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Notch signaling regulates neuroepithelial stem cell maintenance and neuroblast formation in *Drosophila* optic lobe development

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ABSTRACT

Notch signaling mediates multiple developmental decisions in *Drosophila*. In this study, we have examined the role of Notch signaling in *Drosophila* larval optic lobe development. Loss of function in Notch or its ligand Delta leads to loss of the lamina and a smaller medulla. The neuroepithelial cells in the optic lobe in *Notch* or *Delta* mutant brains do not expand but instead differentiate prematurely into medulla neuroblasts, which lead to premature neurogenesis in the medulla. Clonal analyses of loss-of-function alleles for the pathway components, including *N*, *Dl*, *Su(H)*, and *E(spl)-C*, indicate that the Delta/Notch/Su(H) pathway is required for both maintaining the neuroepithelial stem cells and inhibiting medulla neuroblast formation while *E(spl)-C* is only required for some aspects of the inhibition of medulla neuroblast formation. Conversely, Notch pathway overactivation promotes neuroepithelial cell expansion while suppressing medulla neuroblast formation and neurogenesis; *numb* loss of function mimics Notch overactivation, suggesting that Numb may inhibit Notch signaling activity in the optic lobe neuroepithelial cells. Thus, our results show that Notch signaling plays a dual role in optic lobe development, by maintaining the neuroepithelial stem cells and promoting their expansion while inhibiting their differentiation into medulla neuroblasts. These roles of Notch signaling are strikingly similar to those of the JAK/STAT pathway in optic lobe development, raising the possibility that these pathways may collaborate to control neuroepithelial stem cell maintenance and expansion, and their differentiation into the progenitor cells.

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Introduction

In *Drosophila*, the central nervous system (CNS) develops through two phases, an embryonic and a post-embryonic phase. While the embryonic nervous system has been extensively studied over the past several decades, the post-embryonic development of the CNS has only recently become a focus of investigation. Certain aspects of the *Drosophila* larval brain make it an attractive model for dissecting the regulatory mechanisms underlying neural stem cell proliferation and maintenance, cell fate determination, cell differentiation, and neuronal diversity in brain development (Wodarz and Näthke, 2007; Doe, 2008; Knoblich, 2008). In the optic lobe region of the larval brain, neuroepithelial cells first expand the stem cell population by symmetric cell division and then differentiate into neuroblasts that undergo asymmetric division to generate medulla neurons (Hofbauer and Campos-Ortega, 1990; Egger et al., 2007). These temporal events resemble those in mouse brain development as the neuroepithelial cells in the ventricular zone also undergo symmetric division to expand the neuroepithelial stem cells and then switch to neurogenic asymmetric cell division to generate neurons of the cerebral cortex (Götz and Huttner, 2005; Huttner and Kosodo, 2005; Kriegstein et al., 2006; Merkle and Alvarez-Buylla, 2006). Thus, the *Drosophila* larval brain provides a simpler and genetically more tractable system to study the regulatory mechanisms underlying brain development, which may be conserved in the vertebrate brains.

The optic lobe originates from the invagination of a small patch of dorsal–lateral ectodermal cells in the head region of late-stage embryo (Hofbauer and Campos-Ortega, 1990; Green et al., 1993). After larval hatching, the neuroepithelial cells start to expand its population, which, by the end of the first instar, becomes separated into two epithelial cell populations, the outer proliferation center (OPC) and the inner proliferation center (IPC) (Fig. 1A); the OPC generates precursor cells for lamina and outer medulla neurons while the IPC generates precursor cells for the lobula complex and inner medulla neurons (Meinertzhagen and Hanson, 1993). Lamina neurogenesis takes place at mid-third instar, on the lateral side of the OPC neuroepithelium when the *hedgehog* signal, delivered by photoreceptor axons from the eye, triggers a final division of lamina precursor cells (LPCs) located on the posterior side of the lamina furrow and the post-mitotic progeny of this division, under the influence of Spitz, another signal delivered by photoreceptor axons, differentiate into lamina neurons (Fig. 1B and C; Huang and Kunes, 1996; Huang et al., 1998). Medulla neurogenesis starts at late-second larval instar when the medial cells of the OPC neuroepithelium lose their epithelial characters and differentiate into medulla neuroblasts, which

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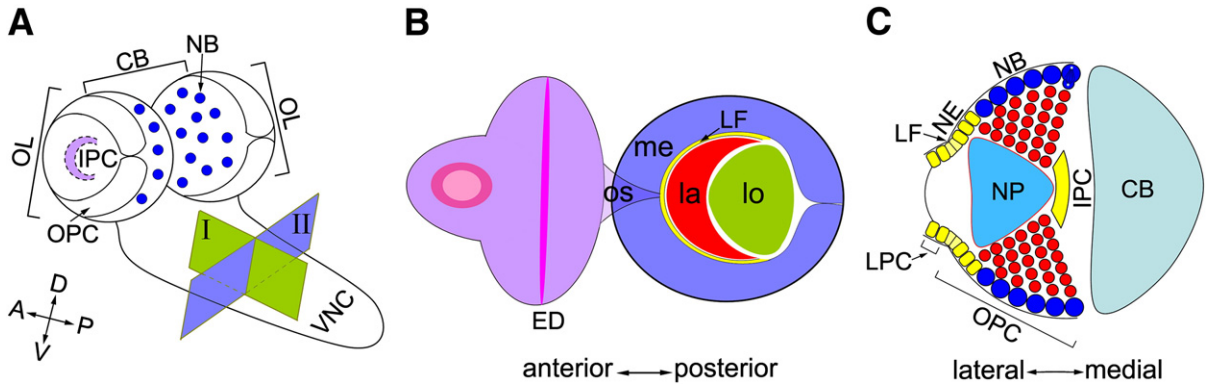


Fig. 1. Schematic diagrams of the larval CNS. A, The larval CNS consists of a pair of brain lobes and the ventral nerve cord (VNC). Each brain lobe is divided into the central brain (CB) and the laterally located optic lobe (OL), which has two proliferation centers, the outer proliferation center (OPC) and the inner proliferation center (IPC). NB: neuroblasts in the central brain. D: dorsal; V: ventral; A: anterior; P: posterior. B, Lateral view of the brain lobe (section plane I in A) showing the three visual processing centers in the optic lobe, the medulla (me), lamina(la) and lobula complex (lo). Along the anterior–posterior axis, the medulla is located anterior to the lamina furrow (LF) and the lamina posterior of the lamina furrow whereas the lobula complex is located posterior of the lamina. The eye imaginal disc (ED) is connected with the brain through the optic stalk (os). C, View of a frontal section of the brain lobe (section plane II in A). Along the lateral–medial axis, the neuroepithalial cells (NE) are located on the lateral side of the OPC, while the medulla neuroblasts (NB) are located on the more medial region of the OPC. The lamina precursor cells (LPC) are located on the posterior side of the lamina furrow. NP: medulla neuropil.

undergo asymmetric cell division generating self-renewing neuroblasts and precursor cells that differentiate into medulla neurons (Fig. 1B and C; Hofbauer and Campos-Ortega, 1990; Meinertzhagen and Hanson, 1993; Nassif et al., 2003; Egger et al., 2007; Hayden et al., 2007). The mechanisms underlying the maintenance and expansion of optic lobe neuroepithelial cells and their differentiation into medulla neuroblasts are poorly understood.

Recently, the JAK/STAT pathway has been implicated in the maintenance of optic lobe neuroepithelial cells and their differentiation into medulla neuroblasts. In loss-of-function JAK/STAT mutant brains, the neuroepithelium in the OPC disintegrates and medulla neuroblasts form prematurely leading to premature neurogenesis in the medulla (Yasugi et al., 2008; W. Wang, Y. Li, L. Zhou, H. Wang, W. Jiang, and H. Luo, unpublished observations), while ectopic activation of JAK signaling suppresses the formation of medulla neuroblasts (Yasugi et al., 2008; W. Wang, Y. Li, L. Zhou, H. Wang, W. Jiang, and H. Luo, unpublished observations). How JAK/STAT signaling may perform this dual role is not clear. In this study, we find that Notch signaling is also required for the maintenance of the OPC neuroepithelium and suppression of medulla neuroblast formation. Loss of function in Notch pathway components causes differentiation of the OPC neuroepithelial cells into medulla neuroblasts and premature neurogenesis; overactivation of Notch signaling leads to the expansion of neuroepithelial cells in the OPC while suppressing medulla neurogenesis. We find that the maintenance of the OPC neuroepithelium and the suppression of medulla neuroblast formation require the Delta/Notch/Su(H) pathway whereas the *E(spl)-C* genes are only required in some aspects of the suppression of medulla neuroblast formation. These phenotypes are strikingly similar to those of JAK/STAT mutants, suggesting that these two pathways may collaborate to maintain neuroepithelial integrity and suppress medulla neuroblast formation.

Materials and methods

Fly stocks

Flies were reared in standard cornmeal food at 25 °C unless otherwise indicated. The following mutations and transgenic fly lines were used. $N^{ts\text{-}1}$ is a temperature-sensitive allele (Bloomington stock 2533), N^{XK11} a strong loss-of-function allele (Struhl et al., 1993), and N^{55E11} a null allele (Couso and Martinez-Arias, 1994). Dl^{6B} is a temperature-sensitive allele (Bloomington stock 5605), Dl^{RF} a strong loss-of-function allele (Bloomington stock 5603) and Dl^{rev10} a null allele (Heitzler and Simpson, 1991). $Su(H)^{\Delta 47}$ is a null allele (Morel and Schweisguth, 2000). $Df(3R)E(spl)^{b32.2}[gro^{+}]$ is a deficiency that deletes all seven members of the *E(spl)-C* and affected an adjacent gene *groucho* (*gro*); however, a transgene carrying wild-type *gro* (gro^{+}) was inserted on this chromosome, thus rescuing the *gro* mutation (Schrons et al., 1992). $numb^{15}$ is a null allele (Berdnik et al., 2002). *E(spl)m8-lacZ* is a lacZ reporter of the *E(spl)m8* gene (Kramatschek and Campos-Ortega, 1994). *UAS-*N^{ACT} encodes the intracellular domain of Notch fused with the transmembrane domain of Delta, which is constitutively active (Doherty et al., 1996). *UAS-Dl* encodes wild-type Delta protein (Bloomington stocks 5612 and 5614). *UAS-*N^{RNAi} encodes a Notch RNAi construct (National Institute of Genetics stock 3936R-3, Japan). Gal4 lines used include *c855a-Gal4* (Hrdlicka et al., 2002) and *c768-Gal4* (Manseau et al., 1997), *inscuteable-Gal4*(*insc-Gal4*) (Betschinger et al., 2006), *elav-Gal4* (Lin and Goodman, 1994), and *hs-Gal4* (Bloomington stock 1799). w^{1118} was used as wild-type control.

Genetic crosses

To assay the effect of loss of *Dl* activity on optic lobe development, Dl^{6B}/*TM6c, Sb, Tb* females were crossed with Dl^{RF}/*TM6c, Sb, Tb* males and the progeny first cultured at 18 °C for about 4 days after egg laying (AEL) to reach the second instar stage and then shifted to 25 °C to inactivate *Dl* activity. Dl^{6B}/Dl^{RF} transheterozygotes were identified as non-Tubby larvae.

For mosaic analyses, N^{XK11}*FRT101*, N^{55E11}*FRT19A*, $Su(H)^{\Delta 47}$*FRT40A*, $numb^{15}$ *FRT40A*, *FRT82B* Dl^{rev10}, or *FRT82B* $Df(3R)E(spl)^{b32.2}[gro^{+}]$ mutant clones were generated using the FRT/FLP method (Xu and Rubin, 1993). N^{55E11} *FL122 FRT19A/FM7a* females were crossed with *FL122 Ub-GFP FRT19A/Y* males, *y* N^{XK11} *FL122 FRT101/FM7, ftz-lacZ* females with *Ub-GFP FRT101/Y* males; $Su(H)^{\Delta 47}$*FRT40A/CyO* or $numb^{15}$ *FRT40A/CyO* females with *y w FL122; Ub-GFP FRT40A/CyO* males; *FRT82B* Dl^{rev10}*/TM3, Sb* females with either *y w hs Flp1/Y; FRT82B Ub-GFP, Minute/TM3, Sb* males or *y w hs Flp1/Y; FRT82B Ub-GFP/TM2* males; *FRT82B* $Df(3R)E(spl)^{b32.2}[gro^{+}]$*/TM6B* females with *y w hs Flp1/Y; FRT82B Ub-GFP, Minute/TM3, Sb* males. Larval progeny from these crosses were subjected to two 1-h heat shocks at 38 °C about 24 and 48 h after larval hatching (ALH) to induce somatic recombination; in some experiments with *numb* mosaic clone induction, heat shock was initiated at about 60-h ALH (early-third instar) to induce clones in which somatic recombination was more likely to occur in medulla neuroblasts. A *Minute* mutation on the right arm of the third chromosome was used to obtain larger mutant clones for Dl^{rev10} and $Df(3R)E(spl)^{b32.2}[gro^{+}]$.

For overexpression and RNAi studies, *UAS-Dl* or *UAS-*N^{RNAi} flies were crossed with *c855a-Gal4*, *c768-Gal4* or *insc-Gal4* flies and the

progenies cultured at 31 °C. Expression of N^{ACT} by any of these drivers caused lethality before hatching; we thus combined *c855a-Gal4*, *c768-Gal4* or *insc-Gal4* with a temperature-sensitive Gal80 repressor construct ($Gal80^{ts}$) to temporally control N^{ACT} expression. *UAS-*N^{ACT} females were crossed with *tub-*$Gal80^{ts}$*/Y; c855a-Gal4/+* males, *tub-*$Gal80^{ts}$*/Y; c768-Gal4/+* males or *tub-*$Gal80^{ts}$*/Y; insc-Gal4/+* males, the progeny from these crosses were first cultured at 18 °C until early-third instar (6 days AEL) and then shifted to 31 °C to inactivate $Gal80^{ts}$ thus allowing N^{ACT} expression. For transient ubiquitous expression, *UAS-*N^{ACT} flies were crossed to *hs-Gal4* flies and the progeny subjected to a 15-min heat shock at 38 °C at early-third instar (60 h ALH) and returned to 25 °C for culture until late-third instar. To induce ectopic expression in clones using the flip-out method (Struhl and Basler, 1993), *UAS-*N^{ACT} females were crossed to *y w hsFlp1/Y; actin<*y^{+}*<Gal4, UAS-GFP* males and first-instar larvae were heat shocked at 38 °C for 30 min and then cultured at 25 °C until late-third instar before dissection.

Immunohistochemistry

Larval CNS was dissected in PBS, fixed with 4.0% paraformaldehyde in PBS/0.1% Triton X-100 at room temperature for 20 min, except for Delta and Notch staining for which CNS specimens were fixed in 2.0% and 4.0% paraformaldehyde, respectively, in PBS without Triton X-100. The samples were blocked in 5% normal goat serum (NGS) in PBS/0.3% Triton X-100 (PTX) and then incubated with primary antibodies at 4 °C overnight. After washing in PTX and blocking with 5% NGS/PTX, the samples were incubated with secondary antibodies at room temperature for 2 h or at 4 °C overnight. The samples were again washed in PTX, mounted in Vectashield mounting medium (Vector), and analyzed using confocal microscopy. The following primary antibodies were used: guinea pig anti-Deadpan (1:1000, gift from J. Skeath), guinea pig anti-Miranda (1:500, gift from C. Doe), rat anti-Miranda (1:500, gift from C. Doe), rabbit anti-PatJ (1:1000, gift from H. Bellen), rat anti-Lethal of Scute (L'sc) (1: 500, gift from A. Carmena), rabbit anti-Asense (1:1,000, gift from Y. Jan), rabbit anti-β-galactosidase (1:1000, Cortex Biochem); rabbit and mouse anti-GFP (1:1000, Molecular Probes); mouse anti-Discs large (Dlg) (4F3, 1:50, Developmental Studies Hybridoma Bank [DSHB]), mouse anti-Notch intracellular domain (C17.9C6, 1:100, DSHB), mouse anti-Delta (C594.9B, 1:100, DSHB), mouse anti-Dachshund (mAbdac2-3, 1:100, DSHB), rat anti-Elav (7E8A10, 1:100, DSHB), mouse anti-Prospero (MR1A, 1:100, DSHB), mouse anti-β-Tubulin (E7, 1:20, DSHB) and mouse anti-Crumbs (Cq4, 1:20, DSHB), rabbit anti-DE-Cadherin (sc-33743, 1:100, Santa Cruz Biotechnology), rabbit anti-aPKC (sc-216, 1:1000, Santa Cruz) and rabbit anti-Su(H) (sc-25761, 1:100, Santa Cruz). Secondary antibodies used were Alexa Fluor-488 goat anti-rabbit (1:200) and Alexa Fluor-488 goat anti-rat (1:200) (Molecular Probes); Cy3-conjugated goat anti-mouse (1:200), Cy3-conjugated goat anti-rat (1:200), Cy5-conjugated goat anti-rat (1:200), and Cy5-conjugated donkey anti-guinea pig (1:200) (Jackson ImmunoResearch Lab).

Confocal images were captured by an Olympus FV500 confocal microscope (60× objective, N.A.1.4) and processed with Imaris (Bitplane) and Adobe Photoshop 7.0 software. Deconvolution was done using AutoDeblur (Bitplane).

Results

Notch signaling is active in the optic lobe neuroepithelial cells

Notch signaling participates in a variety of developmental processes in *Drosophila* (Artavanis-Tsakonas et al., 1999). However, the role of Notch signaling in the larval brain is not well understood. To determine whether Notch signaling plays a role in larval optic lobe development, we first examined in more detail the expression patterns of Notch pathway components in larval brains.

Notch expression was assessed using the mouse monoclonal antibody C17.9C6 developed against the intracellular region of Notch. As has been shown previously, Notch protein is expressed in neuroblasts and their progeny in the ventral nerve cord (VNC) and central brain at late-third instar (Johansen et al., 1989; Kidd et al., 1989; Fehon et al., 1991; Almeida and Bray, 2005). In the optic lobe, Notch is strongly expressed in the neuroepithelial cells in the OPC and IPC (Fig. 2D′); its expression is down-regulated in the medial cells in the OPC that have become medulla neuroblasts (Fig. 2D′, medial to the arrowheads). Interestingly, Notch protein is also expressed in medulla neurons and their axons, as well as in the medulla neuropil (Fig. 2D′; Fig. S1A′). In addition, Notch is strongly expressed in the lamina (Fig. S1B).

Delta expression was assessed using C594.9B, a monoclonal mouse antibody against the extracellular domain of Delta. Delta is expressed in the neuroblasts and their progeny in the VNC and central brain (Kooh et al., 1993; Cornbrooks et al., 2007). Delta is strongly expressed in the neuroepithelial cells in the OPC and IPC (Fig. 2H′); in the OPC, its expression appears stronger in the medial neuroepithelial cells that border the medulla neuroblasts (Fig. 2H′, lateral to the arrowheads). Delta expression is detected at a lower level in medulla neuroblasts but becomes stronger in newly generated medulla neurons, although not detected in mature medulla neurons or their axons (Fig. 2H′). In the lamina, Delta is weakly expressed in the LPCs and anterior lamina cells; its expression becomes stronger as punctuate dots in the more posterior lamina cells in contrast to strong and uniform Notch expression in the lamina (Fig. S1C).

The Suppressor of Hairless protein (Su(H)) mediates Notch signaling in most cases examined. We used a rabbit polyclonal antibody raised against Su(H) to examine its expression in the larval CNS. The protein is ubiquitously expressed in the optic lobe, central brain, and VNC and is localized both in the cytoplasm and in the nucleus (Fig. S1D). Thus, the major Notch signaling components are all expressed in the optic lobe and in particular in the OPC and IPC neuroepithelial cells.

To examine whether Notch signaling is active in the optic lobe, we examined the expression of a *lacZ* reporter for the *E(spl)m8* gene (Kramatschek and Campos-Ortega, 1994). *E(spl)m8* belongs to the Enhancer of Split gene complex (*E(spl)-C*) that are activated in response to Notch signaling activity (Jennings et al., 1994). We find that *E(spl)m8-lacZ* is expressed in the OPC and IPC, and like Delta, the expression is stronger in the medial neuroepithelial cells in the OPC (Fig. 2L′). The reporter is also detected in medulla neuroblasts, newly generated medulla neurons and their axons (Fig. 2L′; Fig. S1E). This reporter profile suggests that the Notch pathway is activated in the optic lobe neuroepithelial cells, medulla neuroblasts, newly generated medulla neurons, and their axons. In contrast, the reporter is not detected in any lamina cells (Fig. S1E). In the central brain and VNC, the reporter is strongly expressed in neuroblasts and their progeny, consistent with a previous report that showed Notch to be active in these regions of the larval CNS (Almeida and Bray, 2005).

During earlier larval stages, both Notch and Delta proteins are expressed in the OPC and IPC neuroepithelia (Fig. 2A′–C′, E′–G′) with Delta being more strongly expressed in the medial neuroepithelial cells (Fig. 2E′–G′). *E(spl)m8-lacZ* is detected in the OPC and IPC neuroepithelia during these larval stages, also with stronger expression in the medial neuroepithelial cells in the OPC (Fig. 2I′–K′). Together, these expression data suggest that the Notch pathway is active during the expansion of neuroepithelial cells in the optic lobe.

Notch signaling is essential for lamina and medulla development in the optic lobe

To address whether Notch signaling is required for optic lobe development, we examined the effects of loss of Notch signaling

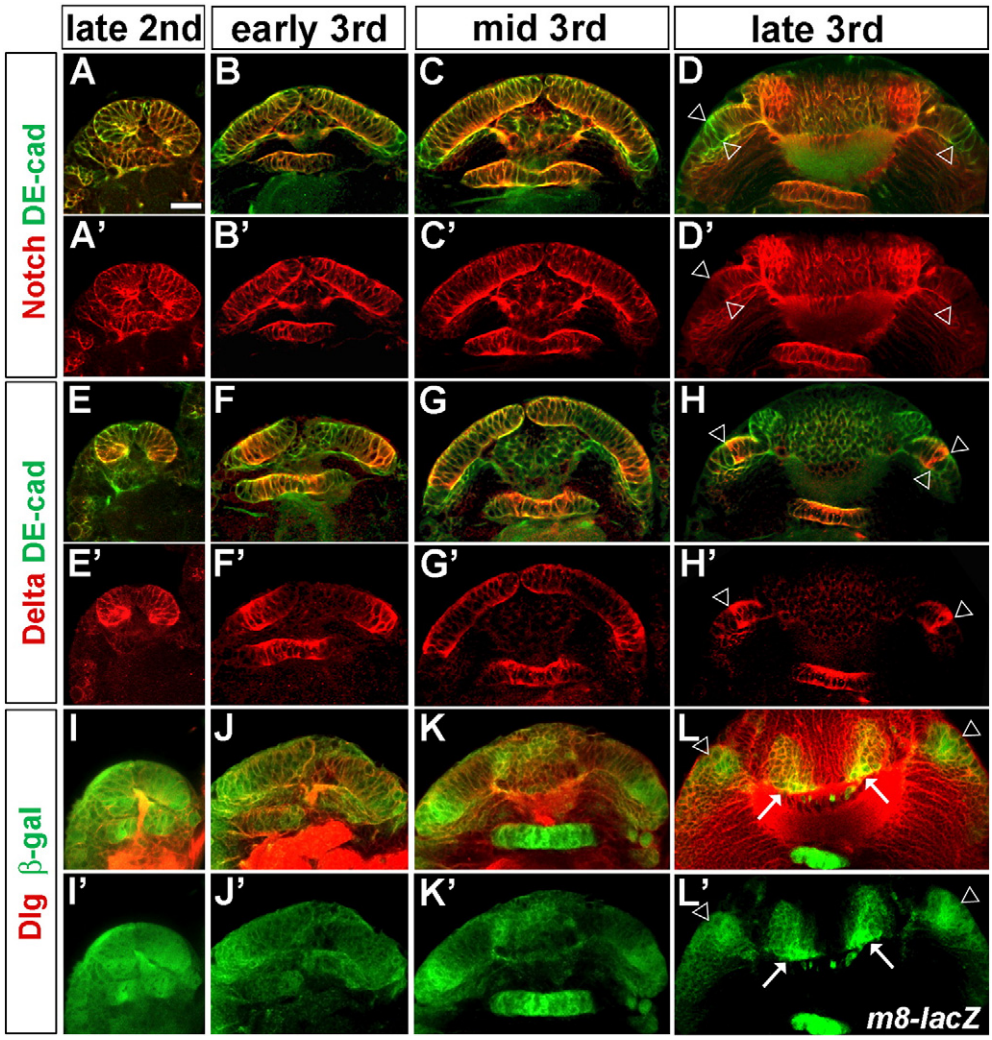


Fig. 2. Notch signaling is active in the optic lobe neuroepithelial cells. A–D′, Notch expression at late-second (A′), early-third (B′), mid-third (C′) and late-third instar (D′). Notch staining is shown in red. E–H′, Delta expression at late-second (E′), early-third (F′), mid-third (G′), and late-third instar (H′). Delta staining is shown in red. I–L′, *E(spl)m8-lacZ* expression at late-second (I′), early-third (J′), mid-third (K′), and late-third instar (L′). LacZ staining is shown in green; the arrows indicate LacZ reporter expression in the IPC neuroblasts. Arrowheads indicate the border between the neuroepithelial cells (NE) and medulla neuroblasts (NB) in the OPC. DE-cadheren is shown in green, Dlg is in red. Lateral is up and medial down. Scale bar: 20 μm.

activity. We used the temperature-sensitive allele $N^{ts\text{-}1}$ to control Notch activity during the larval stages. $N^{ts\text{-}1}$ is fully active at 25 °C but becomes inactivated at non-permissive temperature (31 °C). $N^{ts\text{-}1}$ mutant animals were first cultured at 25 °C for 1 day to allow completion of embryogenesis and then shifted to 31 °C to inactivate Notch activity; late-third instar N^{ts} mutant brains were analyzed. We used an anti-Dachshund (Dac) antibody to visualize the lamina and an anti-Elav antibody to reveal differentiated neurons in the lamina, medulla, and lobula complex. Inactivation of Notch under these culture conditions strongly affected optic lobe development. The lamina was absent (Fig. 3C′, compare with wild type in Fig. 3A′, 100%, $n=32$) while the medulla is smaller than wild type (Fig. 3D, compare with wild type in Fig. 3B, 100%, $n=37$). The central brain and VNC appeared normal (data not shown).

Because $N^{ts\text{-}1}$ mutant animals also had very small eye imaginal discs (not shown), which might affect lamina development (Selleck and Steller, 1991), we used the UAS/GAL4 system (Brand and Perrimon, 1993) to express a *UAS-*N^{RNAi} construct more specifically in the optic lobe using two different Gal4 drivers. *c855a-Gal4* is active in the OPC neuroepithelial cells and lamina precursor cells (Egger et al., 2007; Fig. S2), while *c768-Gal4* is mainly active in the OPC neuroepithelial cells, as well as in some cells in the central brain and VNC (Manseau et al., 1997; Fig. S2); both Gal4 lines start to be expressed in the optic lobe neuroepithelial cells at the end of the first larval instar (Fig. S2). RNAi knockdown of Notch expression in the optic lobe using these Gal4 drivers led to a complete loss of lamina (Fig. 3E′, 100%, $n=41$) and a smaller medulla cortex (Fig. 3F, compare with wild type in Fig. 3B; 100%, $n=47$), similar to the defects in $N^{ts\text{-}1}$ mutant brains. The eye imaginal disc appeared normal and the central brain and VNC were not affected in these N^{RNAi} knockdown experiments (data not shown). Thus, we conclude that the defects in lamina and medulla development resulted from the optic lobe rather than from the eye. To address whether Notch is specifically required in the optic lobe neuroepithelial cells, Notch expression was knocked down by RNAi using the *insc-Gal4* or *elav-Gal4* driver, both of which are active in the neuroblasts in the optic lobe, central brain and VNC but not in the OPC neuroepithelial cells (Betschinger et al., 2006; Hayden et al., 2007). Notch RNAi driven by these Gal4 drivers did not cause any defects in the development of the optic lobe, central brain or VNC (Fig. 9D, and data not shown).

Since Delta is expressed in the optic lobe neuroepithelial cells in a pattern that overlaps with Notch expression, it may serve as a ligand to activate Notch in the neuroepithelial cells. To assay the effect of loss of *Dl* function, we employed the temperature-sensitive allele Dl^{6B} in combination with the strong loss-of-function allele Dl^{RF}. Dl^{6B}/Dl^{RF} transheterozygous animals were first cultured at 18 °C until early-

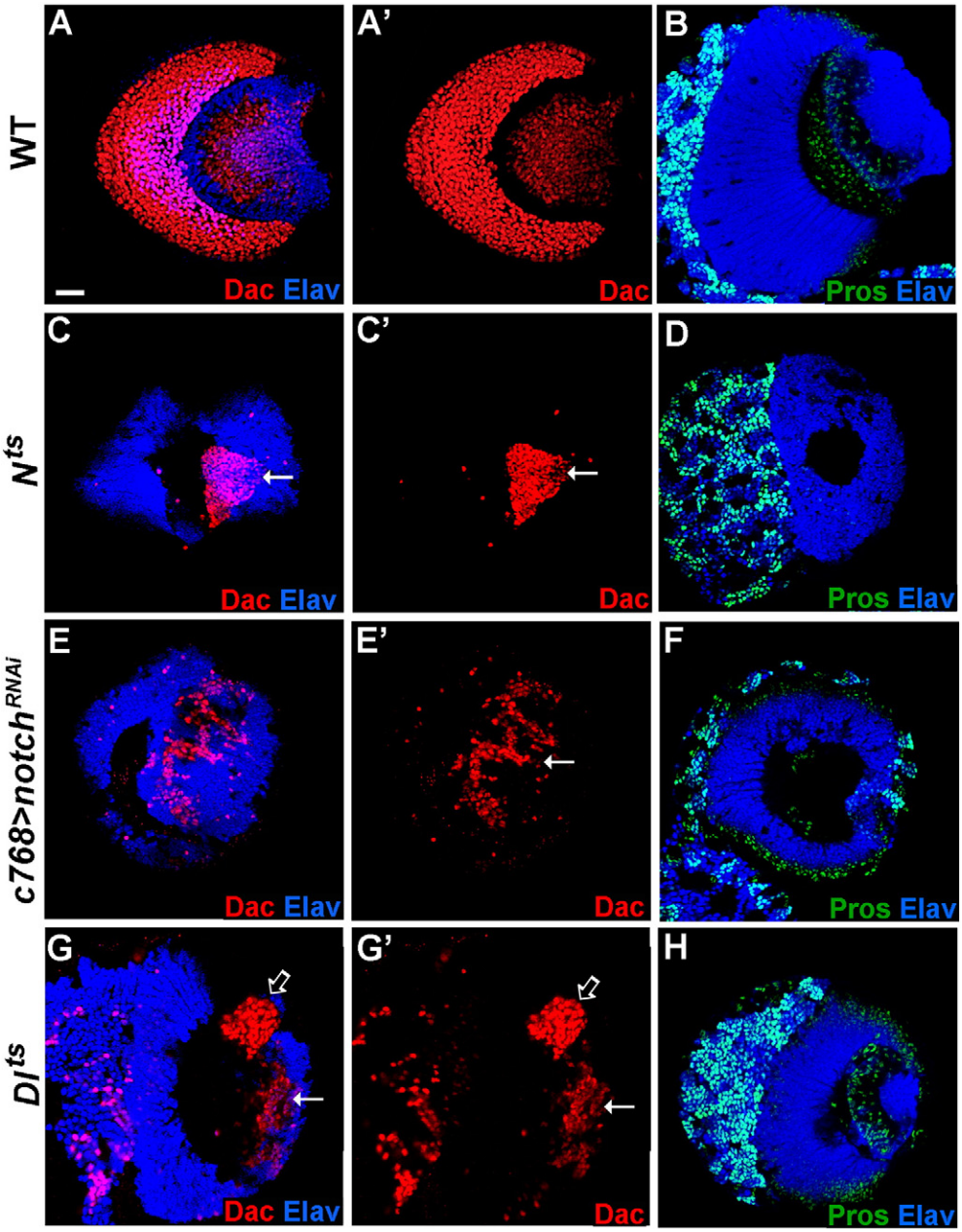
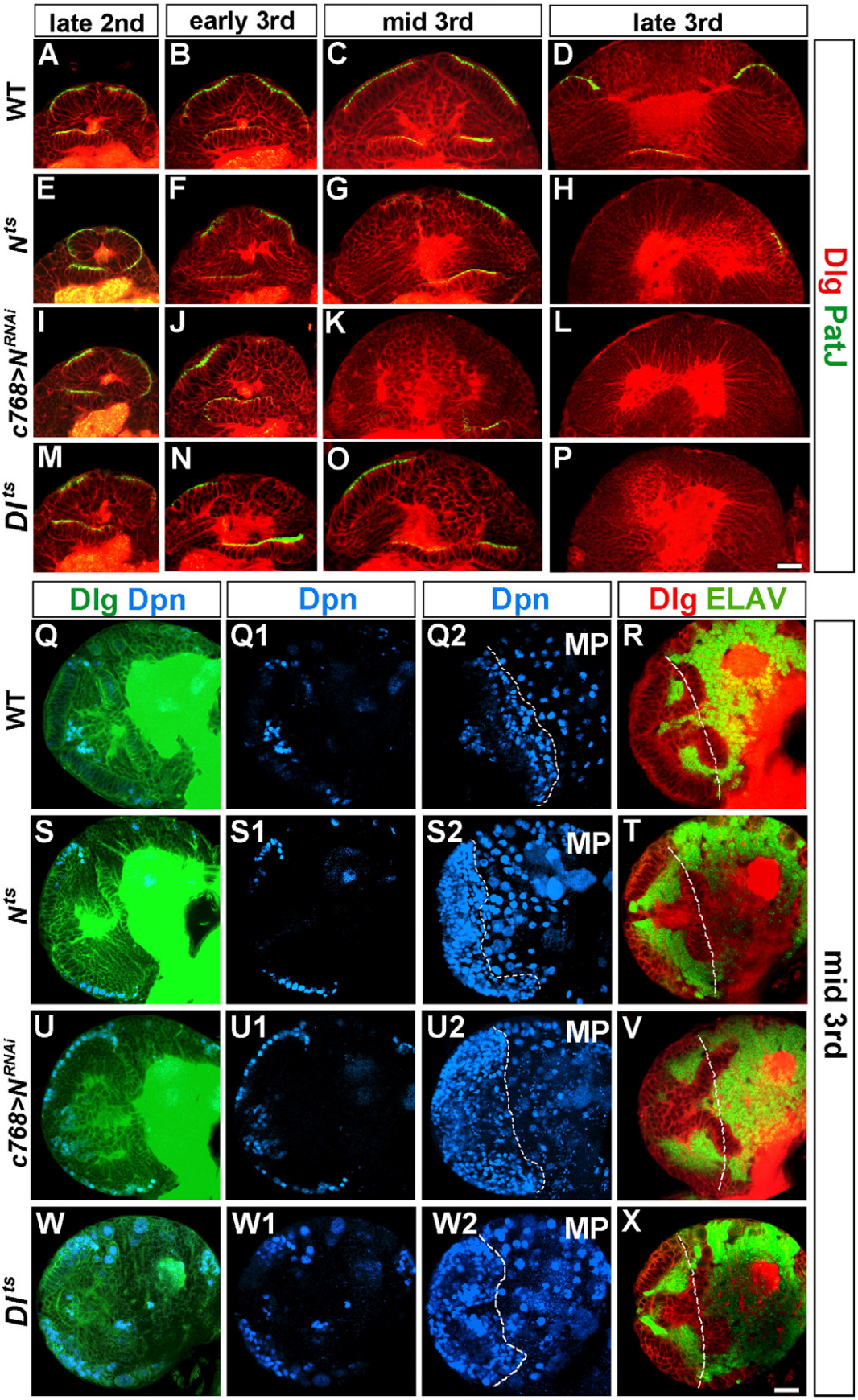


Fig. 3. Loss of Notch signaling activity leads to defects in lamina and medulla development. Brain lobes from late-third instar larvae were stained with anti-Dachshund (Dac, red) to visualize the lamina, anti-Elav (blue) to visualize the medulla cortex and anti-Prospero (Pros, green) to outline the border between the optic lobe and the central brain. A, A′, B, Wild-type brain lobes showing the crescent shaped lamina (A, A′) and the dome-shaped medulla cortex (B). C, C′, D, N^{ts} brain lobes. The lamina is absent (C, C′) and the medulla smaller than wild type (D, compare with wild type in B). Arrow indicates Dac-positive cells in the lobula complex. E, E′, F, Brain lobes in which Notch expression was knocked down by RNAi using the *c768-Gal4* driver. This also leads to loss of lamina (E, E′) and a smaller medulla (F, compare with wild type in B). G, G′, H, Dl^{ts} brain lobes. The lamina is severely reduced (G, G′, the larger arrow indicates lamina cells) and the medulla smaller than wild type (H). Anterior is to the left and dorsal up. Scale bar: 20 μm.

Fig. 4. Notch signaling is required for the maintenance of neuroepithelial integrity in the optic lobe. A–P, Time-courses of neuroepithelial maintenance and expansion. Brain lobes were stained with PatJ (green) and Dlg (red) to reveal the morphology of neuroepithelial cells in the optic lobe. A–D, Wild-type brain lobes obtained from late-second (A), early-third (B), mid-third (C), and late-third instar larvae (D). E-H, N^{ts} brain lobes obtained from late-second (E), early-third (F), mid-third (G), and late-third instar larvae (H). The OPC neuroepithelium did not expand and was largely lost by mid-third instar (G), and there were very few neuroepithelial cells in the OPC at late-third instar (H). I–L, Brain lobes obtained from late-second (I), early-third (J), mid-third (K), and late-third instar (L) larvae in which *N* expression was knocked down by RNAi using the *c768-Gal4* driver. The OPC neuroepithelium did not expand and was completely lost by mid-third instar (K). M–P, Dl^{ts} brain lobes obtained from late-second (M), early-third (N), mid-third (O), and late-third instar larvae (P). The OPC neuroepithelium was partially lost by mid-third instar (O) and completely lost at late-third instar (P). Lateral is up and medial down. Scale bar in P: 20 μm for A–P. Q–X, Medulla neurogenesis at mid-third instar. Brain lobes were stained with the markers indicated, and neuroblasts were identified with anti-Dpn (blue), neurons with anti-ELAV (green). Q, Q1, Q2, R, Wild-type brain lobes; S, S1, S2, T, N^{ts} brain lobes; U, U1, U2, V, brain lobes in which *N* expression was knocked down by RNAi using *c768-Gal4*; W, W1, W2, X, Dl^{ts} brain lobes. At mid-third instar, the wild-type optic lobe has a limited number of neuroblasts (Q, Q1, Q2) and a limited number of medulla neurons (R) whereas the number of neuroblasts in N^{ts} (S, S1, S2), N^{RNAi} (U, U1, U2) or Dl^{ts} brain lobes (W, W1, W2) was increased and there was a corresponding increase in the number of medulla neurons in N^{ts} (T), N^{RNAi} (V), or Dl^{ts} brains (X). Q2, S2, U2, and W2 are maximal projection (MP) images of all confocal sections of a brain lobe. Dashed lines indicate the border between the optic lobe and the central brain. Lateral is to the left and dorsal up. Scale bar in X: 20 μm for Q–X.


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early 3rd
mid 3rd
late 3rd
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Dlg PatJ
Dlg Dpn
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mid 3rd

second instar and then shifted to 25 °C. These culture conditions allowed us to obtain some Dl^{6B}/Dl^{RF} animals that survived to late-third instar. Similar to loss of *N* function, *Dl* mutant brains developed no lamina or a very incomplete lamina (Fig. 3G′, the larger arrow indicates lamina cells; 100%, $n=14$) and had a smaller medulla cortex (Fig. 3H; 100%, $n=22$) as compared with wild type (Fig. 3B). Together, these results indicate that Delta-Notch signaling is required for lamina and medulla development in the optic lobe.

Notch signaling maintains neuroepithelial integrity in the optic lobe

The absence of lamina and an underdeveloped medulla in *N* or *Dl* mutant larval brains suggested to us a possible deficiency in the maintenance/expansion of the neuroepithelial cells in the OPC as they are the progenitor cells that generate both lamina and medulla neurons. To examine potential abnormalities in neuroepithelial integrity in *N* or *Dl* mutant brains, we used epithelial cell markers to

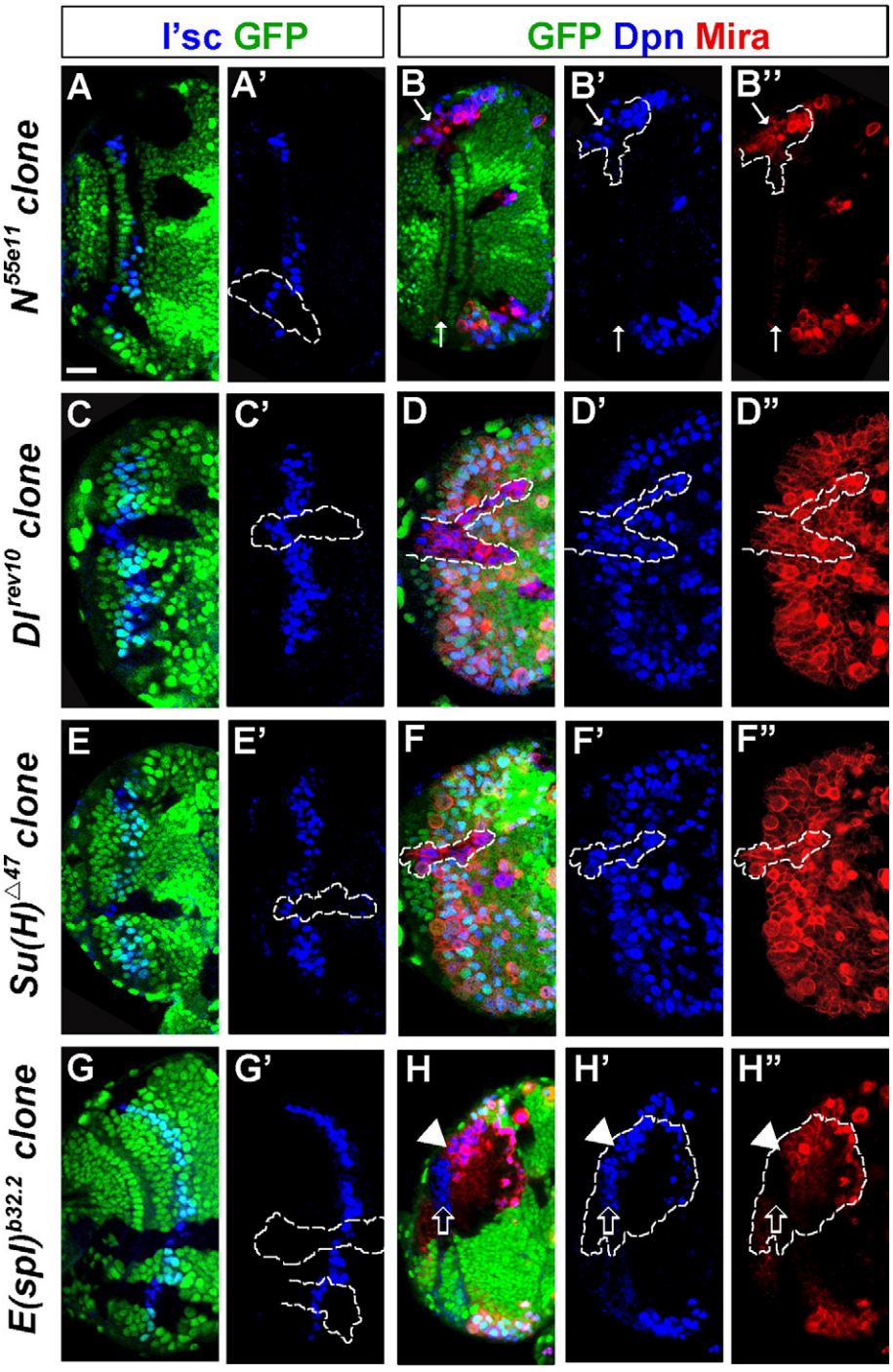


Fig. 5. Loss of Notch pathway activity in mosaic clones leads to premature neuroblast formation. Brain lobes from late-third instar larvae were stained with the antigens indicated; the clones are marked by the lack of GFP expression and by dashed lines. A–B″, *N* mosaic clones. The proneural protein L'sc was expressed prematurely in the clone (A, A′) and neuroblasts, which express Dpn and Mira formed prematurely (B, B′, B″). Note that ectopic neuroblasts are located in the lamina furrow (small arrows) and into the lamina area. C–D″, *Dl* mosaic clones. L'sc was expressed prematurely in the clone (C, C′), and neuroblasts formed prematurely in the clone (D, D′, D″). E–F″, *Su(H)* mosaic clones. L'sc was expressed prematurely in the clone (E, E′), and neuroblasts formed prematurely (F, F′, F″). G–H″, *E(spl)-C* mosaic clones. L'sc was expressed prematurely in the clone (G, G″); Dpn was prematurely expressed (H, H′) but Mira was not expressed prematurely (H, H″, Dpn^{+} cells between the large arrow and the arrowhead did not express Mira). Lateral is to the left and dorsal up. Scale bar: 20 μm.

follow the course of neuroepithelial cell expansion and maintenance in the mutant optic lobes.

Neuroepithelial cells in the OPC are columnar in shape and express apical cell polarity markers such as PatJ, aPKC, and Crumbs and adherens junction proteins DE-Cadherens and Armadillo (Egger et al., 2007; W. Wang, Y. Li, L. Zhou, H. Wang, W. Jiang, and H. Luo, unpublished observations), whereas medulla neuroblasts are rounded, express the pan-neural marker protein Deadpan (Dpn), and down-regulate the expression of the apical markers (Egger et al., 2007). In the wild type, the neuroepithelial cells in the OPC undergo symmetric cell division to expand the neuroepithelial cell population during first and second instars and reach a maximal number by mid-third instar (Fig. 4A–C); the neuroepithelium then gradually reduces its size due to the differentiation of medial neuroepithelial cells into medulla neuroblasts and lamina neurogenesis on the lateral side of the OPC. Thus, by late-third instar, the neuroepithelium is reduced to a quite narrow band around the lamina furrow (Fig. 4D); eventually all neuroepithelial cells in the OPC differentiate into medulla and lamina neurons in early pupal stages (Hofbauer and Campos-Ortega, 1990).

In N^{ts-1} mutant brains, the neuroepithelial cells in the OPC were found at the second-instar stage and appeared normal with regard to epithelial cell morphology and apical marker expression (Fig. 4E, 100%, $n=8$). However, the neuroepithelium did not expand subsequently and had largely disappeared by mid-third instar (Fig. 4F and G, 100%, $n=44$). By contrast, the neuroepithelium in the wild type had expanded dramatically during this period (Fig. 4C). By late-third instar, there were very few neuroepithelial cells in the N^{ts-1} mutant OPC (Fig. 4H, $n=51$). Accompanying the loss of neuroepithelial cells during early to mid-third instar, an increased number of medulla neuroblasts appeared in comparison with wild type (Fig. 4S, S1, S2, compare with Fig. 4Q, Q1, Q2; 100%, $n=23$) suggesting that the neuroepithelial cells in the mutant brains had prematurely differentiated into medulla neuroblasts. Correspondingly, medulla neurogenesis occurred prematurely in N^{ts-1} mutant optic lobes as reflected by more Elav-positive neurons in the mutant medulla cortex compared with wild type at mid-third instar (Fig. 4T, compare with Fig. 4R; 100%, $n=20$). N^{ts-1} mutant brains had very few medulla neuroblasts by late-third instar in contrast to a large number of medulla neuroblasts in wild-type optic lobes (not shown). N^{RNAi} knockdown using either *c768-Gal4* or *c855a-Gal4* similarly led to disappearance of neuroepithelial cells in the OPC during early to mid-third instar (Fig. 4J and K; 100%, $n=35$), premature formation of medulla neuroblasts (Fig. 4U, U1, U2, compare with wild type in Fig. 4Q, Q1, Q2; 100%, $n=14$) and premature neurogenesis (Fig. 4V, compare with wild type in Fig. 4R; 100%, $n=20$).

Similar to loss of *N* function, loss of *Dl* also resulted in defects in neuroepithelial maintenance/expansion. The Dl^{6B}/Dl^{RF} animals were first cultured at 18 °C until early-second instar and then shifted to 25 °C to inactivate *Dl* activity; the brains were dissected and analyzed at different larval stages. *Dl* mutant brains had a relatively normal neuroepithelium in the OPC at late-second instar (Fig. 4M, $n=10$); however, the neuroepithelial cells were partially lost by mid-third instar (Fig. 4N and O, 100%, $n=14$) and no neuroepithelial cells were found at late-third instar (Fig. 4P, 100%, $n=9$). Again, medulla neuroblasts were generated prematurely during early to mid-third instar in *Dl* mutant brains (Fig. 4W, W1, W2, compare with wild type in Fig. 4Q, Q1, Q2, 100%, $n=8$) leading to premature neurogenesis (Fig. 4X, compare with wild type in Fig. 4R, 100%, $n=6$); very few medulla neuroblasts were found at late-third instar (data not shown). Taken together, these results indicate that Delta-Notch signaling is required for the maintenance and expansion of neuroepithelial cells in the OPC and that loss of Delta-Notch function led to early depletion of neuroepithelial cells in the OPC, premature neuroblast formation, and premature neurogenesis in the medulla.

Notch signaling inhibits neuroblast formation in the medulla

Mosaic clones of strong loss-of-function or null alleles for Notch pathway components were induced to further assess the effect of removing Notch signaling activity on optic lobe development. For *N* mosaic analyses, we used the strong loss-of-function allele N^{XK11} and the null allele N^{55E11}. Clones were induced at late-first or early-second instar and the mosaic brains analyzed at late-third instar. Similar effects were observed for both *N* alleles. When *N* mutant clones were located in the OPC neuroepithelium, the proneural protein Lethal of scute (L'sc) was expressed prematurely in the clones (Fig. 5A, A′, 66.7%, $n=9$), which in wild-type brain is expressed as a 1–2 cell-wide stripe of medial neuroepithelial cells that foreshadow their differentiation into medulla neuroblasts (Yasugi et al., 2008); indeed, neuroblast-specific markers Dpn (Fig. 5B, B′, 58.6%, $n=29$) and Miranda (Fig. 5B, B″, 64.7%, $n=17$) were prematurely expressed in a number of mutant cells in the clones. Many *N* mutant clones were found located in deeper layers of the medulla cortex (Fig. S3A–C), which presumably originated from the neuroepithelial cells in the OPC; such mutant clones usually contained a number of ectopic $Dpn^{+}/Mira^{+}$ cells that behaved like neuroblasts as they had asymmetric Miranda localization during mitosis, generated GMC progeny ($Prospero^{+}$) and neurons ($Elav^{+}$) (Fig. S3A–C, $n=17$).

In *N* mutant clones located in the OPC neuroepithelium, the epithelial characters of *N* mutant cells were affected as they often had lost or reduced expression of apical markers such as PatJ (Fig. 6B, B′, 51.9%, $n=27$) and adopted rounded or irregular cell morphology rather than columnar epithelial cell morphology (Fig. 6A, A′, compare with wild-type epithelial cells in A″; 57.9%, $n=19$), suggesting that the adherens junction might be disrupted in *N* mutant epithelial cells. To address whether loss of neuroepithelial cell integrity is always associated with neuroblast formation, we co-stained *N* mosaic brain lobes with Dpn and DE-cad or PatJ. In a total of 18 *N* mutant clones that were localized in the OPC neuroepithelium, we found that the mutant cells in 9 clones (50%) had both changed cell morphology to rounded or irregular shape and transformed into neuroblasts (Dpn-positive) (Fig. S4A, A′, A″) while in 5 clones (28%), the mutant cells had only changed cell morphology or lost PatJ expression but did not transform into neuroblasts (Dpn-negative) (Fig. S4B, B′, B″). There were still another 4 clones (22%) in which the mutant cells did not show any change in either neuroepithelial cell morphology or cell fate, these clones were usually located in the lateral-most part of the OPC neuroepithelium (not shown). We conclude that loss of *N* function in mosaic clones cell autonomously led to premature neuroblast formation and loss of epithelial cell characters and that the loss of epithelial integrity is not always linked with premature neuroblast formation.

Dl mutant clones were induced using the null allele Dl^{rev10} (Heitzler and Simpson, 1991). A *Minute* mutation on the right arm of the third chromosome allowed us to frequently recover quite large Dl^{rev10} mutant clones due to a growth advantage given to the mutant cells. The overall results were similar to those for *N* mutant clones. L'sc expression was induced prematurely (Fig. 5C, C′, 83.3%, $n=6$), and neuroblasts were generated prematurely in Dl^{rev10} clones located in the OPC neuroepithelium (Fig. 5D, D′, D″, 71.4%, $n=42$), and like *N* mutant clones, many *Dl* mutant clones were also found in the deeper layers of the medulla cortex in which the ectopic neuroblasts underwent asymmetric divisions to generate GMC progeny and neurons (Fig. S3D–F, $n=30$).

The mutant cells in the *Dl* mosaic clone located in the OPC neuroepithelium had more rounded or irregular morphology (Fig. 6C, C′, compare with wild-type epithelial cells in C″; 87.5%, $n=24$) and had reduced or lost expression of PatJ (Fig. 6D, D′, 72.7%, $n=33$), aPKC and Crumbs (not shown). Co-staining of *Dl* mutant cells with Dpn and DE-cad or PatJ showed that the mutant cells in 46% of the clones ($n=26$) had both changed cell morphology and become neuroblasts (Fig. S4C, C′, C″), while in 31% of the clones, the mutant cells exhibited only cell morphology change or loss of PatJ

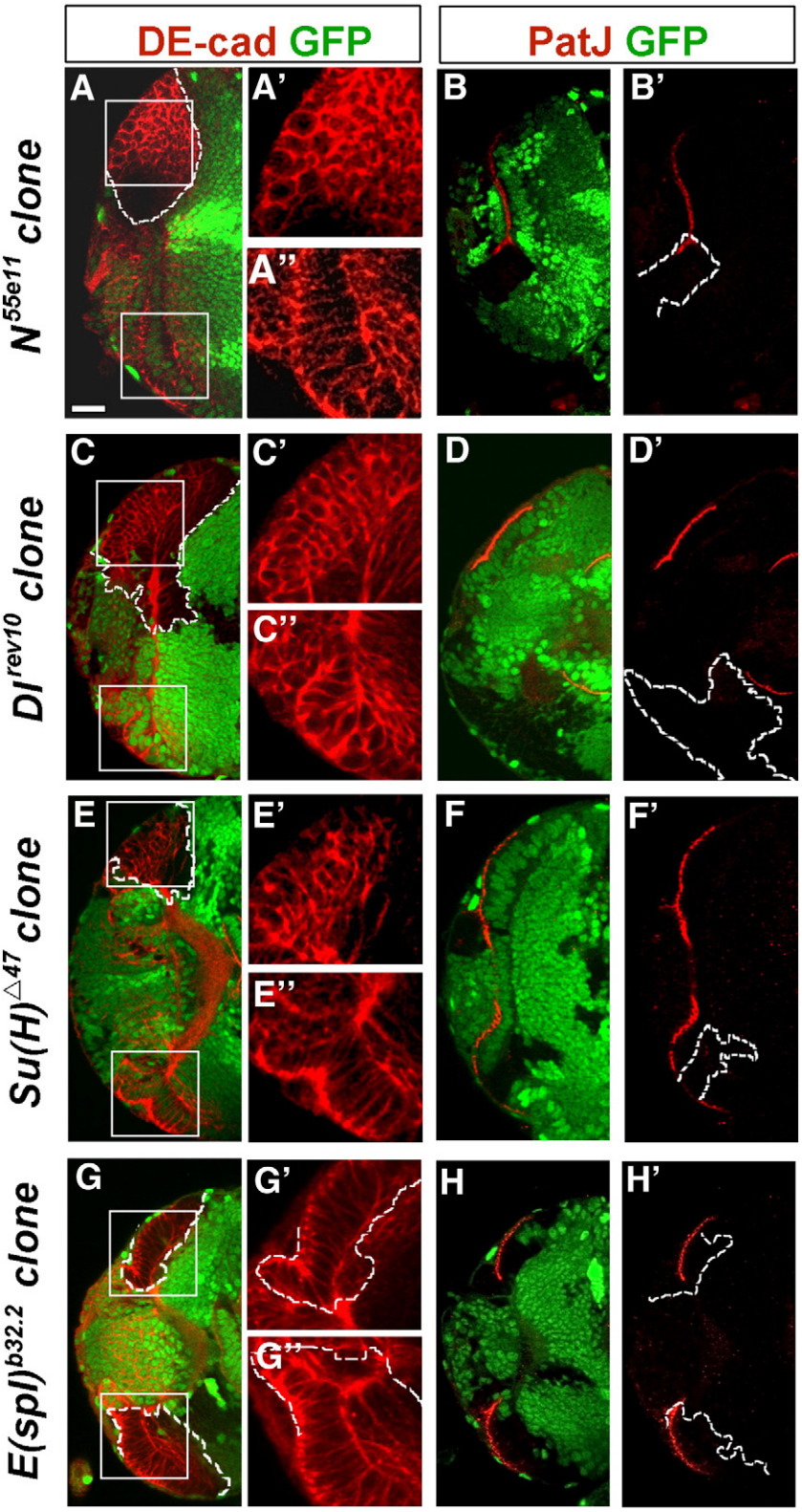


Fig. 6. Loss of Notch pathway activity in mosaic clones disrupts epithelial cell integrity. Brain lobes from late-third instar larvae were stained with the antigens indicated; the clones are marked by the lack of GFP expression and by dashed lines. A–B′, *N* mosaic clones. The mutant cells in the clone changed their morphology to rounded shape (A, A′, compare with wild-type epithelial cells in A″), and the apical marker PatJ was lost in the clone (B, B′). C–D′, *Dl* mosaic clones. The mutant cells in the clone changed their morphology to rounded or irregular shape (C, C′, compare with wild-type epithelial cells in C″), and PatJ was lost in the clone (D, D′). E–F′, *Su(H)* mosaic clones. The mutant cells in the clone changed their morphology to irregular shape (E, E′, compare with wild-type epithelial cells in E″), and PatJ was reduced in the clone (F, F′); G–H′, *E(spl)-C* mosaic clones. The mutant cells in the clone retained columnar epithelial cell morphology (G, G′, G″), and PatJ expression was unchanged (H, H′). Lateral is to the left and dorsal up. Scale bar: 20 μm.

expression but did not become neuroblasts (Fig. S4D, D′, D″), and in 23% of the clones located in the lateral-most part of the OPC neuroepithelium, the mutant cells changed neither cell morphology nor cell fate (not shown). Thus, like Notch, loss of Delta activity in mosaic clones also results in premature neuroblast formation and loss of epithelial cell characters.

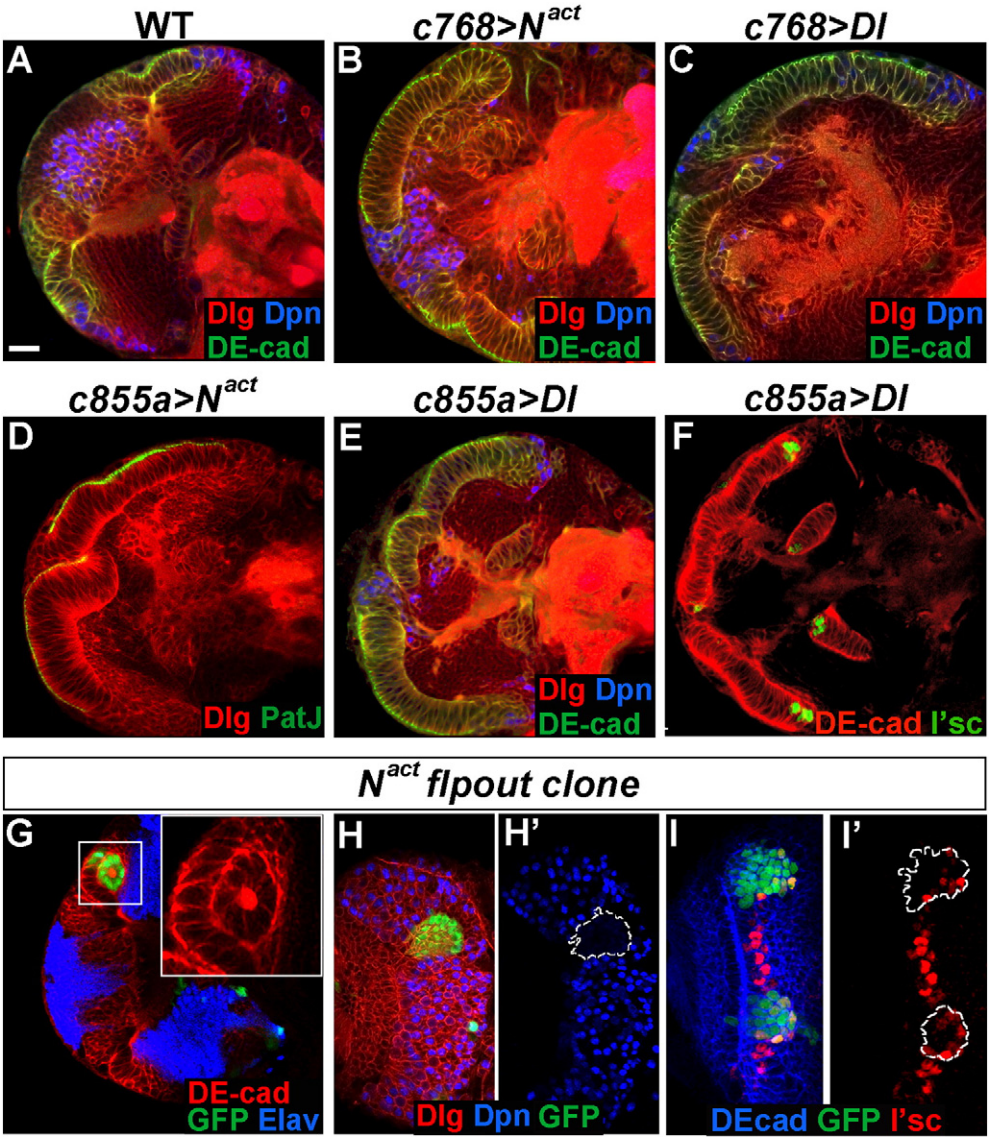


Fig. 7. Ectopic activation of Notch signaling promotes growth of the optic lobe neuroepithelium and inhibits neuroblast formation. Brain lobes obtained from late-third instar larvae were stained with the antigens indicated; flip-out clones are marked by GFP and dashed lines. Compared with wild-type (A), ectopic N^{ACT} (B, D) or *Dl* expression (C, E, F) driven by either *c768-Gal4* (B, C) or *c855a-Gal4* (D, E, F) lead to an expansion of neuroepithelial cells. L'sc expression was restricted to the medial most cells in the expanded neuroeptihelium (F). G–I′, N^{ACT} expression in flip-out clones generates ectopic neuroepithelial cells which form rosettes and are located underneath wild type neuroepithelial cells (G, enlarged view of the rosette in inset), and the expression of L'sc (I, I′) and Dpn (H, H′) was suppressed in the clones. Lateral is to the left and dorsal up. Scale bar: 20 μm.

To test whether the canonical Notch signaling pathway suppresses medulla neuroblast formation and maintains epithelial integrity, we examined clones of cells mutant for *Su(H)*, which acts downstream of Notch to induce target gene expression. Clones of cells mutant for the null allele $Su(H)^{\Delta 47}$ were induced and the mutant cells in clones located in the OPC exhibited phenotypic similarities to those of *N* and *Dl* mutants. Again, L'sc was prematurely expressed (Fig. 5E, E′, 71.4%, $n=7$) and neuroblasts were prematurely induced in $Su(H)^{\Delta 47}$ mutant clones (Fig. 5F, F′, F″, 75%, $n=40$); the mutant cells had reduced apical marker expression (Fig. 6F, F′, 79%, $n=57$) and adopted irregular cell morphology (Fig. 6E, E′, compare with wild-type epithelial cells in E″; 78.6%, $n=28$). Co-staining of $Su(H)^{\Delta 47}$ mutant clones with Dpn and DE-cad or PatJ showed that the mutant cells in 51% of the clones ($n=45$) had changed both cell morphology and become neuroblasts (Fig. S4E, E′, E″), whereas in 20% of the clones, the mutant cells only changed cell morphology but did not become neuroblasts (Fig. S4F, F′, F″), and in 29% of the clones located in the lateral-most part of the OPC neuroepithelium, the mutant cells did not change neuroepithelial cell morphology or cell fate (not shown).

The *E(spl)-C* encodes seven members of structurally similar bHLH proteins and are direct target genes of Notch pathway activation (Jennings et al., 1994). To analyze the role of *E(spl)-C* in optic lobe development, we made use of a deficiency that deleted all seven genes, $Df(3R)E(spl)^{b32.2}[gro^{+}]$ (Schrons et al., 1992). Analyses of *E(spl)-C* mosaic clones indicate that this gene complex is involved in inhibiting medulla neuroblast formation. In *E(spl)-C* mutant clones located in the OPC, L'sc was prematurely expressed (Fig. 5G, G′, 81.8%, $n=11$), and Dpn^{+} cells were prematurely generated (Fig. 5H, H′, 86.7%, $n=30$). However, in contrast to the Notch pathway components shown above, the *E(spl)-C* mutant cells did not express Miranda prematurely (Fig. 5H, H″, $Dpn^{+}/Mira^{-}$ cells between the arrow and arrowhead; 100%, $n=17$) and the mutant cells underwent symmetric cell divisions (Fig. S5A′, 100%, $n=43$) in contrast to the asymmetric cell division of medulla neuroblasts; these mutant cells may represent an early stage of differentiation from neuroepithelial cells to medulla neuroblasts. Furthermore, the mutant cells in the clones retained normal epithelial cell morphology (Fig. 6G, G′, G″, 100%, $n=16$), and PatJ and aPKC expression were unaffected (Fig. 6H, H′, and data not shown; 100%,

$n=15$). Co-staining with Dpn and DE-cad or PatJ showed that while mutant cells in the clones prematurely expressed Dpn, they still retained the columnar epithelial morphology and apical marker expression (Fig. S5B–C″, 81.3%, $n=16$).

From these mosaic clone data and together with the results obtained from *N* and *Dl* mutant brains, we conclude that the activity of the canonical Notch pathway from Delta/Notch/Su(H) to the target genes *E(spl)-C* inhibits medulla neuroblast formation, while the maintenance of the epithelial cell characters requires only Delta, Notch and Su(H), but not *E(spl)-C*.

Activation of Notch signaling promotes the growth of the OPC neuroepithelium

To address whether Notch pathway activation is sufficient to promote neuroepithelial cell growth in the OPC, we ectopically expressed *Dl* and *N* using the *c855a-Gal4* or the *c768-Gal4* driver. *Dl* overexpression led to an expansion of neuroepithelial cells in the OPC (Fig. 7C, E, F, 100%, $n=50$). While wild-type brains have a band of neuroepithelial cells in the lateral region of the OPC at late-third instar (Fig. 7A), *Dl*-overexpressing brains had neuroepithelial cells covering the entire OPC (Fig. 7C and E); at the same time, L'sc expression was suppressed in the expanded neuroepithelium and limited to the medial edge of the neuroepithelium (Fig. 7F), and the mutant brains had fewer medulla neuroblasts (Fig. 7C and E) and a smaller medulla as compared with wild type (not shown). We also attempted to overexpress N^{ACT} in the OPC; however, N^{ACT} expression driven by the *c855a-Gal4* or the *c768-Gal4* driver caused early lethality. Thus, N^{ACT} expression was achieved by combining a $Gal80^{ts}$ construct with these drivers to temporally control N^{ACT} expression in the optic lobe (see Materials and methods). N^{ACT} overexpression also led to an expansion of neuroepithelial cells while suppressing medulla neurogenesis (Fig. 7B and D, 100%, $n=18$). In addition, ectopic N^{ACT} expression in flip-out clones in the OPC also led to ectopic neuroepithelial cells, which formed rosettes separated from the neuroepithelium and expressed epithelial cell markers (Fig. 7G, inset and data not shown; 100%, $n=45$); L'sc and Dpn expression was suppressed in the clones (Fig. 7H–I′, 100%, $n=52$). Overexpression of full-length Notch did not promote neuroepithelial cell expansion nor did it inhibit medulla neurogenesis (data not shown). Thus, activation of Notch signaling appears sufficient to promote the growth of the OPC neuroepithelium.

Since Notch activation suppresses medulla neuroblast formation, it is possible that an increase in neuroepithelial cells in *Dl* or N^{ACT}-overexpressing brains was solely due to a suppression of differentiation of neuroepithelial cells into medulla neuroblasts. We quantified the number of neuroepithelial cells per confocal section of *Dl* or N^{ACT}-overepexressing brains obtained from late-third instar larvae and compared these with the maximal numbers of neuroepithelial cells per confocal section of wild-type brains obtained from mid-third instar larvae and found that there was a twofold increase of neuroepithelial cells per confocal section in the mutant (95.8 ± 7.7, $n=12$) as compared with wild type (53.1 ± 3.0, $n=12$). This twofold increase could be explained by the suppression of neuroepithelial cell differentiation into medulla neuroblasts if the neuroepithelial cells continue to divide at a constant proliferation rate. Alternatively, the increased number of neuroepithelial cells in *Dl* or N^{ACT}-overexpressing brains might result from an increased rate of cell proliferation. To test whether Notch activation might accelerate cell cycle progression in the neuroepithelial cells, we induced flip-out clones for wild-type cells and for N^{ACT}-expressing cells and compared the number of cells in the mutant clones with those of control clones. We found that N^{ACT}-expressing cells had similar rates of cell proliferation to wild-type cells. When clones

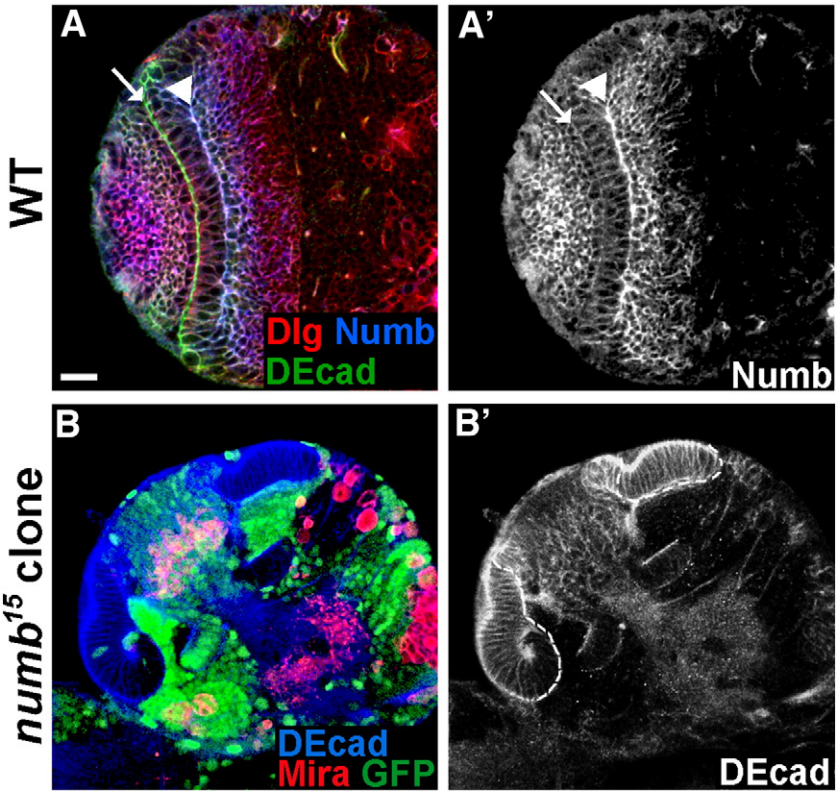


Fig. 8. Numb loss of function leads to the expansion of neuroepithelial cells. A, A′, Expression pattern of Numb protein in the optic lobe. Numb is expressed in the OPC neuroepithelial cells with strong basal localization; it is also expressed in the medulla neuroblasts and their progeny, and in the lamina (A′). Arrow and arrowhead indicate the apical and basal domain of the neuroepithelial cell, respectively. B, B′, $numb^{15}$ mosaic clones were induced in the optic lobe, and brain lobes obtained from late-third instar larvae were stained with the antigens indicated. The clone of $numb^{15}$ mutant cells is marked by the lack of GFP expression (B) and dashed line (B′). In clones located in the OPC neuroepithelium, mutant cells maintained neuroepithelial cell identity and did not differentiate into neuroblasts ($Mira^{-}$). Lateral is to the left and dorsal up. Scale bar: 20 μm.

were induced in early-second instar and assayed at mid-third instar, both wild-type cell clones and N^{ACT} expressing clones had on average divided about 4.5 times (25 cells) per clone per day, and a similar proliferation rate for both control and N^{ACT}-expressing cells was also observed for clones induced at mid-third instar and assayed at late-third instar. In addition, there was no difference in cell size in N^{ACT}-expressing

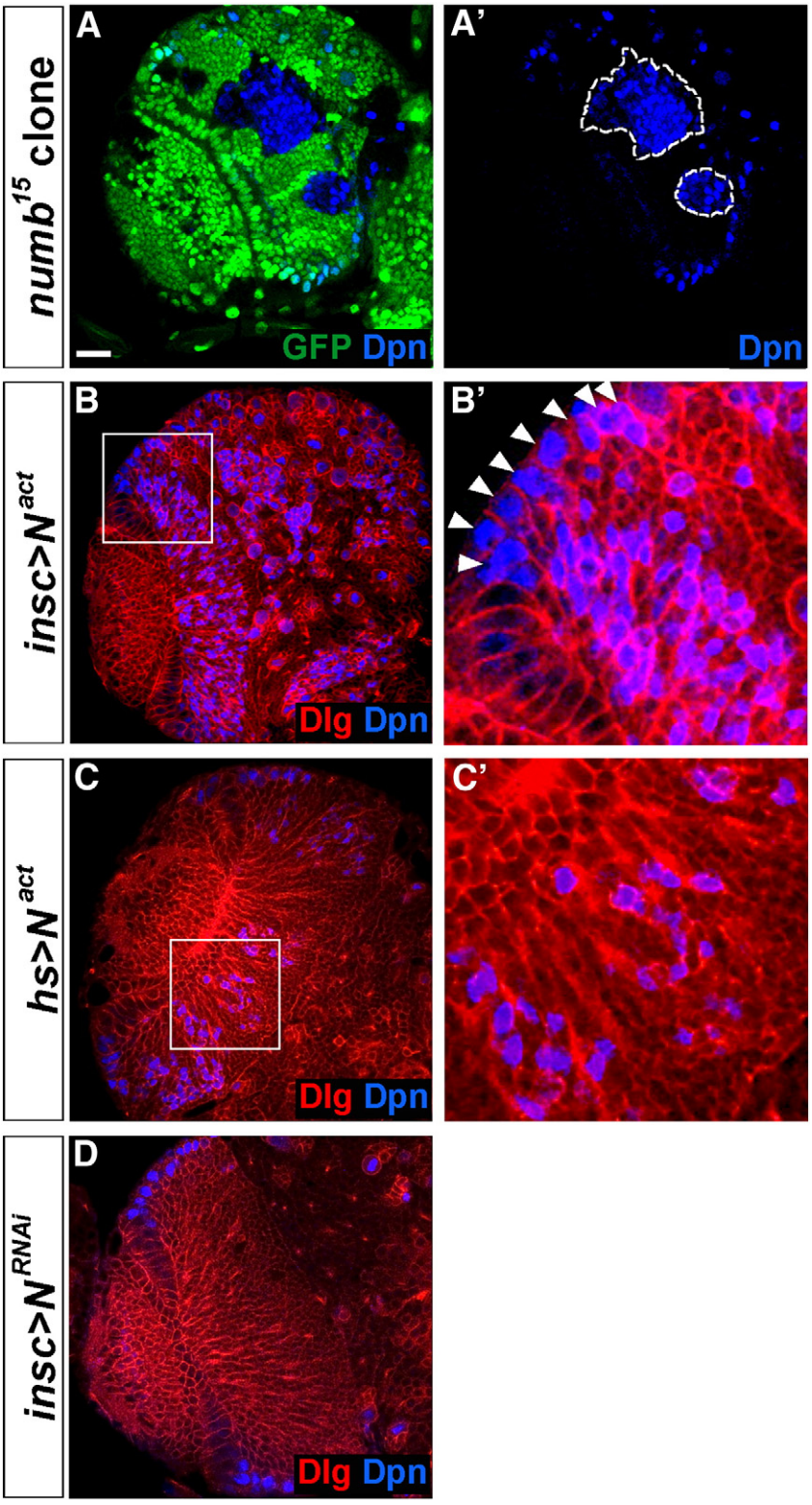


Fig. 9. Induction of ectopic neuroblasts by ectopic Notch activation. Brain lobes obtained from late-third instar larvae were stained with the antigens indicated; the clones are identified by the lack of GFP and dashed line. A, A′, *numb*^{15} mosaic clones that were induced in late-second to early-third instar stage contain numerous neuroblasts, which are identified by anti-Dpn staining (blue). B, B′, Ectopic expression of N^{ACT} driven by *insc-Gal4* that is active in the medulla neuroblasts/GMCs but not in the neuroepithelial cells in the OPC resulted in ectopic neuroblasts in the medulla cortex. These ectopic medulla cortex neuroblasts are smaller in size than the medulla neuroblasts in the OPC (indicated by arrowheads in B′). C, C′, Ectopic expression of N^{ACT} driven by *hs-Gal4* resulted in ectopic neuroblasts in the medulla cortex. D, Knockdown of *N* expression in the neuroblasts by RNAi using *insc-Gal4* did not affect the formation of medulla neuroblasts. Lateral is to the left and dorsal up. Scale bar: 20 μm.

cells as compared with wild-type cells and no apoptosis was induced by N^{ACT} overexpression (not shown). Thus, wild-type neuroepithelial cells appear to divide at a constant rate during early to late larval stages and N^{ACT} overexpression did not accelerate cell cycle progression. The growth signal that promotes cell cycle progression appears to be distinct from the Notch signal. Thus, we conclude that the expansion of neuroepithelial cells in *Dl* or N^{ACT}-overexpressing brains is due to two contributing factors, maintaining neuroepithelial stem cell identity and suppressing their differentiation into medulla neuroblasts.

The role of numb in neuroepithelial cell maintenance in the optic lobe

Numb has been shown to negatively regulate Notch signaling activity in embryonic neuroblasts and in the sensory organ precursors (SOP) (Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996; Buescher et al., 1998). Numb is expressed in the optic lobe and is localized basally in the OPC neuroepithelial cells (Fig. 8A, A′). To test whether Numb might also negatively regulate Notch signaling activity in the optic lobe, we induced *numb* mosaic clones using the null allele $numb^{15}$ (Berdnik et al., 2002). When clones were induced during the first instar or early-second instar stages, we found that *numb* mutant cells in the clone maintained neuroepithelial cell identity until late-third instar (Fig. 8B, B′, 100%, $n=22$), and the differentiation of neuroepithelial cells into medulla neuroblasts was inhibited as revealed by the absence of Mira and Dpn expression in the mutant cells (Fig. 8B′ and data not shown). These effects of *numb* loss of function are similar to ectopic Notch activation in the OPC neuroepithelium, suggesting that Numb may negatively regulate Notch signaling activity in the OPC neuroepithelium.

Ectopic neuroblasts can be induced by ectopic Notch signaling activity

When $numb^{15}$ mosaic clones were induced at later larval stages (late-second to early-third instar when medulla neuroblasts have started to form), we observed a large number of ectopic Dpn^+ cells in the clones that were typically located in deeper layers of the medulla cortex (Fig. 9A, A′, 100%, $n=16$); these Dpn^+ cells appeared to be ectopic neuroblasts, as they were mitotically active as assayed by labeling with phosphorylated histone H3 (a mitotic marker) and had asymmetric localization of Miranda during mitosis (not shown). However, the sizes of these ectopic neuroblasts are in the range of ganglion mother cells (GMCs) or neurons, rather than the larger size of medulla neuroblasts in the OPC, and unlike medulla neuroblasts, these ectopic neuroblasts only occasionally generated progeny that expressed the Prospero protein ($Pros^+$) and virtually no neurons ($Elav^+$) were generated by these neuroblasts. When N^{ACT} was ectopically expressed using *insc-Gal4*, which, in the optic lobe, is only expressed in medulla neuroblasts and their progeny but not in the OPC neuroepithelial cells (Betschinger et al., 2006), we also observed ectopic Dpn^+ cells in the medulla cortex (Fig. 9B, B′, 100%, $n=11$). Ectopic Dpn^+ cells were also generated when N^{ACT} expression was induced under *hs-Gal4* control by a brief heat shock at mid-third instar (Fig. 9C, C′, 100%, $n=8$). Thus, ectopic Notch pathway activation seemed to promote neuroblast proliferation in the optic lobe. This finding apparently contradicts with a role of Notch signaling in inhibiting medulla neuroblast formation. However, ectopic Notch activity may not reflect a normal requirement of the Notch pathway in medulla neuroblast proliferation/self-renewal. To test this possibility, we used RNAi to knock down *N* expression specifically in the neuroblasts using *insc-Gal4* and found that this did not affect medulla neuroblast formation or their self-renewing ability (as judged by a normal medulla cortex in these RNAi-treated brains) (Fig. 9D, 100%, $n=7$). Thus, we conclude that while Notch signaling is not required for medulla neuroblast proliferation/self-renewal, ectopic Notch pathway activation can generate ectopic neuroblasts in the medulla.

Discussion

In this study, we find that Notch signaling plays an essential role in the maintenance and expansion of neuroepithelial cells in the optic lobe; it also inhibits medulla neuroblast formation. Clonal analyses of several pathway components indicate that this dual function bifurcates downstream of Su(H) with E(spl)-C only partly involved in the inhibition of medulla neuroblast formation but not the maintenance and expansion of neuroepithelial stem cells.

In the optic lobe, Notch signaling plays a role analogous to lateral inhibition during embryonic CNS development. However, the selection of neuroblasts in the OPC neuroepithelium is an all-or-none process rather than selecting individual neuroblasts from the neuroepithelium. Medulla neuroblasts are generated in a wave progressing in a medial to lateral direction in the OPC neuroepithelium with all cells at a particular position along the medial–lateral axis differentiating into neuroblasts (Yasugi et al., 2008). Interestingly, this wave of medulla neuroblast formation coincides with the down-regulation of both Delta and Notch expression in the medial cells in the OPC, which might reduce Notch signaling activity, thereby allowing medulla neuroblasts to form. What factors drive the recession of both Delta and Notch expression in the OPC neuroepithelium along the medial–lateral axis is not known. When Notch signaling is inactivated, neuroepithelial cells in the OPC change cell morphology and differentiate into medulla neuroblasts prematurely. Our results indicate that Notch signaling actively controls neuroepithelial integrity, possibly by regulating the adherens junction (AJ), since in Notch pathway mutant mosaic clones in the OPC, the apical determinants PatJ, Crumbs and aPKC are cell autonomously reduced or lost and the mutant cells change to rounded or irregular morphology (Fig. 6, Fig. S4, and data not shown). Further experiments will be needed to determine how Notch signaling activity affects the maintenance of neuroepithelial integrity, particularly the stability of the adherens junction.

Is neuroblast formation also actively inhibited by Notch signaling or simply a default state of neurogenic epithelial cells? In the latter model, Notch signaling may only maintain neuroepithelial integrity and promote their expansion while medulla neuroblasts form when the neuroepithelial integrity is disrupted. The argument against this model is that changes in neuroepithelial integrity are not always accompanied with cell fate changes. In *N*, *Dl* or *Su(H)* mosaic clones located in the OPC neuroepithelium, we found in about 25% of the clones, the mutant cells changed morphology or lost apical marker expression but did not become neuroblasts (Dpn-negative) (Fig. S4), whereas in *E(spl)-C* mosaic clones, Dpn^+ cells were prematurely induced, which indicate that the cells begin to differentiate into neuroblasts, but these cells still retained columnar epithelial cell morphology and apical marker expression (Fig. 6G–H′; Fig. S5). This suggests that the suppression of neuroblast formation by Notch signaling activity is separable from the maintenance of neuroepithelial integrity and that medulla neuroblast formation is actively suppressed by Notch signaling. A possible scenario is that activation of the Notch pathway turns on the *E(spl)-C* genes, which in turn suppress proneural gene expression in the optic lobe neuroepithelia. Indeed, at least one member in the *E(spl)-C* genes, *E(spl)m8*, appears to be activated in the neuroepithelial cells by the Notch pathway, as the *E(spl)m8-lacZ* reporter is expressed in a pattern similar to Delta and Notch expression in the OPC and IPC (Fig. 2). E(spl)m8 protein and possibly additional members of the E(spl)-C may suppress the expression of proneural genes in the optic lobe. The proneural genes of the *achaete-scute* complex (as-c) comprise four members, *achaete*, *scute*, L'sc, and *asense* (Campuzano and Modollel, 1992; García-Bellido and de Celis, 2009). *achaete* is not expressed in the optic lobe, but *scute* is expressed in both the neuroepithelial cells and neuroblasts in the OPC (Egger et al., 2007) implying that *scute* expression in the neuroepithelial cells is not suppressed by Notch signaling activity. By

contrast, *asense* is only expressed in the neuroblast and GMCs (Egger et al., 2007) and L'sc is transiently detected in an advancing stripe of neuroepithelial cells of 1–2 cells wide that are just ahead of newly formed medulla neuroblasts (Yasugi et al., 2008). Thus, E(spl)-C proteins may suppress L'sc and/or *ase* expression, the release of this suppression may allow the neuroepithelial cells to begin to differentiate into medulla neuroblasts. It should be noted, however, that the removal of the *E(spl)-C* activity does not seem to be sufficient to allow full differentiation of neuroepithelial cells into medulla neuroblasts (Fig. 5G–H″), which suggests that additional factors downstream of Notch signaling may be involved in the suppression of medulla neuroblast formation.

The phenotypes of Notch pathway mutants are reminiscent of those of JAK/STAT mutants (Yasugi et al., 2008; W. Wang, Y. Li, L. Zhou, H. Wang, W. Jiang, and H. Luo, unpublished observations). For example, inactivation of either pathway led to early depletion of the OPC neuroepithelium; either pathway inhibits neuroblast formation, and ectopic activation of either pathway promotes the growth of the OPC neuroepithelium. The remarkable phenotypic similarities in Notch and JAK signaling mutant brains suggest that these pathways may act in a linear relationship such that activation of one pathway is relayed to the second, perhaps by inducing the expression of a ligand. Alternatively, these pathways may act in parallel and converge onto some key downstream effectors or target genes. Further experiments will be needed to test whether Notch interacts with JAK/STAT and if it does, to find out where the interaction occurs during the development of the optic lobe.

The roles of Notch signaling in mammalian brain development have been studied intensely. Many Notch pathway components have been examined in knockout mice, which showed defects in brain development (Yoon and Gaiano, 2005). Mice deficient for *Notch1* (de la Pompa et al., 1997; Hitoshi et al., 2002; Lütolf et al., 2002; Yang et al., 2004; Yoon et al., 2004), *Dll* (Grandbarbe et al., 2003) or *Cbf* (de la Pompa et al., 1997) all display precocious neurogenesis during early stages of nervous system development. This has led to the view that the role of Notch signaling in the mouse brain is to maintain the progenitor state and inhibit neurogenesis. However, it is not clear from these studies whether the premature neurogenesis in Notch signaling mutant mice was caused by premature differentiation of neuroepithelial stem cells into neurons or by premature differentiation of neuroepithelial stem cells into progenitor cells, which then generated neurons. In fact, it has been proposed that Notch activation can promote the differentiation of neuroepithelial stem cells into radial glial cells, the progenitor cells that generate the majority of neurons in the cerebral cortex (Malatesta et al., 2003; Anthony et al., 2004). This is based on the observation that ectopic Notch activation using activated forms of Notch1 and Notch3 (N^{ICD}) caused an increase in radial glial cells as compared to control (Gaiano et al., 2000; Dang et al., 2006). The radial glial cells resemble medulla neuroblasts in the *Drosophila* optic lobe in that they are both derived from neuroepithelial stem cells and undergo asymmetric division to self-renew and generate neurons, although morphologically radial glial cells are still polarized while medulla neuroblasts have lost epithelial characters and are rounded in shape. Based on our results, we suggest that Notch signaling maintains the pool of neuroepithelial stem cells and promotes their expansion in both *Drosophila* and mammals and that the precocious neurogenesis in Notch signaling mutant brains arise due to premature differentiation of the neuroepithelial stem cells into the progenitor cells.

However, ectopic Notch activation may indeed promote progenitor cell proliferation in the brain. We observed ectopic neuroblasts in the medulla cortex when N^{ACT} was ectopically expressed by the neuroblast/GMC driver *insc-Gal4* (Fig. 9B, B′), by ubiquitous expression using *hs-Gal4* (Fig. 9C, C′), or when $numb^{15}$ mosaic clones were induced at later larval stages when neuroblasts normally begin to form (Fig. 9A, A′). Since our results have shown that the Notch pathway is not essential for medulla neuroblast formation or self-renewal (Fig. 9D), the ectopic neuroblasts are a novel phenotype solely induced by ectopic Notch signaling activity. This is consistent with Notch activation promoting ectopic neuroblast formation in the central brain and VNC (Wang et al., 2006; Bowman et al., 2008) without being required for neuroblast self-renewal in these regions of the CNS (Almeida and Bray, 2005; Lee et al., 2006a; and mosaic clone data not shown); and Notch has been shown to be an oncogene in mammals (Wilson and Radtke, 2006). Since the sizes of the ectopic neuroblasts were in the range of GMC or neurons (Fig. 9B′, C′), they may resemble the transit-amplifying (TA) neuroblasts that are found in the dorsal–medial region of the central brain (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). The origin of these ectopic neuroblasts in the medulla cortex is not clear, but it is unlikely that they are derived from differentiated medulla neurons as ectopic expression of N^{ACT} using *elav-Gal4*, which is active in medulla neurons, did not result in ectopic neuroblasts (not shown) and by the fact that ectopic neuroblasts can be induced in $numb^{15}$ mosaic clones, which could only arise from mitotically active cells that include neuroepithelial cells, medulla neuroblasts, and ganglion mother cells (GMCs), but not neurons. The ectopic neuroblasts could be generated by a transformation of GMCs into a neuroblast identity as suggested for ectopic neuroblasts in *brat* mutant central brains (Lee et al., 2006b; Bowman et al., 2008). Ectopic Notch signaling activity may even directly promote the expansion of neuroblasts after they have differentiated from the neuroepithelial cells in the OPC. In either case, ectopic Notch signaling activity may block the normal path of neuronal differentiation and lock the cells in a proliferative state. This is indeed what we have observed in $numb^{15}$ mosaic clones in which numerous ectopic neuroblasts were induced in the medulla cortex without generating medulla neurons. Perhaps ectopic Notch signaling activity may also promote the proliferation of neural progenitors in vertebrates, such as the radial glial cells in the mouse brain.

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