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Notch signaling regulates neural precursor allocation and binary neuronal fate decisions in zebrafish

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Notch signaling plays a well-described role in regulating the formation of neurons from proliferative neural precursors in vertebrates but whether, as in flies, it also specifies sibling cells for different neuronal fates is not known. Ventral spinal cord precursors called pMN cells produce mostly motoneurons and oligodendrocytes, but recent lineage-marking experiments reveal that they also make astrocytes, ependymal cells and interneurons. Our own clonal analysis of pMN cells in zebrafish showed that some produce a primary motoneuron and KA′ interneuron at their final division. We investigated the possibility that Notch signaling regulates a motoneuron-interneuron fate decision using a combination of mutant, transgenic and pharmacological manipulations of Notch activity. We show that continuous absence of Notch activity produces excess primary motoneurons and a deficit of KA′ interneurons, whereas transient inactivation preceding neurogenesis results in an excess of both cell types. By contrast, activation of Notch signaling at the neural plate stage produces excess KA′ interneurons and a deficit of primary motoneurons. Furthermore, individual pMN cells produce similar kinds of neurons at their final division in *mib* mutant embryos, which lack Notch signaling. These data provide evidence that, among some postmitotic daughters of pMN cells, Notch promotes KA′ interneuron identity and inhibits primary motoneuron fate, raising the possibility that Notch signaling diversifies vertebrate neuron type by mediating similar binary fate decisions.

KEY WORDS: *olig2*, Neural precursors, Motoneurons, Interneurons, Cell lineage, Lateral inhibition

INTRODUCTION

Neuronal diversity is one of the fundamental features of the nervous system. For example, the spinal cord, the simplest part of the vertebrate central nervous system (CNS), has three main classes of neurons – motoneurons, projection neurons and interneurons (Altman and Bayer, 1984) – and these can be subdivided into many distinct subtypes (Lewis, 2006). Neuronal diversity is achieved through various developmental strategies. For example, neural precursors are specified differentially by spatial cues. In the ventral neural tube, different types of neurons are generated from distinct neural precursor domains, which are specified at different positions on the dorsoventral axis by a morphogenic gradient of Sonic hedgehog (Shh) (Jessell, 2000). Also, intrinsic signals that change over time can direct the formation of different neurons from common precursors. The best-described example is that of fly neuroblasts, whereby temporal transition in the expression of transcription factors specifies neuronal identity (Brody and Odenwald, 2002; Pearson and Doe, 2004). Similar mechanisms could operate in vertebrates. Foxg1, a winged helix transcription factor, is a key temporal specification regulator for telencephalic corticogenesis because it suppresses early-born Cajal-Retzius cell fate in vivo (Hanashima et al., 2004), and experimental reduction of Foxg1 expression restores fate plasticity of late cortical progenitors (Shen et al., 2006).

In addition to spatial and temporal cues, signals that act asymmetrically in pairs of newly divided sibling cells can specify them for different fates (Gotz and Huttner, 2005). In many instances, asymmetric division produces one postmitotic neuron and one proliferative precursor. However, asymmetric divisions can also produce two different kinds of neurons. Again, this is best understood in flies where ganglion mother cells (GMCs) undergo a terminal, asymmetric division to produce two distinct neurons. For example, the MP neural precursor divides asymmetrically to produce two different interneurons called dMP2 and vMP2 (Spana et al., 1995).

A critical mechanism for regulating neural cell diversity is cell-cell communication mediated by Notch receptors and membrane-bound Notch ligands. Through lateral inhibition, Notch signaling limits the number of cells within a precursor domain that adopt neuronal fates. In the fly embryonic CNS, loss of Notch signaling results in the formation of excess neurons at the expense of epidermal cells (Campos-Ortega, 1995). Notch also influences asymmetric fate decisions. In the absence of Notch signaling, both daughters of the MP neural precursor develop as dMP2 (Spana and Doe, 1996). Thus, in flies, Notch signaling regulates neural fate through at least two mechanisms. First, through lateral inhibition it regulates the number of cells specified for neural fate. Second, through binary fate specification it directs sibling cells for different neuronal fates.

In zebrafish embryos, primary motoneurons that are ablated soon after they are born are replaced (Appel et al., 2001), showing that lateral inhibition also operates in vertebrates to regulate neural development. Numerous lines of evidence indicate that, as in flies, Notch receptors and their ligands mediate vertebrate lateral inhibition. For example, expression of dominant-negative forms of Delta ligands causes formation of excess early-born neurons in frog, fish and chicken embryos with concomitant decreases in proliferative cells and later-born neurons and glia (Appel and Eisen, 1998; Chitnis et al., 1995; Dornseifer et al., 1997; Dorsky et al., 1997; Haddon et al., 1998; Henrique et al., 1997). Consistent with these data, zebrafish embryos homozygous for mutations of *mind bomb* (*mib*), which encodes a ubiquitin ligase necessary for efficient Notch signaling, have excess early-born neurons and depletion of

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neural precursors (Itoh et al., 2003), and Notch mutant mice upregulate expression of proneuronal transcription factors, indicative of premature and excess neuronal development (de la Pompa et al., 1997; Ishibashi et al., 1995). By contrast, expression of constitutively active forms of Notch block neuronal development and seemingly maintain neural cells in a precursor state (Coffman et al., 1993; Dorsky et al., 1995; Gaiano et al., 2000; Park and Appel, 2003). These observations helped foster the view that in vertebrates Notch diversifies neural fate by determining, through lateral inhibition, whether a cell differentiates or remains as a precursor (Gaiano and Fishell, 2002). It is not yet clear whether Notch signaling mediates asymmetric neuronal fate specification in vertebrates because the detailed cell lineage information necessary for identifying binary fate decisions is lacking.

Previously, we showed that a subset of *olig2*$^{+}$ ventral spinal cord precursors produce primary motoneurons and KA′ interneurons at their final division (Park et al., 2004). Here we describe a series of experiments designed to test the role of Notch signaling in primary motoneurons and KA′ interneuron specification. Our results provide evidence that Notch signaling operates at two important levels in vertebrate neuronal specification. First, through lateral inhibition, it regulates the number of neural precursors that give rise to specific subsets of neurons. Second, it specifies sibling cells for different neuronal fates.

MATERIALS AND METHODS

Fish lines and maintenance

Embryos were raised at 28.5°C in egg water or embryo medium (EM) and staged according to hours post-fertilization (hpf) and morphological criteria (Kimmel et al., 1995). *mib*^{ta52b} mutant fish (Jiang et al., 1996; Schier et al., 1996), *Tg(olig2:egfp)*^{VU12} (Park et al., 2004; Shin et al., 2003), *Tg(hsp70l:XdnSu(H)myc)*^{VU21} (Latimer et al., 2005), *Tg(hsp70:GAL4)*, *Tg(UAS:Notch1a*^{ac}*-myc)* (Scheer et al., 2002) and *Tg(hsp70l:gal4vp16)*^{VU22} fish were used for this study.

Generation of *Tg(hsp70l:gal4vp16)*^{VU22} transgenic fish

We first substituted the zebrafish *heat shock cognate 70-kd protein, like* (*hsp70l*) promoter (Shoji et al., 1998) for the CMV promoter of pCS2. Next, we inserted *gal4vp16* cDNA (Koster and Fraser, 2001) into the *phsp70l* vector, creating *phsp70l:gal4vp16*. We then transferred a fragment containing the *hsp70l:gal4vp16* fused to SV40 poly(A) into the *pINmega* vector (Latimer et al., 2005), which contains *I-SceI* recognition sequences, thus creating *pINmega-hsp70l:gal4vp16*. To produce *Tg(hsp70l:gal4vp16)* fish, we used the *I-SceI*-mediated transgenesis strategy (Thermes et al., 2002) as described previously (Latimer et al., 2005). To identify germline transformed founders, G0 fish were crossed to homozygous *Tg(UAS:Notch1a*^{ac}*-myc)* fish and the embryos heat shocked by incubation at 10 hpf for 30 minutes at 36°C. *Tg(hsp70l:gal4vp16);Tg(UAS:Notch1a*^{ac}*-myc)* double-transgenic embryos were identified by morphological defects and confirmed by anti-Myc immunohistochemistry. To establish stable lines we mated G0 founders to wild-type fish, raised the embryos to adulthood and screened them by the same method.

In situ hybridization, immunohistochemistry, BrdU and TUNEL assays

isl2 (Appel et al., 1995) and *her4* (Takke et al., 1999) antisense RNA probes were generated using Digoxigenin RNA Labeling Kits (Roche). In situ hybridization was performed as described previously (Hauptmann and Gerster, 2000).

For immunohistochemistry, we used the following primary antibodies: mouse anti-BrdU [G3G4, 1:1000, Developmental Studies Hybridoma Bank (DSHB)], anti-c-Myc (Ab-1, 1:100, Oncogene Research Products), mouse anti-HuC/D (1:20, Molecular Probes), mouse anti-Isl (39.4D5, 1:200, DSHB), rabbit anti-phosphorylated histone H3 (1:1000, Upstate Biotechnology) and rabbit anti-GABA (1:1000, Sigma). For fluorescent detection of antibody labeling, we used Alexa Fluor 568 goat anti-mouse, Alexa Fluor 568 goat anti-rabbit conjugate, Alexa Fluor 647 goat anti-mouse conjugate and Alexa Fluor 647 goat anti-rabbit conjugate (all 1:200, Molecular Probes).

For BrdU labeling, manually dechorionated embryos were incubated in BrdU solution (10 mM BrdU and 15% DMSO in EM) for 20 minutes on ice. After washing three times, the embryos were placed in EM, incubated until the appropriate stages at 28.5°C and then fixed with 4% paraformaldehyde. The fixed embryos were immersed in 2 M HCl for 30 minutes and then processed for anti-BrdU and anti-GABA immunohistochemistry at the same time.

For the TUNEL assay, embryos were fixed, dechorionated manually and dehydrated in 100% methanol. The embryos were rehydrated in a graded TBS (10×: 1 M Tris base, 1.5 M NaCl, pH 7.5) series and washed (3×5 minutes) in TBST (1×TBS, 4% Triton X-100). Embryos were rinsed (2×5 minutes) in 1×TTase buffer (5×: 125 mM Tris buffer, 1 M Na cacodylate, 1.25 mg/ml BSA, 1% Tween 20, pH 6.6 at room temperature) without $CoCl_2$. Embryos were preincubated in reaction solution [1×TTase buffer, 1 mM $CoCl_2$, 0.5 μl terminal transferase (Roche), 0.25 μl tetramethyl-rhodamine-5-dUTP (Roche)] on ice, in the dark. Incubation was continued at room temperature for 24 hours. Embryos were washed in TBST (2×5 minutes) followed by the immunohistochemistry procedure.

In situ hybridization images were collected using a QImaging Retiga Exi color CCD camera mounted on a compound microscope and imported into Photoshop (Adobe). Image manipulations were restricted to levels, curve and contrast adjustments. Fluorescence images were collected using a Zeiss LSM510 laser scanning confocal microscope.

Heat-shock induction

To induce expression of the *Xenopus laevis* dominant-negative form of Suppressor of Hairless fused to the Myc tag (XdnSu(H)myc), embryos were collected from matings of *Tg(hsp70l:XdnSu(H)myc);Tg(olig2:egfp)* homozygous adults and raised at 28.5°C. At 6 hpf, 9.5 hpf or 11 hpf, embryos were placed in a prewarmed beaker containing 35 ml of EM in a 39.5°C circulating water bath for 30 minutes and then transferred to fresh EM and incubated at 28.5°C until fixing stage (20 hpf and 25 hpf). Similarly, to induce expression of constitutively active Notch1a fused to the Myc tag (Notch1a^{ac}-myc), embryos were collected from matings of heterozygous *Tg(hsp70l:gal4vp16)* and homozygous *Tg(UAS:Notch1a*^{ac}*-myc);Tg(olig2:egfp)* adults and raised at 28.5°C. At 11 hpf, embryos were transferred to EM either at 34°C (mild induction) or 36°C (strong induction) for 30 minutes and then returned to 28.5°C until the appropriate stage for fixing. Approximately half of the embryos should inherit all three transgenes, which we confirmed by morphological differences, anti-Myc immunocytochemistry and EGFP fluorescence.

DAPT treatment

DAPT treatments were performed as previously described (Geling et al., 2002). DAPT (Calbiochem) was reconstituted with dimethyl sulfoxide (DMSO) to make a stock concentration of 10 mM. Aliquots were diluted to 100 μM in EM. Embryos were dechorionated manually and placed in an agarose-coated plate, which contained the DAPT solution, at 6 hpf. Half of the embryos were washed with EM at 11 hpf. All embryos were returned to 28.5°C until appropriate stage for fixing. Control embryos were treated with EM containing 1% DMSO only.

Single-cell labeling

Embryos were collected from matings of *mib*$^{ta52b+/-}$*;Tg(olig2:egfp)* adults and raised at 28.5°C. At 10 hpf, embryos were dechorionated manually and mounted, dorsal side upwards, in 3% methyl cellulose (1500 centipoises, Sigma) submerged in EM on a depression slide. The slide was mounted on a fixed-stage compound microscope and viewed using a 40× water-immersion objective. An injection pipette was backloaded with 2 ml of 5% tetramethyl-rhodamine dextran (M_r 10,000, Molecular Probes) solution dissolved in 0.2 M KCl, filled completely with 0.5 M KCl and connected to the electrode, which was connected to an amplifier. To establish an electrical circuit, a ground wire was placed in the depression slide. The pipette was positioned on a single EGFP^{+} cell and labeled by an electric pulse. Embryos with single labeled cells were transferred individually to EM with 0.5% penicillin/streptomycin (Gibco) in 24-well plates and raised in the dark at

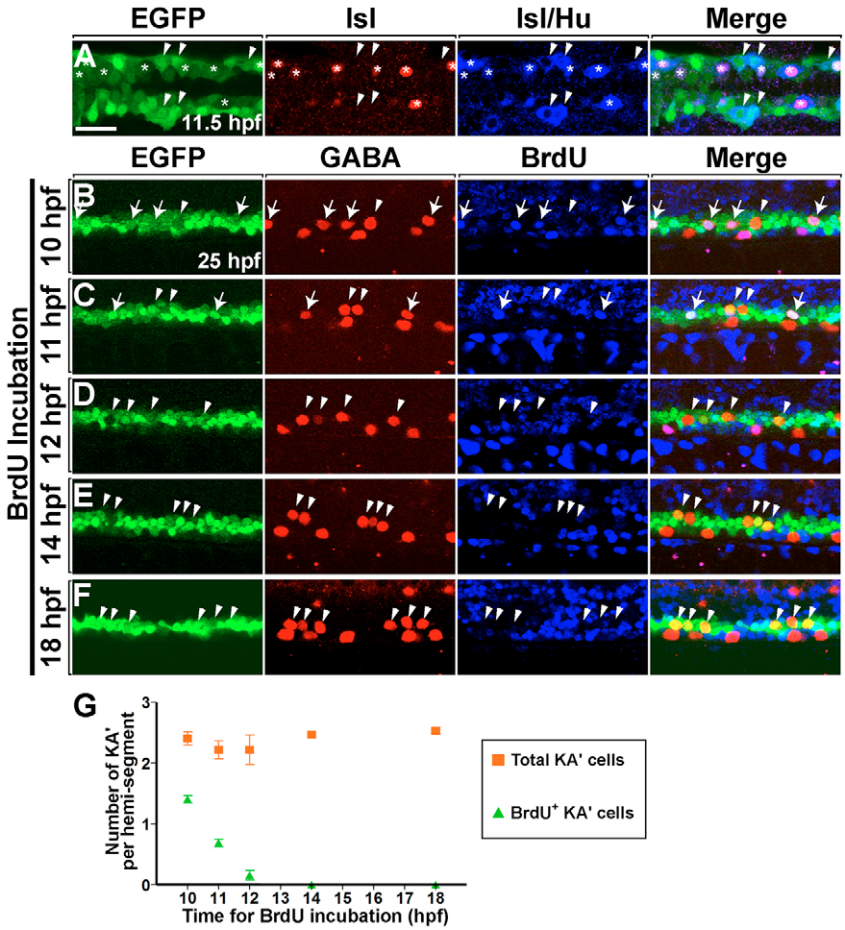

Fig. 1. Neural plate *olig2*:EGFP$^+$ precursors generate PMNs and KA′ interneurons at the same time. (**A**) Dorsal view of the posterior neural plate at 11.5 hpf (five-somite stage) and (**B-F**) lateral view of 6- to 12-somite regions of spinal cord at 25 hpf in *Tg(olig2:egfp)* zebrafish embryos. (A) Heterogeneous primary neuronal populations within the EGFP$^+$ domain. Asterisks and arrowheads mark EGFP$^+$ Hu$^+$ Isl$^+$ PMNs and EGFP$^+$ Hu$^+$ Isl$^-$ interneurons, respectively. Isl protein is nuclear, whereas Hu is cytoplasmic. (B-F) Embryos were incubated with BrdU at successive timepoints (as shown to the left of each panel) and labeled with anti-GABA (red) and anti-BrdU (blue) antibodies. Arrows and arrowheads mark BrdU$^+$ and BrdU$^-$ KA′ interneurons, respectively. (B) Four KA′ interneurons were formed from EGFP$^+$ precursors that underwent S phase at 10 hfp (arrows). Arrowhead marks BrdU$^-$ KA′ interneuron, indicating that the postmitotic cell was formed before or after 10 hpf. (C) Two BrdU$^+$ KA′ interneurons (arrows) and two BrdU$^-$ KA′ interneurons (arrowheads) were detected in the embryos that were incubated with BrdU at 11 hpf. (D,E,F) Embryos incubated with BrdU at 12, 14 and 18 hpf. No BrdU$^+$ KA′ interneurons were evident. (**G**) Average of all KA′ cells (squares) versus BrdU$^+$ KA′ cells (triangles) (n=4 animals each). S phase for the last KA′ interneurons produced occurs between 10 and 12 hpf. Error bars represent s.e.m. Scale bar: 25 μm.

28.5°C. At 24-30 hpf, labeled cells of mutant embryos were analyzed using a Zeiss LSM510 Meta laser scanning confocal microscope. All clones occupied the mid-trunk region of the spinal cord (somite levels 6-15).

RESULTS

Medial neural plate cells produce different types of neurons at the same time

Previously, we defined a subpopulation of pMN cells proximal to the neural plate midline from which individual precursors give rise to early-born primary motor neurons (PMNs), a few later-born secondary motor neurons (SMNs), and two types of GABAergic interneurons called KA′ and VeLD (Park et al., 2004). Notably, about two-thirds of proximal pMN cells that proliferated produced two distinct types of neurons, raising the possibility that a binary fate decision mechanism produces different neurons from some pMN precursors undergoing terminal divisions. As a first test of this idea, we labeled 11.5 hour-post-fertilization (hpf), five-somite stage *Tg(olig2:egfp)* embryos with anti-Isl antibody, which marks PMNs (Myers et al., 1986; Park et al., 2002), and anti-Hu antibody, which labels newly born neurons (Marusich et al., 1994). This revealed two types of neurons within the proximal pMN domain, EGFP$^+$ Hu$^+$ Isl$^+$ PMNs and EGFP$^+$ Hu$^+$ Isl$^-$ neurons (Fig. 1A). EGFP$^+$ Hu$^+$ Isl$^-$ neurons might be GABAergic VeLD and KA′ interneurons because they are born from *olig2*$^+$ cells that also produce PMNs (Park et al., 2004). However, VeLD and KA′ interneurons are not GABA-immunoreactive until about 20 hpf (data not shown), well after PMNs are evident by gene expression (Appel et al., 1995; Inoue et al., 1994; Tokumoto et al., 1995). Consequently, we determined the birth date of KA′ and VeLD interneurons to ascertain whether they are born at the same time as PMNs. Beginning at 10 hpf, we pulse-labeled *Tg(olig2:egfp)* embryos with BrdU at successive time points and then analyzed BrdU and GABA labeling at 25 hpf. 58% of KA′ cells incorporated BrdU when labeling occurred at 10 hpf (Fig. 1B,G). The portion of KA′ cells incorporating BrdU fell to 31% at 11 hpf (Fig. 1C,G) and 7% at 12 hpf (Fig. 1D,G). No KA′ cells incorporated BrdU when labeling was at 14 and 18 hpf (Fig. 1E,F,G). These data show that most KA′ precursors undergo a final round of DNA synthesis between 10 and 12 hpf, which correlates with the birth dates of PMNs between 9 and 16 hpf (Myers et al., 1986). Thus, birth date, gene expression and lineage data are all consistent with the idea that two different types of neurons can arise from pMN precursors undergoing terminal division.

mib mutant embryos produce excess PMNs and have a deficit of KA′ interneurons

In zebrafish, loss of Notch signaling results in the formation of excess PMNs (Appel et al., 2001; Haddon et al., 1998). If Notch signaling mediates a binary decision between PMN and KA′ interneuron fates, Notch signaling-deficient embryos should have a deficit of KA′ interneurons. To test this, we used gene expression to examine PMNs and KA′ interneurons in embryos mutant for the *mind bomb* (*mib*) gene, which encodes an E3 ubiquitin ligase necessary for efficient Notch signaling (Itoh et al., 2003). In the ventral spinal cord of wild-type embryos, *isl2* RNA expression marks 1-2 cells, which are CaP and VaP PMNs, for an average of about 1.3 cells per hemisegment (Fig. 2A,E) (Appel et

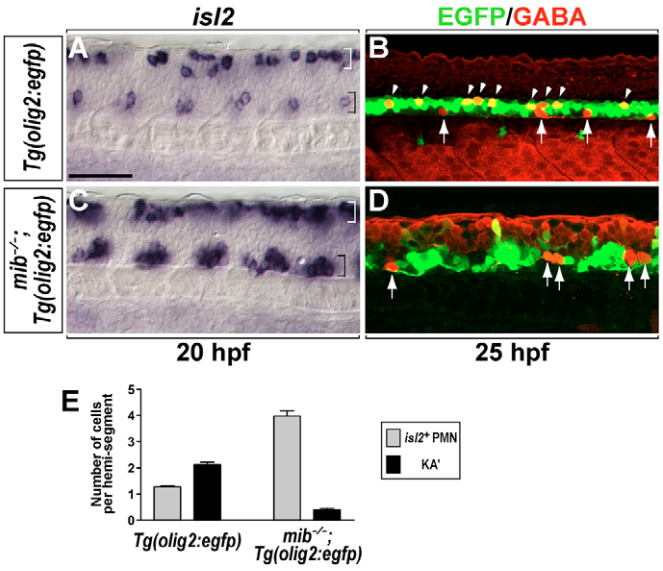

Fig. 2. Loss of Notch signaling causes formation of excess PMNs and a deficit of KA′ interneurons. (**A-D**) Lateral views of the spinal cord at the 6- to 12-somite region of zebrafish embryos, with anterior to left and dorsal at the top. (A) A subset of PMNs (CaP and VaP) and Rohon-Beard neurons of *Tg(olig2:egfp)* embryos expressed *isl2* in the ventral spinal cord (black bracket) and dorsal spinal cord (white bracket) at 20 hpf, respectively. (B) In *Tg(olig2:egfp)* embryos, KA′ interneurons (arrowheads) were detected by anti-GABA antibody (red) within EGFP$^+$ cells at 25 hpf. EGFP$^-$ GABA$^+$ cells (arrows) in ventral spinal cord are KA″ interneurons (Park et al., 2004). (C) In *mib*$^{-/-}$*;Tg(olig2:egfp)* embryos, *isl2*$^+$ PMNs formed as clusters in each hemisegment in the ventral spinal cord (black bracket). (D) *mib*$^{-/-}$*;Tg(olig2:egfp)* embryos did not produce EGFP$^+$ GABA$^+$ KA′ interneurons, although EGFP$^-$ GABA$^+$ KA″ interneurons were still present (arrows). (**E**) Mean of the number of PMNs (gray bar) and KA′ interneurons (black bar) per hemisegment in *Tg(olig2:egfp)* and *mib*$^{-/-}$*;Tg(olig2:egfp)* embryos (n=7). Error bars represent s.e.m. Scale bar: 50 μm.

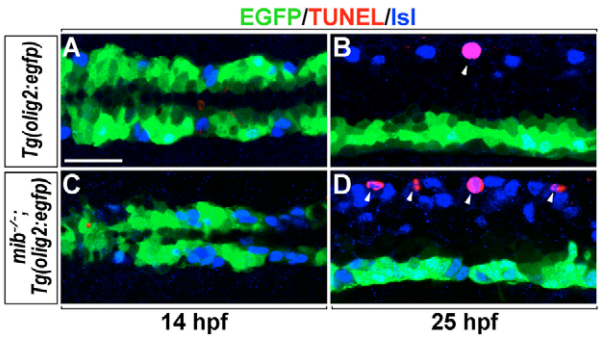

Fig. 3. Deficit of KA′ interneurons is independent of apoptosis in *mib*$^{-/-}$ zebrafish embryos. Dorsal views of the posterior neural plate at 14 hpf (**A**,**C**) and lateral views of spinal cord at 25 hpf (**B**,**D**) in *Tg(olig2:egfp)* (A,B) and *mib*$^{-/-}$*;Tg(olig2:egfp)* embryos (C,D). Green, blue and red channels mark EGFP$^+$ cells, Isl$^+$ neurons and TUNEL$^+$ cells, respectively. Neither control nor *mib*$^{-/-}$ embryos had EGFP$^+$ TUNEL$^+$ cells at 14 hpf or 25 hpf (A-D). TUNEL$^+$ Rohon-Beard neurons (arrowheads) were detected in control and *mib*$^{-/-}$ embryos (B,D) at 25 hpf. Scale bar: 25 μm.

al., 1995). In *mib* mutant embryos, about 4.0 *isl2*$^+$ cells formed per hemisegment (Fig. 2C,E). To count KA′ interneurons, we scored EGFP$^+$ GABA$^+$ cells in wild-type *Tg(olig2:egfp)* and *mib;Tg(olig2:egfp)* mutant embryos. Whereas wild-type embryos had an average of 2.1 KA′ interneurons per hemisegment (Fig. 2B,E), *mib* mutant embryos had an average of only 0.4 KA′ cells per hemisegment (Fig. 2D,E). Thus, loss of Notch signaling had complementary effects on PMNs and KA′ interneurons, producing an excess of the former and a deficit of the latter, consistent with the possibility that Notch signaling regulates specification of these cell fates in a binary fashion.

Although disruption of a Notch-mediated fate decision might account for the deficit of KA′ interneurons in *mib* mutant embryos, alternative explanations are that these cells were eliminated by apoptotic death, or neural plate cells differentiated directly into PMNs without dividing. To discriminate between these possibilities we performed cell death and proliferation assays. At 14 hpf, no EGFP$^+$ cells were labeled by the apoptotic-cell-death marker TUNEL in either wild-type *Tg(olig2:egfp)* or *mib;Tg(olig2:egfp)* mutant embryos (Fig. 3A,C). Similarly, TUNEL labeling was not evident in EGFP$^+$ cells of wild-type or mutant embryos at 25 hpf (Fig. 3B), although *mib* mutant embryos had an elevated number of TUNEL$^+$ dorsal sensory neurons (Fig. 3D). To investigate cell division, we labeled wild-type *Tg(olig2:egfp)* and *mib;Tg(olig2:egfp)* mutant embryos with anti-phosphorylated histone H3 (PH3) antibody, which marks M-phase cells, and with anti-Hu antibody. At 11 hpf, wild-type embryos had an average of 11.8 EGFP$^+$ PH3$^+$ cells (see Fig. S1A,I in the supplementary material). In *mib* mutant embryos, an average of 7.8 EGFP$^+$ cells were PH3$^+$ (see Fig. S1B,I in the supplementary material). At 12 hpf, wild-type and *mib* embryos averaged 11.5 and 7.8 EGFP$^+$ PH3$^+$ cells, respectively (see Fig. S1C,D,I in the supplementary material). Subsequently, mitotic EGFP$^+$ cells were maintained until at least 18 hpf in both wild-type and *mib* mutant embryos, although the number of double-labeled cells declined at similar rates (see Fig. S1E-I in the supplementary material). Thus, *mib* mutant embryos had a population of dividing EGFP$^+$ cells during the period of motoneuron and KA′ interneuron birth. Together, these data indicate that neither cell death nor absence of cell division accounts for the deficit of KA′ interneurons in *mib* mutant embryos.

Transient inhibition of Notch signaling during early neurogenesis produces excess PMNs and KA′ interneurons

There are at least two, non-mutually exclusive mechanisms by which Notch signaling might regulate the number of PMNs and KA′ interneurons. First, through lateral inhibition, Notch signaling might limit the number of neural precursors that undergo a terminal division to produce PMNs and KA′ interneurons. Second, Notch might subsequently regulate a binary fate decision for PMN and KA′ interneuron fate. To discriminate between these possibilities, we performed two independent series of conditional loss-of-function experiments of Notch signaling, with the idea that lateral inhibition and binary fate specification might be temporally separated processes. First, we used DAPT, a pharmacological inhibitor of γ-secretase (Dovey et al., 2001), which effectively interferes with Notch pathway functions when applied to zebrafish embryos (Geling et al., 2002; Latimer et al., 2005). Whereas DMSO-treated control embryos had averages of 1.3 *isl2*$^+$ PMNs and 2.1 GABA$^+$ KA′ interneurons per hemisegment (Fig. 4A,B,K), embryos that were incubated

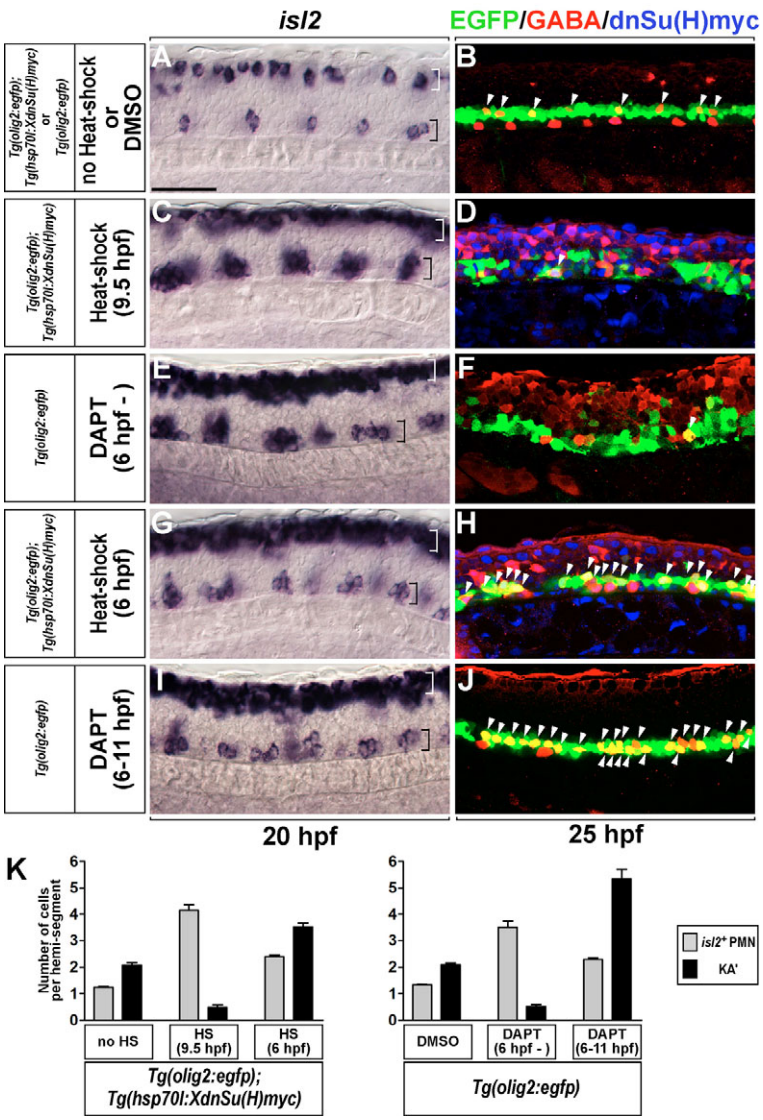

Fig. 4. Transient inactivation of Notch signaling produces excess PMNs and KA′ interneurons. (**A-J**) Lateral views of the spinal cord of zebrafish embryos at the 6- to 12-somite region, with anterior to the left and dorsal at the top. All embryos carried the *Tg(olig2:egfp)* transgene. (C,D,G,H) *Tg(olig2:egfp);Tg(hsp70l:XdnSu(H)myc)* double-transgenic embryos. (A,C,E,G,I) *isl2* expression at 20 hpf. White and black brackets mark dorsal and ventral spinal cord, respectively. (B,D,F,H,J) KA′ cells (arrowheads) were detected by anti-GABA antibody (red) within EGFP$^+$ cells (green) at 25 hpf. (A,B) Control embryos. (C,D) Induction of XdnSu(H)Myc by heat shock at 9.5 hpf resulted in excess PMNs with reduction of KA′ interneurons (arrowhead). XdnSu(H)Myc protein was detected by anti-Myc antibody (blue). (E,F) Embryos incubated continuously with DAPT had excess PMNs and a deficit of KA′ interneurons (arrowhead). (G,H) Double-transgenic embryos heat shocked at 6 hpf formed excess PMNs (black bracket) and KA′ interneurons (arrowheads). (I,J) Embryos incubated with DAPT from 6 to 11 hpf had excess PMNs (black bracket) and KA′ cells (arrowheads). (**K**) Mean of the number of PMNs (gray bar) and KA′ interneurons (black bar) per hemisegment (*n*=7) in A-J. Error bars represent s.e.m. Scale bar: 50 μm.

continuously in DAPT solution beginning at 6 hpf had excess PMNs (average 3.5 *isl2*$^+$ PMNs per hemisegment) and a deficit of KA′ interneurons (average 0.5 cells per hemisegment) at 20 hpf and 25 hpf, respectively (Fig. 4E,F,K). Thus, continuous DAPT incubation effectively phenocopied the *mib* mutant phenotype (compare Fig. 4A,B,E,F,K with Fig. 2). Next, we attempted to inhibit and then restore Notch activity by incubating embryos in DAPT solution from 6-11 hpf followed by incubation in drug-free embryo medium until 20 hpf or 25 hpf. To determine if removal from DAPT restored Notch signaling, we examined expression of the Notch target gene *her4* (Takke et al., 1999). At 12 hpf, posterior neural plate expression of *her4* was greatly reduced, relative to control embryos, in embryos treated with DAPT continuously from 6 hpf (see Fig. S2A,B in the supplementary material), whereas *her4* appeared to be expressed at intermediate levels in embryos treated with DAPT from 6-11 hpf, indicating recovery of Notch signaling (see Fig. S2C in the supplementary material). In contrast to continuous DAPT treatment, DAPT treatment from 6-11 hpf produced an excess of both PMNs and KA′ interneurons (average 2.3 and 5.3 cells per hemisegment, respectively) (Fig. 4I,J,K).

Because DAPT is not a specific inhibitor of Notch signaling, we performed experiments using a transgenic line that expresses a dominant-negative form of the *Xenopus laevis* Suppressor of Hairless transcription factor (XdnSu(H)myc) under control of the zebrafish *heat shock cognate 70-kd protein, like* (*hsp70l*) promoter (Latimer et al., 2005). For control experiments, non-transgenic embryos were heat shocked at 39.5°C for 30 minutes or transgenic embryos were raised at normal temperature. Control embryos showed normal expression of *her4* at 11 and 12 hpf (see Fig. S2D,G in the supplementary material) and had an average of 1.2 *isl2*$^+$ PMNs and 2.1 KA′ interneurons per hemisegment (Fig. 4K). When heat shocked at 9.5 hpf, *Tg(hsp70l:XdnSu(H)myc)* embryos did not express *her4* at 11 and 12 hpf (see Fig. S2E,H in the supplementary material) and had an average of 4.1 *isl2*$^+$ PMNs and 0.5 KA′ interneurons per hemisegment, similar to *mib* mutant embryos and wild-type embryos that were continuously incubated in DAPT (compare Fig. 4C,D with Fig. 2; Fig. 4E,F). To inhibit Notch signaling transiently, as with the DAPT washout experiment, we heat shocked embryos at 6 hpf, reasoning that the level of XdnSu(H)myc protein would subside through dilution by cell division or degradation, eventually relieving Notch signaling

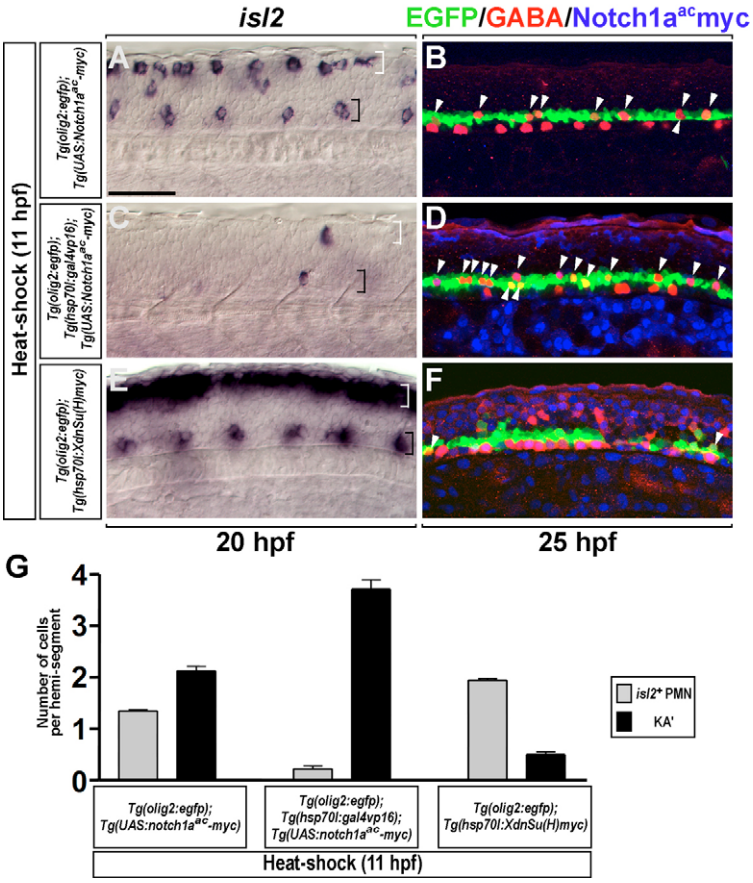

Fig. 5. Conditional activation of Notch signaling produces excess KA′ interneurons at the expense of PMNs. (**A-F**) Lateral views of the spinal cord of zebrafish embryos at the 6- to 12-somite region, with anterior to the left and dorsal at the top. (A,B) Control *Tg*(UAS:Notch1a^{ac}*-myc)*;*Tg(olig2:egfp)*, (C,D) *Tg(hsp70l:gal4vp16)*;*Tg*(UAS:Notch1a^{ac}*-myc)*;*Tg(olig2:egfp)* and (E,F) *Tg(hsp70l:*XdnSu(H)*myc)*;*Tg(olig2:egfp*) embryos were heat shocked at 11 hpf. (A,B) Control embryos showed normal numbers of *isl2*$^{+}$ PMNs (black bracket), dorsal Rohon-Beard sensory neurons (white bracket) and GABAergic KA′ cells (arrowheads). (C) *isl2*$^{+}$ PMNs (black bracket) and Rohon-Beard neurons (white bracket) were almost absent from heat-shocked triple-transgenic embryos. (D) Heat-shocked triple-transgenic embryos had excess KA′ interneurons (arrowheads). (E,F) Heat-shocked *Tg(hsp70l:*XdnSu(H)*myc)*;*Tg(olig2:egfp*) embryos had excess PMNs (black bracket), excess Rohon-Beard neurons (white bracket) and a deficit of KA′ interneurons (arrowheads). (**G**) Mean of the number of PMNs (gray bar) and KA′ interneurons (black bar) per hemisegment (*n*=7) in A-F. Error bars represent s.e.m. Scale bar: 50 μm.

inhibition several hours after heat shock. In fact, whereas transgenic embryos heat shocked at 6 hpf did not express *her4* at 11 hpf (see Fig. S2F in the supplementary material), a low level of *her4* expression was evident at 12 hpf (see Fig. S2I in the supplementary material), indicating recovery of Notch signaling. Following heat shock at 6 hpf, transgenic embryos had an average of 2.4 *isl2*$^{+}$ PMNs and 3.5 KA′ interneurons (Fig. 4G,H,K), consistent with DAPT treatment from 6-11 hpf. Thus, both the pharmacological and transgenic experiments show that continuous inhibition of Notch activity produces excess PMNs and a deficit of KA′ interneurons, whereas transient inhibition during early neural development results in an excess of both PMNs and KA′ cells. These results are consistent with the possibility that Notch provides two temporally separable functions in PMN and KA′ interneuron development.

Activation of Notch signaling promotes formation of excess KA′ interneurons at the expense of PMNs

Uniform expression of constitutively active Notch blocks formation of early-born neurons, in accordance with its proposed role in mediating lateral inhibition (Appel et al., 2001; Chitnis et al., 1995; Itoh et al., 2003; Takke et al., 1999). Nevertheless, if Notch signaling regulates a binary decision between PMN and KA′ fates, as indicated by the above loss-of-function data, then forced Notch activity should promote formation of KA′ interneurons at the expense of PMNs. We tested this using a transgenic *hsp70l*:GAL4VP16 driver to express a constitutively active form of the Notch1a intracellular domain (Notch1a^{ac}). Heat shocking *Tg(hsp70l:gal4vp16);Tg(UAS:Notch1a^{ac}-myc)* embryos at 36°C for 30 minutes at 11 hpf produced stronger morphological defects than those previously described using a *hsp70*:GAL4 driver (Scheer et al., 2002) (data not shown). These embryos did not form *isl2*$^{+}$ PMNs or GABAergic neurons (data not shown), consistent with previous observations that Notch activity blocks neurogenesis (Itoh et al., 2003; Takke et al., 1999). Next, we treated 11-hpf embryos using a milder heat-shock condition of 34°C for 30 minutes to induce transgene expression at an intermediate level. Control *Tg(UAS:myc-Notch1a-intra);Tg(olig2:egfp)* embryos formed about 1.3 *isl2*$^{+}$ PMNs and 2.1 KA′ interneurons per hemisegment (Fig. 5A,B,G), whereas *Tg(hsp70l:gal4vp16);Tg(UAS:Notch1a^{ac}-myc);-Tg(olig2:egfp)* transgenic embryos had an average of 0.2 *isl2*$^{+}$ PMNs and 3.7 KA′ interneurons per hemisegment, respectively (Fig. 5C,D,G). By contrast, *Tg(hsp70l:XdnSu(H)myc);-Tg(olig2:egfp)* embryos heat shocked at 11 hpf had an average of 1.9 *isl2*$^{+}$ PMNs and 0.5 KA′ interneurons per hemisegment (Fig. 5E,F,G). Thus, activation and inhibition of Notch activity during the period in which PMNs and KA′ interneurons are born have reciprocal effects on PMN and KA′ interneuron number, consistent with a role for Notch signaling in mediating binary neuronal fate decisions.

Individual neural plate cells produce identical sibling neurons in *mib* mutant embryos

To investigate the role of Notch signaling at the level of individual cells, we labeled single EGFP^{+} neural plate cells of *mib;Tg(olig2:egfp)* mutant embryos by iontophoresis at 10 hpf and determined the identities of clonal descendents at 24-30 hpf by observing axonal projections. Of ten successful experiments, two

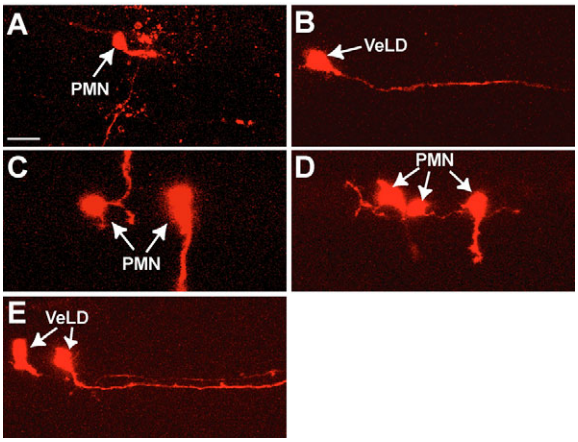

Fig. 6. Individual *olig2*:EGFP$^+$ neural plate cells produce single types of neurons in *mib*$^{-/-}$ embryos. (**A-E**) Lateral views of the spinal cord of 24- to 30-hpf *mib*$^{-/-}$;*Tg(olig2:egfp)* zebrafish embryos, with anterior to left and dorsal at the top. In each case, a single EGFP$^+$ cell was labeled with vital dye (red) at 10 hpf. (A,B) Labeled cells that did not divide but differentiated into a PMN and VeLD. (C,D) Examples of individual EGFP$^+$ clones that produced only PMNs, as determined by axons that projected out of the spinal cord to muscle. (E) Example of an EGFP$^+$ clone that produced two VeLD interneurons. Scale bar: 20 μm.

Table 1. Summary of *olig2*:EGFP$^+$ clones in *mib*$^{-/-}$ embryos

	PMN	SMN	KA′	VeLD	Total
Case 1	1				1
Case 2				1	1
Case 3	2				2
Case 4	2				2
Case 5	2				2
Case 6				2	2
Case 7				2	2
Case 8				2	2
Case 9				2	2
Case 10	3				3

Each case is an individual clone that labeled single *olig2*:EGFP$^+$ cells of the neural plate in *mib* mutant embryos. The number of each cell type within a clone is indicated.

labeled cells did not divide but differentiated as a PMN and VeLD interneuron (Fig. 6A,B), seven divided once to produce two neurons each, and one gave rise to three neurons (Table 1). By comparison, when we similarly labeled 18 neural plate cells of wild-type embryos, five did not divide, five produced two neurons and eight gave rise to three to five neurons (Park et al., 2004). Thus, clonal size was reduced in *mib* mutant embryos, consistent with our proliferation data presented above. Notably, the neuronal composition of clones was also affected by disruption of Notch signaling. Of the seven labeled cells that divided once in *mib* mutant embryos, all produced two identical neurons, which were either PMNs or VeLD interneurons (Fig. 6C,E). The three-cell clone consisted entirely of PMNs (Fig. 6D). In this case, the labeled cell probably divided once to make a PMN and a cell that divided again to make two PMNs. By contrast, 12 of 13 cells that divided in wild-type embryos produced neurons of different types, including four of the five cells that divided once (Park et al., 2004). Together, these data indicate that Notch signaling acts to extend the proliferative state of some precursors and, at terminal division, acts to specify sibling cells for different neuronal fates.

DISCUSSION

Understanding the mechanisms that coordinately specify the many different types of cells in the vertebrate nervous system is a fundamental problem in developmental biology. Here we built on our previous investigation of neural cell lineages (Park et al., 2004) to test the role of Notch signaling activity in regulating neural cell specification in zebrafish. Our work establishes that, in addition to its well-known role as a lateral inhibition signal to limit the number of precursors that exit the cell cycle and develop as neurons, Notch signaling specifies sister cells for different neuronal fates in the vertebrate CNS.

The neural plate of frog and zebrafish embryos has been an important model for investigation of mechanisms that regulate vertebrate neurogenesis. Both frog and fish embryos produce a relatively small number of early-born primary neurons, which are first evident within the neural plate. Primary neurons are organized into three longitudinal bands on either side of the neural plate midline. PMNs occupy a medial band, interneurons are distributed in an intermediate band and sensory Rohon-Beard neurons lie within a band near the lateral edge of the neural plate. Within each band, neurons are intermixed with proliferative neural precursors in a salt-and-pepper fashion. PMNs that are ablated before they undergo axogenesis are replaced (Appel et al., 2001), suggesting that lateral inhibition regulates allocation of neural precursors for PMN fate. As in insects, lateral inhibition is mediated by Notch signaling. Disruption of Notch signaling results in the formation of excess primary neurons at the expense of precursors and later-born neurons and glia, whereas forced expression of constitutively active Notch prior to the onset of neurogenesis blocks neuronal development (Appel et al., 2001; Chitnis et al., 1995; Coffman et al., 1993; Dorsky et al., 1995; Henrique et al., 1997; Park and Appel, 2003). Thus, Notch, via lateral inhibition, maintains neural cells in a precursor state.

Although a role for Notch signaling as a lateral inhibitory mechanism is well established, this is likely to be an incomplete view of Notch function in vertebrate neural development. A major limitation to a full understanding of Notch function and cell fate diversification is that the neural cell lineage remains poorly described. Many models of spinal cord development portray single types of neurons produced from distinct precursor subdomains aligned on the dorsoventral axis (Briscoe and Ericson, 2001; Jessell, 2000; Shirasaki and Pfaff, 2002). It is now becoming apparent that each precursor population produces various cell types. For example, pMN precursors produce oligodendrocytes in addition to motoneurons (Richardson et al., 2000; Rowitch, 2004). Recent analysis of transgenically marked lineages showed that *Olig2*$^+$ pMN cells also give rise to astrocytes and ependymal cells (Masahira et al., 2006). Our own analysis of clones resulting from individually labeled *olig2*$^+$ precursors in zebrafish showed that these cells also produce a small number of interneurons (Park et al., 2004). Thus, vertebrate neural precursors can give rise to various cell types, raising the possibility that, as in insects, Notch functions to diversify the fates of differentiating cells that have a common origin.

Notably, our clonal analysis revealed that some *olig2*$^+$ precursors produce a PMN and KA′ interneuron at their last division (Park et al., 2004). Consistent with this observation, we showed that PMNs were often adjacent to neurons that did not express motoneuron markers during early neurogenesis and KA′ interneurons were born at the same time as PMNs. Formation of excess PMNs in the absence of Notch signaling is already well documented. Here we showed that these same embryos had a deficit of KA′ interneurons and that the deficit did not stem from cell death or failure of

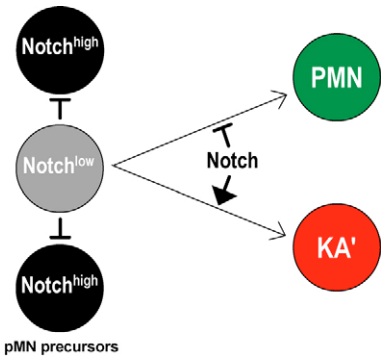

Fig. 7. Summary of sequential roles of Notch signaling in neuronal specification in zebrafish. First, Notch mediates lateral inhibition among *olig2*$^+$ pMN precursors to limit the number that exit the cell cycle and differentiate as neurons. Second, different levels of Notch activity in sibling neurons specifies different neuronal fates. Here, cells with relatively high-level Notch signaling develop as KA′ interneurons, whereas those with lower activity develop as primary motoneurons (PMNs).

precursors to divide. Blocking Notch activity continuously using a pharmacological inhibitor phenocopied the *mib* mutation, in producing excess PMNs and a deficit of KA′ interneurons, as did transgenically inducing Notch inhibition to coincide with the time at which PMNs and KA′ interneurons are born. All these results are consistent with the possibility that Notch signaling regulates binary neuronal fate decisions. Curiously, embryos in which Notch signaling was inhibited prior to neurogenesis but active during the time that PMNs and KA′ interneurons were born had an excess of both PMNs and KA′ interneurons. One possible explanation for this result is that transient Notch inactivation disrupts lateral inhibition, committing more precursors to lineages that produce PMNs and KA′ cells, but restoration of signaling activity subsequently regulates binary neuronal fate specification within these lineages resulting in an excess of both cell types.

If inhibition of Notch signaling results in excess PMNs and a deficit of KA′ interneurons by disrupting a binary neuronal fate decision, then timed, ubiquitous activation should produce the reciprocal result. This is indeed what we found. Transgenic embryos that were induced to express constitutively active Notch1a just prior to PMN and KA′ interneuron birth had few PMNs and more KA′ interneurons. As noted above, Notch signaling activity is commonly considered to keep precursor cells in a proliferative, undifferentiated state. Our results suggest that, once neural precursors undergo terminal division, differential Notch activity within daughter cells can specify them for different neuronal fates.

As a final test of the role of Notch signaling in neuronal specification, we analyzed the clonal descendents of labeled neural plate cells in *mib* mutant embryos. Whereas wild-type clones nearly always consisted of different kinds of neurons (Park et al., 2004), *mib* mutant clones always consisted of the same kinds of neurons. This is similar to the situation in flies, in which MP neural precursors, which normally produce dMP2 and vMP2 interneurons, form only dMP2 cells in the absence of Notch activity (Spana and Doe, 1996). Additionally, *mib* mutant clones tended to have fewer total cells than wild-type clones. Thus, Notch signaling serves both to expand cell number by maintaining some cells in a proliferative state, and to diversify neuronal fate by specifying sister neurons for different neuronal identities.

Our observations are most consistent with the model shown in Fig. 7. Within neural precursor populations, lateral inhibitory signaling mediated by Notch receptors and their ligands regulates the allocation of precursors for neuronal fate. In the standard model of lateral inhibition, precursors with high levels of Notch activity remain as precursors, whereas those that have less activity undergo a final division and give rise to neurons. Subsequently, differential Notch activity specifies some *olig2*$^+$ sibling neurons for different fates. In particular, cells with relatively high Notch activity develop as KA′ interneurons, whereas those with lower activity develop as PMNs. Although we focused on just two kinds of neurons, Notch is likely to regulate other kinds of binary neuronal fate decisions. Consistent with this, four of our *mib* mutant clones consisted of two VeLD interneurons, whereas in wild-type embryos VeLD interneurons share lineages with PMNs and KA′ interneurons. Specification of the full range of neuronal fates is likely to require the integration of Notch activity with other signaling molecules such as Sonic hedgehog.

How do sibling neurons have different levels of Notch activity? In flies, asymmetric distribution of Numb to one of two sibling neurons blocks Notch signaling, causing the two siblings to take different fates (Spana and Doe, 1996). Numb proteins are asymmetrically localized within vertebrate neural cells (Cayouette and Raff, 2003; Cayouette et al., 2001; Shen et al., 2002; Wakamatsu et al., 1999; Zhong et al., 1996) and loss-of-function experiments in mice reveal that they have key roles in regulating neurogenesis (Petersen et al., 2002; Petersen et al., 2004; Zhong et al., 2000; Zilian et al., 2001). Lineage analysis performed in cultures revealed that the formation of morphologically different neurons coincides with asymmetric Numb distribution (Shen et al., 2002). In zebrafish, a Numb:EGFP fusion protein distributes uniformly around the cell membrane of dividing cells during the neural plate stage (Reugels et al., 2006). Although this suggests that neuronal fate might not be influenced by asymmetric localization of Numb, overexpression of fusion protein could swamp localization signals or Numb might only be asymmetrically distributed to a small number of cells.

Is binary fate specification a general mechanism for neuronal diversification in vertebrates? Recent cell lineage-tracing experiments show, for example, that neuronal progenitors can divide asymmetrically in culture (Kawaguchi et al., 2004; Shen et al., 2002), that zebrafish retinal progenitors expressing *ath5* (*atoh7* – ZFIN) divide once to produce a retinal ganglion cell and a distinct postmitotic cell (Poggi et al., 2005), and that some dorsal spinal cord dILA and dlLB neurons arise from neuronal progenitors by asymmetric division in chick (Wildner et al., 2006). Additionally, elimination of Notch1 signaling in the mouse retina results in the formation of excess early-born cone photoreceptors at the expense of other early- and late-born retinal cell types (Jadhav et al., 2006; Yaron et al., 2006), which could reflect a role in mediating binary fate decisions in the retina as we have shown for spinal cord. Thus, similar to insects, binary fate decisions regulated by Notch signaling are likely to contribute to the production of diverse neurons from common precursors in vertebrates.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/10/1911/DC1

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