

## Notch1 is required for neuronal and glial differentiation in the cerebellum

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### SUMMARY

The mechanisms that guide progenitor cell fate and differentiation in the vertebrate central nervous system (CNS) are poorly understood. Gain-of-function experiments suggest that Notch signaling is involved in the early stages of mammalian neurogenesis. On the basis of the expression of Notch1 by putative progenitor cells of the vertebrate CNS, we have addressed directly the role of Notch1 in the development of the mammalian brain. Using conditional gene ablation, we show that loss of Notch1 results in premature onset of neurogenesis by neuroepithelial cells of the midbrain-hindbrain region of

the neural tube. Notch1-deficient cells do not complete differentiation but are eliminated by apoptosis, resulting in a reduced number of neurons in the adult cerebellum. We have also analyzed the effects of Notch1 ablation on gliogenesis *in vivo*. Our results show that Notch1 is required for both neuron and glia formation and modulates the onset of neurogenesis within the cerebellar neuroepithelium.

Key words: Notch, Neurogenesis, CNS, Cerebellum, Apoptosis, Mouse

### INTRODUCTION

The nervous system of vertebrates is derived from the neuroepithelial cells of the neural tube. The early embryonic neuroepithelium contains cells that are considered to have stem cell-like properties in that they are mitotically active, can self renew, and have the potential to generate all of the differentiated cells of the adult central nervous system (CNS) (Gage et al., 1995; Kalyani et al., 1997; McKay, 1997; Qian et al., 1997; Vescovi et al., 1993). The neural tube is patterned early in development into defined regions that will develop into the vesicles and structures of the more mature CNS. Within these vesicles the fate of the neuroepithelial cells is regulated to generate neurons and glial cells in a spatially and temporally well-defined manner. The midbrain-hindbrain boundary is an important organizer in the anterior region of the developing CNS (Wassef and Joyner, 1997). This so-called isthmus structure gives rise to signals that include Fibroblast Growth Factor 8 (FGF8) and which regulate anterior-posterior patterning and formation of the caudal midbrain and the cerebellum (Martinez et al., 1999).

The differentiation of multipotent neural progenitor cells isolated from the embryonic nervous system can be modulated by growth factor treatment *in vitro* (Johe et al., 1996; Qian et al., 1997; Reynolds and Weiss, 1992; Reynolds and Weiss, 1996; Williams et al., 1997). However, the molecules that regulate neuroepithelial cell differentiation and determine cell fate in the mammalian neural tube are poorly defined. In invertebrates such as *Drosophila*, the signaling molecule Notch is involved in developmental decisions in many tissues, including the nervous

system, through a process of lateral inhibition (Kimble and Simpson, 1997). Notch is required to generate the correct number of neuronal precursor cells of the sensory organs and also regulates their fate by interacting with transmembrane ligands Delta and Serrate expressed on neighboring cells (Heitzler and Simpson, 1991; Muskavitch, 1994).

Notch regulates the expression of a cascade of transcription factors belonging to the basic helix-loop-helix (bHLH) family (Artavanis-Tsakonas et al., 1999; Kalyani et al., 1997; Robey, 1997). Recent data have shown that Notch receptor signaling is regulated by a complex process of proteolysis (Chan and Jan, 1998; Kopan and Goate, 2000; Lendahl, 1998). Multiple extracellular cleavages are rapidly followed by a ligand-induced intracellular, juxtamembrane proteolysis via a  $\gamma$ -secretase that may include members of the presenilin family (Kopan and Goate, 2000; Selkoe, 2000). The cleaved intracellular domain of the Notch activates *Enhancer of Split*-related (*E(Spl)*) genes (the *Hes* family in vertebrates) in a complex including *Suppressor of Hairless*-like (*Su(H)*) nuclear factors (the vertebrate CBF1, Su(H), LAG1 family). Subsequently, the *Hes* proteins suppress expression of the proneural bHLH transcription factors related to the *Drosophila Achaete-Scute* complex genes. These proneural genes are pivotal in the induction of neurogenesis and regulation of neuronal differentiation (Cau et al., 2000; Nieto et al., 2001; Ohtsuka et al., 2001; Satow et al., 2001; Lee, 1997).

Vertebrate neuroepithelial cells require precise regulation of differentiation and cell fate determination. Hence, it has been proposed that Notch signaling may play a role in vertebrate neurogenesis (Artavanis-Tsakonas et al., 1999). Gain-of-

function experiments performed in *Xenopus laevis* and chicken have provided strong indications that Notch signaling can maintain neural progenitor cells in an undifferentiated state (Austin et al., 1995; Chitnis et al., 1995; Coffman et al., 1993; Wakamatsu et al., 1999). Overexpression of an intrinsically active form of Notch or the ligand Delta blocked progenitor cell differentiation and reduced neurogenesis at early stages (Ahmad et al., 1997; Austin et al., 1995; Chitnis et al., 1995; Coffman et al., 1993; Wakamatsu et al., 1999). In other studies, strong overexpression of Delta in chick retinal and *Xenopus* otic vesicle progenitors mimicked a reduction in Notch signaling, resulting in an increased neuronal differentiation of isolated progenitor cells (Dorsky et al., 1997; Henrique et al., 1997). Furthermore, overexpression of a dominant negative Delta blocked Notch signaling in clustered cells and resulted in premature differentiation of retinal and otic vesicle progenitors (Austin et al., 1995; Dorsky et al., 1997; Henrique et al., 1997). These findings have been supported by antisense oligonucleotide experiments to reduce *Notch1* expression in the retina of chicken embryos, which caused an increase in neurons without affecting neuroepithelial cell proliferation (Austin et al., 1995). Gain-of-function experiments in the rat retina and mouse telencephalon have also indicated that Notch signaling in neural progenitors may regulate neuronal versus glial differentiation of multipotent progenitors (Furukawa et al., 2000; Gaiano et al., 2000; Lundkvist and Lendahl, 2001). This hypothesis has recently been supported by overexpression of a constitutively active Notch in the zebrafish retina resulting in an instructive signal for gliogenesis (Scheer et al., 2001). A similar gliogenic effect can also be induced in cultured murine neural progenitors in vitro by overexpression of intrinsically active Notch3 (Tanigaki et al., 2001).

*Notch1* expression in the mouse neuroepithelium starts around embryonic day 9 (E9) and continues in the ventricular zone and subventricular zone cells at later developmental stages (Del Amo et al., 1992; Reaume et al., 1992; Weinmaster et al., 1991). Inactivation of the *Notch1* gene in mice resulted in a developmental arrest at the four to six somite stage and death around E9.5 (Conlon et al., 1995; Swiatek et al., 1994). In addition, a potential precocious expression of the neurogenic bHLH transcription factor Mash1 (Ascl1 – Mouse Genome Informatics) was observed (de la Pompa et al., 1997). However, the in vivo role of Notch1 in mammalian neurogenesis and the fate of Notch1-deficient neuroepithelial cells remain unclear due to the early lethality of Notch1-deficient animals. Thus, we have conditionally ablated the *Notch1* gene from the mouse neural tube neuroepithelium using the Cre-Lox system. *Notch1* was inactivated specifically in neuroepithelial cells of the midbrain-hindbrain boundary and the consequences of *Notch1* ablation on the neuroepithelial cells and their fate was followed through to adulthood. Our approach allowed us to analyze the effects of the loss of Notch1 function on a restricted population of neuroepithelial cells, and revealed a pivotal role for Notch1 in the differentiation of mouse cerebellar progenitor cells.

## MATERIALS AND METHODS

### Generation of mice and breeding

Mice carrying LoxP-flanked *Notch1* alleles have been described previously (Radtke et al., 1999) and mice carrying the Cre-

recombinase under the transcriptional control of the *engrailed 2* promoter enhancer were kindly provided by A. Joyner (Zinyk et al., 1998). The ROSA26-R cre-reporter (*R26R*) transgenic line was kindly provided by P. Soriano (Soriano, 1999). Embryos were generated by timed-mating counting the morning after pairing as embryonic day 0.5. As ablation of the *engrailed 2* gene also results in cerebellar foliation defects (Millen et al., 1994), we addressed the possibility that the abnormalities seen in our homozygous Floxed *Notch1 En2-Cre* animals may be caused by effects of the *En2-Cre* transgene on endogenous *engrailed 2* activity. Therefore, we analyzed heterozygous Floxed *Notch1 En2-Cre* animals and *En2-Cre* transgenic animals but could not find defects in cerebellar development. Hence, we exclude the possibility that the *En2-Cre* transgene induced the reduced cerebellar vermis seen in our mutants. Furthermore, we have observed an identical phenotype using a second line of *En2-Cre* animals that shows a similar expression pattern in the cerebellum (Zinyk et al., 1998). This indicates that the reduction in cerebellar neurons is caused by the loss of Notch1 rather than an integration effect of the *En2-Cre* transgene. The analysis was performed with animals carrying the *En2-Cre* transgene and homozygous Floxed *Notch1* alleles. We have also observed an identical phenotype in *En2-Cre* transgenic animals hemizygous for Floxed and null-*Notch1* alleles.

### In situ hybridization analysis of gene expression

Embryos and brains were isolated and frozen in OCT (TissueTech) on dry ice. Frozen sections (20 µm) were thaw mounted onto Superfrost slides (Mettler), air-dried and fixed in 4% paraformaldehyde. Histological analysis was performed by staining sections for 30 seconds in hematoxylin (Sigma) followed by dehydration in alcohol and embedding in Eukitt (Plano). The midline sagittal sections of the adult cerebellum were orientated between the peduncles. The embryos were mounted and cross-sections cut perpendicular to the neural tube at the level of the isthmus flexure (Fig. 2A). The constriction of the neural tube at the isthmus between the midbrain and the cerebellum was used as the landmark for the anterior aspect of the presumptive cerebellum, and the point of maximum opening of the roof of the fourth ventricle as the posterior margin of the cerebellar primordium. In situ RNA hybridization was performed with digoxigenin-labeled RNA probes for calbindin D 28k, Dll1, Dll3, Hes1, Hes3, Hes5, Jagged1, Jagged2, Mash1, Math1 (Atoh1 – Mouse Genome Informatics) and Notch1, overnight at 72°C in buffer containing 50% formamide, and detected using an anti-DIG-AP antibody according to manufacturer's instructions (Roche Diagnostics). Consecutive sections covering the entire cerebellar primordium were analyzed with three different probes, Xgal staining or antibodies. Whole-mount in situ RNA hybridization was performed according to Wilkinson (Wilkinson, 1992).

### Immunofluorescence and cell fate analysis

Immunofluorescent analysis of protein expression was performed with antibodies against  $\beta$ -tubulinIII (TuJ1) (1:200; Sigma), neurofilament 160 (1:100; Sigma), nestin (1:30; R401, Developmental Studies Hybridoma Bank, University of Iowa), and calbindin D 28k (1:200; Sigma). Sections were fixed in 4% paraformaldehyde and blocked in PBS 0.2% Tween20 containing 5% goat serum. Antibodies were diluted in blocking buffer and incubated overnight at 4°C. Bound antibody was detected with Cy3- or FITC-conjugated goat anti-mouse Ig (Jackson Labs) and goat anti-rabbit Ig (Jackson Labs). Analysis of  $\beta$ -galactosidase activity was performed by Xgal staining according to Zinyk et al. (Zinyk et al., 1998). Expression of  $\beta$ -galactosidase was detected with anti- $\beta$ -galactosidase antibodies (1:100; Cappel). Bromodeoxyuridine (BrdU) was injected into time-mated females and the embryos were excised after 3 hours. BrdU detection was performed with an FITC-conjugated anti-BrdU antibody according to the manufacturer's instructions (Roche Diagnostics). Apoptotic cell death was analyzed by TUNEL staining using biotin-labeled UTP and an FITC-conjugated streptavidin complex according to the

manufacturer's instructions (Roche Diagnostics). Images were collected using an Axiophot microscope (Zeiss) in conjunction with a ProgRes 3008 (Jenoptik) or Hamamatsu (Hamamatsu Photonics) CCD camera; image processing was performed with NIH Image and Adobe Photoshop 5.0 software. Confocal microscopy was performed with a Leica confocal microscope and Imaris software (Bitplane AG, Technopark Zurich, Switzerland).

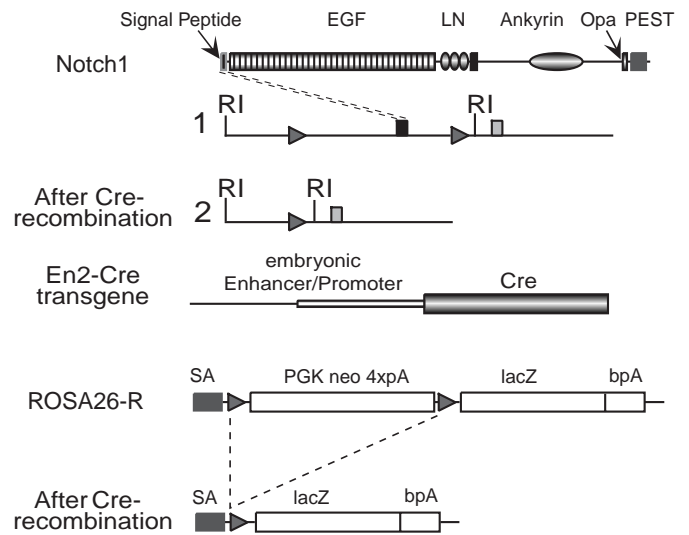
### Isolation and culture of cerebellar glial cells

Postnatal day 4 (P4) mice were sacrificed by decapitation, the cerebellum removed and the vermis region separated from the hemispheres under sterile conditions. The tails of the sacrificed animals were used for DNA isolation and the genotype of the animals analyzed by PCR. The vermis of the individual animals were dissociated and incubated in Ringer's solution containing 0.25% trypsin for 30 minutes at 37°C. The cells were collected by centrifugation and resuspended in 6 ml Basal Medium Eagle (BME) (Gibco) containing 10% fetal calf serum (FCS). The cells were triturated with a fire-polished Pasteur pipette and passed through a 50  $\mu$ m cell sieve. The cells from the vermis of one animal were plated on six 35 mm dishes coated with poly-L-lysine. There were no obvious differences in the plating efficiency between mutant and control cells. The cells were cultured for 5 days in BME, 10% FCS, and half of the medium was replaced every second day. The cells were fixed and immunostained with anti-GFAP (glial fibrillary acidic protein) antibodies (Sigma) in conjunction with anti- $\beta$ -galactosidase antibodies or Xgal staining. The cultures from mutant animals contained considerably fewer cells than those from control animals, which likely reflects the 50% reduction in the size of the cerebellar vermis of the mutants compared to control littermates at P4 (Fig. 8A,B).

## RESULTS

To address the role of Notch1 in the development and maintenance of the CNS we conditionally inactivated the *Notch1* gene in midbrain-hindbrain neuroepithelial cells from E9. The *Notch1* gene encodes a type 1 membrane receptor of approximately 300 kDa with an N-terminal signal peptide, 36 epidermal growth factor (EGF) repeats and conserved lin/Notch repeats forming the extracellular, ligand-binding domain (Fig. 1). The intracellular portion of Notch1 contains ankyrin-like motifs as well as a polyglutamine (Opa) region and a PEST protein stability sequence. The Floxed *Notch1* allele was generated by introducing LoxP sequences upstream and downstream of the first translated exon encoding the signal peptide (Fig. 1). Recombination between the LoxP sites ablates the first coding exon of the *Notch1* gene and results in a null allele (Radtke et al., 1999) (Fig. 1). Mice homozygous for the Floxed *Notch1* allele show no abnormal phenotype and were used to analyze the function of Notch1 by temporal and spatial gene ablation.

The ablation of Notch1 from the neuroepithelial cells of the developing CNS was restricted by combining homozygous Floxed *Notch1* alleles and a Cre-recombinase transgene expressed under the control of the *engrailed 2* promoter enhancer (*En2-Cre*) (Fig. 1). This approach circumvents the early embryonic lethality observed in Notch1-deficient animals (Conlon et al., 1995; de la Pompa et al., 1997; Swiatek et al., 1994). In addition, we followed the fate of Cre-recombinase-expressing cells and their progeny by including the ROSA26 Cre-reporter allele (*R26R*) in the analysis (Soriano, 1999). The *R26R* allele consists of a Floxed PGK neo cassette containing

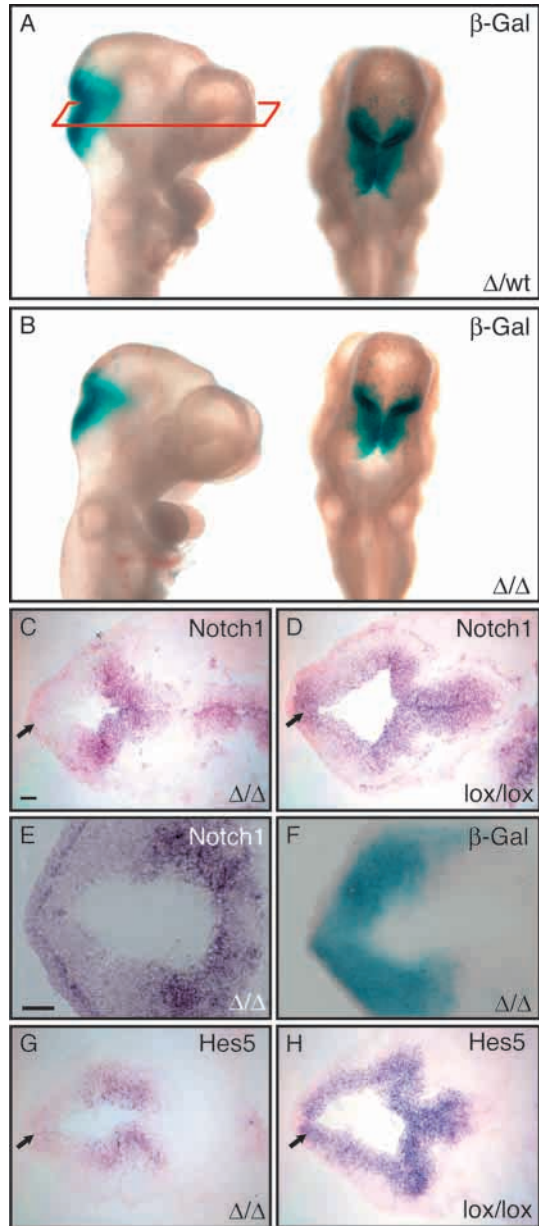


**Fig. 1.** Notch1 and the transgenic constructs. Notch1 is a type-I transmembrane protein with a cleavable signal peptide followed by 36 EGF-like repeats and three Lin/Notch domains (LN). The intracellular domain contains ankyrin-like repeats, a polyglutamine stretch (Opa) and a protein stability PEST sequence. The first coding exon of the *Notch1* gene was flanked by LoxP sequences (triangles) to generate the Floxed *Notch1* allele (1) and the null allele for *Notch1* generated after Cre-induced recombination (2). *EcoRI* (RI) restriction sites flanking the Floxed coding exon are shown. The Cre-recombinase was driven from the *engrailed-2* promoter enhancer (*En2-Cre*), which restricted expression to the neuroepithelium of the midbrain-hindbrain boundary (Zinyk et al., 1998). The ROSA26-R Cre-reporter allele (*R26R*) was used in combination with the *En2-Cre* transgene and Floxed *Notch1* alleles for cell fate tracing. The *R26R* comprises a Floxed PGK promoter driving a neomycin cassette containing four polyadenylation sequences (4XpA) and a  $\beta$ -galactosidase coding region (*lacZ*) with polyadenylation site (bpA) introduced into the ROSA26 locus by homologous recombination (Soriano, 1999). Cre-mediated recombination deletes the PGK neo cassette and results in constitutive expression of  $\beta$ -galactosidase.

four polyadenylation sites and a  $\beta$ -galactosidase gene downstream of the distal LoxP site (Fig. 1). In the absence of Cre-recombinase the expression of  $\beta$ -galactosidase mRNA is blocked by the PGK neo polyadenylation sites. In the presence of Cre-recombinase the LoxP sequences flanking the PGK neo cassette are recombined and  $\beta$ -galactosidase is expressed (Soriano, 1999).

### Cre-mediated recombination is restricted to the neuroepithelial cells of the midbrain-hindbrain region

We have used the *En2-Cre* transgene to drive expression of Cre-recombinase in the embryonic nervous system. Previous experiments revealed that the Tg22 line of *En2-Cre* animals shows recombinase activity within the midbrain-hindbrain region of the neural tube, starting at E9 (Zinyk et al., 1998). We have confirmed this restricted expression by combining the *En2-Cre* and *R26R* alleles. At E10,  $\beta$ -galactosidase-expressing cells can be detected by whole-mount preparations in the midbrain-hindbrain neuroepithelium and progenitor cells within the medial aspect of the cerebellar primordium of control animals (Fig. 2A).



Analysis of triple transgenic mutant animals carrying homozygous Floxed *Notch1* alleles, the *En2-Cre* transgene and the *R26R* reporter showed a similar, restricted appearance of  $\beta$ -galactosidase-expressing cells in the midbrain-hindbrain region at E10 (Fig. 2B). Anatomical changes in midbrain-hindbrain boundary formation were not observed in the mutant embryos and expression of the caudal midbrain-rostral-hindbrain domain marker *Engrailed 1* in the mutants was indistinguishable from that in control embryos (Fig. 2A,B and data not shown). To confirm the ablation of the *Notch1* gene we analyzed the expression of *Notch1* mRNA on cryosections of mutant and control embryos at E10. Sections of embryos were prepared perpendicular to the axis of the neural tube at the level of the midbrain-hindbrain region and cerebellar primordium (plane of section is indicated on the whole-mount preparation shown in Fig. 2A). Sections hybridized with the *Notch1* probe revealed a strong reduction in *Notch1* expression within the cerebellar primordium in the dorsal aspect of the

**Fig. 2.** Determination of the *En2-Cre* expression domain using the *R26R* reporter and restricted ablation of *Notch1* signaling. (A) Heterozygous Floxed *Notch1* ( $\Delta$ /wt) animals carrying an *En2-Cre* and *R26R* allele showed a restricted distribution of Cre-expressing cells (blue staining) and their progeny in the midbrain-hindbrain region of the developing brain at E10. The plane of the sections used in the subsequent analysis is depicted in red. (B) Homozygous Floxed *Notch1* ( $\Delta$ / $\Delta$ ) littermates carrying an *En2-Cre* and *R26R* allele showed a similar distribution of cells expressing  $\beta$ -galactosidase (blue) in the midbrain-hindbrain region of the brain to control embryos at E10. (C) In situ RNA hybridization on cross-sections through the neural tube, dorsal to the left and ventral to the right, of E10 mutant animals ( $\Delta$ / $\Delta$ ) showed normal expression of *Notch1* mRNA in the ventral region of the hindbrain neural tube but loss of expression in the medial cerebellar primordium (arrow). (D) Control Floxed *Notch1* (*lox/lox*) animals without an *En2-Cre* transgene showed normal distribution of *Notch1* transcripts throughout the dorsal and ventral region of the hindbrain neural tube including the cerebellar primordium (arrow). (E) The downregulation of *Notch1* expression in the medial portion of the cerebellar primordium of the mutants coincides with the region of recombination as indicated by expression of  $\beta$ -galactosidase from the *R26R* allele on adjacent sections (F). (G) *Hes5* expression was strongly reduced in the medial cerebellar primordium of mutant embryos ( $\Delta$ / $\Delta$ ) (arrow). By contrast, control embryos (*lox/lox*) showed homogeneous expression of *Hes5* in the cerebellar primordium (H). Scale bars: in C, 100  $\mu$ m for C,D,G,H; in E 100  $\mu$ m for E,F.

neural tube of mutant compared to control animals (arrows in Fig. 2C,D). The reduced expression of *Notch1* in the mutants was restricted to the medial portion of the cerebellar neuroepithelium and overlapped with the expression of  $\beta$ -galactosidase in the triple transgenic mutants (Fig. 2E,F). On the basis of these data, we consider  $\beta$ -galactosidase-expressing cells in homozygous Floxed *Notch1*, *En2-Cre*, *R26R* animals as being *Notch1*-ablated. However, different recombination events even in the same cell may differ in their efficiency (Vooijs et al., 2001).

Notch signaling in invertebrates regulates the expression of a cascade of bHLH transcription factors. The first genes of this cascade in mammals are the *E(Spl)*-related *Hes* genes. Activation of Notch signaling induces the expression of *Hes* genes and particularly *Hes1* and *Hes5* in the nervous system. We have analyzed the expression of *Hes1*, *Hes3* and *Hes5* in the cerebellar primordium by in situ RNA hybridization following ablation of *Notch1*. Although no marked changes in *Hes1* and *Hes3* expression were observed in the mutant compared to control embryos at E10 (data not shown), expression of *Hes5* was drastically reduced in the cerebellar primordium compared with control animals (arrows in Fig. 2G,H). These data are consistent with *Notch1* gene ablation in the cerebellar primordium resulting in downregulation of the *Notch1* signal and the loss of *Hes5* gene expression.

#### Ablation of *Notch1* induces promiscuous Delta-like ligand expression in the cerebellum

On the basis of data from other experimental systems, Notch and its ligands are thought to be reciprocally regulated within the same cell (Kimble and Simpson, 1997). An increased Notch signal results in downregulation of ligand expressed by the receiving cell. By analogy, reduced Notch activity induces ligand expression by the signaling cell. Therefore, we

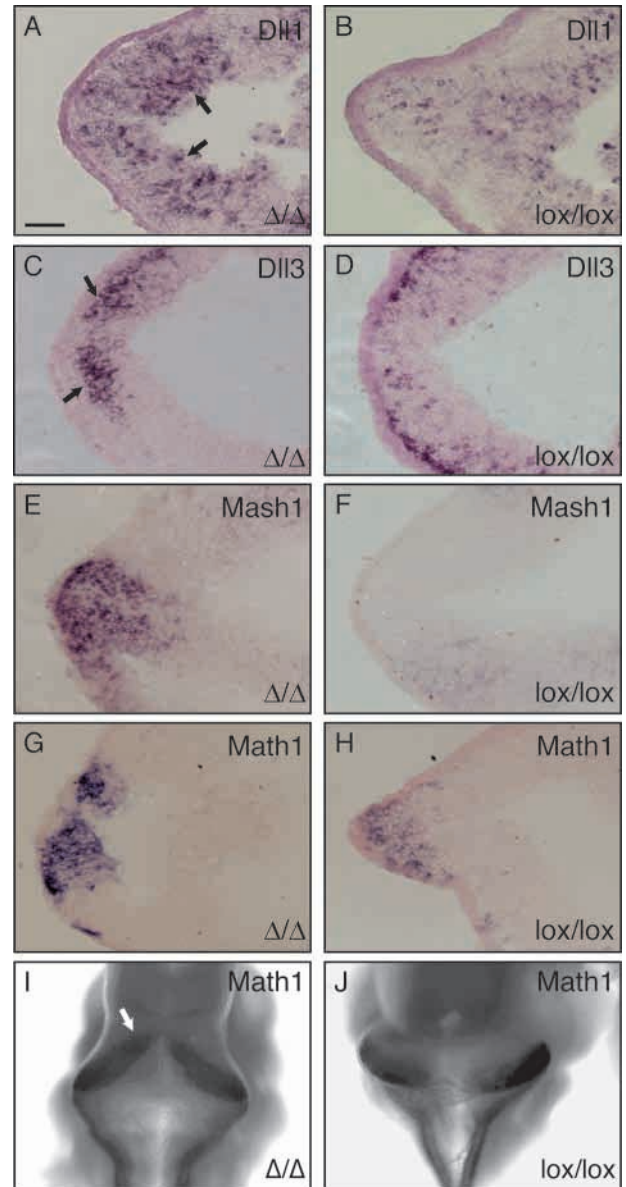
examined the fate of the *Notch1*-ablated cells within the neuroepithelium of the cerebellar primordium by in situ RNA hybridization with probes for the *Notch1* ligands Delta-like1 and 3 (*Dll1* and *Dll3*) and *Jagged1* and 2 on sections of E10 embryos. Mutant embryos showed an upregulation of *Dll1* within the neuroepithelium of the medial portion of the cerebellar primordium (arrow in Fig. 3A,B). In addition, cells expressing high levels of *Dll3* were observed in the dorsal aspect of the medial cerebellum of E10 mutants (arrows in Fig. 3C). However, in contrast to the cells with increased *Dll1* expression that resided within the ventricular zone of the cerebellar primordium, the cells precociously expressing *Dll3* were found towards the basal aspect of the neuroepithelium in a position analogous to differentiating cells (Fig. 3C,D) (Dunwoodie et al., 1997). Ectopic expression, upregulation or abnormal distribution of the ligands *Jagged1* and *Jagged2* were not observed at E10 (data not shown). Taken together, the increased expression of *Notch* ligands observed in the mutants supports the spatially restricted reduction of *Notch* signaling in the neuroepithelium of the cerebellar primordium.

### Ablation of *Notch1* induces early onset of neurogenesis in the cerebellum

To determine the identity of the cells that had initiated precocious differentiation within the cerebellum, we analyzed the expression of the proneural genes *Mash1* and *Math1*. *Mash1* is a mouse orthologue of the *Drosophila Achaete-Scute* genes and has been shown to be expressed in the cerebellar primordium, commencing around E12 (Guillemot and Joyner, 1993). This expression of *Mash1* is likely to be associated with putative Purkinje cell precursors. At E10, we observed precocious expression of *Mash1* mRNA in cells within the dorsal aspect of the medial cerebellum of mutant embryos in positions similar to those of the cells expressing *Dll1* and *Dll3* (Fig. 3E,F). These cells likely represent *Notch1*-ablated neuroepithelial cells that have prematurely differentiated into neuronal precursors. In addition, we also observed an increased expression of *Math1* in E10 mutants (Fig. 3G,H). *Math1* in the cerebellum is the earliest known marker for granule cell precursor cells and is required for their determination (Ben-Arie et al., 1997). It is unclear whether the observed expression represents an expansion of the *Math1* expression domain within the neuroepithelium or whether the granule cell precursor cells have started rostral migration over the cerebellar primordium prematurely. Whole-mount in situ RNA hybridization using probes for *Mash1* (data not shown) and *Math1* (Fig. 3I,J) confirmed the medial and rostral appearance of differentiating cells in the mutant compared to control animals. These data are in agreement with the precocious expression of *Mash1* observed in *Notch1*-deficient mice and validate our conditional gene-ablation approach (de la Pompa et al., 1997).

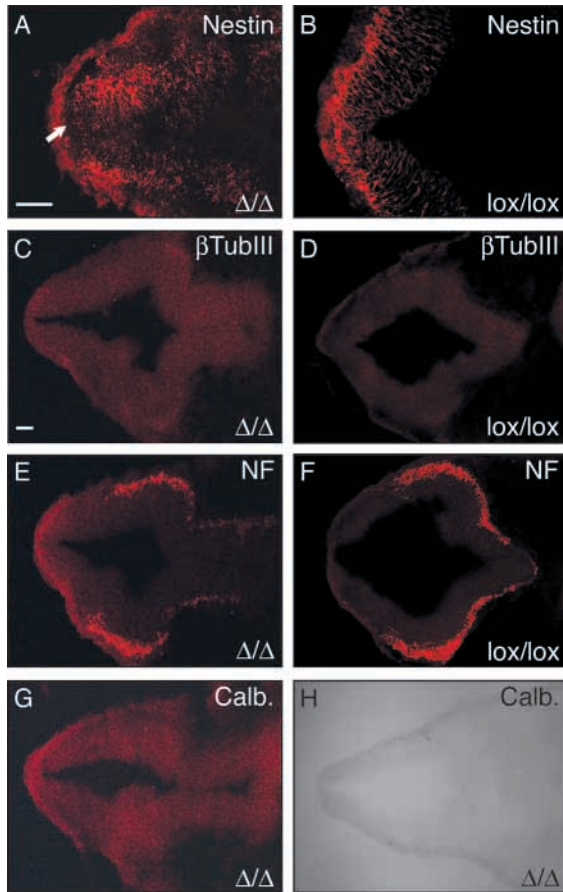
### *Notch1*-deficient cells leave the progenitor cell pool but fail to generate differentiated neurons

To determine the fate of the cells that initiate premature differentiation, we analyzed the expression of neural differentiation markers. Consecutive sections stained for *Mash1* mRNA (Fig. 3E) and nestin (Fig. 4A,B), an intermediate filament protein expressed by multipotent neuroepithelial cells, revealed that the differentiating cells had downregulated nestin (arrow in Fig. 4A) and left the



**Fig. 3.** Expression patterns of *Notch* signaling cascade components at E10. In situ RNA hybridization on cross-sections through the neural tube, dorsal to the left and ventral to the right, of E10 mutant (A,C,E,G) and control (B,D,F,H) embryos show an upregulation of the *Notch* ligands *Dll1* (arrows, A) and *Dll3* (arrows, C) in the medial aspect of the mutant cerebellar primordium. *Mash1* (E,F) and *Math1* (G,H) expression were also upregulated in the mutant cerebellar primordium compared to control littermates. (I,J) Whole-mount in situ RNA hybridization confirmed the ectopic expression of *Math1* in the mutant compared with control embryos (arrow, I). Scale bars: in A, 100  $\mu$ m for A-D; in E, 100  $\mu$ m for E-H.

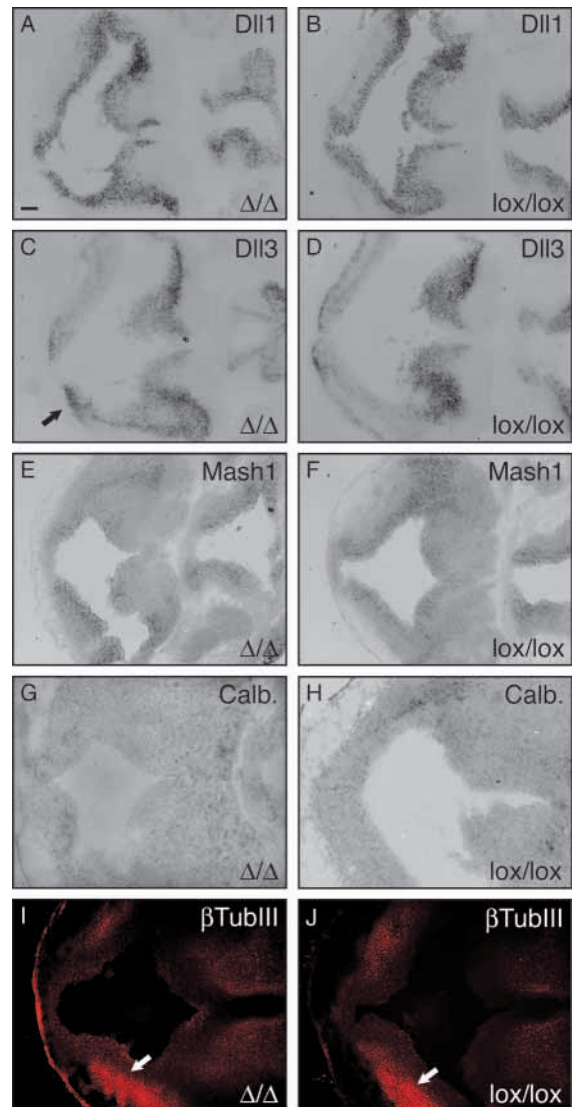
neuroepithelial cell pool at E10. Immunofluorescence with antibodies against pan-neuronal markers such as  $\beta$ -tubulin III (TuJ1) (Fig. 4C,D) and neurofilament 160 kDa (Fig. 4E,F) identified differentiating cells outside the cerebellar primordium of both mutant and control animals, but did not reveal aberrant neurons within the mutant animals. We then analyzed the expression of calbindin D 28k, which is upregulated in postmitotic Purkinje cells between E14 and E16



**Fig. 4.** Differentiation state of cells within the cerebellar primordium. Immunofluorescent analysis of cross-sections through the neural tube at E10, dorsal to the left and ventral to the right, shows a reduction in the expression of the intermediate filament protein nestin in the medial aspect of the mutant cerebellar primordium (arrow, A) compared with control littermates (B). Examination of neuronal differentiation genes, such as those encoding  $\beta$ -tubulinIII (TuJ1) (C,D), and neurofilament 160 (E,F), failed to reveal ectopic formation of differentiated neurons in the cerebellar primordium between E10 mutant and control animals. Calbindin D 28k expression could not be detected in E10 mutant animals at the protein (G) or RNA (H) levels, indicating the absence of precociously differentiated postmitotic Purkinje cells. Bars in A for A,B and in C for C-H are 100  $\mu$ m.

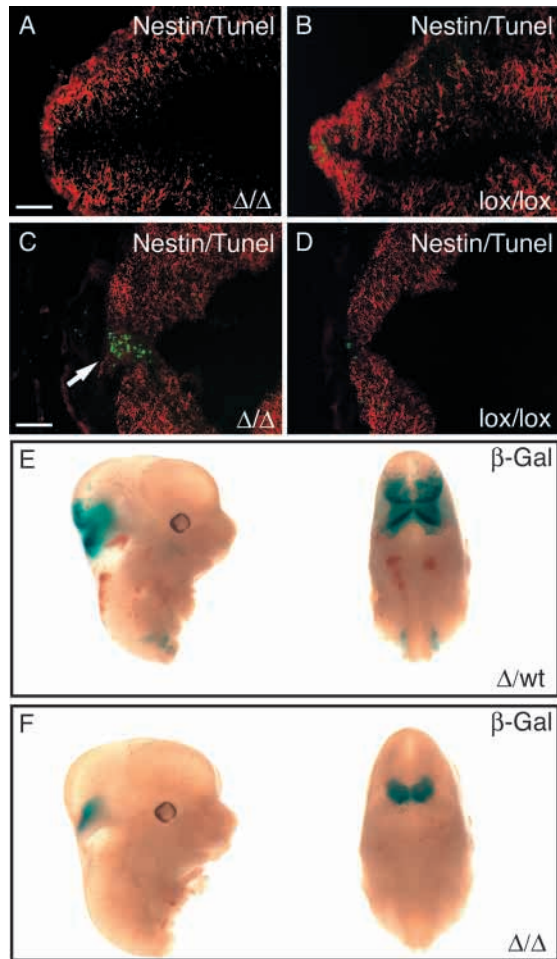
and serves as a cell-type marker in the cerebellum. Calbindin D 28k expression could not be detected at the protein (Fig. 4G) or mRNA (Fig. 4H) levels within the dorsal aspect of the cerebellar primordia of mutant or control embryos (data not shown) at E10. These data suggest that the Notch1-deficient cells had initiated differentiation by E10 but had not progressed to immature neurons or Purkinje cells expressing calbindin D 28k. Owing to the previously shown role of Notch proteins in the regulation of cell fate in invertebrates, we also analyzed the expression of other markers such as GFAP, A2B5 and RC2, which is expressed by radial glial cells during late embryogenesis. We did not find increased expression of these markers in the mutant compared with control animals, suggesting that the neuroepithelial cells had not undergone an apparent neuronal-to-glial lineage fate switch (data not shown).

At E12.5, increased expression of Dll1 was no longer



**Fig. 5.** Expression patterns of Notch signaling cascade components and neuronal markers calbindin D 28k and  $\beta$ -tubulinIII (TuJ1) at E12.5. In situ RNA hybridization on cross-sections through the neural tube, dorsal to the left and ventral to the right, of E12.5 mutant ( $\Delta/\Delta$ ) (A,C,E,G) and control embryos (lox/lox) (B,D,F,H) show that the extended expression of Dll1 (A,B) observed in E10 mutants compared with controls was no longer seen at E12.5 and the induction of Dll3 (arrow, C,D) and *Mash1* (E,F) was detectable but less pronounced than at E10. In situ RNA hybridization did not reveal precocious expression of calbindin D 28k (G,H) in the mutant compared with control animals. Expression of the pan-neuronal marker  $\beta$ -tubulin (TuJ1) (I,J) revealed differentiated cells in the lateral aspects of the cerebellum (arrows) but no precocious neurogenesis in the mutants. Scale bar: 100  $\mu$ m.

evident (Fig. 5A,B). *Mash1* expression within the medial cerebellum was less pronounced, as normal *Mash1* expression had started in the lateral domains of the cerebellar primordium (Fig. 5E,F). The altered expression of Dll3 seen at E10 was still detectable at E12.5, although less prominent than at earlier ages (arrow in Fig. 5C,D). Analysis of calbindin D 28k (Fig. 5G,H) and  $\beta$ -tubulinIII (TuJ1) (Fig. 5I,J) expression at E12.5 did not reveal increased or promiscuous neuronal or Purkinje



**Fig. 6.** Cell survival and fate analysis in the mutant cerebellum. TUNEL analysis (green) for dying cells and nestin (red) for neuroepithelial progenitor cells in the cerebellum failed to show a difference between mutant (A) and control animals (B) at E10. However, at E12.5, mutant animals (C) contained a greatly increased number of TUNEL-positive cells (green; arrow) within the medial aspect of the cerebellar primordium compared to control animals (D). These TUNEL-positive cells were in a position analogous to differentiating neuroblasts and had downregulated expression of nestin. (E,F) The lineage tracing Cre-reporter transgene *R26R* revealed an extensive number of  $\beta$ -galactosidase-expressing cells in the midbrain-hindbrain region of control animals carrying heterozygous Floxed *Notch1*, *En2-Cre* and *R26R* transgenes (E). A substantial reduction in the number of  $\beta$ -galactosidase-expressing cells was observed in the E12.5 mutant embryos (F; homozygous Floxed *Notch1*, *En2-Cre* and *R26R*), compared with control embryos. Scale bars: in A, 100  $\mu$ m for A,B; in C, 50  $\mu$ m for C,D.

cell differentiation. Furthermore, analysis of glial markers also did not identify aberrant glial differentiation at E12.5 (data not shown). Hence, although the *Notch1*-ablated cells had initiated differentiation within the cerebellum at E10 they had not progressed to express markers of differentiated neural cells by E12.5.

### **Notch1 ablation from the neuroepithelium results in increased cell death**

To elucidate the fate of *Notch1*-ablated cells, we examined cell

proliferation and cell death. BrdU was injected into time-mated females and the embryos were excised 3 hours later. The expression of *Mash1* mRNA and the incorporation of BrdU into proliferating cells was analyzed on consecutive sections covering the entire cerebellar primordium of mutant and control embryos at E10 and E12.5. Although the *Mash1*-expressing cells had initiated a premature differentiation program, there were no obvious differences in BrdU incorporation within the medial cerebellar primordium at E10 or E12.5 (data not shown).

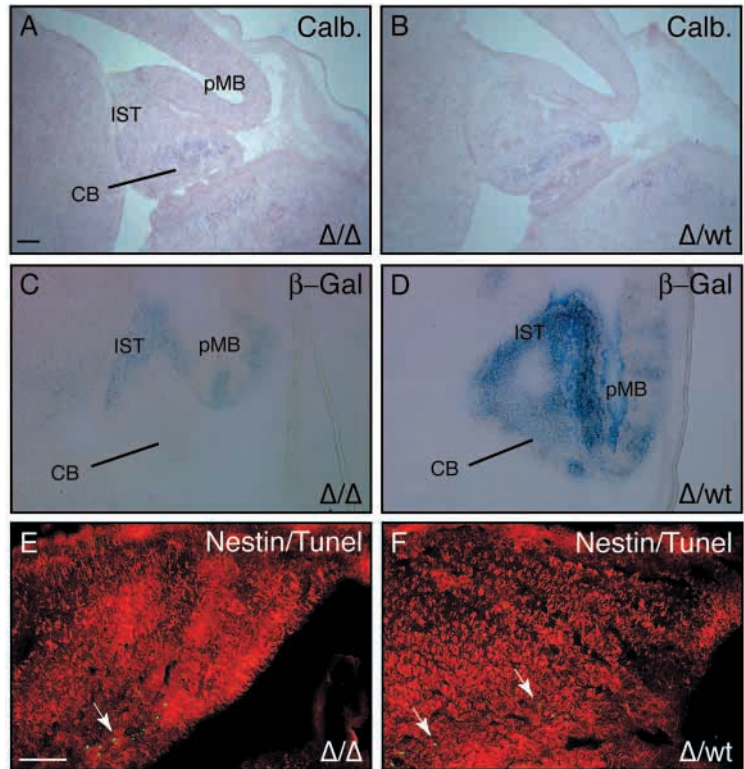
TUNEL analysis for apoptotic cells within the neuroepithelium failed to reveal significant differences between mutant and control embryos at E10 (Fig. 6A,B). This supports the equivalent distribution and numbers of  $\beta$ -galactosidase-expressing cells observed in E10 mutant and control embryos (Fig. 2,B). However, when TUNEL staining was performed on sections of E12.5 embryos, a strong increase in the number of apoptotic cells was observed within the mutant cerebellum compared with control embryos (arrow in Fig. 6C,D). The TUNEL-positive cells were mainly restricted to the medial, presumptive ectopically differentiating cells, with few TUNEL-positive cells within the lateral domains of the cerebellum in all mutants examined. Double immunofluorescence with nestin antibodies and TUNEL staining showed that the apoptotic cells in the medial cerebellar primordium of the mutant lie outside the neuroepithelium in the differentiation zone and express low levels of nestin (Fig. 6C,D). Additional TUNEL analysis of embryos between E10 and E12.5 indicated that increased apoptosis starts in the mutants around E11 and peaks at E12 with approximately sixfold more TUNEL-positive cells per section in the mutants (43 TUNEL-positive cells/section; an average from three embryos) than in controls (seven TUNEL-positive cells/section; an average from three embryos). Furthermore, in contrast to control animals, where apoptosis was similar in medial and lateral regions of the cerebellar primordium (seven and ten TUNEL-positive cells/section, respectively), the increased apoptosis seen in the mutants was restricted to the medial domain of the cerebellum (43 versus four TUNEL-positive cells/section, medial and lateral, respectively).

### **Notch1 ablation from the neuroepithelium results in cell loss**

The increased cell death observed in the mutant embryos at E12.5 suggests that *Notch1*-ablated cells are eliminated before undergoing terminal differentiation. Therefore, we analyzed the distribution of  $\beta$ -galactosidase-expressing cells in E12.5 embryos. In contrast to E10, where mutant and control embryos were indistinguishable (Fig. 2A,B), at E12.5, mutant embryos displayed fewer  $\beta$ -galactosidase-expressing cells in the midbrain-hindbrain region (Fig. 6E,F). These data support the interpretation that the ablation of *Notch1* from neuroepithelial cells induces premature initiation of a differentiation program and subsequent cell death.

Owing to the apparent loss of *Notch1*-deficient cells in the cerebellum of mutant embryos at E12.5, we addressed the consequences of the *Notch1* ablation in E15 embryos. Immature Purkinje cells start to express calbindin D 28k between E14 and E16 (Hatten et al., 1997). Normal differentiation progresses in a lateral-to-medial fashion (Altman and Bayer, 1985). In E15 *Notch1* mutant embryos,

**Fig. 7.** Differentiation and fate of Notch1-ablated cells in the cerebellar primordium at E15. (A,B) In situ RNA hybridization for calbindin D 28k expression on sagittal sections of E15 mutant (A; homozygous Floxed *Notch1* *En2-Cre*) and control embryos (B; heterozygous Floxed *Notch1* *En2-Cre*) show no differences in the expression of the Purkinje cell marker. (C,D) Cell fate analysis of mutant (C) and control animals (B) at E15 using the lineage tracer Cre-reporter transgene *R26R* revealed an absence of  $\beta$ -galactosidase expressing cells from the cerebellum (CB) of mutant compared with control embryos, where most of the cerebellar cells had undergone *R26R* recombination. Some  $\beta$ -galactosidase-expressing cells were detected in the isthmus (IST) and posterior midbrain (pMB) region of the E15 mutant brain. However, the control embryos also showed substantially more  $\beta$ -galactosidase-expressing cells in these brain regions. (E,F) TUNEL analysis (green; arrows) for dying cells and nestin (red) for neuroepithelial progenitor cells on cross-sections of the hemi-cerebellum (midline to the right and dorsal to the top) at E15 showed no significant difference between mutant (E) and control animals (F). Scale bars: in A, 100  $\mu$ m for A-D; in E, 100  $\mu$ m for E,F.



the expression of calbindin D 28k reflected that of control embryos, with a pronounced lateral-to-medial distribution and no obvious increase in the medial aspects of the mutant cerebellum (Fig. 7A,B). Analysis of E12.5 embryos had revealed a highly reduced population of  $\beta$ -galactosidase-expressing cells in the region of the midbrain-hindbrain boundary of mutant compared to control embryos (Fig. 6E,F). Therefore, we addressed whether the remaining  $\beta$ -galactosidase-positive cells at E12.5 are insensitive to the loss of Notch1 by analyzing E15 embryos (Fig. 7C,D). Control embryos (heterozygous Floxed *Notch1*, *En2-Cre*, *R26R*) showed extensive expression of  $\beta$ -galactosidase by most of the cells throughout the medial cerebellum and isthmus region of the brain (Fig. 7D). By contrast,  $\beta$ -galactosidase expression in the mutants was restricted to a few cells within the isthmus and posterior midbrain with no  $\beta$ -galactosidase-expressing cells detectable in the developing cerebellum (Fig. 7C). This suggests that the residual Notch1-ablated cells found in the cerebellum at E12.5 had been eliminated by E15. TUNEL analysis of the cerebellar primordia of E15 embryos showed no difference between mutant animals (16 TUNEL-positive cells/section; an average from 20 sections of three embryos) and control animals (14 TUNEL-positive cells/section; an average from 15 sections of two embryos) (arrows in Fig. 7E,F). Thus, Notch1-deficient cells within the cerebellar primordium are eliminated by apoptosis before E15.

### Purkinje cell number is reduced in the early postnatal cerebellum

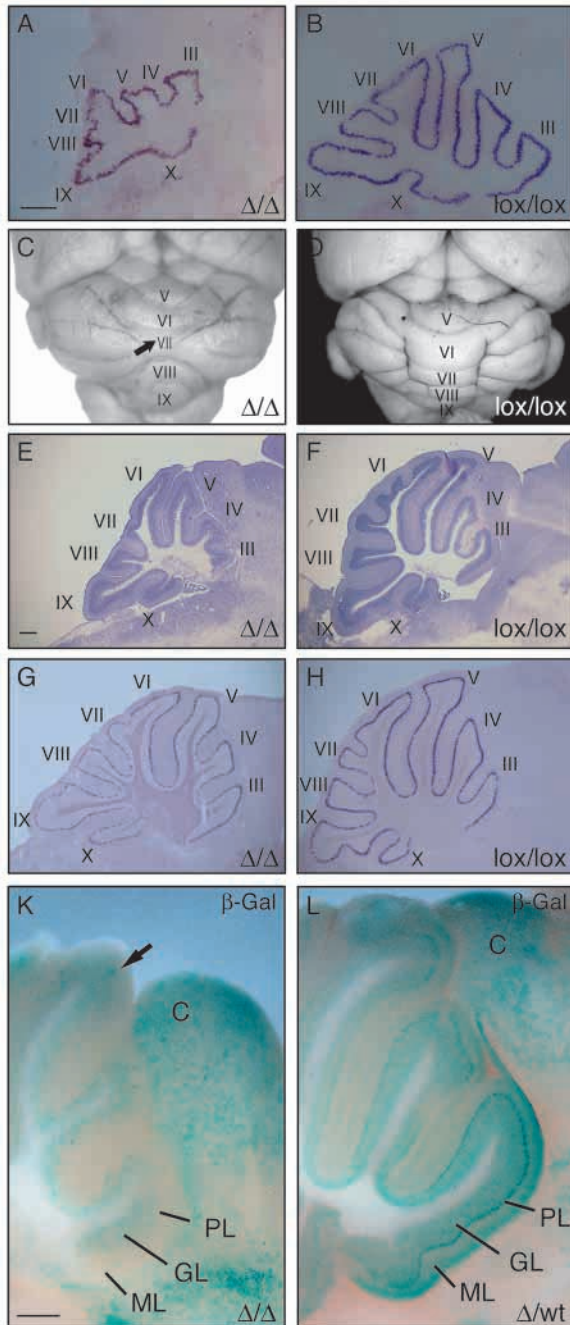
Owing to the apparent loss of Notch1-ablated cells in the embryonic brain, we analyzed cerebellar formation in early postnatal animals. At P4, the cerebellum is undergoing active lobulation in the mouse. The Purkinje cells are arranged into a dense cell layer that becomes extended in the first three postnatal weeks as the number of granule cells in the internal granule cell layer increases. Hematoxylin analysis of brains of P4 mutant animals revealed a marked reduction in the size of the cerebellum (data not shown). Therefore, we performed in situ RNA hybridization with a calbindin D 28k probe to

identify Purkinje cells and to reveal the extent of cerebellar lobulation. The cerebellum of P4 mutant animals showed a severe reduction in the extent of lobulation compared with control littermates (Fig. 8A,B). As in the control animals the Purkinje cells of the mutants were arranged into a dense multicellular layer; however, the total length of the layer was dramatically reduced. Although the Purkinje cell layer is approximately 50% shorter in the mutants, the thickness and hence the density of the cells appeared not to be affected compared with control littermates (Fig. 8A,B). This finding reflects the increased apoptosis observed in the cerebellum of the mutants during embryonic development that is likely to be due, at least in part, to a loss of Purkinje cells.

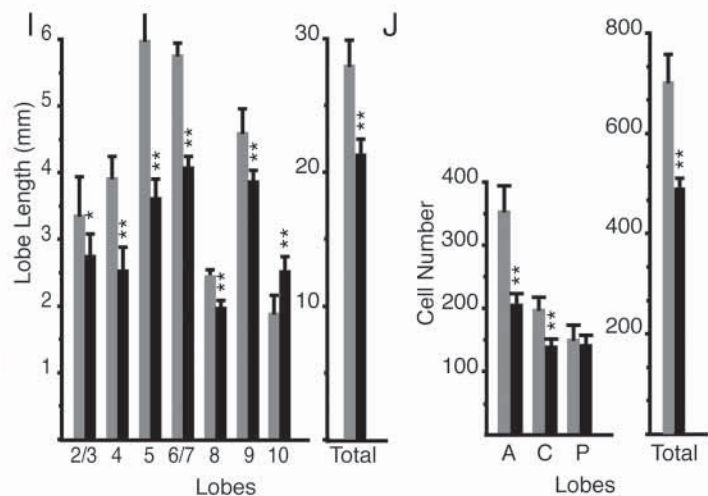
### Conditional ablation of Notch1 results in a reduction in neurons in the adult cerebellum

Mutant animals carrying homozygous Floxed *Notch1* alleles and the *En2-Cre* transgene survived to adulthood and showed no obvious abnormalities compared with control littermates, even beyond one year of age. Macroscopic examination of adult mutant brains revealed a severe reduction in size and foliation of the cerebellar vermis compared with littermates devoid of the *En2-Cre* transgene or heterozygous for the Floxed *Notch1* allele (Fig. 8C,D), consistent with the observations made at P4. Histological examination of sagittal brain sections confirmed that the cerebellar lobes of the mutant animals were severely reduced in length and degree of foliation (Fig. 8E). The major abnormalities were apparent in the anterior (II-V) and central (VI-VIII) lobes. Cerebellar lobes IV, V and VI were reduced in length (approximately 40%) and lobe VI showed no secondary foliation at the distal extremity (compare Fig. 8E,F). Lobe VII was either absent or fused to the posterior aspect of lobe VI. Lobes VIII and IX were





**Fig. 8.** Morphological analysis of the postnatal cerebellum and determination of cell loss. Examination of the cerebellum of P4 animals by in situ RNA hybridization with a calbindin D 28k probe (A,B) showed a significant reduction in the length of the Purkinje cell layer in mutant (A) compared with control (B) animals (reduced by 50%) without obvious changes in cell density. (C-F) Morphological analysis of the cerebellum from adult homozygous Floxed *Notch1* *En2-Cre* mutant (C,E) and control, homozygous Floxed *Notch1*, littermates (D,F) revealed a marked reduction in the size and foliation of the medial cerebellum (arrow, C). Histological examination of midline sagittal sections showed aberrant lobulation of the adult mutant cerebellum (E) with a loss of lobe VII compared with control (F) littermates. (G-I) In situ RNA hybridization with a calbindin D 28K probe (G,H) and quantification of lobe length (I), revealed a significant (\*;  $P < 0.05$  Student's *t*-test) reduction in the length of lobe 2/3 and a highly significant (\*\*;  $P < 0.01$  Student's *t*-test) reduction in the other lobes of the mutant (black bars) with the exception of lobe 10. Owing to the loss of lobe 7, lobe 6 and 7 were measured together, from the posterior end of lobe 5 to the anterior end of lobe 8. Analysis of Purkinje cell number and density (J; data not shown) in adult animals showed a highly significant (\*\*) reduction in cell number within the anterior (A, lobes 2-5) and central (C, lobes 6-8) lobes of the mutant (black bars) as well as over the total cerebellum, but no significant difference in the posterior lobes (P, lobes 9 and 10). (K,L) Lineage tracing using the Cre-reporter transgene *R26R* confirmed that few *Notch1*-deficient, β-galactosidase-expressing Purkinje cells were apparent in the medial cerebellum of adult mutants (homozygous Floxed *Notch1* *En2-Cre* and *R26R*) (arrow, K), compared with control animals carrying heterozygous Floxed *Notch1* *En2-Cre* and *R26R* transgenes (L). C, inferior colliculus; GL, granule cell layer; ML, molecular layer; PL, Purkinje cell layer. Scale bars: in A, 1 mm for A,B; in E, 1 mm for E-H; in K, 1 mm for K,L.



moderately reduced in length but were less affected than anterior structures (Fig. 8G,I). However, measuring the thickness of the molecular and internal granule cell layer failed to reveal significant differences between mutant and control animals. Hence, the overall structure and organization of the cell layers within these lobes were not affected (Fig. 8E).

The phenotypes ranged from animals with reduced lobe length and number to some rare cases where the vermal region of the cerebellum was virtually absent (data not shown). This spatially restricted phenotype mirrors the *En2-Cre* activation pattern, which has been shown to be expressed from E9 onwards in a population of progenitors that give rise to the vermis of the cerebellum (Zinyk et al., 1998). Lineage tracing experiments with the *En2-Cre* transgenic animals (Tg22) and

a Cre-reporter allele revealed recombination activity in all cells of the medial cerebellar vermis, with little activity detected in the hemispheres (Zinyk et al., 1998). This expression pattern does not reflect the entire expression domain of the endogenous *engrailed-2* and might be due to integration effects on the transgene or a lack of specific elements in the transgene promoter. However, it enabled us to focus on a subpopulation of cells within the developing cerebellum.

#### **Notch1 ablation leads to a reduced number of Purkinje cells in the adult cerebellum**

Purkinje cells are important regulators of cerebellar lobulation and cell number. Thus, we determined the number and density of Purkinje cells in the vermis of mutant (Fig. 8G) and control

(Fig. 8H) mice using *in situ* RNA hybridization with a calbindin D 28k probe. We counted calbindin D 28k-positive cell bodies per lobe and length of the Purkinje cell layer on ten consecutive 20  $\mu$ m midline sagittal sections. Purkinje cell density in mutants ( $22.94 \pm 0.94$  cells/mm) was slightly, but significantly ( $P < 0.05$ ; Student's t-test) lower than in control animals ( $25.08 \pm 0.75$  cells/mm). Furthermore, the length of the individual lobes (Fig. 8I) and the number of Purkinje cells per lobe (Fig. 8J) were strongly reduced in the mutants, which explains the reduced size and lobulation of the cerebellum (Fig. 8E). The granule cell layer of the mutants showed normal thickness and density of cells compared with control animals (Fig. 8E,F). Owing to the overall smaller size of the medial cerebellum, this suggests that the number of granule cells is also reduced. However, this reduction may be secondary to the loss of Purkinje cells, which regulate granule cell precursor proliferation by secretion of the mitogenic factor Sonic hedgehog (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999).

### Notch1-ablated cells are absent from the adult cerebellum of mutant mice

The increased cell death observed in the mutant cerebellar primordium up to E12.5 and the reduced size of the medial cerebellum at postnatal stages support the hypothesis that cells from which Notch1 expression had been ablated were eliminated by apoptosis. This interpretation was corroborated by the obvious reduction in the number of  $\beta$ -galactosidase-expressing cells in the hindbrain region of mutant E12.5 and E15 embryos carrying the *R26R* allele (Fig. 6E,F and Fig. 7C,D). To address whether Notch1-deficient cells were present in the adult cerebellum, we analyzed the expression of  $\beta$ -galactosidase in the cerebellum of the mutants carrying the *R26R* allele compared with control animals. As predicted, Purkinje cells within the medial cerebellum of control animals carrying the *En2-Cre* and *R26R* transgenes expressed  $\beta$ -galactosidase (Fig. 8L). By contrast, only occasional  $\beta$ -galactosidase-expressing cells were detected in the cerebellum of adult homozygous Floxed *Notch1* animals carrying the *En2-Cre* and *R26R* transgenes (Fig. 8K). These cells may represent either Notch1-ablated cells that escaped apoptosis during embryonic development, or incomplete Cre-recombination of the two Floxed *Notch1* alleles. Interestingly, the prevalence of  $\beta$ -galactosidase-positive, putative granule cells was also reduced in the cerebellum of mutants compared with control animals (Fig. 8K,L). Thus, the dying cells observed in the mutant embryonic primordium may not only be immature Purkinje cells but also putative granule cell precursors.

### Notch1-ablation results in a loss of cerebellar glial cells

Notch signaling in the developing CNS may also regulate neuronal versus glial cell fate choices of putative stem cells (Furukawa et al., 2000; Gaiano et al., 2000). Therefore, we addressed whether ablation of Notch1 from the cerebellar neuroepithelium results in changes in glia formation. Initially, we analyzed GFAP-expressing cells in the cerebellum of adult mutant and heterozygous animals. Anti-GFAP antibodies labeled the radial processes of Bergmann glia in the molecular layer as well as astroglial cells in the internal granule cell layer (Fig. 9A,B). No obvious differences were observed in GFAP

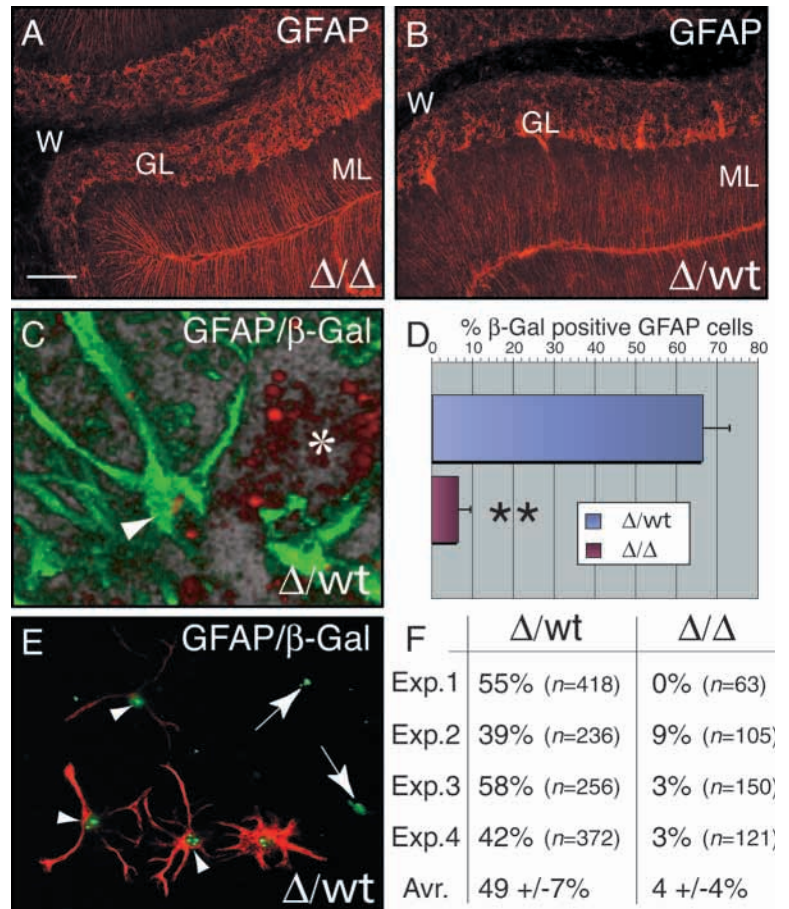
expression or glial cell distribution in the cerebellum of mutant compared to control animals. Therefore, we assessed whether the *En2-Cre* transgene is expressed in cells that generate the glia of the cerebellum. We immunostained midline sagittal cerebellar sections from adult control animals carrying *En2-Cre* and *R26R* alleles and one wild-type *Notch1* gene with antibodies against GFAP and  $\beta$ -galactosidase. Anti-GFAP antibodies labeled Bergmann glia in the cerebellum with their processes in the molecular layer and cell bodies intermingled with Purkinje cells within the Purkinje cell layer (arrowhead in Fig. 9C). Immunostaining for the  $\beta$ -galactosidase enzyme showed a distinct, punctate pattern within Purkinje cells (asterisk in Fig. 9C) similar to the distribution of the Xgal precipitate seen in the cerebellum by detection of  $\beta$ -galactosidase activity. A large proportion of the GFAP-positive Bergmann glia also showed  $\beta$ -galactosidase immunoreactivity when analyzed by confocal microscopy (arrowhead in Fig. 9C). We quantified the percentage of GFAP  $\beta$ -galactosidase double-positive cells on midline sagittal sections from adult triple mutant and control animals. In control animals with one wild-type *Notch1* allele,  $66 \pm 6\%$  of the GFAP-positive cells had undergone recombination compared with only  $7 \pm 3\%$  of the GFAP-positive cells in the mutants, which indicates a highly significant reduction in recombined GFAP-positive cells in the mutants ( $P < 0.01$ ; Student's t-test; Fig. 9D).

To support the quantification of the GFAP  $\beta$ -galactosidase double-positive cells on cerebellar sections, we isolated cells from the cerebellar vermis of early postnatal control (heterozygous Floxed *Notch1*, *En2-Cre*, *R26R*) and mutant (homozygous Floxed *Notch1*, *En2-Cre*, *R26R*) animals and assessed GFAP and  $\beta$ -galactosidase expression *in vitro*. In agreement with the *in vivo* data,  $49 \pm 9\%$  of the GFAP-positive cells from control animals also expressed  $\beta$ -galactosidase (arrowheads in Fig. 9E) but only  $4 \pm 4\%$  of the GFAP-positive cells expressed  $\beta$ -galactosidase in cultures from mutant littermates (Fig. 9F). These data confirm the substantial and highly significant ( $P < 0.01$ ; Student's t-test) reduction in the percentage of glial cells derived from putative Cre-expressing progenitors in the mutant compared with control animals (Fig. 9F). In addition, we quantified the proportion of all of the cells isolated from the control and mutant animals that had undergone recombination. Although  $74 \pm 18\%$  of cells isolated from control animals expressed  $\beta$ -galactosidase, only  $2 \pm 1\%$  of the cells from the mutant animals had undergone recombination. These data confirm the reduction in  $\beta$ -galactosidase-expressing cells observed on sections of the cerebellum from mutant compared with control animals.

## DISCUSSION

Using a conditional loss-of-function approach we have shown that Notch1 plays a central role in the mouse neuroepithelium to maintain cerebellar neural precursor cells in an undifferentiated state and to regulate the onset of their differentiation. On the basis of the expression pattern of Notch1 in the neuroepithelial cells throughout the neural tube, it is likely that it also plays a similar role in other regions of the developing mammalian CNS. Downregulation of Notch1 appears to be sufficient to induce neurogenesis in the developing cerebellar neuroepithelium but does not allow the

**Fig. 9.** Effects of Notch1 ablation on glial cell formation. (A,B) Immunofluorescent analysis of GFAP expression in the vermis of adult mutant (A) and control (B) animals revealed no obvious differences in the glial cell population or density of Bergmann glia fibers, suggesting that although the size of the vermis is reduced, the proportion and distribution of glial cells is not substantially affected in the mutants. (C) Confocal analysis of midline sagittal sections of adult control (heterozygous Floxed *Notch1*, *En2-Cre*, *R26R*) cerebellum immunostained with anti-GFAP (green) and anti- $\beta$ -galactosidase (red) antibodies revealed co-expression in a population of Bergmann glial cells (arrowhead). The soma of a Purkinje cell expressing  $\beta$ -galactosidase but negative for GFAP is also indicated (asterisk). (D) Quantification of GFAP  $\beta$ -galactosidase double-positive cells on midline sagittal sections through the cerebellar vermis showed a highly significant (\*\* $P < 0.01$ ; Student's t-test) reduction in recombined glial cells in the mutant ( $7 \pm 3\%$ ; homozygous Floxed *Notch1*, *En2-Cre*, *R26R* ( $\Delta/\Delta$ ) compared with the control animals ( $66 \pm 6\%$ ; heterozygous Floxed *Notch1*, *En2-Cre*, *R26R* ( $\Delta/wt$ )). (E) Isolation of glial cells from the vermis region of the cerebellum of P4 control animals and immunostaining with anti-GFAP (red) and anti- $\beta$ -galactosidase (green) antibodies supported the presence of glial cells derived from Cre-recombinase-expressing progenitor cells (arrowheads).  $\beta$ -Galactosidase-expressing GFAP-negative, putative neurons are indicated (arrows in E). (F) Quantification of four individual experiments to identify GFAP  $\beta$ -galactosidase-expressing cells in control ( $\Delta/wt$ ) and mutant ( $\Delta/\Delta$ ) animals. The percentage of GFAP  $\beta$ -galactosidase double-positive cells is shown, as is the total number of GFAP-positive cells in each culture ( $n$ ) and the average of all four experiments (Avr.). Note that the total number of cells in the cultures from mutants was lower than that from control animals due to the 50% reduction in size of the mutant vermis at P4. GL, granule cell layer; ML, molecular layer; W, white matter. Scale bar in A, 0.5 mm for A,B.



progression to immature neurons. Instead, the affected cells are eliminated by apoptotic cell death, which leads to a reduction in the number of cerebellar neurons later in development.

Ablation of Notch1 from cerebellar neuroepithelial cells results in increased Dll1 and Dll3 expression and neural progenitor cell differentiation, comparable to the effects of loss of Notch function in the *Drosophila* sensory organ. On the basis of the patchy distribution of Dll1 and the low expression levels of other Notch ligands in the normal cerebellar primordium at E10 (Fig. 3B, data not shown), it is plausible that lateral signaling between cerebellar neuroepithelial cells may involve Dll1 activation of Notch1. Precocious Dll3 expression is also induced in the mutant cells between E10 and E12.5, similar to the normal upregulation of Dll3 by differentiating cells later in development. It remains to be clarified whether progenitor cells switch Notch ligand expression from Dll1 to Dll3 as differentiation progresses or if these ligands mark precursors of independent cell lineages in the cerebellum.

The precocious neurogenesis in conditional Notch1 mutants leads to early upregulation of the proneural genes *Mash1* and *Math1*, but does not affect the spatially restricted expression pattern of these genes normally seen later in development (Ben-Arie et al., 1997; Guillemot and Joyner, 1993). Mechanistically, these findings can be interpreted to indicate that neuroepithelial cells of the cerebellum are patterned before the ablation of Notch1, or that Purkinje cell and granule cell

determination factors are acting on the mutant neuroepithelial cells at E10. The identities of molecules that may regulate neuroepithelial cell fate in the cerebellum in vivo are not known. However, targeted gene ablation studies implicate *Math1* as a potential granule cell determination factor in vivo (Ben-Arie et al., 1997). Furthermore, in vitro experiments suggest that bone morphogenetic proteins (BMPs) derived from the roof plate may induce *Math1* expression and granule cell fate in the cerebellum (Alder et al., 1999). Hence, a coordinated action of Notch1 and BMP signaling may regulate cerebellar neuroepithelial cell differentiation and granule cell fate determination.

Using TUNEL and immunostaining in combination with the *R26R* allele, we have shown that Notch1-ablated cells undergo apoptosis before differentiating into immature neurons. This may be due to the aberrant, premature differentiation of Notch1-deficient neuroepithelial cells. Alternatively, the apoptotic cell death of putative neuroblasts may also be caused by a continuous requirement for Notch1. Indeed, recent evidence suggests that Notch signaling may be required by postmitotic cortical neurons to regulate dendrite formation (Berezovska et al., 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999). However, as the Notch1-deficient cells fail to generate  $\beta$ -tubulinIII or neurofilament 160-positive neurons in our animals, aberrant neurite formation cannot explain the observed cell death. It appears more likely that a

lack of crucial trophic support for the precociously differentiating cells leads to their elimination.

The loss of neurons in the medial aspect of the cerebellar vermis reflects the expression pattern of the *En2-Cre* transgene (Tg22) as determined by recombination-mediated lineage tracing (Zinyk et al., 1998). This restricted pattern of the *En2-Cre* transgene expression circumvents the early embryonic lethality of the Notch1-deficient mice and, by utilizing the *R26R* reporter allele, allowed us to trace the Notch1-ablated cells in the mutant cerebellum in the context of nonablated cells. Although the vermis of the mutant animals was strongly reduced in size, the remaining cells were organized into layers with apparently normal cytoarchitecture. In addition, the cerebellar hemispheres formed normally. This restricted phenotype was reflected in the lack of obvious behavioral abnormalities in the mutants. The origin of the remaining, non-Cre-recombined cells within the cerebellar vermis of the mutants is unknown.

It has been proposed that the developing CNS contains a population of multipotent progenitor cells that give rise to neurons during early development and then switch to a gliogenic fate later (Qian et al., 2000). During late embryonic and early postnatal development of the cerebellum, glial progenitors proliferate and differentiate into the astroglial cells of the cerebellum, including the Bergmann glia (Altman and Bayer, 1997). We show by fate tracing experiments with the *R26R* reporter allele that the *En2-Cre* transgene is expressed by cerebellar glia or their precursors. Our results indicate that, although the distribution and density of glial cells in the mutant cerebellum was not obviously affected, Notch1-deficient neuroepithelial cells may be unable to generate mature glial cells. The apparent reduction in Notch1-deficient glia in the cerebellum of our mutant animals reflects the dramatic underrepresentation of Notch1-deficient cells in the cerebellum of mutant animals. Our loss-of-function data is also supported by recent gain-of-function experiments showing that increased Notch activity can suppress neurogenesis in the mouse embryonic cortex and both the rat and Zebrafish retina, and promote glial formation (Furukawa et al., 2000; Gaiano et al., 2000; Scheer et al., 2001). Thus, ablation of Notch1 from the cerebellar neuroepithelium may restrict multipotent progenitors to a non-glial-cell fate. Indeed, we have shown that Notch1-ablated cells in the cerebellar primordium commence aberrant neuronal differentiation and are subsequently removed by apoptosis. As a consequence, the population of gliogenic cells in the cerebellum of our mutants may also be diminished. However, loss of Notch1-deficient glial cells in the mutants may be the result of a specific block of differentiation or an indirect effect of neuronal loss. Hence, Notch1 signaling is required, directly or indirectly, for the generation of mature glial cells in the cerebellum.

Overexpression of Notch signaling components in isolated neural crest cells indicates that regulation of Notch function is important for the formation of the peripheral nervous system (PNS). Notch1 activation suppresses neurogenesis and can induce gliogenesis in the PNS (Morrison et al., 2000; Wakamatsu et al., 2000), whereas overexpression of the antagonistic protein Numb results in apoptosis (Wakamatsu et al., 2000). Interestingly, the Numb-induced apoptosis in avian neural crest cells could be rescued by neurotrophins (Wakamatsu et al., 2000). By analogy, the apoptotic death of precociously differentiating Notch1-deficient cells in our

mutants may also be due to a lack of trophic support in the early cerebellar primordium.

In conclusion, we show that Notch1 regulates the differentiation of neuroepithelial cells in the cerebellum and that precociously differentiating Notch1-deficient cells are eliminated by apoptosis. In addition, Notch1-deficient progenitor cells fail to generate differentiated glial cells in vivo. Our findings have wide-ranging implications for Notch functions in other regions of the developing CNS and suggest that the mechanisms regulating the differentiation of putative stem cells in the adult CNS may also be under the control of Notch signaling pathways.

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