Zhou and Anderson, 2002). Thus, we examined expression of zebrafish *olig2* and *ngn1* to gain further insight into the basis of the mutant phenotypes described above. In 24 hpf wild-type embryos, a discrete group of ventral spinal cord cells uniformly expressed *olig2* (Fig. 2A) (Park et al., 2002). By contrast, *ngn1* was expressed in a mosaic throughout the dorsoventral spinal cord axis (Fig. 2B). Double RNA in situ hybridization showed that a subset of *olig2*$^{+}$ cells expressed *ngn1* (Fig. 2C). These expression patterns are consistent with immunolocalization of Olig2 and Ngn2 proteins in bird and rodent embryos (Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001; Zhou and Anderson, 2002) and consistent with the idea that motoneurons develop from *olig2*$^{+}$, *ngn1*$^{+}$ cells, whereas oligodendrocytes arise from *olig2*$^{+}$, *ngn1*$^{-}$ cells.

At 24 hpf, spinal cord cells of *dla*$^{-/-}$;*dld*$^{-/-}$ and *mib*$^{-/-}$ embryos did not express *olig2* or *ngn1* (H.-C. Park, unpublished). One explanation for this observation is that prematurely differentiating neurons of mutant embryos did not maintain *olig2* and *ngn1* expression. Thus, we examined mutant embryos at early stages of neurogenesis. At 10.5 hpf, medial neural plate cells of wild-type embryos, which occupy ventral spinal cord upon completion of neurulation, expressed *olig2* (Park et al., 2002). All embryos produced by intercrosses of *dla*$^{+/-}$;*dld*$^{+/-}$ adults appeared to express *olig2* RNA similarly at 10.5 hpf (data not shown). At a comparable stage, the excess primary motoneuron phenotype of *dla*$^{-/-}$ and *mib*$^{-/-}$ embryos was evident (Appel et al., 2001). However, by the seven-somite stage (12 hpf), anterior ventromedial neural keel cells of double mutant embryos lacked detectable levels of *olig2* transcripts (Fig. 2E), whereas wild-type cells maintained *olig2* expression along the length of the neural keel (Fig. 2D). *mib*$^{-/-}$ embryos expressed *olig2* similarly to *dla*$^{-/-}$;*dld*$^{-/-}$ embryos (H.-C. Park, unpublished).

Excess neural plate cells of Notch signaling deficient zebrafish embryos expressed *ngn1*, consistent with the excess primary neuron phenotype of the same embryos (Appel et al., 2001). Similarly, at 12 hpf, when *olig2* expression was absent

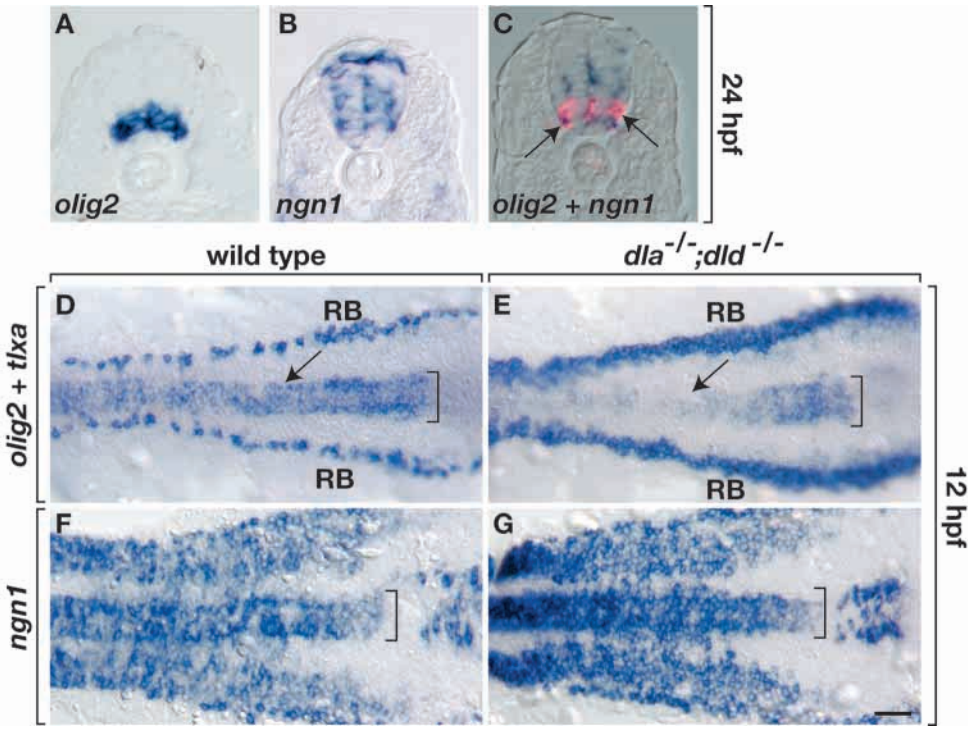

Fig. 2. Delta-Notch signaling maintains *olig2* expression and inhibits *ngn1* expression. (A-C) Transverse sections, dorsal up, of 24 hpf embryos. Whereas a discrete group of ventral spinal cord cells expressed *olig2* (A), cells that expressed *ngn1* were scattered throughout the spinal cord (B). In situ double RNA hybridization revealed that a subset of *olig2*$^{+}$ cells (C, red) expressed *ngn1* (blue, arrows). (D-G) Dorsal views, anterior left, of 12 hpf embryos. (D,E) Embryos hybridized with probes for *olig2* and *tlxa*. Brackets indicate *olig2* expression. *tlxa* expression marks prospective Rohon-Beard (RB) sensory neurons. In wild-type embryos, *olig2* expression was maintained throughout the length of the trunk neural keel (D, arrow). In *dla*$^{-}$*;dld*$^{-}$ mutant embryos, identified by the excess Rohon-Beard phenotype, anterior neural keel cells did not maintain *olig2* expression (E). (F,G) Embryos hybridized with probe to reveal *ngn1* expression. Brackets indicate region of neural keel that expressed *olig2*. Excess cells in *dla*$^{-}$*;dld*$^{-}$ mutant embryos expressed *ngn1* at high level (G) compared to wild type (F). Scale bar: 20 μm (A-C); 50 μm (D-G).

from the anterior neural keel, the density of *ngn1*$^{+}$ cells was greater in *dla*$^{-/-}$*;dld*$^{-/-}$ embryos than in wild-type (Fig. 2F,G). Taken together, these data indicate that Delta-Notch signaling creates a combinatorial bHLH protein code by inhibiting *ngn1* expression and maintaining *olig2* expression.

Notch signaling promotes OPC specification and inhibits oligodendrocyte differentiation

One interpretation of our data is that the requirement for Notch signaling in formation of spinal cord oligodendrocytes indirectly arises from the well-known role of Notch signaling in maintaining neural precursor populations. In this view, Notch signaling prevents neuronal differentiation of a subset of precursors, which then might be specified for oligodendrocyte development by another, instructive signal. An alternative, but not mutually exclusive, possibility is that Notch signaling actively promotes specification of spinal cord oligodendrocytes. To test this possibility, we used a transgenic GAL4/UAS system controlled by a heat-shock promoter to drive ubiquitous expression of a constitutively active form of zebrafish Notch1a (Notch1a^{ac}). Previous studies showed that Notch1a^{ac} expressed in this way persisted for at least 17 hours, effectively blocked neurogenesis and promoted formation of Müller glia in the retina (Scheer et al., 2001; Scheer et al., 2002).

We first established that heat induction of Notch1a^{ac} expression inhibits the formation of spinal cord neurons during embryonic periods relevant to OPC specification. When we induced Notch1a^{ac} expression repeatedly at 10, 24 and 36 hpf, to ensure a high level of Notch1a^{ac} expression, we found that transgenic embryos lacked most spinal cord neurons, marked by Hu immunofluorescence, at 48 hpf (Fig. 3). Labeling to

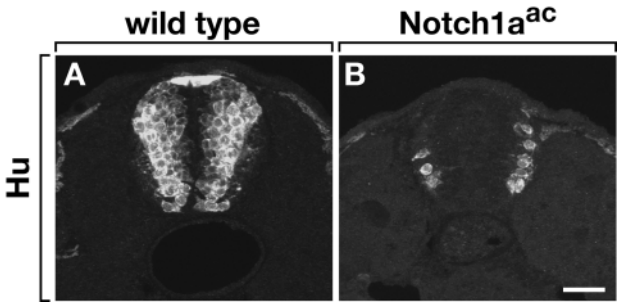

Fig. 3. Notch1a^{ac} expression blocks neurogenesis. Transverse sections, dorsal to top, labeled by immunocytochemistry to reveal Hu proteins. (A) Wild-type control embryo revealing normal distribution of neurons at 48 hpf. (B) 48 hpf transgenic embryo, in which Notch1a^{ac} expression was induced at 10, 24 and 36 hpf, had a deficit of neurons. Scale bar: 20 μm.

detect the Myc epitope fused to Notch1a^{ac} confirmed that cells strongly expressed Notch1a^{ac} at 48 hpf (H.-C. Park, unpublished). Those neurons present in heat-shocked embryos probably were born before Notch1a^{ac} accumulated to high level. Consistent with this, when we induced Notch1a^{ac} at mid-gastrulation (8 hpf), no spinal cord neurons were present at 24 hpf (H.-C. Park, unpublished). Thus, prolonged Notch1a^{ac} expression effectively inhibits spinal cord neurogenesis.

We next examined *olig2* expression in transgenic embryos. Heat induction of Notch1a^{ac} expression at 8 and 24 hpf did not alter spinal cord *olig2* expression at 26 hpf, although *olig2* expression was induced in somite cells (Fig. 4B,E). However,

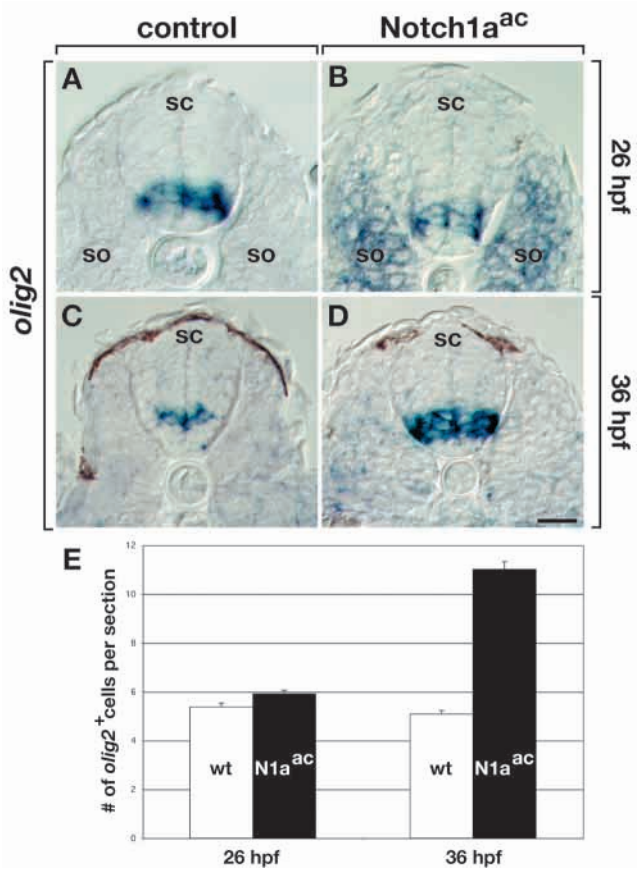

Fig. 4. Notch1a^{ac} increases the number of ventral spinal cord cells that express *olig2*. Transverse sections, dorsal to top, labeled by *olig2* RNA hybridization. (A) Wild-type control embryo revealing normal distribution of *olig2*$^{+}$ cells at 26 hpf. (B) 26 hpf transgenic embryo in which Notch1a^{ac} was induced at 8 and 24 hpf expressed *olig2* similarly to wild type in spinal cord (sc) but expressed *olig2* ectopically in somites (so). (C) Wild-type control embryo revealing normal distribution of *olig2*$^{+}$ cells at 36 hpf. (D) Excess spinal cord cells expressed *olig2* at 36 hpf in transgenic embryo that was heat shocked at 8 and 24 hpf. (E) Quantification of *olig2*$^{+}$ cells at 24 and 36 hpf in wild-type (wt) and Notch1a^{ac}-expressing embryos. Bars indicate the average number of *olig2*$^{+}$ cells per transverse section. Data were obtained from 20 sections from each of five control and five heat-shocked transgenic embryos for each time point. P<0.00000001 by Student's t test. Scale bar: 20 μm.

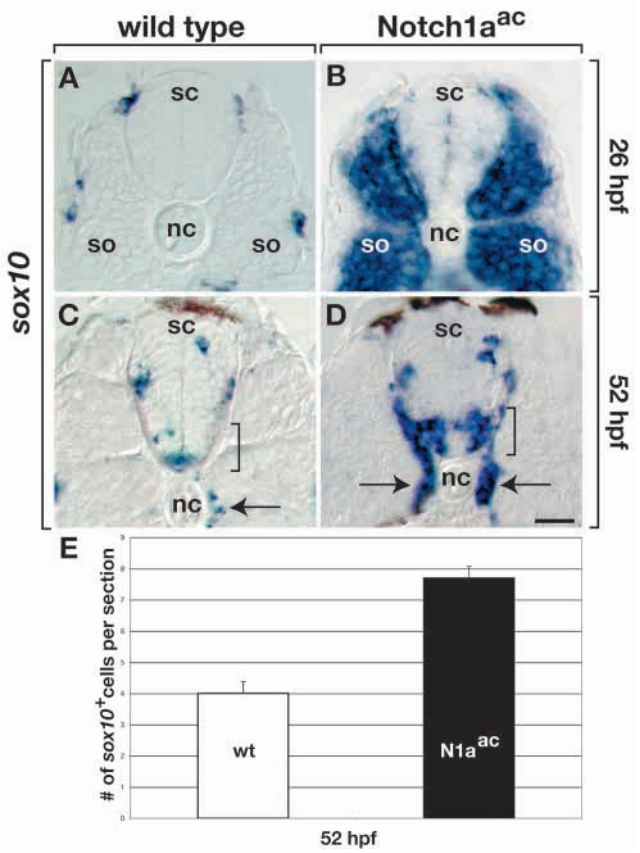

Fig. 5. Notch1a^{ac} promotes OPC specification. Transverse sections, dorsal to top, labeled by *sox10* RNA hybridization. (A) 26 hpf wild-type embryo. Spinal cord (sc), somite (so) and notochord (nc) cells did not express *sox10*. (B) Transgenic embryo in which Notch1a^{ac} was induced at 8 and 24 hpf. Somite cells expressed *sox10* but spinal cord and notochord cells did not. (C) 52 hpf wild-type embryo hybridized with *sox10* probe. *sox10*$^{+}$ cells had mostly dispersed from ventral spinal cord (bracket). Neural crest-derived cells on the medial migratory pathway also expressed *sox10* (arrow). (D) Transgenic embryo in which Notch1a^{ac} was induced at 8, 24 and 36 hpf. Excess ventral spinal cord cells expressed *sox10* (bracket) as did presumptive neural crest cells (arrows). (E) Quantification of *sox10*$^{+}$ cells at 52 hpf in wild-type (wt) and Notch1a^{ac}-expressing embryos. Bars indicate the average number of *sox10*$^{+}$ cells per transverse section. Data were obtained from 20 sections from each of 5 control and 5 heat-shocked transgenic embryos. P<0.00000001 by Student's t test. Scale bar: 20 μm.

at 36 hpf, transgenic embryos that had been heat shocked at 8 and 24 hpf had a 2.2-times increase in the number of *olig2*$^{+}$ ventral spinal cord cells, although *olig2* transcripts were no longer evident in somites (Fig. 4D,E). At 48 hpf, the number of *olig2*$^{+}$ spinal cord cells in transgenic embryos that had been heat shocked at 8, 24 and 36 hpf did not appear to differ significantly from control embryos (H.-C. Park, unpublished). These data raise several possibilities. First, the ectopic somite expression of *olig2* indicates that *olig2* might be a regulatory target of the Notch signaling pathway. However, Notch activity alone was not sufficient to promote *olig2* expression in spinal cord. Second, the increase in the number of spinal cord cells that expressed *olig2* in transgenic embryos, compared with the wild type, occurred between 24 and 36 hpf. This is the period when secondary motoneurons, which arise from *olig2*$^{+}$ precursors, differentiate. As Notch1a^{ac} expression blocked the formation of neurons, we interpret our data to mean that Notch activity maintains a population of proliferative *olig2*$^{+}$ precursors during the time of secondary motoneuron formation. Finally, the apparent downregulation of *olig2* by 48 hpf in transgenic embryos corresponds to the time that *olig2* is downregulated and OPCs are specified in normal embryos (Park et al., 2002). One possible explanation for this observation is that Notch1a^{ac} drives *olig2*$^{+}$ precursors into the OPC developmental pathway at 48 hpf.

To test whether Notch activity can promote formation of OPCs, we examined *sox10* expression. Although somites of 26 hpf embryos that had been heat shocked at 8 and 24 hpf strongly expressed *sox10*, spinal cord cells did not (Fig. 5B),

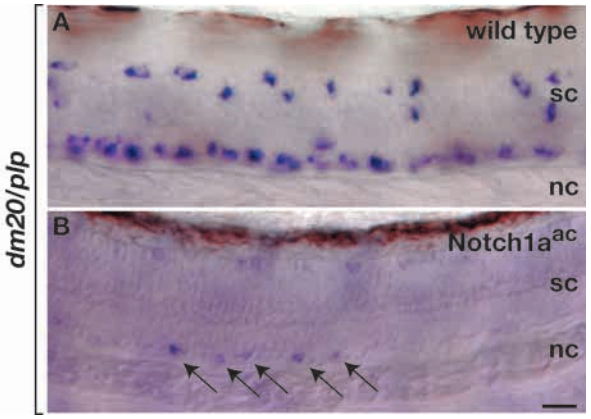

Fig. 6. Notch1a^{ac} inhibits oligodendrocyte differentiation. Side views of 3.5 dpf embryos, dorsal to top. (A) Wild-type embryo showing normal distribution of *plp1/dm20*$^{+}$ cells in spinal cord (sc). (B) Transgenic embryo in which Notch1a^{ac} was induced at 60 hpf. The number of *plp1/dm20*$^{+}$ cells was greatly reduced. Arrows indicate *plp1/dm20*$^{+}$ cells in ventral spinal cord. Notochord indicated by nc. Scale bar: 20 μm.

indicating that Notch1a^{ac} is not sufficient to promote premature formation of OPCs. 52 hpf transgenic embryos that were heat shocked at 8, 24 and 36 hpf had a 1.9-times excess of spinal cord *sox10*$^{+}$ cells compared with control embryos (Fig. 5D,E). The excess *sox10*$^{+}$ spinal cord cells occupied positions at which motoneurons normally reside. Because the increase in the number of *sox10*$^{+}$ spinal cord cells is similar to the increase in *olig2*$^{+}$ cells at 36 hpf, these data support the possibility that continuous Notch signaling directs ventral spinal cord cells that normally develop as neurons into the oligodendrocyte pathway. In contrast to embryos assayed at 26 hpf, somites of Notch1a^{ac}-expressing embryos of 52 hpf embryos did not express *sox10*, indicating that Notch activity cannot continuously promote *sox10* expression in mesodermal cells. However, these embryos had many *sox10*$^{+}$ cells adjacent to the spinal cord and notochord (Fig. 5D). In wild-type embryos, *sox10*$^{+}$ cells that occupy similar positions originate in neural crest (Dutton et al., 2001) (Fig. 5C). Thus, Notch activity might promote formation of neural crest or particular subsets of neural crest, consistent with previous data showing that Notch signaling is required for neural crest formation in zebrafish (Cornell and Eisen, 2000) and that Notch activity can promote formation of Schwann cells from cultured neural crest stem cells (Morrison et al., 2000). Taken together, our data show that Notch signaling can promote *sox10* expression and oligodendrocyte specification in the spinal cord, but that these functions are limited to the normal time and place of oligodendrocyte development.

Because experiments using cultured cells showed that Notch activity can block OPC differentiation (Wang et al., 1998; John et al., 2002), we induced Notch1a^{ac} expression at 60 hpf, after OPC specification, and assayed expression of *dm20/plp1*, which marks premyelinating oligodendrocytes, at 3.5 dpf. Compared with control embryos, few cells in experimental embryos expressed *dm20/plp1* (Fig. 6A,B). Thus, by driving ubiquitous Notch1a^{ac} expression after OPC specification, we prevented the progression of OPCs to a more mature developmental stage. Taken together, our data provide in vivo evidence that Notch signaling first promotes OPC specification and then inhibits their differentiation into mature oligodendrocytes.

DISCUSSION

Here, we presented two tests of a hypothesis that Delta-Notch signaling regulates specification of a common population of neural precursors for neuronal and oligodendrocyte fates. First, we found that mutant zebrafish embryos that had reduced Notch signaling activity failed to maintain the proliferative neural precursor population, had excess neurons and lacked oligodendrocytes. Second, we found that transgenic, conditional expression of a constitutively active form of Notch blocked neuronal development and promoted formation of excess OPCs in ventral spinal cord. Our data support a model in which Delta-Notch signaling prevents ventral spinal cord precursors from developing as neurons and then, acting with other temporally and spatially limited factors, specifies them for oligodendrocyte fate.

Delta-Notch signaling is required for OPC specification

Because mouse embryos that are homozygous for null mutations of *Delta* or *Notch* genes die at early stages of neural development, there are few data that address the requirement of Notch signaling for vertebrate CNS glial specification. Recently, this limitation was circumvented through analysis of mice in which *Notch1* was conditionally inactivated in the cerebellum. These mice prematurely expressed neuronal markers and had reduced number of mutant cerebellar cells that expressed the glial marker GFAP (Lutolf et al., 2002). In an alternative approach, neurospheres were derived from *Delta-like 1* mutant mice. After culturing, mutant neurospheres produced excess neurons and a deficit of oligodendrocytes and astrocytes compared with controls (Grandbarbe et al., 2003). Additionally, retinas of mice that were homozygous for a mutation of *Hes5*, which encodes a downstream effector of Notch signaling, had fewer Müller glia than the wild type (Hojo et al., 2000). These observations are consistent with the idea that Delta-Notch signaling regulates neuronal-glial fate decisions.

Several lines of evidence point toward a role for Delta-Notch signaling in regulating specification of motoneuron and oligodendrocyte fates in zebrafish. First, prospective primary motoneurons were usually replaced when they were removed at the 11-somite stage (Appel et al., 2001) but not at the 13-somite stage (Eisen et al., 1989). This is similar to observations that ablated neuroblasts were replaced by neighboring cells in grasshoppers (Doe and Goodman, 1985) and raised the possibility that primary motoneurons, like grasshopper neuroblasts, inhibit neighboring precursors from adopting the same fate. Second, prospective primary motoneurons expressed higher levels of *dla* and *dld* than neighboring cells (Appel and Eisen, 1998; Haddon et al., 1998), indicating that Notch ligands are present at the right time and place to regulate specification of cells that arise in close proximity to primary motoneurons. Third, mutant zebrafish that had reduced levels of Notch signaling had excess primary motoneurons and a concomitant deficit of later-born secondary motoneurons,

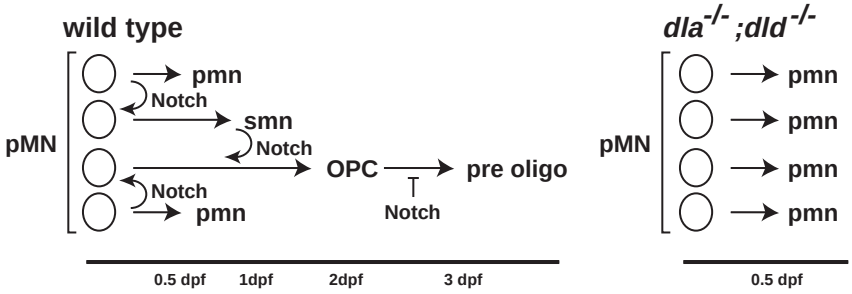

Fig. 7. Model for Delta-Notch regulated specification of pMN precursors for motoneuron and oligodendrocyte fates. Primary motoneurons (pmn) are the first cells to differentiate and activate Notch in neighboring cells via Delta. Notch activation maintains these cells as precursors during the period of primary motoneuron specification. Downregulation of Delta expression in differentiating primary motoneurons results in loss of Notch activity in some precursors, which then adopt secondary motoneuron (smn) fate. These cells upregulate Delta expression, maintaining high Notch activity in remaining precursors, which are specified as OPCs. Maintenance of high Notch activity in OPCs inhibits their differentiation into premyelinated oligodendrocytes (pre oligo). In *dla*$^{-/-}$;*dld*$^{-/-}$ embryos, which have reduced levels of Notch activity, excess pMN precursors are specified as primary motoneurons at the expense of secondary motoneurons and oligodendrocytes. This model does not account for possible lineage relationships between different cell types.

showing that Delta-Notch signaling regulates specification of neural precursors for different neuronal fates (Appel et al., 2001). Finally, medial neural plate cells, which occupy ventral spinal cord upon completion of neurulation, gave rise to primary motoneurons and oligodendrocytes (Park et al., 2002). Thus, Delta proteins expressed by primary motoneurons could regulate specification of nearby cells for oligodendrocyte fate.

Here, we showed that *dla*$^{-/-}$;*dld*$^{-/-}$ and *mib*$^{-/-}$ embryos did not produce OPCs or premyelinating oligodendrocytes. Additionally, neural precursors prematurely exited the cell cycle and differentiated as neurons in these embryos. As secondary motoneurons and oligodendrocytes arise after primary motoneurons, one interpretation of our data is that Notch signaling prevents a subset of ventral spinal cord precursors from developing as primary motoneurons, enabling them to take later neuronal or oligodendrocyte fates. In this view, downregulation of *delta* gene expression during primary motoneuron differentiation would result in a decrease of Notch activity in neighboring precursors. A release from Notch-mediated inhibition soon after primary motoneuron specification might allow a cell to develop as a secondary motoneuron, whereas a later release might result in oligodendrocyte development (Fig. 7). Thus, temporal regulation of Notch signaling might underlie the temporal switch in production of primary motoneurons to secondary motoneurons to oligodendrocytes.

A switch between production of neurons and glial cells was proposed to be regulated by bHLH proteins (Vetter, 2001). In the ventral spinal cord, motoneuron and oligodendrocyte precursors expressed Olig bHLH proteins, which are structurally similar to proneural Ngns (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000). During the period of motoneuron production, a subset of Olig^{+} cells expressed Ngns (Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001) (data shown here). Later, Ngn expression subsided, coincident with the time at which oligodendrocytes are thought to be specified (Zhou et al., 2001). These observations, coupled with various functional tests, led to the proposal that Ngn and Olig proteins create a simple bHLH protein code in which Ngn and Olig expression together specify motoneuron development and Olig alone, upon Ngn downregulation, specifies oligodendrocyte development (Zhou et al., 2001; Zhou and Anderson, 2002).

Our data provide evidence supporting the importance of a bHLH protein code to motoneuron and oligodendrocyte specification and show that Delta-Notch signaling is required to establish the code. We have shown that the failure to restrict *ngn1* expression to a subset of medial neural plate cells in Notch signaling deficient zebrafish embryos correlated with formation of excess neurons, consistent with other observations that Notch signaling inhibits proneural genes expression and neuronal development in vertebrate and invertebrate embryos. Furthermore, *dla*$^{-/-}$;*dld*$^{-/-}$ and *mib*$^{-/-}$ embryos failed to maintain a proliferative population of *olig2*$^{+}$ cells. We interpret this to mean that, in the absence of Delta-Notch mediated inhibition, uniformly high levels of Ngns cause all *olig2*$^{+}$ neural precursors to stop dividing and differentiate as neurons at the expense of oligodendrocytes. Thus, in normal embryos, high levels of Notch activity prevents *ngn* gene expression in a subset of *olig2*$^{+}$ neural precursors, reserving them to produce other cell types, such as oligodendrocytes, at a later time. In this view, Delta-Notch signaling might play a purely permissive role in neural cell fate diversification, by regulating the ability of neural precursors to respond to other instructive signals.

Delta-Notch signaling promotes oligodendrocyte specification

Another possibility we sought to test is that Notch activity might specify neural precursors for oligodendrocyte fate. Although several reports already addressed this possibility, the data are ambiguous. For example, Notch activity blocked formation of oligodendrocytes from cultured adult hippocampus-derived progenitors (AHPs) (Tanigaki et al., 2001). However, this study assessed oligodendrocyte development using only markers that reveal late stages of oligodendrocyte differentiation. Because Notch activity inhibits oligodendrocyte differentiation (see below), OPCs might have been produced from AHPs but not detected in these experiments. In a different cell culture assay, Notch activity promoted formation of both OPCs and astrocytes, apparently from common precursors, within neurospheres (Grandbarbe et al., 2003). However, there are no data that support the presence of a common precursor of oligodendrocytes and astrocytes in vivo, leaving open the question of whether Notch signaling specifies OPCs during development. Finally, expression of constitutively active Notch, alone, by electroporation was not sufficient to promote OPC formation in chick spinal cords (Zhou et al., 2001).

Here, we used a transgenic, conditional expression system

to drive high levels of Notch1a^{ac} in intact zebrafish embryos throughout the period of neurogenesis. These embryos did not prematurely nor ectopically express OPC markers. However, they had an approximately twofold excess of *olig2*$^{+}$ and *sox10*$^{+}$ ventral spinal cord cells at 36 and 52 hpf, respectively. By contrast, spinal cord cells of *dla*$^{-/-}$;*dld*$^{-/-}$ and *mib*$^{-/-}$ embryos initiated but did not maintain *olig2* expression and never expressed *sox10*. Because Notch1a^{ac} simultaneously blocked neuronal development, we interpret our results to mean that Notch diverts precursors that would otherwise develop as ventral spinal cord neurons toward an oligodendrocyte fate (Fig. 7). In contrast to canonical models of Notch signaling, which suggest that Notch activity must be downregulated for precursor cells to enter a developmental pathway, our work shows that continuous Notch activity causes ventral spinal cord precursors to develop as oligodendrocytes instead of neurons.

Our observation that Notch1a^{ac} expression promoted formation of excess OPCs only in ventral spinal cord at the normal time of OPC specification indicates that Notch must act with other spatially and temporally regulated factors. One candidate is Olig2, as co-electroporation of plasmids that encode Olig2 and constitutively active Notch promoted ectopic OPC development in chick embryos (Zhou et al., 2001). However, in zebrafish, OPCs do not appear to be specified for at least 36 hours after initiation of *olig2* expression. The Notch pathway must be active in a subset of these cells throughout the period of *olig2* expression to block motoneuron development. This raises the possibility that the factor that controls the timing of oligodendrocyte specification is downstream of *olig2*.

One important problem our data do not address is the nature of motoneuron and oligodendrocyte precursors. At one extreme, pMN precursors might be multipotent, having equivalent potential to develop as primary motoneurons, secondary motoneurons or oligodendrocytes. At the other extreme, pMN precursors might be restricted to a single fate. A third alternative is that some precursors produce primary and secondary motoneurons whereas others produce only oligodendrocytes. Lineage analysis performed in chick embryos showed that motoneurons and oligodendrocytes can share a precursor but left unresolved the question of when these cell types become separated in the lineage (Leber et al., 1990). A second problem that must be considered is whether pMN cells give rise only to motoneurons and oligodendrocytes or whether they also produce other cell types. Various kinds of ventral spinal cord interneurons in zebrafish embryos have been described by morphology but their origin is unknown (Bernhardt et al., 1990; Bernhardt et al., 1992; Hale et al., 2001). A complete understanding of the mechanism by which Delta-Notch signaling regulates motoneuron, oligodendrocyte and possibly interneuron specification will require careful cell lineage analysis combined with conditional manipulation of Notch signaling activity.

Delta-Notch signaling couples oligodendrocyte specification and differentiation

Significantly, various data indicate that Notch signaling inhibits oligodendrocyte differentiation. In vitro experiments showed that Notch activity could inhibit differentiation of purified OPCs (Wang et al., 1998), selective inactivation of Notch1a in OPCs in vivo caused their premature differentiation (Genoud et al., 2002) and oligodendrocytes became myelinated too soon in mice heterozygous for a *Notch1* mutation (Givogri et al., 2002). Additionally, astrocytes in demyelinating lesions of human multiple sclerosis patients upregulated Jagged1, a Notch ligand, and Jagged1 blocked maturation of purified human OPCs (John et al., 2002). Our observation that Notch1a^{ac} inhibited expression of *plp1/dm20* in vivo provides support for the idea that Notch signaling regulates maturation of oligodendrocytes. Taken together with our demonstration that Delta-Notch signaling promotes OPC formation, our work indicates that Notch signaling both promotes specification of neural precursors for oligodendrocyte fate and subsequently regulates their differentiation (Fig. 7). Thus, Notch activity couples the control of oligodendrocyte specification and differentiation, which might help to match the development of myelinating oligodendrocytes to their target axons.

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