KEEPING NEURAL PROGENITORS ON A SHORT LEASH: DISTINGUISH INTERMEDIATE PROGENITORS FROM STEM CELLS IN DEVELOPING *DROSOPHILA* OPTIC LOBE AND CENTRAL BRAIN

by

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To

my parents
Yijun Li and Xiongyi Weng

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ABSTRACT

Producing intermediate progenitors is one of the critical strategies for stem cells to amplify their output and generate diversity, allowing stem cells to meet demanding requirement during development, homeostasis and tissue repair. In contrast to stem cells, intermediate progenitors possess restricted developmental potential limiting the number and types of their progeny. Failure to establish or maintain restricted potential can perturb tissue development and homeostasis, and probably contributes to tumor initiation. My thesis work revealed the mechanisms that distinguish intermediate progenitors from stem cells during the neurogenesis in both central brain and optic lobe of fruit fly *Drosophila* larval brain.

By examining neuroepithelial stem cell lineage in larval optic lobe, I showed that the restricted potential of intermediate progenitors is established through highly ordered sequential steps precisely paced by a spatial fluctuation of Notch activity. I identified the intermediate cell types during the differentiation of neuroepithelial stem cells into intermediate progenitors and Notch activities in those cell types indicate that while down-regulation of Notch is required for generating intermediate progenitors, up-regulation of Notch immediately before the transition is critical to prevent premature differentiation. Notch signaling plays two roles in this process: maintains the neuroepithelial stem cell identify via downstream target *aop* and prevents premature

differentiation into intermediate progenitors by raising the threshold of response to *pointP1*.

Using central brain type II neuroblast lineages as the model system, I showed that the restricted potential of intermediate neural progenitors (INPs) needs to be actively maintained after correct specification and identified a novel transcription factor *earmuff* as the major factor maintaining restricted potential and preventing dedifferentiation of INPs. *earmuff* mutant shows ectopic generation of neural stem cells although the asymmetric division of neural stem cells or INPs and the specification of INPs are completely normal. *earmuff* mutant INPs can dedifferentiate back into a neural stem cell state, functionally indistinguishable from normal neural stem cells. Earmuff uses two independent mechanisms to restrict the potential of intermediate neural progenitors: promoting nuclear localization of Prospero to limit proliferation and antagonizing Notch signaling to prevent dedifferentiation.

CHAPTER I

Introduction

The developmental potential of stem cells and progenitor cells must be functionally distinguished to ensure the generation of diverse cell types while maintaining the stem cell pool throughout the lifetime of an organism. In contrast to stem cells, progenitor cells possess restricted developmental potential, allowing them to give rise to only a limited number of post-mitotic progeny. Failure to establish or maintain restricted progenitor cell potential can perturb tissue development and homeostasis, and likely contributes to tumor initiation. How progenitor cell potential is restricted remains largely unknown due to their short-lived nature. The fruit fly *Drosophila* larval brain, which consists of the central brain and optic lobe, possesses well-defined lineages of neural stem cells that generate progenitor cells in a highly reproducible pattern (Figure 1.1). These lineages provide an excellent *in vivo* system for studying regulation of the progenitor cell potential at a single-cell resolution. Conservation in gene function between flies and mammals suggests that molecular mechanisms that regulate progenitor cell potential in *Drosophila* neural stem cell lineages might be similarly employed during vertebrate neurogenesis.

Central brain neuroblasts generate neural progenitor cells with distinct developmental potential

All neural stem cells in the central brain (called neuroblasts) undergo repetitive asymmetric divisions to self-renew and to generate a neural progenitor cell with limited developmental potential. The cortex of a mitotic central brain neuroblast is highly polarized, and the role of this polarity in neuroblast asymmetric division has been extensively reviewed (Doe, 2008; Gonczy, 2008; Knoblich, 2008; Wu et al., 2008). Discrete protein complexes are assembled in the apical and basal cortical domains. In telophase, the apical protein complexes segregate into the self-renewing neuroblast, whereas the basal protein complexes segregate into the neural progenitor cell. Both genetic and correlative live imaging studies indicate that the apical protein complexes have dual functions: promoting neuroblast identity and targeting the basal protein complexes into the neural progenitor cell. The basal protein complexes function specifically in restricting the neural progenitor cell potential (Cabernard and Doe, 2009). Two classes of central brain neuroblast lineages (types I and II) can be unambiguously identified based on the progenitor progeny generated and the combination of cell fate markers expressed (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008) (Figure 1.1).

Notch signaling pathway during neurogenesis in *Drosophila*

Notch signaling pathway is evolutionarily conserved and plays a critical role in diverse processes in different tissues at multiple stages during development and is also involved in the maintenance of many self-renewing adult tissue (Bray, 2006; Kopan and Ilagan, 2009). In most circumstances, it mediates communication between physically contacted cells via membrane bound receptor and ligand. While there are multiple Notch receptors and ligands in mammals, there are only one Notch receptor and two ligands Delta and Serrate in *Drosophila*. Notch siganling is often used to make the binary cell-fate decisions but is also implied to directly regulate the maintenance of stem cells.

Notch receptor precursor is post-translationally processed by enzymes in endoplasmic reticulum and Golgi. Activation of Notch signaling pathway requires the receptor proteolysis induced by ligand binding. The proteolytic cleavage releases Notch intracellular domain which enters the nucleus to activate transcription of target genes via binding to transcriptional factor CSL and other co-activators (Figure 1.2).

There are three modes in regulating Notch activity to make different cell fates. The lateral inhibition mediated by Notch signaling selects neural precursors from a group of equivalent cells in neuroectoderm during embryogenesis. During this process, feedback mechanism amplifies the small difference in Notch activity between cells and the cell with the lowest Notch activity will become the neural precursor. Another way of making differential Notch activity is to differentially segregate regulators of Notch signaling into two daughter cells during asymmetric cell division. In *Drosophila* peripheral nervous system, the sensory organ precursor asymmetrically divides and segregates Notch inhibitor Numb into one daughter cell and thus produces two daughter cells with distinct fates. Notch signaling can also be activated between two populations of cells and leads to compartmentalization of the developing tissue. For example, Notch signaling segregates the dorsal and ventral parts of the wing imaginal discs.

Notch was initially identified and studied as an important factor for neurogenesis in *Drosophila*. Examination of Notch reporter activity shows that Notch is activated in larval central brain and optic lobe (Almeida and Bray, 2005; Bowman et al., 2008; Reddy et al., 2010). Its activity in different cell types in the larval brain will be discussed in the following sections.

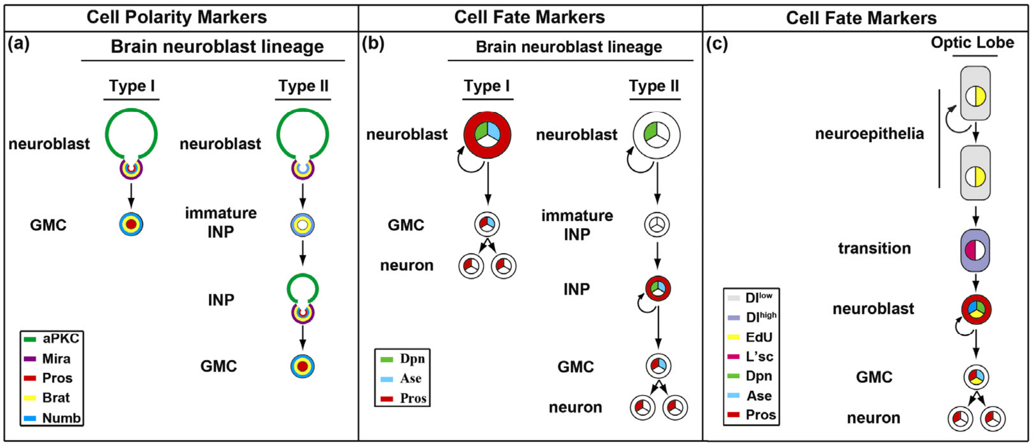

Figure 1.1. Neural stem cell lineages in the developing *Drosophila* larval brain. (a) The apical and basal protein complexes unequally segregate during asymmetric divisions of neural stem/progenitor cells in the type I and type II neuroblast lineages in the larval brain. Abbreviation: aPKC: atypical Protein Kinase C; Mira: Miranda; Pros: Prospero; Brat: Brain tumor. (b) The cell fate markers allow unambiguous identification of neural stem/progenitor cells in the type I and type II neuroblast lineages in the larval brain. Abbreviation: Dpn: Deadpan; Ase: Asense; Pros: Prospero. (c) The cell fate markers allow unambiguous identification of neuroepithelial stem cells and progenitor cells in the optic lobe. Abbreviation: Dl: Delta; EdU: 5-ethynyl-2´ -deoxyuridine; L'sc: Lethal of scute; Dpn: Deadpan; Ase: Asense; Pros: Prospero.

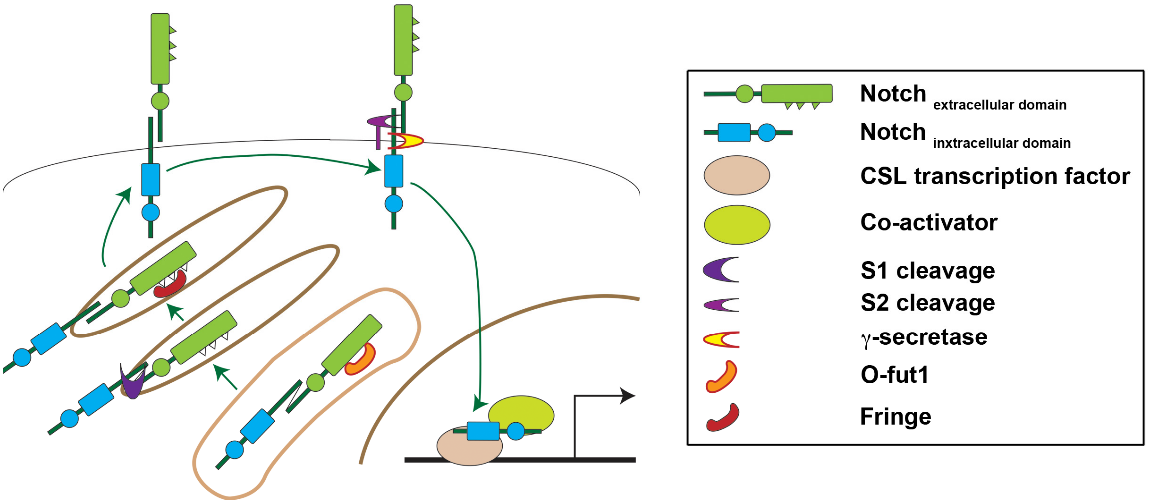

Figure 1.2. Activation of Notch signaling pathway

A simplified cartoon summarizes processing and activation of the Notch receptor protein.

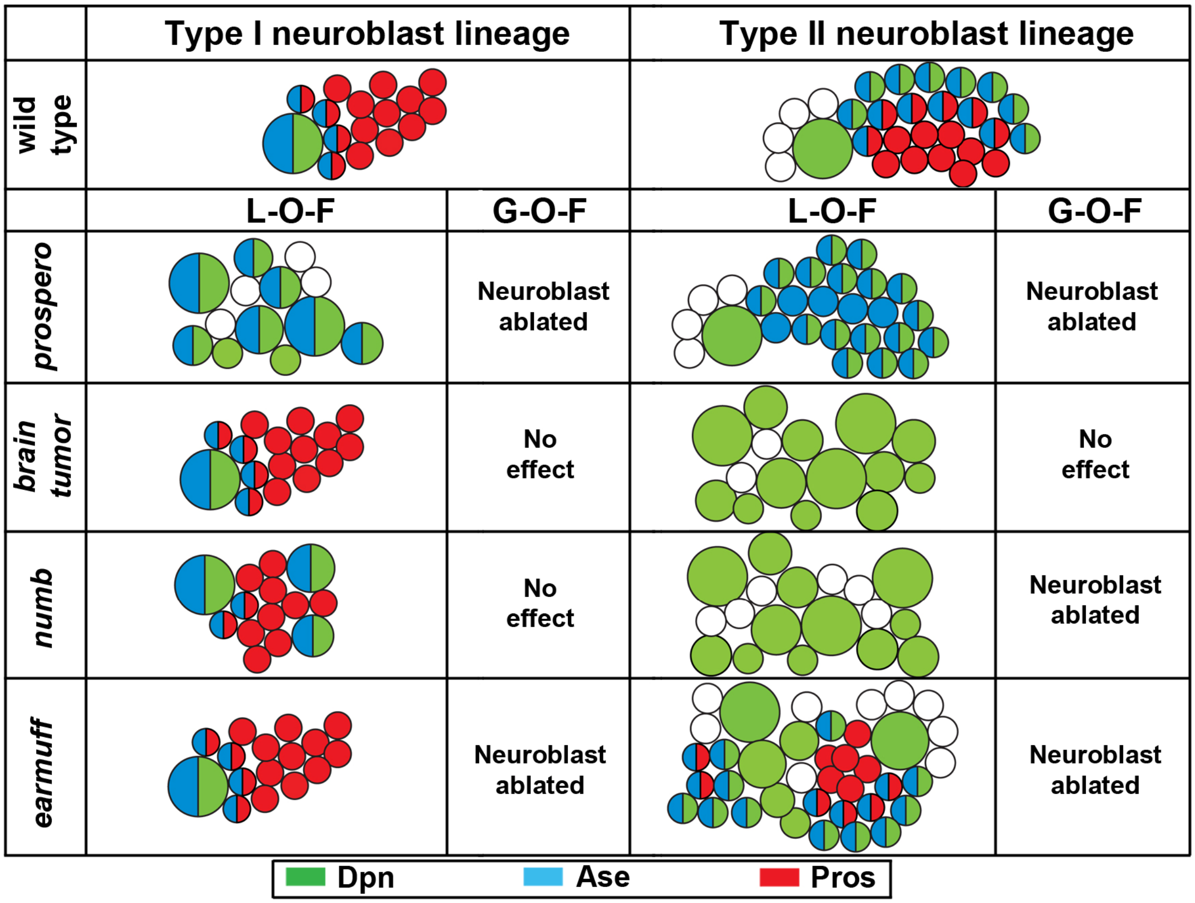

Figure 1.3. A summary of the fate of cells derived from type I and II neuroblasts lacking or over-expressing key proteins required to restrict the progenitor cell potential. Nuclear proteins Dpn, Ase and Pros are used to distinguish different cell types: type I neuroblasts are Dpn$^+$Ase$^+$ whereas type II neuroblasts are Dpn$^+$Ase$^-$. Abbreviation: L-O-F: loss-of-function; G-O-F: gain-of-function; Dpn: Deadpan; Ase: Asense; Pros: Prospero.

Neuroblasts and neural progenitor cells in the type I lineage

A type I neuroblast divides asymmetrically to generate a self-renewing daughter neuroblast and a neural progenitor cell called a ganglion mother cell (GMC) which divides once to produce two post-mitotic neurons (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). During this asymmetric division, the basal proteins Brain tumor and Prospero exclusively segregate into the GMC by binding to the scaffolding protein Miranda, while Numb partitions into the GMC independently of Miranda. The basal proteins remain asymmetrically segregated into GMCs during asymmetric divisions of a *brain tumor* mutant type I neuroblast, while genetic clones derived from single *brain tumor* mutant type I neuroblasts always contain one neuroblast and many neurons per clone (figure 2). Thus, Brain tumor is either dispensable or functionally redundant with other proteins in restricting the GMC potential.

prospero encodes a homeodomain transcription factor, and plays a key role in specifying neuronal and glial cell types in the developing nervous system (Choksi et al., 2006; Chu-Lagraff et al., 1991; Freeman and Doe, 2001; Manning and Doe, 1999). Although Prospero is expressed in neuroblasts, it is kept out of neuroblast nuclei by the combination of nuclear exclusion and Miranda-binding mediated cytoplasmic sequester (Ikeshima-Kataoka et al., 1997; Matsuzaki et al., 1998; Schuldt et al., 1998; Shen et al., 1997). The Miranda-Prospero complex localizes to the basal cortex of a mitotic neuroblast in metaphase and asymmetrically segregates into the GMC in telophase. Upon completion of cell division, Miranda becomes proteolytically degraded, and Prospero is released from the cortex and localizes to the GMC nuclei (Wang et al., 2006). Nuclear Prospero restricts the GMC potential by suppressing genes that promote the neuroblast identity and activating genes that promote differentiation and cell cycle exit (Choksi et al., 2006; Li and Vaessin, 2000). While mitotic *prospero* mutant type I neuroblasts exhibit normal apical-basal cortical polarity, *prospero* mutant type I neuroblast lineage clones contain almost exclusively neuroblasts at the expense of neurons (Bello et al., 2006; Betschinger et al., 2006; Choksi et al., 2006; Lee et al., 2006; Weng et al., 2010) (figure 2). Over-expression of Prospero leads to constitutive accumulation of Prospero in neuroblast nuclei, triggering premature loss of neuroblasts. These data indicate that Prospero is necessary and sufficient to restrict the GMC potential.

Numb, an evolutionarily-conserved protein essential for proper neuronal fate specification in the developing nervous system, unequally partitions into GMCs via a Miranda-independent mechanism during asymmetric divisions of neuroblasts (Rhyu et al., 1994; Spana and Doe, 1996; Spana et al., 1995; Uemura et al., 1989; Zhong et al., 1996). Eighty-five percent of *numb* mutant type I neuroblast lineage clones contain more than one neuroblast per clone despite asymmetric segregation of Miranda into GMCs (Bowman et al., 2008; Wang et al., 2007)(figure 2). Furthermore, mutations that perturb asymmetric segregation of Numb into GMCs lead to formation of ectopic neuroblasts, a phenotype that can be suppressed by over-expression of Numb in neuroblasts(Wang et al., 2007; Wirtz-Peitz et al., 2008). Thus, Numb likely restricts the GMC potential independent of Prospero. Fly and mouse studies have shown that Numb suppresses Notch signaling in the developing nervous system, raising the possibility that Numb might restrict the GMC potential by antagonizing Notch signaling. Expression of multiple *Notch* reporters is detectable in neuroblasts but is undetectable in GMCs in the wild type brain (Almeida and Bray, 2005; Bowman et al., 2008). Additionally, ectopic expression of a constitutively active form of Notch ($\mathrm{Notch}_{\mathrm{intra}}$) perturbs neuroblast asymmetric divisions, leading to a massive increase in neuroblasts at the expense of neurons (Almeida and Bray, 2005; Bowman et al., 2008). Unlike Prospero, ectopic expression of Numb or knock-down of the *Notch* function by RNA interference is insufficient to trigger premature loss of type I neuroblasts (Bowman et al., 2008). Thus, inhibition of the Notch signaling by Numb is necessary but not sufficient to limit the GMC potential.

Neuroblasts and neural progenitors in the type II lineage

A type II neuroblast divides asymmetrically to self-renew and generate an intermediate neural progenitor cell (INP), previously referred to as a transit amplifying GMC, a secondary neuroblast or an intermediate progenitor (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008)(Figure 1). A newly-born INP is immature and it is arrested in the G2 phase of the cell cycle and must undergo maturation, during which it acquires restricted developmental potential prior to resuming proliferation (Bowman et al., 2008). The mature INP divides asymmetrically several times, each time self-renewing by producing a daughter INP and a GMC. The basal proteins Brain tumor and Numb, inherited from the asymmetrically dividing parental neuroblasts, establish the restricted developmental potential in an immature INP (Bowman et al., 2008). In contrast to type I lineages, Pros is not expressed in type II neuroblasts but in INPs and segregated into GMCs. These sequential mechanisms and/or other unidentified mechanisms ensure that INPs are short-lived and can only generate progeny destined for terminal differentiation.

Establishment of the restricted developmental potential in INPs

While a wild-type type II neuroblast clone always contains one neuroblast, 3-5 immature INPs and 20-30 INPs, a *brain tumor* mutant type II neuroblast clone contains almost exclusively neuroblasts (Bowman et al., 2008) (figure 2). Interestingly, a mitotic *brain tumor* mutant type II neuroblast shows normal apical-basal cortical polarity and asymmetric segregation of Numb into immature INPs. Thus, ectopic type II neuroblasts in the *brain tumor* mutant brain likely arise from de-differentiation of immature INPs that fail to acquire restricted developmental potential despite inheriting Numb. These data suggest that Brain tumor likely functions parallel to Numb to promote restriction of the INP potential. Over-expression of Brain tumor does not effect the expression of a *Notch* reporter in neuroblasts, and removal of *brain tumor* does not alter binary cell fate determination in the sensory organ precursor lineage, a system highly sensitive to the loss of *Notch* function (Bowman et al., 2008). Together, these data strongly suggest that Brain tumor is necessary but not sufficient to restrict the INP developmental potential.

Despite showing normal apical-basal cortical polarity and asymmetric segregation of Brain tumor into immature INPs, *numb* mutant type II neuroblast clones consist of mostly neuroblasts (Bowman et al., 2008; Wang et al., 2007) (Figure 1.2). Thus, ectopic type II neuroblasts in the *numb* mutant brain might arise from de-differentiation of immature INPs due to aberrant activation of the Notch signaling mechanism. Indeed, ectopic expression of $\text{Notch}_{\text{intra}}$ leads to ectopic type II neuroblasts at the expense of immature INPs, whereas over-expression of Numb or knock-down of the *Notch* function by RNA interference results in the premature loss of type II neuroblasts (Bowman et al., 2008). Thus, by antagonizing Notch, Numb is necessary and sufficient to establish the restricted developmental potential in immature INPs. Taken together, Brain tumor and Numb function non-redundantly to establish the INP potential during maturation.

Optic lobe neuroepithelial stem cells generate two types of neural progenitor cells

Neuroepithelial stem cells in the developing optic lobe initially undergo symmetric divisions to expand the stem cell population, then differentiate into neural progenitors that generate terminally-differentiated neurons through limited rounds of asymmetric divisions (Egger et al., 2007) (Figure 1.1). This dynamic mechanism allows rapid generation of a large number of post-mitotic progeny from a relatively small population of stem cells, and is widely used in the context of development and regeneration (Farkas and Huttner, 2008; Kriegstein and Alvarez-Buylla, 2009). Failure to properly restrict the developmental potential in neuroepithelial stem cells and their progenitor progeny might contribute to childhood tumors of epithelial origin (Dubuc et al., 2010; Hadjipanayis and Van Meir, 2009). Thus, understanding how developmental potential is precisely specified in neuroepithelial stem cells and neural progenitor cells will likely provide novel insight into development and tumorigenesis.

The functional property of neuroepithelial stem cells changes dynamically in the outer proliferation center of the developing optic lobe. Prior to the third larval instar, most neuroepithelial stem cells predominantly undergo symmetric divisions to expand the stem cell population, forming a C-shaped swath flanked with few neuroblasts at the medial edge bordering the central brain. In the third larval instar, neuroepithelial stem cells progressively transition into neuroblasts from the medial edge toward the lateral edge of the optic lobe, leading to narrowing of the neuroepithelia and widening of the neuroblast swath (Egger et al., 2007; Nassif et al., 2003; Yasugi et al., 2008). Neuroblasts in the optic lobe share many parallels with INPs in the central brain, including expression of similar cell fate markers and asymmetric segregation of similar cell polarity proteins. A neuroblast in the optic lobe also undergoes limited rounds of asymmetric divisions to regenerate and to produce a GMC that gives rise to two terminally-differentiated progeny (Egger et al., 2007; Lee et al., 2006)]. However, the molecular mechanism that restricts the neuroblast potential in the optic lobe has yet to be investigated and will not be discussed further. Below, we will focus on the molecular mechanism that regulates the neuroepithelial stem cells.

Comparative expression profiling of micro-dissected neuroepithelia and neuroblasts from the optic lobe suggests that the Notch signaling mechanism likely plays a key role in maintaining the neuroepithelial stem cell identity (Egger et al., 2010). Removal of *Notch* function triggers premature transition from neuroepithelia to neuroblasts, whereas constitutive activation of Notch signaling prevents the transition. Thus, down-regulation of Notch signaling is necessary and sufficient for the transition from neuroepithelia to neuroblasts in the larval optic lobe.

How is the Notch signaling spatially and temporally regulated in the developing optic lobe allowing synchronous transition from neuroepithelial stem cells to neuroblasts in a medial-to- lateral manner? Neuroepithelial stem cells become transiently arrested in cell cycle prior to reaching the transition zone where they lose their epithelial characteristics and assume the stereotypical round neuroblast morphology(Reddy et al., 2010). *delta*, encoding a Notch ligand, is expressed at a high level in 1-2 rows of cells that are among those transiently arrested in cell cycle (Reddy et al., 2010; Yasugi et al., 2008). Since Delta activates Notch signaling non-cell autonomously and suppresses Notch signaling cell autonomously, over-expression or removal of *delta* leads to both inhibition and acceleration of neuroblast formation within the same clone. This result suggests that the coordinated change between the level of Delta and the Notch signaling provides the cue that times the transition from neuroepithelia to neuroblasts. Interestingly, the proneural gene *lethal of scute* is also highly expressed in 1-2 rows of cells that are among those transiently arrested in cell cycle (Reddy et al., 2010; Yasugi et al., 2008). While removal of the *lethal of scute* function mildly delays the transition of neuroepithelial stem cells to neuroblasts, over-expression of *lethal of scute* suppresses Notch signaling and promotes premature transition. The dynamic integration of Delta and Lethal of scute specifies the transition from neuroepithelia to neuroblasts spatially in the optic lobe by repressing the Notch signaling.

The swath of neuroblasts widens synchronously from the medial edge toward the lateral edge of the developing optic lobe, suggesting that the transition from neuroepithelia to neuroblasts might also be temporally coordinated. Intriguingly, the output of the Janus kinase (Jak/Stat) signaling mechanism coincides with the timing of neuroepithelia transitioning into neuroblasts: Jak/Stat signaling is the highest at the lateral edge and the lowest at the medial edge. Removal of the components in the Jak/Stat signaling mechanism leads to precocious transition of neuroepithelia into neuroblasts, while constitutive activation of the Jak/Stat signaling delays the transition (Yasugi et al., 2008). In addition, inactivation of the Fat-Hippo signaling mechanism delays the transition from neuroepithelia to neuroblasts, whereas constitutive activation of the Fat-Hippo signaling accelerates the transition at the medial edge of neuroepithelia (Reddy et al., 2010). Taken together, the Jak/Stat and the Fat-Hippo signaling mechanisms provide temporal control of the transition from neuroepithelia to neuroblasts. More experiments will be necessary to elucidate whether these two signaling pathways promote the transition through Notch or independent of Notch.

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References

Almeida, M.S., and Bray, S.J. (2005). Regulation of post-embryonic neuroblasts by Drosophila Grainyhead. Mechanisms of Development *122*, 1282-1293.

Bello, B., Reichert, H., and Hirth, F. (2006). The brain tumor gene negatively regulates neural progenitor cell proliferation in the larval central brain of Drosophila. Development *133*, 2639-2648.

Bello, B.C., Izergina, N., Caussinus, E., and Reichert, H. (2008). Amplification of neural stem cell proliferation by intermediate progenitor cells in Drosophila brain development. Neural Develop *3*, 5.

Betschinger, J., Mechtler, K., and Knoblich, J.A. (2006). Asymmetric segregation of the tumor suppressor brat regulates self-renewal in Drosophila neural stem cells. Cell *124*, 1241-1253.

Boone, J.Q., and Doe, C.Q. (2008). Identification of Drosophila type II neuroblast lineages containing transit amplifying ganglion mother cells. Dev Neurobiol.

Bowman, S.K., Rolland, V., Betschinger, J., Kinsey, K.A., Emery, G., and Knoblich, J.A. (2008). The tumor suppressors Brat and Numb regulate transit-amplifying neuroblast lineages in Drosophila. Dev Cell *14*, 535-546.

Bray, S.J. (2006). Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol *7*, 678-689.

Cabernard, C., and Doe, C.Q. (2009). Apical/Basal Spindle Orientation Is Required for Neuroblast Homeostasis and Neuronal Differentiation in Drosophila. Dev Cell *17*, 134-141.

Choksi, S.P., Southall, T.D., Bossing, T., Edoff, K., de Wit, E., Fischer, B., van Steensel, B., Micklem, G., and Brand, A.H. (2006). Prospero Acts as a Binary Switch between Self-Renewal and Differentiation in

Drosophila Neural Stem Cells. *11*, 775-789.

Chu-Lagraff, Q., Wright, D.M., McNeil, L.K., and Doe, C.Q. (1991). The prospero gene encodes a divergent homeodomain protein that controls neuronal identity in Drosophila. Development *113*, 79-85.

Doe, C.Q. (2008). Neural stem cells: balancing self-renewal with differentiation. Development *135*, 1575-1587.

Dubuc, A., Northcott, P., Mack, S., Witt, H., Pfister, S., and Taylor, M. (2010). The Genetics of Pediatric Brain Tumors. Current Neurology and Neuroscience Reports *10*, 215-223.

Egger, B., Boone, J., Stevens, N., Brand, A., and Doe, C. (2007). Regulation of spindle orientation and neural stem cell fate in the Drosophila optic lobe. Neural Development *2*, 1.

Egger, B., Gold, K., and Brand, A. (2010). Notch regulates the switch from symmetric to asymmetric neural stem cell division in the Drosophila optic lobe. Development.

Farkas, L.M., and Huttner, W.B. (2008). The cell biology of neural stem and progenitor cells and its significance for their proliferation versus differentiation during mammalian brain development. Current Opinion in Cell Biology *20*, 707-715.

Freeman, M.R., and Doe, C.Q. (2001). Asymmetric Prospero localization is required to generate mixed neuronal/glial lineages in the Drosophila CNS. Development *128*, 4103-4112.

Gonczy, P. (2008). Mechanisms of asymmetric cell division: flies and worms pave the way. Nat Rev Mol Cell Biol *9*, 355-366.

Hadjipanayis, C.G., and Van Meir, E.G. (2009). Brain cancer propagating cells: biology, genetics and targeted therapies. Trends in molecular medicine *15*, 519-530.

Ikeshima-Kataoka, H., Skeath, J.B., Nabeshima, Y., Doe, C.Q., and Matsuzaki, F. (1997). Miranda directs Prospero to a daughter cell during Drosophila asymmetric divisions. Nature *390*, 625-629.

Knoblich, J.A. (2008). Mechanisms of Asymmetric Stem Cell Division. Cell *132*, 583-597.

Kopan, R., and Ilagan, M.X.G. (2009). The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism. Cell *137*, 216-233.

Kriegstein, A., and Alvarez-Buylla, A. (2009). The Glial Nature of Embryonic and Adult Neural Stem Cells. Annual Review of Neuroscience *32*, 149-184.

Lee, C.Y., Wilkinson, B.D., Siegrist, S.E., Wharton, R.P., and Doe, C.Q. (2006). Brat is a Miranda cargo protein that promotes neuronal differentiation and inhibits neuroblast self-renewal. Dev Cell *10*, 441-449.

Li, L., and Vaessin, H. (2000). Pan-neural Prospero terminates cell proliferation during Drosophila neurogenesis. Genes & Development *14*, 147-151.

Manning, L., and Doe, C.Q. (1999). Prospero distinguishes sibling cell fate without asymmetric localization in the Drosophila adult external sense organ lineage. Development *126*, 2063-2071.

Matsuzaki, F., Ohshiro, T., Ikeshima-Kataoka, H., and Izumi, H. (1998). miranda localizes staufen and prospero asymmetrically in mitotic neuroblasts and epithelial cells in early Drosophila embryogenesis. Development *125*, 4089-4098.

Nassif, C., Noveen, A., and Hartenstein, V. (2003). Early development of the Drosophila brain: III. The pattern of neuropile founder tracts during the larval period. The Journal of Comparative Neurology *455*, 417-434.

Reddy, B.V.V.G., Rauskolb, C., and Irvine, K.D. (2010). Influence of Fat-Hippo and Notch signaling on the proliferation and differentiation of Drosophla optic neuroepithelia. Development.

Rhyu, M.S., Jan, L.Y., and Jan, Y.N. (1994). Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. Cell *76*, 477-491.

Schuldt, A.J., Adams, J.H.J., Davidson, C.M., Micklem, D.R., Haseloff, J., Johnston, D.S., and Brand, A.H. (1998). Miranda mediates asymmetric protein and RNA localization in the developing nervous system. Genes & Development *12*, 1847-1857.

Shen, C.P., Jan, L.Y., and Jan, Y.N. (1997). Miranda is required for the asymmetric localization of Prospero during mitosis in Drosophila. Cell *90*, 449-458.

Spana, E.P., and Doe, C.Q. (1996). Numb Antagonizes Notch Signaling to Specify Sibling Neuron Cell Fates. Neuron *17*, 21-26.

Spana, E.P., Kopczynski, C., Goodman, C.S., and Doe, C.Q. (1995). Asymmetric localization of numb autonomously determines sibling neuron identity in the Drosophila CNS. Development *121*, 3489-3494.

Uemura, T., Shepherd, S., Ackerman, L., Jan, L.Y., and Jan, Y.N. (1989). numb, a gene required in determination of cell fate during sensory organ formation in Drosophila embryos. Cell *58*, 349-360.

Wang, H., Cai, Y., Chia, W., and Yang, X. (2006). Drosophila homologs of mammalian TNF/TNFR-related molecules regulate segregation of Miranda/Prospero in neuroblasts. EMBO J *25*, 5783-5793.

Wang, H., Ouyang, Y., Somers, W.G., Chia, W., and Lu, B. (2007). Polo inhibits progenitor self-renewal and regulates Numb asymmetry by phosphorylating Pon. Nature *449*, 96-100.

Weng, M., Golden, K.L., and Lee, C.Y. (2010). dFezf/Earmuff maintains the restricted developmental potential of intermediate neural progenitors in Drosophila. Dev Cell *18*, 126-135.

Wirtz-Peitz, F., Nishimura, T., and Knoblich, J.A. (2008). Linking Cell Cycle to Asymmetric Division: Aurora-A Phosphorylates the Par Complex to Regulate Numb Localization. Cell *135*, 161-173.

Wu, P.-S., Egger, B., and Brand, A.H. (2008). Asymmetric stem cell division: Lessons from Drosophila. Seminars in Cell & Developmental Biology *19*, 283-293.

Yasugi, T., Umetsu, D., Murakami, S., Sato, M., and Tabata, T. (2008). Drosophila optic lobe neuroblasts triggered by a wave of proneural gene expression that is negatively regulated by JAK/STAT. Development *135*, 1471-1480.

Zhong, W., Feder, J.N., Jiang, M.-M., Jan, L.Y., and Jan, Y.N. (1996). Asymmetric Localization of a Mammalian Numb Homolog during Mouse Cortical Neurogenesis. Neuron *17*, 43-53.

CHAPTER II

Spatial Changes in *Notch* Signaling Coordinates Maintenance and Differentiation of the *Drosophila* Larval Optic Lobe Neuroepithelia

Summary

A dynamic balance between stem cell maintenance and differentiation paces generation of post-mitotic progeny during tissue morphogenesis, but the mechanisms remain largely unknown. Recent studies show that *Notch* signaling play a critical role in the developing *Drosophila* larval optic lobe where neuroepithelia differentiate into neuroblasts, but in which cell types *Notch* function and the molecular mechanism involved are unclear. We showed here that Notch has two functions during this process: maintains non-differentiating neuroepithelia and prevent premature differentiation of the neuroepithelia committed to differentiation. Inactivation of *Notch* signaling led to the entire swath of neuroepithelial cells in the outer proliferation center prematurely differentiating into neuroblasts resulting in a fragmented neuronal network in the developing optic lobe. We showed that a low level of *Notch* signaling functions to maintain the neuroepithelial cell identity by suppressing the expression of *pointedP1* gene through the transcriptional repressor Anterior open. An increase in *Notch* signaling near the medial edge of neuroepithelia coincides with transient cell cycle arrest but

precedes the expression of PointedP1. This high level of *Notch* signaling defines neuroepithelial cells that are in the process of differentiating into optic lobe neuroblasts by raising their threshold of the response to PointedP1. Down-regulation of *Notch* signaling combined with a high level of PointedP1 at the medial edge of neuroepithelia trigger a synchronous transition from differentiating neuroepithelial cells to future neuroblasts. Thus, spatial changes in *Notch* signaling orchestrate a dynamic balance between maintenance and differentiation of neuroepithelial cells that paces generation of neurons during larval optic lobe neurogenesis.

Introduction

During mammalian cortical neurogenesis, neural stem cells initially divide symmetrically to expand their population, and then divide asymmetrically to produce layer-specific neurons (Kriegstein and Alvarez-Buylla, 2009). Thus, a precise control of stem cell maintenance versus differentiation directly impinges on the pace of generating post-mitotic progeny in a developing tissue, but the underlying mechanisms remain virtually unknown. In the fly larval optic lobe, neuroepithelial cells divide symmetrically to expand their population during the first two larval instars, and then progressively differentiate into neuroblasts that divide asymmetrically to generate neurons (Egger et al., 2007). Several recent studies show that down-regulation of *Notch* signaling is necessary and sufficient for differentiation of neuroepithelia (Egger et al., 2010; Ngo et al., 2010; Orihara-Ono et al., 2011; Reddy et al., 2010; Wang et al., 2011; Yasugi et al., 2010).

However, these studies also reported that the *Notch* activity becomes up-regulated prior to differentiation of neuroepithelial cells. One study suggests that activation of the EGF receptor triggers increased *Notch* signaling, and proposes that *Notch* and *EGF* signaling function cooperatively to assure the directional progression of differentiation (Yasugi et al., 2010). However, how *Notch* and *EGF* might function in concert to regulate differentiation of neuroepithelia cannot be fully understood until several fundamental questions are addressed. For instance, what is the functional distinction between neuroepithelial cells actively differentiating and the rest of neuroepithelia? What is the molecular mechanism by which *Notch* maintains the identity of neuroepithelial cells? What purpose does up-regulation of *Notch* signaling serve in neuroepithelial cells prior to becoming neuroblasts?

The *Notch* signaling pathway is highly conserved and regulates many key developmental processes (Bray, 2006; Okajima and Irvine, 2002). The Notch receptor precursor is post-translationally modified by enzymes such as O-fucosyltransferase in the ER and then undergoes the S1 proteolytic cleavage in the Golgi prior to being trafficked to the membrane. Ligand binding triggers the S2 and S3 proteolytic cleavage of the Notch precursor protein, and releases the intracellular domain, which activates its target gene transcription. Recent studies have suggested two distinct mechanisms by which the ligand can regulate *Notch* signaling (del Álamo et al., 2011; Sprinzak et al., 2010). The ligand can activate *Notch* signaling in the adjacent cell, so that the level of the ligand correlates with the level of signaling output. However, the ligand can also inactivate

Notch signaling in the same cell such that above the threshold-level of the ligand inhibits *Notch* signaling. *cis*-inhibition of *Notch* signaling by the ligand can sharply define the boundary between cells that show activated *Notch* signaling and the adjacent cells that lack activated *Notch* signaling. The combination of *cis*-inhibitory and *trans*-activation of *Notch* signaling provides a precise mechanism to limit the zone of the Notch activity.

In this study, we describe a genetic screen that allows systematic identification of mutations that perturb differentiation of neuroepithelia into neuroblasts in the larval optic lobe. We specifically focus our study on the role of *Notch* signaling in the maintenance and differentiation of neuroepithelia in the outer proliferation center. We show that a low level of *Notch* signaling maintains the identity of neuroepithelial cells through Anterior open (Aop)-dependent repression of the *pointedP1* (*pntP1*) gene. An increase in *Notch* signaling located near the medial edge of neuroepithelia functionally defines neuroepithelial cells that are actively differentiating into future optic lobe neuroblasts. Transient up-regulation of *Notch* signaling paces progressive differentiation of the entire swath of neuroepithelia into future neuroblasts in a medial-to-lateral orientation by increasing the threshold of response to PntP1. Finally, the combination of abrupt down-regulation of *Notch* signaling and a high level of PntP1 triggers a synchronous transition from differentiating neuroepithelial cells to future neuroblasts. Our data strongly suggest that spatial changes in *Notch* signaling dynamically balance maintenance and differentiation of neuroepithelial cells during larval optic lobe morphogenesis.

Results

Differentiation of neuroepithelia into neuroblasts in the outer proliferation center

The larval optic lobe consists of the outer and the inner proliferation center that each consists of neuroepithelial cells and neuroblasts (Egger et al., 2007). We used expression of *GH146-gal4* or the apical marker PatJ as the marker for neuroepithelial cells and Deadpan (Dpn) as the marker for neuroblasts to unambiguously identify these cells in the whole-mount larval optic lobe (Figure 2.1 A). The outer proliferation center is located on the lateral surface of the optic lobe, and contains neuroepithelial cells that form a single-cell layer, C-shaped swath and progressively differentiate into neuroblasts from the medial edge (Egger et al., 2007). In this study, we presented two distinct views for visualizing neuroepithelia in the developing outer proliferation center. First, a lateral projection of confocal z-sections shows the overall morphology of the neuroepithelia in the outer proliferation center (Figures 2.1 B and C). Second, a single confocal optical section illustrates the interface between neuroepithelia and neuroblasts at a single cell level (Figures 2.1 B' and D). Neuroblasts derived from neuroepithelial cells generate a large number of neurons that give rise to a funnel-shape neuropil as revealed by staining using Phalloidin (Figure 2.1 E).

The transition from a neuroepithelial cell into a neuroblast occurs at the medial edge of neuroepithelia. Thus, defining the identity of cells at the medial edge of neuroepithelia and those immediately adjacent to them is pivotal for investigating

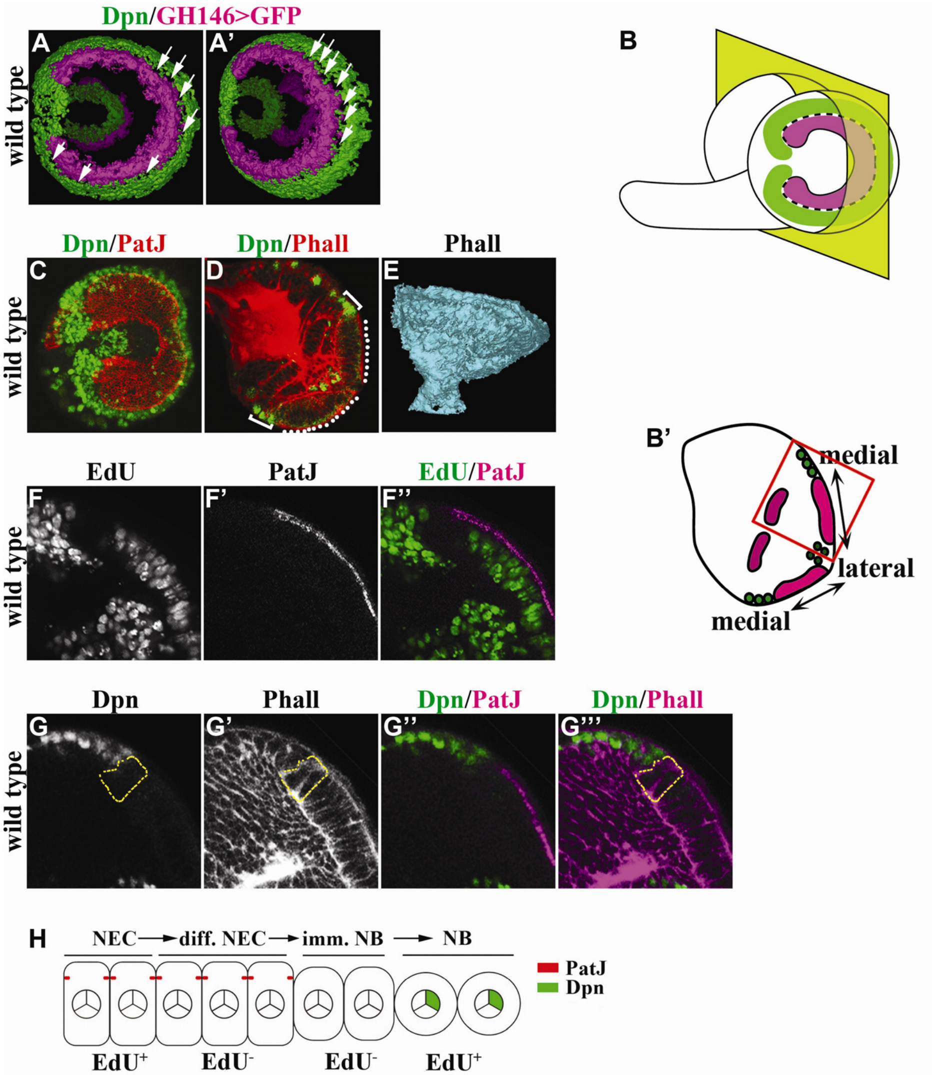

Figure 2.1. Identification of intermediate cell types during differentiation of neuroepithelial cells into neuroblasts

(A-A') Three-dimensionally reconstructed views of the outer and inner proliferation center in the third larval instar optic lobe. The expression pattern of *GH146-gal4* marks neuroepithelia in the outer (bright colored) and inner (faded colored) proliferation center, whereas expression of Deadpan (Dpn) marks neuroblasts. White arrows indicate immature neuroblasts (see below for more detail).

(B-B') Visualization of the developing larval optic lobe. (B) A lateral view of a wild type larval optic lobe shows the overall morphology of the outer proliferation center, which consists of a C-shape swath of neuroepithelia (magenta) surrounded by neuroblasts (green). The medial edge of neuroepithelia separating neuroepithelial cells and neuroblasts is indicated by the black & white dotted line. (B') An anteroposterior single confocal optical section of a wild type larval brain reveals neuroepithelia in the inner and outer proliferation center. In this view, optic lobe neuroblasts flank the medial edge of neuroepithelia in the outer proliferation center. The higher magnification image of neuroepithelia and neuroblasts boxed in red is used in subsequent figures to illustrate the effects of removing or increasing the function of a gene.

(C) A lateral view of a wild type larval optic lobe stained for PatJ and Dpn reveals the swath of neuroepithelia flanked by neuroblasts.

(D) An anteroposterior single confocal optical section of a wild type larval brain stained for Phalloidin (Phall) and Dpn shows neuroepithelia in the outer proliferation center (white dotted line) flanked by neuroblasts (white bracket).

(E) A three-dimensionally reconstructed confocal z-series of a wild type optic lobe stained for Phall reveals the axon bundles that constitute the neuropil.

(F-F'') Differentiating neuroepithelial cells and immature neuroblasts are transiently arrested in cell cycle. Differentiating neuroepithelial cells (white arrow) located at the medial edge of neuroepithelia and their adjacent immature neuroblasts (yellow arrow) do not incorporate EdU following a 3-hour pulse labeling. The area shown corresponds to the red box in B'.

(G-G''') Immature neuroblasts do not maintain the epithelial cell morphology and do not express the neuroblast marker. Immature neuroblasts (outlined in dotted yellow line) located immediately adjacent to the medial edge of neuroepithelia lack expression of PatJ and Dpn. The area shown corresponds to the red box in B'.

(H) A cartoon summarizes the intermediate cell types during differentiation of neuroepithelia. NEC: neuroepithelial cells; diff. NEC: differentiating neuroepithelial cells; imm. NB: immature neuroblast; NB: neuroblast.

the mechanisms that regulate maintenance or differentiation of neuroepithelial cells. Both cells at the medial edge of neuroepithelia and those immediately adjacent to them are functionally distinct from the rest of neuroepithelial cells and neuroblasts as they can be unambiguously identified by the absence of EdU incorporation following a 3-hour pulse labeling (Figure 2.1 F). We named cells at the medial edge of neuroepithelia differentiating neuroepithelial cells and those immediately adjacent to them immature neuroblasts based on cell morphology and cell fate markers (Figure 2.1 G). Differentiating neuroepithelial cells maintained the epithelial cell morphology and expressed PatJ, but lacked Dpn expression. In contrast, immature neuroblasts no longer maintained the epithelial cell morphology, and did not express either PatJ or Dpn. Thus, differentiation of neuroepithelia occurs in the following sequence: neuroepithelial cells -> differentiating neuroepithelial cells -> immature neuroblasts -> neuroblasts (Figure 2.1 H).

Identification of genes required for proper development of optic lobe neuroepithelia

To gain insight into the mechanisms that regulate differentiation of neuroepithelia, we screened for zygotic lethal mutations induced by transposable P-element or ethyl methane sulfonate (EMS) that led to aberrant morphology in the third larval instar optic lobe (Figure 2.1S A-I). We used expression of the *PCNA::3XEmGFP* transgene, which encodes a fusion protein containing the amino-terminus of the PCNA protein and three copies of the <u>em</u>erald <u>g</u>reen <u>f</u>luorescent <u>p</u>rotein driven by its own

S-phase enhancer, to rapidly visualize the larval optic lobe (Thacker et al., 2003). The EmGFP expression pattern in brain neuroblasts and optic lobe neuroepithelial cells was indistinguishable from the pattern of BrdU incorporation following a 3-hour pulse labeling, indicating that PCNA::3XEmGFP reliably and reproducibly labeled all proliferating cells (Figure 2.1S J-K; data not presented). From this genetic screen, we identified three phenotypic categories namely optic lobe absent, optic lobe expanded and optic lobe prematurely lost. We confirmed that the mutant phenotypes are indeed linked to these genes by complementation tests with previously characterized mutant alleles.

Neuroepithelia in the third instar optic lobe absent mutant larvae were indistinguishable from those in the first instar wild type larvae, and were unable to produce neurons despite continuous growth of the organism (Figure 2.1S A-C). Importantly, brain neuroblasts in these mutant larvae remained proliferative and can generate neurons indicating that these mutations did not result in global defects in proliferation and differentiation. We mapped these mutations to the *connector enhancer of KSR* (*cnk*) and *corkscrew* (*csw*) genes, which have been shown to function in receptor tyrosine kinase signaling (Kolch, 2005; Perkins et al., 1992; Therrien et al., 1998; Van Vactor et al., 1996). Consistent with a recently published study, removal of the *EGF* function also leads to the "optic lobe absent " phenotype (Yasugi et al., 2010). Thus, the receptor tyrosine kinase signaling cascade functions to initiate neuroepithelial cell proliferation in the developing larval optic lobe.

The third instar optic lobe expanded mutant larvae showed excessive expansion of optic lobe neuroepithelia and reduction in optic lobe neuroblasts compared to similarly staged wild type larvae (Figure 2.1S D-F). Importantly, optic lobe neuroblasts in the homozygous optic lobe expanded mutant larvae underwent asymmetric divisions to produce neurons, indicating that this mutation does not perturb neuronal differentiation. We mapped this mutation to the *fat* gene, which encodes a critical activator of *hippo* signaling that regulates tissue growth (Zhao et al., 2010). A recent study shows that *fat-hippo* signaling triggers transient cell cycle arrest in cells near the medial edge of neuroepithelia, and inactivation of the *hippo* signaling delays differentiation of neuroepithelial cells (Reddy et al., 2010). Thus, transient cell cycle arrest induced by *fat-hippo* signaling is required for efficient differentiation of neuroepithelia.

The third instar optic lobe prematurely lost mutant larvae lacked neuroepithelia in the outer proliferation center, and instead possessed a swath of neuroblasts (Figure 2.1S G-I). Importantly, these mutant optic lobes possessed expansive and organized neuroepithelia in the second larval instar, but lost neuroepithelia in the third larval instar. This result strongly suggests that the premature loss of neuroepithelia occurred following depletion of maternally deposited gene product as larval development progressed. We mapped these two mutations to the *o-fucosyltransferase 1* (*o-fut1*) and *anterior pharynx defective 1* (*aph-1*) genes, which encode key components required for activating the precursor form of the Notch protein (Bray, 2006; Francis et al., 2002; Okajima and Irvine, 2002) (Figure 2.1S J). The newly isolated *o-fut1*^{2834} mutant allele carried a single nucleotide substitution

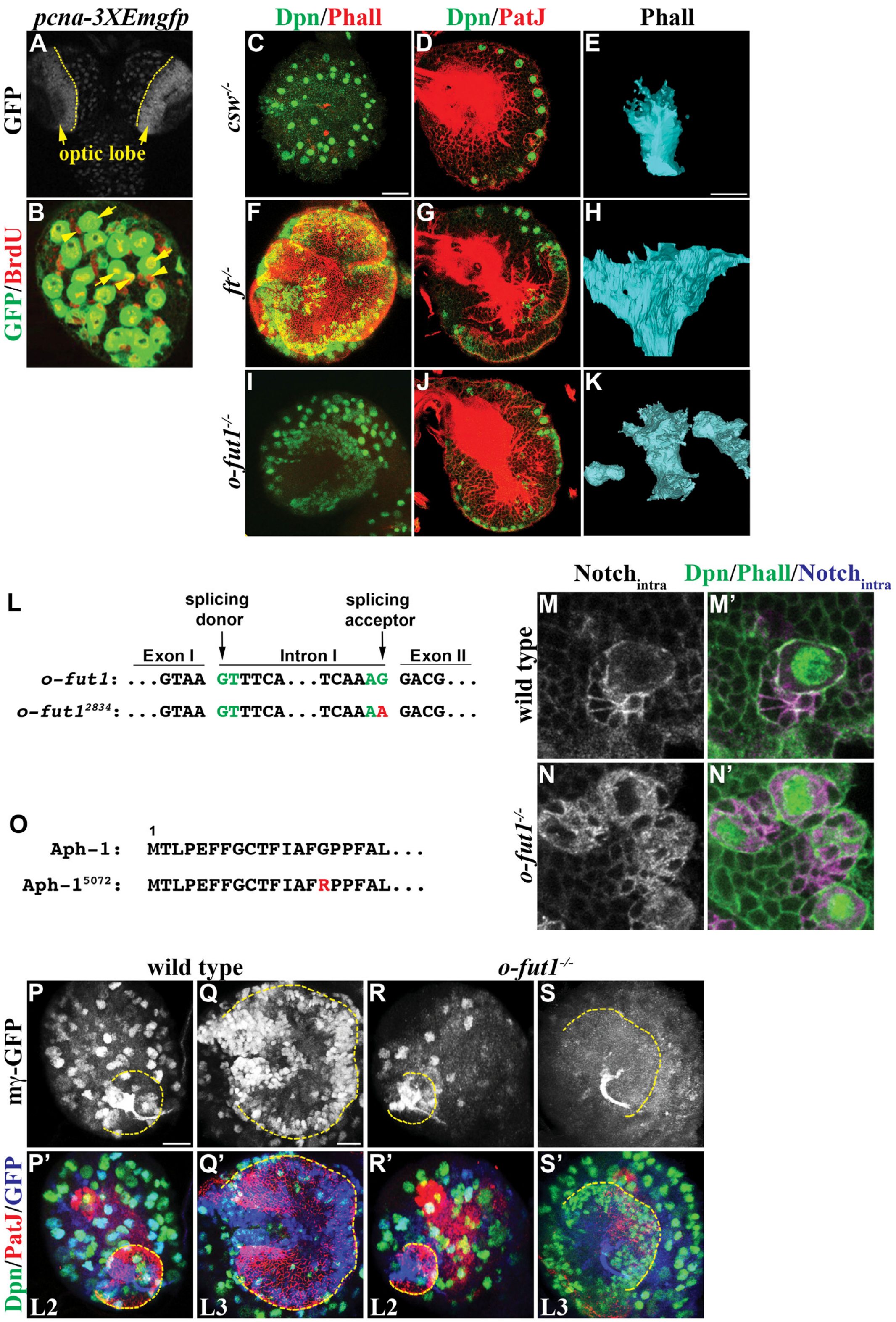

Figure 2.1S. A genetic screen that leads to identification of the new *o-fut1* and *aph-1* mutant alleles

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(A-B) PCNA-3xEmGFP allows rapid identification of optic lobe neuroepithelial cells. (A) The expression of a *pcna-3XEmgfp* transgene is detectable in neuroblasts in the brain and in neuroepithelial cells and neuroblasts in the optic lobe (yellow arrow). The yellow dotted line separated the central brain from the optic lobe. The scale bar = 50 μm. (B) PCNA-3XEmGFP co-localizes with BrdU incorporated during a 3-hour pulse labeling in brain neuroblasts (yellow arrow). Yellow arrowhead = ganglion mother cell. The scale bar = 10 μm.

(C-E) The optic lobe absent mutant. (C) The lateral view of a third larval instar *csw* mutant optic lobe reveals absence of optic lobe neuroepithelia and neuroblasts. The scale bar = 20 μm. (D) The anteroposterior single confocal optical section of a third larval instar *csw* mutant optic lobe shows a small cluster of primitive optic lobe neuroepithelial cells. (E) The three-dimensional reconstructed axon bundle revealed by the Phalloidin staining in a third larval instar *csw* mutant optic lobe.

(F-H) The optic lobe expanded mutant. (F) The lateral view of a third larval instar *ft* mutant optic lobe reveals dramatically expanded optic lobe neuroepithelia at the expense of neuroblasts. (G) The anteroposterior single confocal optical section of a third larval instar *ft* mutant optic lobe shows partially overlapped optic lobe neuroepithelia. (E) The three-dimensional reconstructed axon bundle revealed by the Phall staining in a third larval instar *ft* mutant optic lobe.

(I-K) The optic lobe premature lost mutant. (I) The lateral view of a third larval instar *o-fut1* mutant optic lobe reveals prematurely formed neuroblasts replacing the swath of neuroepithelia. (J) The anteroposterior single confocal optical section of a third larval instar *o-fut1* mutant optic lobe shows prematurely formed neuroblasts located on the surface of the larval optic lobe. (E) The three-dimensional reconstructed axon bundle revealed by the Phall staining in a third larval instar *o-fut1* mutant optic lobe.

(L) The *o-fut1*^{2834} mutant allele contains a nucleotide substitution (G->A; highlighted in red) at the splicing acceptor site in the first intron of the *o-fut1* transcript.

(M-N) Failure to properly process the Notch protein in the *o-fut1* mutant neuroblast. (M-M') The Notch protein is abundantly detected in the cortex of a wild type brain neuroblast. (N-N') The Notch protein fails to localize to the cortex, and instead, localizes in the cytoplasm of *o-fut1* mutant brain neuroblasts.

(O) The Aph-1^{5072} mutant protein contains an amino acid substitution (G->R; highlighted in red) at the amino acid residue 15.

(P-S) Inactivation of *Notch* signaling correlates with premature differentiation of neuroepithelia in the *o-fut1* mutant. (P-Q) E(spl)mγ-GFP is detectable in the central brain and optic lobe in the second (L2) and the third (L3) larval instar. The dotted yellow line indicates the boundary between the larval optic and the larval brain. (R-S) E(spl)mγ-GFP is detectable in (L2), but becomes undetectable in L3. The scale bar = 20 μm.

disrupting the splicing acceptor site in the first intron of the *o-fut1* transcript (Figure 2.1S K). Consistent with the function of O-fut1 in activating *Notch* signaling, the Notch protein aberrantly accumulated in the cytosol of *o-fut1* mutant brain neuroblasts (Figure 2.1S L-M). Furthermore, the newly isolated *aph-1*^{5072} mutation led to substitution of glycine with arginine in the first transmembrane domain of the Aph-1 protein (Figure 2.1S N). Finally, we tested if depletion of the maternally deposited *o-fut1* and *aph-1* gene product led to inactivation of *Notch* signaling by examining expression of the *Notch* reporter transgene *E(spl)mγ-GFP*. In agreement with the timing of neuroepithelia loss in these mutants, E(spl)mγ-GFP was clearly detectable in the optic lobe of second instar *o-fut1* or *aph-1* mutant larvae, but was undetectable in the third instar mutant larval optic lobe (Figure 2.1S O-R; data not presented). Together, these data support the hypothesis that *Notch* signaling maintains the identity of neuroepithelial cells, and that inactivation of *Notch* signaling lead to premature differentiation of neuroepithelia into neuroblasts.

***Notch* functions as a key regulator to maintain the identity of neuroepithelial cells**

We directly tested if *Notch* is required for maintenance of neuroepithelia by taking two complementary approaches. We first reduced the function of *Notch* globally in all neuroepithelial cells by over-expressing an inducible *UAS-Notch*$_{RNAi}$ transgene driven by the neuroepithelia-specific *GH146-gal4* driver. Knocking-down the function of *Notch* led to formation of neuroblasts throughout the entire neuroepithelia in the outer proliferation center, identical to the *o-fut1* or *aph-1* mutant phenotype (Figure 2.2 A-B; N

= 7 per genotype). This result prompted us to test if *Notch* functions cell autonomously to maintain the identity of neuroepithelial cells. We induced the GFP-marked mosaic clone derived from wild type or *Notch* mutant neuroepithelial cells at 24 hours after hatching when the larval optic lobe consists of mostly neuroepithelial cells. 72 hours after clone induction, we determined the fate of the progeny in the clone by the cell identity markers PatJ and Dpn, and the position of the clone relative to the remaining neuroepithelia. In the wild type clone, neuroblasts generated by neuroepithelial cells all located medially to neuroepithelia on the surface of the optic lobe and produced neuron progeny incorporated into the optic lobe medulla (Figure 2.2 C; 100%, N = 9 clones). Although most *Notch* mutant clones also contained neuroblasts and their progeny, these cells delaminated inward away from the rest of neuroepithelia and located deep in the developing medulla (Figure 2.2 D; 85.7%; N = 21 clones). Thus, *Notch* functions cell autonomously to maintain the identity of neuroepithelial cells. We next tested if down-regulation of *Notch* signaling might be necessary for differentiation of neuroepithelia. We over-expressed a *UAS-Notch*$_{intra}$ transgene driven by an *Actin-Gal4* driver in the GFP-marked genetic clone derived from neuroepithelial cells induced at 48 hours after hatching. We assessed the identity of cells within the clone by using the neuroblast marker Dpn and cell cortex marker Phalloidin 48 hours after clone induction. All control clones lacking the *UAS-Notch*$_{intra}$ transgene expanded medially and contained mixed neuroepithelial cells, neuroblasts and their progeny (Figure 2.2 E; N = 10). In contrast, constitutively activated *Notch* signaling led to unrestrained expansion of neuroepithelial cells at the expense of neuroblasts in the clone, which expanded beyond the medial edge of neuroepithelia as determined by the identity of neighboring cells (Figure 2.2 F; N = 8). Thus, *Notch* functions cell autonomously to maintain the neuroepithelial cell identity, and down-regulation of *Notch* is necessary for formation of neuroblasts.

The mutation in the *lethal (3) malignant brain tumor* (*l(3)mbt*) gene leads to soma-to-germline transformation of neuroepithelial cells in the optic lobe (Janic et al., 2010). We generated the *o-fut1; l(3)mbt* double mutant to test if *Notch* is also required for maintenance of these transformed neuroepithelial cells. The optic lobe in the *l(3)mbt* mutant larva contained almost exclusively PatJ$^+$ neuroepithelial cells at the expense of neurons as indicated by the lack of Elav expression (Figure 2.2 G; 100%, N = 6). In contrast, the optic lobe in the *o-fut1; l(3)mbt* mutant larva contained mostly Elav$^+$ neurons with very few PatJ$^+$ neuroepithelial cells (Figure 2.2 H; 100%, N = 6). This result indicates that the soma-to-germline transformed neuroepithelial cells in the *l(3)mbt* mutant optic lobe require *Notch* signaling to maintain their identity. Thus, *Notch* functions as a key regulator in maintaining the neuroepithelial cell identity in the larval optic lobe.

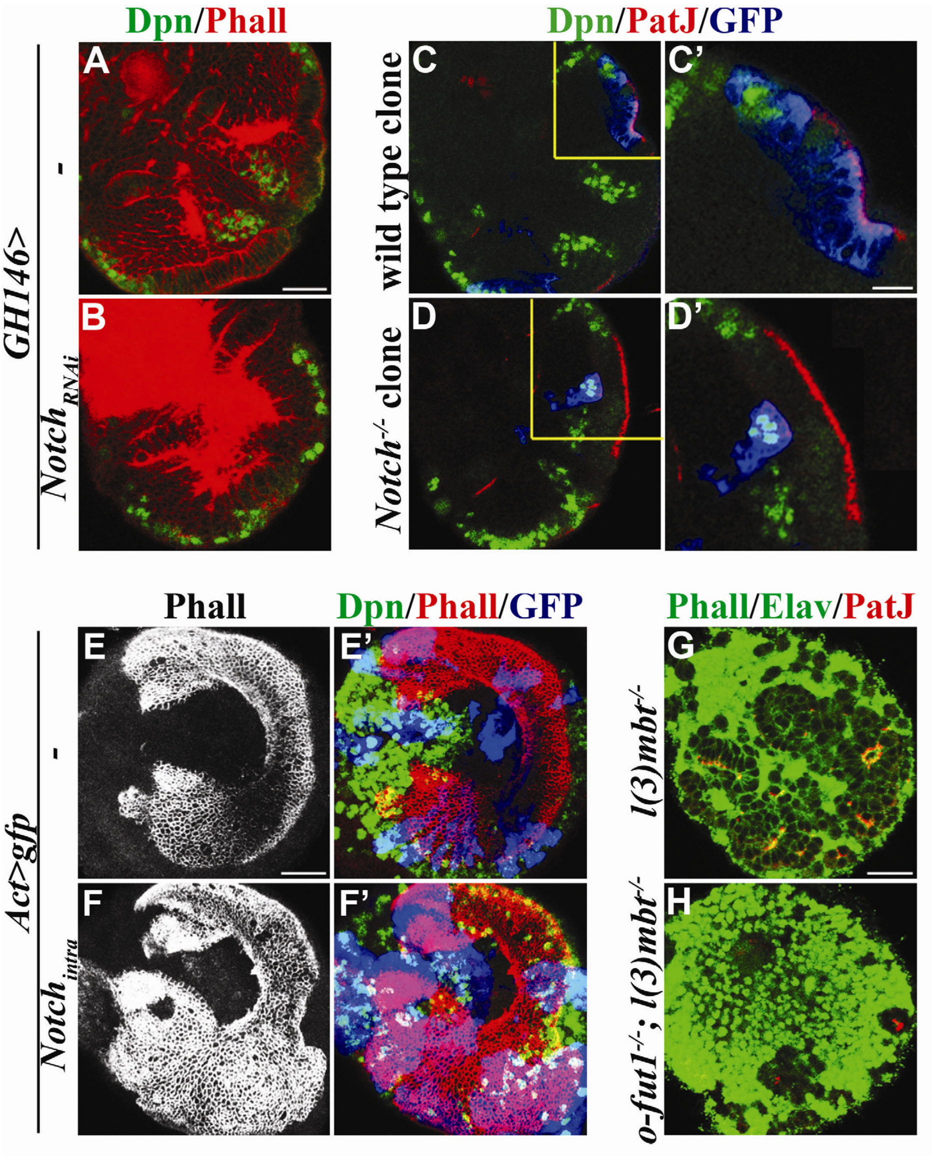

Figure 2.2. A low level of *Notch* signaling maintains the identity of neuroepithelial cells

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(A-B) *Notch* is necessary to maintain the identity of neuroepithelial cells. (A) An anteroposterior single confocal optical section of a control larval optic lobe shows neuroepithelia in the outer proliferation center flanked by neuroblasts. (B) A similar confocal optical section of an optic lobe with the function of *Notch* knocked-down by RNAi shows premature formation of neuroblasts, replacing neuroepithelial cells in the outer proliferation center (the scale bar = 20 μm).

(C-D) *Notch* functions cell autonomously to maintain the neuroepithelial cell identity. (C-C') A GFP-marked mosaic clone derived from wild type neuroepithelial cells expands and contains neuroblasts located medially from neuroepithelia. The higher magnification image of the boxed area is shown in C'. (D-D') A mosaic clone derived from *Notch* mutant neuroepithelial cells delaminates inward away from the rest of neuroepithelia and contains neuroblasts located deep in the cortex of future medulla. The higher magnification image of the boxed area is shown in D'. The scale bar = 10 μm.

(E-F) Down-regulation of *Notch* is necessary for differentiation of neuroepithelial cells. (E) A larval optic lobe containing GFP-marked mosaic clones derived from wild type neuroepithelial cells shows synchronous differentiation from neuroepithelia into neuroblasts. The higher magnification image is shown in E'. (F) A larval optic lobe containing mosaic clones derived from neuroepithelial cells over-expressing $Notch_{intra}$ shows unrestrained expansion of neuroepithelia at the expense of neuroblasts. The higher magnification image of the boxed area is shown in F'. The scale bar = 20 μm.

(G-H) *Notch* is necessary for maintenance of somatic-to-germline transformed neuroepithelial cells in the *l(3)mbt* mutant. (G) The *l(3)mbt* mutant larval optic lobe shows over-expansion of neuroepithelia at the expense of neurons as indicated by the lack of Elav-expressing cells. (H) Neuroepithelial cells in the *o-fut1*; *l(3)mbt* mutant larval optic lobe fail to maintain their identity. The scale bar = 20 μm.

***Notch* signaling becomes up-regulated in differentiating neuroepithelial cells but down-regulated in immature neuroblasts**

Precise regulation of the *Notch* activity is pivotal for maintenance and differentiation of neuroepithelial cells, so we characterize the spatial profile of *Notch* signaling in neuroepithelia by examining the expression of a *Notch* reporter transgene *E(spl)mγ-GFP*. We detected E(spl)mγ-GFP expression, which indicates activated *Notch* signaling, only in cells located at and near the medial edge of neuroepithelia (Figure 2.3 A). The spatial pattern of activated *Notch* signaling based on E(spl)mγ-GFP expression contradicted with our data showing that *Notch* functions globally to maintain the identity of neuroepithelial cells in the outer proliferation center. One possible explanation might be that cells located laterally from the medial edge of neuroepithelia show a low level of activated *Notch* signaling. We tested this hypothesis by over-expressing $Notch_{intra}$ ubiquitously in the neuroepithelial cell genetic clone induced 72 hours after hatching in larvae that carry the *E(spl)mγ-GFP* transgene. 24 after clone induction, we assessed the identity of cells within the RFP-marked clone. Transient activation of *Notch* signaling led to cell autonomous expression of E(spl)mγ-GFP in cells within the clone located laterally from the medial edge of neuroepithelia (Figure 2.3 A-B; 100%, N = 8). Thus, neuroepithelial cells located laterally from the medial edge neuroepithelia can indeed express E(spl)mγ-GFP in response to a high level of activated *Notch* signaling. This result supports our hypothesis that most neuroepithelial cells do not show detectable

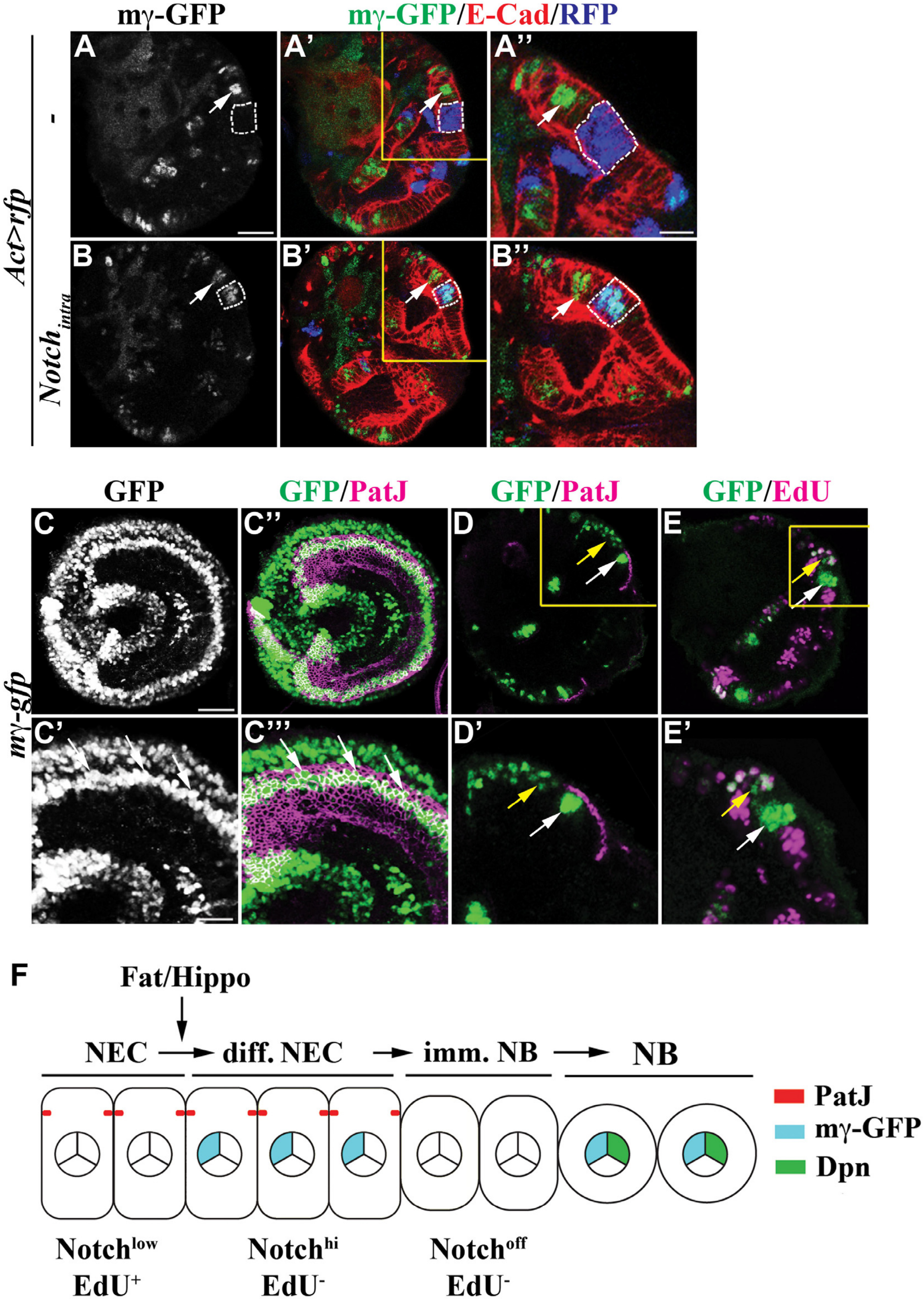

Figure 2. 3. Spatial changes in *Notch* signaling coincide with differentiation of neuroepithelia

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(A-B) *Notch* signaling appears to be low throughout most of neuroepithelia. (A-A") Cells in the RFP-marked wild type genetic clone (outlined in dotted white line) located laterally from the medial edge of neuroepithelia lack expression of E(spl)mγ-GFP. GFP expression is only detectable in neuroepithelial cells near the medial edge of neuroepithelia (white arrow). The scale bar = 20 μm. The higher magnification image of the boxed area is shown in A". The scale bar = 10 μm. (B) Cells in the RFP-marked genetic clone derived from neuroepithelial cells over-expressing $Notch_{intra}$ (outlined in dotted white line) located laterally from the medial edge of neuroepithelia show robust expression of E(spl)mγ-GFP. GFP expression in neuroepithelial cells near the medial edge of neuroepithelia (white arrow). The higher magnification image of the boxed area is shown in B".

(C-C"') *Notch* signaling becomes drastically increased in neuroepithelial cells near the medial edge of neuroepithelia. (C and C") E(spl)mγ-GFP expression (white arrow) co-localizes with PatJ in neuroepithelial cells near the medial edge of neuroepithelia. The scale bar = 20 μm. The higher magnification image of the cells expressing E(spl)mγ-GFP is shown in C' and C"'. The scale bar = 10 μm.

(D-E) *Notch* signaling becomes drastically increased in differentiating neuroepithelial cells. (D) Although E(spl)mγ-GFP expression co-localizes with PatJ at the medial edge of neuroepithelia (white arrow), it is undetectable in the adjacent immature optic lobe neuroblast. The higher magnification image of the boxed area showing cells expressing E(spl)mγ-GFP is shown in D'. (E) E(spl)mγ-GFP expression does not co-localize with EdU incorporated into neuroepthelia and neuroblasts following a 3-hour pulse labeling. The higher magnification image of the boxed area showing cells expressing E(spl)mγ-GFP is shown in E'.

(F) A cartoon summarizes spatial changes in *Notch* signaling during differentiation of neuroepithelial cells into neuroblasts. NEC: neuroepithelial cells; diff. NEC: differentiating neuroepithelial cells; imm. NB: immature neuroblast; NB: neuroblast.

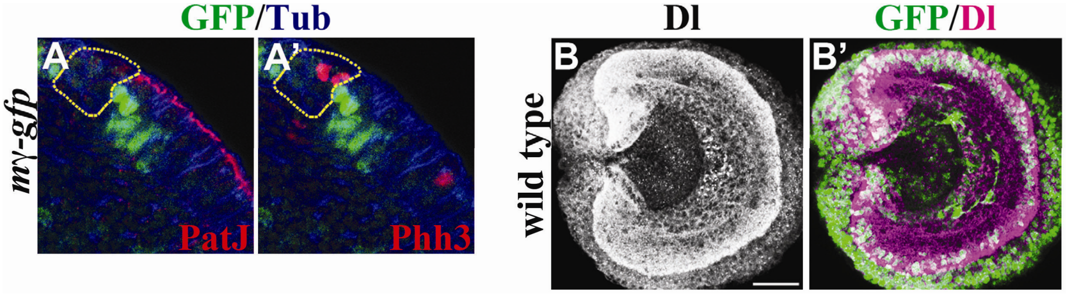


Figure 2.3S. E(spl)mγ-GFP marks differentiating neuroepithelial cells

(A) E(spl)mγ-GFP is detected in differentiating neuroepithelial cells located at the medial edge of neuroepithelia but is absence from the neighboring immature neuroblast.

(B) An elevated level of the Delta protein largely overlaps with the expression of E(spl)mγ-GFP in differentiating neuroepithelial cells, but the expression of Delta peaks in the immature neuroblast. The scale bar = 50 μm.

E(spl)mγ-GFP expression most likely due to a relatively low level of *Notch* signaling that is insufficient to activate the *E(spl)mγ-GFP* reporter transgene.

The spatial expression pattern of E(spl)mγ-GFP led us to hypothesize that *Notch* signaling might become up-regulated in differentiating neuroepithelial cells. Specifically, E(spl)mγ-GFP expression was detected across two to three PatJ$^+$ neuroepithelial cells located one- to two-cell away from the medial edge of neuroepithelia, but was undetectable in the adjacent PatJ$^-$ immature neuroblasts (Figures 2.3 C-D and 2.2S A; N = 8). Following a 3-hour pulse labeling, we did not detect any EdU incorporation in the cells that expressed E(spl)mγ-GFP (Figures 2.3E and 2.2S A). These data indicate that the *E(spl)mγ-GFP* transgene is expressed in differentiating neuroepithelial cells, but is not expressed in immature neuroblasts (Figure 2.3 F). Thus, *Notch* signaling becomes up-regulated in differentiating neuroepithelial cells, but rapidly down-regulated in immature neuroblasts.

Delta regulates spatial changes in *Notch* signaling

Delta activates the Notch receptor in a wide variety of developmental processes, so we tested if the expression of Delta correlates with spatial changes in *Notch* signaling in neuroepithelia. We determined the spatial expression pattern of Delta by co-localizing the endogenous protein using a specific antibody with PatJ and E(spl)mγ-GFP. We detected a low level of the Delta protein throughout neuroepithelial cells that lacked E(spl)mγ-GFP expression (Figures 2.4 A and 2.2S B). Delta expression increased sharply in differentiating neuroepithelial cells colocalizing with the expression of E(spl)mγ-GFP (Figures 2.4 A and 2.2S B). Delta expression peaked in immature neuroblasts immediately adjacent to differentiating neuroepithelial cells, and became undetectable in the rest of immature neuroblasts (Figures 2.4 A and 2.2S B). Thus, the spatial expression profile of Delta closely resembles the spatial pattern of activated *Notch* signaling in neuroepithelia.

Since removal of the *Delta* function abolishes its intricate regulation on *Notch* signaling, we tested how increased function of *Delta* might alter dynamic changes in *Notch* signaling spatially. We transiently over-expressed a *UAS-Delta* transgene driven by *Actin-Gal4* for 24 hours in the RFP-marked genetic clone derived from neuroepithelial cells in larvae that carry an *E(spl)mγ-GFP* transgene. We first assessed how increased function of *Delta* might effect the expression of *E(spl)mγ-GFP* in the clone located laterally from differentiating neuroepithelial cells. Over-expression of Delta led to aberrant expression of E(spl)mγ-GFP non-cell autonomously in the clone located laterally from differentiating neuroepithelial cells (Figure 2.4 B; 100%, N = 6). Thus, Delta can *trans*-activate *Notch* signaling in neuroepithelial cells that normally do not express E(spl)mγ-GFP, implicating that a low level of Delta might maintain a low level of activated *Notch* signaling throughout neuroepithelia. This result is consistent with reduced function of *Delta* by globally over-expressing a dominant negative form of Delta led to premature differentiation of neuroepithelia (Reddy et al., 2010; Wang et al., 2011). In contrast, over-expression of Delta in differentiating neuroepithelial cells F;

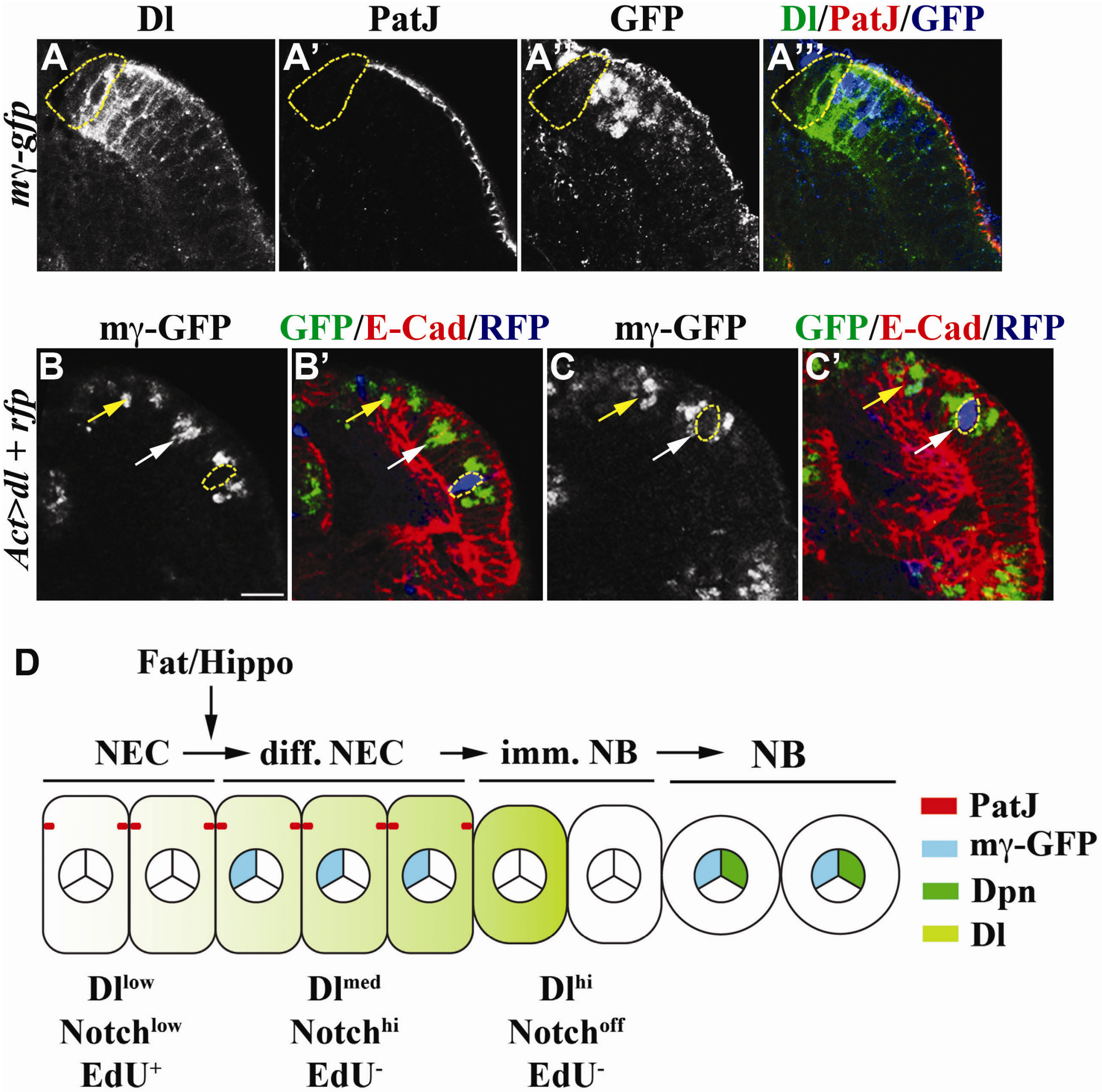


Figure 2.4. Delta likely regulates spatial changes in *Notch* signaling in neuroepithelia

Figure 2.4. Delta likely regulates spatial changes in *Notch* signaling in neuroepithelia

(A) The expression pattern of Delta largely correlates with spatial changes in *Notch* signaling. Delta is expressed in a low level throughout neuroepithelia, but rises dramatically in differentiating neuroepithelial cells co-localizing with E(spl)mγ-GFP expression. Delta expression peaks in the immature neuroblast immediately adjacent to differentiating neuroepithelial cells (outlined in dotted yellow line).

(B-C) Delta regulates spatial changes in *Notch* signaling. (B) Transient over-expression of Delta in the RFP-marked genetic clone (outlined in dotted yellow line) located laterally from differentiating neuroepithelial cells (white arrow) activates cell non-autonomous expression of E(spl)mγ-GFP. The yellow arrow indicates optic lobe neuroblasts. The scale bar = 10 μm. (C) Transient over-expression of Delta in the RFP-marked genetic clone (outlined in dotted yellow line) located in differentiating neuroepithelial cells (white arrow) triggers cell autonomous inhibition of E(spl)mγ-GFP expression.

(D) A cartoon summarizes the spatial expression pattern of Delta expression and the *Notch* activity during differentiation of neuroepithelial cells.

strikingly abolished E(spl)mγ-GFP expression in a cell autonomous manner (Figure 2.3 100%, N = 10). Therefore, a high level of Delta is sufficient to *cis*-inhibit *Notch* signaling in differentiating neuroepithelial cells where *Notch* signaling is transiently up-regulated. This result suggests that the peak level of Delta expression immediately adjacent to the medial edge of neuroepithelia might down-regulate *Notch* signaling necessary for the transition from differentiating neuroepithelial cells into immature neuroblasts. Taken together, we propose that *trans*-activation of a lower level of *Notch* signaling by Delta contributes to maintenance of neuroepithelial cell identity whereas *cis*-inhibition of a high level of *Notch* signaling by Delta triggers the transition from differentiating neuroepithelial cells to neuroblasts (Figure 2.4 D).

Notch maintains the identity of neuroepithelial cells via Aop

We took a candidate gene approach to test if *aop* might be a target of *Notch* during maintenance of the neuroepithelial cell identity for the following reasons. First, the synchrony of neuroepithelial cell differentiation progressing across the entire neuroepithelial swath resembles the morphogenic furrow sweeping across the larval eye-antennal imaginal disc (Doroquez and Rebay, 2006; Hsiung and Moses, 2002). *Notch* plays an important role in regulating eye morphogenesis by directly activating the *aop* gene (Rohrbaugh et al., 2002). Second, a recent study showed that *Notch* regulate the maintenance of maintaining adult muscle progenitor cells during metamorphosis also by directly activating *aop* gene expression (Krejcí et al., 2009; Rohrbaugh et al., 2002). We attempted to investigate if Aop is expressed in neuroepithelia by using a specific antibody, but failed to detect any specific expression pattern presumably due to a very low level of the endogenous Aop protein (data not presented). As an alternative approach, we analyzed the expression of an *aop-lacZ* reporter transgene that is expressed in a pattern largely mimicking the endogenous Aop protein in the developing eye disc (Rohrbaugh et al., 2002). We detected a low level of Aop-lacZ throughout neuroepithelia in a non-overlapping pattern with E(spl)mγ-GFP, indicating that *aop* expression likely become abrogated in differentiating neuroepithelial cells (Figure 2.5 A; N = 5). Thus, the expression of *aop-lacZ* is consistent with our hypothesis that *Notch* might maintain the identity of neuroepithelial cells by activating *aop*.

We next tested if *aop* functions to maintain neuroepithelia by taking two complementary approaches. We first induced the mosaic clone derived from *aop* mutant neuroepithelial cells 24 hours after hatching, and determined the identity of cells in the GFP-marked clone 72 hours after clone induction. While wild type cells outside of the clone maintained expression of the neuroepithelial cell marker PatJ, all cells within the *aop* mutant clone expressed the neuroblast marker Dpn (Figure 2.5 B). The high efficiency in *aop* mutant clone induction led to widespread premature formation of neuroblasts replacing neuroepithelia in the outer proliferation center (Figure 2.5 B). This result indicates that *aop* functions to maintain neuroepithelia prompting us to test if over-expression of *aop* might prevent differentiation of neuroepithelial cells into neuroblasts. We over-expressed a *UAS-aop* transgene driven by *Actin-Gal4* in the neuroepithelial cell genetic clone induced at 48 hours after hatching. We then assessed the identity of cells within the clones that located medially from the edge of neuroepithelia as determined by the fate of surrounding cells 48 hours after clone induction. Cells inside and outside of the control clone lacking the *UAS-aop* transgene synchronously transitioned from differentiating neuroepithelial cells into neuroblasts, and expressed the marker Dpn (Figure 2.2 E). In contrast, 91% of the *aop* over-expressing clone contained ten or more rows of neuroepithelial cells protruding from the medial edge of neuroepithelia (Figure 2.5 C; N = 11). This result indicates that *aop* is necessary for maintaining neuroepithelial cell identity, and is consistent with *aop* acting downstream of *Notch* for maintenance of neuroepithelia.

We directly tested if over-expression of *aop* can prevent premature differentiation of *Notch* mutant neuroepithelial cells. We over-expressed *aop* ubiquitously in the mosaic clone derived from *Notch* mutant neuroepithelial cells induced 24 hours after hatching. We then assessed the identity of cells within the GFP-marked clone 72 hours after clone induction. *Notch* mutant neuroepithelial cells prematurely differentiated into neuroblasts, and delaminated inward from the rest of wild type neuroepithelia (Figure 2.2 D). In contrast, over-expression of *aop* allowed *Notch* mutant neuroepithelial cells to maintain their identity, and differentiated into neuroblasts located medially from the edge of neuroepithelia (Figure 2.5 D; 82%, N = 11). Thus, over-expression of Aop suppresses premature differentiation of *Notch* mutant neuroepithelial cells, strongly suggesting that *aop* functions downstream of *Notch* to maintain neuroepithelia.

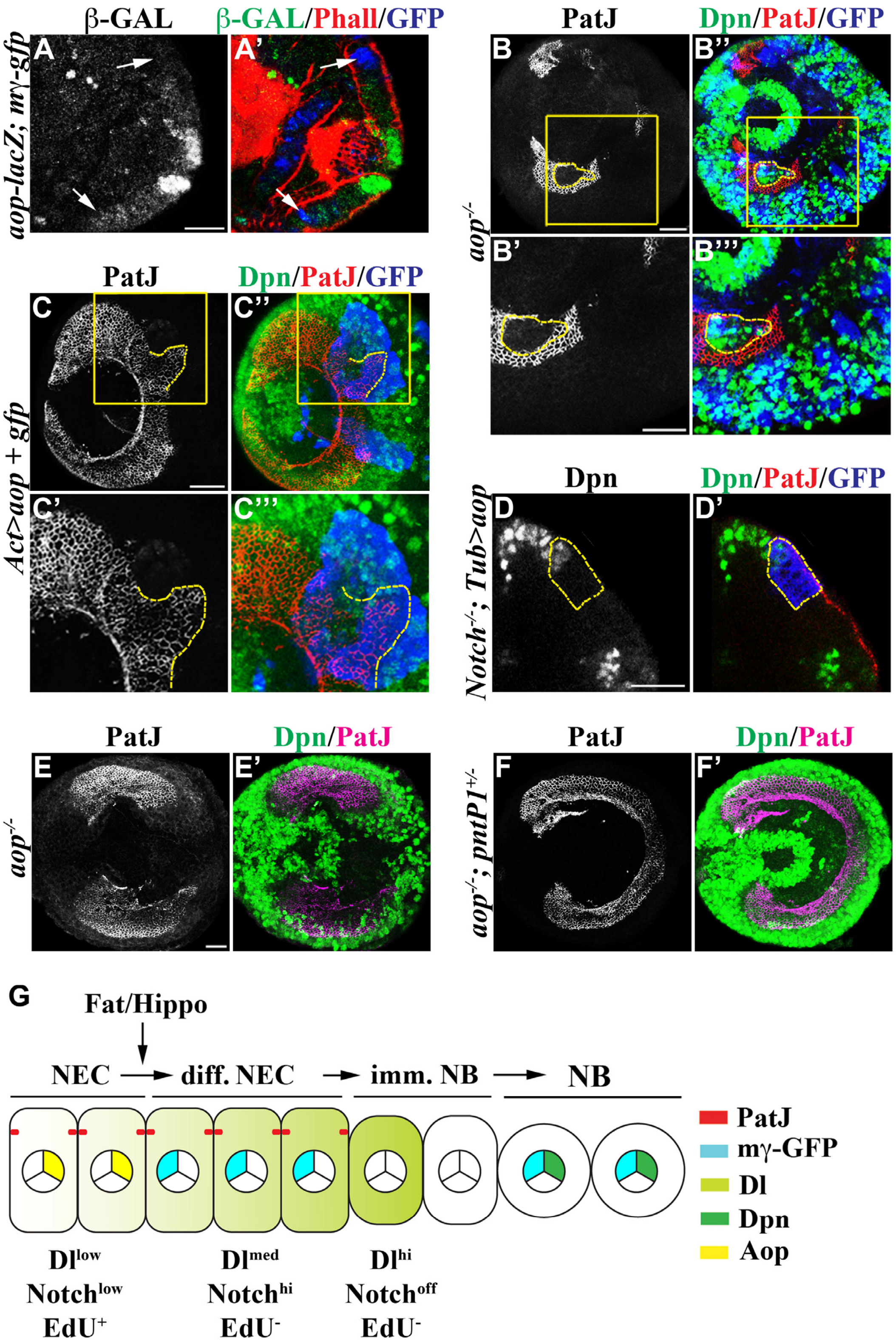


Figure 2.5. Aop functions downstream of *Notch* signaling to maintain the identity of neuroepithelial cells

Figure 2.5. Aop functions downstream of *Notch* signaling to maintain the identity of neuroepithelial cells

(A) Aop is likely expressed in a low level through neuroepithelia. A low level of *aop-lacZ* expression can be detected in a non-overlapping pattern with E(spl)mγ-GFP in neuroepithelia (white arrow). The scale bar = 20 μm.

(B) Aop is necessary for maintaining the identity of neuroepithelial cells. *aop* mutant neuroepithelial cells in the GFP-marked mosaic clones prematurely differentiate into neuroblasts perturbing the swath of neuroepithelia in the outer proliferation center. The scale bar = 20 μm. Higher magnification of the boxed area showing an *aop* mutant neuroepithelial cell clone is shown in B' and B'''. The scale bar = 20 μm.

(C) Aop is sufficient to promote the identity of neuroepithelial cells. Neuroepithelial cells over-expressing *aop* in the GFP-marked genetic clone (outlined in dotted yellow line) fail to differentiate into neuroblasts leading to protrusion of neuroepithelia surrounded by optic lobe neuroblasts. The scale bar = 20 μm. The higher magnification image of the boxed area is shown in C' and C'''.

(D) Over-expression of Aop suppresses premature differentiation of *Notch* mutant neuroepithelial cells. Ectopic expression of Aop prevents *Notch* mutant neuroepithelial cells marked by the expression of GFP from prematurely differentiating into neuroblasts and allows them to maintain their identity (outlined in dotted yellow line). The scale bar = 20 μm.

(E-F) Aop maintains the identity of neuroepithelial cells by repressing the *pntP1* gene. (E) Neuroepithelial cells prematurely differentiate into neuroblasts perturbing the swath of neuroepithelia in the *aop* hypomorphic mutant optic lobe. The scale bar = 20 μm. (F) Reduced function of *pntP1* prevents premature differentiation of neuroepithelial cells and restores a continuous swath of neuroepithelia in the *aop* hypomorphic mutant optic lobe.

(G) A cartoon summarizes the expression pattern of Aop during differentiation of neuroepithelial cells.

The expression of *aop-lacZ* is undetectable in differentiating neuroepithelial cells, and over-expression of *aop* prevents differentiation of neuroepithelia. These data led us to hypothesize that a successful transition from differentiating neuroepithelial cells into neuroblasts requires a mechanism that inactivates the function of *aop*. Activated receptor tyrosine kinases can phosphorylate the Aop protein, and alleviates its repression on the *pntP1* gene that encodes a transcriptional activator critical for expression of the downstream genes in the signaling cascade (Lai and Rubin, 1992). Thus, we tested if increased *pntP1* function might be the underlying cause that led to premature differentiation of *aop* mutant neuroepithelial cells. In 66.7% of the $aop^{1/yan1}$ hypomorphic mutant larvae, the neuroepithelia swath in the outer proliferation center became interrupted by aberrant formation of Dpn^{+} neuroblasts indicating that neuroepithelial cells prematurely differentiate into neuroblasts (Figure 2.5 E; N = 12). In contrast, heterozygosity of the $pnt^{\Delta 33}$ mutation (a *pntP1*-specific allele) prevented premature differentiation of neuroepithelial cells and restored an uninterrupted neuroepithelia swath in 100% of $aop^{1/yan1}$ mutant larvae (Figure 2.5 F; N = 12). Thus, we conclude that Aop maintains the identity of neuroepithelial cells by repressing *pntP1*.

Up-regulation of *Notch* signaling increases the threshold of response to PntP1 in differentiating neuroepithelial cells

Down-regulation of the *aop* function leads to de-repression of *pntP1* and is critical for generation of neuroblasts, so we hypothesized that *pntP1* regulates differentiation of neuroepithelial cells. We first examined if the endogenous PntP1 co-localizes with E(spl)mγ-GFP in differentiating neuroepithelial cells by immunofluorescent staining. While PntP1 expression was undetectable in neuroepithelial cells that lacked E(spl)mγ-GFP, PntP1 largely co-localized with E(spl)mγ-GFP in differentiating neuroepithelial cells (Figure 2.6 A). In addition, we reproducibly detected PntP1 expression in immature neuroblasts immediately adjacent to differentiating neuroepithelial cells (Figure 2.6 A). Together, *pntP1* is expressed during the transition from differentiating neuroepithelial cells into neuroblasts, supporting the hypothesis that *pntP1* regulates differentiation of neuroepithelia.

We tested if *pntP1* is required for the transition from differentiating neuroepithelia by generating neuroepithelial cell mosaic clones using the *pntP1*-specific *pnt*$^{\Delta 33}$ mutant allele. We induced the negatively marked clone 48 hours after hatching, and assessed the identity of cells within the clone spanning across the medial edge of neuroepithelia by using Delta as the marker. In the wild type optic lobe, Delta expression became drastically up-regulated in differentiating neuroepithelial cells and peaked in the neighboring immature neuroblasts, but its expression was sparse in the rest of immature neuroblasts (Figure 2.4 A and 2.2S B). Multiple rows of cells in the *pntP1* mutant clone maintained robust expression of Delta despite being located beyond the medial edge of neuroepithelia as determined by sparse Delta expression in the surrounding cells (Figure 2.6 B). This result indicates that *pntP1* is necessary for a timely transition from differentiating neuroepithelial cells into neuroblasts. Nevertheless, the transition from neuroepithelial cell to neuroblast still happened as Delta expression in the cells located most medially in the clone were down-regulated, indicating that additional parallel signaling mechanisms must exit. The *pnt* locus encodes at least two protein isoforms P1 and P2, and PntP2 competes with Aop for binding to the promoter of various target genes including *pntP1* when activated by the receptor tyrosine kinase signaling cascade (Doroquez and Rebay, 2006; Hsiung and Moses, 2002). Thus, we tested if *pntP2* might be required for differentiation of neuroepithelia. We decided against generating mosaic neuroepithelial cell clones using the *pntP2*-specific $pnt^{\Delta 78}$ allele because it failed to complement the $pnt^{\Delta 33}$ mutant allele. As an alternative, we knocked-down the function of both *pntP1* and *pntP2* transcription units by over-expressing a $UAS\text{-}pnt_{RNAi}$ transgene ubiquitously in the neuroepithelial cell mosaic clone induced 48 hours after hatching. We chose to assess the fate of cells within the GFP-marked clone that located medially from the edge of neuroepithelia as determined by the fate of surrounding cells. While cells directly adjacent to the clone adopted the neuroblast identity, 77.8 % of the *pnt* mutant clone contained ten or more rows of neuroepithelial cells protruding beyond the medial edge of neuroepithelia (Figure 2.6 C; N = 9). This result strongly suggests that simultaneously reducing the function of *pntP1* and *pntP2* prevented differentiation of neuroepithelial cells more efficiently than removing the function of *pntP1* alone. Thus, a successful transition from differentiating neuroepithelial cells to neuroblasts requires both *pntP1*-dependent and -independent mechanisms.

We extended out analyses to test if over-expression of *pntP1* might be sufficient to induce premature differentiation of neuroepithelial cells. We transiently over-expressed a *UAS-pntP1* transgene driven by *Actin-Gal4* for 16 hours in the neuroepithelial cell genetic clone induced at 72 hours after hatching. We then assessed whether cells within the GFP-marked clone located laterally from differentiating neuroepithelial cells might show up-regulation of Delta. While wild type cells located immediately adjacent to the clone showed a very low level of Delta expression, cells within the clone showed significantly elevated expression of Delta (Figure 2.6 D; N = 16). We next prolonged the duration of *pntP1* over-expression to 48 hours and assessed the identity of the cells within the GFP-marked clone. Consistently, cells in the clone delaminated inward from the surrounding wild type neuroepithelia and expressed the neuroblast marker Dpn (Figure 2.6 E; N = 12). Thus, over-expression of *pntP1* is sufficient to trigger premature differentiation of neuroepithelial cells.

Since the expression of *pntP1* is necessary for the transition from differentiating neuroepithelial cells into neuroblasts, we tested if constitutively activated *Notch* signaling prevents differentiation of neuroepithelia by repressing expression of *pntP1*. We over-expressed $Notch_{intra}$ for 48 hours in cells within the neuroepithelial cell genetic clone induced at 48 hours after hatching. We then assessed the identity of cells within the GFP-marked clone located at the edge of neuroepithelia. Unexpectedly, most cells in the clone resembled differentiating neuroepithelial cells in that they maintained the epithelial cell morphology and expressed PntP1 (Figure 2.6 F; N = 10). Thus, differentiating neuroepithelial cells cannot transition into neuroblasts in the presence of constitutively activated *Notch* signaling despite expressing PntP1. This result was surprising and led us to hypothesize that transient up-regulation of *Notch* signaling in differentiating neuroepithelial cells might raise the threshold of their response to PntP1. We tested this hypothesis by co-expressing $Notch_{intra}$ and *pntP1* in cells within the GFP-marked clone derived from neuroepithelial cells. In agreement with our hypothesis, cells in 80% of the clone assumed the neuroblast fate as indicated by the absence of the epithelial cell morphology and expression of the neuroblast marker Dpn (Figure 2.6 G; N = 15). Taken together, we conclude that transient activation of *Notch* signaling raises the threshold of the response to PntP1 in differentiating neuroepithelial cells.

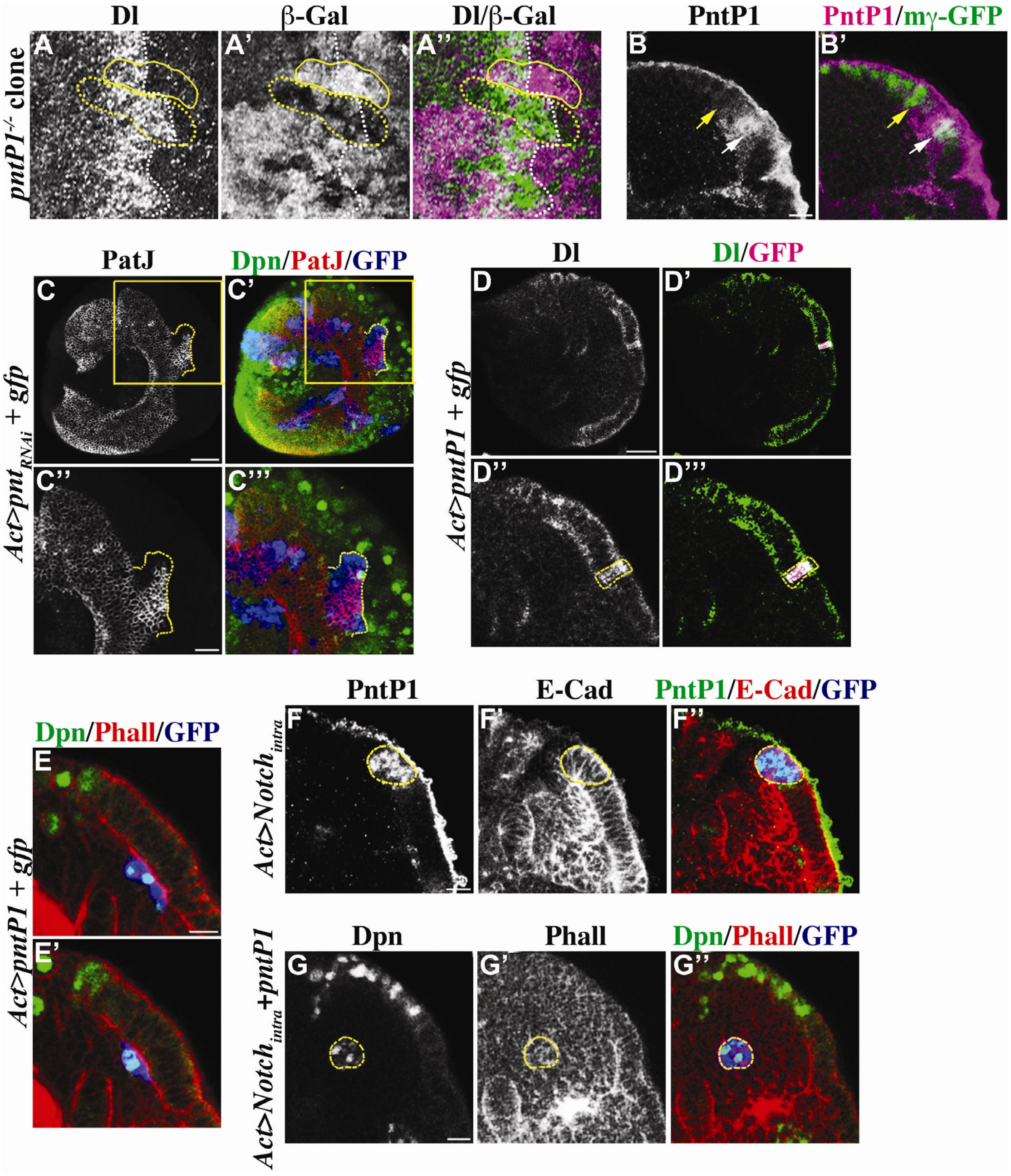


Figure 2.6. PntP1 promotes the transition from differentiating neuroepithelial cells to neuroblasts

Figure 2.6. PntP1 promotes the transition from differentiating neuroepithelial cells to neuroblasts

(A) *pntP1* is required for timely differentiation of neuroepithelia. Neuroepithelial cells in the negatively marked *pntP1* mutant mosaic clone (outline in dotted yellow line) show a significant delay in transitioning from differentiating neuroepithelial cells into neuroblasts as indicated by prolonged expression of a high level of Delta. In contrast, neuroepithelial cells in the wild type control twin-spot clone (outlined in solid yellow line) down-regulate Delta expression and transition into neuroblasts synchronously as the surrounding cells outside of the clone. Down-regulation of Delta expression indicates a successful transition from differentiating neuroepithelial cells into neuroblasts (white dotted line).

(B) PntP1 becomes transiently up-regulated in differentiating neuroepithelial cells. The expression of PntP1 largely co-localizes with E(spl)mγ-GFP in differentiating neuroepithelial cells (white arrow), but is undetectable in optic lobe neuroblasts (yellow arrow). Please note that the antibody against PntP1 shows non-specific background staining on the surface of the brain. The scale bar = 10 μm.

(C) *pnt* is necessary for differentiation of neuroepithelial cells. Neuroepithelial cells with reduced function of both *pntP1* and *pntP2* isoforms marked by the expression of GFP fail to differentiate into neuroblasts leading to protrusion of neuroepithelia surrounded by optic lobe neuroblasts. The scale bar = 20 μm. The higher magnification image of the boxed area is shown in C" and C"'. The scale bar = 10 μm.

(D-E) *pntP1* is sufficient to induce differentiation of neuroepithelial cells. (D-D"') Cells transiently over-expressing *pntP1* in the GFP-marked genetic clone (yellow arrow) located laterally from differentiating neuroepithelial cells (white arrow) show dramatic up-regulation of Delta. The scale bar = 20 μm. The higher magnification image of neuroepithelia containing the clone is shown in D" (outlined in dotted yellow line). (E) Cells over-expressing *pntP1* marked by expression of GFP delaminate inward away from the rest of neuroepithelia, and prematurely differentiate into neuroblasts. A superficial optical section is shown in E, and a distal optical section is shown in E'. The scale bar = 10 µm.

(F-G) Constitutively activated *Notch* signaling de-sensitizes neuroepithelial cells from PntP1. (F) Neuroepithelial cells over-expressing $Notch_{intra}$ marked by expression of GFP (outlined in dotted yellow line) accumulate at the medial edge of neuroepithelia and show expression of PntP1. However, constitutively activated *Notch* signaling prevents the transition from differentiating neuroepithelial cells into neuroblasts. The scale bar = 10 µm. (G) Co-expression of PntP1 triggers GFP-marked neuroepithelial cells that exhibit constitutively activated *Notch* signaling to differentiate into neuroblasts (outlined in yellow). The scale bar = 10 µm.

Discussion

A precise balance between neuroepithelial cell maintenance and differentiation coordinates neuronogenesis and morphogenesis in the developing optic lobe. A dysregulated transition from neuroepithelial cells into neuroblasts perturbs formation of the neuronal network, which will almost certainly lead to visual impairment of the adult fly. In this study, we provide evidence that spatial changes in the level of *Notch* signaling regulate the balance between maintenance and differentiation of neuroepithelial cells. A low level of *Notch* signaling maintains the neuroepithelial cell identity by triggering Aop-dependent repression of the *pntP1* gene. Transient up-regulation of the *Notch* signaling at the onset of neuroepithelial cell differentiation prevent precocious formation of immature neuroblasts by raising the threshold of response to PntP1. Finally, abrupt down-regulation of *Notch* signaling together with a high level of PntP1 trigger the transition from differentiating neuroepithelial cells into immature neuroblasts at the medial edge of neuroepithelia. Thus, interplay between spatial changes in *Notch* signaling and transient up-regulation of *pntP1* orchestrates synchronous and progressive formation of neuroblasts in a medial-to-lateral orientation across the entire neuroepithelial swath (Figure 2.7).

Maintenance of the optic lobe neuroepithelium

Maintenance of the neuroepithelial cell identity requires a low level of *Notch* signaling, which triggers Aop-dependent repression of the *pntP1* gene. Consistent with recent

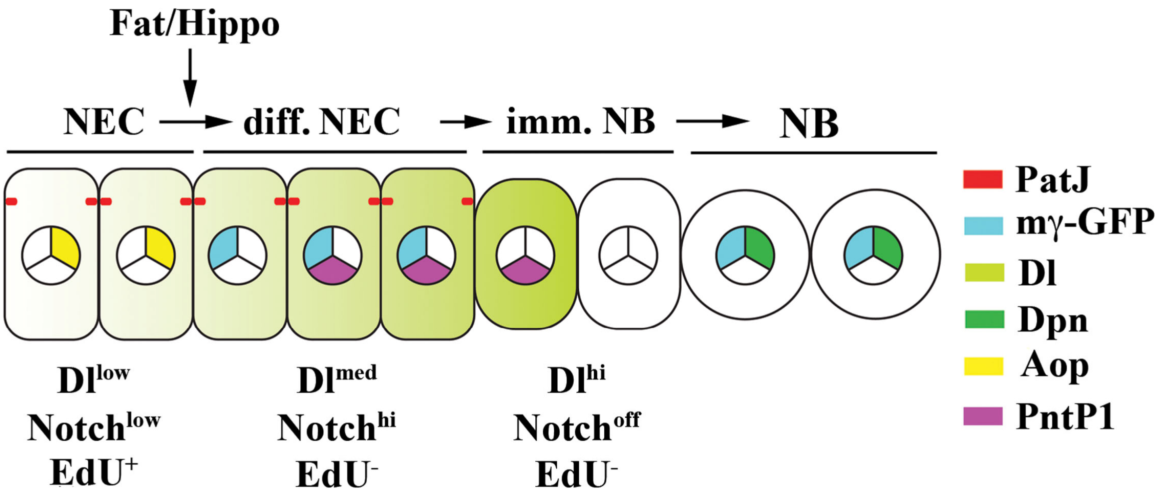


Figure 2.7. A model summarizing spatial changes in *Notch* signaling during differentiation of neuroepithelial cells

studies, we showed that *Notch* plays a key role in maintaining the identity of neuroepithelial cells despite the lack of *Notch* reporter transgene expression. Importantly, constitutively activated *Notch* signaling is sufficient to trigger robust cell autonomous expression of E(spl)mγ-GFP in cells located laterally from differentiating neuroepithelial cells. Thus, neuroepithelial cells that are yet ready to differentiate are competent to express the *E(spl)mγ-GFP* reporter transgene in response to a high level of *Notch* signaling. In agreement with this result, a low level of Delta expression, which can *trans*-activate *Notch* signaling, is also detected throughout neuroepithelia. Taken together, maintenance of the neuroepithelial cell identity requires a low level of *Notch* signaling.

We propose that *Notch* maintains the identity of neuroepithelial cells by activating Aop-dependent repression of the *pntP1* gene. The Suppressor of Hairless protein, which is necessary for activating transcription of *Notch* targets genes, directly binds to the promoter of the *aop* gene (Krejcí et al., 2009; Rohrbaugh et al., 2002). Consistently, removing the *Notch* or *aop* function triggered premature differentiation of neuroepithelia whereas over-expressing *Notch* or *aop* prevented neuroepithelial cell differentiation. Importantly, over-expression of *aop* suppressed premature differentiation of *Notch* mutant neuroepithelial cells. Thus, Aop functions downstream of *Notch* to maintain the identity of neuroepithelial cells. Aop functions as an evolutionarily conserved transcriptional repressor in the absence of activated receptor tyrosine kinase signaling by suppressing the expression of *pntP1* (Doroquez and Rebay, 2006). Consistent with this regulatory mechanism, heterozygosity of the *pntP1* gene completely suppressed premature differentiation of neuroepithelial cells in a hypomorphic *aop* mutant genetic background. Therefore, Aop maintains the identity of neuroepithelial cells by repressing *pntP1*. In the future, analyses of *Notch* and *pntP1* double mutants will be necessary to confirm that *Notch* maintains neuroepithelial cell identity by repressing the *pntP1* gene.

The transition from differentiating neuroepithelia to immature neuroblasts

Notch signaling first becomes transiently up regulated in differentiating neuroepithelial cells, and then abruptly down-regulated at the medial edge of neuroepithelia where the transition to neuroblasts occurs. One possibility might be that up-regulation of *Notch* signaling prevents differentiating neuroepithelial cells from precociously transitioning into immature neuroblasts by desensitizing these cells from responding to PntP1. While constitutively activated *Notch* signaling blocked differentiation of neuroepithelial cells despite expressing PntP1, co-expression of a *UAS-pntP1* transgene overcame the blockade and allowed these cells to transition into immature neuroblasts. Such mechanism potentially permits only differentiating neuroepithelial cells showing the highest level of receptor tyrosine signaling, which should also express the highest level of PntP1, to transition into immature neuroblasts. This regulatory mechanism will be consistent with a recent study showing that the EGF ligand is processed and secreted by cells near the medial edge of the optic lobe neuroepithelia (Yasugi et al., 2010). As a result of simple diffusion, differentiating neuroepithelial cells at the medial edge of neuroepithelia will likely express the highest level of PntP1, and consequently, only these cells can transition into immature neuroblasts. Taken together, *EGF* signaling likely creates a vector field establishing the directionality of differentiation whereas *Notch* signaling refines the functional output of *EGF* signaling by raising the threshold of PntP1.

Many important questions arise from this highly plausible mechanism by which the interplay between *Notch* and *EGF* signaling paces synchronous differentiation of neuroepithelial cells into neuroblasts one row at a time. This model will predict that immature neuroblasts immediately adjacent to differentiating neuroepithelial cells should secrete the processed EGF ligand. However, the antibody specific for the Rhomboid (Rho) protease is currently unavailable, and a genomic fragment encompassing the *rho-1* locus tagged with YFP did not show detectable expression in the larval optic lobe (Weng and Lee, unpublished observation). Identification of the cell type from which the processed EGF ligand is released will be key to verify this model. In addition, a recent study shows that *pntP1* is a direct target of Notch *in vivo* raising the possibility that up-regulation of *Notch* might directly activate transcription of the *pntP1* gene in differentiating neuroepithelial cells (Krejcí et al., 2009). Analyses of the promoter in the *pntP1* gene will be critical to address regulation of the spatial-specific expression of PntP1. Finally, more experiments will be needed to rule out the possibility that the transition from differentiating neuroepithelial cells into neuroblasts might occur simply due to a decrease in the threshold of response to PntP1 following *cis*-inhibition of *Notch* signaling by Delta.

Other signaling mechanisms that affect differentiation of neuroepithelial cells

The *fat-hippo* signaling mechanism controls tissue growth by regulating proliferation and cell death, and promotes timely differentiation of optic lobe neuroepithelial cells (Reddy et al., 2010; Zhao et al., 2010). While inactivation of *fat-hippo* signaling delays differentiation of the optic lobe neuroepithelia, removal of the downstream effecter *yorkie* only accelerates differentiation of neuroepithelial cells near the medial edge of the optic lobe neuroepithelia (Reddy et al., 2010). Thus, *fat-hippo* signaling likely functions as a gatekeeper to prevent over-growth of the optic lobe neuroepithelia by triggering transient cell cycle arrest. Intriguingly, transient cell cycle arrest precedes increased *Notch* signaling in differentiating neuroepithelial cells. Detailed studies in the future will be necessary to determine if activation of the *fat-hippo* signaling might contribute to increased *Notch* signaling in differentiating neuroepithelial cells.

Materials and Methods

o-fut1^{2834}, *aph-1*^{5072} and *fat*^{3477} alleles were recovered from EMS mutagenesis following a standard protocol. *Drosophila* cultures were kept at 25°C on standard cornmeal food. Other mutant alleles and transgenes used in this study include *pcna-3XEmgfp* (R. Duronio), *o-fut1*^{4R6} (K. Matsuno), , *l(3)mbt*^{GM76} and *l(3)mbt*^{GM79} (R. Lehmann), *E(spl)mγ-gfp* (S. Bray), *UAS-Notch*$_{intra}$ (G. Struhl), *aop-lacZ* (Z.-C. Lai), *hs-flp; FRT40A, tub-gal80; tub-gal4, UAS-mCD8-GFP* (H. Reichert), *FRT19A, tub-gal80, hs-flp; UAS-mcd8::GFP* (C. Doe), *rho-1–YFP* (B. Shilo). The following stocks were obtained from the Bloomington Stock Center: *tub-gal4, hs-flp(F38)*, *act-FRT-CD2-FRT-gal4, UAS-GFP*, *act-FRT-CD2-FRT-gal4, UAS-mRFP*, *UAS-mCD8-GFP*, *tub-GAL80*^{ts}, *GH146-gal4*, *UAS-delta*, *UAS-aop.WT*, *UAS-pntP1*, *N*^{55e11}, *FRT19A*, *aop*^{yan1}, *aop*^{1}, *aph-1*^{D35}, *pnt*$^{\Delta33}$, *csw*^{G0170}, *cnk*^{E2083}, *cnk*^{k16314}, *ft*$^{G-rv}$.

Immunofluorescent microscopy and antibodies

Larval brains were dissected in Schneider's medium and fixed in PBS containing 0.3% Triton X-100 (PBS-T) containing 4% Formaldehyde for 23 min at room temperature; quickly washed in PBS-T; incubated in primary antibodies diluted in PBS-T for 3-4 hrs at the room temperature or overnight at 4°C. After quick washing with PBS-T, the specimen was incubated in secondary antibodies in PBS-T at the room temperature or overnight at 4^{o}C. After quick washing in PBS-T, the specimen was equilibrated in Prolong Gold (Molecular Probe). Antibodies used in this study include rat Dpn (1:1), guinea pig Dpn (1:2500, J. Skeath), rabbit PatJ (1:2000, H. Bellen), rabbit PntP1 (1:1000, J. Skeath), L'(sc) (A. Carmena), rat -tubulin (1:100, Serotec), rabbit phosphorylated-histone H3 (1:1000, Upstate), rabbit -gal (1:1000, ICN/Cappel), mouse -gal (1:100, Sigma), chicken GFP (1:2000, Aves Lab), rabbit RFP (1:100, Rockland), mouse BrdU (1:100, BD Biosciences), mouse Elav (DSHB), mouse dE-Cad (DSHB), mouse Notch$_{intra}$ (1:100, DSHB). Secondary antibodies were from Molecular Probes and Jackson Labs (details are available upon request). To stain cortical actin, rhodamine Phalloidin or Alexa 488 Phalloidin (Molecular Probes) was used as 1:100 in PBS-T. The images were acquired on a Leica SP5 scanning confocal microscope. 3-D reconstruction of the fly larval optic lobe was performed by using the Mimics software (Materialise).

EdU staining

Larvae were aged for 72 hr after hatching, and were pulse labeled for 3 hrs by feeding on the Kankel-White media containing 50 µg/ml EdU (5-ethynyl-2'deoxyuridine) (Daul et al., 2010). Half of the larvae were processed for staining immediately following the pulse; remaining larvae were transferred to standard media for a 12 hr EdU-free chase. Larvae were dissected and processed for antibody staining. Incorporated EdU was detected by Click-iT fluorescent dye azide reaction as described in the Click-iT product literature (Invitrogen).

This chapter presents the following manuscript submitted for review:

Weng M., Haenfler J., Lee C.-Y. Spatial changes in *Notch* signaling coordinates maintenance and differentiation of the *Drosophila* larval optic lobe neuroepithelia

Reference

Bray, S.J. (2006). Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol *7*, 678-689.

Daul, A.L., Komori, H., and Lee, C.-Y. (2010). EdU (5-ethynyl-2'-deoxyuridine) labeling of Drosophila mitotic neuroblasts. Cold Spring Harb Protoc *2010*, pdb.prot5461.

del Álamo, D., Rouault, H., and Schweisguth, F. (2011). Mechanism and significance of cis-inhibition in Notch signalling. Curr Biol *21*, R40-47.

Doroquez, D.B., and Rebay, I. (2006). Signal Integration During Development: Mechanisms of EGFR and Notch Pathway Function and Cross-Talk. Crit Rev Biochem Mol Biol *41*, 339-385.

Egger, B., Boone, J.Q., Stevens, N., Brand, A.H., and Doe, C. (2007). Regulation of spindle orientation and neural stem cell fate in the Drosophila optic lobe. Neural Dev *2*, 1.

Egger, B., Gold, K.S., and Brand, A.H. (2010). Notch regulates the switch from symmetric to asymmetric neural stem cell division in the Drosophila optic lobe. Development *137*, 2981-2987.

Francis, R., McGrath, G., Zhang, J., Ruddy, D.A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M.C.*, et al.* (2002). aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation. Dev Cell *3*, 85-97.

Hsiung, F., and Moses, K. (2002). Retinal development in Drosophila: specifying the first neuron. Hum Mol Genet 2002 May 15;11(10):1207-14 Review *Hum Mol Genet*, 1207-1214.

Janic, A., Mendizabal, L., Llamazares, S., Rossell, D., and Gonzalez, C. (2010). Ectopic expression of germline genes drives malignant brain tumor growth in Drosophila. Science *330*, 1824-1827.

Kolch, W. (2005). Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nat Rev Mol Cell Biol *6*, 827-837.

Krejcí, A., Bernard, F., Housden, B.E., Collins, S., and Bray, S.J. (2009). Direct response to Notch activation: signaling crosstalk and incoherent logic. Sci Signal *2*, ra1.

Kriegstein, A., and Alvarez-Buylla, A. (2009). The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci *32*, 149-184.

Lai, Z.-C., and Rubin, G.M. (1992). Negative control of photoreceptor development in Drosophila by the product of the yan gene, an ETS domain protein. Cell *70*, 609-620.

Ngo, K.T., Wang, J., Junker, M., Kriz, S., Vo, G., Asem, B., Olson, J.M., Banerjee, U., and Hartenstein, V. (2010). Concomitant requirement for Notch and Jak/Stat signaling during neuro-epithelial differentiation in the Drosophila optic lobe. Dev Biol *346*, 284-295.

Okajima, T., and Irvine, K.D. (2002). Regulation of notch signaling by o-linked fucose. Cell *111*, 893-904.

Orihara-Ono, M., Toriya, M., Nakao, K., and Okano, H. (2011). Downregulation of Notch mediates the seamless transition of individual Drosophila neuroepithelial progenitors into optic medullar neuroblasts during prolonged G1. Dev Biol *351*, 163-175.

Perkins, L.A., Larsen, I., and Perrimon, N. (1992). corkscrew encodes a putative protein tyrosine phosphatase that functions to transduce the terminal signal from the receptor tyrosine kinase torso. Cell *70*, 225-236.

Reddy, B.V.V.G., Rauskolb, C., and Irvine, K.D. (2010). Influence of Fat-Hippo and Notch signaling on the proliferation and differentiation of Drosophila optic neuroepithelia. Development *137*, 2397-2408.

Rohrbaugh, M., Ramos, E., Nguyen, D., Price, M., Wen, Y., and Lai, Z.-C. (2002). Notch Activation of yan Expression Is Antagonized by RTK/Pointed Signaling in the Drosophila Eye. Current Biology *12*, 576-581.

Sprinzak, D., Lakhanpal, A., LeBon, L., Santat, L.A., Fontes, M.E., Anderson, G.A., Garcia-Ojalvo, J., and Elowitz, M.B. (2010). Cis-interactions between Notch and Delta generate mutually exclusive signalling states. Nature *465*, 86-90.

Thacker, S.A., Bonnette, P.C., and Duronio, R.J. (2003). The contribution of E2F-regulated transcription to Drosophila PCNA gene function. Curr Biol *13*, 53-58.

Therrien, M., Wong, A.M., and Rubin, G.M. (1998). CNK, a RAF-binding multidomain protein required for RAS signaling. Cell *95*, 343-353.

Van Vactor, D., O'Reilly, A.M., and Neel, B.G. (1996). Genetic analysis of protein tyrosine phosphatases. Curr Opin Genet Dev *8*, 112-126.

Wang, W., Liu, W., Wang, Y., Zhou, L., Tang, X., and Luo, H. (2011). Notch signaling regulates neuroepithelial stem cell maintenance and neuroblast formation in Drosophila optic lobe development. Dev Biol *350*, 414-428.

Yasugi, T., Sugie, A., Umetsu, D., and Tabata, T. (2010). Coordinated sequential action of EGFR and Notch signaling pathways regulates proneural wave progression in the Drosophila optic lobe. Development *137*, 3193-3203.

Zhao, B., Li, L., and Guan, K.-L. (2010). Hippo signaling at a glance. J Cell Sci *123*, 4001-4006.

CHAPTER III

dFezf/Earmuff Maintains the Restricted Developmental Potential of Intermediate Neural Progenitors in *Drosophila*

Summary

To ensure normal development and maintaining homeostasis, the extensive developmental potential of stem cells must be functionally distinguished from the limited developmental potential of transit amplifying cells. Yet the mechanisms that restrict the developmental potential of transit amplifying cells are poorly understood. Here we show that the evolutionarily conserved transcription factor dFezf/Earmuff (Erm) functions cell-autonomously to maintain the restricted developmental potential of the intermediate neural progenitors generated by type II neuroblasts in *Drosophila* larval brains. Although *erm* mutant intermediate neural progenitors are correctly specified and show normal apical-basal cortical polarity, they can de-differentiate back into a neuroblast state, functionally indistinguishable from normal type II neuroblasts. Erm restricts the potential of intermediate neural progenitors by activating Prospero to limit proliferation and by antagonizing Notch signaling to prevent de-differentiation. We conclude that Erm dependence functionally distinguishes intermediate neural progenitors from neuroblasts in the *Drosophila* larval brain, balancing neurogenesis with stem cell maintenance.

Introduction

Tissue development and homeostasis often require stem cells to transiently expand the progenitor pool by producing transit amplifying cells. Yet the developmental potential of transit amplifying cells must be tightly restricted to ensure generation of differentiated progeny and to prevent unrestrained proliferation that might lead to tumorigenesis (Morrison and Kimble, 2006; Pontious et al., 2008; Vescovi et al., 2006). Transit amplifying cells are defined by their limited developmental capacity, a feature specified during fate determination (Farkas et al., 2008; Hodge et al., 2008; Sessa et al., 2008). It is unknown whether an active mechanism is required to maintain restricted developmental potential in transit amplifying cells after specification. Here we use intermediate neural progenitors (INPs) in developing *Drosophila* larval brains as a genetic model to investigate how restricted developmental potential is regulated in transit amplifying cells.

A fly larval brain hemisphere contains eight type II neuroblasts that undergo repeated asymmetric divisions to self-renew and to generate immature INPs (Figure 3.1A) (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). Immature INPs are unstable in nature and are mitotically inactive, and lack the expression of Deadpan (Dpn) and Asense (Ase) (Figure 3.1S A). Immature INPs commit to the INP fate through maturation, a differentiation process necessary for specification of the INP identity (Figure 3.1 A). INPs express Dpn and Ase, and undergo 8-10 rounds of asymmetric divisions to self-renew and to produce ganglion mother cells (GMCs) that typically generate two neurons (Figure 3.1S A) (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). While 5-6 immature INPs and 1-2 young INPs are always in direct contact with their parental neuroblasts, the older INPs become progressively displaced from their parental neuroblasts over time (Figure 3.7 A) (Bowman et al., 2008).

During asymmetric divisions of type II neuroblasts, the basal proteins Brain tumor and Numb are exclusively segregated into immature INPs, and function cooperatively, but non-redundantly, to ensure that immature INPs undergo maturation and commit to the INP fate (Boone and Doe, 2008; Bowman et al., 2008). *brain tumor* or *numb* mutant type II neuroblasts generate immature INPs that fail to mature and do not commit to the INP fate (Figure 3.3S). Instead, *brain tumor* or *numb* mutant immature INPs adopt their parental neuroblast fate leading to supernumerary type II neuroblasts. Thus, *brain tumor* and *numb* specify the INP fate, and the ectopic expansion of type II neuroblasts in these mutant genetic backgrounds occurs due to failure to properly specifying the INP fate. Although Brain tumor is also asymmetrically segregated into GMCs during asymmetric divisions of INPs, the mosaic clones in *brain tumor* mutant INPs contain only differentiated neurons (Bowman et al., 2008). This result indicates that Brain tumor is dispensable for maintaining the restricted developmental potential of INPs. How restricted developmental potential is maintained in INPs is currently unknown.

To identify genes that regulate self-renewal of neuroblasts, we conducted a genetic screen for mutants exhibiting ectopic larval brain neuroblasts (C.-Y. Lee and C.Q. Doe, unpublished). One mutation, *l(2)5138*, specifically resulted in massive expansion of neuroblasts in the brain but did not affect neuroblasts on the ventral nerve cord (Figure 3.1S B-D). We mapped the *l(2)5138* mutation to the 22B4-7 chromosomal interval that contains the *earmuff* (*erm*) gene (Pfeiffer et al., 2008). The *erm* transcripts are first detected at the embryonic stage 4-6 in the specific domain preceding formation of the embryonic brain, and remain highly expressed in the brain throughout development (Chintapalli et al., 2007; Pfeiffer et al., 2008). Here, we report that Erm functions to restrict the developmental potential of INPs by promoting Prospero-dependent termination of proliferation and suppressing Notch-mediated de-differentiation. By restricting their developmental potential, Erm ensures that INPs generate only differentiated neurons during *Drosophila* neurogenesis.

Results

Earmuff prevents abnormal expansion of neural progenitors in type II neuroblast lineages

All neuroblasts in *l(2)5138* homozygous mutant brains were proliferative, expressed all known neuroblast markers, and lacked neuronal and glial markers (Figure 3.1 B-G; Figure 3.1S B-D; data not shown). We mapped the *l(2)5138* mutation to the *erm* gene, which encodes a homolog of the vertebrate Forebrain embryonic zinc-finger family (Fezf) transcription factors (Hashimoto et al., 2000; Matsuo-Takasaki et al., 2000). The *l(2)5138* mutants contained a single A->T nucleotide change in the *erm* coding region, leading to the substitution of a leucine for a conserved histidine in the third C_2H_2 zinc-finger domain (data not presented). Consistent with its predicted molecular function, ectopic expression of Erm transgenic proteins tagged with a HA epitope at the amino- or carboxyl-terminus driven by neuroblast-specific *Wor-Gal4* was detected in the nuclei of neuroblasts (data not presented). However, the expression of the HA-tagged Erm transgenic protein bearing the identical leucine to histidine substitution as in the *l(2)5138* mutant was undetectable, suggesting that the mutant Erm protein is unstable (data not presented). We conclude that *l(2)5138* is a mutant allele of *erm*.

To determine whether *erm* mutant brains have ectopic type I and/or type II neuroblasts, we analyzed the expression pattern of Ase and Prospero (Pros), which are only expressed in type I neuroblasts (Figure 3.1S A) (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). We found that *erm* mutant brains contained over 20-fold more type II neuroblasts (Dpn$^+$Ase$^-$) than wild type brains with no significant change in the number of type I neuroblasts (Dpn$^+$Ase$^+$) (Figure 3.1 F-H). Next, we analyzed the localization of Prospero in mitotic neuroblasts in larval brains expressing GFP induced by *Ase-Gal4* (*Ase>GFP*), which mimicked the expression pattern of the endogenous Ase protein (Bowman et al., 2008). In *erm* mutant larval brains, all mitotic type I neuroblasts (GFP$^+$) showed formation of basal Prospero crescents, but none of the mitotic type II neuroblasts (GFP$^-$) showed the expression of Prospero (Figure 3.1 I-J; n=20). Furthermore, GFP-marked *erm* mutant type II neuroblast clones consistently contained

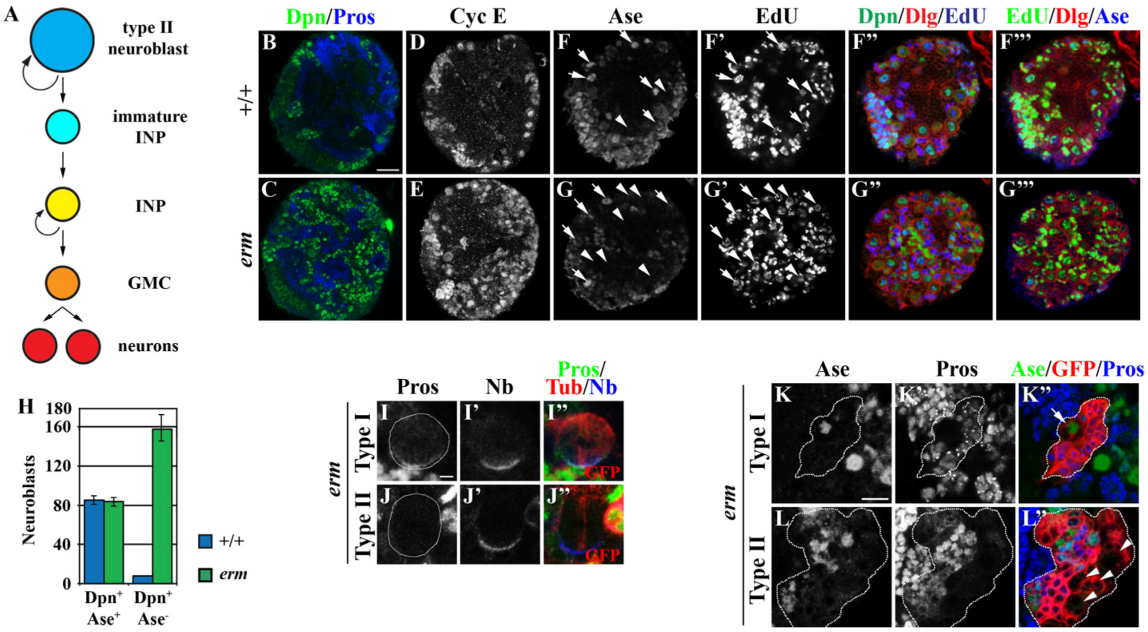


Figure 3.1. ***erm* mutant brains show ectopic type II neuroblasts**

(A) A summary of type II neuroblast lineage.

(B-H) While wild type (+/+) and *erm* mutant brains contained a similar number of type I neuroblasts (Dpn$^+$CycE$^+$Ase$^+$EdU$^+$; white arrows), *erm* mutant brains contained ectopic type II neuroblasts (Dpn$^+$CycE$^+$Ase$^-$EdU$^+$; white arrowheads). Scale bar, 20μm.

(I-J) In *erm* mutant brains expressing GFP driven by *Ase-Gal4*, Prospero (Pros) always co-localized with Numb (Nb) in metaphase type I neuroblasts (GFP$^+$; white circle), but never in type II neuroblasts (GFP$^-$; white circle). Scale bar, 2μm.

(K-L) *erm* mutant type I neuroblast clones (white circle) always contained a single neuroblast (white arrow), but *erm* mutant type II neuroblast clones (white circle) always contained multiple neuroblasts (white arrowheads).

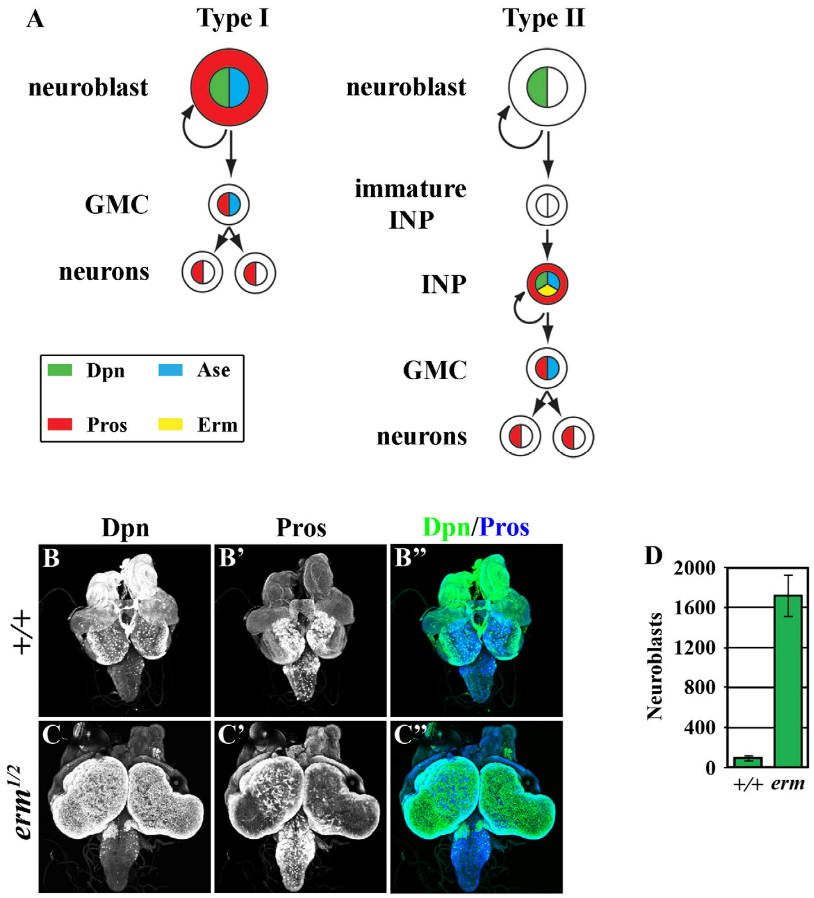


Figure 3.1S. *erm* mutant brains show a dramatic increase in neuroblasts

(A) A type I neuroblast generates a daughter type I neuroblast and a GMC that produces two neurons. A type II neuroblast generates a daughter type II neuroblast and an immature INP that becomes an INP undergoing limited rounds of asymmetric divisions. (B-C) *erm* mutant larvae showed dramatically enlarged brain lobes containing supernumerary neuroblasts compared to similarly staged wild type control larvae.

(D) *erm* mutant larval brains contained more than 10-fold increase in ectopic neuroblasts (Dpn$^+$Pros$^-$).

multiple type II neuroblasts, whereas *erm* mutant type I neuroblast clones always contained single type I neuroblasts and neurons (Figure 3.1 K-L). We conclude that *erm* mutant brains exhibit an abnormal expansion of type II neuroblasts.

erm regulates the developmental potential of INPs

To determine the cellular origin of ectopic type II neuroblasts in *erm* mutant brains, we analyzed the identity of cells in the GFP-marked clones derived from wild type or *erm* mutant type II neuroblasts using specific cell fate markers. At 30 hrs after clone induction, wild type and *erm* mutant neuroblast clones appeared indistinguishable, containing single parental neuroblasts (Dpn$^+$Ase$^-$; $\geq$ 10 μm) in direct contact with 5-6 immature INPs (Dpn$^-$Ase$^-$) while most of the INPs (Dpn$^+$Ase$^+$; $\geq$ 6 m) were 1 cell or more away from the parental neuroblasts (Figure 3.2 A-B). At 48 hrs after clone induction, the overall size of both wild type and *erm* mutant neuroblast clones has increased significantly due to an increase in cell number reflecting continuous asymmetric divisions of the parental neuroblasts. In both wild type and *erm* mutant clones, the parental neuroblasts remained surrounded by 5-6 immature INPs while INPs and differentiated neurons (Dpn$^-$Ase$^-$Pros$^+$) can be found several cells away from the parental neuroblasts (Figure 3.2 C-F; Figure 3.2S A-F). However, *erm* mutant clones contained fewer INPs (16±4; n=10 brains) than the wild type clones (21±4; n=10 brains). Importantly, *erm* mutant clones consistently contained 4-6 smaller ectopic type II neuroblasts (Dpn$^+$Ase$^-$; 6-8 m in diameter) (Figure 3.2 F and 3.2S F). Thus, Erm is dispensable for both generation and maturation of immature INPs.

Ectopic type II neuroblasts in 48-hr *erm* mutant clones were always several cells away from the parental neuroblasts (Figure 3.2 F and 3.2S F). This result strongly suggests that ectopic type II neuroblasts in *erm* mutant clones likely originate from INPs and Erm likely functions in INPs. However, we could not assess the spatial expression pattern of the endogenous Erm protein in larval brains due to lack of a specific antibody and low signals by fluorescent RNA *in situ* (data not presented). Alternatively, we analyzed the expression of the *R9D* series of *Gal4*-transgenes in which Gal4 is expressed under the control of overlapping *erm* promoter fragments (Pfeiffer et al., 2008). The expression of *R9D11-Gal4* was clearly detected in INPs, but was undetectable in type II neuroblasts and immature INPs even when two copies of the *UAS-mCD8-GFP* transgenes were driven by two copies of *R9D11-Gal4* at 32^{o}C for 72 hrs after larval hatching (Figure 3.2 G; Figure 3.2S G). Consistently, the expression of *Erm-Gal4* was virtually undetectable in *brain tumor* mutant brains that contain thousands of type II neuroblasts and immature INPs (Figure S2H). While the expression of *UAS-erm* induced by the neuroblast-specific *Wor-Gal4* driver led to premature loss of type II neuroblasts, expression of *UAS-erm* driven by *Erm-Gal4* failed to exert any effects on type II neuroblasts (data not presented). Importantly, targeted expression of the fly Erm, mouse Fezf1 or Fezf2 transgenic protein driven by *R9D11-Gal4* restored the function of Erm and efficiently rescued the ectopic neuroblast phenotype in *erm* mutant brains (Figure 3.2S I-L). Therefore, *R9D11-Gal4* (*Erm-Gal4*) contains the enhancer element sufficient

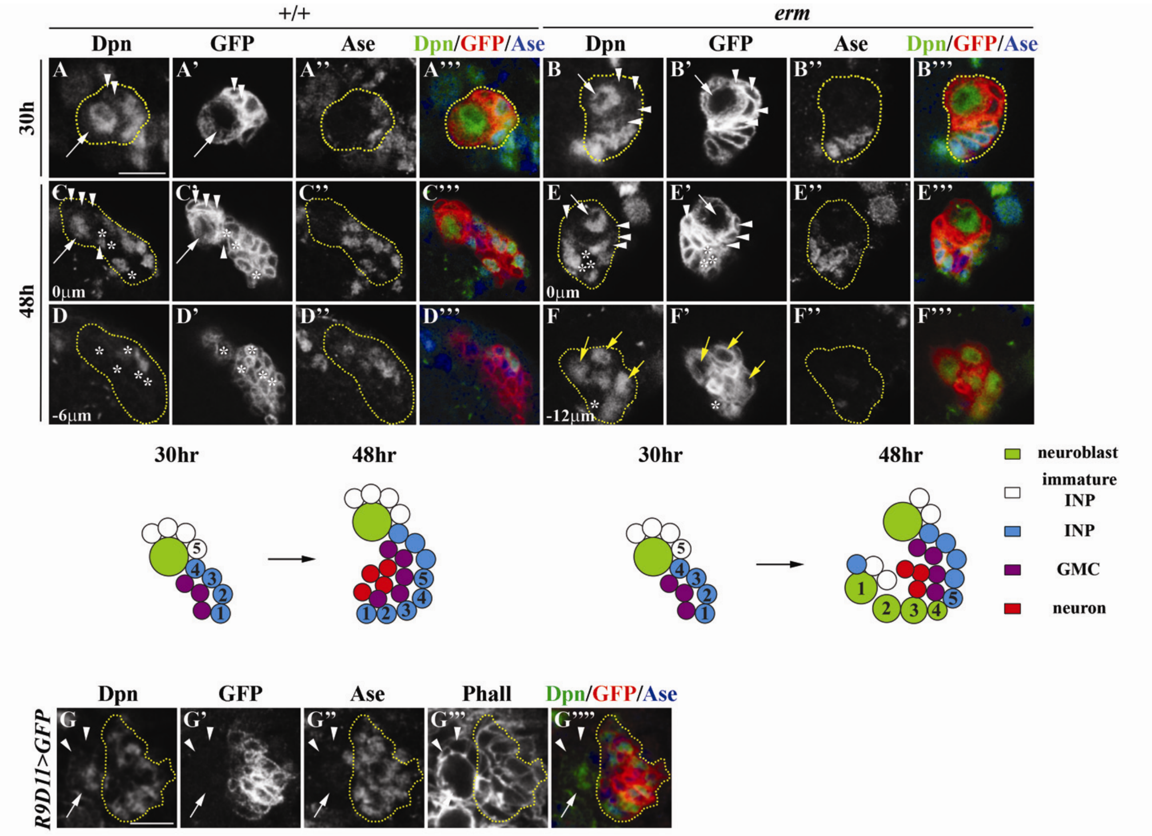
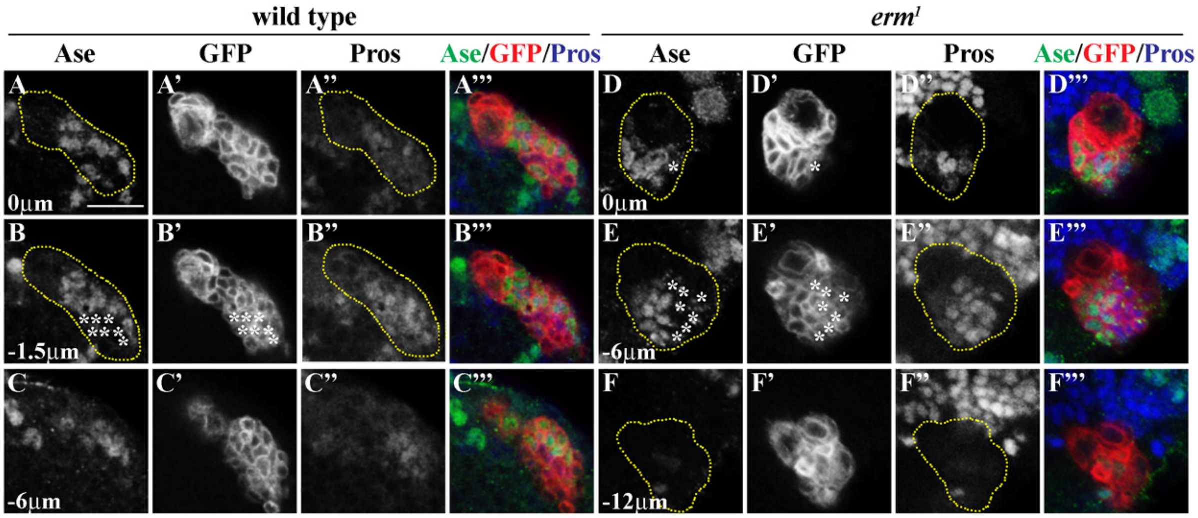


Figure 3.2. Erm maintains the limited developmental potential of INPs

(A-B) At 30 hours after clone induction, both wild type (+/+) and *erm* mutant neuroblast clones (yellow circles) contained a single parental neuroblast (white arrows) directly surrounded by immature INPs (white arrowheads) and 1-2 young INPs (Dpn^{+}Ase^{+}).

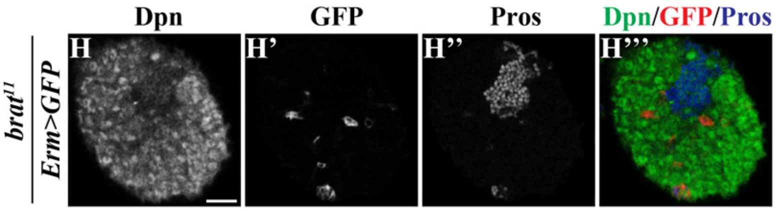
(C-F) At 48 hours after clone induction, wild type (+/+) neuroblast clones (yellow circles) contained a single parental neuroblast (white arrows) in direct contact with immature INPs (white arrowheads) and young INPs (Dpn^{+}Ase^{+}). Older INPs were away from their parental neuroblasts and were surrounded by GMCs (white asterisks) and neurons (Dpn^{-}Ase^{-}). In contrast, the *erm* mutant clones contained ectopic type II neuroblast-like cells (f, yellow arrows) further from the parental neuroblasts than most INPs and neurons. A summary diagram is shown below.

(G) *R9D11-Gal4* (*Erm-Gal4*) was undetectable in type II neuroblasts (white arrow) and immature INPs (white arrowheads), but was clearly detected in INPs. All scale bars, 10 µm.



G

	Central Brain
9D2-Gal4	3 type I neuroblast lineages
9D3-Gal4	a few neurons & 1 type I neuroblast
9D4Gal4	very few neurons
9D5-Gal4	no expression
9D6-Gal4	neurons
9D8-Gal4	few type I neuroblasts
9D9-Gal4	few neurons
9D10-Gal4	INPs
9D11-Gal4	INPs



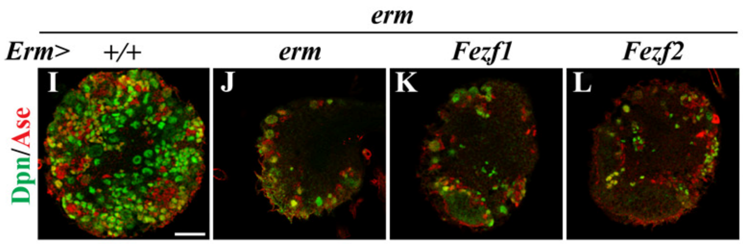


Figure 3.2S. ***erm*** **prevents INPs from reverting into type II neuroblasts**

Figure 3.2S. *erm* prevents INPs from reverting into type II neuroblasts

(A-F) At 48 hrs after clone induction, *erm* mutant type II neuroblast clones (yellow circles) contained fewer INPs (Ase^+Pros^-), GMCs (Ase^+Pros^+) and neurons (Ase^-Pros^+; white asterisks) compared to similarly staged wild type neuroblast clones.

(G) The expression pattern of the *R9D* series of *Gal4* lines

(H) The expression of *Erm-Gal4* in *brain tumor* (*brat*) mutant brains was undetected in ectopic type II neuroblasts and immature INPs despite a dramatic increase in their population.

(I-L) Over-expression of *erm*, *Fezf1* and *Fezf2* induced by *Erm-Gal4* efficiently restored the Erm function and rescued the ectopic neuroblast phenotype in *erm* mutant brains.

to restore the Erm function in INPs leading to suppression of ectopic type II neuroblasts in *erm* mutant brains.

erm mutant INPs de-differentiate back into type II neuroblasts

Mutant clonal analyses and over-expression studies strongly suggest that Erm functions to suppress reversion of INPs back into a neuroblast state. Here, we directly tested if INPs in *erm* mutant brains can de-differentiate back into type II neuroblasts. We induced gal-marked lineage clones originating exclusively from INPs via FRT-mediated recombination. We targeted a short pulse of flipase (FLP) expression in INPs by heat-shocking larvae carrying a *UAS-flp* transgene under the control of *Erm-Gal4* and *tub-Gal80^{ts}* at 30°C for 1 hour (see Experimental procedures for detail). At 72 hours after heat-shock, INP clones in wild type brains contained only differentiated neurons (Dpn^{-}Ase^{-}) (Figure 3.3 A). In contrast, INP clones in *erm* mutant brains contained one or more type II neuroblasts as well as immature INPs, INPs, GMCs and neurons (Figure 3.3 B-C). This result indicates that while INPs in wild type larval brains can only give rise to neurons, INPs in *erm* mutant brains can de-differentiate into type II neuroblasts that can give rise to all cell types found in a normal type II neuroblast lineage. We conclude that Erm functions to maintain the restricted developmental potential of INPs and prevents them from de-differentiating back into a neuroblast state.

We further assessed if the de-differentiated type II neuroblasts in *erm* mutant brains displayed multiple functional characteristics of normal type II neuroblasts.

Apical-basal cell polarity: All mitotic type II neuroblasts in wild type and *erm* mutant brains showed normal establishment and maintenance of cortical polarity by asymmetrically localizing and segregating atypical Protein Kinase C (aPKC), Pins, Miranda and Numb (data not presented).

Proliferation profile: All wild type and *erm* mutant type II neuroblasts could be labeled with a 3-hour pulse of the thymidine analog EdU (Figure 3.1F' and G'), and incorporated EdU can be chased into INPs following a 12-hour EdU-free chase (Figure 3.3 D-E).

***prospero* and *earmuff* promoter activity:** While all type I neuroblasts in wild type and *erm* mutant brains expressed *Pros-Gal4* but lacked *Erm-Gal4* expression, none of the type II neuroblasts in wild type and *erm* mutant brains showed detectable expression of *Pros-Gal4* or *Erm-Gal4* (Figure 3.3 F-G; data not presented).

Formation of glial chambers: Individual neuroblast lineages are surrounded by the cortex glial membrane forming distinct chambers (Pereanu et al., 2005). A wild type brain hemisphere contained eight glial chambers encapsulating eight individual type II neuroblast lineages (Figure 3.3 H). In contrast, an *erm* mutant brain hemisphere contained more than 50 glial chambers each containing one or more type II neuroblasts and their presumptive progeny (Figure 3.3 I).

Taken together, INPs in *erm* mutant brains de-differentiate back into apparently normal neuroblasts that can establish ectopic type II neuroblast lineages.

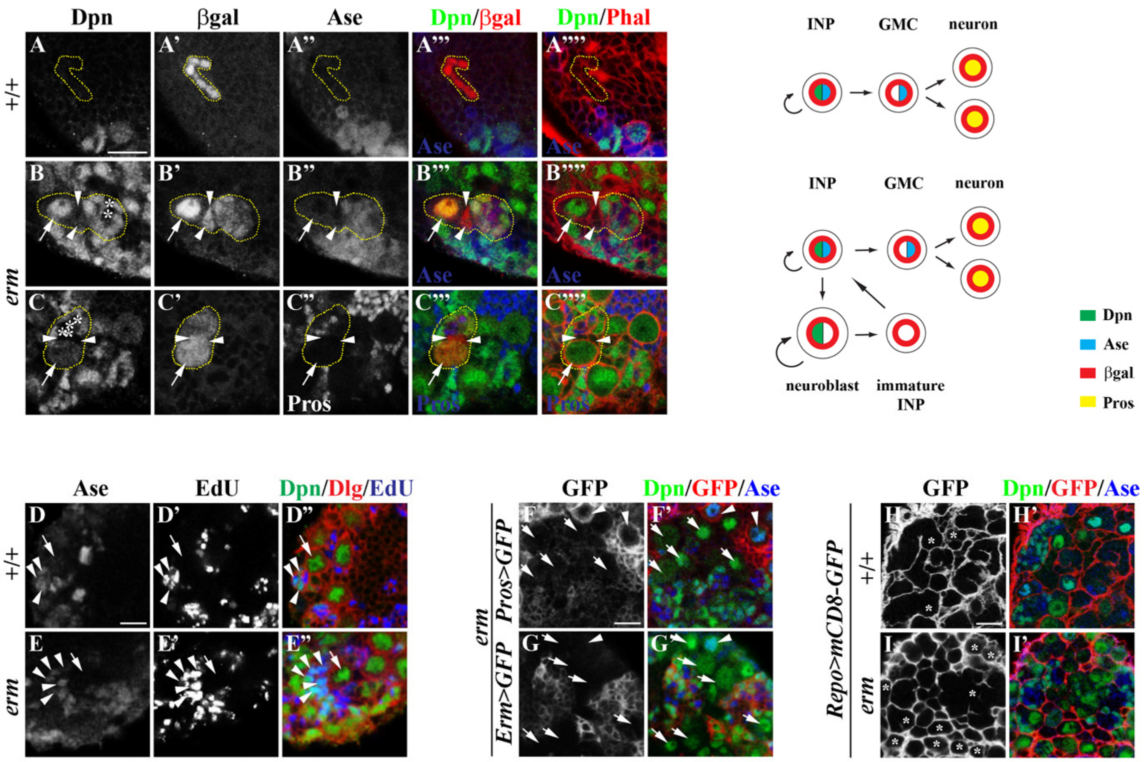


Figure 3.3. ***erm*** **suppresses the de-differentiation of INPs**

(A-C) A wild type (+/+) INP only generated neurons (Dpn$^-$Ase$^-$), but an *erm* mutant INP generated de-differentiated neuroblasts (white arrows), immature INPs (white arrowheads) and INPs (Dpn$^+$Ase$^+$), GMCs (B, white asterisks) and neurons (C, white asterisks). A lineage clone is circled in yellow, and a summary diagram is shown on the right.

(D-I) Similar to wild type type II neuroblasts, ectopic type II neuroblasts in *erm* mutant brains lost incorporated EdU (neuroblasts, white arrows; INPs, white arrowheads) (D-E), did not express *Pros-Gal4* and *Erm-Gal4* (type I neuroblast, white arrowheads; type II neuroblasts, white arrows) (F-G), and established ectopic neuroblast lineages (white asterisks) surrounded by glial membrane (H-I). All scale bars, 10 µm.

erm mutant INPs exhibit normal apical-basal cortical polarity

Dysregulation of apical-basal polarity can lead to failure in differentiation and result in ectopic neuroblasts at the expense of GMC formation (Betschinger et al., 2006; Lee et al., 2006a; Lee et al., 2006b; Lee et al., 2006c; Wang et al., 2006). To determine if the de-differentiation of INPs in *erm* mutant brains might be due to defects in cortical polarity, we assayed apical-basal polarity by examining the localization of aPKC, Miranda, Prospero and Numb in larval brains expressing GFP driven by *Ase-GAL4* (*Ase>GFP*). Mitotic INPs (GFP^+) in *erm* mutant brains showed the same asymmetric localization of aPKC, Miranda, Prospero and Numb as in wild type brains (Figure 3.4 A-B; data not presented). Thus, we conclude that INPs in *erm* mutant brains de-differentiate while displaying normal cortical polarity.

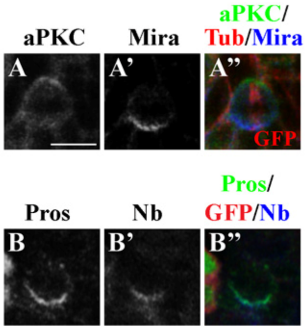


Figure 3.4. *erm* mutant INPs show normal apical-basal polarity

(A-B) Metaphase INPs in *erm* mutant brains expressing GFP induced by *Ase-Gal4* showed asymmetric localization of aPKC, Miranda (Mira), Pros and Numb (Nb). The scale bar, 5 μ m.

Erm restricts proliferation through activating Prospero-dependent cell cycle exit

To determine how Erm maintains the restricted developmental potential of INPs, we performed microarray analyses and found that *prospero* mRNA was drastically reduced in *erm* mutant brains compared to the control brains (Weng and Lee, unpublished). We confirmed that the relative level of *prospero* mRNA was indeed reduced by 60-70% in *erm* mutant brain extracts by using real-time PCR (data not presented). These data supported that Erm is necessary for proper transcription of *prospero*, and prompted us to test if over-expression of Erm might be sufficient to induce ectopic Prospero expression. We induced a short pulse of Erm expression in brain neuroblasts by shifting larvae carrying a *UAS-erm* transgene under the control of *Wor-Gal4* and *tub-Gal80*^{ts} to from 25^{o}C to 30^{o}C. A 3.5-hour pulse of Erm expression was sufficient to induce nuclear localization of Prospero in larval brain neuroblasts (Figure 3.5 A). Consistent with nuclear Prospero promoting termination of neuroblast proliferation, ectopic expression of Erm induced by *Wor-Gal4* resulted in decreased neuroblasts compared to wild type brains (Figure 3.5 B). Thus, we conclude that over-expression of Erm can restrict neuroblast proliferation by triggering nuclear localization of Pros.

Our data suggest that Erm might restrict the developmental potential of INPs in part by limiting their proliferation through activating Prospero-dependent cell cycle exit. If so, we predict that over-expression of Erm should induce ectopic nuclear Prospero in INPs and over-expression of Prospero should suppress ectopic neuroblasts in *erm* mutant brains. In wild type brains, 9.6% of INPs (32/325) showed nuclear localization of Prospero. However, over-expression of Erm driven by *Erm-Gal4* led to nuclear localization of Prospero in 41.5% of INPs (105/253), likely restricting their proliferation potential and resulting in some parental type II neuroblasts surrounded only by differentiated neurons (Figure 3.5 C-D). Importantly, ectopic expression of Prospero induced by *Erm-Gal4* efficiently suppressed ectopic neuroblasts and restored neuronal differentiation in *erm* mutant brains (Figure 3.5 E-F). Thus, Erm likely restricts the proliferation of INPs by promoting nuclear localization of Prospero. To confirm that Prospero indeed functions downstream of Erm to restrict the proliferation of INPs, we performed genetic epistatic analyses. Consistent with previously published results, *prospero* mutant type I neuroblast clones contained ectopic type I neuroblasts (Figure 3.5 G) (Bowman et al., 2008). In contrast, *prospero* mutant type II neuroblast clones exhibited accumulation of ectopic INPs while maintaining single parental neuroblasts (Figure 3.5 H). Furthermore, over-expression of Erm failed to suppress ectopic INPs in *prospero* mutant type II neuroblast clones, consistent with Prospero functioning downstream of Erm (Figure 3.5 I). These results indicate that blocking differentiation is not sufficient to trigger the de-differentiation of INPs back into type II neuroblasts. Thus, Erm restricts the proliferation of INPs dependent of Prospero function, but suppresses the de-differentiation of INPs independent of Prospero.

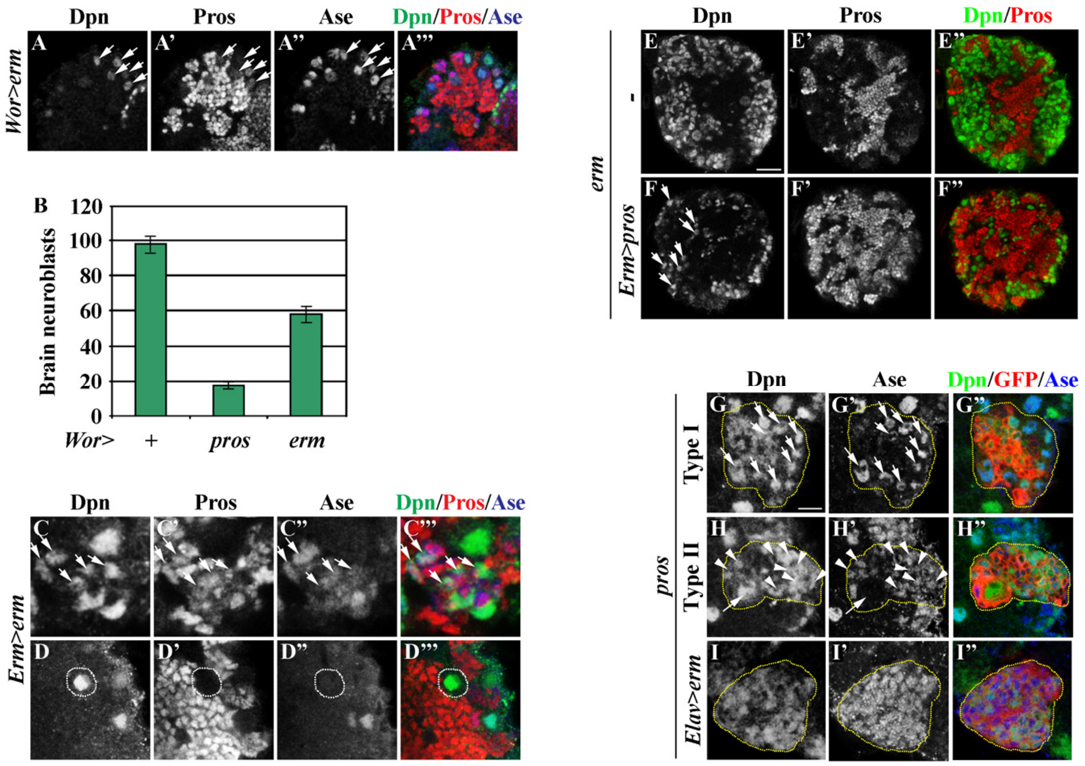


Figure 3.5. Erm restricts the proliferation of INPs by promoting nuclear Prospero

(A) A 3.5-hour pulse of Erm expression induced by *Wor-Gal4* was sufficient to trigger Pros localization in neuroblast nuclei (white arrows). (B) Ectopic expression of Erm or Pros driven by *Wor-Gal4* was sufficient to terminate neuroblast proliferation prematurely. (C-D) Ectopic expression of Erm induced by *Erm-Gal4* triggered a significant increased in INPs that exhibited nuclear Pros (white arrows), likely leading them to exit cell cycle prematurely and resulting in some type II neuroblasts (white circle) surrounded only by neurons. Scale bar, 10µm.

(E-F) Over-expression of Pros induced by *Erm-Gal4* suppressed ectopic neuroblasts and restored neuronal differentiation in *erm* mutant brains. Scale bar, 20 µm.

(G-H) *pros* mutant type I neuroblast clones contained ectopic neuroblasts (white arrows). *pros* mutant type II neuroblast clones contained single type II neuroblast (white arrow) but showed dramatic over-proliferation of INPs (white arrowheads).

(I) Over-expression of Erm failed to suppress over-proliferation of INPs in *pros* mutant type II neuroblast clones. Scale bar, 10 µm.

Erm suppresses de-differentiation through antagonizing Notch signaling

Previous studies showed that over-expression of constitutively active Notch ($\mathrm{Notch}^{\mathrm{intra}}$) in both type I and II neuroblasts is sufficient to trigger ectopic neuroblasts (Bowman et al., 2008; Wang et al., 2006). Here, we tested whether Erm suppresses the de-differentiation of INPs by inhibiting Notch signaling. Indeed, knock-down of Notch function by RNAi in *erm* mutant brains led to a dramatic reduction in ectopic type II neuroblasts compared to *erm* mutant brains alone (Figure 3.6 A-B). Complementarily, ectopic expression of constitutively active Notch ($\mathrm{Notch}^{\mathrm{intra}}$) induced by *Erm-Gal4* transforms INPs into ectopic type II neuroblasts (Figure 3.6 C). Thus, reduced Notch function suppresses the de-differentiation of INPs in *erm* mutant brains while ectopic activation of Notch induces the de-differentiation of INPs. We next tested if Erm suppresses the de-differentiation of INPs by antagonizing a Notch-activated mechanism. Co-expression of Erm under the control of *Erm-Gal4* is sufficient to suppress ectopic neuroblasts induced by the expression of $\mathrm{Notch}^{\mathrm{intra}}$ (Figure 3.6 D). Thus, we conclude that Erm can suppress the de-differentiation of INPs by negatively regulating a Notch-activated signaling mechanism.

Discussion

The limited developmental potential of transit amplifying cells is generally thought to be specified during fate determination (Farkas et al., 2008; Hodge et al., 2008; Sessa et al., 2008). In this study, we report a mechanism that actively maintains the restricted developmental potential of transit amplifying cells after specification of their

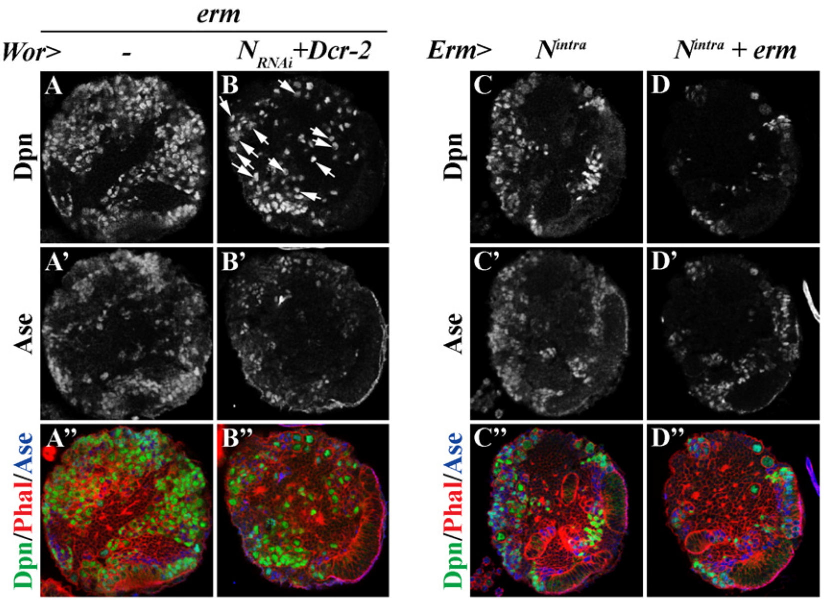


Figure 3.6. Erm suppresses the de-differentiation of INPs through negatively regulating Notch signaling

(A-B) Knocking-down Notch function by RNAi suppressed ectopic neuroblasts (white arrows) in *erm* mutant brains.

(C-D) Ectopic expression of Erm under the control of *Erm-Gal4* suppressed ectopic neuroblasts induced by constitutive activation of Notch signaling. Scale bar, 20 μm.

identity. We show that the evolutionarily conserved transcription factor Erm/Fezf functions to maintain the restricted developmental potential of INPs by limiting their proliferation potential and suppressing their de-differentiation capacity (Figure 3.7). Combining proper specification of the transit amplifying cell identity and active maintenance of their restricted developmental potential will ensure the generation of differentiated progeny and prevent aberrant expansion of stem cells.

The lineage clones derived from single INPs in *erm*^{1}/*erm*^{2} mutant brains contain de-differentiated neuroblasts, immature INPs, INPs, GMCs and neurons (Figure 3.3 B-C). Several mechanisms could lead to the diversity of cells within the clones. First, INPs in *erm* mutant brains might generate GMCs and neurons initially due to the presence of maternally deposited Erm. However, *erm* transcripts are undetectable in both adult male and female germlines by microarray analyses and in stage 1-3 embryos by RNA *in situ* (Chintapalli et al., 2007) (http://flybase.org/reports/FBgn0031375.html; data not presented). Furthermore, the *erm*^{1}/*erm*^{2} allelic combination resulted in little to no zygotic Erm in the brain because the *erm*^{1} mutation likely leads to the production of an unstable Erm protein, whereas the *erm*^{2} mutation deletes the entire *erm* open reading frame (data not presented). Additionally, the ectopic neuroblast phenotype in *erm*^{1}/*erm*^{2} mutant brains can be observed as early as 36-48 hrs after larval hatching (data not presented). Thus, generation of GMCs and differentiated neurons by INPs in *erm*^{1}/*erm*^{2} mutant brains is unlikely due to the maternal effect. Alternatively, *erm* may promote GMC differentiation in the type II neuroblast lineage, and in *erm* mutant brains, GMCs might de-differentiate

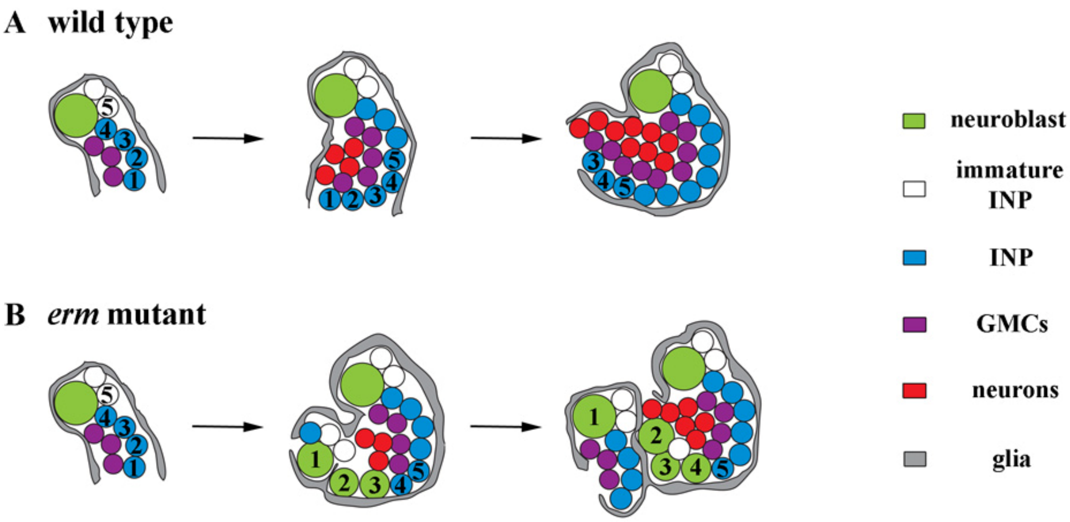


Figure 3.7. ***erm*** **maintains the restricted developmental potential of INPs**

(A) Wild type INPs undergo limited rounds of asymmetric divisions to generate neurons prior to exiting from the cell cycle, and remain in the same glial chamber as their parental type II neuroblasts.

(B) Some *erm* mutant INPs fail to terminate proliferation and de-differentiate back into their parental type II neuroblast fate. These de-differentiated neuroblasts can establish ectopic type II neuroblast lineages and form ectopic glial chambers.

back into neuroblasts. If so, we would predict an ectopic accumulation of INPs in similarly staged mosaic clones derived from *erm* mutant type II neuroblasts as compared to wild type clones. However, 48-hr *erm* mutant single neuroblast clones consistently contained fewer INPs when compared to the wild type clones (Figure 3.2 C-F). In addition, blocking GMC differentiation by removing Prospero function resulted in ectopic accumulation of INPs but did not lead to ectopic neuroblast formation (Figure 3.5 H). Therefore, the diversity of cells within *erm* mutant clones is also unlikely due to blocking GMC differentiation. We favor the interpretation that *erm* mutant INPs de-differentiate into apparently normal neuroblasts that can give rise to all cell types found in a type II neuroblast lineage. Consistently, the de-differentiated neuroblasts in *erm* mutant brains exhibited normal cortical polarity and proliferation potential (Figure 3.3 & 3.4). Furthermore, the de-differentiated neuroblasts in *erm* mutant brains also lost the expression of *Pros-Gal4* and *Erm-Gal4* and established ectopic type II neuroblast lineages encapsulated by the cortex glial membrane (Figure 3.3 & 3.4). Thus, we conclude that Erm likely restricts the developmental potential of INPs by limiting proliferation and suppressing de-differentiation.

Although mutations in *erm*, *brain tumor* and *numb* genes all lead to ectopic type II neuroblasts, these proteins appear to regulate INPs at distinct steps in the type II neuroblast lineage (Figure 3.3S). Numb and Brain tumor function cooperatively, but non-redundantly, to ensure that immature INPs undergo maturation and commit to the INP fate (Boone and Doe, 2008; Bowman et al., 2008). While ectopic expression of

Numb induces premature differentiation of type II neuroblasts and immature INPs (Haenfler, Golden and Lee, unpublished), over-expression of Numb is not sufficient to suppress ectopic neuroblasts in *brain tumor* mutant brains (Komori and Lee, unpublished). Thus, Numb likely promotes differentiation of immature INPs whereas Brain tumor likely prevents immature INPs, which are unstable in nature, from adopting their parental neuroblast fate. More studies will be necessary to discern whether ectopic neuroblasts in *brain tumor* mutant brains arise from de-differentiation of partially differentiated immature INPs or failure of immature INPs to initiate differentiation. In contrast, immature INPs in *erm* mutant brains mature into functional INPs that exhibit normal cortical polarity and proliferation potential and can generate GMCs and neurons (Figure 3.2 A-F, 3.3S, 3.3D-E and 3.4). Additionally, over-expression of Brain tumor or Numb in INPs was not sufficient to suppress ectopic neuroblasts in *erm* mutant brains (data not presented). Finally, lineage clones derived from single INPs in *erm* mutant brains always contain ectopic type II neuroblasts, multiple immature INPs, INPs, GMCs and neurons (Figure 3.3 B-C). These results indicate that Erm is dispensable for maturation of immature INPs and is not within the genetic hierarchy specifying the INP identity. Instead, Erm maintains the restricted developmental potential of INPs after specification of their identity.

Prospero encodes a homeodomain transcription factor, and nuclear Prospero has been shown to trigger cell cycle exit and GMC differentiation (Choksi et al., 2006; Doe et al., 1991; Maurange et al., 2008). In the wild type brain, 9.6% of INPs showed nuclear

Prospero and were likely undergoing differentiation (data not presented). *prospero* mutant type II neuroblast clones showed ectopic accumulation of INPs but contained single neuroblasts indicating that blocking differentiation is not sufficient to trigger the de-differentiation of INPs (Figure 3.5 H). Thus, Prospero restricts the proliferation potential of INPs but does not suppress de-differentiation of INPs.

While ectopic expression of Prospero in INPs can restore neuronal differentiation in *erm* mutant brains, targeted expression of Erm in neuroblasts or INPs was sufficient to induce rapid nuclear localization of Prospero in these cells and terminate their proliferation (Figure 3.5). In wild type brains, Prospero is sequestered in a basal crescent by the adaptor protein Miranda in mitotic neural progenitors (Ikeshima-Kataoka et al., 1997; Shen et al., 1997). Interestingly, mitotic neural progenitors including neuroblasts and INPs transiently over-expressing Erm also showed basal localization and segregation of Miranda and Prospero (data not presented). As such, Erm likely restricts the proliferation potential of INPs by indirectly promoting nuclear localization of Prospero. Therefore, Prospero does not localize in the nuclei of mitotically active INPs which express Miranda, but localizes in the nuclei of GMCs that do not express Miranda.

How does Erm suppress the de-differentiation of INPs? Our results show that reduced Notch function can efficiently suppress ectopic neuroblasts in *erm* mutant brains while constitutive activation of Notch signaling induced the de-differentiation of INPs (Figure 3.6 A-C). Importantly, co-expression of Erm is sufficient to suppress the de-differentiation of INPs triggered by expression of constitutively active Notch^{intra}

(Figure 3.6 D). Together, these results strongly suggest that Erm prevents the de-differentiation of INPs by antagonizing a Notch-activated mechanism through interfering with the assembly of the Notch transcriptional activator complex or inhibiting the expression of Notch targets. Intriguingly, the amino terminus of all Fezf proteins contains an engrailed homology 1 domain. This domain can mediate direct interaction with the conserved transcriptional co-repressor Groucho that can function as a co-repressor of Notch signaling (Cinnamon and Paroush, 2008; Copley, 2005; Jeong et al., 2006; Levkowitz et al., 2003; Shimizu and Hibi, 2009). Additional experiments will be needed to discern how Erm antagonizes Notch-activated de-differentiation of INPs.

Materials and Methods

Fly genetics and transgenes

A total of six *erm* alleles were recovered from EMS mutagenesis following a standard protocol. *erm*^{2} was generated by FRT-based high-resolution deletion method and verified by PCR (Parks et al., 2004). The cDNA for *CG31670* was obtained from the *Drosophila* Genome Resource Center, sequenced and cloned into the *pUAST-HA* vector for germline transformation. Mouse *fezf1* and *fezf2* cDNAs were sequenced (M. Hibi), and were cloned into the *pUAST-HA* vector for germline transformation. *Drosophila* cultures were kept at 25^{o}C on standard cornmeal food. Other mutant alleles and transgenes used in this study include *brat*^{11} (Lee et al., 2006c), *pros*^{17}, *FRT82B* (Lee et al., 2006c), *aPKC*^{k06403} (Lee et al., 2006b); *pins*^{62} (Lee et al., 2006b), *UAS-pros* (Hirata et al., 1995), *Wor-gal4* (Lee et al., 2006b), *Ase-gal4* (Zhu et al., 2006), and *R9D-Gal4* lines (Pfeiffer et al., 2008). The *UAS-Notch*$_{RNAi}$ lines were obtained from the Vienna *Drosophila* Resource Center. *Oregon R*, *elav-gal4 (C155), hs-flp, UAS-mCD8-GFP, FRT40A, tub-gal80*, *FRT82B, hs-flp(F38)*, *act-FRT-Stop-FRT-lacZ*, *UAS-flp*, *tub-GAL80*^{ts}, *UAS-dcr-2, UAS-Notch*^{intra}, *Repo-Gal4* flies were obtained from Bloomington *Drosophila* Stock Center.

Immunofluorescent staining and antibodies

Antibody staining was performed as previously described (Lee et al., 2006b). The rabbit Ase antibody was raised against a previously described synthetic peptide (Brand and Perrimon, 1993). Other antibodies used in this study include guinea pig Ase (1:100; J. Knoblich), rat Wor (1:1), rat Dpn (1:1), guinea pig Dpn (1:2500, J. Skeath), mouse Pros (1:100), rat Mira (1:100); guinea pig Mira (1:400), guinea pig Numb (1:3000, J. Skeath); rat Pins (1:500), rabbit Scrib (1:2500), mouse Elav(1:50, DSHB), mouse Dlg (1:100, DSHB), mouse Repo (1:50, DSHB), mouse BrdU (1:50, Roche), rabbit β-gal (1:1000, ICN/Cappel), rat α-Tub (1:100, Sigma), rat mCD8 (1:100, Caltag), rabbit GFP (1:1000, Torreypine), mouse HA (1:1000, Covance), rat HA (1:2000, Roche). Secondary antibodies were from Molecular Probes (details are available upon request). The confocal images were acquired on a Leica SP5 scanning confocal microscope with AOBS.

Edu pulse-chase

Larvae were aged for 72 hr after hatching, and were pulse labeled for 3 hrs by feeding on the Kankel-White media containing 50 μg/mL EdU (5-ethynyl-2'deoxyuridine) (Lee et al., 2006c). Half of the larvae were processed for staining immediately following the pulse; remaining larvae were transferred to standard media for a 12 hr EdU-free chase. Larvae were dissected and processed for antibody staining as previously described (Lee et al., 2006b). Incorporated EdU was detected by Click-iT fluorescent dye azide reaction as described in the Click-iT product literature (Invitrogen).

Lineage clonal analysis

We initially performed genetic clonal analyses of INPs using *Ase-Gal4* by crossing *erm*^{1}*, Actin-FRT-Stop-FRT-lacZ/CyO, Actin-GFP* flies to *erm*^{2}*, Ase-Gal4/CyO, Actin-GFP; UAS-flp, tub-Gal80*^{ts} flies. At 24 hours after hatching, *erm*^{1}*/erm*^{2} larvae were shifted to 31°C for 48 hours to inactivate Gal80^{ts} allowing FRT-mediated recombination to induce permanently marked lineage clones. The expression level of *Ase-Gal4* is very low (Bowman et al., 2008) allowing us to induce genetic clones at a very low frequency. However, due to the prolonged incubation time at the non-permissive temperature, clones derived from two neighboring INPs sometimes became overlapped resulting in appearance of a "large" clone. We repeated this experiment by using *Erm-Gal4*, whose expression level was significantly higher compared to *Ase-Gal4* (Weng and Lee, data not presented). We crossed *erm*^{1}*, Actin-FRT-Stop-FRT-lacZ/CyO, Actin-GFP; Erm-Gal4* flies to *erm*^{2}*/CyO, Actin-GFP; UAS-flp, tub-Gal80*^{ts} flies. At 24 hours after hatching, *erm*^{1}*/erm*^{2} larvae were shifted to 31°C for 1 hour to induce positively marked genetic clones derived from single INP. Larvae were returned back to 25°C for 72 hours prior to processing larval brains for antibody staining.

Mutant clonal analyses

We induced mosaic clones derived from *erm*^{1} and *pros*^{17} mutant neuroblasts by following a previously established protocol (Lee et al., 2006c; Lee and Luo, 2001).

Over-expression of Notchintra

Over-expression of Notch$^{\text{intra}}$ in INPs in larval brains was accomplished by crossing *UAS-Notch*^{intra}*/CyO, Actin-GFP; tub-Gal80*^{ts} flies to *Erm-Gal4* flies. GFP$^-$ larvae were allowed to hatch at 25°C, and were then shifted to 31°C for 72 hrs. Larval brains were dissected and processed for antibody staining. Co-overexpression of Erm and Notch$^{\text{intra}}$ was carried out following an identical protocol.

Real-time PCR

Late third instar larval brains were dissected free of surrounding tissues. Total RNA is extracted following the standard Trizol RNA isolation protocol and cleaned by the Qiagen RNeasy kit. cDNA was transcribed using First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche).Quantitative PCR was performed by using SYBR-green. Resulted data was analyzed by the comparative CT method and the relative mRNA expression is presented.

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References

Bello, B.C., Izergina, N., Caussinus, E., and Reichert, H. (2008). Amplification of neural stem cell proliferation by intermediate progenitor cells in Drosophila brain development. Neural Develop *3*, 5.

Betschinger, J., Mechtler, K., and Knoblich, J.A. (2006). Asymmetric segregation of the tumor suppressor brat regulates self-renewal in Drosophila neural stem cells. Cell *124*, 1241-1253.

Boone, J.Q., and Doe, C.Q. (2008). Identification of Drosophila type II neuroblast lineages containing transit amplifying ganglion mother cells. Dev Neurobiol *68*, 1185-1195.

Bowman, S.K., Rolland, V., Betschinger, J., Kinsey, K.A., Emery, G., and Knoblich, J.A. (2008). The Tumor Suppressors Brat and Numb Regulate Transit-Amplifying Neuroblast Lineages in Drosophila. Dev Cell *14*, 535-546.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Chintapalli, V.R., Wang, J., and Dow, J.A.T. (2007). Using FlyAtlas to identify better Drosophila models of human disease. Nat Genet *39*, 715-720.

Choksi, S.P., Southall, T.D., Bossing, T., Edoff, K., de Wit, E., Fischer, B.E., van Steensel, B., Micklem, G., and Brand, A.H. (2006). Prospero acts as a binary switch between self-renewal and differentiation in Drosophila neural stem cells. Dev Cell *11*, 775-789.

Cinnamon, E., and Paroush, Z. (2008). Context-dependent regulation of Groucho/TLE-mediated repression. Curr Opin Genet Dev *18*, 435-440.

Copley, R.R. (2005). The EH1 motif in metazoan transcription factors. BMC Genomics *27*, 169.

Doe, C.Q., Chu-LaGraff, Q., Wright, D.M., and Scott, M.P. (1991). The prospero gene specifies cell fates in the Drosophila central nervous system. Cell *65*, 451-464.

Farkas, L.M., Haffner, C., Giger, T., Khaitovich, P., Nowick, K., Birchmeier, C., Pääbo, S., and Huttner, W.B. (2008). Insulinoma-associated 1 has a panneurogenic role and promotes the generation and expansion of basal progenitors in the developing mouse neocortex. Neuron *60*, 40-55.

Hashimoto, H., Yabe, T., Hirata, T., Shimizu, T., Bae, Y., Yamanaka, Y., Hirano, T., and Hibi, M. (2000). Expression of the zinc finger gene fez-like in zebrafish forebrain. Mech Dev *97*, 191-195.

Hirata, J., Nakagoshi, H., Nabeshima, Y., and Matsuzaki, F. (1995). Asymmetric segregation of the homeodomain protein Prospero during Drosophila development. Nature *377*, 627-630.

Hodge, R.D., Kowalczyk, T.D., Wolf, S.A., Encinas, J.M., Rippey, C., Enikolopov, G., Kempermann, G., and Hevner, R.F. (2008). Intermediate progenitors in adult hippocampal neurogenesis: Tbr2 expression and coordinate regulation of neuronal output. J Neurosci *28*, 3707-3717.

Ikeshima-Kataoka, H., Skeath, J.B., Nabeshima, Y., Doe, C.Q., and Matsuzaki, F. (1997). Miranda directs Prospero to a daughter cell during Drosophila asymmetric divisions. Nature *390*, 625-629.

Jeong, J.Y., Einhorn, Z., Mercurio, S., Lee, S., Lau, B., Mione, M., Wilson, S.W., and Guo, S. (2006). Neurogenin1 is a determinant of zebrafish basal forebrain dopaminergic neurons and is regulated by the conserved zinc finger protein Tof/Fezl. Proc Natl Acad Sci U S A *103*, 5143-5148.

Lee, C.Y., Andersen, R.O., Cabernard, C., Manning, L., Tran, K.D., Lanskey, M.J., Bashirullah, A., and Doe, C.Q. (2006a). Drosophila Aurora-A kinase inhibits neuroblast self-renewal by regulating aPKC/Numb cortical polarity and spindle orientation. Genes Dev *20*, 3464-3474.

Lee, C.Y., Robinson, K.J., and Doe, C.Q. (2006b). Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. Nature *439*, 594-598.

Lee, C.Y., Wilkinson, B.D., Siegrist, S.E., Wharton, R.P., and Doe, C.Q. (2006c). Brat is a Miranda cargo protein that promotes neuronal differentiation and inhibits neuroblast self-renewal. Dev Cell *10*, 441-449.

Lee, T., and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. Trends Neurosci *24*, 251-254.

Levkowitz, G., Zeller, J., Sirotkin, H.I., French, D., Schilbach, S., Hashimoto, H., Hibi, M., Talbot, W.S., and Rosenthal, A. (2003). Zinc finger protein too few controls the development of monoaminergic neurons. Nat Neurosci *6*, 28-33.

Matsuo-Takasaki, M., Lim, J.H., Beanan, M.J., Sato, S.M., and Sargent, T.D. (2000). Cloning and expression of a novel zinc finger gene, Fez, transcribed in the forebrain of Xenopus and mouse embryos. Mech Dev *93*, 201-204.

Maurange, C., Cheng, L., and Gould, A.P. (2008). Temporal transcription factors and their targets schedule the end of neural proliferation in Drosophila. Cell *133*, 891-902.

Morrison, S.J., and Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer. Nature *441*, 1068-1074.

Parks, A.L., Cook, K.R., Belvin, M., Dompe, N.A., Fawcett, R., Huppert, K., Tan, L.R., Winter, C.G., Bogart, K.P., Deal, J.E*., et al.* (2004). Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. Nat Genet *36*, 288-292.

Pereanu, W., Shy, D., and Hartenstein, V. (2005). Morphogenesis and proliferation of the larval brain glia in Drosophila. Dev Biol *283*, 191-203.

Pfeiffer, B.D., Jenett, A., Hammonds, A.S., Ngo, T.T., Misra, S., Murphy, C., Scully, A., Carlson, J.W., Wan, K.H., Laverty, T.R*., et al.* (2008). Tools for neuroanatomy and neurogenetics in Drosophila. Proc Natl Acad Sci U S A *105*, 9715-9720.

Pontious, A., Kowalczyk, T., Englund, C., and Hevner, R.F. (2008). Role of intermediate progenitor cells in cerebral cortex development. Dev Neurosci *30*, 24-32.

Sessa, A., Mao, C.A., Hadjantonakis, A.K., Klein, W.H., and Broccoli, V. (2008). Tbr2 directs conversion of radial glia into basal precursors and guides neuronal amplification by indirect neurogenesis in the developing neocortex. Neuron *60*, 56-69.

Shen, C.P., Jan, L.Y., and Jan, Y.N. (1997). Miranda is required for the asymmetric localization of Prospero during mitosis in Drosophila. Cell *90(3):449-58.*, 90(93):449-458.

Shimizu, T., and Hibi, M. (2009). Formation and patterning of the forebrain and olfactory system by zinc-finger genes Fezf1 and Fezf2. Dev Growth Differ *51*, 221-231.

Vescovi, A.L., Galli, R., and Reynolds, B.A. (2006). Brain tumour stem cells. Nat Rev Cancer *6*, 425-436.

Wang, H., Somers, G.W., Bashirullah, A., Heberlein, U., Yu, F., and Chia, W. (2006). Aurora-A acts as a tumor suppressor and regulates self-renewal of Drosophila neuroblasts. Genes Dev *20*, 3453-3463.

Zhu, S., Lin, S., Kao, C.F., Awasaki, T., Chiang, A.S., and Lee, T. (2006). Gradients of the Drosophila Chinmo BTB-zinc finger protein govern neuronal temporal identity. Cell *127(2):409-22*, 409-422.

CHAPTER IV

Conclusions and Perspectives

Intermediate progenitor cells undertake the tasks of stem cells in producing a large number of differentiated cells in a short time, generating groups of differentiated cells of a particular subtype, and likely contributing to the organization of the tissue. By distributing the effort to intermediate progenitor cells, stem cells also save themselves from genomic instability due to excessive DNA replication. Furthermore, it was proposed that generating intermediate progenitor cells may be a mechanism underlying the transformation of smooth brain cortex surface to highly folded cortex surface seen in primates during the evolution. However, the benefit of having intermediate progenitor cells comes along with a challenge: to impose another layer of regulation along the pathway of differentiation. Compared to stem cells and differentiated cells, intermediate progenitor cells possess intermediate capacity and thus may need a sophisticated mechanism to specify and maintain their developmental potential at a desired level. This thesis studied neural intermediate progenitor cells in two independent systems: optic lobe neuroblasts generated by neuroepithelial stem cells in fly larval optic lobe and intermediate neural progenitors generated by central brain neuroblasts in fly larval central brain. The former system was used to study the specification of neural progenitors and the later one was used to study the maintenance of neural progenitor after the correct specification.

Chapter II of this thesis presents the mechanisms that specify the intermediate progenitor cells during the differentiation of symmetrically dividing neuroepithelial stem cells into asymmetrically dividing non-epithelial intermediate progenitor cells (optic lobe neuroblasts) in *Drosophila* larval optic lobe. It shows that the specification of intermediate progenitor cells is a multi-step process that involves multiple signaling pathways and the precision of the transition is achieved by generating intermediate cell types and creating sharp boundary between cells of different status.

Unambiguous identification of the intermediate cell types is critical to delineate the steps that specify the optic lobe neuroblasts. The transition from neuroepithelial cells into neuroblasts happens only at the medial edge of neuroepithelial sheet. Therefore, at a given time point, the array of cells from lateral to medial side represent all the steps a neuroepithelial cell takes to become a neuroblast. Using cell morphology and molecular markers independent of any singling pathways, I showed that differentiation of neuroepithelia in larval optic lobe occurs in the following sequence: neuroepithelial cells -> differentiating neuroepithelial cells -> immature neuroblasts -> neuroblasts. I found that optic lobe neuroepithelial cells start their differentiation by undergoing cell cycle arrest and do not resume proliferation until they become the optic lobe neuroblasts. The differentiating neuroepithelial cells were defined as those cells that enter cell cycle arrest but still maintain epithelial cell morphology. Immediately adjacent to differentiating neuroepithelial cells are immature neuroblasts that have lost epithelial character but still arrested in cell cycle.

By examining Notch reporter activity and Notch mutant phenotype, I found that the progression of differentiation is paced by the spatial fluctuation of Notch signaling activity which in turn is patterned by EGFR signaling: Notch activity is low but necessary in neuroepithelial cells; it is greatly upregulated in differentiating neuroepithelia but sharply suppressed in immature neuroblasts; it reappears at a medial level in neuroblasts. Furthermore, I showed that low Notch activity maintains the proliferating neuroepithelial cell identity by allowing the expression of *anterior open* gene which in turn inhibit the expression of *pntP1*, the major downstream target of EGFR signaling; the upregulation of Notch activity in differentiating neuroepithelia prevents premature differentiation while allows the expression of *ptnP1*; only when Notch activity is eliminated through Delta-immediated cis-inhibition, PntP1 can drive the transition of neuroepithelial cells into immature neuroblasts. Such dynamics of Notch activity is patterned by a gradient of Delta expression: low Delta maintains a low Notch activity; increased Delta triggers upregulated Notch activity in differentiating neuroepithelia while high level of Delta at the peak of the gradient cis-inhibits Notch activity and allows the formation of immature neuroblasts. The activated EGFR ligand forms a gradient similar to Delta and its downstream target PntP1 is sufficient to induce Delta expression cell-autonomously. Therefore the gradient of Delta is likely induced by the gradient of EGFR signaling.

The comparison with other systems reveals mechanisms controlling the specification of neural progenitor cells. Similar to the mechanisms described in this chapter, it was shown that EGFR and Notch signaling together keeps the population balance between neural stem cell and neural progenitor cells in developing mouse cortex. While Notch is required for neural stem cell maintenance, EGFR is responsible for the proliferation and migration of neural progenitor cells (Alexson et al., 2006; Breunig et al., 2007; Hitoshi et al., 2002; Lillien and Raphael, 2000). Interestingly, ectopic expression of EGFR specifically in neural progenitor cells reduced the neural stem cell population non-cell-autonomously while hypomorphic EGFR mutant shows increased neural stem cell population (Aguirre et al., 2010). However more definitive in vivo evidence, such as change in stem cell population when EGFR is specifically removed from neural progenitor cells, is needed to prove the physiological relevance of this non-cell-autonomous mechanism. In fly larval optic lobe, it has been shown that the source of secreted EGFR ligand is around the transition zone but no cell fate marker was examined to confirm the cell type that secret EGFR ligand (Yasugi et al., 2010). Thus it will be important to determine the cell type of EGFR ligand source and test the non-cell-autonomous role of EGFR in neural progenitor cells.

Studies in Chapter III demonstrate that the restricted potential of intermediate progenitor cells needs to be actively maintained after correct specification and identify *earmuff* as the major regulator that maintains the restricted potential of INPs in fly larval central brain via two independent mechanisms: limiting proliferation by promoting nuclear localization of Prospero and antagonizing Notch signaling to suppress dedifferentiation.

Extensive examination of *earmuff* mutant phenotype indicated that the ectopic neuroblasts in *earmuff* mutants originate from the dedifferentiation of correctly specified INPs in the type II neuroblast lineages. *earmuff* mutants showed massive ectopic generation of neuroblasts in the central brain of fly larvae without affecting the asymmetric division of either neuroblasts or INPs. Lineage tracing of type II neuroblasts at different time points revealed that earlier born INPs in *earmuff* mutant lineage appeared to be replaced by ectopic cells resembling the parental neuroblasts suggesting the INP origin of ectopic neuroblasts and the INP specificity of *earmuff* expression and function. Indeed, the expression pattern reported by the gal4 driver under the control of *earmuff* promoter was restricted in the mature INPs but not in neuroblasts or immature INPs. In addition, *earmuff* phenotype can be rescued by over-expressing Earmuff with the same driver indicating that *earmuff* function was specifically required in INPs. Importantly, specific labeling of single INP confirmed that *earmuff* mutant INPs eventually dedifferentiated and generated all types of progeny normally found in a wild type type II neuroblast lineage, suggesting the dedifferentiated neuroblasts physiologically behave like normal neuroblasts. Further examination confirmed that those dedifferentiated neuroblasts are indistinguishable from wild type neuroblasts in all assessable aspects.

The mechanisms for *earmuff* to maintain the restricted potential of INPs lie in two aspects: restricting the proliferation and suppressing the dedifferentiation. A small percentage of INPs showed nuclear Prospero and Prospero loss of function led to unrestrained proliferation of INPs, suggesting Prospero mediated cell cycle exit may be the mechanism to terminate INPs. The frequency of nuclear Prospero in INPs was significantly increased upon *earmuff* expression and over-expression of Prospero specifically in INP suppressed the dedifferentiation of *earmuff* mutant INPs, suggesting *prospero* may function downstream of the *earmuff* to limit the proliferation of INPs. However regulating *prospero* isn't the only function of *earmuff*, since Prospero loss of function leads to continued proliferation of INPs but not dedifferentiation of INPs seen in *earmuff* mutants. I found that knocking down Notch could also suppress *earmuff* phenotype and constitutively active Notch (N_{intra}, Notch intracellular domain) expressed specifically in INPs but not neuroblasts was sufficient to induce the dedifferentiation phenotype like *earmuff* loss of function, suggesting *earmuff* may suppress Notch signaling in INPs. Consistently, over-expression of Earmuff was able to suppress $Notch_{intra}$ induced dedifferentiation, suggesting that as a transcription factor *earmuff* may interfere with Notch's function, likely competing in regulating Notch's targets.

One critical question about the dedifferentiation is whether it is simply caused by acquiring the sensitivity or accessibility to signals that maintain stem cells or it happens via a specific pathway that is not necessary for stem cell self-renewal. To understand the mechanism underlying the dedifferentiation, identification of *earmuff* target genes is critical. Screening for enhancer and suppressor in a sensitized *earmuff* mutant background will help identify additional players in the dedifferentiation process. As Earmuff is a putative transcriptional factor with very conserved DNA binding domains, biochemical approaches can be used to identify the direct targets and help dissect the pathways in detail.

In addition, further characterization of dedifferentiation process is needed. Firstly, do *earmuff* mutant INPs dedifferentiate before they reach the terminal division? Though unrestrained proliferation isn't enough for dedifferentiation as demonstrated in *prospero* mutant, it is unclear whether it is necessary. Secondly, do INPs in the process of dedifferentiation proliferate and generate INPs before they gain all the features of neuroblasts? *earmuff* mutant INPs loss INP markers before their sizes can reach the size of a neuroblast indicating that dedifferentiation indeed proceeds in a multi-step manner. It will be interesting to know which step is critical and will help reveal the minimum requirement for behaving as neural stem cells.. Thirdly, is being wrapped by cortex glial cells the reason or the result of dedifferentiation? Although many dedifferentiated neuroblasts, like wild type neuroblasts, have cortex glial cells wrapping their lineage, the ectopic neuroblasts that have not generated INPs usually do not have glia contact and some smaller lineages share the glial chamber with other lineages, indicating a correlation between the development of the ectopic lineage and the glia acquisition.

In summary, using *Drosophila* larval central nervous system as the model system, this thesis work probed the mechanisms that underlying the specification and maintenance of neural progenitor cells. Although much has been known about the mechanisms that maintain stem cells, little is known about how neural progenitor cells are specified and maintained. Yet, intermediate progenitor cells is critical for stem cell to accomplish its overwhelming task at generating all the cells in an organism and mis-regulation of intermediate progenitor cell potential can be devastating for the development and homeostasis of the organism. One would expect more discoveries in the delicate mechanisms regulating intermediate progenitor cells which will advance our understanding of stem cell biology in general and help formulate the therapeutical strategies for developmental defects and degenerative diseases.

References

Aguirre, A., Rubio, M.E., and Gallo, V. (2010). Notch and EGFR pathway interaction regulates neural stem cell number and self-renewal. Nature *467*, 323-327.

Alexson, T.O., Hitoshi, S., Coles, B.L., Bernstein, A., and van der Kooy, D. (2006). Notch Signaling Is Required to Maintain All Neural Stem Cell Populations – Irrespective of Spatial or Temporal Niche. Developmental Neuroscience *28*, 34-48.

Breunig, J.J., Silbereis, J., Vaccarino, F.M., Šestan, N., and Rakic, P. (2007). Notch regulates cell fate and dendrite morphology of newborn neurons in the postnatal dentate gyrus. Proceedings of the National Academy of Sciences *104*, 20558-20563.

Hitoshi, S., Alexson, T., Tropepe, V., Donoviel, D., Elia, A.J., Nye, J.S., Conlon, R.A., Mak, T.W., Bernstein, A., and van der Kooy, D. (2002). Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. Genes & Development *16*, 846-858.

Lillien, L., and Raphael, H. (2000). BMP and FGF regulate the development of EGF-responsive neural progenitor cells. Development *127*, 4993-5005.

Yasugi, T., Sugie, A., Umetsu, D., and Tabata, T. (2010). Coordinated sequential action of EGFR and Notch signaling pathways regulates proneural wave progression in the Drosophila optic lobe. Development *137*, 3193-3203.

APPENDIX

Identification of Neural Stem Cells in the Drosophila Larval Brain

Summary:

The balance between self-renewal and differentiation must be tightly regulated in somatic stem cells to ensure proper tissue generation and to prevent tumor-like overgrowth. A Drosophila larval brain lobe consists of the central brain and the optic lobe, and possesses three well-defined neural stem cell lineages that generate differentiated cells in a highly reproducible pattern. Unambiguous identification of various cell types in these stem cell lineages is pivotal for studying the regulation of neural stem cells and progenitor cells at a single-cell resolution. This chapter will describe the methodology for collection and processing of larval brains for examination by fluorescence confocal microscopy.

1. Introduction

The central brain occupies the medial half of a fly larval brain lobe, and contains neural stem cells (called neuroblasts) that undergo repetitive asymmetric divisions to self-renew and to generate a neural progenitor cell with limited developmental potential (Sousa-Nunes et al., 2010). Two distinct larval brain neuroblast lineages (types I and II) can be unambiguously identified based on the progenitor progeny generated and the combination of cell fate markers expressed (Weng and Lee, 2011). A type I neuroblast divides asymmetrically to self-renew and to generate a neural progenitor cell called a ganglion mother cell (GMC), which divides once to produce two post-mitotic neuron. In contrast, a type II neuroblast divides to self-renew and to produce an immature intermediate neural progenitor cell (INP), which acquires restricted developmental potential during maturation and undergoes limited rounds of asymmetric divisions to regenerate and to produce GMCs. While all neuroblasts express the molecular marker Deadpan (Dpn), a type I neuroblast also expresses Asense (Ase) whereas a type II neuroblast expresses PointedP1 (PntP1) (Bowman et al., 2008) (Komori and Lee, unpublished). An immature INP expresses a high level of PntP1, but following maturation, an INP expresses Dpn and Ase. Finally, a GMC shows nuclear localization of Ase and Prospero (Pros) whereas an immature neuron expresses Pros only.

The optic lobe occupies the lateral half of a fly larval brain lobe, and contains two single-cell layers of neuroepithelial stem cells that form the inner and the outer proliferation center (Sousa-Nunes et al., 2010; Weng and Lee, 2011). Neuroepithelial cells in the outer proliferation center are located on the surface of the optic lobe, and initially divide symmetrically to expand their population. In third larval instar, these neuroepithelial cells progressively differentiate into lamina precursor at the lateral edge and into medulla neuroblasts at the medial edge. The apical complex protein PatJ specifically labels all neuroepithelial cells while the expression of the Notch reporter E(spl)m -GFP labels differentiating neuroepithelial cells at the medial edge and medulla neuroblasts. Medulla neuroblasts express molecular markers including Dpn and Ase.

2. Materials

2.1. Reagents

1. Fix solution: 4% formaldehyde, 0.1M PIPES (pH=6.9) , 0.3% TritionX-100, 20mM EGTA, 1mM $MgSO_4$. Fix solution should be prepared fresh every time.

2. 10XPBS: 1.37M NaCl, 27mM KCl, 100mM Na_2HPO_4, 20mM KH_2PO_4. Dissolve and adjust pH to 7.4 with concentrated HCl. Bring the volume up to 1 L with dH_2O and sterilize. Store at room temperature.

3. 10% Triton X-100: 100% Trition X-100 diluted in sterilized water.

3. PBST (500 ml): 1X PBS, 0.3% TritonX-100. Store at room temperature.

4. 10X Glycine: 1M Glycine, 2% sodium azide, 1XPBS.

5. Block solution: 1X PBST, 0.1% Normal goat serum, 1X Glycine. Prepare fresh and keep on ice.

6. 70% Glycerol: 100% Glycerol diluted in sterilized water.

7. Prolong Gold Anti-fade mounting medium (Invitrogen)

8. Schneider's Insect Medium (Sigma-Aldrich)

2.2 Equipment

1. 22 x 22 mm coverslips, #1 thickness

2. 24 x 40 mm coverslips

3. Dissection dishes

4. Fine-tipped forceps (2 pairs)

5. Fine micro knife with thickness about 0.15 mm

6. Microfuge tubes (0.5 ml)

7. Microscope slides

8. Nutator or rocker

9. Pipettes & sterile tips

3. Methods

3.1. Dissection of Larval Brains

1. Fill wells of dissection dishes with 200-400 μl cold Schneider's medium.
2. Dissect larvae by rolling them onto their dorsal side so the denticle belts are facing up.
3. Using a pair of forceps, gently grasp the larva just posterior of the midpoint. With the second pair of forceps, grasp the anterior end of the larva with one tip pushing mouth hook inwards and the other tip outside on the cuticle.
4. Carefully tear the cuticle at the tip of second pair of forceps while slowly drawing the body away from the mouthpart. The brains will remain attached to the head and be clearly visible among the gut and salivary glands. Remove excess tissues, but leave the brains attached to the mouth hooks. (see **Note 1-2**)
5. Place the brains in a 0.5 ml tube containing cold Schneider's medium. (see **Note 3**)

3.2. Fixation and Staining

1. Remove Schneider's medium from the tube containing the brains.
2. Add 500 μl fixative to the brains and incubate with rocking for 23 minutes at room temperature.
3. Quickly wash the brains for three times in ~500 μl of PBST.
4. Incubate the brains in primary antibodies diluted in PBST for 3 hours at the room temperature or overnight at 4°C. (see **Note 4-7**)
5. Quickly wash the brains three times in PBST.

6. Incubate the brains in secondary antibodies overnight at 4°C. Secondary antibodies are typically diluted 1:200-500 in PBST. Wrap the tube with foil to keep the brains protected from light after this point. (see **Note 4-7**)

8. Quickly wash the brains three times in PBST.

9. Equilibrate the brains in Prolong Gold at the room temperature. Samples can be stored in the dark at room temperature or 4°C.

3.3. Mounting Samples

3.3.1. Taking a Z-stack from posterior to anterior side

1. Adhere two 22 x 22 mm coverslips to a slide with a small amount of 70% glycerol, leaving a ~5 mm space between them. (see **Note 8**)

2. Transfer the brains to a slide using a pipette with the tip cut off.

3. Remove all excess tissues including discs from each brain with forceps.

4. Orient the brains so that the ventral side is down. Arrange the brains in an array for easy tracking during confocaling. (see **Note 9**)

5. Place a 24 x 40 mm coverslip over the samples and back-fill the space between the slide and coverslip by pipetting a small amount of mounting medium along the edge of the coverslip.

3.3.2. Taking a Z-stack from lateral to medial side

1. Adhere two 22 x 22 mm coverslips to a slide with a small amount of 70% glycerol, leaving a ~5 mm space between them.

2. Transfer one brain to the slide using a pipette with the tip cut off. (see **Note 10**)

3. Remove all excess tissues including the discs from each brain with forceps.

4. Using one pair of forceps to hold the brain and cut the brain lobes apart from the ventral nerve cord using a micro knife. (see **Note 11**)

5. Place a 24 x 40 mm coverslip over the samples and back-fill the space between the slide and coverslip by pipetting a small amount of mounting medium along the edge of the coverslip. Do not seal the coverslip with nail polish. (see **Note 12**)

4. Notes

1. Leaving the brains connected to the mouth hooks will help the brains sink to the bottom of the tube during washes and their dark color will make it easier to see the brains while pipetting solutions in the tubes.

2. Leaving the ventral nerve cord intact will aid in preferred orientation of the brain on the slide: the brain can rest steadily on its ventral surface.

3. Fix the brain within 20 minutes following dissection to prevent protein degradation.

4. Four conditions are compared in Figure 3. Skipping the blocking step has no effect on the quality of the staining.

5. Conditions for primary antibody incubation are dependent on the specific antibody being used. In general, primary antibody incubation at room temp for 3 hours and secondary antibody incubation at 4°C overnight give a good balance between quality and efficiency for the majority of the antibodies. However, some primary antibodies work significantly better when incubated at the room temperature for 3 hours than at 4°C overnight or the reverse. Thus different staining conditions should be tested when the staining quality is not satisfactory.

6. Due to the thickness of the brain, it is recommended to incubate secondary antibodies

at 4^{o}C overnight for complete penetration, especially for anti-rabbit secondary antibodies.

7. Phalloidin (Invitrogen) is a high-affinity filamentous actin probe conjugated with specific fluorophores. It is useful to mark the cell cortex as shown in Figure 1. To do this, incubate the samples with phalloidin diluted 1:100 in PBST for 30 minutes at room temperature after step 5 of the fixation and staining protocol.

8. Building a bridge using cover slips provides the space to maintain larval brains without destroying their shape.

9. Orienting the brain with the ventral surface down will give a better confocal quality since this leaves the brain at a higher position than the ventral nerve cord. The 24 x 40 mm coverslip will apply pressure on the brain and result in a larger angle between nerve cord and the brain lobe and leave posterior side up. This is especially important for examining type II neuroblast lineages for them being located at posterior side.

10. It is preferred to mount one brain per slide, as it is hard to keep track of multiple lobes when rolling the brains under coverslip.

11. Despite being discarded, an intact ventral nerve cord helps in positioning the brain during cutting, thus resulting in a clean cut and a smoother lobe surface. This will aid in rolling the brain to the desired orientation during confocaling.

12. Put the slide on the confocal microscope stage and find the brain lobes under low magnification. Slowly and gently slide the coverslip to roll the brain lobe to the desired orientation determined by visualizing markers like PatJ and E(spl)m -GFP under an epifluorescent scope.

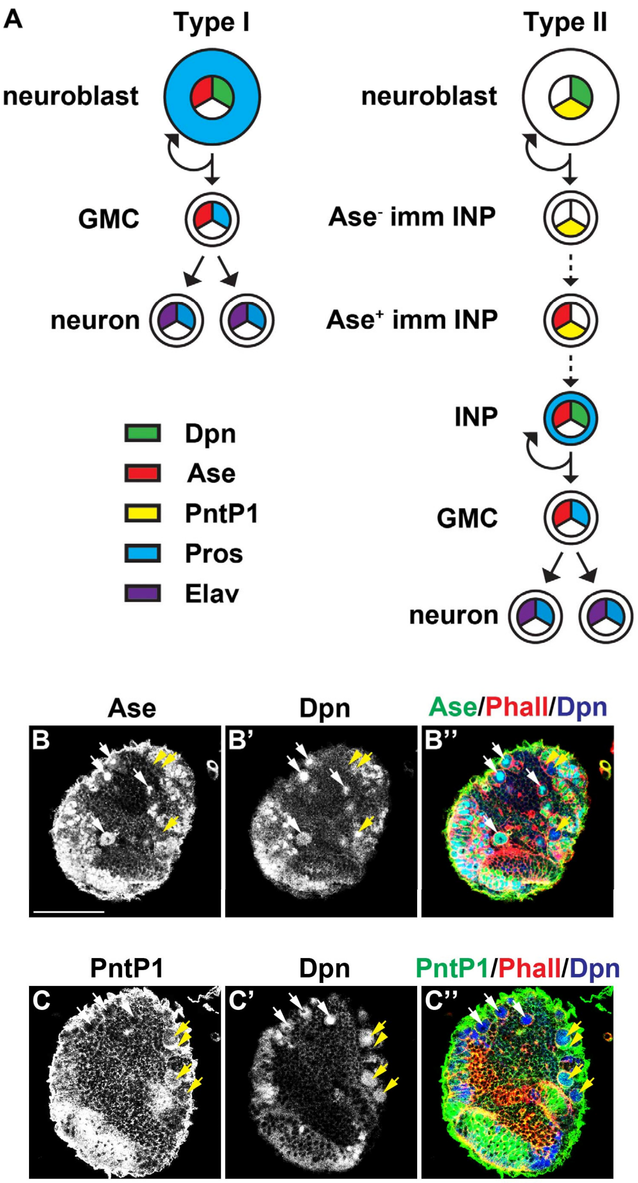

Figure 1. Neuroblast lineages in the fly larval central brain. (A) A cartoon summarizes the expression profile of molecular markers in the type I neuroblast and type II neuroblast lineage. Imm INP: immature INP. (B-C) A third instar larval brain was stained with antibodies against Dpn, Ase and PntP1. The cell cortex was marked by phalloidin. White arrows indicate type I neuroblasts whereas yellow arrows indicate type II neuroblasts and yellow arrowheads indicate immature INPs.

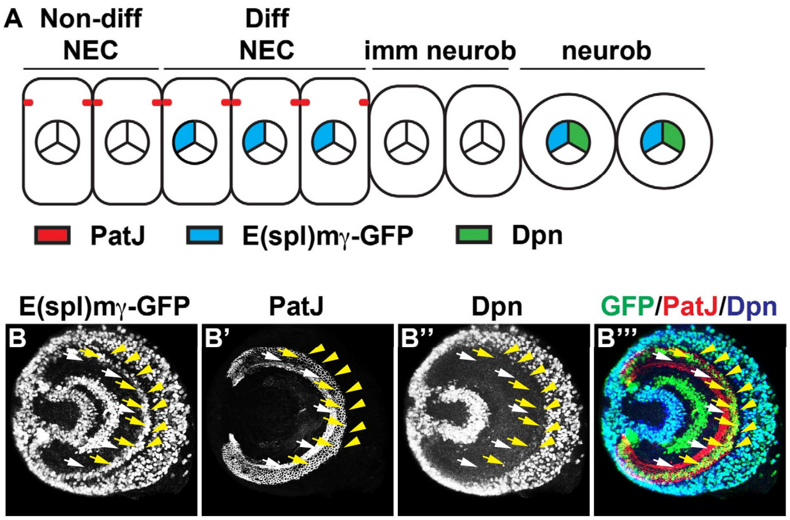

Figure 2. Neuroepithelial cells and neuroblasts in the fly larval optic lobe. (A) A cartoon summarizes the expression profile of molecular markers in neuroepithelial cells and neuroblasts. Non-diff NEC: non-differentiating neuroepithelial cells. Diff NEC: differentiating neuroepithelial cells. imm neurob: immature neuroblasts. Neurob: neuroblasts. (B) A third instar larval optic lobe was stained with antibodies against PatJ, E(spl)mγ-GFP and Dpn. White arrows indicate non-differentiating neuroepithelial cells whereas yellow arrows indicate differentiating neuroepithelial cells and yellow arrowheads indicate immature neuroblasts.

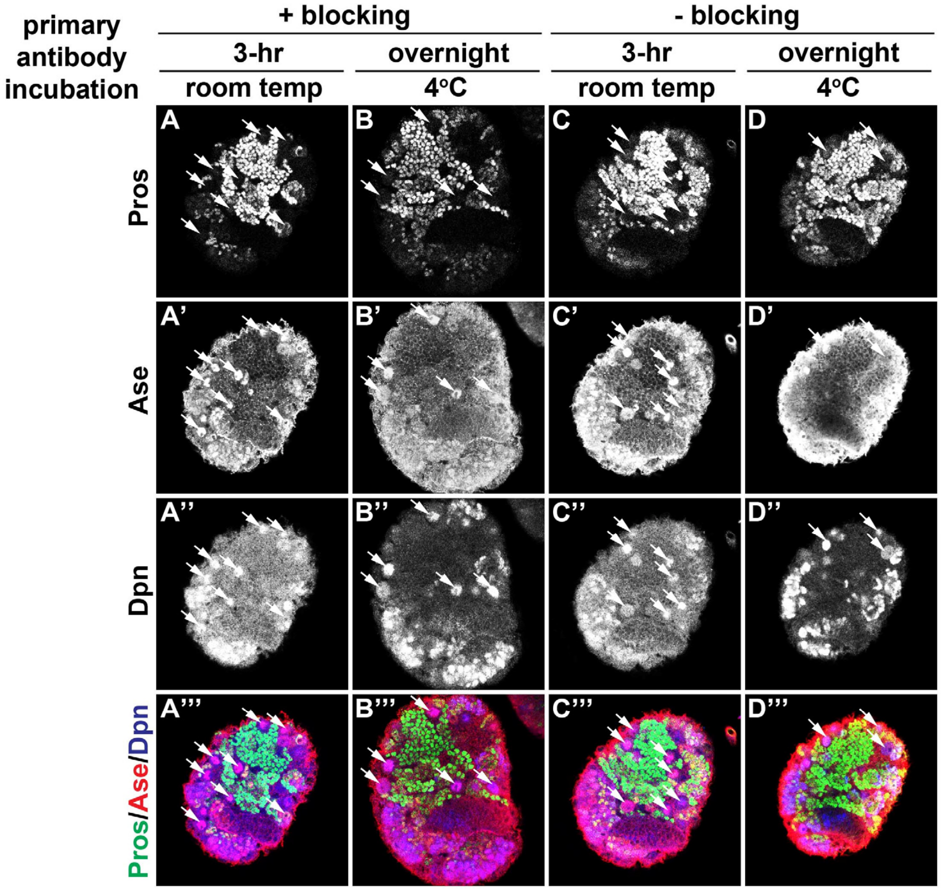

Figure 3. Comparative analyses of four distinct primary antibody staining protocols. (A-D) Third instar larval brains were stained with Dpn, Pros and Ase. White arrows indicate neuroblasts.

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References

Bowman, S. K., Rolland, V., Betschinger, J., Kinsey, K. A., Emery, G. and Knoblich, J. A. (2008). The Tumor Suppressors Brat and Numb Regulate Transit-Amplifying Neuroblast Lineages in Drosophila. Dev Cell 14, 535-46.

Sousa-Nunes, R., Cheng, L. Y. and Gould, A. P. (2010). Regulating neural proliferation in the Drosophila CNS. Curr Opin Neurobiol 20, 50-7.

Weng, M. and Lee, C. Y. (2011). Keeping neural progenitor cells on a short leash during Drosophila neurogenesis. Curr Opin Neurobiol 21, 36-42.