THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 4, pp. 2580–2592, January 25, 2013
© 2013 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.

The E3 Ligase Mind Bomb-1 (Mib1) Modulates Delta-Notch Signaling to Control Neurogenesis and Gliogenesis in the Developing Spinal Cord*

Received for publication, July 5, 2012, and in revised form, December 4, 2012 Published, JBC Papers in Press, December 5, 2012, DOI 10.1074/jbc.M112.398263

Kyungjoon Kang, Donghoon Lee, Seulgi Hong, Sung-Gyoo Park, and Mi-Ryoung Song[1]

From the School of Life Sciences, Bioimaging Research Center and Cell Dynamics Research Center, Gwangju Institute of Science and Technology, Oryong-dong, Buk-gu, Gwangju 500-712, Republic of Korea

Background: Mib1 is a ubiquitin ligase that modifies Delta, a ligand for the Notch signaling pathway.
Results: Absence of Mib1 results in a reduced number of neural progenitors, spinal interneurons, and astrocytes.
Conclusion: Mib1 controls neurogenesis and gliogenesis in the spinal cord.
Significance: Novel insights about the role of Mind bomb1 in the regulation of early spinal cord development via Delta signaling are presented.

The Notch signaling pathway is essential for neuronal and glial specification during CNS development. Mind bomb-1 (Mib1) is an E3 ubiquitin ligase that ubiquitinates and promotes the endocytosis of Notch ligands. Although Mib1 is essential for transmitting the Notch signal, it is still unclear whether it is a primary regulator of Notch ligand activity in the developing spinal cord. In *Mib1* conditional knock-out mice, we observed depletion of spinal progenitors, premature differentiation of neurons, and unbalanced specification of V2 interneurons, all of which mimic the conventional Notch phenotype. In agreement with this, the reduction of progenitors in the absence of *Mib1* led to a loss of both astrocytes and oligodendrocytes. Late removal of *Mib1* using a drug-inducible system suppressed glial differentiation, suggesting that Mib1 continues to play a role in the formation of late progenitors mainly designated for gliogenesis. Finally, misexpression of Mib1 or *Mib1* deletion mutants revealed that the ring domain of Mib1 is required for the specification of V2 interneurons in the chick neural tube. Together, these findings suggest that Mib1 is a major component of the signal-sending cells required to provide Notch ligand activity for specifying neurons and glia in the spinal cord.

Notch signaling is one of the fundamental pathways that specify the fates of diverse cell types during embryonic development based on cell-to-cell interaction, mediated by Delta ligands and Notch receptors. Notch signaling converts initially homogeneous progenitors into cells with different neurogenic potential by lateral inhibition; cells expressing Delta adopt neuronal fates and simultaneously prevent Notch-expressing adjacent cells from becoming neurons. The role of Notch in controlling the neurogenic potential was initially suggested by classic genetic studies using *Drosophila*; disruption of the Notch pathway resulted in the production of extra neurons and a reduction in the number of progenitors (1). In zebrafish and mice, down-regulation of *Notch* or *Delta* led to premature and excessive neuronal differentiation, whereas ectopic expression of Delta inhibited neurogenesis (2–4). Thus, the role of the Notch signal in creating a balance between maintenance of neural progenitors and neuronal differentiation is evolutionarily conserved.

In addition to the general role of Notch in regulating neuronal fates, the Notch signal has been proposed to serve a more specific and refined role in the vertebrate nervous system. For instance, multiple neuronal subtypes are derived from distinct progenitor populations along the dorsal-ventral axis of the spinal cord (5). Previous studies have shown that the fate of interneuronal subtypes is compromised when Notch activity is altered (6–8). The binary choice between V2a and V2b interneurons, both derived from p2 ventral progenitors, is sensitive to the Notch signal. When Notch activity is absent, as demonstrated in *Notch1* conditional mutant mice or *Presenilin* null mice, more V2a interneurons and less V2b interneurons are generated. Together, cell lineage and genetic studies suggest that Notch-expressing cells turn into inhibitory V2b interneurons marked by Gata3, whereas Delta-expressing cells become excitatory V2a interneurons labeled by Chx10 (7, 8).

In the developing CNS, gliogenesis begins after neurogenesis and produces astrocytes and oligodendrocytes in the late period of embryonic development (9). Because Notch maintains progenitor populations, it could potentially contribute to glia formation by reserving a progenitor pool for gliogenesis. Consistent with this idea, misexpression of NICD,[2] an active form of Notch, induces radial glial cells, a population recently identified as embryonic neural stem cells that later transform into astrocytes (10, 11). Likewise, the Delta-Notch interaction between intermediate progenitor cells and radial glia maintains the stem cell potential of radial glial cells (12). Furthermore, the formation of other specialized glial cells, including Müller glia in the

* This work was supported by National Research Foundation of Korea Grants 2011-0029179, NRF-2008-313-E00343, and 2012-0002097, Cell Dynamics Research Center Grant 2012-0000768, and the BioImaging Research Center and “Basic Research Projects in High-tech Industrial Technology” Project at Gwangju Institute of Science and Technology (to M.-R. S.).
[1] To whom correspondence should be addressed. Tel.: 82-62-715-2508; Fax: 82-62-715-2484; E-mail: msong@gist.ac.kr.

[2] The abbreviations used are: NICD, Notch intracellular domain; iKO, drug-inducible knock-out; cKO, conditional knock-out; E, embryonic day; GFAP, glial fibrillary acidic protein.

retina and Bergman glia in the cerebellum, is also promoted by active Notch (13, 14). In contrast, it is still unclear how Notch signaling affects oligodendrocyte development. Notch is known to inhibit oligodendrocyte differentiation, whereas oligodendrocyte precursor cells appear to be induced by the Notch signal (2, 15–17).

Notch signaling is initiated by interaction between the Notch receptor and DSL family ligands (Delta, Serrate, Lag-2). This triggers a series of protease-driven cleavages of the Notch receptor. As a result, intracellular fragments of Notch (NICD) are produced and translocated to the nucleus where they directly activate gene transcription together with other transcriptional activators (18). Studies over the past several decades have identified numerous core elements of the Notch pathway, mostly implicated in signal transduction following receptor activation. Nevertheless, our understanding of the regulation of Delta within signal-sending cells, the key first event triggering Notch signaling, is still limited. In the signal-sending cells, DSL family ligands are present on the cell membrane and become endocytosed to be incorporated into vesicles. Endocytosis of the Delta ligands appears to be required for Notch signal transduction because blockade of endocytosis in *Drosophila* perturbs Notch signal transduction (19). Genetic screens in *Drosophila* and zebrafish have identified two genes, *neuralized* (*Neur*) and *mind bomb-1* (*Mib1*), both of which are E3 ubiquitin ligases that ubiquitinate and promote endocytosis of DSL family ligands (20, 21). Elimination of *Mib1* in zebrafish and mice resulted in premature differentiation of neurons, a phenotype common to mutants in which Notch signaling is defective (12, 21). Although it is not yet known whether Mib1 is involved in the maturation of Delta ligand by recycling or trafficking, or provides a pulling-force for cleavage, it is clear that it is essential for Notch signaling (22, 23).

In this study, we investigated the role of Mib1 in neurogenesis and gliogenesis during spinal cord development. The absence of Mib1 resulted in premature differentiation of neurons and mis-specification of spinal V2 interneurons. In addition, radial glia and later differentiation of astrocytes and oligodendrocytes were inhibited in *Mib1* mutant mice. Down-regulation of *Mib1* late in development using a drug-inducible system resulted in various extents of reduction of mature glia. The developmental defects found in *Mib1* mutant mice were identical to those of other Notch-related mutants, supporting the view that Mib1 is a critical regulator of Notch signaling in the developing spinal cord.

EXPERIMENTAL PROCEDURES

Animals—*Mib1* floxed (f) mice and *Nes-Cre ERT2* transgenic mice have been described previously (24, 25). *Nestin-Cre* transgenic mice were obtained from The Jackson Laboratory. *Mib1* conditional knock-out (cKO) mice were generated by mating *Mib1*$^{f/f}$ homozygous mice with *Mib1*$^{f/+}$;*Nestin-Cre* mice. *Mib1* drug-inducible knock-out (iKO) mice were produced by mating *Mib1*$^{f/f}$ homozygous mice with *Mib1*$^{f/+}$;*Nes-Cre ERT2* mice. To delete the flox allele, tamoxifen (5 mg/ml) was administered intraperitoneally daily for 3 days as indicated.

Expression Constructs—Mouse *Mib1* was generated by PCR from mouse cDNA and cloned into the *pCAGGS1* vector. The *Mib1dn* and *Mib1ΔRD3* constructs lack all the ring finger domains (amino acids 781–1006) and the third domain (amino acids 959–1006), respectively (21). The *NICD* construct was purchased from Addgene. The plasmid *Dll1* was kindly provided by Dr. S. Sockathanan. Mouse *Dll1*, *Dll4*, and *Delta* mutants were cloned into *pCAGGS1* with the FLAG tag at the C terminus. *Dll4*$^{\Delta ICD}$ is a deletion mutant that contains amino acids 1–555 of Dll4. *Dll4*$^{\Delta ICD}$*-Ub-HA* was designed to have Ub-HA conjugated with Dll4$^{\Delta ICD}$ in-frame (21).

Chick in Ovo Electroporation—Chick eggs (Hwasun Hyangsang Farm) were incubated in a humidified chamber, and DNA constructs were injected into the lumens of Hamburger Hamilton (HH) stage 10–12 spinal cords. Electroporation was performed using a square wave electroporator (BTX). Co-electroporation resulted in >80% of cells expressing all constructs. Incubated chicks were harvested and analyzed at HH stage 20–25.

In Utero Electroporation—Timed pregnant mice were ethically anesthetized with inhalation anesthetics, isoflurane combined with oxygen/nitrous oxide. CAG promoter-based plasmid DNAs (up to 1 μg/μl) were injected into the lateral ventricles of embryos. Pulse conditions were 5 pulses separated by 950 ms at 40 V for E13.5 embryos. Embryonic brains were harvested at E16.5 and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. After PBS washing and cryoprotection, brains were sectioned coronally at 12 μm for immunohistochemistry.

Immunohistochemistry and in Situ Hybridization—Embryos were fixed in 4% paraformaldehyde for immunohistochemistry. Antibodies used in this study were as follows: guinea pig anti-Olig2 (26); rabbit anti-Hb9 (27); guinea pig anti-Chx10 (28); monoclonal anti-Gata3 (Santa Cruz Biotechnology); rabbit anti-En1 (Dr. Jessell); rabbit anti-Sox1 (Cell Signaling Technology); rabbit anti-NFIA (Active Motif); rat anti-Nestin (Chemicon); rabbit anti-p-Vimentin (MBL); rabbit anti-GFAP (Dako); and rabbit anti-GFP (Invitrogen) antibodies. Fluorophore-conjugated species-specific secondary antibodies were used as recommended (The Jackson Laboratory and Invitrogen). For endogenous Dll1 staining, autoclaved antigen retrieval and signal amplification using TSA kit (Molecular Probes) were performed. The slides were processed at 121 °C in 0.01 M tri-sodium citrate buffer, pH 6.0, for 10 min and immunostained with anti-Dll1 antibody (H-265, Santa Cruz Biotechnology) at 1:100 (29).

For *in situ* hybridization, transverse sections were hybridized with digoxigenin-labeled probes specific for each gene amplified from mouse or chick embryonic cDNA using an Advantage cDNA PCR kit (Clontech).

Ubiquitination Assay—HEK293T cells were transiently transfected with *pCAGGS1-Dll1-flag*, *pcDNA3Ub-HA*, *pEGFP-Mib1*, and *pCAGSS1-Mib1dn* as indicated (21). *pcDNA3-Ub-HA* was kindly provided by Dr. Y. J. Yoo. After 30 h, cells were lysed in IP lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) with 10 mM iodoacetamide (Sigma), 20 μM MG132 (Biomol), and protease inhibitor mixture (Calbiochem). Lysates were immunoprecipitated with anti-FLAG M2 affinity gel (Sigma). Immunoprecipitates were washed and analyzed by Western blot analysis using anti-FLAG

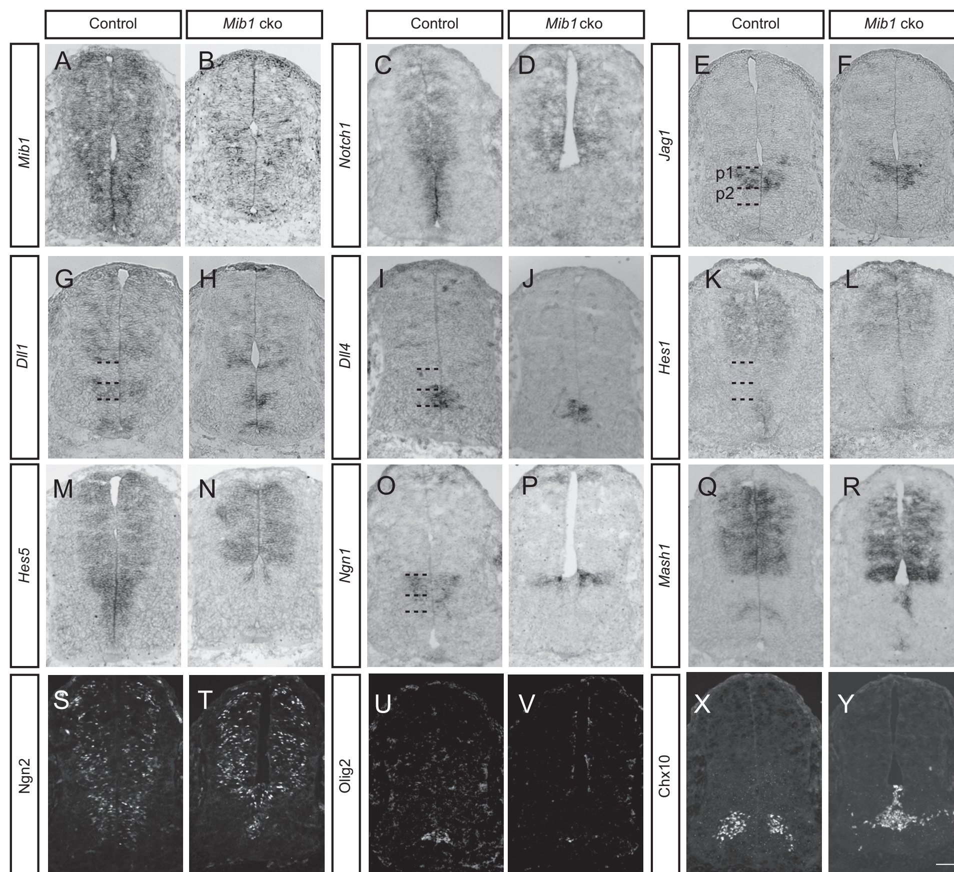

FIGURE 1. **Patterning of the neural tube in E11.5 *Mib1* mutant mice.** *A–R*, expression of *Mib1, Notch1, Jag1, Dll1, Dll4, Hes1, Hes5, Ngn1,* and *Mash1* was examined in E11.5 *Mib1* mutant mice by *in situ* hybridization. Progenitor domains were marked by *dashed lines*. *S–Y*, protein expression of Ngn2, Olig2, and Chx10 was examined in adjacent sections. *Scale bar* in *Y*, 200 μm for *A–Y*.

M2 peroxidase (Sigma), rat anti-HA peroxidase (Roche Applied Science), or anti-α-tubulin (AbD Serotec) antibodies.

Subcellular Localization Analysis—Dll4, Dll4-flag, Mib1wt-GFP (30), or *Mib1dn-Myc* was transfected in COS7 cells. Primary antibodies used for immunocytochemistry were anti-FLAG (Sigma), anti-Myc, and anti-GFP (Invitrogen) antibodies.

Primary Cortical Culture—Primary astrocytes were cultured from the 1- to 3-day-old Sprague-Dawley rat cortices. Cortices were dissected, dissociated using papain (Worthington Biochemical Corp.), and grown on poly-D-lysine (Sigma)-coated culture dishes for 2 weeks in neurobasal medium (Invitrogen), 10% fetal bovine serum (Hyclone). To deplete microglia and meningeal cells, cortical cultures were incubated with serum-free neurobasal medium for 2 days before use. Cells were trypsinized and re-plated into poly-D-lysine-coated coverslips. Neocortices from E14.5 $Mib1^{f/f}$ mouse embryos were dissected and dissociated and were grown on astrocyte-plated coverslips. Cultures were maintained in neurobasal medium with B27 (Invitrogen). GFP or Cre recombinase-expressing retrovirus was added to the culture after plating. Coverslips were fixed and immunostained at 3 days *in vitro*.

Quantification and Statistical Analysis—Cells were counted in immunostained transverse sections containing the brachial spinal cords of various embryonic stages as indicated. At least three embryos were harvested from each of three independent experiments and used for quantification. Cell counts are shown as mean ± S.E. Unpaired Student's *t* tests were used for statistical analysis.

RESULTS

Delta-Notch Signaling Components Are Expressed in the Progenitor Domains of the Spinal Cord—To define the location of Notch signaling in the developing spinal cord, we examined the expression of Notch-Delta signaling components in E11.5 mouse spinal cords by *in situ* hybridization. Three Notch receptors (Notch1, Notch2, and Notch3) and five DSL ligands (Jagged1, Jagged2, Jagged3, Dll1, and Dll4) are present in the neural tube (31). Expression of *Mib1*, a major component required for Delta trafficking and thereby transmitting the Notch signal, and a Notch effector gene, *Hes5,* were also examined. At E11.5, *Mib1* transcript was found throughout the progenitor domains along the entire dorsoventral axis (Fig. 1*A*). Progenitors tended to express higher levels of *Mib1* as they proceed laterally within the progenitor domains where its substrates Delta and Jagged co-exist. *Notch1* mRNA was relatively uniformly expressed in the ventricular zone along the dorsovental axis of the spinal cord (Fig. 1*C*). Notch ligands *Jag1, Dll1,* and *Dll4* were expressed in distinct ventral progenitor domains in a mutually exclusive fashion as follow: *Jagged1* in the p1 domain and *Dll1* and *Dll4* in the p0 and p2 domains (Fig. 1, *E, G,* and *I*). *Hes1* was restricted to the dorsal ventricular zone, and *Hes5* was highly

expressed in all ventral progenitor domains, implying that Delta-Notch signaling is active in the majority of ventral progenitors (Fig. 1, *K* and *M*).

Defective Notch Signaling in Mib1 Mutant Mice—*Mib1* mutant mice were severely growth-retarded around E9.5 and die around E11.5, which makes it impossible to examine the role of Mib1 in neural tube patterning (30). To overcome this problem, we crossed conditional *Mib1* mutant mice with *Nestin-Cre* transgenic mice to selectively eliminate *Mib1* within neural progenitors (12). Although the *Mib1* conditional knockout mice developed hemorrhages in the forebrain that affected brain development before E15.5, the spinal cord remained intact, which allowed us to examine the overall patterning of the spinal cord. We confirmed that *Mib1* transcript was significantly down-regulated in *Mib1* mutant mice as expected (Fig. 1*B*). We next examined the expression of Notch signaling components, including *Notch1, Jag1, Dll1, Dll4, Hes1,* and *Hes5* in E11.5 *Mib1* mutant spinal cords by *in situ* hybridization. Expression of *Notch1, Dll1,* and *Hes5* was significantly reduced in the ventral spinal cord, indicating that Notch activity was low. Furthermore, the ventral midline was slightly distorted due to extinction of the progenitor domain (Fig. 1, *D, P,* and *R*). *Jag1, Dll4,* and *Hes1* expression was unaltered in the mutant (Fig. 1, *F, J,* and *L*). We also compared the expression of the proneural genes *Ngn1, Mash1,* and Ngn2 together with the pMN domain marker Olig2 and the V2a interneuron marker Chx10 (Fig. 1, *O–Y*). Expression of *Ngn1, Mash1,* Ngn2, and Olig2 was greatly reduced in the ventral progenitor domain, although expression of Chx10 was prematurely increased in the progenitor domain. Together, the low Notch activity and depletion of ventral progenitors in the *Mib1* mutant indicate that Mib1 is essential for the Notch signaling that maintains progenitor cell populations.

Mib1 Is Essential for Internalization and Ubiquitination of Delta—Mib1 is an E3 ligase that mono-ubiquitinates the Delta ligand (21). Because the RING domain is critical for its enzymatic activity, we generated deletion mutant constructs that either lack all three RING domains (*Mib1dn*) or the third one (*Mib1ΔRD3*) (21). We co-transfected Dll1 and ubiquitin into 293T cells, immunoprecipitated *Dll1*, and measured its ubiquitin level. Without Mib1, the level of ubiquitin in the immunoprecipitate was almost undetectable (Fig. 2*A*, *2nd lane*). When Mib1 was introduced with Dll1 and ubiquitin, a significant level of ubiquitin in Dll1 was detected (Fig. 2*A*, *3rd lane*). However, co-transfection of *Mib1dn* or *Mib1ΔRD3* greatly inhibited ubiquitination of Dll1 (Fig. 2*A*, *4th* and *5th lanes*). The weak ubiquitination level in the presence of Mib1ΔRD3 is likely due to the residual activity coming from the other two intact RING domains.

We next tested whether ubiquitination of Delta by Mib1 induces internalization of Delta, a process necessary for Delta signaling transduction. To analyze subcellular localization of Delta, we co-transfected *Dll4* and *Mib1* into COS7 cells. When *Dll4* and *Mib1* were introduced, both were internalized and co-existed in intracellular vesicles (Fig. 2, *B, B′*, and *B″*). However, co-transfection of Dll4 with Mib1dn resulted in diffuse distribution of both proteins in the plasma membrane and cytoplasm. Together, ubiquitination of Dll1 by Mib1 internalizes Dll1 into the cells (Fig. 2, *C, C′*, and *C″*).

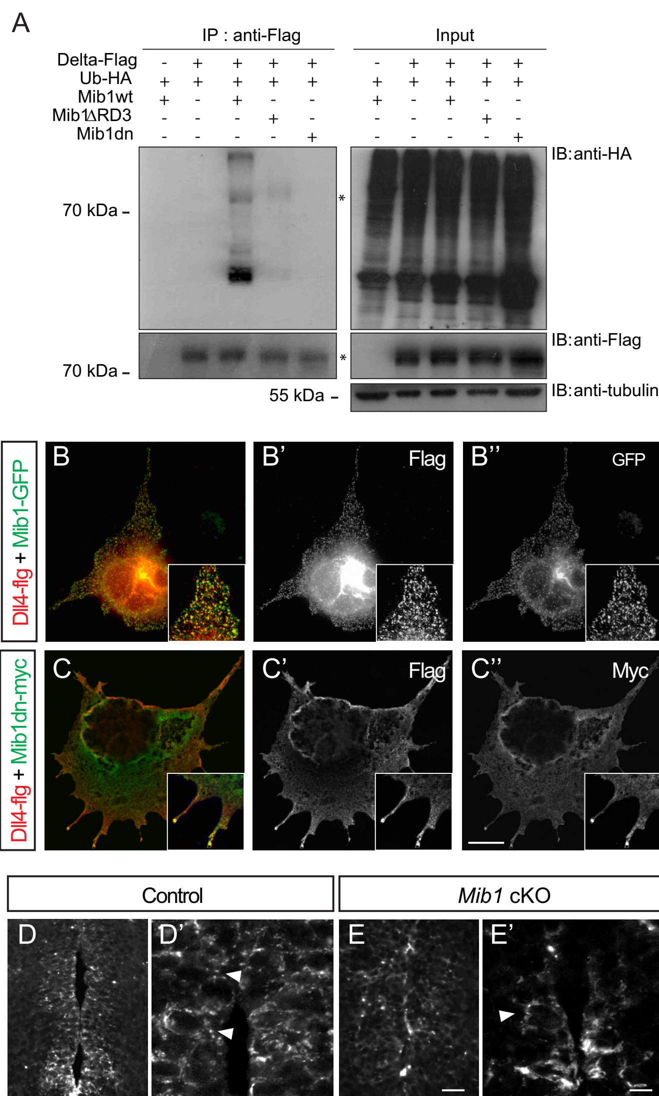

FIGURE 2. **Mib1 is critical for ubiquitination and internalization of Delta1.** *A*, ubiquitination of Dll1 by Mib1 *in vitro*. Delta-FLAG, ubiquitin-HA (*Ub-HA*), Mib1, Mib1ΔRD3, or Mib1dn was transfected in 293T cells as indicated. Cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody and immunoblotted (*IB*) with anti-HA or anti-FLAG antibody as shown. *Asterisk* marks the predicted size of Delta protein. Anti-tubulin antibody was used as a loading control. *B* and *C*, distribution of Dll4 and Mib1 or Mib1dn in COS7 cells. Dll4 and Mib1 co-localize in intracellular vesicles (*B, B′*, and *B″*). Dll4 is present in the plasma membrane when Mib1dn is introduced (*C, C′*, and *C″*). *D* and *E′*, localization of Notch1 was assessed in E12.5 *Mib1* mutant mice by immunohistochemistry. Notch1 expression is broad and occasionally internalized into puncta in the control (*D′*, *arrowheads*), although it is reduced and mostly localized in the membrane in *Mib1* mutant mice (*E′*, *arrowhead*). *Scale bar* in *C″* is 15 μm for *B* and *C″*; in *E*, 50 μm for *D* and *E*; and in *E′*, 50 μm for *D′* and *E′*.

If Dll1 is mainly ubiquitinated by Mib1 and becomes internalized, we expect to see mis-localization of Dll1 in *Mib1* mutant mice. To test our hypothesis, we examined expression of endogenous Delta1 in E11.5 *Mib1* mutant spinal cord. In littermate control, Delta expression was restricted to the ventricular zone similar to the distribution of *Mib1* transcript (Figs. 1 and 2, *D* and *D′*). In a high magnification view, Delta1 is localized in the plasma membrane in some cells, although it is present in intracellular vesicles in others, showing a salt and pepper pattern (32). In E11.5 *Mib1* mutant, expression of Delta1 was reduced in the ventricular zone and mostly localized

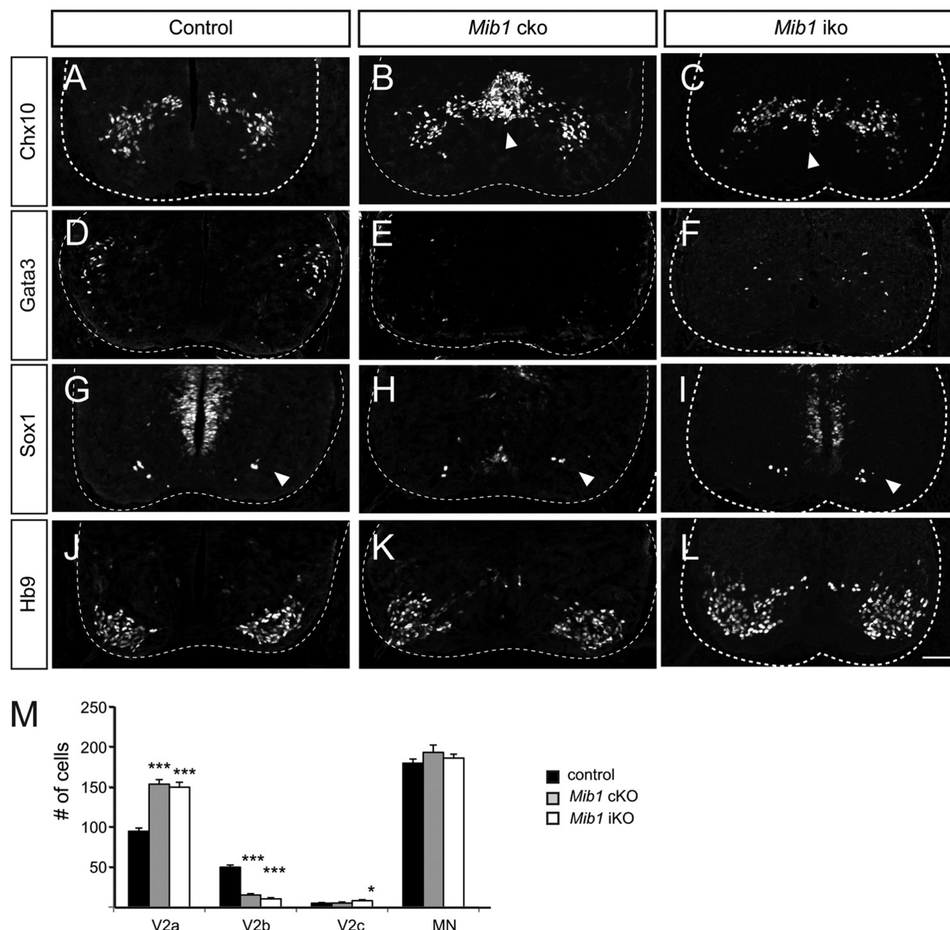

FIGURE 3. **Interneuron specification in the ventral neural tube is perturbed when *Mib1* is down-regulated.** *A–L*, expression of Chx10, Gata3, Sox1, and Hb9 in E12.5 *Mib1* cKO, iKO, and littermate controls. For *Mib1* iKO, tamoxifen was injected daily from E10 to E12. Premature Chx10$^+$ V2a interneurons appeared in the ventricular zone close to the midline in *Mib1* cKO and iKO mice (*B* and *C, arrowheads*). Numbers of Gata3$^+$ V2b interneurons are reduced, although those of Sox1$^+$ V2c interneurons (*G–I, arrowheads*) and Hb9$^+$ motor neurons are mostly unchanged. *M,* numbers of cells expressing individual markers. Means ± standard errors are shown. *Asterisks* indicate statistically significant compared with controls (*, $p < 0.05$; ***, $p < 0.001$ in the unpaired Student's *t* tests). *Scale bar* in *L*, 200 μm for *A–L*.

in the plasma membrane (Fig. 2, *E* and *E′*). Thus, it is likely that internalization of Delta is defective when Mib1 is absent.

Mis-specification of Neuronal Subtypes in the Ventral Spinal Cord without Mib1—Recent studies suggest that Notch signaling specifies selective neuronal subtypes, including V2a and V2b interneurons in the p2 domain within the developing spinal cord (7, 8). In *Notch1* conditional knockouts and *presenilin 1* null mice where Notch signaling is compromised, p2 progenitors become V2a interneurons instead of V2b interneurons. To determine whether the absence of *Mib1,* like other Notch-related genetic defects, influences the choice between V2a and V2b interneurons, we examined the neuronal subtypes in the ventral spinal cord of *Mib1* mutants. In addition to the conditional mutant mice (cKO), we examined a drug-inducible *Mib1* mutant mice (iKO) containing the *Nes-Cre* allele that allows drug-dependent elimination of *Mib1* (25). When tamoxifen was administered from E10 to E12, *Mib1* expression was almost undetectable at E12.5 (data not shown). Expression of Chx10, Gata3, and Sox1, which label V2a, V2b, and V2c interneurons, respectively, was examined by immunohistochemistry in E12.5 spinal cord (Fig. 3). We found that the ventral progenitors were virtually absent in *Mib1* mutant mice, as indicated by the loss of the progenitor marker Sox1 and the appearance of the postmitotic markers Chx10 and Gata3 in the ventricular zone where only progenitors are supposed to be located (Fig. 3, *B, C, E, F, H,* and *I*). This is consistent with the phenotype found in *Notch1* mutants, which display premature differentiation of neurons with reduced numbers of neural precursors (8). In the absence of *Mib1,* the number of V2a neurons increased (153.1 ± 5.437, $p < 0.001$) as compared with littermate controls (94.3 ± 4.89). The number of V2b interneurons declined (15 ± 1.592, $p < 0.001$) as compared with littermate controls (49.6 ± 2.633) (Fig. 3, *A–F* and *M*). To locate V2c interneurons, a population recently shown to originate from the p2 domain, we examined the expression of Sox1 (33). To focus on postmitotic V2c interneurons and eliminate Sox1$^+$ progenitors, we only counted cells that expressed both Sox1 and NeuN, a postmitotic neuronal marker (data not shown). Sox1$^+$ V2c interneurons were slightly increased in *Mib1*-inducible mutant mice (8 ± 1.125) as compared with the littermate controls (4.47 ± 0.583) (Fig. 3, *G–I* and *M*).

Expression of progenitor markers that label most progenitor cells such as Sox1, Sox2, and NFIA revealed that numbers of progenitors were reduced in a broad region of the ventral spinal

cord, including the p2 domain. To examine in more detail the specification of other ventral cell types in the absence of *Mib1*, we assessed the expression of additional markers such as Hb9, *Evx1,* and En1, which label motor neurons and V0 and V1 interneurons, respectively. We observed expansion of $En1^+$ V1 interneurons and no change in motor neurons and V0 neurons in *Mib1* mutant mice, although premature expression of Hb9 in the progenitor domain was found (Fig. 3, *J–L* and *M*, and data not shown). These results suggest that Mib1 mainly controls the fate specification of V1 and V2 interneurons but is dispensable for generation of motor neurons and V0 interneurons.

Specification of Glial Progenitors Is Compromised in Mib1 Mutant Spinal Cords—In addition to maintaining neural progenitors, the Notch signal is implicated in gliogenesis because the late progenitor pools preserved by the Notch signal retain the potential to generate glial cells after neurogenesis. Consistent with this, previous studies showed that activation of the Notch receptor triggered the generation of radial glia and astrocytes in the cortex (10). To examine whether the late progenitors that give rise to glial cells are under the influence of Mib1, we examined the development of glial progenitors in *Mib1* mutants, using markers for progenitors or immature astrocytes such as NFIA, Nestin, p-Vimentin, Sox2, and *Fgfr3* (34–36). NFIA is known to be present in progenitors as well as postmitotic motor neurons (34). Expression of NFIA in the progenitor domain was significantly reduced in E12.5 *Mib1* mutant spinal cords, although its expression within the motor column was unchanged (Fig. 4, *A–C* and *P*). Examination of Nestin expression showed that radial processes were reduced in number and disorganized when *Mib1* was absent. We also observed a similar reduction in p-Vimentin, Sox2, and *Fgfr3* transcripts in the ventral spinal cord, consistent with the depletion of ventral progenitors in Fig. 3 (Fig. 4, *D–P*). Together our results suggest that Mib1-mediated Notch signaling is necessary for specification of glial cells.

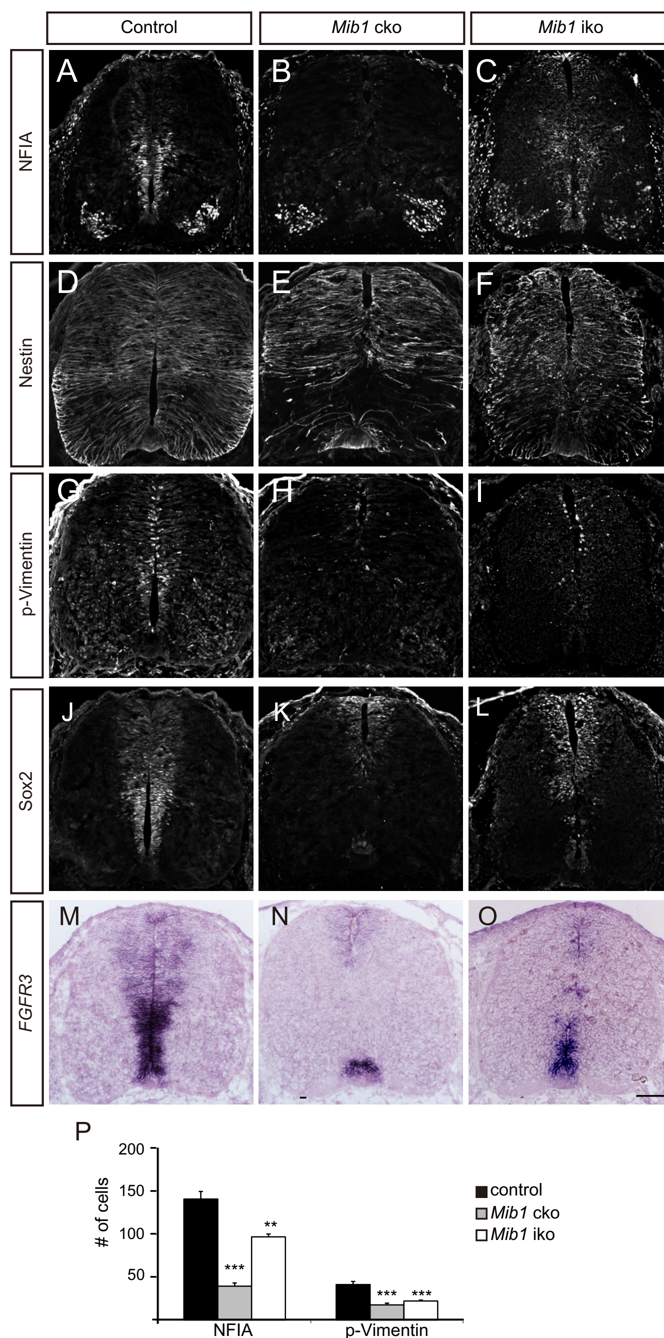

FIGURE 4. **Assessment of glial progenitors in *Mib1* mice.** *A–O,* expression of NFIA, Nestin, p-Vimentin, Sox2, and *Fgfr3* in E12.5 *Mib1* mutants and controls. Drug was injected daily from E10 to E12 for *Mib1* iKO. *P,* numbers of cells expressing NFIA and p-Vimentin. *Asterisks* indicate significant differences compared with controls (**, $p < 0.01$; ***, $p < 0.001$ in the unpaired Student's *t* test). Means ± S.E. are shown. *Scale bar* in *O*, 300 μm for *A–O*.

Mib1 Continues to Play a Role in the Later Period of Astrocytic Development—There are two types of astrocytes defined by their final destinations, morphology and marker expression, $GFAP^+$ white matter astrocytes and $GFAP^-$ gray matter astrocytes. In both types, immature astrocytes arise in the progenitor domain and migrate laterally. To decide whether the initial misspecification of astrocytic progenitors in the absence of *Mib1* affects the terminal differentiation of astrocytes, we monitored expression of GFAP in E17.5 *Mib1* mutant spinal cords. In littermate controls, $GFAP^+$ astrocytes with fibrous morphology were located within the white matter along the entire edge of the spinal cord. In contrast, GFAP expression in the *Mib1* conditional mutant was significantly reduced in the white matter (Fig. 5, *B* and *G*). A very low level of disorganized GFAP immunoreactivity was found in the dorsal gray matter where GFAP expression is normally absent, indicating that astrocyte differentiation is either compromised or delayed (Fig. 5, *B* and *G*). The expression of *AldhL1*, a marker that labels both gray matter and white matter astrocytes, revealed that numbers of both types of astrocyte were significantly reduced in the absence of *Mib1* (Fig. 5*L*) (37). Thus, it seemed likely that the initial loss of glial progenitors in the absence of *Mib1* has an ongoing suppressive effect on astrocyte differentiation.

Recent studies have suggested that the Notch signal remains active in mature astrocytes and neurons (37–39). Consistent with this, *Mib1* expression persisted among late progenitors when gliogenesis occurred (data not shown). Thus, it is possible that Mib1 plays a role in transmitting the Notch signal during late gliogenesis or the differentiation process. To locate the potential critical period for Notch signaling during glial differentiation, we eliminated *Mib1* at various stages of embryonic development using tamoxifen-inducible Cre transgenic mice

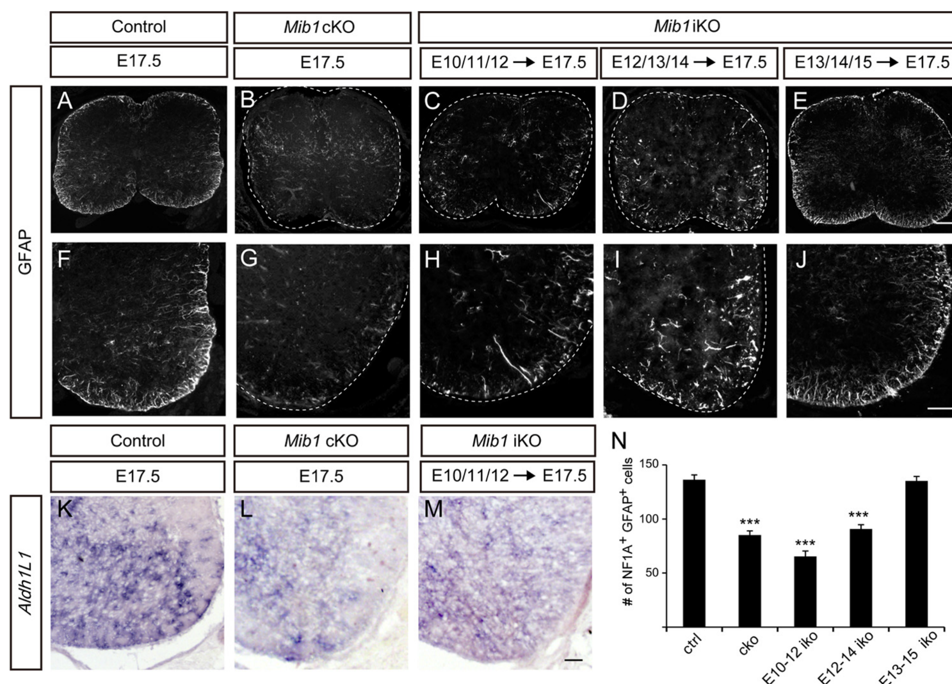

FIGURE 5. **Mib1 continues to be required for astrocyte generation as determined by removal of *Mib1* at different developmental times.** *A–E*, expression of GFAP in E17.5 spinal cords of *Mib1* mutants and controls. Drug was administered at different time points as indicated. *F–J*, higher magnification views of *A–E*. *K–M*, expression of *AldhL1* in *Mib1* mutant and control animals. *AldhL1* transcripts are reduced in the *Mib1* mutant mice to a similar extent as GFAP. *N*, numbers of cells co-expressing NFIA and GFAP to quantify mature astrocytes. Means ± S.E. are shown. *Asterisks* indicate statistically significant difference compared with control (***, $p < 0.001$ in the unpaired Student's *t* test). *Scale bars* in *E*, 400 μm for *A–E*; in *J*, 100 μm for *F–J*; in *M*, 100 μm for *K–M*.

(25). We injected tamoxifen into the pregnant mice for 3 days at either E10 to E12, E12 to E14, or E13 to E15, and we harvested the embryos at E17.5. GFAP and *AldhL1* expression was significantly reduced when the drug was applied from E10 to E12 (E10–12 *Mib1* iKO) (Fig. 5, *C, H, M,* and *N*). We only observed a partial reduction of GFAP-expressing astrocytes in E12–14 iKO mice (33.4%) and almost no reduction in E13–15 iKO mice (0.8%) (Fig. 5, *D, E, I,* and *J*). Thus, it appears that, in addition to the early role of Mib1 during neurogenesis, this protein continues to be required for proper glial differentiation at least until around E14.

Generation of Oligodendrocytes Is Compromised in the Absence of Mib1—To decide whether Mib1 also plays a role in oligodendrocyte development, we examined the production and differentiation of oligodendrocytes by assessing the expression of Olig2, a marker of oligodendrocytes. Oligodendrocytes are derived from the pMN domain after motor neurons were generated. Oligodendrocyte precursors migrate and populate the entire region of the spinal cord. At E13.0, Olig2$^+$ cells in the pMN domain were reduced in both *Mib1* cKO and iKO mutants injected between E10 and E12 (Fig. 6, *A–C* and *N*). We also examined the number of oligodendrocytes in the *Mib1* iKOs at E17.5. When the drug was delivered between E10 and E12 or E12 and E14, the number of oligodendrocytes was greatly reduced (67.5 and 44.4% compared with the control) (Fig. 6, *C, F, G, K, L,* and *O*). In contrast, it was unchanged when the drug was applied between E13 and E15 (Fig. 6, *H, M,* and *O*). This suggests that at least the early period of oligodendrocyte development (until ~E14) was dependent on the Mib1-Notch signal.

Functional Interaction between Mib1 and Other Notch Signaling Components during Neural Development—It is known that Mib1 induces the endocytic trafficking of DSL family ligands that trigger activation of the Notch signal pathway (3). To characterize the molecular events behind this, we generated *Mib1* deletion constructs and tested their ability to induce Notch signaling in chicks by in ovo electroporation (21, 40). Misexpression of wild-type Mib1 did not have any effect on V2 interneuron specification, suggesting that Mib1 is not sufficient to initiate Notch signaling without Delta (Fig. 7, *A* and *E*). Mib1dn, a deletion mutant lacking the C-terminal RING domain, induced Chx10$^+$ V2a interneurons at the expense of Gata3$^+$ V2b interneurons when misexpressed in chick neural tubes (Chx10, 114.6%; Gata3, 57.4%) (Fig. 7, *B, F,* and *Q*). Mib1ΔRD3, which does not contain the third terminal RING domain, caused mis-specification of V2 interneurons to a greater extent, indicating that the last RING domain is critical for Mib1 activity (Chx10, 114.6%; Gata3, 70.7%) (Fig. 7, *C, G,* and *Q*). Furthermore, ectopic V2a interneurons were observed in the ventricular zone dorsal to the p2 domain in both Mib1dn and Mib1ΔRD3-expressing neural tubes. This indicates that premature differentiation of V2a interneurons occurs as in *Mib1* mice (Fig. 7, *B, C,* and *G*). In contrast, activation of Notch signaling by introducing the NICD resulted in an increase in V2b interneurons and a reduction in V2a interneurons (Chx10, 88.6%; Gata3, 143.3%) (Fig. 7, *D, H,* and *Q*). Next, we electroporated *Dll1* that reduced both V2a and V2b interneurons without changing the ratio between them (Chx10, 73.4%; Gata3, 81.5%) (Fig. 7, *I, M,* and *Q*). The reduction of cell numbers in V2 interneurons in the presence of Dll1 could be due to early deple-

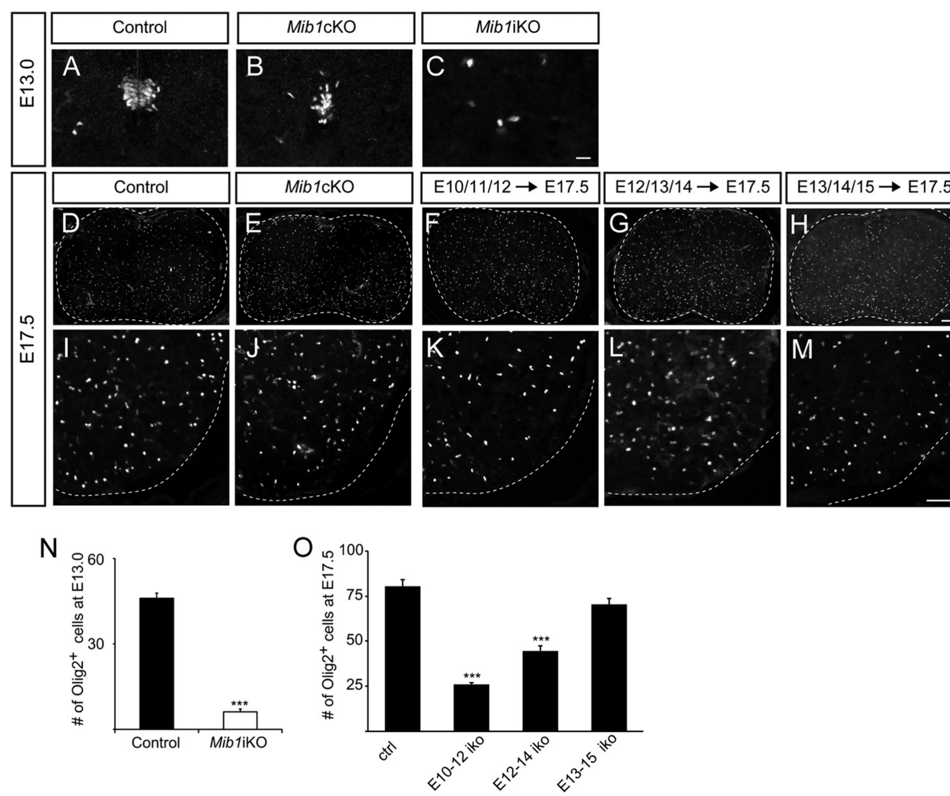

FIGURE 6. **Oligodendrocyte generation is affected in *Mib1* mice.** *A–C,* expression of Olig2 in the spinal cord of *Mib1* iKO injected between E10 and E12 and controls at E13.0. Oligodendrocyte progenitors in the pMN domain are depleted in the mutants as indicated by Olig2 expression. *D–H,* expression of Olig2 in E17.5 spinal cords of *Mib1* and controls. Drug was administered at different time points as indicated. *I–M,* higher magnification views of *D–H*. *N* and *O,* quantification of Olig2-expressing cells. Means ± S.E. are shown. *Asterisks* indicate statistically significant differences compared with control (***, $p < 0.001$ in the unpaired Student's *t* test). *Scale bar* in *C*, 50 μm for *A–C*; in *H*, 200 μm for *D--H*; in *M*, 100 μm for *I–M*.

tion of p2 progenitors. Misexpression of Dll4 also showed no significant difference in a ratio between V2a and V2b interneurons (data not shown).

The fact that introduction of exogenous Dll ligands alone is relatively ineffective in altering V2 interneuron identity prompted us to reason that it may require additional modification such as ubiquitination. To test this, we generated Dll4Δicd that lacks an intracellular domain where Mib1 interacts (21). When we electroporated *Dll4Δicd*, both V2a and V2b interneurons were reduced, but the ratio between V2a and V2b was unchanged (Chx10, 73.0%; Gata3, 66.1%) (Fig. 7, *J, N,* and *Q*). This suggests that Delta activity to assign V2 interneuron specification may require Mib1. If the dominant-negative effect by Mib1dn is mainly due to Delta, one would expect that conjugating a mono-ubiquitin with Delta would rescue the dominant-negative effect of Mib1dn. To test this, we fused Dll4Δicd with ubiquitin to generate Dll4Δicd-Ub (21). Introduction of *Dll4Δicd-Ub* alone induced more V2b interneurons and lesser V2a interneurons, indicating that mono-ubiquitination of Dll4 is sufficient to transmit Notch signaling without Mib1 (Chx10, 81.0%; Gata3, 126.5%) (Fig. 7, *K, O,* and *Q*).

We next electroporated *Δicd-Ub* with *Mib1dn*. As expected, Δicd-Ub rescued the phenotype of Mib1dn to produce more V2b interneurons at the expense of V2a interneurons (Chx10, 96.8%; Gata3, 101.4%) (Fig. 7, *L, P,* and *Q*). Together, these results suggest that an ability of Mib1 to ubiquitinate Delta ligand via the RING domain is essential for V2 interneuron specification.

Cell-autonomous Role of Mib1 in Neuronal Specification—When Notch-Delta signaling occurs, the Delta-expressing cell, the signal-sending cell, becomes a neuron, whereas the Notch-expressing cell, the signal-receiving cell, remains a progenitor. Although we had demonstrated premature neuronal differentiation when Mib1 was absent, as a result of failure to transmit the Notch signal, it was unclear whether the cells with reduced Mib1 activity maintained their neurogenic ability when Delta activity could be compromised. To test this, we isolated neuronal progenitors from E14.5 *Mib1^f/f^* animals and infected them with either a GFP or Cre retrovirus. To provide Notch signals from the neighborhood, we grew progenitors at low density on top of primary astrocytes, cells in which Notch signaling is known to be active (37, 41). If Mib1 was required for delivering but not receiving the Notch signal, the *Mib1*-deficient cells would remain progenitors. If Mib1 was also necessary for receiving the Notch signal, they would become neurons. At 3 days *in vitro*, most cells infected by GFP virus became neurons, as determined by MAP2 immunostaining (88.5% ± 3.073%) (Fig. 8, *A–E*). In contrast, the *Mib1*-deficient cells infected by Cre virus generated fewer neurons (59.9% ± 2.16%). There was no significant difference in Nestin$^+$ progenitors between the two groups, indicating that Delta-expressing cells lacking *Mib1* can receive the Notch signal and hence tend to remain as progenitors.

To test the cell-autonomous role of Mib1 *in vivo*, we electroporated *Gfp* or *Mib1dn* plasmids into E13.5 mouse embryonic brains and harvested them at E16.5. The number of neural pro-

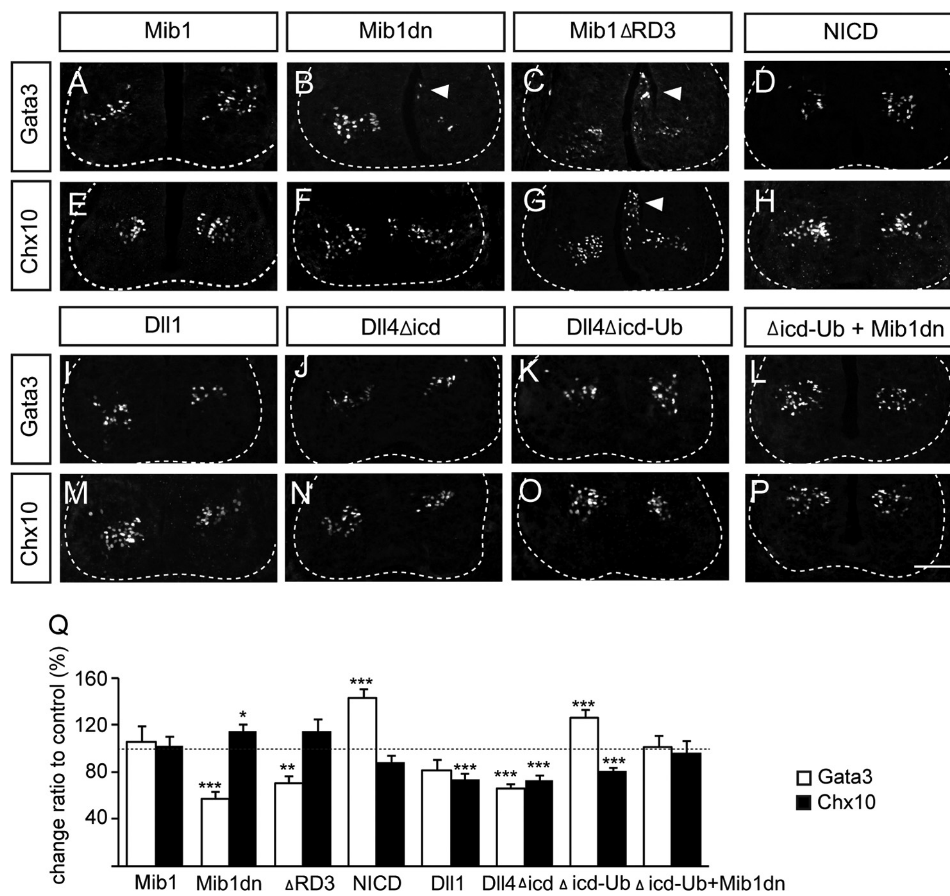

FIGURE 7. **Assessment of the requirement for Mib1 activity for specifying V2 interneurons.** *A–P*, misexpression of Mib1, Mib1dn, Mib1ΔRD3, NICD, Dll1, Δicd, Δicd-Ub, or Δicd-Ub + Mid1dn in the chick neural tube by *in ovo* electroporation. Generation of $Chx10^+$ V2a interneurons or $Gata3^+$ V2b interneurons was measured. Note that ectopic V2 interneurons are found in the progenitor zone dorsal to the p2 domain (*C* and *H, arrowheads*). *Q*, % change compared with control. Means ± S.E. are shown. *Asterisks* indicate significant difference compared with control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ in the unpaired Student's *t* test). *Scale bar* in *P*, 50 μm for *A–P*.

genitors located in the ventricular zone was counted by co-labeling them with the neural progenitor marker, Ngn2 (Fig. 8, *F–H*). We found that the density of $Ngn2^+GFP^+$ cells was reduced when Mib1dn was expressed. Although detailed mechanisms need to be further characterized, our result suggests that Mib1 is involved in the neuronal specification of Delta-expressing cells in a cell-autonomous manner.

DISCUSSION

Mib1 Is a Major Mediator of Notch-Delta Signaling—Numerous signaling components of the Notch-Delta signaling pathway have been identified (42). Nevertheless, the constituents of the signal-sending cells that function along with Delta and modulate its activity are less well known. One of the major steps in the activation of Delta ligands is their ubiquitination, which promotes internalization and signal transmission to the neighboring cells (21, 23). At least two ubiquitin ligases, Mind bomb (Mib) and Neuralized (Neur), are known to ubiquitinate DSL family members Delta and Jagged in the CNS (20, 21). Jagged ligands are expressed in the p1 domain, whereas Delta ligands are expressed in the p2 domain. The domain-specific and complementary expression of Delta and Jagged ligands suggests that individual ligands may deliver differential Notch signals. Nevertheless, we found that Mib1 is uniformly expressed in the ventral spinal cord, suggesting that it is a common ligase in the spinal cord that modifies several DSL ligands (data not shown). In contrast, *Neur* appears to play only a minor role in the developing neural tube because the elimination of both *Neur1* and *Neur2* does not cause any obvious developmental defects (24).

Mib1 is also known to ubiquitinate other proteins that are mainly known in different cellular context (40, 43). Although we cannot completely eliminate the possibility that these proteins may participate, several lines of evidence suggest that defective modification of Delta by Mib1 could be a major factor for the phenotype in *Mib1* mutant mice. First, we observed premature neuronal differentiation and mis-specification of selective interneurons when *Mib1* was absent. Development of radial glia, astrocytes, and oligodendrocytes was perturbed, all of which exactly phenocopy the mutants with defective Notch signaling (Fig. 8*I*) (7, 12). Second, Yoon *et al.* (12) demonstrated that the *Mib1* mutant phenotype is rescued by activating Notch signaling using mouse genetics, indicating that the Notch receptor is downstream of Mib1. Third, expression of DAPK, another substrate of Mib1, is unchanged in the *Mib1* mutant nervous system, which suggests that DAPK may not be relevant (44). Finally, we and others showed that introduction of the mono-ubiquitinated form of Delta is sufficient to rescue the defects caused by the dominant-negative Mib1 (21). Taken

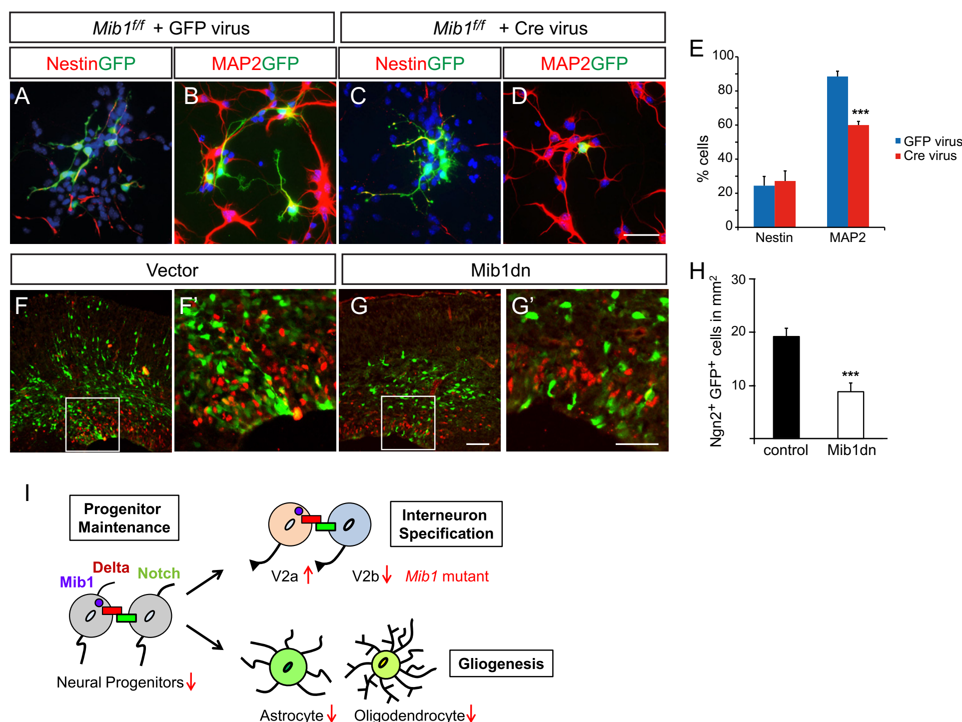

FIGURE 8. **Cell autonomous role of Mib1 in neuronal specification.** *A–D*, representative images of cortical neuronal cultures from E14.5 *Mib$^{f/f}$* animals infected by GFP or Cre-GFP retroviruses and grown on top of astrocytes for 3 days *in vitro*. Coverslips were immunolabeled for GFP and Nestin or MAP2 as indicated. *E*, quantification of Nestin and MAP2-expressing GFP$^+$ cells. *F–G′*, misexpression of Mib1dn or vector in E13.5 embryos by *in utero* electroporation. Pups were harvested at E16.5 and immunostained for Ngn2 (*red*) and GFP (*green*). *H*, quantification of Ngn2$^+$ GFP$^+$ cells per mm^2. Means ± S.E. are shown. *Asterisks* indicate significant differences compared with control (***, $p < 0.001$ in the unpaired Student's *t* test). *Scale bars* in *D*, 100 μm for *A–D*; in *G*, 200 μm for *F* and *G*; in *G′*, 100 μm for *F′* and *G′*. *I*, schematic model depicting the roles of Mib1 in spinal neuron development. Mib1 maintains neural progenitors and regulates V2a-V2b interneuron specification as well as gliogenesis.

together, our findings suggest that Mind bomb-1 is the major mediator that transmits Delta-Notch signaling during CNS development.

Mib1 Conveys Delta-Notch Signaling for Interneuron Identity—In addition to its pan-neuronal role in neurogenesis, Delta-Notch signaling assigns cell identity to the specific interneuronal subtypes in the spinal cord, such as in the binary choice between V2a and V2b interneurons in the ventral spinal cord (6–8, 45). In mutants where Delta-Notch signaling is compromised, there is an imbalance in the ratio of the interneurons that share lineages (7, 8). Recently, another V2 interneuron called V2c, originating from the same p2 progenitors, has been identified. Fate mapping and analysis of marker expression revealed that V2c interneurons are in the same lineage as V2b but later segregate and display distinct sets of transcription factors (33, 46). In this study, we examined the production of all three V2 interneurons, as well as other ventral cell types, in *Mib1* mutant mice. As expected, abnormal expansion of V2a interneurons and the loss of V2b interneurons were observed, supporting the view that Mind bomb-1 is the major mediator of Notch signaling in the spinal cord. Interestingly, the generation of V2c interneurons was only slightly altered in the *Mib1* mutants although they derive from the same progenitors as V2b interneurons (33). V2c neurons are far fewer in number than V2a or V2b interneurons. This argues against a simple binary cell fate decision that should generate equal numbers of V2b and V2c interneurons. Thus, the V2c identity may not be directly controlled by Notch activity, unlike the other V2 interneurons. One possibility is that two different pools of p2 progenitors exist that generate either V2a/V2b neurons or V2b/V2c neurons. An alternative possibility is that transiently amplifying p2 progenitors exist that preferentially generate greater numbers of V2a and V2b neurons; Notch would then dictate the choice between V2a and V2b neurons whereas other factors such as Foxn4 or Sox1 would occasionally induce the V2c identity, independent of Notch signaling (45).

Several lines of evidence suggest that the role of Notch signaling in cell fate specification may not be restricted to V2 interneurons (6, 8). An imbalance of neuronal subtypes was reported involving other cell types such as dorsal interneurons and ventral cell types when Notch signaling was defective (6, 8, 47, 48). For instance, the generation of motor neurons in the pMN domain, a domain next to the p2 domain, is inhibited by Jagged2 activity (48). Likewise, we observed the premature appearance of motor neuron markers in chick neural tubes when a dominant-negative Mib1 was introduced (data not shown). In addition, premature extinction of the ventricular zone in the p1 domain as well as the p2 domain was found in *Mib1* mutant mice. Finally, the glial defects found in *Mib1* mutant mice suggest that Notch signaling influences gliogenesis in multiple ventral progenitor domains. In the absence of *Mib1*, the numbers of astrocytes, which arise in multiple ventral domains, and of oligodendrocytes, which originate from the pMN domain, were both reduced. Together, these observations suggest that Mib1 plays a major role in cell specification in the ventral spinal cord.

Spatiotemporal Segregation of Neurogenesis and Gliogenesis Is Controlled by Mib1-mediated Notch Signaling—During neural development, neural progenitors generate neurons and glia at different times and locations (9). Given that Notch inhibits neuronal differentiation and maintains neural progenitors, it seems likely that its early action in neurogenesis influences the behavior of reserved progenitors that later give rise to glial cells. In this way, the Notch signal appears to be the major pathway that links neurogenesis and gliogenesis by controlling the timing of progenitor competence.

Astrocytes and oligodendrocytes are two major glial cell types that arise in different regions of the developing spinal cord. In the cortex, astrocytes are generated in the dorsal telencephalon, whereas oligodendrocytes are produced in the basal forebrain area (9, 49). Likewise, in the spinal cord, astrocytes arise in the ventral progenitor domains after the appearance of neurons. Oligodendrocytes mainly originate in the pMN domain, and some from the dorsal spinal cord in later development. Thus, the region-specific generation of glial cells may be regulated by Notch signaling, in the same way as it affects spinal interneuronal identity. In *Mib1* mutant ventral spinal cords, we observed a significant loss of radial glial cells, the precursors of astrocytes. Consistent with this, the number of astrocytes in both the gray and white matter of the spinal cords was reduced in *Mib1* mutant mice. It is noteworthy that elimination of *Mib1* in drug-inducible *Mib1* mutant animals reduced numbers of astrocytes and oligodendrocytes at least up to ~E14, indicating that Notch continues to act in late glial progenitors.

In addition to its primary role in astrocytes, Notch is thought to serve a dual role in oligodendrocyte generation; it promotes oligodendrocyte precursor cell development and suppresses later differentiation of oligodendrocytes (2, 15, 16, 48). This binary action of Notch was confirmed *in vitro* where astrocytes were induced and oligodendrocytes were reduced in a stepwise manner in clonal neurosphere stem cell cultures (50). Consistent with this, in *Mib1* mutants, we first observed a reduction of Olig2 expression in the pMN domain after motor neurons had arisen. Likewise, the number of mature oligodendrocytes in the entire spinal cord was reduced in *Mib1* mutant mice. This was observed only when *Mib1* was eliminated in the early period between E10 and 12, a similar time window to the one in which astrocyte specification is perturbed. Together, these findings indicate that Mind bomb-1 mediates the Notch signaling that controls the generation of astrocytes and oligodendrocytes during the early period of glial development.

Cell Autonomous Role of Mib1 in Neuron-Glial Specification—There are two mechanisms by which Delta ligands control Notch signaling activity as follows: they trans-activate Notch in surrounding cells and cis-inhibit Notch in their own cells (51–54). Previous studies have shown that endocytic internalization of Mib1 is essential for transmitting Notch signals to neighboring cells (21). However, it is unclear whether the inhibition of Notch within the same cells is also modulated by endocytic events or ubiquitin modification by Mib1. Previous studies indicated that the cis-inhibition process might not require Mib1 (23, 55). First, Dll3, which does not undergo ubiquitination, acts primarily on cis-inhibition but not transactivation (56). Next, co-expression of Mib1 and Dll1 does not inhibit the cis-inhibitory activity of Delta ligands *in vitro* (21). Furthermore, transplantation experiments in zebrafish have demonstrated that *Mib1*-deficient cells surrounded by wild-type cells do not readily become neurons because they can still accept Notch signals without Mib1 (21). Finally, a *Serrate* mutant that cannot interact with Mib1 still cis-inhibits Notch activity in *Drosophila* (57). Using low titer retroviral infections and lineage tracing of progenitors, we demonstrated that *Mib1*-deficient cells can receive Notch signal and remain as progenitors. Interestingly, the portion of cells destined to become neurons were reduced without Mib1, implying that the neurogenic potential in *Mib1*-deficient cells could be compromised. Recently, the roles of Notch are rapidly expanding to mature neurons and the adult brain (58, 59). It is tempting to say that Mib1 may participate in later events in neuronal maturation in a similar context, which need to be further characterized in future studies.

Acknowledgements—We are grateful to Dr. Young Yoon Kong for Mib1 flox mice and Dr. Kageyama for Nes-CreERT2 mice. We thank members of the Song laboratory for helpful discussions and encouragement; Choon Soon Park and Jeong Ja Lee for excellent technical assistance; and the BioImaging Research Center and Systems Biology Research Center at Gwangju Institute of Science and Technology for the confocal imaging facility.

REFERENCES

1. Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M. E. (1995) Notch signaling. *Science* **268,** 225–232
2. Park, H. C., and Appel, B. (2003) Delta-Notch signaling regulates oligodendrocyte specification. *Development* **130,** 3747–3755
3. Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995) Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene *Delta*. *Nature* **375,** 761–766
4. Haddon, C., Smithers, L., Schneider-Maunoury, S., Coche, T., Henrique, D., and Lewis, J. (1998) Multiple *delta* genes and lateral inhibition in zebrafish primary neurogenesis. *Development* **125,** 359–370
5. Jessell, T. M. (2000) Neuronal specification in the spinal cord. Inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1,** 20–29
6. Mizuguchi, R., Kriks, S., Cordes, R., Gossler, A., Ma, Q., and Goulding, M. (2006) Ascl1 and Gsh1/2 control inhibitory and excitatory cell fate in spinal sensory interneurons. *Nat. Neurosci.* **9,** 770–778
7. Peng, C. Y., Yajima, H., Burns, C. E., Zon, L. I., Sisodia, S. S., Pfaff, S. L., and Sharma, K. (2007) Notch and MAML signaling drives Scl-dependent interneuron diversity in the spinal cord. *Neuron* **53,** 813–827
8. Yang, X., Tomita, T., Wines-Samuelson, M., Beglopoulos, V., Tansey, M. G., Kopan, R., and Shen, J. (2006) Notch1 signaling influences v2 interneuron and motor neuron development in the spinal cord. *Dev. Neurosci.* **28,** 102–117
9. Kriegstein, A., and Alvarez-Buylla, A. (2009) The glial nature of embryonic and adult neural stem cells. *Annu. Rev. Neurosci.* **32,** 149–184
10. Gaiano, N., Nye, J. S., and Fishell, G. (2000) Radial glial identity is promoted by Notch1 signaling in the murine forebrain. *Neuron* **26,** 395–404
11. Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S., and Kriegstein, A. R. (2001) Neurons derived from radial glial cells establish radial units in neocortex. *Nature* **409,** 714–720
12. Yoon, K. J., Koo, B. K., Im, S. K., Jeong, H. W., Ghim, J., Kwon, M. C., Moon, J. S., Miyata, T., and Kong, Y. Y. (2008) Mind bomb 1-expressing intermediate progenitors generate notch signaling to maintain radial glial cells. *Neuron* **58,** 519–531
13. Bernardos, R. L., Lentz, S. I., Wolfe, M. S., and Raymond, P. A. (2005) Notch-Delta signaling is required for spatial patterning and Müller glia differentiation in the zebrafish retina. *Dev. Biol.* **278,** 381–395

14. Eiraku, M., Tohgo, A., Ono, K., Kaneko, M., Fujishima, K., Hirano, T., and Kengaku, M. (2005) DNER acts as a neuron-specific Notch ligand during Bergmann glial development. *Nat. Neurosci.* **8,** 873–880
15. Taylor, M. K., Yeager, K., and Morrison, S. J. (2007) Physiological Notch signaling promotes gliogenesis in the developing peripheral and central nervous systems. *Development* **134,** 2435–2447
16. Wang, S., Sdrulla, A. D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., Weinmaster, G., and Barres, B. A. (1998) Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* **21,** 63–75
17. Kim, H., Shin, J., Kim, S., Poling, J., Park, H. C., and Appel, B. (2008) Notch-regulated oligodendrocyte specification from radial glia in the spinal cord of zebrafish embryos. *Dev. Dyn.* **237,** 2081–2089
18. Pierfelice, T., Alberi, L., and Gaiano, N. (2011) Notch in the vertebrate nervous system. An old dog with new tricks. *Neuron* **69,** 840–855
19. Seugnet, L., Simpson, P., and Haenlin, M. (1997) Requirement for dynamin during Notch signaling in *Drosophila* neurogenesis. *Dev. Biol.* **192,** 585–598
20. Yeh, E., Zhou, L., Rudzik, N., and Boulianne, G. L. (2000) Neuralized functions cell autonomously to regulate *Drosophila* sense organ development. *EMBO J.* **19,** 4827–4837
21. Itoh, M., Kim, C. H., Palardy, G., Oda, T., Jiang, Y. J., Maust, D., Yeo, S. Y., Lorick, K., Wright, G. J., Ariza-McNaughton, L., Weissman, A. M., Lewis, J., Chandrasekharappa, S. C., and Chitnis, A. B. (2003) Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev. Cell* **4,** 67–82
22. Weinmaster, G., and Fischer, J. A. (2011) Notch ligand ubiquitylation. What is it good for? *Dev. Cell* **21,** 134–144
23. Chitnis, A. (2006) Why is delta endocytosis required for effective activation of notch? *Dev. Dyn.* **235,** 886–894
24. Koo, B. K., Yoon, M. J., Yoon, K. J., Im, S. K., Kim, Y. Y., Kim, C. H., Suh, P. G., Jan, Y. N., and Kong, Y. Y. (2007) An obligatory role of mind bomb-1 in notch signaling of mammalian development. *PLoS ONE* **2,** e1221
25. Imayoshi, I., Ohtsuka, T., Metzger, D., Chambon, P., and Kageyama, R. (2006) Temporal regulation of Cre recombinase activity in neural stem cells. *Genesis* **44,** 233–238
26. Thaler, J. P., Koo, S. J., Kania, A., Lettieri, K., Andrews, S., Cox, C., Jessell, T. M., and Pfaff, S. L. (2004) A postmitotic role for Isl-class LIM homeodomain proteins in the assignment of visceral spinal motor neuron identity. *Neuron* **41,** 337–350
27. Thaler, J., Harrison, K., Sharma, K., Lettieri, K., Kehrl, J., and Pfaff, S. L. (1999) Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. *Neuron* **23,** 675–687
28. Thaler, J. P., Lee, S.-K., Jurata, L. W., Gill, G. N., and Pfaff, S. L. (2002) LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions. *Cell* **110,** 237–249
29. Kawaguchi, D., Yoshimatsu, T., Hozumi, K., and Gotoh, Y. (2008) Selection of differentiating cells by different levels of delta-like 1 among neural precursor cells in the developing mouse telencephalon. *Development* **135,** 3849–3858
30. Koo, B. K., Lim, H. S., Song, R., Yoon, M. J., Yoon, K. J., Moon, J. S., Kim, Y. W., Kwon, M. C., Yoo, K. W., Kong, M. P., Lee, J., Chitnis, A. B., Kim, C. H., and Kong, Y. Y. (2005) Mind bomb 1 is essential for generating functional Notch ligands to activate Notch. *Development* **132,** 3459–3470
31. Lindsell, C. E., Boulter, J., diSibio, G., Gossler, A., and Weinmaster, G. (1996) Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. *Mol. Cell. Neurosci.* **8,** 14–27
32. Caprioli, A., Goitsuka, R., Pouget, C., Dunon, D., and Jaffredo, T. (2002) Expression of Notch genes and their ligands during gastrulation in the chicken embryo. *Mech. Dev.* **116,** 161–164
33. Panayi, H., Panayiotou, E., Orford, M., Genethliou, N., Mean, R., Lapathitis, G., Li, S., Xiang, M., Kessaris, N., Richardson, W. D., and Malas, S. (2010) Sox1 is required for the specification of a novel p2-derived interneuron subtype in the mouse ventral spinal cord. *J. Neurosci.* **30,** 12274–12280
34. Deneen, B., Ho, R., Lukaszewicz, A., Hochstim, C. J., Gronostajski, R. M., and Anderson, D. J. (2006) The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. *Neuron* **52,** 953–968
35. Pringle, N. P., Yu, W. P., Howell, M., Colvin, J. S., Ornitz, D. M., and Richardson, W. D. (2003) Fgfr3 expression by astrocytes and their precursors. Evidence that astrocytes and oligodendrocytes originate in distinct neuroepithelial domains. *Development* **130,** 93–102
36. Bani-Yaghoub, M., Tremblay, R. G., Lei, J. X., Zhang, D., Zurakowski, B., Sandhu, J. K., Smith, B., Ribecco-Lutkiewicz, M., Kennedy, J., Walker, P. R., and Sikorska, M. (2006) Role of Sox2 in the development of the mouse neocortex. *Dev. Biol.* **295,** 52–66
37. Cahoy, J. D., Emery, B., Kaushal, A., Foo, L. C., Zamanian, J. L., Christopherson, K. S., Xing, Y., Lubischer, J. L., Krieg, P. A., Krupenko, S. A., Thompson, W. J., and Barres, B. A. (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J. Neurosci.* **28,** 264–278
38. Alberi, L., Liu, S., Wang, Y., Badie, R., Smith-Hicks, C., Wu, J., Pierfelice, T. J., Abazyan, B., Mattson, M. P., Kuhl, D., Pletnikov, M., Worley, P. F., and Gaiano, N. (2011) Activity-induced Notch signaling in neurons requires Arc/Arg3.1 and is essential for synaptic plasticity in hippocampal networks. *Neuron* **69,** 437–444
39. Shimada, I. S., Borders, A., Aronshtam, A., and Spees, J. L. (2011) Proliferating reactive astrocytes are regulated by Notch-1 in the peri-infarct area after stroke. *Stroke* **42,** 3231–3237
40. Berndt, J. D., Aoyagi, A., Yang, P., Anastas, J. N., Tang, L., and Moon, R. T. (2011) Mindbomb 1, an E3 ubiquitin ligase, forms a complex with RYK to activate Wnt/β-catenin signaling. *J. Cell Biol.* **194,** 737–750
41. Givogri, M. I., de Planell, M., Galbiati, F., Superchi, D., Gritti, A., Vescovi, A., de Vellis, J., and Bongarzone, E. R. (2006) Notch signaling in astrocytes and neuroblasts of the adult subventricular zone in health and after cortical injury. *Dev. Neurosci.* **28,** 81–91
42. de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J., and Conlon, R. A. (1997) Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124,** 1139–1148
43. Jin, Y., Blue, E. K., and Gallagher, P. J. (2006) Control of death-associated protein kinase (DAPK) activity by phosphorylation and proteasomal degradation. *J. Biol. Chem.* **281,** 39033–39040
44. Barsi, J. C., Rajendra, R., Wu, J. I., and Artzt, K. (2005) Mind bomb1 is a ubiquitin ligase essential for mouse embryonic development and Notch signaling. *Mech. Dev.* **122,** 1106–1117
45. Del Barrio, M. G., Taveira-Marques, R., Muroyama, Y., Yuk, D. I., Li, S., Wines-Samuelson, M., Shen, J., Smith, H. K., Xiang, M., Rowitch, D., and Richardson, W. D. (2007) A regulatory network involving Foxn4, Mash1, and delta-like 4/Notch1 generates V2a and V2b spinal interneurons from a common progenitor pool. *Development* **134,** 3427–3436
46. Li, S., Misra, K., and Xiang, M. (2010) A Cre transgenic line for studying V2 neuronal lineages and functions in the spinal cord. *Genesis* **48,** 667–672
47. Yeo, S. Y., and Chitnis, A. B. (2007) Jagged-mediated Notch signaling maintains proliferating neural progenitors and regulates cell diversity in the ventral spinal cord. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 5913–5918
48. Rabadán, M. A., Cayuso, J., Le Dréau, G., Cruz, C., Barzi, M., Pons, S., Briscoe, J., and Martí, E. (2012) Jagged2 controls the generation of motor neuron and oligodendrocyte progenitors in the ventral spinal cord. *Cell Death Differ.* **19,** 209–219
49. Rowitch, D. H. (2004) Glial specification in the vertebrate neural tube. *Nat. Rev. Neurosci.* **5,** 409–419
50. Grandbarbe, L., Bouissac, J., Rand, M., Hrabé de Angelis, M., Artavanis-Tsakonas, S., and Mohier, E. (2003) Delta-Notch signaling controls the generation of neurons/glia from neural stem cells in a stepwise process. *Development* **130,** 1391–1402
51. del Álamo, D., Rouault, H., and Schweisguth, F. (2011) Mechanism and significance of cis-inhibition in Notch signalling. *Curr. Biol.* **21,** R40–R47
52. de Celis, J. F., and Bray, S. (1997) Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the *Drosophila* wing. *Development* **124,** 3241–3251
53. Klein, T., Brennan, K., and Arias, A. M. (1997) An intrinsic dominant negative activity of serrate that is modulated during wing development in *Drosophila*. *Dev. Biol.* **189,** 123–134

54. Micchelli, C. A., Rulifson, E. J., and Blair, S. S. (1997) The function and regulation of cut expression on the wing margin of *Drosophila*. Notch, Wingless, and a dominant negative role for Delta and Serrate. *Development* **124,** 1485–1495
55. Schweisguth, F. (2004) Regulation of notch signaling activity. *Curr. Biol.* **14,** R129–R138
56. Ladi, E., Nichols, J. T., Ge, W., Miyamoto, A., Yao, C., Yang, L. T., Boulter, J., Sun, Y. E., Kintner, C., and Weinmaster, G. (2005) The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands. *J. Cell Biol.* **170,** 983–992
57. Glittenberg, M., Pitsouli, C., Garvey, C., Delidakis, C., and Bray, S. (2006) Role of conserved intracellular motifs in Serrate signalling, cis-inhibition, and endocytosis. *EMBO J.* **25,** 4697–4706
58. Nishimura, T., Yamaguchi, T., Tokunaga, A., Hara, A., Hamaguchi, T., Kato, K., Iwamatsu, A., Okano, H., and Kaibuchi, K. (2006) Role of numb in dendritic spine development with a Cdc42 GEF intersectin and EphB2. *Mol. Biol. Cell* **17,** 1273–1285
59. Giniger, E. (1998) A role for Abl in Notch signaling. *Neuron* **20,** 667–681