TEMPORAL CHANGES IN NEURAL PROGENITOR COMPETENCE

by

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DISSERTATION ABSTRACT

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Drosophila neural stem cells (neuroblasts) are a powerful model system for investigating stem cell self-renewal, specification of temporal identity, and progressive restriction in competence. Notch signaling is a conserved cue that is an important determinant of cell fate in many contexts across animal development; for example mammalian T cell differentiation in the thymus and neuroblast specification in *Drosophila* are both regulated by Notch signaling. However, Notch also functions as a mitogen, and constitutive Notch signaling potentiates T cell leukemia as well as *Drosophila* neuroblast tumors. While the role of Notch signaling has been studied in these and other cell types, it remains unclear how stem cells and progenitors change competence to respond to Notch over time. Notch is required in type II neuroblasts for normal development of their transit amplifying progeny, intermediate neural progenitors (INPs). Here we find that aging INPs lose competence to respond to constitutively active Notch signaling. Moreover, we show that reducing the levels of the old INP temporal transcription factor Eyeless/Pax6 allows Notch signaling to promote the de-differentiation of INP progeny into ectopic INPs, thereby creating a proliferative mass of ectopic progenitors in the brain. These findings provide a new system for studying

progenitor competence, and identify a novel role for the conserved transcription factor Eyeless/Pax6 in blocking Notch signaling during development.

This dissertation includes previously published, co-authored material.

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CHAPTER I

CHANGING COMPETENCE IN NEURAL STEM CELLS DURING DEVELOPMENT

INTRODUCTION

Understanding the genetic and molecular mechanisms that allow stem cells to generate distinct cell types over time is critical to our broader understanding of animal development and how to reprogram adult stem cells to regenerate tissues damaged from injury or disease. It is well known that extrinsic niche-derived cues can alter stem cell self-renewal and differentiation (G. Huang, Ye, Zhou, Liu, & Ying, 2015; Mooney et al., 2015; Pauklin & Vallier, 2015; Serio, 2014), but stem cells often have heterogeneous responses to a single cue (Figure 1.1), and how stem cells change their competence to respond to a specific cue has only recently been characterized. Here I use the term "competence" to describe the ability of a stem cell to respond to an extrinsic or intrinsic cue – for example, a progenitor at one stage of development may be competent to proliferate in response to active Notch signaling, but the same progenitor at a later state of development may be non-competent to respond to the same Notch signal. There are many ways a stem cell might change its competence to respond to a cue, but recently the role of epigenetic remodeling of the stem cell genome has emerged as an important process in controlling stem cell competence. Here I focus on evidence that neural stem cells change competence over time to generate different responses to a single cue; and highlight examples in which changes in stem cell competence are due to epigenetic modifications. I highlight how findings in multiple model organisms demonstrate that

changes in stem cell competence are relevant for generating neuronal diversity during embryogenesis, as well as preventing tumorigenesis in adult stem cells.

To maintain the focus of this chapter, I do not cover work in non-neural stem cells, which has been reviewed elsewhere (Segales, Perdiguero, & Munoz-Canoves, 2015). I hope to convey how the gain and loss of stem cell competence via known or likely epigenetic modification is a conserved developmental strategy to generate neuronal diversity from a relatively small pool of neural stem cells.

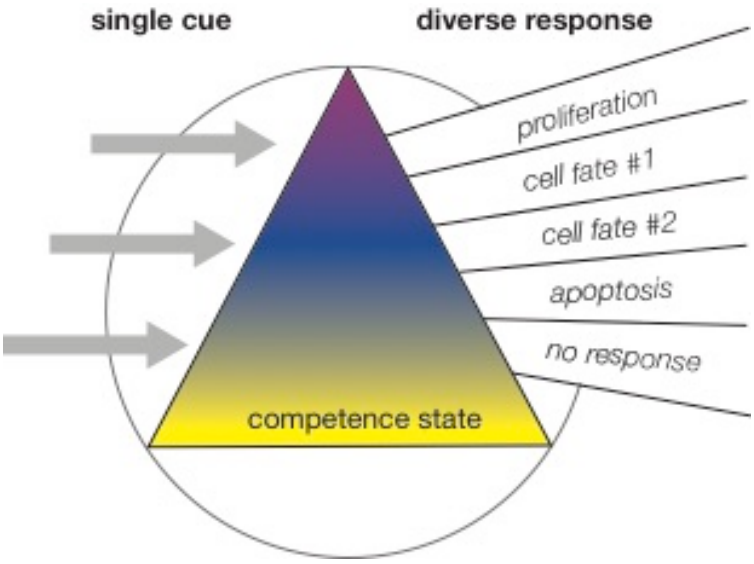


Figure 1.1. Competence.
Illustration showing how a single developmental cue (intrinsic factor or extrinsic signaling pathway) can produce multiple outcomes depending on stem cell competence. Note that stem cell competence can change over time (vertical axis), and that in some cases a potent signal can generate no response if the cell has lost competence to respond.

Chapter II of this dissertation contains previously published, co-authored material reproduced here with the permission Dylan R. Farnsworth, Omer Ali Bayraktar and Chris Q. Doe.

DROSOPHILA

The Drosophila CNS is generated by neural stem cells called neuroblasts, which undergo a series of asymmetric divisions to generate progeny with a more restricted fate. The most common "type I" neuroblast lineage produces ganglion mother cells (GMCs) which undergo a terminal division to generate a pair of neurons or glia; these neuroblasts can be found in the embryo, the larval optic lobe, and the larval central brain (Figure 1.2a,b) (Bayraktar, Boone, Drummond, & Doe, 2010b; Kang & Reichert, 2015; Viktorin, Riebli, & Reichert, 2013). The rarer Type II neuroblasts are located in the dorsomedial region of the central brain (Figure 1.2a)(Bayraktar et al., 2010b; Ito, Masuda, Shinomiya, Endo, & Ito, 2013; J. S. Yang et al., 2013). Type II neuroblasts produce a series of intermediate neural progenitors (INPs) that each divide asymmetrically to generate 4-6 GMCs which make 8-12 neurons and thus they give rise to large clones of neurons that contribute to the adult central complex (Figure 1.2c) (B. Bello, Reichert, & Hirth, 2006; Boone & Doe, 2008; Bowman et al., 2008a). In this section, I will discuss how (a) embryonic type I neuroblasts lose competence to respond to early temporal transcription factors due to changes in subnuclear gene position, (b) larval type I neuroblasts lose competence to respond to oncogenic mutations, (c) larval INPs lose competence to respond to Notch signaling, (d) larval type II neuroblasts use Trithorax to maintain competence to generate INPs, and (e) sensory neuron progenitors change competence to respond to Notch signaling.

Aging embryonic neuroblasts lose competence to respond to early temporal transcription factors via subnuclear genome reorganization and PRC1/2 complex function

Embryonic neuroblasts of the ventral nerve cord (VNC) can be uniquely identified by their position, molecular markers, and the stereotyped clone of neural progeny they produce (Skeath & Thor, 2003). Individual neuronal identity is determined by the spatial identity of the parental neuroblast in combination with "temporal transcription factors" which are sequentially expressed by most neuroblasts as they progress through their lineage. The temporal transcription factor cascade is Hunchback (Hb; Ikaros in mammals), Kruppel (Kr), Nubbin/Pou domain 2 (Pdm), and Castor (Casz1 in mammals). Loss of Hb or Kr leads to failure to specify the neurons born during these expression windows, whereas forced misexpression of Hb or Kr results in ectopic first-born or second-born neuron subtypes (Isshiki, Pearson, Holbrook, & Doe, 2001; Kanai, Okabe, & Hiromi, 2005; Novotny, Eiselt, & Urban, 2002), in part by Hb positively regulating its own expression (Kohwi, Lupton, Lai, Miller, & Doe, 2013). However, pulses of Hb or Kr later in embryonic neuroblast lineages fail to induce early neuronal fates: the neuroblast has lost competence to respond to these transcription factors (Cleary & Doe, 2006; Pearson & Doe, 2003). Recent work has shown that loss of competence to respond to Hb is due to movement of the *hb* locus to the nuclear lamina in aging neuroblasts, thereby preventing ectopic Hb protein from inducing endogenous hb transcription (Kohwi et al., 2013). In contrast, loss of competence to respond to Kr is due to activity of Polycomb repressive complex (PRC) activity (Touma, Weckerle, & Cleary,

2012), presumably by making Kr target genes inaccessible. It will be interesting to see if both mechanisms are linked: do both Hb and Kr target genes move to the nuclear lamina? Is this movement a cause or consequence of PRC recruitment to these loci?

Larval type I neuroblasts lose competence to respond to oncogenic mutations

Drosophila embryonic and early larval type I neuroblasts coordinately express two RNA-binding proteins (Imp/IGF2BP and Lin-28) and a transcription factor (Chinmo); they are all down-regulated in neuroblasts during the second half of larval life (Liu et al., 2015; Narbonne-Reveau et al., 2016; Zhu et al., 2006). Recent work has shown that this suite of factors gives neuroblasts competence to form malignant tumors in response to several oncogenic mutations, including mutants in transcription factors (Prospero, Nerfin-1), and an RNA-binding protein (Brain tumor; Brat). Importantly, older neuroblasts during the second half of larval life are still proliferating but have little or no response to the same oncogenic mutations (Narbonne-Reveau et al., 2016). The normal function of Chinmo, Lin-28 and Imp is to specify early-born larval neurons and glia (Liu et al., 2015; Zhu et al., 2006), but they also open a competence window for "single hit" tumor formation; it is unknown if these two functions are related. This suite of proteins is unlikely to act on a single locus or a highly specific process because they provide tumor-forming competence to a diverse array of oncogenic mutations, including mutants in two different transcription factors (Prospero and Nerfin-1) and an RNA-binding protein (Brain tumor). All three proteins are conserved in mammals (Bell et al., 2013; Dykes et al., 2014; M. Yang et al., 2015), raising the question of whether they may have a similar

function, and making it important to determine their mechanism of action in both Drosophila and mammals.

Larval type II neuroblasts require Trithorax to maintain competence to produce INPs

Larval type II neuroblasts divide asymmetrically to generate a series of INPs that produce an average of 10 neurons each, whereas larval type I neuroblasts make GMCs that only produce a pair of neurons (B. Bello et al., 2006; Boone & Doe, 2008; Bowman et al., 2008a). How do type II neuroblasts generate INPs rather than GMCs? Recent work demonstrated that type II neuroblasts require the Buttonhead (Btd) transcription factor to maintain INP production, and that Trithorax (member of the SET1/MLL histone methyltransferase complex) is required to maintain the *btd* locus in a permissive chromatin state, allowing its expression in type II neuroblasts (Komori, Xiao, Janssens, Dou, & Lee, 2014). Loss of Trithorax led to lack of Btd, and loss of either Trithorax or Btd led to type II neuroblasts switching to GMC production (Komori et al., 2014) (Figure 1.2e). They next showed that the loss of type II neuroblast identity was specifically caused by a loss of Trx histone methylation activity. Similarly, RNAi knockdown of several members of the SET1/MLL histone methyltransferase complex that co-purified with Trx also led to loss of INP production from type II neuroblasts (Komori et al., 2014). Conversely, the Brahma/histone deacetylase 3/Earmuff (Brm/HDAC3/Erm) complex is required to maintain INP identity and prevent dedifferentiation into type II neuroblasts (Koe et al., 2014) (Figure 2e). Collectively, these results show that type II neuroblast

competence to produce INPs, and ability of INPs to initiate a program of differentiation, is regulated by the cell type-specific actions of multiple chromatin remodeling complexes.

Aging INPs lose competence to proliferate in response to Notch signaling

Type II neuroblasts divide asymmetrically to produce a series of INPs, which have a limited ability to proliferate, dividing only 4-6 times. Recently, it has been shown that aging INPs express a series of three temporal transcription factors: Dichaete (Sox family), Grainy head (CP2 family), and Eyeless (Pax family), which are important for generating neuronal and glial diversity within the short INP lineages (Bayraktar & Doe, 2013a). An interesting question is what limits INP proliferation to 4-6 divisions, when their parental neuroblast can divide ~50 times. Recent work has shown that the chromatin remodeler Osa (SWI/SNF complex member) and Prdm family member Hamlet also limit INP proliferation (Eroglu et al., 2014). Osa is required for expression of Hamlet in INPs (but not in other cell types of the lineage), and reducing Osa or Hamlet levels in INPs led to extension of INP lineages(Figure 1.2f,g). This is not due to derepression of Notch target genes (none were upregulated by transcriptional profiling of *osa* mutant INPs), but rather due to changes in INP temporal transcription factor expression: prolonged Grainy head and reduced Eyeless (Eroglu et al., 2014). These data suggest a model in which INP chromatin remodeling is required for proper expression of the anti-proliferation factor Eyeless, which helps terminate INP proliferation.

How might Eyeless restrict INP proliferation? Many stem cells and progenitors require Notch signaling to maintain proliferation, so we asked whether Eyeless limits

Notch signaling in aging INPs. It is well known that misexpression of the Notch intracellular domain (NICD), a potent inducer of Notch target gene expression (Borggrefe et al., 2016), in Type II NBs and young Eyeless-negative INPs results in tumor formation (Bowman et al., 2008a; Farnsworth, Bayraktar, & Doe, 2015; Weng, Golden, & Lee, 2010b; Xiao, Komori, & Lee, 2012) (Figure 1.2h). In contrast, we found that NICD expression in old Eyeless+ INPs had no effect on the fate or proliferation of INPs, even when the exact same promoter was used to drive expression to ensure equal levels of Notch activity (Farnsworth et al., 2015) (Figure 1.2i) (see Chapter II of this dissertation). Furthermore, removal of the late temporal transcription factor Eyeless restored competence to generate ectopic cells by de-repressing Notch target genes in INP progeny (Figure 1.2j). Thus, aging INPs lose competence to respond to Notch signaling, and Eyeless is required to block Notch-induced proliferation in old INP progeny (Farnsworth et al., 2015). How does Ey block Notch signaling? An attractive model is that Ey recruits SWI/SNF proteins to prevent activation of Notch target genes in GMCs (Housden, Li, & Bray, 2014; San-Juan & Baonza, 2011). Consistent with this model, murine Pax6 protein directly binds the SWI/SNF-related BAF complex to promote neuronal differentiation in murine adult neural progenitors (Ninkovic et al., 2013). In addition, a BAF subunit switch triggers the transition from proliferation to differentiation in mammalian neural progenitors (Lessard et al., 2007), raising the possibility that both Drosophila and mammals use similar pathways to regulate progenitor choice of differentiation or proliferation.

Aging sensory neuron progenitors change competence to respond to Notch signaling

Drosophila olfactory receptor neurons (ORNs) are specified from neuronal progenitors called sensory organ precursors (SOP), which undergo three rounds of division to generate 8 cells, three of which are distinct ORNs. One ORN is specified by absence of Notch signaling (Nab) while two ORNs are specified by high level Notch signaling (Naa and Nba) – in the absence of Notch signaling these two neurons take alternate fates (Endo et al., 2012). How does one signal, Notch, generate two different ORN fates? The authors found that only Naa expressed the Prdm member Hamlet, and that Hamlet was necessary and sufficient to induce Naa identity including axon projection to the appropriate olfactory glomeruli and odorant receptor expression (Endo et al., 2012). How is Notch signaling and Hamlet expression integrated to generate distinct ORN fates? Genetic experiments indicate that Hamlet suppresses Notch activity, and biochemical data support this conclusion. Hamlet directly binds the CtBP co-repressor, and this binding is required for Hamlet suppression of Notch signaling. Furthermore, forced expression of Hamlet in a *Drosophila* cell line resulted in altered chromatin structure at Notch target loci, likely through enhancing H3K27 tri-methylation (associated with a repressive chromatin state) and diminishing H3K4 tri-methylation (associated with an active chromatin state). For example, Hamlet expression decreased the Notch nuclear effector Su(H) occupancy at the Notch target gene *E(spl)m3*. Thus, Hamlet appears to bias Notch signaling by creating repressive chromatin structure around at least one Notch target gene, such that high Notch signaling without Hamlet gives the Nba fate, partial or differential Notch signaling with Hamlet gives the Naa fate, and no Notch signaling gives

the Nab fate (Endo et al., 2012). These findings illustrate (a) how histone modifications can drive changes in competence by altering the local chromatin structure of target genes important for neuronal specification and function, and (b) how neuronal diversity can be expanded in a stem cell lineage through changes in competence while reusing the same extrinsic cue.

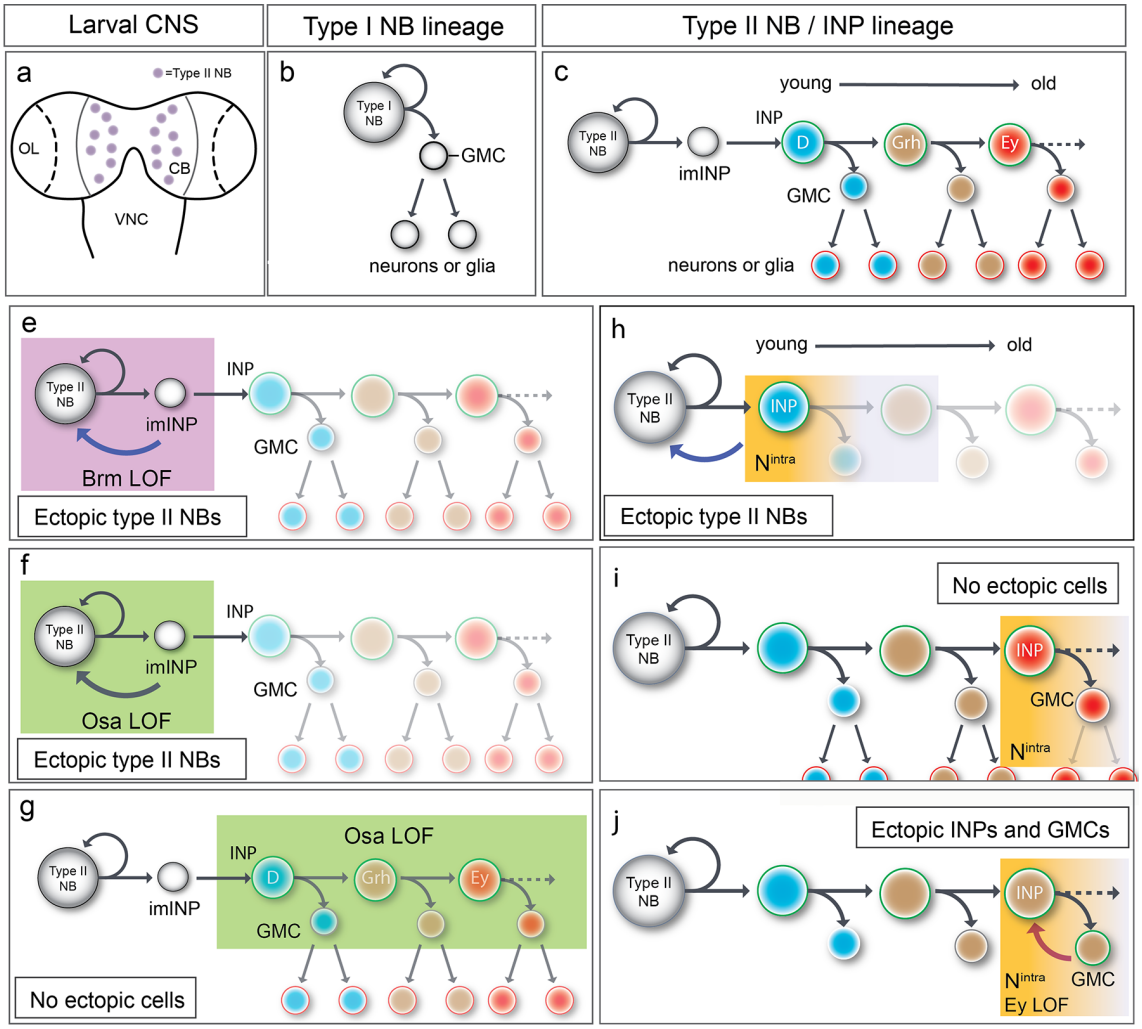


Figure 1.2. Changing competence in *Drosophila* neural stem cells and progenitors. (a-c) Drosophila neural stem cells in the central brain (a) undergo a type I lineage (b) or a more elaborate type II lineage (c). (e-g) Epigenetic regulation of Drosophila neural stem cells alters their ability to respond to Notch signaling, which is normally present in stem cell progeny but suppressed by Brm (e) or Osa (f,g), Eyeless (i,j) or other Notch pathway repressors (h).

MOUSE

Competence to respond to extrinsic cues depends on Sox2-regulated chromatin state in neural progenitor lineages

The Sox family of transcription factors are important for maintaining stem cell/progenitor identity in many contexts (Miyagi, Kato, & Okuda, 2009). Sox2 loss of function results in premature expression of neuronal differentiation genes, and Sox2 overexpression represses neuronal differentiation (Graham, Khudyakov, Ellis, & Pevny, 2003). However, recent work reveals that Sox2 also has a role in promoting competence of young neurons to initiate neuronal differentiation in response to extrinsic Wnt signaling. Conditional Sox2 deletion in adult hippocampal neural progenitor cells (NPCs) has shown that Sox2 limits Polycomb Repressive Complex 2 (PRC2) activity to maintain a "poised" bivalently marked H3K4me3/H3K27me3 chromatin state at neuronal differentiation loci such as NeuroD1 and Bdnf (Amador-Arjona et al., 2015). In this manner, Sox2 prevents the formation of a "closed" H3K27me3 chromatin state, which would otherwise block Wnt-induced expression of neuronal differentiation loci could be rescued by targeted re-expression of NeuroD1 (Amador-Arjona et al., 2015). The authors propose a model where Sox2 limits PRC2 activity to maintain a poised chromatin state at neuronal differentiation genes, thereby giving them competence to respond to Wnt-induced expression and subsequent neuronal differentiation (Figure 1.3).

Interestingly, this is a different mode of Sox2 action than the authors previously described for maintaining progenitor identity of hippocampal NPCs. In that study, they found that Sox2 recruited the TRRAP/GCN5 histone acetyltransferase complex to

maintain “open” H3K9ac chromatin at the *LIN28* locus, allowing this self-renewal promoting gene to be expressed in NPCs (Cimadamore, Amador-Arjona, Chen, Huang, & Terskikh, 2013).

Sox2 is also required to maintain the proliferative potential of retinal progenitor cells by modulating responsiveness to the Notch signaling pathway (Taranova et al., 2006). Conditional mutations and knockdown of Sox2 resulted in decreased expression of the Notch1 receptor, and chromatin immunoprecipitation experiments showed association of Sox2 and the *Notch1* locus. The authors propose a model in which Sox2 promotes Notch1 receptor expression in retinal progenitors, giving them competence to respond to Notch ligands and activating expression of Notch target genes such as Hes-5, which are important for maintaining the proliferative capacity of retinal progenitors (Taranova et al., 2006).

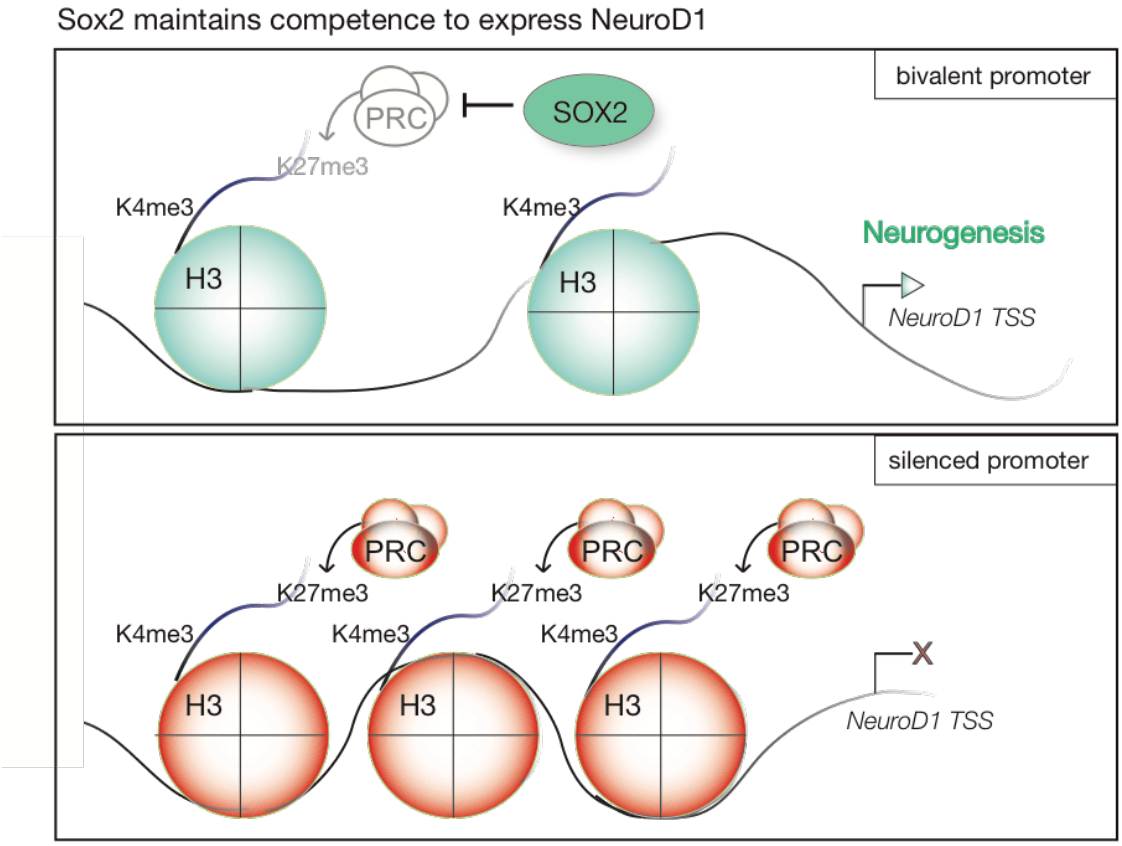


Figure 1.3. Sox2 prevents the Polycomb Repressive Complex (PRC) from silencing proneural genes such as NeuroD1 via histone H3 lysine 27 trimethylation (K27me3).

Epigenetic silencing of Notch target genes restricts INP competence to respond to Notch

Neural stem cells (NSCs) in the germinal zone of developing mammalian cortex have active Notch signaling via the canonical CBF1/SuH/Lag-1 (CSL) nuclear effector, and express target genes such as Hes-5 to maintain proliferation and block differentiation. In contrast, NSC progeny called intermediate neural progenitors (INPs) are exposed to Notch ligands but fail to express Notch target genes including a CSL reporter construct or Hes5, and thus initiate neuronal differentiation (Mizutani, Yoon, Dang, Tokunaga, &

Gaiano, 2007; Tiberi et al., 2012). What limits INP competence to respond to Notch/CSL signaling? Recent work has shown that the Bcl6 oncogene is required to blunt Notch signaling in INPs. Bcl6 is detected at low levels in NSCs and high levels in INPs, where it reduces occupancy of the Mam-1 co-activator protein at the *Hes5* locus, increases occupancy of the Sirt-1 deacetylase, leading to silencing of the Hes5 gene (Tiberi et al., 2012). The authors conclude that epigenetic silencing of the *Hes5* locus blocks productive Notch signaling in INPs leading to neuronal differentiation. It will be interesting to compare this mechanism to that of Eyeless blocking Notch signaling in Drosophila old INPs (see above); perhaps in both cases loss of competence to respond to Notch will be due to epigenetic silencing of specific Notch target genes.

The role of extrinsic cues and epigenetic modification in subdividing a single progenitor competence window

In the developing mammalian hindbrain, Nkx2.2+ progenitors produce motor neurons (MNs) during early neurogenesis, and then switch to making serotonergic neurons (5HTNs). The homeodomain transcription factor Phox2b is expressed in young progenitors during MN production, and *Phox2b* mutant progenitors fail to make MNs and instead prematurely give rise to 5HTNs, showing that young progenitors have an intrinsic competence to generate 5HTNs (Pattyn et al., 2003). More recently, Dias *et al.* identified TGFβ signaling as a temporally regulated cue that downregulates Phox2b expression; reduced TGFβ signaling delayed the MN-to-5HTN switch, altering the number of neurons in each population (Dias, Alekseenko, Applequist, & Ericson, 2014). This system illustrates how temporal regulation of cell fate determinates (e.g. Phox2b) can

subdivide a single competence window to generate neuronal diversity, and how an extrinsic cue can determine the timing of the switch between neuronal cell types.

In contrast, a different mechanism times the neuronal-to-glial switch that occurs in many regions of the murine CNS. Although the switch requires an extrinsic cue, in this case the CNTF/LIF cytokine, there is also a requirement for epigenomic modification. Early cortical progenitors are exposed to CNTF, yet they still produce neurons (Derouet et al., 2004; Uemura et al., 2002). Similarly, young cortical progenitors were less competent to produce glia than older progenitors when exposed to gliogenic cytokines in culture (He et al., 2005). Even over-expression of CNTF in young cortical progenitors only generates a slight increase in glial production (Barnabe-Heider et al., 2005). What prevents CNTF from inducing gliogenesis in young progenitors? It appears that at least one key glial differentiation gene, *Gfap*, is highly methylated and thus epigenetically silenced in young cortical progenitors; elimination of DNA methyltransferase 1 (Dnmt1) activity leads to robust precocious production of $GFAP^+$ astrocytes in response to CNTF (Fan et al., 2005; Takizawa et al., 2001).

ZEBRAFISH

Insight into how progenitors change competence to respond to extrinsic cues has come from studies in the developing zebrafish spinal cord. Zebrafish lateral floor plate progenitors (LFPs) require Hedgehog (Hh) signaling to maintain proliferation (Hudish et al., 2016). Progenitors stop dividing and initiate differentiation by diminishing their response to Hh signaling; this is achieved, at least in part, by a regulatory network that restricts apical cilia formation – a process implicated in perceiving Hh signaling (P.

Huang & Schier, 2009), and controlled by apically restricted proteins – a hallmark of asymmetrically dividing neural stem cells and progenitors (Lee, Robinson, & Doe, 2006). Previous work revealed that expression of the apically restricted Par proteins, Pard3 and Prkci, are repressed by the expression of miR-219 (Hudish, Blasky, & Appel, 2013). The authors put forth a model where spinal cord progenitors in the early embryo proliferate in response to Hh signaling, but the onset of miR-219 expression leads to Par protein repression and loss of apical cilia, thereby rendering progenitors non-competent to respond to Hh signaling and triggering differentiation. This model is supported by the observations that miR-219 knockdown caused an extension of Hh signaling, as measured by *patched2* expression and an increased number of Sox2+ progenitors in the spinal cord. Furthermore, these effects of miR-219 knockdown could be rescued by treating embryos with cyclopamine, an inhibitor of Hh signaling. Importantly, expression of Shh ligands in the developing spinal cord does not diminish from one to three days post fertilization (dpf), although expression of *patched2* is lost by 3 dpf, suggesting that progenitors are no longer competent to respond to Shh ligands. Thus, microRNAs regulate the competence of neural progenitors to respond to Hh signaling, leading to a transition from proliferation to neurogenesis.

Another example of altered neural progenitor competence comes from the analysis of Kolmer-Agduhr (KA") interneuron development. LFPs generate KA" neurons via combinatorial interactions between the Notch and Hh signaling pathways (P. Huang, Xiong, Megason, & Schier, 2012). Notch signaling is required transiently to maintain LFP progenitors and to convey competence to respond to Hh, which is required in LFP progenitors for the subsequent specification of KA" interneurons (P. Huang et al., 2012).

Early activation of Hh caused the formation of ectopic KA" interneurons, while late activation the Hh effector Gli1 inhibited the differentiation of LFP progeny into KA" interneurons. Thus, Hh signaling could only promote the specification of KA" interneurons in LFP progenitors where Notch signaling was active (P. Huang et al., 2012). How Notch signaling provides competence to respond to Hh remains unknown, but Notch signaling is known to alter chromatin state (Schwanbeck, 2015; Wang, Zang, Liu, & Aster, 2015) and it a likely mechanism for altering progenitor competence in this system.

BRIDGE

Work in many systems has now shown that epigenetic remodeling can alter neural stem cell competence, thereby resulting in a single neural stem cell generating diverse progeny in response to a common signaling pathway (e.g. Notch). This allows a limited number of highly conserved signaling pathways to generate myriad cell fate outcomes during neural development. Future work will be needed to identify factors that trigger chromatin alterations, the precise nature of the alterations at a genome-wide level, and the mechanism by which these changes lead to distinct neuronal and glial cell types. A better understanding of how temporally regulated changes in stem cell epigenomes bias the response to signaling pathways is likely to help guide in vitro production of neural cell types for clinical neurotherapeutics.

CHAPTER II

AGING NEURAL PROGENITORS LOSE COMPETENCE TO RESPOND TO MITOGENIC NOTCH SIGNALING

INTRODUCTION

Development of complex structures like the human central nervous system (CNS) requires the production of a staggering diversity of cell types from a relatively small pool of progenitors. Spatial cues generate progenitor diversity, whereas subsequent temporal cues allow single progenitors to produce a series of distinct neuronal and glial cell types (Jessell, 2000; Kohwi & Doe, 2013). Recently it has become clear that progenitors change competence to respond to spatial and temporal cues, potentially allowing a single cue to generate distinct outputs (Boije, MacDonald, & Harris, 2014; Groves & LaBonne, 2014; Kohwi & Doe, 2013; Li, Chen, & Desplan, 2013; Livesey & Cepko, 2001). For example, mammalian cortical progenitors gradually lose competence to form early-born cell types. When developmentally advanced progenitors are transplanted into their native region in younger hosts, they fail to produce the deep layer neurons typically born in this cortical environment (Frantz & McConnell, 1996). Similarly, Drosophila embryonic neuroblasts (NBs) are initially competent to respond to the early temporal transcription factors Hunchback or Krüppel, but later lose competence to respond to these cues (Cleary & Doe, 2006; Kohwi et al., 2013; Pearson & Doe, 2003). Although there has been excellent progress on identifying spatial and temporal patterning cues, much less is

known about how progenitors change competence. Do progenitors pass through discrete competence windows where distinct cells types are born in response to the same cue? What are the mechanisms that restrict competence? Are there many mechanisms, or might there be a small number of highly conserved mechanisms?

Drosophila neural progenitors are a model system to investigate how competence to respond to cell fate cues changes over time. Drosophila neuroblasts arise in the early embryo and can persist throughout larval stages. Most neuroblasts undergo a "type I" mode of division in which they divide asymmetrically to generate a series of smaller ganglion mother cells (GMCs) that each produces a pair of neurons or glia (**Figure 2.1A**). There are well-characterized spatial and temporal patterning cues that act on embryonic type I neuroblasts to generate neural diversity, as well as evidence for at least two distinct neuroblast competence windows that may produce different responses to early temporal identity factors [reviewed in (Kohwi & Doe, 2013; Li, Chen, et al., 2013; Pearson & Doe, 2004; Skeath & Thor, 2003; Sousa-Nunes, Cheng, & Gould, 2010)].

More recently, our lab and others have identified eight larval neuroblasts per brain lobe that undergo a more complex "type II" mode of division (**Figure 2.1A'**). Type II neuroblasts generate a series of smaller intermediate neural progenitors (INPs) that act as transit amplifying cells; each INP undergoes a series molecularly asymmetric divisions to self-renew and produce about six GMCs, each of which makes a pair of neurons or glia (**Figure 2.1A''**) (B. C. Bello, Izergina, Caussinus, & Reichert, 2008; Boone & Doe, 2008; Bowman et al., 2008b). Type I and II neuroblasts can also be distinguished by molecular markers; type I neuroblasts contain the transcription factors Deadpan (Dpn), Worniu (Wor), and Asense (Ase) whereas the type II neuroblasts contain Dpn, Wor, and

Pointed P1 (PntP1). Spatial and temporal patterning factors acting on larval neuroblasts have been identified (Bertet et al., 2014; Kao, Yu, He, Kao, & Lee, 2012; Li, Erclik, et al., 2013; Maurange, Cheng, & Gould, 2008; Sen, Biagini, Reichert, & VijayRaghavan, 2014; Zhu et al., 2006), and we have recently identified three INP temporal transcription factors: Dichaete (D), Grainy head (Grh), and Eyeless (Ey) (Bayraktar & Doe, 2013b). Despite this progress, currently nothing is known about how larval neuroblasts or INPs change competence to respond to cell fate or mitogenic cues.

Here we established a new system for investigating progenitor competence, INPs of the type II neuroblast lineages. In type II neuroblasts, Notch signaling is active and is required to maintain neuroblast identity and proliferation (Bowman et al., 2008b; Wang et al., 2006; Weng, Golden, & Lee, 2010a; Wirtz-Peitz, Nishimura, & Knoblich, 2008; Xiao et al., 2012). This is a highly conserved function, as Notch signaling also promotes self-renewal and proliferation of mammalian neural progenitors and stem cells (Harrison et al., 2010; Luo, Renault, & Rando, 2005; Mizutani et al., 2007; Ohishi, Katayama, Shiku, Varnum-Finney, & Bernstein, 2003; Yoon & Gaiano, 2005). Drosophila type II neuroblasts divide asymmetrically to produce immature INPs that lack active Notch signaling due in part to partitioning of the Notch inhibitor Numb selectively into the newborn INP. Overexpression of the Notch intracellular domain ($Notch^{intra}$) can bypass this block and induce de-differentiation of the new-born INP back into a type II neuroblast, leading to "neuroblast tumors" (Bowman et al., 2008b; Song & Lu, 2011; Weng et al., 2010a; Xiao et al., 2012). Here we investigate how INPs change competence to respond to Notch signaling over time. We confirm that expression of constitutively active $Notch^{intra}$ in young INPs results in the formation of neuroblast tumors, but in

striking contrast old INPs have no detectable response to precisely the same level of Notch^{intra}. Thus, INP competence to respond to Notch signaling changes over time, although the mechanism preventing old INPs from responding to Notch^{intra} remains unknown. Here, we identify a second mechanism that prevents GMCs from responding to Notch signaling: reducing the level of the old INP temporal transcription factor Eyeless/Pax6 resulted in de-differentiation of GMCs into INPs, leading to a proliferative mass of INP/GMC cell types that failed to initiate neuronal/glial differentiation. This defines a new role for the conserved Eyeless/Pax6 transcription factor in preventing progenitors from responding to Notch signaling.

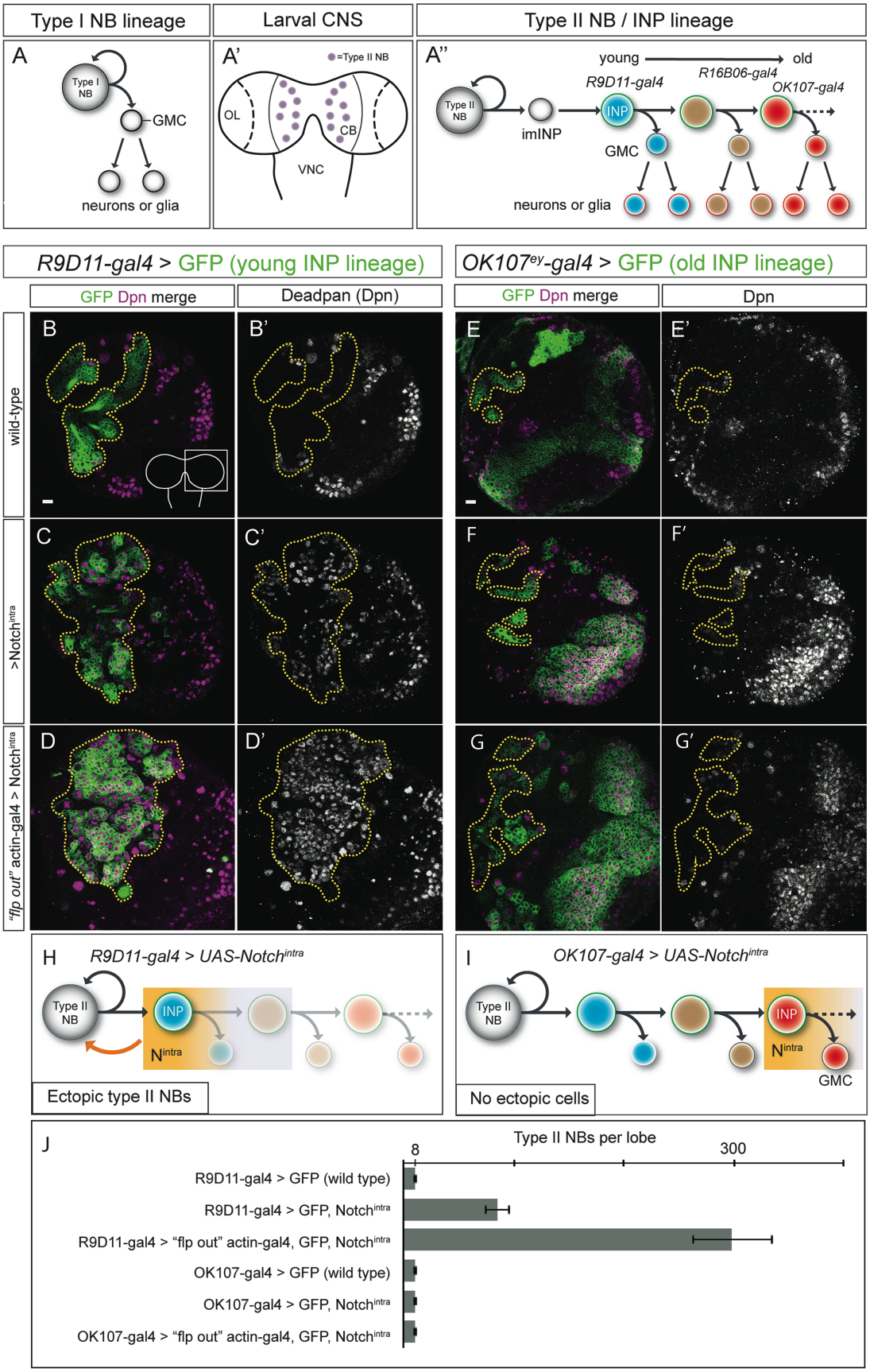
RESULTS

Old INPs lose competence to respond to Notch^{intra} signaling

As a starting point for our studies, we confirmed previous reports showing that constitutively active Notch (Notch^{intra}) in young INPs triggered INP de-differentiation into ectopic Dpn+ Ase- type II neuroblasts (**Figure 2.1C-C'** and data not shown; quantified in **Figure 2.1J**) [and see Figure 6C in (Weng et al., 2010a)]. Next, to determine whether old INPs remained competent to de-differentiate into type II neuroblasts in response to Notch signaling, we expressed Notch^{intra} using *OK107-gal4*, which is specifically expressed in old INPs within type II lineages (Bayraktar & Doe, 2013b). As expected, expression of GFP alone in old INPs did not produce any ectopic Dpn+ Ase- Type II neuroblasts (**Figure 2.1E** and data not shown; quantified in **Figure 2.1J**). Interestingly, expression of Notch^{intra} alone in old INPs also did not generate any ectopic neuroblasts (**Figure 2.1F**; quantified in **Figure 2.1J**), in contrast to its potent

induction of ectopic neuroblasts when expressed in young INPs. There are two possible interpretations of these results: (a) the *OK107-gal4* line produced lower levels of Notch^{intra} compared to *R9D11-gal4*, leading to insufficient Notch^{intra} to induce neuroblast identity; or (b) old INPs have lost competence to respond to Notch^{intra}.

Fig 2.1. Old INPs lose competence to respond to Notch (Next page).
(A) Eight type II NBs are found in the central brain (CB) of each larval brain lobe (OL = optic lobe, VNC = ventral nerve cord).
(A'-A'') Summary of type I and type II NB cell lineages. Type I NBs self-renew and produce GMCs which divide to make two neurons or glia. Type II NBs make INPs which transit amplify their lineage. *R9D11-gal4* is expressed in young INPs and their progeny but not the parental NB, whereas *OK107-gal4* is expressed in old INPs and their progeny but not other cells in the lineage.
(B-B') Wild type third instar larvae expressing GFP in young INP lineages (*R9D11-gal4 UAS-GFP)* show the normal number of Dpn+ Ase- type II neuroblasts (8±0 per lobe; n=3).
(C-C') Expression of constitutively active Notch in young INPs (*R9D11-gal4 UAS-Notch^{intra} UAS-GFP)* produces ectopic Dpn+ Ase- type II neuroblasts (34±1 per lobe, n=3).
(D-D') A permanent lineage tracing system in young INPs (*UAS-Flp, UAS-FRT-Stop-FRT-actin-gal4, UAS-Notch^{intra})* standardized expression of *UAS-Notch^{intra}*. This also produced ectopic type II NBs.
(E-E') Old INPs are labeled by *OK107-gal4* driving membrane GFP, without generating ectopic type II neuroblasts (8±0 per lobe; n=3).
(F-F') Old INPs do not generate ectopic Dpn+ NBs in response to constitutive Notch signaling (8±0; n=3).
(G-G') Using *OK107-gal4, UAS-Flp, UAS-FRT-Stop-FRT-actin-gal4, UAS-Notch^{intra}* to standardize *UAS-Notch^{intra}* expression levels did not produce ectopic Dpn+ NBs (8±0 per lobe; n=3).
(H, I, J) Summary and quantification of results. Images are a single, one micron plane through a whole brain lobe. Yellow outline = INP lineages in central brain.
All panels show third instar larvae; scale bar = 10 μm.


Type I NB lineage
Larval CNS
Type II NB / INP lineage
A
Type I NB
GMC
neurons or glia
A'
=Type II NB
OL
CB
VNC
A''
young
old
R9D11-gal4
R16B06-gal4
OK107-gal4
Type II NB
imINP
INP
GMC
neurons or glia
R9D11-gal4 > GFP (young INP lineage)
OK107ey-gal4 > GFP (old INP lineage)
GFP Dpn merge
Deadpan (Dpn)
GFP Dpn merge
Dpn
wild-type
>Notchintra
"flp out" actin-gal4 > Notchintra
B
B'
C
C'
D
D'
E
E'
F
F'
G
G'
H
R9D11-gal4 > UAS-Notchintra
Type II NB
INP
Nintra
Ectopic type II NBs
I
OK107-gal4 > UAS-Notchintra
Type II NB
INP
Nintra
GMC
No ectopic cells
J
Type II NBs per lobe
8
300
R9D11-gal4 > GFP (wild type)
R9D11-gal4 > GFP, Notchintra
R9D11-gal4 > "flp out" actin-gal4, GFP, Notchintra
OK107-gal4 > GFP (wild type)
OK107-gal4 > GFP, Notchintra
OK107-gal4 > "flp out" actin-gal4, GFP, Notchintra

To ensure equal Notch^{intra} levels in young or old INPs, we used a "flp out" expression method (Bayraktar & Doe, 2013b). We used the young INP *R9D11-gal4* line or the old INP *OK107-gal4* line to drive expression of *UAS-Flp*, which catalyzes excision of transcriptional stop sequences in the *actin-FRT-stop-FRT-gal4* gene. Thus, this method results in permanent expression of *actin-gal4* in either young INPs or old INPs, thereby ensuring equal levels of expression of the *UAS-Notch^{intra}* gene. As expected, *actin-gal4* driving *UAS-Notch^{intra}* in young INPs induced a large number of ectopic Dpn+ Ase- Type II neuroblasts (**Figure 2.1D** and data not shown; quantified in **Figure 2.1J**; summarized in **Figure 2.1H)**. In contrast, *actin-gal4* driving *UAS-Notch^{intra}* in old INPs did not generate any Dpn+ Ase- neuroblasts (**Figure 2.1G** and data not shown; quantified in **Figure 2.1J**; summarized in **Figure 2.1I**). In addition, Notch^{intra} protein levels are indistinguishable among these genotypes (**Figure S1**). We conclude that old INPs have lost competence to form neuroblasts in response to Notch signaling.

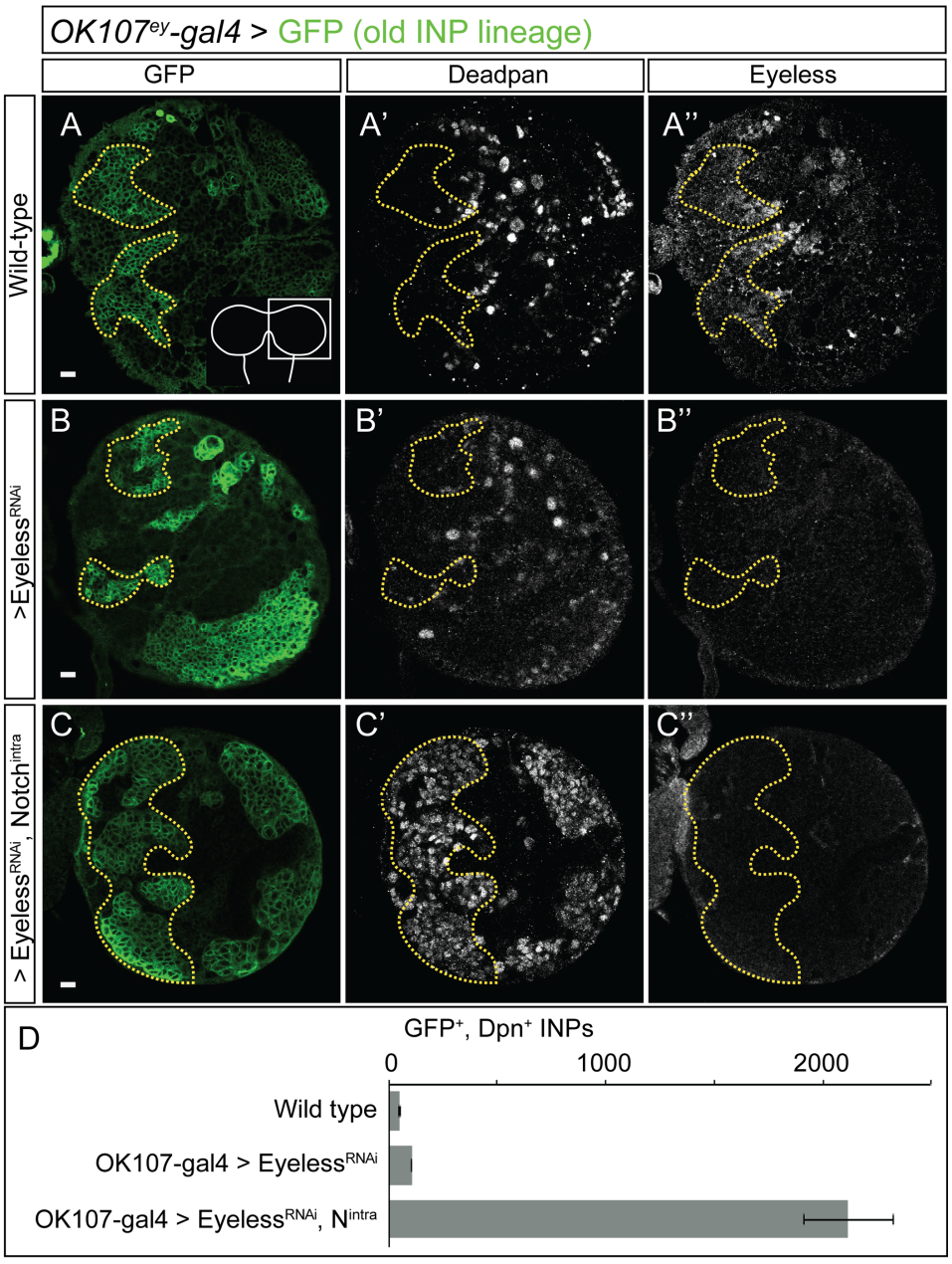
Eyeless restricts the competence of old INPs, or their progeny, to respond to Notch^{intra} signaling

We have shown that young and old INPs differ in their competence to respond to Notch signaling. What might be the cause of these differences? The recent identification of the transcription factor Eyeless expressed in old INPs provides a good candidate. We hypothesized that Eyeless may block Notch signaling in old INPs or their progeny.

We have previously shown that loss of Eyeless causes old INPs to delay the termination of their lineages by several additional divisions, but no ectopic neuroblasts or INPs are formed (Bayraktar & Doe, 2013b). To test whether loss of Eyeless increased the

competence of old INPs to respond to Notch signaling, we used our previously well-characterized *UAS-eyeless*^{RNAi} transgene (Bayraktar & Doe, 2013b) to eliminate all detectable Eyeless protein concurrent with expression of *UAS-Notch*^{intra} (*OK107-gal4*, *UAS-mCD8-GFP, UAS*-Notch^{intra} , *UAS-eyeless*^{RNAi}). Confirming previous findings (Bayraktar & Doe, 2013b), Eyeless RNAi removes all detectable Eyeless protein without generating any ectopic Dpn+ Ase- neuroblasts and very few Dpn+ Ase+ INPs (**Figure 2.2A,B** and data not shown; quantified in **Figure 2.2D**). In contrast, removing all detectable Eyeless together with expression of Notch^{intra} led to the formation of many ectopic Dpn+ neuroblasts or INPs (**Figure 2.2C**; quantified in **Figure 2.2D**). There are several possible explanations for the observed phenotype: (a) the ectopic Dpn+ cells could arise from the *OK107-gal4* expressing optic lobe or mushroom body that have migrated into medial brain regions where the type II lineages are located; (b) the ectopic Dpn+ cells could be due to Notch^{intra} in the optic lobe or mushroom body lineages, leading to indirect effects on the type II lineages; or (c) the ectopic Dpn+ cells could be due to the action of Notch^{intra} within the type II lineages.

Figure 2.2. Eyeless restricts the competence of old INPs to respond to Notch signaling . (Next page).
(A-C) Overexpression of Notch in Eyeless-negative old INPs generates ectopic Deadpan+ presumptive INPs. (A-A'') *OK107-gal4* driving membrane GFP labels old INPs that express Eyeless and Deadpan. (B-B'') *OK107-gal4 UAS-eyeless*^{RNAi} results in efficient knockdown of Ey in old INPs, but does not generate ectopic Deadpan+ NBs or INPs. (C-E) Constitutive Notch signaling in Eyeless-negative old INPs (*OK107-gal4, UAS-eyeless*^{RNAi}, *UAS-Notch*^{intra}) generates many ectopic Dpn+ (C) presumptive INPs expressing Grh (D-E) in the dorsomedial brain.
Images are a single, one micron plane through a whole brain lobe (A-D) or zoomed in to the dorsal-anterior central brain (E).
All panels show third instar larvae; scale bar = 10 μm.


OK107ey-gal4 > GFP (old INP lineage)
GFP
Deadpan
Eyeless
Wild-type
>EylessRNAi
> EylessRNAi, NotchIntra
A
A'
A''
B
B'
B''
C
C'
C''
D
GFP+, Dpn+ INPs
0
1000
2000
Wild type
OK107-gal4 > EylessRNAi
OK107-gal4 > EylessRNAi, NIntra

To distinguish between Notch^{intra} acting directly or indirectly on type II lineages, we used the *R16B06-gal4* line. *R16B06-gal4* contains an *eyeless* fragment driving *gal4* expression (Manning et al., 2012; Pfeiffer et al., 2008) and can be used to target Notch^{intra} expression specifically to old Eyeless+ INPs without additional larval brain expression in the optic lobe or mushroom body (**Figure S2**). Using *R16B06-gal4* to drive expression of GFP alone or Notch^{intra} alone did not produce any ectopic Dpn+ cells (**Figure 2.3A-B**; quantified in **Figure 2.3F**; summarized in **Figure 2.3G**). In contrast, using *R16B06-gal4* to express *UAS-GFP UAS-eyeless^{RNAi} UAS-Notch^{intra}* together in old INPs produced many ectopic Dpn+ cells (**Figure 2.3C-C')**; quantified in **Figure 2.3F**; summarized in **Figure 2.3G**), which we provisionally assign an INP identity because most cells have the Dpn+ Ase+ molecular profile of INPs (**Figure 2.3D-D''')**. This is in contrast to the ectopic Dpn+ Ase- Type II neuroblasts formed from young INPs dedifferentiating in response to Notch (**Figure 2.3E-E''').** We conclude that Eyeless restricts the competence of old INPs, or their progeny, to respond to Notch^{intra} signaling.

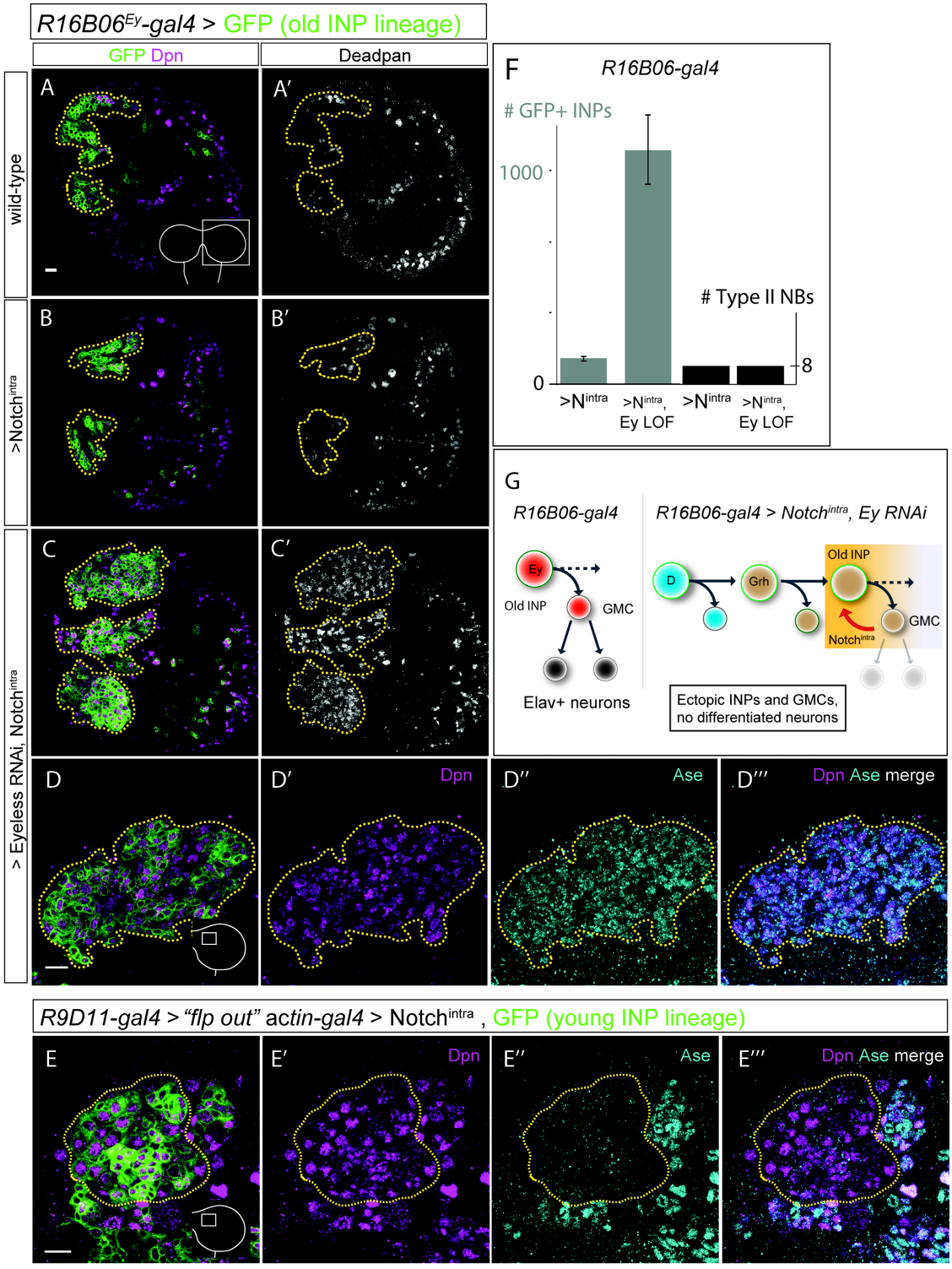

R16B06Ey-gal4 > GFP (old INP lineage)
GFP Dpn
Deadpan
wild-type
>Notchintra
> Eyeless RNAi, Notchintra
A
A′
B
B′
C
C′
D
D′
Dpn
D″
Ase
D‴
Dpn Ase merge
F
R16B06-gal4
GFP+ INPs
1000
0
Type II NBs
8
>Nintra
>Nintra, Ey LOF
>Nintra
>Nintra, Ey LOF
G
R16B06-gal4
R16B06-gal4 > Notchintra, Ey RNAi
Ey
Old INP
GMC
Elav+ neurons
D
Grh
Old INP
GMC
Notchintra
Ectopic INPs and GMCs, no differentiated neurons
R9D11-gal4 > "flp out" actin-gal4 > Notchintra , GFP (young INP lineage)
E
E′
Dpn
E″
Ase
E‴
Dpn Ase merge

Figure 2.3. Old INPs labeled by R16B06-gal4 also lose competence to respond to Notch (Previous page).
(A-A') Old INPs in the central brain are labeled by *R16B06-gal4* driving membrane-bound GFP.
(B-B') Old INPs labeled by *R16B06-gal4* do not produce ectopic Dpn+ cells in response to constitutive notch signaling (*R16B06-gal4, UAS-Notch*^{intra}).
(C-C') When Eyeless knockdown is coupled with constitutive Notch signaling in old INPs (*R16B06-gal4, UAS-eyeless*^{RNAi}, *UAS-Notch*^{intra}), many ectopic Dpn+ cells are produced.
(D-D''') The ectopic cells produced from constitutive Notch signaling coupled with Ey knockdown in old INPs labeled by *R16B06-gal4* have an INP-like identity (Dpn+ Ase+). (E-E''') Ectopic cells produced from constitutive Notch expression in young INPs are Dpn+ but do not express Ase, indicating a Type II NB-like identity. (F,G) Summary of results. Images are a single, one micron plane through a whole brain lobe (A-C) or zoomed in to the dorsal-anterior central brain (D-E). All panels show third instar larvae; scale bar = 10 μm.

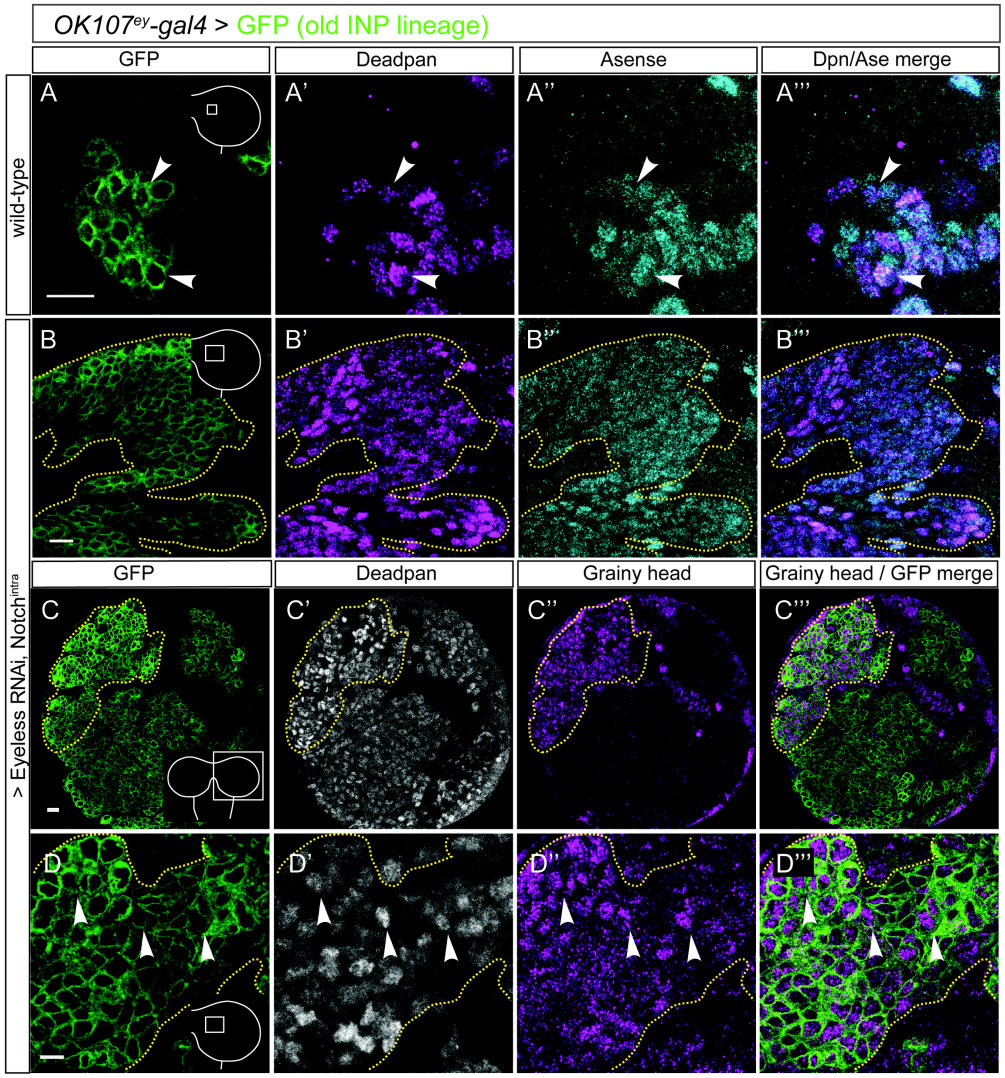


Figure 2.4. Notchintra in old INPs lacking Eyeless generates ectopic INPs and GMCs. (next page).

(A-B) Constitutive Notch signaling in Eyeless-negative old INPs (*OK107-gal4, UAS-eyeless*^{RNAi}, *UAS-Notch*^{intra}) generates many ectopic Dpn+ Grh+ cells. (C) presumptive INPs expressing Grh (D-E) in the dorsomedial brain.

Images are a single, one micron plane through a whole brain lobe (A-D) or zoomed in to the dorsal-anterior central brain (E).(C) Wild-type old INPs normally express Dpn and Asense (Ase).

(D) Overexpression of Notch in old INPs generates ectopic Dpn^{+} Ase^{+} INPs. Images are a single, one micron plane zoomed in to the dorsal-anterior central brain (D-F).

All panels show third instar larvae; scale bar = 10 μm.

Eyeless blocks Notch^{intra} from inducing GMC-to-INP dedifferentiation

Next, we wanted to verify the INP identity of the ectopic Dpn+ cells induced by Notch^{intra}, and determine their developmental origin. Using the old INP lines *R16B06-gal4* or *OK107-gal4* to concurrently eliminate Eyeless protein and induce Notch^{intra}, we find the vast majority of ectopic cells are Dpn+ Ase+ consistent with an INP identity (**Figure 2.4A,B**). In addition, most of the ectopic cells were also Grh+ (**Figure 2.4C,D**) consistent with the molecular profile of Eyeless-negative INPs (Bayraktar & Doe, 2013b). We conclude that the majority of the ectopic cells induced by Notch in old Eyeless-negative INP lineages have the molecular characteristics of INPs.

The large number of ectopic INPs could form by two mechanism: via symmetric cell divisions to expand the INP pool (i.e. one INP produces two INPs following mitosis), or via a normal asymmetric cell division to generate a self-renewed INP and a GMC that subsequently de-differentiates into an INP (similar to the role of Notch^{intra} in promoting young INP de-differentiation into a type II neuroblast). To distinguish these alternatives we assayed mitotic INPs to determine if they performed a symmetric or asymmetric cell division. Wild type INPs are phospho-histone H3 (PH3) positive during mitosis (**Figure 2.5A'''**), and divide asymmetrically

to localize the Miranda scaffolding protein and Prospero transcription factor cargo to the basal cortex (**Figure 2.5A-A''**) thereby partitioning Prospero into the GMC daughter cell, where it enters the nucleus at interphase. We find that the Notch-induced ectopic INPs also undergo asymmetric cell division, forming Miranda/Prospero crescents during mitosis (**Figure 2.5B-B''**), are PH3+ and localize Prospero to the nucleus during interphase. Furthermore, Pros+ GMCs can be identified throughout the proliferative mass

(Figure 2.5C). Interestingly, nuclear Prospero is insufficient to drive neuronal differentiation in this population (see next section). Thus, INPs undergo asymmetric division to generate INP and GMC daughter cells, although the GMC fate does not appear to be maintained. We propose that loss of Eyeless allows $Notch^{intra}$ to induce GMC > INP de-differentiation.

Next, we determined whether the GMCs in the $Eyeless^{RNAi}$ $Notch^{intra}$ expressing population always de-differentiate or whether they can sometimes produce differentiated neurons. In wild type, the pan-neuronal Elav protein is detected in all neurons but not in neuroblasts or INPs (B. C. Bello et al., 2008; Boone & Doe, 2008; Bowman et al., 2008b; Robinow & White, 1988), and as expected we observe Elav+ neurons within *R16B06-gal4,"flp-out," UAS-GFP* permanently marked old INP lineages (**Figure 2.6A,B**; quantified in **Figure 2.6E**). In contrast, the $Eyeless^{RNAi}$ $Notch^{intra}$ population contained few or no Elav+ neurons (**Figure 2.6C,D**; quantified in **Figure 2.6E**). In addition, this population never expressed markers for differentiated neurons derived from old INPs like Twin of Eyeless (Toy) or from young INPs like Brain-specific homeobox (Bsh) (data not shown). We conclude that loss of Eyeless allows $Notch^{intra}$ to induce GMC > INP de-differentiation which maintains INP proliferation and nearly completely blocks neuronal differentiation (summarized in **Figure 2.6F**). This highlights the loss of competence that INPs undergo as they age, and identifies a novel function for the conserved Eyeless/Pax6 transcription factor: to block Notch signaling.

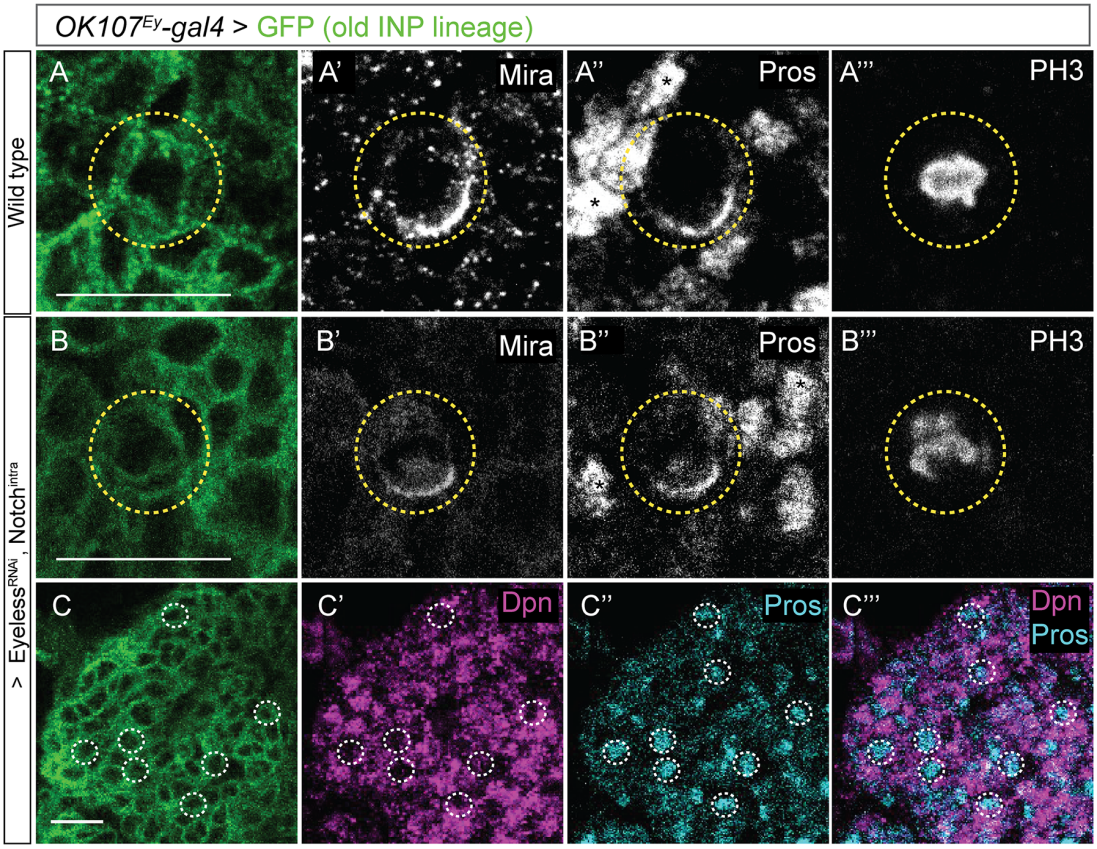


Figure 2.5. Asymmetrical cell division is maintained in ectopic INP-like cells. (A-A''') Wild-type INPs expressing *OK107-gal4 UAS-GFP* are GFP+ (A) and divide asymmetrically with basally localized crescents of Miranda (Mira; A') and Prospero (Pros; A'') (white arrow marks basal crescent). The GFP+ cells marked by yellow dashed lines are in interphase (Pros+, PH3-). (B-B''') Ectopic INP-like cells also asymmetrically localize Pros and Mira and have PH3+ chromosomes. (C-C''') Pros+, Dpn- GMC-like cells are found in the proliferating mass generated from constitutive Notch signaling in old INPs where Eyeless is knocked down. All panels show third instar larvae; scale bar = 10 μm.

Eyeless blocks Notch^{intra} from inducing direct target gene expression

Old INP lineages are non-responsive to the potent Notch^{intra} mitogenic signal, at least in part due to the presence of the Eyeless/Pax6 transcription factor. Where in the Notch signaling pathway does Eyeless act? We can conclude it acts after ligand binding and proteolytic cleavage of Notch, because these steps are bypassed by overexpression of Notch^{intra}; furthermore, we've shown that nuclear import of Notch^{intra} is normal (**Figure S1**). Furthermore, gene expression driven by a synthetic Notch response element (Housden et al., 2014) was observed when Notch^{intra} was expressed in old INPs, indicating that the Notch^{intra} protein is functional (**Figure S3)**. Does Eyeless block expression of Notch direct target genes in GMCs? There are four proposed direct Notch target genes in the larval CNS: *E(spl)mγ, dpn*, *hey*, and *Myc* (Almeida & Bray, 2005; Housden et al., 2014; Monastirioti et al., 2010; San-Juan & Baonza, 2011; Song & Lu, 2011). Here we focus on Dpn and E(spl)mγ because their expression has been detected in INPs, and Myc because it is detected in neuroblasts (Song & Lu, 2011). In contrast, Hey is detected only in a subset of post-mitotic neurons (Monastirioti et al., 2010) and is not likely to be relevant to the GMC > INP dedifferentiation step.

In wild type, Eyeless+ old INPs normally express the Notch target genes *dpn*, *E(spl)mγ*, and the *NRE-GFP* Notch reporter gene whereas these genes are not expressed in GMC progeny (**Figure 2.7A-A'''**, see also **Figure S3)**. Similarly, forced expression of Notch^{intra} in old INPs results in Notch target gene expression in INPs but not GMCs (**Figure 2.7B-B'''**, see also **Figure S3B,B'**; data not shown). In contrast, forced expression of Notch^{intra} in old INPs that lack Eyeless (Eyeless^{RNAi} Notch^{intra}) results in Dpn expression in both INPs as well as some GMCs (**Figure 2.7C-C''', quantified in**

2.7D). We conclude that Eyeless functions in GMCs to prevent Notch^{intra} from activating target gene expression.

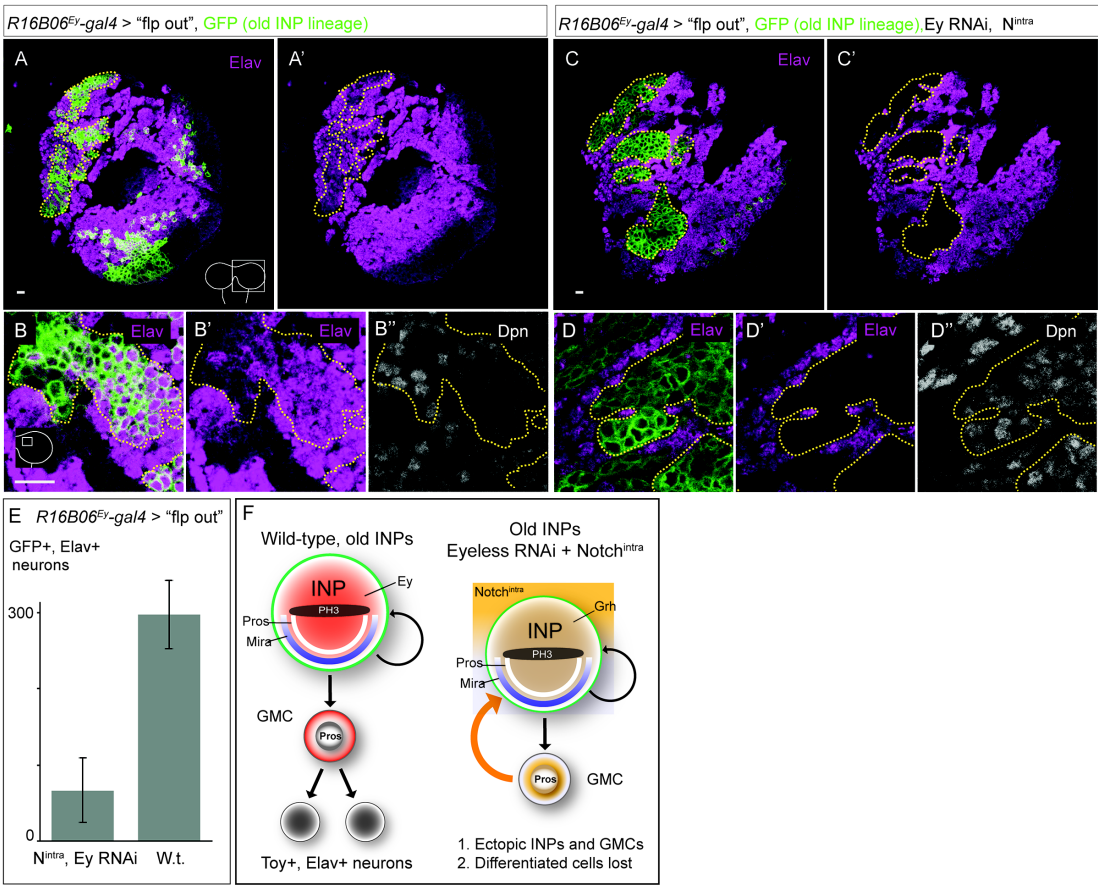


Figure 2.6. Notch signaling induces GMC to INP de-differentiation within old INP lineages in the absence of Eyeless.
(A-B) Old INPs lineages are permanently labeled by *R16B06-gal4 "flp-out"* driving membrane GFP. (A-A') Wild-type, old INP lineages labeled with GFP produce differentiated neurons marked by Elav. (B-B') High-magnification images show Dpn+ INPs and Elav+ neurons in these GFP+ lineages.
(C-C') Eyeless knockdown and constitutive Notch signaling in old INPs produces ectopic cells at the expense of Elav+ differentiated cells. (D-D') High magnification images show striking loss of Elav+ cells in GFP+, old INP lineages, while many ectopic cells express Dpn+.
(E) Quantification of Elav+ neurons in GFP+ old INP lineages.
(F) Model of asymmetric cell division in wild-type and ectopic INP-like cell phenotype for old INPs responding to Notch in the absence of Eyeless.
All panels show third instar larvae; scale bar = 10 μm.

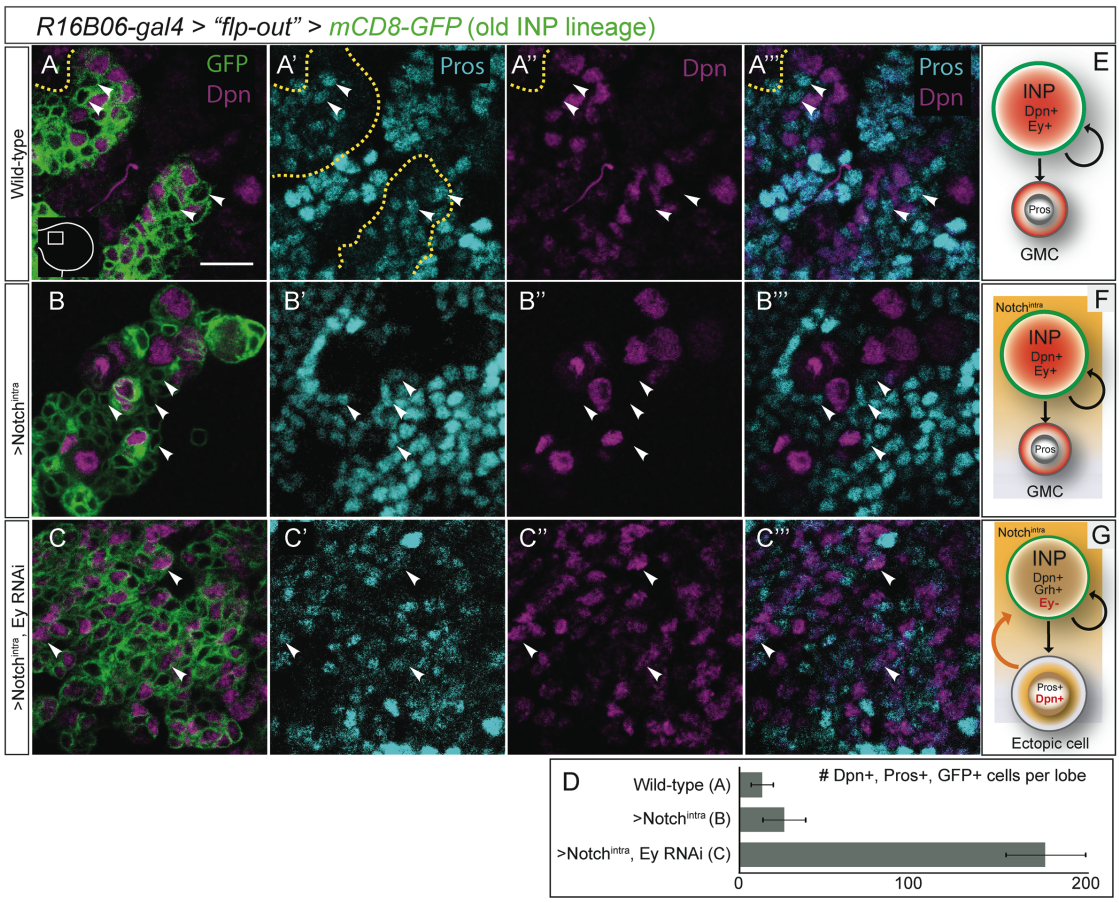


Figure 2.7. Derepression of Deadpan in old INP progeny is induced by loss of Eyeless and constitutive Notch signaling.
(A-A''') Wild-type, old INPs give berth to GMC progeny that express Pros but not Dpn. (B-B''') Constitutive Notch signaling in old INPs and their progeny (UAS-N^{intra}) does not induce expression of Dpn. (C-C''') Loss of Ey function and constitutive Notch signaling in old INPs and their progeny produce many ectopic GMC-like cells which express Pros and have derepressed Dpn. (D-F) Schematic of results. (G) Quantification of cells with nuclear Pros and Dpn per brain lobe. (A-B) White arrows show Pros+, Dpn- GMCs. (C) Arrows show ectopic Pros+, Dpn+ double positive cells. All panels show third instar larvae; scale bar = 10 μm.

DISCUSSION

Here we report three new findings. First, we show that young INPs undergo an INP > neuroblast dedifferentiation in response elevated Notch signaling, whereas old INPs are completely resistant to elevated Notch signaling; thus, old INPs lose competence to generate tumors in response to Notch signaling. Second, we show Notch signaling can induce GMC > INP dedifferentiation in the absence of the late INP temporal transcription factor Eyeless/Pax6. Third, we show that Eyeless/Pax6 blocks Notch signaling by preventing transcriptional activation of several direct target genes.

Why do old INP lineages lack competence to respond to potent $Notch^{intra}$ signaling? A simple model is old INPs may undergo chromatin remodeling to silence Notch target genes. The SWI/SNF chromatin remodeling complex helps commit INPs to a limited proliferative potential and prevent their dedifferentiation into neuroblasts (Eroglu et al., 2014; Koe et al., 2014). These factors are expressed throughout the lifespan of INPs, and may directly silence Notch target genes.

We have shown that $Notch^{intra}$ can promote GMCs > INP dedifferentiation, but that this effect of $Notch^{intra}$ can be completely blocked by the conserved Eyeless/Pax6 transcription factor. How does Eyeless block Notch signaling? One model is that Eyeless recruits the SWI/SNF complex to block activation of the Notch target genes *Dpn* and *E(spl)mγ* - which are normally expressed in INPs but not GMCs (Housden et al., 2014; San-Juan & Baonza, 2011) - preventing them from becoming transcriptionally activated by Notch signaling. Supporting this notion, the Eyeless-related Pax6 protein binds the SWI/SNF-related BAF complex to regulate the expression of neurogenic transcription factors in murine adult neural progenitors (Ninkovic et al., 2013). In addition, a switch in

BAF subunits has been shown to direct the transition from proliferation to differentiation in mammalian neural progenitors (Lessard et al., 2007), raising the possibility that both Drosophila and mammals use similar pathways to regulate progenitor choice of differentiation or proliferation.

Our finding that Eyeless can block the activity of constitutively active Notch^{intra} signaling raises several questions. First, why does Eyeless block expression of the Notch target genes *dpn* and *E(spl)mγ* in GMCs but not INPs? An attractive model is that there is a co-factor present in GMCs but not INPs (such as Prospero) that acts with Eyeless to block Notch target gene expression. Consistent with this model is the observation that reducing Prospero from GMCs results in dedifferentiation into neuroblasts that express the Notch target genes *dpn*, *E(spl)mγ*, and *Myc* (Bayraktar, Boone, Drummond, & Doe, 2010a; Bowman et al., 2008b; Choksi et al., 2006; Lai & Doe, 2014). Second, can misexpression of Notch target genes bypass the tumor suppressor function of Eyeless? We misexpressed the Notch target genes *dpn*, *E(spl)mγ*, and *Myc* in old INPs, but we detected no ectopic INPs (data not shown); perhaps two or more target genes, or a currently unknown Notch target gene, are required to induce a GMC>INP dedifferentiation. Third, why doesn't loss of Eyeless alone trigger GMC dedifferentiation? One possibility is that endogenous Notch signaling is too low to induce dedifferentiation either due to absence of a Notch pathway component or lack of access to ligand. Fourth, can misexpression of Eyeless block Notch^{intra}-induced young INP > neuroblast dedifferentiation? We attempted to answer this question by misexpressing Notch^{intra} and Eyeless together in young INPs (*R9D11-gal4 UAS-GFP UAS-Notch^{intra} UAS-Eyeless*). Surprisingly, the young INPs had no detectable Eyeless protein (**Figure**

S4), although they had high GFP levels and despite *UAS-GFP* and *UAS-Eyeless* being coexpressed, due to an unknown mechanism blocking Eyeless translation in young INPs. Consequently, the expected "neuroblast tumor" phenotype was observed and we could not determine the role of Ey in blocking young INP tumors. The mechanism preventing Eyeless protein expression is an interesting area for future investigation, particularly to determine if a similar mechanism is used to regulate its mammalian ortholog, Pax6.

Notch signaling is well conserved and has been shown to initiate diverse cell fate outcomes in a context dependent fashion. For example, constitutively active Notch signaling in hematopoietic stem cells (HSCs) in mouse bone marrow is sufficient to generate extra-thymic T cells (Allman et al., 2001), but the competence to respond to Notch in these cells requires functional pre-T cell receptor (TCR) signaling. Furthermore, restoration of competence to respond to Notch in TCR mutant HSCs with a TCR transgene and active Notch1 signaling potentiates these tissues to form T cell leukemia (Allman et al., 2001). In addition, the transcription factor Ikaros has been shown to control the availability of Notch targets genes during T cell differentiation and loss of Ikaros generates T cell leukemias in mice (Geimer Le Lay et al., 2014). The tumor suppressor function of Ikaros in controlling the response to Notch signaling in T cells is strikingly similar to the function of Eyeless we report here. Similar to Type II neuroblasts, T cell precursors rely on endogenous levels of Notch signaling to properly specify progeny, but are also sensitive to Notch as a mitogen, and must maintain homeostatic proliferation through the careful regulation of Notch signaling (Geimer Le Lay et al., 2014). In the case of pre-T cells, it appears that competence to respond to Notch is established by TCR expression, and final T cell differentiation requires Notch

signaling provided in the thymus, spatially controlling T cell development. Thus, in Drosophila as well as mammalian tissues, Notch signaling must be precisely regulated to ensure normal development. In addition, it is clear that cells also regulate their competence to respond to Notch, enabling multiple, context-dependent outcomes from a single extrinsic cue.

Eyeless and its mammalian ortholog Pax6 were initially defined as master regulators of eye development, and have since been shown to play essential roles in other cell types (Georgala, Carr, & Price, 2011). Eyeless was recently identified as a temporal identity factor in INPs, and is essential for proper development of the *Drosophila* adult central complex (Bayraktar & Doe, 2013b). Pax6 expression is a reliable marker of mammalian cortical progenitors and is under both spatial and temporal control. Both Pax6/Eyeless transcription factors and Notch signaling are well conserved between *Drosophila* and mammals. Understanding how these factors interact to regulate progenitor competence may provide insight into mammalian neural development and tissue repair following injury or disease.

METHODS

Fly genetics. Mutant larvae were generated in vial collections incubated at 28-30°C using 3-5 day old females. Larvae were collected at third instar for dissection based on a combination of timing and morphology.

Immunohistochemistry. Larval brains were fixed in 4% paraformaldehyde in PBST (phosphate-buffered saline plus 0.3% Triton-X100; Sigma Aldrich) for 25 min at room temperature. Normal goat and donkey serum (5%) in PBST was used as a pre-staining blocking solution and staining buffer. Primary antibody staining was performed overnight at 4°C. The following primary antibodies were used: chicken antibody to GFP (1:2000; Aves Laboratories, Tigard, OR), rat antibody to Dpn (1:50; Doe lab), rabbit antibody to Ase (1:2000; C.-Y. Lee lab, Univ. Michigan), guinea pig antibody to D (1:500; J. Nambu), rabbit antibody to Ey (1:3500; U. Walldorf), guinea pig antibody to Mira (1:1000; Doe lab), mouse antibody to Pros (1:1000; Doe lab), guinea pig antibody to Toy (1:500; U. Walldorf) and mouse antibody to $Notch^{intra}$ (1:50; Developmental Studies Hybridoma Bank, Iowa City, Iowa). Secondary antibody staining was performed at room temperature for two hours (1:500; Molecular Probes, Eugene, OR, or Jackson Immunoresearch, West Grove, PA). After staining, brains were kept at 4°C in Vectashield (Vector Laboratories, Burlingame, CA) prior to imaging.

Imaging and analysis. Images were obtained using a Zeiss LSM710 confocal microscope. Image processing and analysis was performed in FIJI (Schindelin et al., 2012).

Author Contributions

DRF did all experiments and co-wrote the manuscript; OAB participated in the characterization of R16B06-gal4 and the design of the study; CQD guided the project and co-wrote the manuscript.

BRIDGE

In this chapter, we demonstrate that aging neural progenitors lose competence to respond to Notch signaling, and that old INP progeny require Eyeless prevent constitutive Notch signaling from inducing de-differentiation and tumor formation. In the next chapter, I examine how the transcriptome of INPs changes over the course of their lifespan.

CHAPTER III

TRANSCRIPTOMIC CHANGES IN AGING INPS

INTRODUCTION

The striking loss of competence we observed in aging INPs to respond to Notch signaling prompted several additional questions. 1. Which Notch target genes are activated in young INPs that enable their dedifferentiation into Type II neuroblasts? 2. Are Notch target genes silenced in old INPs, and if so, through what mechanism? 3. How does Eyeless prevent old INP progeny from dedifferentiating into INP-like cells that form hyperplastic masses? To address these questions, I developed a platform to profile young and old INPs for genes that are being actively transcribed. My goal was to compare gene expression in aging INPs so that I might capture global changes in the transcriptome of INPs over time. Within these data, I looked for genes that are transcribed in young INP, but silenced in old INPs (and vise versa). In addition, I looked for known targets of Notch signaling that show temporal changes in expression over the course of INPs' lifespan.

I turned to DNA adenine methyltransferase identification (DamID) based techniques to profile changes in gene expression in aging INPs. These methods rely on coupling a DNA adenine methyltransferase (Dam) enzyme to a DNA binding protein of interest to determine where in the genome the protein of interest interacts (van Steensel, Delrow, & Henikoff, 2001; van Steensel & Henikoff, 2000). When a DNA-binding protein:Dam fusion molecule binds DNA, the Dam enzyme will methylate adenosine nucleotides in the vicinity of the interaction, leaving a footprint that can be purified and sequenced. The purification of methylated regions of the genome bound by the protein of

interest is obtained by digesting purified genomic DNA with the restriction enzyme DpnI, which will only cut at GATC tetranucleotide sequences where adenosine has been methylated. This digested DNA can be ligated to adapters for PCR and sequencing that will return a library of reads representative of genomic regions where the protein of interest was bound (Marshall, Southall, Cheetham, & Brand, 2016).

Since our aim was to profile genes that are actively, and differentially, transcribed as INPs progress through their lifespan, I utilized a DamID platform where RNA polymerase II (RNA Pol II) is fused to Dam. This method, named Targeted DamID (TaDa) was recently developed by Andrea Brand's laboratory (Marshall et al., 2016), and enables the expression of RNA Pol II:Dam fusion proteins in a cell and developmental stage specific manner using the Gal4-UAS system. When the RNA Pol II:Dam fusion protein is expressed in target cells, it methylates regions of DNA where it interacts, leaving a trace of actively transcribed genes. In addition, paused polymerases at the promoter of genes that are not actively transcribed will also leave a methylation signature that can be recovered during sequencing. To control for non-specific methylation of DNA, a parallel control experiment was conducted for each Gal4 driver in which UAS-Dam (no DNA binding protein is fused) is expressed. These samples are treated identically and fully sequenced to provide a critical baseline for non-specific Dam methylation of the genome and the calculation of a false discovery rate. Thus, the data generated by TaDa are similar to those produced in an RNA-seq experiment, but have the advantage of working with genomic DNA, which is less prone to degradation than RNA, and does not require isolation of the targeted cells, but achieves cell and developmental stage specific profiling using genetic reagents.

I chose two Gal4 drivers that express specifically in young (R9D11-gal4) and old (R16B06-gal4) INPs (see Chapter II) to control expression of UAS-RNA Pol II:Dam. In addition, I added a temperature sensitive, tubulin-Gal80 transgene to enable additional temporal control of RNA Pol II:Dam expression to a 24h pulse at the end of larval life. This enabled us to obtain information about RNA Pol II occupancy and infer actively transcribed genes in young versus old INPs. The expression of these two Gal4 lines has been previously characterized (Figure S2), but it is worth noting here that based on UAS-GFP expression in L3 brains, it is likely that the GMC and neuronal progeny of INPs will also be profiled in our TaDa experiments (Figure 3.1). In addition, there is potential overlap in the timing of R9D11-gal4 expression termination and the initiation of R16B06-gal4 expression, so I could recover some old INP specific genes also detected as actively transcribed in our R9D11-gal4 data set. The reciprocal outcome (young INP genes in old INP TaDa profile), however, should not be possible.

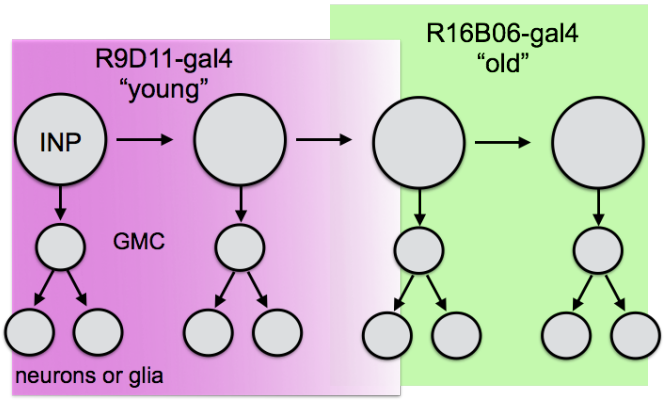


Figure 3.1 Expression of R9D11-gal4 and R16B06-gal4 in young and old INPs and their progeny. There is the potential for partial overlap in the timing of their expression as well as labeling GMC and neuronal progeny.

RESULTS

Targeted DamID accurately profiles gene expression in young INPs

To validate the effectiveness of TaDa in profiling gene expression in aging INPs, I first looked for the specific expression of genes that have been previously characterized with antibody staining as temporally restricted to young INPs and not expressed in old INPs. To do this, I looked for actively transcribed genes in the R9D11-gal4 derived sequences that were absent in the R16B06-gal4 derived sequences. I identified 744 such genes. Strikingly, essentially every known gene that had previously been shown to have specific expression in young INPs relative to old INPs was accurately captured in our TaDa data set (Table 3.1). Importantly, none of these genes were determined to be expressed in old INPs using R16B06-gal4 driven TaDa. Thus, this method appears to accurately capture temporally expressed genes in young INPs.

Gene name	Previously characterized expression in young INPs
Dichaete	Bayraktar and Doe (2013) Nature
Earmuff	Weng et al. (2010) Dev. Cell
Klumpfuss	Xiao et al. (2012) Development
Numb	Bowman et al. (2008) Dev. Cell
Hamlet	Eroglu et al. (2014) Cell
Buttonhead	Xie et al. (2014) eLife

Table 3.1. TaDa recovers young INP-specific gene expression.
A selection of genes identified as actively transcribed using R9D11-gal4 driven TaDa that are absent from the profile obtained using R16B06-gal4. These genes have been previously validated using antibody staining for expression in young INPs.

R9D11-gal4 driven TaDa identifies two previously uncharacterized Notch target genes, Hey and E(spl)m-alpha, specifically expressed in young INPs.

The previously described (see Chapter II) loss of competence to respond to Notch signaling I observed in old INPs prompted us to look for targets of Notch signaling that were differentially expressed in young and old INPs. Perhaps endogenous Notch signaling in INP lineages would reveal expression of a Notch target in young INPs that is silenced in old INPs. Strikingly, a Hes family gene, *Hey*, which has previously been described as a target of Notch (Monastirioti et al., 2010), was found to be actively transcribed only in our R9D11-gal4 TaDa sample, and was absent from the R16B06-gal TaDa results. In addition, a member of the enhancer of split complex, *E(spl)m-alpha* (Jennings, Preiss, Delidakis, & Bray, 1994*)*, was also unique to the R9D11-gal4 drive TaDa sample (Table 3.2). Thus, two bona fide targets of Notch signaling appear to be differentially expressed between young and old INPs; the first such examples currently identified (see discussion below).

Multiple Notch target genes are identified in both R9D11-gal4 and R16B06-gal driven TaDa samples.

I also asked whether known targets of Notch signaling were expressed in both young and old INPs. To this end, I compared the expression of all genes in the R9D11-gal4 and R16B06-gal4 driven samples and found 1758 genes that were actively transcribed in both – suggesting that they are expressed throughout INP lifespan (Table 3.3). These included Notch target genes previously known to be expressed throughout INP lifespan, including the Hes family member Deadpan, the bHLH transcription factor,

Myc, and E(spl)m-gamma (Herranz et al., 2014; Jennings et al., 1994; Palomero et al., 2006; San-Juan & Baonza, 2011). In addition, several members of the enhancer of split locus for whom expression in INPs had not been previously characterized were also found in both of our TaDa samples. These included, E(spl)m7, E(spl)m8 and E(spl)m-beta (Table 3.3). Thus, I confirm here that multiple Notch target genes, some known and others novel, are expressed in both young and old INPs.

Old INP-specific transcription factors, Grainyhead and Eyeless, were found not to be actively transcribed using R16B06-gal driven TaDa.

I identified 597 genes that were actively transcribed in the R16B06-gal4 sample and were absent from R9D11-gal4 driven TaDa. To validate the specificity and accuracy of the R16B06-gal4 driven TaDa experiment, I asked whether two previously identified transcription factors, Eyeless and Grainyhead, were detected using this method. Surprisingly, these genes were determined not to be actively transcribed in old INPs using TaDa. Similarly, they were also absent from the R9D11-gal4 driven TaDa sample. There are two possible explanations of this result: 1. R16B06-gal4 driven TaDa failed to detect endogenous transcription of *Eyeless* and *Grainyhead* in old INPs (i.e. false negative). 2. *Eyeless* and *Grainyhead* are not transcribed in old INPs and the Eyeless and Grainyhead protein detected in old INPs using antibody staining is translated from mRNA that has been transcribed earlier in the development of these lineages (i.e. in the maternal Type II neuroblast). With existing data I can not rule out either of these possibilities at this time (see discussion below).

Distinct translation initiation factors are detected using R16B06-gal4 TaDa

To address the molecular mechanisms through which aging INPs lose competence to respond to Notch signaling, I asked if old INPs change the post-transcriptional regulation of mRNAs. One way this occurs is through the regulation of translation. To determine if aging INPs might differentially regulate translation of mRNAs, I looked for eukaryotic Initiation Factors (eIFs) that were uniquely present in the R16B06-gal4 driven TaDa sequences. Strikingly, I identified eIF2D, eIF3-S9 and eIF3-gamma as actively transcribed in the R16B06-gal4 driven TaDa sample and absent from the sequences obtained using R9D11-gal4 (Table 3.2). By comparison, five eIFs were detected as actively transcribed in both samples: eIF2B-gamma, eIF3-S8, eIF-4a, eIF5 and eIF-5A (Table 3.3). Thus, old INPs specifically express three regulators of translation, not present in young INPs, that could influence post-transcriptional gene expression in old INPs.

R9D11 only			
Gene name	polii	gatc.num	FDR
Earmuff	0.91	54	1.64E-18
Klumpfuss	0.38	100	5.29E-11
Dichaete	1.46	12	1.07E-08
Numb	0.39	68	4.29E-08
Hamlet	0.27	108	7.52E-07
Buttonhead	1.21	15	1.58E-08
Hey	0.60	10	2.00E-03
E(spl)malpha-BFM	0.61	6	9.54E-03

16B06 only			
Gene name	polii	gatc.num	FDR
eIF2D	0.50	10	6.15E-04
eIF3-S9	0.34	17	7.62E-04
eIF3ga	0.44	9	2.25E-03

Table 3.2 Selected genes with differential transcription in young and old INPs.
Grey box contains genes unique to R9D11-gal4 (young) driven TaDa sample; white background shows those unique to R16B06-gal4 (old) driven sample. Polii is a metric of expression level, gatc.num indicates the number of DpnI cut sites mapped by sequences, FDR = false discovery rate.

	9D11			16B06		
Gene name	polii	gatc.num	FDR	polii	gatc.num	FDR
Deadpan	1.14	18	2.58E-09	0.72	18	4.48E-08
Asense	2.32	12	1.47E-13	0.53	12	1.39E-04
E(spl)m7-HLH	2.24	9	1.52E-10	1.22	9	5.78E-08
E(spl)m8-HLH	2.16	3	2.61E-05	0.94	3	1.64E-03
E(spl)mbeta-HLH	2.24	7	7.14E-09	1.11	7	3.62E-06
E(spl)mgamma-HLH	3.36	4	4.83E-09	1.12	4	1.47E-04
eIF2B-gamma	0.62	11	9.44E-04	0.37	11	2.96E-03
eIF3-S8	0.53	25	2.18E-05	0.53	25	1.11E-07
eIF-4a	1.27	19	8.06E-11	0.80	19	2.40E-09
eIF5	0.84	11	6.66E-05	0.99	11	1.48E-07
eIF-5A	0.76	4	9.81E-03	0.60	4	6.71E-03

Table 3.3 Selected genes transcribed in both young and old INPs.
Grey box contains parameters from R9D11-gal4 (young) driven TaDa sample; white background shows those from R16B06-gal4 (old) driven sample. Polii is a metric of expression level, gatc.num indicates the number of DpnI cut sites mapped by sequences, FDR = false discovery rate.

DISCUSSION

TaDa yields high quality transcriptomes of INPs at different ages in their lineage.

In this study, I set out to determine which genes are differentially transcribed between young and old INPs. The TaDa method I employed relies upon RNA Pol II interactions with DNA during transcription to present a genome wide analysis of actively transcribed genes with cell and developmental stage specificity. I chose two Gal4 drivers that specifically express in young (R9D11-gal4) and old (R16B06-gal4) to enable a direct comparison of their transcriptomes. Genes whose expression was previously described as specific to young INPs using antibody staining correlated nicely with the young INP-specific transcriptome I obtained using TaDa. This validates the method and indicates that I profiled the correct cells at the anticipated age. However, it is likely that I am also capturing the transcriptome of the GMC and neuronal progeny of INPs too, due to the duration of gal4 expression and the potential for perdurance of RNA Pol II-Dam protein into daughter cells. In addition, each Gal4 driver has off target expression in the optic lobe, which will contaminate the INP transcripome with "false positives," where genes not expressed in INPs at all will appear in the TaDa sequences. Nevertheless, the verification of essentially every known young INP-specific gene is very encouraging that this method can be used to identify transcripts *de novo* that could underlie the loss of competence to respond to Notch in aging INPs.

Thus far, our analysis has identified two targets of Notch signaling whose expression appears confined to young INPs. Could these genes be responsible for young INP's forming large tumors in response to constitutive Notch signaling? Does their

repression cause the loss of competence to respond to Notch we observed in old INPs? Further genetic analysis and antibody staining will be required to determine if the targets of Notch signaling identified here indeed play a role in enabling young INP competence to Notch signaling. It would also be interesting to probe for epigenetic changes at the *Hey* and *enhancer of split* loci to see if chromatin remodeling is responsible for silencing these genes in old INPs.

Are Eyeless and Grainyhead regulated post-transcriptionally in INP lineages?

As I mentioned in the results section above, the absence of *Eyeless and Grainyhead* transcripts in the R16B06-gal driven sample was surprising and has two possible explanations. 1. R16B06-gal4 driven TaDa failed to detect endogenous transcription of *Eyeless* and *Grainyhead* in old INPs (i.e. false negative). 2. *Eyeless* and *Grainyhead* are not transcribed in old INPs and the Eyeless and Grainyhead protein detected in old INPs using antibody staining is translated from mRNA that has been transcribed earlier in development of these lineages (i.e. in the maternal Type II neuroblast). Currently, I cannot rule out the first possibility, though many genes known to be expressed in both young and old INPs were efficiently detected using R16B06-gal driven TaDa, including, *Deadpan, Ase and e(spl)m-gamma.* These observations strongly argue against a catastrophic, technical issue with this sample. How then might the transcripts of *Grainyhead* and *Eyeless* have been missed, when I know protein from these genes is present in old INPs?

The second explanation, that the timing of Eyeless and Grainyhead protein expression is post-transcriptionally regulated, is supported by the observation that

expression of a UAS-Eyeless transgene in young INPs failed to produce Eyeless protein (Appendix I, Figure S4). This negative result suggest that young INPs actively prevent *Eyeless* transcripts from being translated into protein. If this were true, I can hypothesize that *Eyeless* is transcribed in Type II NBs and inherited by their daughter cells, but actively prevented from being translated until late in INPs' lifespan. This would be an exciting molecular-genetic mechanism to explore in INP lineages. Furthermore, our identification of distinct classes of eIF proteins expressed in young and old INPs provides and interesting starting point to investigating translational control of temporal identity factors in neural progenitor lineages.

METHODS

Fly genetics and larval brain dissection

I performed the following crosses. *UAS-RNA Pol II-Dam (II)* and *UAS-Dam (II)* flies were the generous gift of Andrea Brand.

1. *ts-tubulin-gal80; R9D11-gal4* X *UAS-RNA Pol II-Dam*
2. *ts-tubulin-gal80; R16B06-gal4* X *UAS-RNA Pol II-Dam*
3. *ts-tubulin-gal80; R9D11-gal4* X *UAS-Dam*
4. *ts-tubulin-gal80; R16B06-gal4* X *UAS-Dam*

Larvae were reared at 18°C until L2, then shifted to 28°C for 24h to repress gal80 expression and enable RNA-pol II-Dam or Dam, expression. At L3 and after 24h in permissive temperature conditions, whole larval CNS was removed and pooled until ~100 brains of each genotype were acquired. Brains were stored at -20°C in PBS until subsequent DNA extraction was performed.

DNA extraction and library preparation

The isolation and genomic DNA and subsequent library preparation was performed according to a protocol generously provided by Tony Southall (Marshall et al., 2016).

Sequencing and bioinformatic analysis

Sequencing was performed on an Illumina NextSeq 500. We obtained single-end, 84 nt reads. To analyze sequencing reads for gene expression, the DamID-seq pipeline (Marshall & Brand, 2015) was utilized. Execution of the pipeline was conducted with standard parameters and a false discovery rate (FDR) of less than 0.01 was required to consider a gene actively transcribed.

CHAPTER IV

CONCLUDING SUMMARY

During development of the central nervous system, an initially small and similar pool of neural progenitor cells divide in response to intrinsic and extrinsic molecular cues to generate a tremendous number of functionally diverse neurons and glia. Spatial cues generate progenitor diversity, whereas subsequent temporal cues allow single progenitors to produce a series of distinct neuronal and glial cell types. Recently, it has become clear that progenitors change competence to respond to spatial and temporal cues, potentially allowing a single cue to generate distinct outputs.

Although there has been excellent progress on identifying spatial and temporal patterning cues, much less is known about how progenitors change competence. Do progenitors pass through discrete competence windows where distinct cell types are born in response to the same cue? What are the mechanisms that restrict competence? Are there many mechanisms, or might there be a small number of highly conserved mechanisms?

Notch signaling is a deeply conserved cell-extrinsic cue that is required for the formation of *Drosophila* intermediate neural progenitors (INPs) – a transit-amplifying lineage of neural progenitors that are crucial for larval neurogenesis and the subsequent development of the adult brain. My research has focused on: 1) Probing for changes in competence to respond to Notch signaling as INPs age, and 2) Understanding the molecular mechanisms that underlie changes in progenitor competence to respond to Notch signaling.

I have shown that old INPs lose competence to respond to Notch signaling. Furthermore, old INPs are known to express the Pax family transcription factor Eyeless, and upon removing Eyeless from old INPs with RNAi, their ganglion mother cell (GMC) progeny form large, hyperplastic tumors in the developing brain. We have demonstrated that this ectopic GMC/INP proliferation is likely due to derepession of the Notch target gene, Dpn. These results establish a new interaction between two conserved developmental cues: Eyeless/Pax6 can block Notch signaling in neural progenitor lineages.

I have also investigated the changes in gene expression that might underlie the loss of competence to respond to Notch signaling we have observed in aging INP lineages. I was fortunate that Eyeless had been identified as temporally expressed in old INPs, and plays a role in blocking Notch signaling in these lineages, but our understanding of this striking loss of competence requires a more complete picture of the genomic changes associated with this transition. To this end, I conducted "Targeted-DamID (TaDa)" experiments to profile and compare the occupancy of RNA Polymerase II in young and old INPs.

TaDa enables expression of a DNA methyl-transferase – RNA Pol II fusion protein, under UAS control, to profile Pol II occupancy in a developmental stage and cell-specific manner. Following massively parallel sequencing of isolated genomic DNA, I compared the Pol II occupancy of young and old INPs. This identified genes correlated with the loss of competence to respond to Notch signaling we observed, as well as other temporally controlled changes in gene expression. Future work should use traditionally

genetic approaches to validate the causative function of these genes in controlling INP competence to respond to Notch.

Collectively, these experiments advance our understanding of the gene expression programs that underlie changes in competence within aging INP lineages. Future work should focus on a mechanistic understanding of progenitor genome plasticity during development and help illuminate the dynamic nature of genomic changes that generate neural diversity.

APPENDIX

SUPPLEMENT TO CHAPTER II

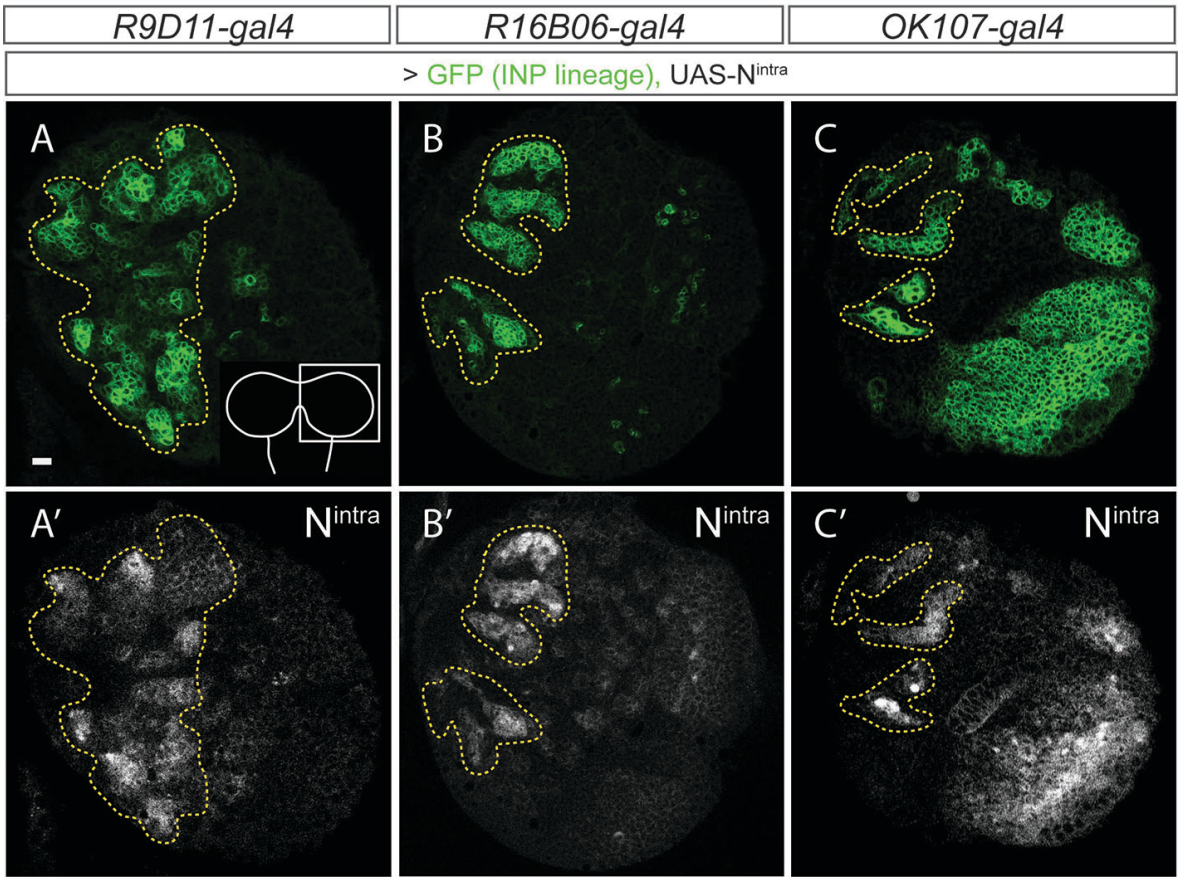


Figure S1, related to Figure 2.1.
Notch^{intra} is nuclear and at qualitatively similar levels when expressed in young or old INP lineages. (A-C') R9D11-gal4, R16B06-gal and OK107-gal4 driving UAS-Notch^{intra} result in efficient Notch^{intra} protein expression, as visualized by antibody staining. Yellow dashed outlines show INP lineages in central brain labeled by each driver. Scale bar = 10 μm.

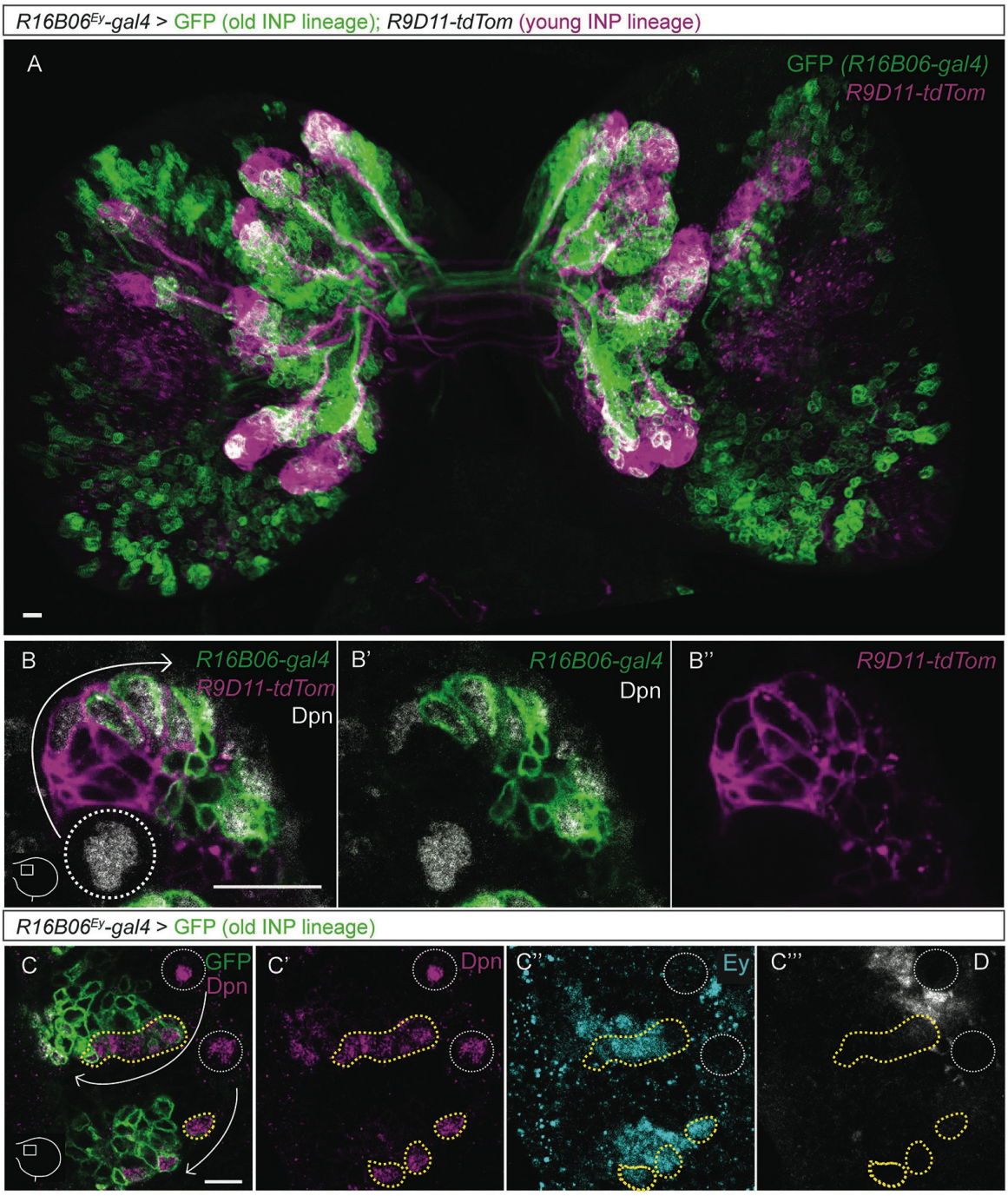


Figure S2, related to Figure 2.3.
R16B06-gal4 labels old INPs in third instar larval brains. (A) Expression pattern of R16B06-gal4 in both brain lobes of third instar larva. R9D11-gal4 marks young INPs and is shown for comparison. (B-B'') High magnification images show Dpn+ INPs distal to their parental Type II NB (white dashed line) are labeled by R16B06-gal4. Arrow indicates direction of age progression in lineage. (C-C''') Distal INPs (yellow dashed line) labeled by R16B06-gal4 express the old INP specific transcription factor Eyeless (Ey) but not the young INP specific transcription factor Dichaete (D). Images are a single, one micron plane. All panels show third instar larvae; scale bar = 10 μm.

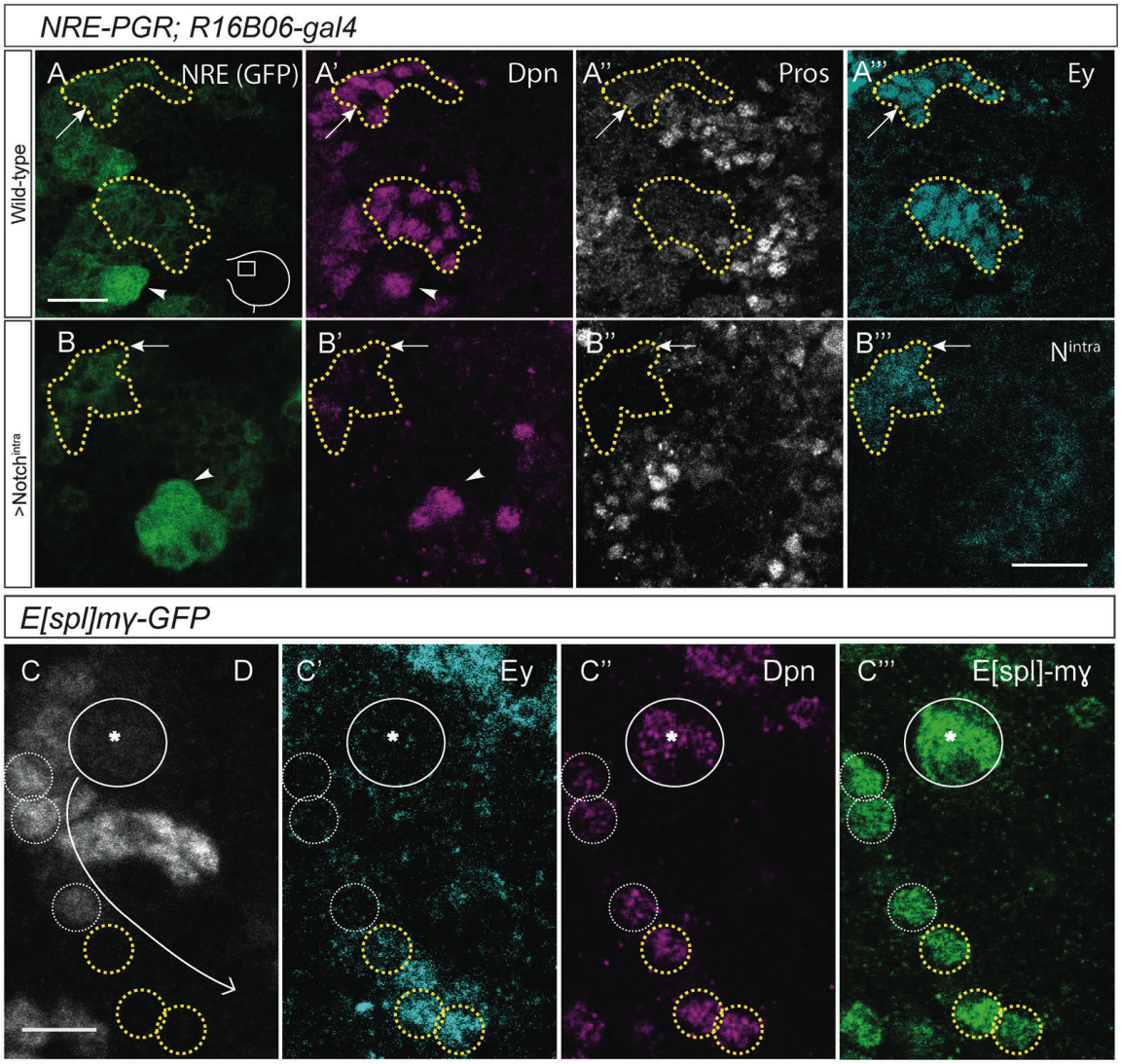


Figure S3, related to Figure 2.7.

Notch^{intra} signaling can induce expression of the Notch response element reporter (NRE-PGR) in old INPs but not in GMCs. (A) In wild type, the NRE reporter is expressed at high levels in Type II neuroblasts (arrowhead) and shows progressively weaker levels in the progeny. There are low levels in old INPs (dashed yellow outline; identified by Ey expression), but is not detectable in Prospero (Pros)+ GMCs (arrow). (B) Expression of Notch^{intra} in the old INPs and their progeny using R16B06-gal4 results in elevated expression of the NRE reporter (dashed yellow outline; compare to level in adjacent cells); only Dpn+ INPs show elevated levels of the reporter, Prospero (Pros)+ GMCs show no detectable expression. (C-C''') The Notch target *E(spl)mγ* is expressed in both young and old INPs. (C) Young INPs expressing Dichaete (small white circles) and (C') old INPs expressing Eyeless (small dashed yellow circles) are positive for Deadpan and the Notch target *E(spl)mγ*-GFP fusion reporter. Asterisk marks Type II NB, arrow indicates direction of lineage from young to old. All panels show third instar larvae; scale bar = 10 μm.

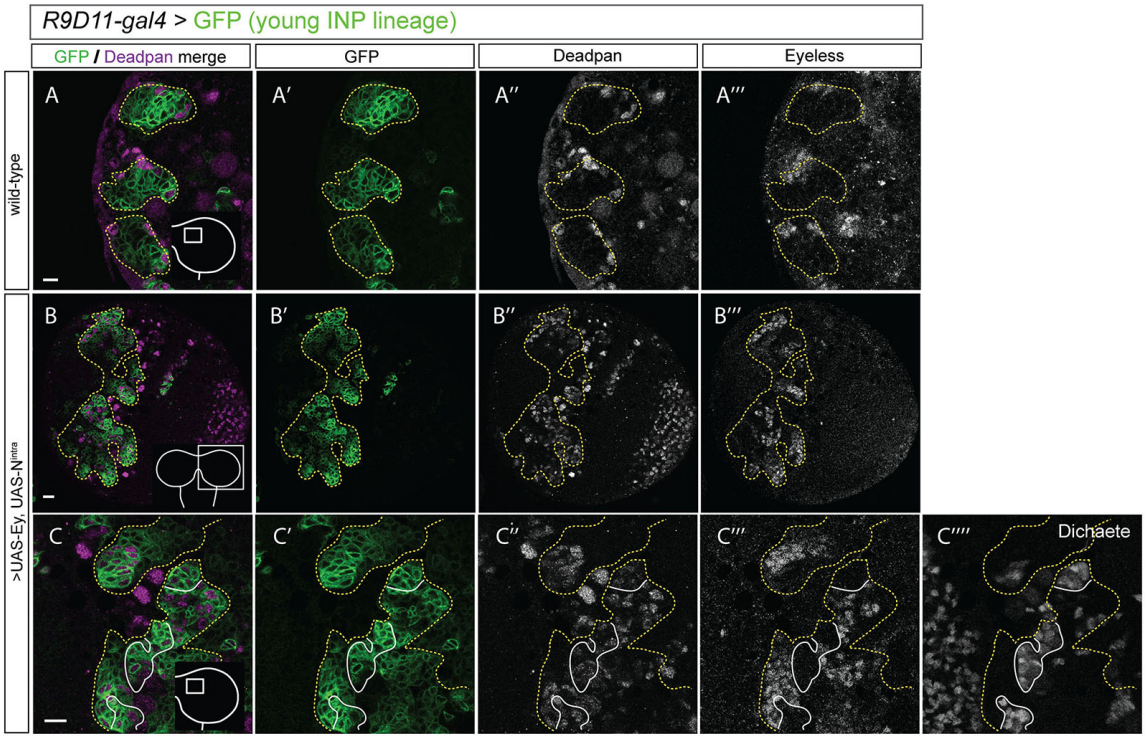


Figure S4, related to Discussion and Figure 2.2.
Misexpression of Eyeless in young INPs does not repress competence to dedifferentiate in response to Notch. (A-A’’’) Young INPs labeled by R9D11-gal4 do not express Eyeless (yellow dashed outline shows young INP lineage). (B-B’’’) Using R9D11-gal4 to drive expression of UAS-Eyeless in young INPs does not suppress the formation of ectopic Type II NB- like cells. (C-C’’’) High magnification images show Eyeless misexpression in GFP+ cells except the youngest, Dichaete+ INPs (white outline). Images are a single, one micron plane. All panels show third instar larvae; scale bar = 10 μm.

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