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Aging Neural Progenitors Lose Competence to Respond to Mitogenic Notch Signaling

Highlights

- Aging INPs lose competence to respond to constitutively active Notch signaling
- The late temporal factor Eyeless blocks Notch-induced target gene expression
- Eyeless blocks Notch-induced INP tumor formation

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In Brief

Notch signaling potentiates tumor formation in flies and mammals. Farnsworth et al. show that aging *Drosophila* intermediate neural progenitors use the temporal identity factor Eyeless/Pax6 to prevent Notch-induced target gene expression and tumorigenesis. Thus, the Eyeless/Pax6 transcription factor has a novel role in blocking Notch signaling.

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Aging Neural Progenitors Lose Competence to Respond to Mitogenic Notch Signaling

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SUMMARY

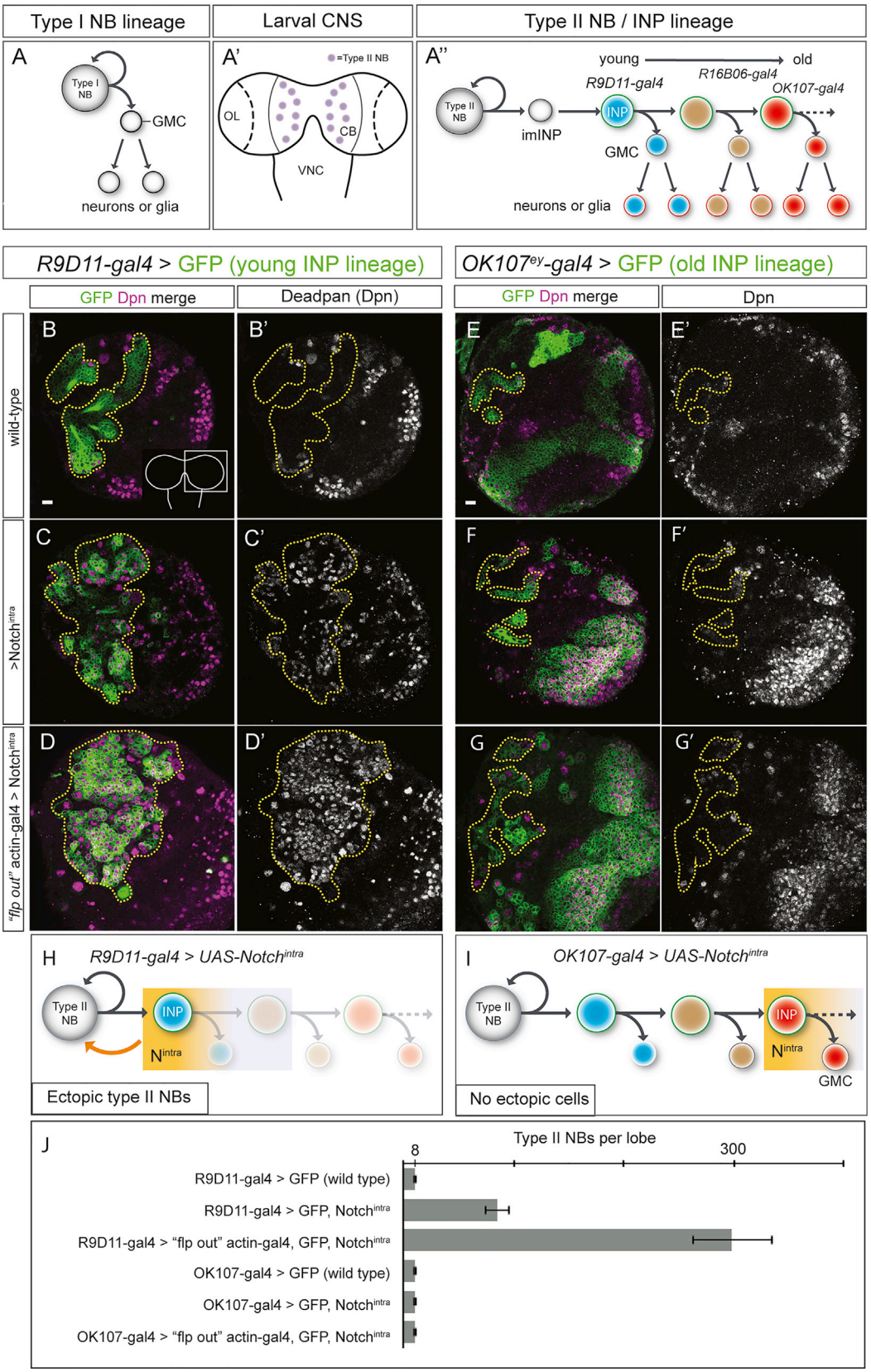
Drosophila neural stem cells (neuroblasts) are a powerful model system for investigating stem cell self-renewal, specification of temporal identity, and progressive restriction in competence. Notch signaling is a conserved cue that is an important determinant of cell fate in many contexts across animal 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.

INTRODUCTION

Development of complex structures like the human 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 [1, 2]. 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 [2–6]. 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 [7]. 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 [8–10]. 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?

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 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 [2, 6, 11–13]).

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 1A′). Type II neuroblasts generate a series of smaller intermediate neural progenitors (INPs) that act as transit-amplifying cells; each INP undergoes a series of molecularly asymmetric divisions to self-renew and produce about six GMCs, each of which makes a pair of neurons or glia (Figure 1A″) [14–16]. 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 [17–22], and we have recently identified three INP temporal transcription factors: Dichaete (D), Grainy head (Grh), and Eyeless (Ey) [23]. Despite this progress, currently nothing is known about how



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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 [16, 24–27]. This is a highly conserved function, as Notch signaling also promotes self-renewal and proliferation of mammalian neural progenitors and stem cells [28–32]. *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 newborn INP back into a type II neuroblast, leading to "neuroblast tumors" [16, 25, 26, 33]. 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 Ey/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 Ey/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 (Figures 1C and 1C′; data not shown; quantified in Figure 1J; see Figure 6C in [25]). 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 [23]. As expected, expression of GFP alone in old INPs did not produce any ectopic Dpn+ Ase− type II neuroblasts (Figure 1E; data not shown; quantified in Figure 1J). Interestingly, expression of $Notch^{intra}$ alone in old INPs also did not generate any ectopic neuroblasts (Figure 1F; quantified in Figure 1J), in contrast to its potent induction of ectopic neuroblasts when expressed in young INPs. There are two possible interpretations of these results: (1) the *OK107-gal4* line produced lower levels of $Notch^{intra}$ compared to *R9D11-gal4*, leading to insufficient $Notch^{intra}$ to induce neuroblast identity, or (2) old INPs have lost competence to respond to $Notch^{intra}$.

To ensure equal $Notch^{intra}$ levels in young or old INPs, we used a "flp out" expression method [23]. 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 1D; data not shown; quantified in Figure 1J; summarized in Figure 1H). In contrast, *actin-gal4* driving *UAS-*$Notch^{intra}$ in old INPs did not generate any Dpn+ Ase− neuroblasts (Figure 1G; data not shown; quantified in Figure 1J; summarized in Figure 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.

Ey 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 Ey expressed in old INPs provides a good candidate. We hypothesized that Ey may block Notch signaling in old INPs or their progeny.

We have previously shown that loss of Ey causes old INPs to delay the termination of their lineages by several additional divisions, but no ectopic neuroblasts or INPs are formed [23]. To test whether loss of Ey increased the competence of old INPs to respond to Notch signaling, we used our previously

Figure 1. Old INPs Lose Competence to Respond to Notch

(A–A″) Summary of type I and type II NB cell lineages. (A) Type I NBs self-renew and produce GMCs, which divide to make two neurons or glia. (A′) Eight type II NBs are found in the central brain of each larval brain lobe (OL, optic lobe; VNC, ventral nerve cord). (A″) 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 and 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 and C′) Expression of constitutively active Notch in young INPs (*R9D11-gal4 UAS-*$Notch^{intra}$ *UAS-GFP*) produces ectopic Dpn+ Ase− type II neuroblasts.

(D and 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 and E′) Old INPs are labeled by *OK107-gal4*-driving membrane GFP without generating ectopic type II neuroblasts (8 ± 0 per lobe; n = 3).

(F and F′) Old INPs do not generate ectopic Dpn+ NBs in response to constitutive Notch signaling (8 ± 0; n = 3).

(G and 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–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. Images are a single, one-micron plane through a whole brain lobe. All panels show third instar larvae; scale bar = 10 μm.

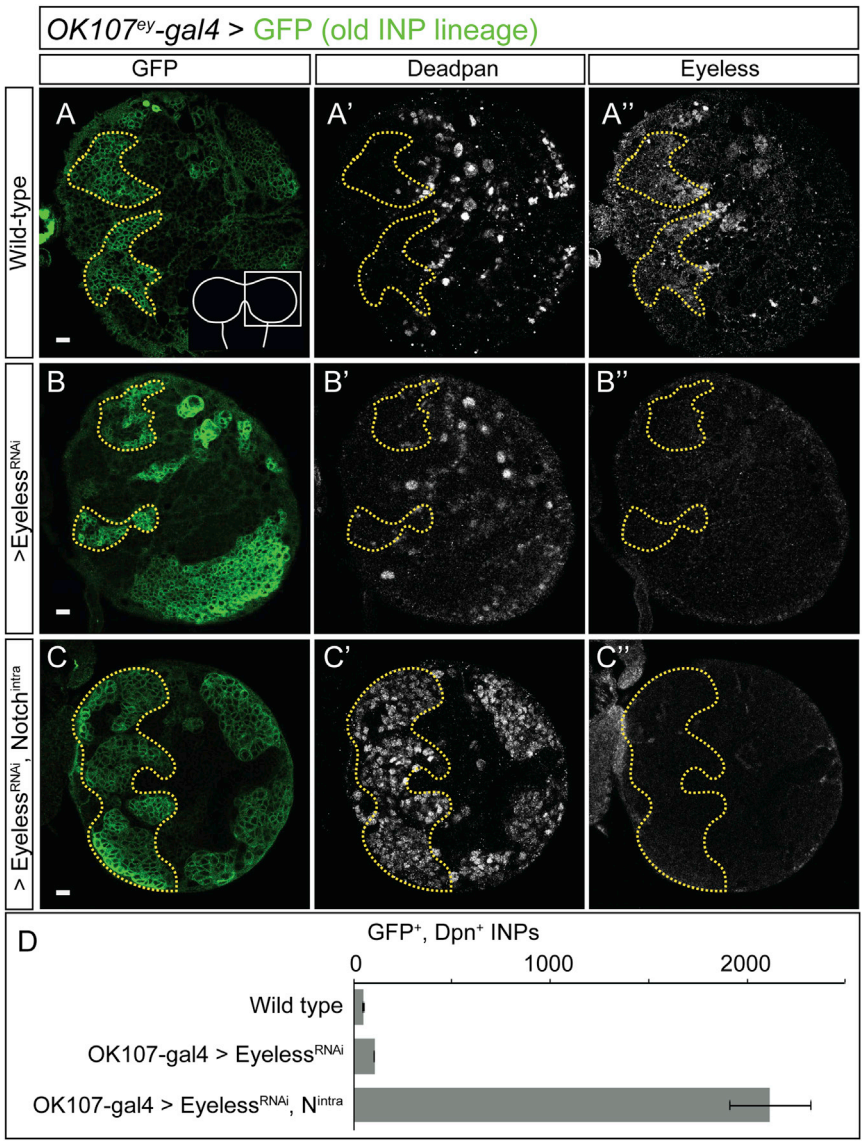

Figure 2. Eyeless Restricts the Competence of Old INPs to Respond to Notch Signaling

(A–A″) *OK107-gal4*-driving membrane GFP labels old INPs that express Dpn and Ey.

(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–C″) Constitutive Notch signaling in Eyeless-negative old INPs (*OK107-gal4*, *UAS-eyeless*^{RNAi}, *UAS-Notch*^{intra}) generates many ectopic Dpn+ presumptive INPs in the dorsomedial brain.

(D) Quantification of results.

Images are a single, one-micron plane through a whole brain lobe. All panels show third instar larvae; scale bar = 10 μm.

well-characterized *UAS-eyeless*^{RNAi} transgene [23] to eliminate all detectable Ey protein concurrent with expression of *UAS-Notch*^{intra} (*OK107-gal4*, *UAS-mCD8-GFP*, *UAS*-Notch^{intra}, and *UAS-eyeless*^{RNAi}). Confirming previous findings [23], Ey RNAi removes all detectable Ey protein without generating any ectopic Dpn+ Ase− neuroblasts and very few Dpn+ Ase+ INPs (Figures 2A and 2B; data not shown; quantified in Figure 2D). In contrast, removing all detectable Ey together with expression of Notch^{intra} led to the formation of many ectopic Dpn+ neuroblasts or INPs (Figure 2C; quantified in Figure 2D). There are several possible explanations for the observed phenotype: (1) 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; (2) 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 (3) the ectopic Dpn+ cells could be due to the action of Notch^{intra} within the type II lineages.

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 [34, 35] and can be used to target Notch^{intra} expression specifically to old Ey+ 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 (Figures 3A and 3B; quantified in Figure 3F; summarized in Figure 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 (Figures 3C and 3C′; quantified in Figure 3F; summarized in Figure 3G), which we provisionally assign an INP identity because most cells have the Dpn+ Ase+ molecular profile of INPs (Figures 3D–3D‴). This is in contrast to the ectopic Dpn+ Ase− type II neuroblasts formed from young INPs de-differentiating in response to Notch (Figures 3E–3E‴). We conclude that Ey restricts the competence of old INPs, or their progeny, to respond to Notch^{intra} signaling.

Ey Blocks Notch^{intra} from Inducing GMC-to-INP De-differentiation

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 Ey protein and induce Notch^{intra}, we find the vast majority of ectopic cells are Dpn+ Ase+, consistent with an INP identity (Figures 4A and 4B). In addition, most of the ectopic cells were also Grh+ (Figures 4C and 4D), consistent with the molecular profile of Ey-negative INPs [23]. We conclude that the majority of the ectopic cells induced by Notch in old Ey-negative INP lineages have the molecular characteristics of INPs.

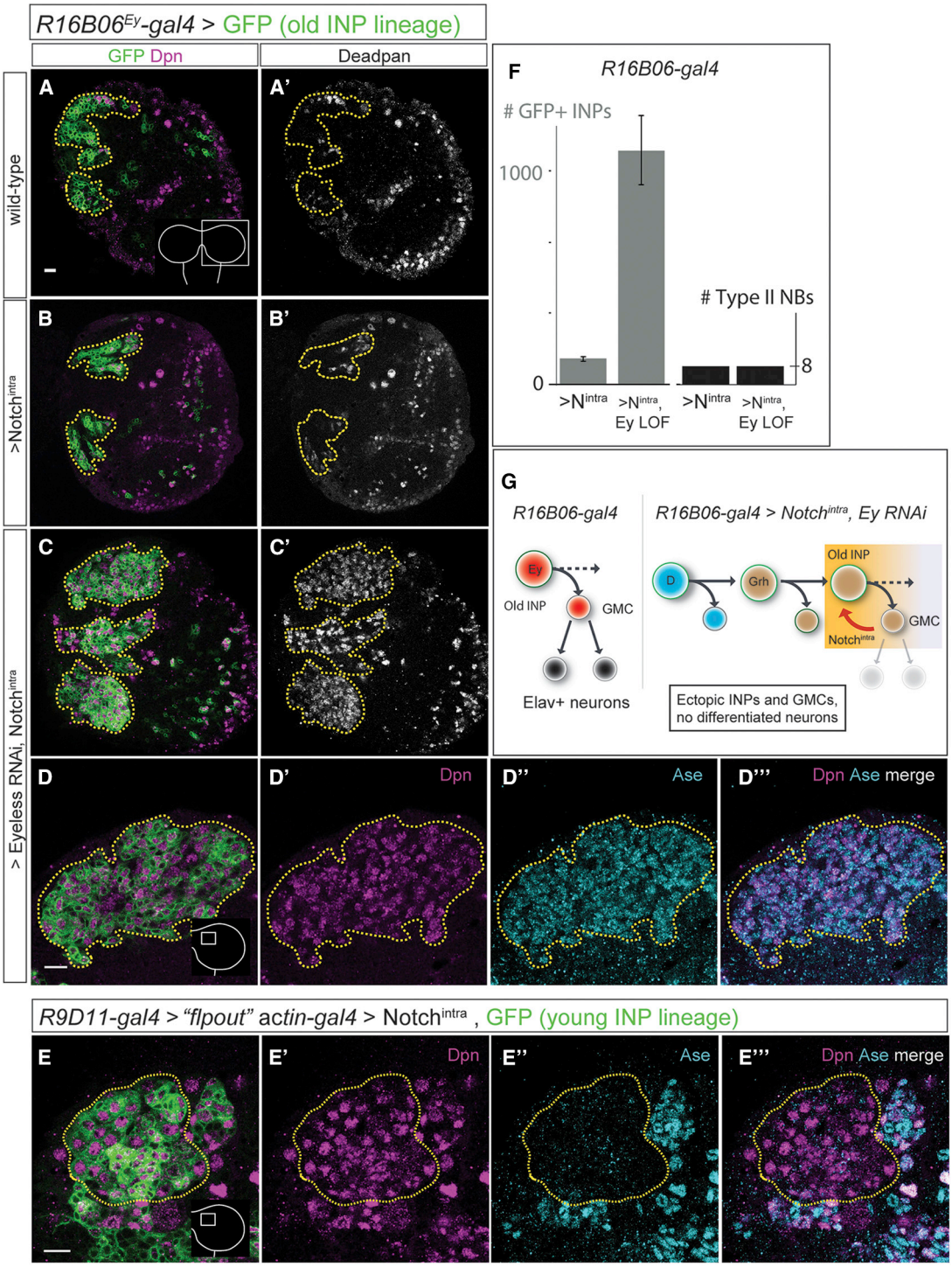

Figure 3. Old INPs Labeled by *R16B06-gal4* Also Lose Competence to Respond to Notch

(A and A′) Old INPs in the central brain are labeled by *R16B06-gal4*-driving membrane-bound GFP.

(B and 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 and 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+).

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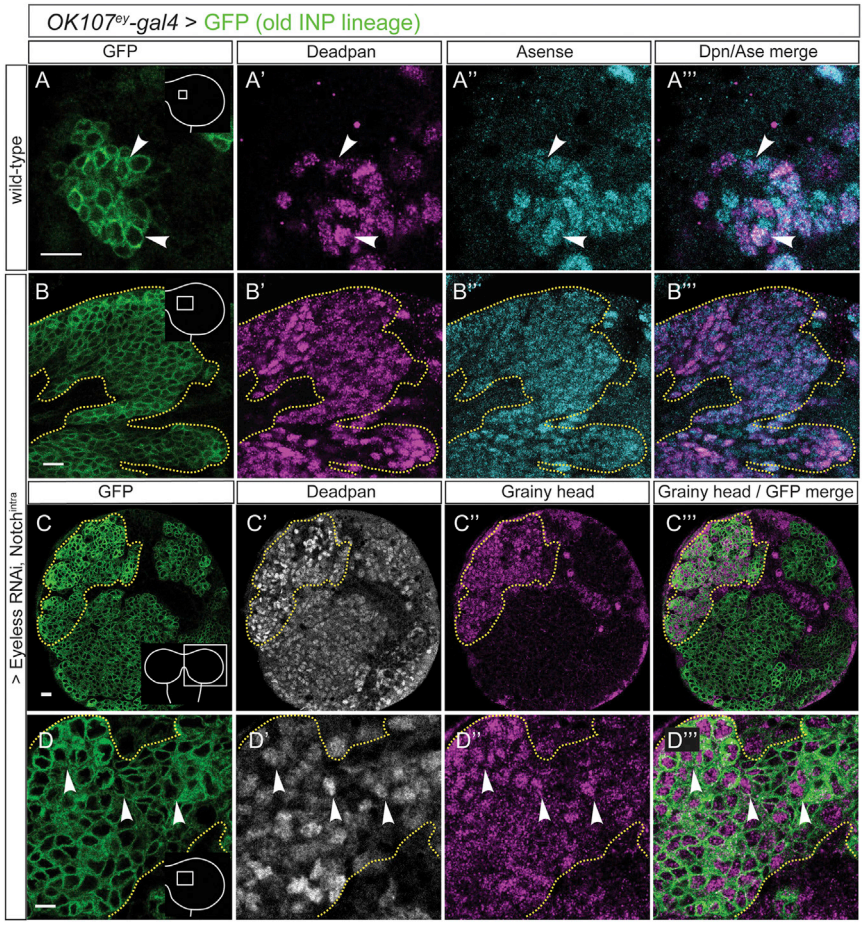

Figure 4. Notch^{intra} in Old INPs Lacking Eyeless Generates Ectopic INPs

(A–A′′′) Wild-type old INPs normally express Dpn and Asense (Ase).

(B–B′′′) Constitutive Notch signaling in Eyeless-negative old INPs (*OK107-gal4*, *UAS-Notch^{intra}*, *UAS-eyeless^{RNAi}*) generates many ectopic Dpn+ Ase+ cells.

(C–D′′′) Presumptive ectopic INPs expressing Grh in the dorsomedial brain.

Images are a single, one-micron plane zoomed in to the dorsal-anterior central brain (A, B, and D) or show one brain lobe (C). All panels show third instar larvae; scale bar = 10 μm.

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 whether they performed a symmetric or asymmetric cell division. Wild-type INPs are phospho-histone H3 (PH3) positive during mitosis (Figure 5A′′′) and divide asymmetrically to localize the Miranda scaffolding protein and Prospero transcription factor cargo to the basal cortex (Figures 5A–5A′′), 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 (Figures 5B–5B′′), are PH3+, and localize Prospero to the nucleus during interphase. Furthermore, Pros+ GMCs can be identified throughout the proliferative mass (Figure 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 Ey allows Notch^{intra} to induce GMC > INP de-differentiation.

Next, we determined whether the GMCs in the Ey^{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 [14–16, 36], and as expected, we observe Elav+ neurons within *R16B06-gal4, "flp-out," UAS-GFP* permanently marked old INP lineages (Figures 6A and 6B; quantified in Figure 6E). In contrast, the Ey^{RNAi} Notch^{intra} population contained few or no Elav+ neurons (Figures 6C and 6D; quantified in Figure 6E). In addition, this population never expressed markers for differentiated neurons derived from old INPs like Twin of Ey (Toy) or from young INPs like brain-specific homeobox (Bsh) (data not shown). We conclude that loss of Ey allows Notch^{intra} to induce GMC > INP de-differentiation, which maintains INP proliferation and nearly completely blocks neuronal differentiation (summarized in Figure 6F). This highlights the loss of competence that INPs undergo as they age and identifies a novel function for the conserved Ey/Pax6 transcription factor: to block Notch signaling.

Ey 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 Ey/Pax6 transcription factor. Where in the Notch-signaling pathway does Ey act? We can conclude it acts after ligand binding and proteolytic cleavage of Notch, because these steps are

(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 and 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 and E). All panels show third instar larvae; scale bar = 10 μm.

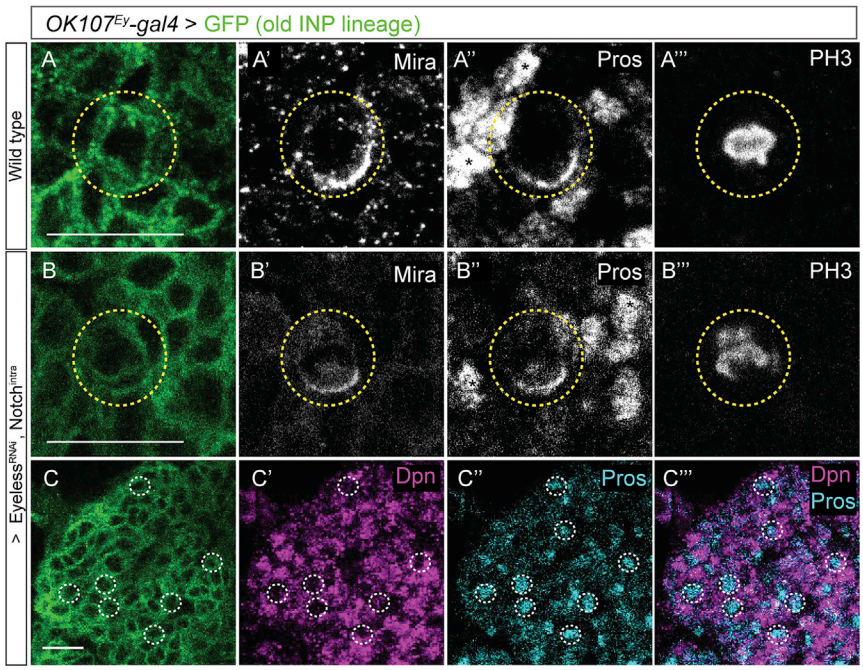

Figure 5. Asymmetrical Cell Division Is Maintained in Ectopic INP-like Cells

(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.

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 [37] was observed when Notch^{intra} was expressed in old INPs, indicating that the Notch^{intra} protein is functional (Figure S3). Does Ey 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* [33, 37–40]. 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 [33]. In contrast, Hey is detected only in a subset of post-mitotic neurons [39] and is not likely to be relevant to the GMC > INP de-differentiation step.

In wild-type, Ey+ 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 (Figures 7A–7A‴; see also Figure S3). Similarly, forced expression of Notch^{intra} in old INPs results in Notch target gene expression in INPs, but not GMCs (Figures 7B–7B‴; see also Figures S3B and S3B′; data not shown). In contrast, forced expression of Notch^{intra} in old INPs that lack Ey (Ey^{RNAi} Notch^{intra}) results in Dpn expression in both INPs as well as some GMCs (Figures 7C–7C‴; quantified in Figure 7D). We conclude that Ey functions in GMCs to prevent Notch^{intra} from activating target gene expression.

DISCUSSION

Here, we report three new findings. First, we show that young INPs undergo an INP > neuroblast de-differentiation in response to 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 de-differentiation in the absence of the late INP temporal transcription factor Ey/Pax6. Third, we show that Ey/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 de-differentiation into neuroblasts [41, 42]. 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 de-differentiation but that this effect of Notch^{intra} can be completely blocked by the conserved Ey/Pax6 transcription factor. How does Ey block Notch signaling? One model is that Ey 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 [37, 38]—preventing them from becoming transcriptionally activated by Notch signaling. Supporting this notion, the Ey-related Pax6 protein binds the SWI/SNF-related BAF complex to regulate the expression of neurogenic transcription factors in murine adult neural progenitors [43]. In addition, a switch in BAF subunits has been shown to direct the transition from proliferation to differentiation in mammalian neural progenitors [44], raising the possibility that both *Drosophila* and mammals use similar pathways to regulate progenitor choice of differentiation or proliferation.

Our finding that Ey can block the activity of constitutively active Notch^{intra} signaling raises several questions. First, why does Ey 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 Ey to block Notch target gene expression. Consistent with this model is the observation that reducing Prospero from GMCs results in de-differentiation into neuroblasts that express the Notch target genes *dpn*, *E(spl)mγ*, and *Myc* [16, 45–47]. Second, can misexpression of Notch target genes bypass

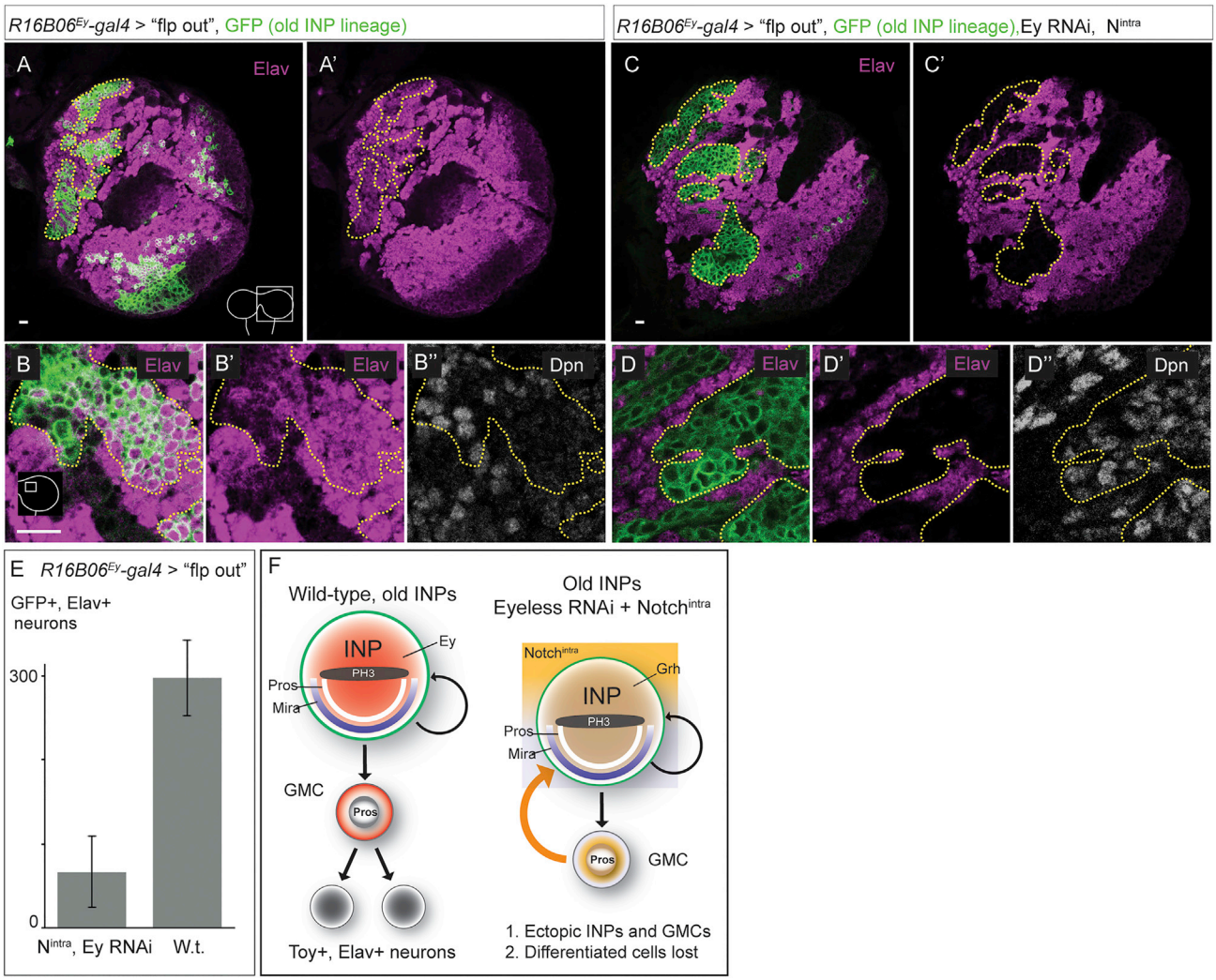

Figure 6. Notch Signaling Induces GMC to INP De-differentiation within Old INP Lineages in the Absence of Eyeless

(A and B) Old INPs lineages are permanently labeled by *R16B06-gal4* "*flp-out*"-driving membrane GFP. (A and A′) Wild-type, old INP lineages labeled with GFP produce differentiated neurons marked by Elav. (B and B′) High-magnification images show Dpn+ INPs and Elav+ neurons in these GFP+ lineages.

(C and C′) Eyeless knockdown and constitutive Notch signaling in old INPs produces ectopic cells at the expense of Elav+ differentiated cells.

(D and D′) High-magnification images show striking loss of Elav+ cells in GFP+, old INP lineages, whereas 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.

the tumor suppressor function of Ey? 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 de-differentiation. Third, why doesn't loss of Ey alone trigger GMC de-differentiation? One possibility is that endogenous Notch signaling is too low to induce de-differentiation either due to absence of a Notch pathway component or lack of access to ligand. Fourth, can misexpression of Ey block Notch^{intra}-induced young INP > neuroblast de-differentiation? We attempted to answer this question by misexpressing Notch^{intra} and Ey together in young INPs (*R9D11-gal4 UAS-GFP UAS-Notch^{intra} UAS-Eyeless*). Surprisingly, the young INPs had no detectable Ey protein (Figure S4), although they had high GFP levels and despite *UAS-GFP* and *UAS-Eyeless* being coexpressed, due to an unknown mechanism blocking Ey 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 Ey protein expression is an interesting area for future investigation, particularly to determine whether 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

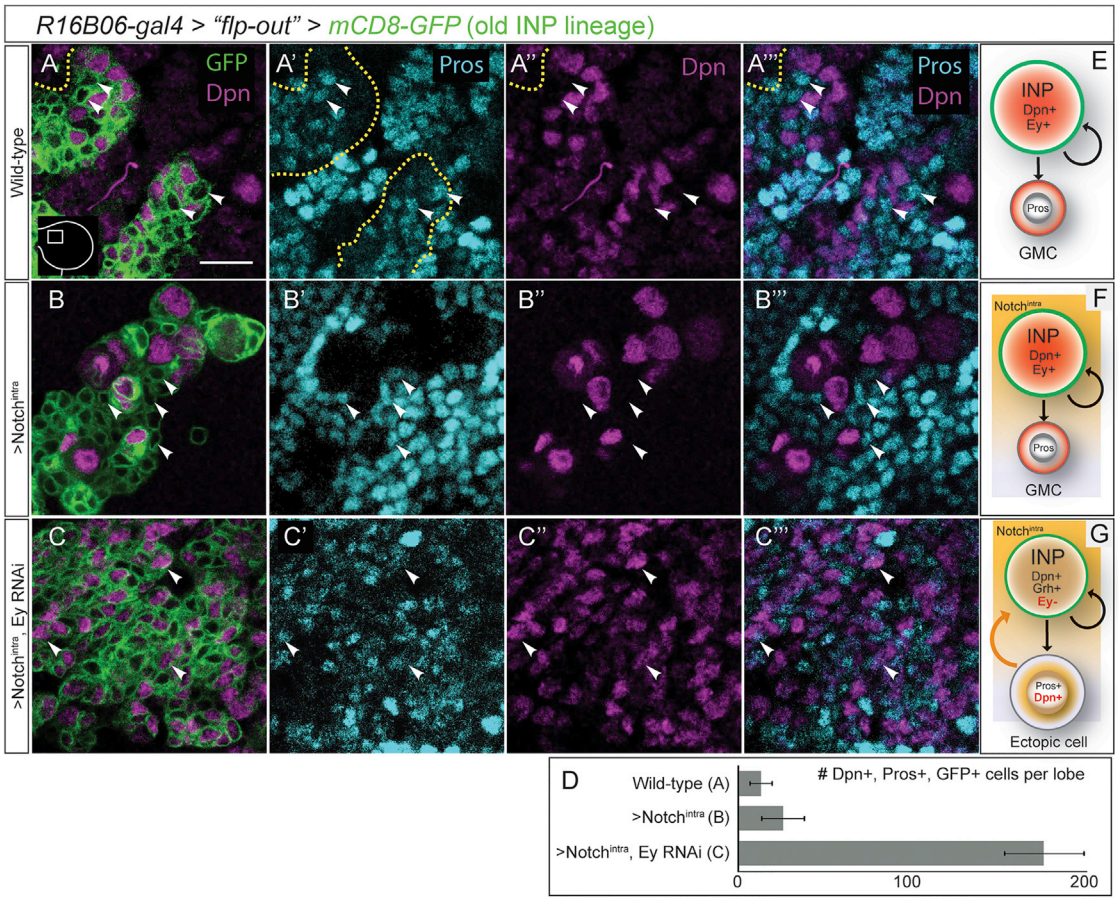

Figure 7. Derepression of Deadpan in Old INP Progeny Is Induced by Loss of Eyeless and Constitutive Notch Signaling

(A–A‴) Wild-type, old INPs give birth 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 that express Pros and have derepressed Dpn.

(D–F) Schematic of results.

(G) Quantification of cells with nuclear Pros and Dpn per brain lobe.

(A and 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.

hematopoietic stem cells (HSCs) in mouse bone marrow is sufficient to generate extra-thymic T cells [48], 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 [48]. 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 [49]. The tumor suppressor function of Ikaros in controlling the response to Notch signaling in T cells is strikingly similar to the function of Ey 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 [49]. 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.

Ey 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 [50]. Ey was recently identified as a temporal identity factor in INPs and is essential for proper development of the *Drosophila* adult central complex [23]. Pax6 expression is a reliable marker of mammalian cortical progenitors and is under both spatial and temporal control. Both Pax6/Ey 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.

EXPERIMENTAL PROCEDURES

Fly Genetics

Mutant larvae were generated in vial collections incubated at 28°C–30°C using 3- to 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 (PBS plus 0.3% Triton X-100; 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 (1:2,000; Aves Laboratories); rat antibody to Dpn (1:50; C.Q.D. lab); rabbit antibody to Ase (1:2,000; C.-Y. Lee lab; Univ. Michigan); guinea pig antibody to D (1:500; J. Nambu); rabbit antibody to Ey (1:3,500; U. Walldorf); guinea pig antibody to Mira (1:1,000; C.Q.D. lab); mouse antibody to Pros (1:1,000; C.Q.D. lab); guinea pig antibody to Toy (1:500; U. Walldorf); and mouse antibody to $Notch^{intra}$ (1:50; Developmental Studies Hybridoma Bank). Secondary antibody staining was performed at room temperature for 2 hr (1:500; Molecular Probes or Jackson Immunoresearch). After staining, brains were kept at 4°C in Vectashield (Vector Laboratories) prior to imaging.

Imaging and Analysis

Images were obtained using a Zeiss LSM710 confocal microscope. Image processing and analysis was performed in FIJI (RRID: SciRes_000137; [51]).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.10.027.

AUTHOR CONTRIBUTIONS

D.R.F. did all experiments and co-wrote the manuscript; O.A.B. participated in the characterization of R16B06-gal4 and the design of the study; and C.Q.D. guided the project and co-wrote the manuscript.

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