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The bHLH factor *deadpan* is a direct target of Notch signaling and regulates neuroblast self-renewal in *Drosophila*

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ABSTRACT

A defining feature of stem cells is their capacity to renew themselves at each division while producing differentiated progeny. How these cells balance self-renewal versus differentiation is a fundamental issue in developmental and cancer biology. The Notch signaling pathway has long been known to influence cell fate decisions during development. Indeed, there is a great deal of evidence correlating its function with the regulation of neuroblast (NB) self-renewal during larval brain development in *Drosophila*. However, little is known about the transcription factors regulated by this pathway during this process. Here we show that *deadpan* (*dpn*), a gene encoding a bHLH transcription factor, is a direct target of the Notch signaling pathway during type II NB development. Type II NBs undergo repeated asymmetric divisions to self-renew and to produce immature intermediate neural progenitors. These cells mature into intermediate neural progenitors (INPs) that have the capacity to undergo multiple rounds of asymmetric division to self-renew and to generate GMCs and neurons. Our results indicate that the expression of *dpn* at least in INPs cells depends on Notch signaling. The ectopic expression of *dpn* in immature INP cells can transform these cells into NBs-like cells that divide uncontrollably causing tumor over-growth. We show that in addition to *dpn*, Notch signaling must be regulating other genes during this process that act redundantly with *dpn*.

Introduction

The development of the *Drosophila* larval central nervous system (CNS) has emerged as a fruitful model system for studying different aspects of stem cell biology. Most of the neurons that constitute the adult brain of the fly originate from the divisions during the larval stage of development of a few hundred stem cell-like precursors called neuroblasts (NBs). Larval NBs are present in the ventral cord, in the optic lobes and in the medial areas of the two brain lobes where they are called central brain (CB) neuroblasts. Unlike embryonic NBs that divide only a few times (Bossing et al., 1996), larval neuroblasts can undergo self-renewal divisions for a longer period of time to give rise to most of the neurons that form the adult fly brain (Urbach and Technau, 2004; Datta, 1995). Considering the lineage of central brain NBs it is possible to distinguish two different types of NBs: the canonical NBs with a type I lineage, where the NB after asymmetric division generate a cell that self-renews, the NB, and a ganglion mother cell (GMC), that divides only once to produce two postmitotic neurons, and the Posterior Asense-Negative (PAN) neuroblasts of type II lineage, which unlike type I NB do not express the bHLH factor Asense. These latter NBs undergo repeated asymmetric divisions to self-renew and to produce immature intermediate neural progenitors that are mitotically inactive and lack the expression of the NB markers Deadpan (Dpn) and Asense (Ase). These cells mature into intermediate neural progenitors (INPs) that express both Dpn and Ase and have the capacity of undergo up to 10 rounds of asymmetric division to self-renew and to generate GMCs and neurons throughout larval development. Each larval brain hemisphere only contains 8 type II NBs and approximately 100 type I NBs (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008).

The asymmetric division of NBs is tightly controlled by the same molecular machinery that regulates asymmetric cell division during embryogenesis (for review, see Doe, 2008; Gonczy, 2008; Knoblich, 2008). Cell fate determinants are differentially segregated during the division of each NBs by a complex of proteins that define the apical–basal polarity of the cell. Thus, apical determinants restrict the expression of Miranda (Mira) and the adapter Partner of Numb (Pon) to the basal cortex, where they recruit the determinants that will segregate into the GMC, such as the homeodomain transcription factor Prospero and the phosphotyrosine-binding (PTB) domain protein Numb (Jan and Jan, 2001; Betschinger and Knoblich, 2004; Doe, 2008; Gonczy, 2008; Knoblich, 2008). Both factors are ultimately required to promote neuronal differentiation, preventing the self-renewal of NBs (Betschinger et al., 2006; Choksi et al., 2006). The function of Numb is required to repress Notch signaling in one of the daughter cells after NBs division (Knoblich, 2008). Thus, the asymmetric sequestration of Numb in the GMC would restricts the activity of Notch signaling to the NBs, where the function of this pathway would be sufficient to promote NB self-renewal

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and suppress differentiation (Wang et al., 2006; Bowman et al., 2008; Weng et al., 2010). This function seems to be restricted to type II NBs, as the ectopic expression of an activated form of N or mutant clones of *numb* induce an excess of this type of NBs, whereas a reduction in Notch signaling reduces its number (Bowman et al., 2008). It has been proposed that the down-regulation of Notch signaling is required to promote maturation of intermediate progenitors, generated after division of type II NBs, into mature INP. In the absence of this down-regulation immature progenitors adopt parental NB fate and continue dividing, causing a tumor over-growth formed by NB-like cells. Numerous data support this model (Wang et al., 2006; Bowman et al., 2008; Weng et al., 2010); however, how Notch signaling exerts this control and which transcription factors are regulated by Notch during this process are important questions that remain unanswered. The function of Notch signaling is also required for the proliferation of the stem cells that constitute the optic lobe. This structure it is formed by a neuroepithelium, in which the stem cell divide symmetrically, and a territory of asymmetrically dividing NBs that generated differentiated neurons. Notch signaling is required to maintain the pattern of symmetric division and the undifferentiated state of neuroepithelial cells, and its down-regulation is necessary for the transition from symmetric to asymmetric differentiative division (Egger et al., 2010). Although, there are differences between the neuroepithelial cells and CB NBs, it is possible that similar molecular mechanisms operate on both processes to control the transition from self-renewal to differentiation.

Here we investigate the function of Notch signaling during larval CB NB self-renewal. We have found that the over-expression of the Hes vertebrate homolog *deadpan* (*dpn*) reproduces the phenotype displayed by the ectopic activation of Notch signaling during NB development. In addition, we have identified a Notch-responsive enhancer contained in the regulatory region of *dpn* that is active in all central brain NBs as well as in the INP cells derived from NBs type II. Our results indicate that the expression of *dpn* in these latter cells depends on Notch signaling. All these data are consistent with a model where *dpn* is a target of Notch signaling at least in the INP cells. We found that the premature expression of *dpn* in immature INP cells can transform these cells into NBs-like cells that divide uncontrollably causing tumor over-growth. Our results also indicate that in addition to *dpn*, Notch signaling must be regulating other genes during this process that act redundantly with *dpn*.

Materials and methods

Genetic strains

The following alleles were used: $Su(H)^{O47}$, N^{55e11}, dpn^{2} and dpn^{7}. The following UAS lines were used: UAS-hey (Supplementary Methods), UAS-N^{int}, UAS-*dpn*, UAS-*prospero K*, UAS-*ase*, UAS-*hairy*, UAS-*E(spl) m8*, UAS-*E(spl)mβ 1-2* and *h8*, UAS-*E(spl)m5.2* and *R4* , UAS-*E(spl)m4* and UAS-*E(spl)β* (de Celis et al., 1996; Ligoxygakis et al., 1998). We used two reporter lines: $P\{PZ\}pros^{10419}$ and *w118; P(E(spl) m4-lac-Z)-96A* (Bailey and Posakony, 1995). The following Gal4 lines were used: wor-*Gal4* and elav-*Gal4*. All of these stocks are described in FlyBase (http://flybase.bio.indiana.edu/). The deficiencies dpn^{Def3D5} and dpn^{Def1D6} completely eliminate *dpn*, CG 33087 and CG34217 (J. Culi, unpublished data; molecular data should be requested to the author).

Generation of mosaics

Mitotic clones were generated by FLP-mediated mitotic recombination (Xu and Rubin, 1993). Clones lacking *dpn* were obtained by crossing FRTG13 dpn^{Def1D6} and FRTG13 dpn^{7} to *hsp-FLP122* tub-*Gal4* UAS-nuc*GFP;* tubP-*Gal80* FRTG13/Cyo flies. Control clones were generated using the FRTG13 UAS-*mCD8-GFP* chromosome. To obtain clones expressing UAS-*dpn* or UAS-N^{intra}, we crossed FRTG13 UAS-*mCD8-GFP*/+; UAS-*X*/+ (where X is *dpn or* N^{intra}) flies with hsp-*FLP122* tub-*Gal4* UAS-*nucGFP;* tubP-*Gal80* FRTG13/Cyo flies. Clones of FRT40A $Su(H)^{O47}$ were generated by crossing these flies with hsp-*FLP122* tub-*Gal4 UAS-nucGFP;* tubP-*Gal80* FRT40A/Cyo flies and *hsp-FLP122 tub-Gal4 UAS-nucGFP; tubP-Gal80 FRT40/+; dpn-reporter/+* flies. Clones of $Su(H)^{O47}$ or dpn^{7} that simultaneously express N^{intra} were generated crossing, FRT40A $Su(H)^{O47}$/+;UAS-N^{intra}/TM6B or FRTG13 dpn^{7}/+; UAS-N^{intra}/TM6B with *hsp-FLP122 tub-Gal4* UAS-*nucGFP;* tubP-*Gal80* FRT40A/Cyo or hsp-*FLP122* tub-*Gal4* UAS-*nucGFP;* tubP-*Gal80* FRTG13/Cyo flies, respectively.

The progeny of these crosses were heat shocked at 37 °C for 1 h between 48 and 72 h after egg-laying. Brains were dissected and analyzed 3 days after the induction of the clones.

Clones of cells expressing Gal4 (Ito et al., 1997) were induced 48–72 h after egg laying by heat shock at 37 °C for 12 min in larvae of the following genotypes: *FLP1.22;* Act5C <FRT*yellow*$^{+}$FRT> *Gal4* UAS-*GFP*/+ UAS-*dpn*/+, and *FLP1.22;* Act5C <FRT *yellow*$^{+}$FRT> *Gal4* UAS-*GFP*/UAS-N^{intra}. The expression of the *prospero Lac-Z* reporter in clones of *dpn*-expressing cells was analyzed in *FLP1.22;* Act5C <FRT*yellow*$^{+}$ FRT> *Gal4* UAS-*GFP*/$P\{PZ\}pros^{10419}$;UAS-*dpn*/+ brains. Clones of cells co-expressing *UAS-dpn* and *UAS-prospero* were induced in larvae of *FLP1.22;* Act5C <FRT *yellow*$^{+}$ FRT> *Gal4* UAS-*GFP*/ UAS- *pros* K;UAS-*dpn*/+.

Clones of *dpn*-expressing cells in N^{ts} background were generated by crossing females N^{ts} *FLP1.22;* Act5C <FRT*yellow*$^{+}$FRT> *Gal4* UAS-*GFP*/Cyo by UAS-*dpn*/*TM6* males, after 48 h at 17 °C, the vials were transfer at restrictive temperature at 29 °C. Clones were induced 48–72 h later by heat shock at 37 °C for 12 min, and males were dissected 72 h later.

Quantitative analysis of clone sizes was done by measuring the volume of the clones using the Volumest tool from the Image J application. The estimated cell size that was used for calculating the number of cells was 60 μm^3.

Immunohistochemistry

Immunostaining of brains was performed according to standard protocols. The following antibodies were used: Guinea pig and rabbit anti-Dpn (diluted 1:1000 and 1:500, respectively) (kindly provided by J. Skeath and Y. Jan, respectively); rabbit and rat anti-Mira (diluted 1:1000 and 1:50, respectively) (kindly provided by C. Doe and Y. Jan, respectively); rat anti-Worniu (kindly provided by C. Doe); rat anti-Geminin (1:50) (kindly provided by H. Richardson); Guinea Pig anti-Ase (1:200) (kindly provided by J. Knoblich) and rabbit anti-phospho-Histone 3 (upstate) (1:1000); Anti-Su(H) (Santa Cruz Biotechnology) (1:1000); mouse anti-β-galactosidase (1:200), mouse and rat anti-Elav (used at 1:50 and 1:100, respectively), mouse anti-Dl (1:50) and mouse anti-Prospero (1:50) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Secondary antibodies (Molecular Probes) were used at dilutions of 1:200.

Results

Notch signaling promotes NB self-renewal via Su(H)

The transcriptional regulation of many genes by the Notch signaling pathway occurs via *Suppressor of Hairless (Su(H))*. To analyze whether the function of Notch signaling during NB development is mediated by this transcription factor, we studied the effects of removing *Su(H)* during NB proliferation. Using the MARCM system we induced clones of mutant cells for the null allele $Su(H)^{O47}$. We found that these clones could be generated in both Ase^{+} type I, and Ase^{-} type II NBs. In all cases, we found a NB per clone expressing the NB markers Dpn and Mira (Fig. 1C–D' and data not shown). Clones originated in Ase^{+} NBs had a size similar to control clones (Fig. 1E). In contrast, $Su(H)^{O47}$ clones generated in type II NBs were smaller than control clones (Fig. 1E) (207 ± 43 cells $n=9$ compared to 366 ± 129 cells $n=10$, $P<0.001$, clones were analyzed 72 h after induction). In addition, the number of

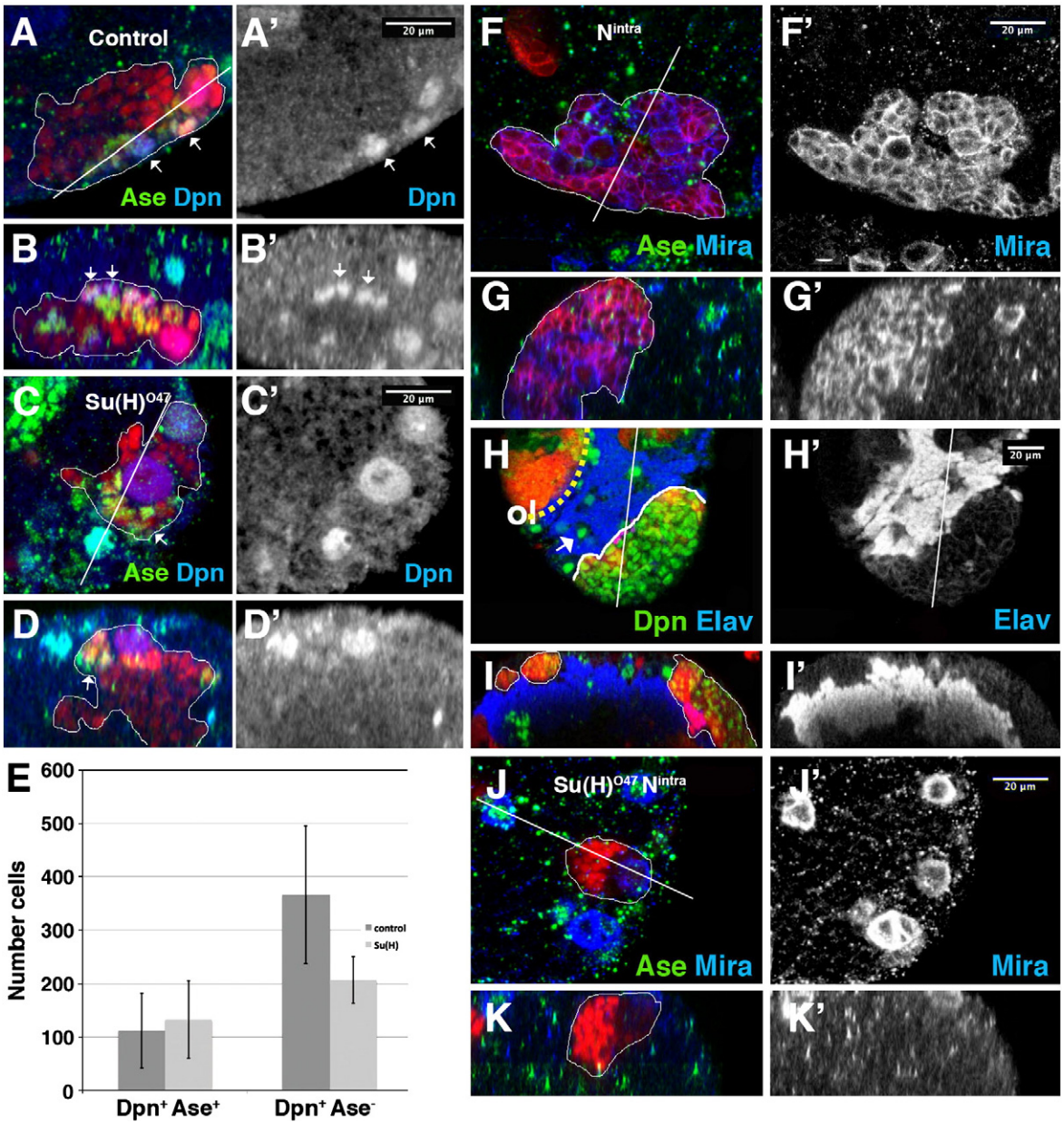


Fig. 1. *Notch* signaling induces outgrowth of NB-like cells via Su(H). (A–D' and F–K') Single focal planes of third instar larval brains containing Control (A–B'), $Su(H)^{047}$ (C–D'), UAS-N^{intra} (F-I') and $Su(H)^{047}$ UAS-N^{intra} (J–K') clones. Clones were always marked with GFP in red. Optical Z-sections of panels A, C, F, H and J are shown in B, D, G, I and K, respectively. White lines indicate the position of the section in each panel. (A–B') Control clones contain several INPs (arrows), characterized by the expression of Dpn (blue in A and B and gray in A' and B') and Ase (green in A and B). (C–D') In clones of $Su(H)^{047}$, Dpn (blue in C and D and gray in C' and D') is expressed in NB but is almost absent in its progeny. We find Ase^+ cells that do not express Dpn (arrows). (E) Quantitative analysis of the size of $Su(H)^{047}$ mutant clones. Clone sizes were analyzed by counting the number of cells in clones derived from type I NB (Dpn^+Ase^+) and type II (Dpn^+Ase^-) NBs (*wt* $n=26$, $Su(H)^{047}$ $n=28$). (F–G') Clones of N^{intra}-expressing cells originated in type II NBs (Ase^- stained in green in F and G) are almost formed by cells that express Mira (blue in F and G and gray in F' and G') and devoid of Ase^+ cells. (H–I') Dpn (green in H and I and gray in H' and I') is ectopically expressed in N^{intra}-expressing cells compared with the expression of Dpn in isolated NBs in wild-type tissue (arrow). Mutant cells do not express Elav (blue in H and I and gray in H' and I'). (J–K') In clones of $Su(H)^{047}$ N^{intra}–expressing cells, the effects produced by the ectopic expression of N^{intra} are suppressed (compare J with F). We only find a single $Mira^+$ (in blue) NB. In this and all subsequent figures the approximate position of the margin between the CB and optic lobe (ol) is marked by a dotted yellow line, and clones are out line in white.

cells expressing Dpn^+ was strongly reduced (3 ± 2 Dpn^+ Ase^+ cells in mutant clones compared to 20 ± 7 Ase^+ Dpn^+ cells in control clones, Fig. 1A–D'), the total number of Ase^+ cells is also reduced in these clones (17 ± 7 Ase^+ in mutant clones, compared to 52 ± 20 in control clones). These data suggest that *Su(H)* could be mediating the function of Notch signaling during NB development. To further study this possible requirement, we induced mutant clones of $Su(H)^{047}$ that simultaneously express an active form of *Notch* (N^{intra}). The lack of *Su(H)* suppressed the effects caused by the ectopic expression of *Notch*. Thus, $Su(H)^{047}$ N^{intra} mutant clones always contain a single NBs, in both type I and type II NBs (Fig. 1J–K'), in contrast to clones of N^{intra}-expressing cells that are predominantly formed by NB-like cells when induced in type II NBs, as seen by the expression of Mira and Dpn, and the lack of expression of the differentiation marker Elav (Fig. 1F–I') (Bowman et al., 2008). These results indicate that Su(H) is the transducer of the function of Notch signaling during type II NBs development. In addition, they suggest that the function of this factor is required to establish the lineage of type II NBs.

The ectopic expression of dpn is sufficient to induces extra type II NBs

We next asked how *Notch* signaling exerts this control and which transcription factors it regulates during this process. We predicted that the ectopic expression of the Notch signaling targets would induce an excess of NB-like cells, as seen upon ectopic activation of the pathway. To this end, we analyzed the effects caused by the ectopic expression of known *Notch* target genes in larval brains, as well as the ectopic expression of other genes that could be potentially involved in the regulation of this process. The best-characterized Notch signaling targets are the group of related genes belonging to the *Enhancer-of-split complex (E(spl)C)*. In *Drosophila*, this complex comprises seven genes encoding bHLH proteins, which mediate different functions of Notch signaling during development (Delidakis and Artavanis-Tsakonas, 1992; Knust et al., 1992). Some of these genes are expressed in NBs and a subset of their progeny (Bailey and Posakony, 1995; Almeida and Bray, 2005), suggesting that they might be required during NB development. In addition to these genes, we also studied two other related genes, *hairy* and *dpn* (Bier et al., 1992; Ohsako et al., 1994; Younger-Shepherd et al., 1992). The products of these genes show strong similarities to the Enhancer of split bHLH proteins. In addition, Dpn expression in some of the cells derived from type II NBs might depend on *Notch* signaling, indicated by the ectopic expression of this factor in clones of N^{intra}-expressing cells (Fig. 1H–I) and by its down-regulation under *Su(H)* mutant conditions in INP cells (Fig. 1C–D). Interestingly, the function of *dpn* is necessary for the proliferation of the optic lobes (OL) (Wallace et al., 2000). In *dpn* mutant brains, cell proliferation is reduced in this region, whereas the ectopic expression of *dpn* induces its over-proliferation (Wallace et al., 2000). Moreover, in a recent paper (Southall and Brand, 2009) have been identified many target genes of Dpn that have been implicated in vertebrates NB cell self-renewal.

All genes analyzed were over-expressed in NBs and their progeny using the *wor-Gal4* and *elav-Gal4* drivers. Of note, we found that *elav-Gal4* is expressed in NBs (in *elav-Gal4*/UAS-*GFP* brains, data not shown), indicating that although anti-Elav only marks differentiated cells, this driver activates the expression of *Gal4* at earlier stages. The over-expression of different members of the *E(spl)* complex or *hairy* did not significantly alter the number of Miranda-positive NBs in third instar larval brains (Table 1 and Supplementary Fig. 1). However, the ectopic expression of *dpn* dramatically increased the number of NBs, as shown by the expression of Mira (Fig. 2B, D, and F). The brains of UAS-*dpn*/*elav-Gal4* larvae were mainly composed of NBs that could be found deep inside the brain forming a compact mass (Fig. 2F'). These brains were double marked for Mira and the neuronal markers Elav and Prospero, revealing that the excess of NBs was at the expense of GMCs and neurons, as indicated by the strong reduction in the number of cells expressing these markers (Fig. 2F–F' and H–H'). Furthermore, in *elav-Gal4*/UAS-*dpn* larvae, a remarkable increase in brain size was detected at the end of larval development (Fig. 2B). Accordingly, the number of mitotic figures was significantly higher in mutant brains (187 ± 9 mitosis $n = 8$) compared with wild-type brains (123 ± 8 mitosis $n = 9$) (Fig. 2H–H'). Interestingly, in the most ventral part of the brain, the effects were much weaker than in the dorsal region (Fig. 2F'). This regional difference could be explained if there are different requirement for *dpn* in distinct NBs. As we mentioned before, the activity of Notch signaling is restricted to type II NBs (Bowman et al., 2008; Weng et al., 2010). To analyze whether ectopic expression of *dpn* induces an excess of type II NBs, we checked the expression of Ase and Mira in UAS-*dpn*/ *elav-Gal4* brains. We found that these brains are filled with type II NB-like cells, as they lack the expression of Ase but maintained the expression of Mira (Fig. 2D). Accordingly, the ectopic expression of *dpn* under the regulation of an *ase-Gal4* driver did not alter the number of NBs in third instar larval brains (data not shown). These effects are similar to those caused by the ectopic activation of Notch signaling during brain development (Bowman et al., 2008). All together, our results suggest that *dpn* might be a target of Notch signaling during type II NB development.

Table 1

	wor-Gal4	*elav-Gal4*
UAS-ase (1)	No Extra NBs	No Extra NBs
UAS-E(spl) m8 (1)	No Extra NBs	No Extra NBs
UAS-E(spl) m5 (2)	No Extra NBs	No Extra NBs
UAS-E(spl) m4 (1)	No Extra NBs	No Extra NBs
UAS-E(spl) mβ (2)	No Extra NBs	No Extra NBs
UAS-E(spl) mδ (2)	No Extra NBs	No Extra NBs
UAS-hairy(1)	No Extra NBs	No Extra NBs
UAS-emc (3)	No Extra NBs	No Extra NBs
UAS-hey (1)	No Extra NBs	No Extra NBs
UAS-dpn (2)	Extra NBs	Extra NBs

Clones of dpn-expressing cells induce over-growth of type II NBs

We next examined whether clones of *dpn*-expressing cells in larval brains reproduced the effects caused by the ectopic activation of *Notch*. Control clones derived from type I NBs always contained a single NB and numerous smaller cells expressing neuronal markers (Fig. 3E–E') (Lee et al., 2006; Betschinger et al., 2006), whereas control clones derived from type II NBs and analyzed 72 h after induction, contained a large Dpn^+ Ase^- NB; 5–6 Ase^- Dpn^- immature INPs closely associated with the NB, several (20 ± 7) Ase^+ Dpn^+ INPs, and a high number of Ase^- Dpn^- $Elav^+$ differentiated neurons with nuclear prospero (Fig. 1A and Fig. 3A–A', C–C'"and E–E'). Clones of *dpn*-expressing cells derived from type I NBs are indistinguishable from control clones (Fig. 3B–B', Supplementary Fig. 2 D–E'"). In contrast, clones resulting from type II NBs were mainly constituted by cells that express Mira and Wor, and they were essentially devoid of cells expressing Elav and Prospero (Fig. 3B–B', D–D", and F–G,' and Supplementary Figs. 2 and 3). In these clones, we only observed few Ase^+ cells (Fig. 3 and Supplementary Fig. 2), suggesting that they are either primary type II NBs or immature INP cells. Type II NBs and immature INP (Ase^- $Elav^-$ pros nuclear$^-$) are only distinguishable by size; immature INPs have a cell diameter of around 5–7 μm, whereas type II NBs are larger than 10 μm (Bowman et al., 2008). Although we found several large NBs in each mutant clone (Fig. 3), most of the cells contained in these clones were smaller than 10 μm (Fig. 3 and Supplementary Fig. 2 and 3), suggesting that they correspond to immature INPs. However, these cells do not behave as normal immature INPs, as it has been proposed that immature INPs are mitotically inactive (Bowman et al., 2008), whereas we observed that multiple small $Mira^+$ cells contained in these clones are in mitosis (Supplementary Fig. 3). In addition, when these mutant clones were generated in type II NB were much larger (4315 ± 1100 cells $n = 8$) than control clones (425 ± 146 cells in type II NBs $n = 15$) and most of the cells that formed them expressed the cell cycle regulator *geminin* (Quinn et al., 2001) (Supplementary Fig. 3). All together, these results indicate that most of the cells contained in clones of *dpn*-expressing cells were dividing. Interestingly, the asymmetric distribution of Mira, observed during metaphase in control NBs, was also found in mitotic *dpn*-expressing cells (Supplementary Fig. 3), suggesting that the mechanisms that control the asymmetric cell division were not perturbed.

Loss of function of dpn only causes mild effects

Next, we addressed whether a reduction of *dpn* results in an alteration in the number of NBs present during larval development, as seen in *N* mutant brains (Wang et al., 2006). To this end, we quantified CB neuroblasts at different times in *dpn* mutant larvae. To completely eliminate the function of *dpn*, we used a heteroallelic combination of the deficiencies dpn^{Def3D5} and dpn^{2}. dpn^{Def3D5} is a small deficiency that also

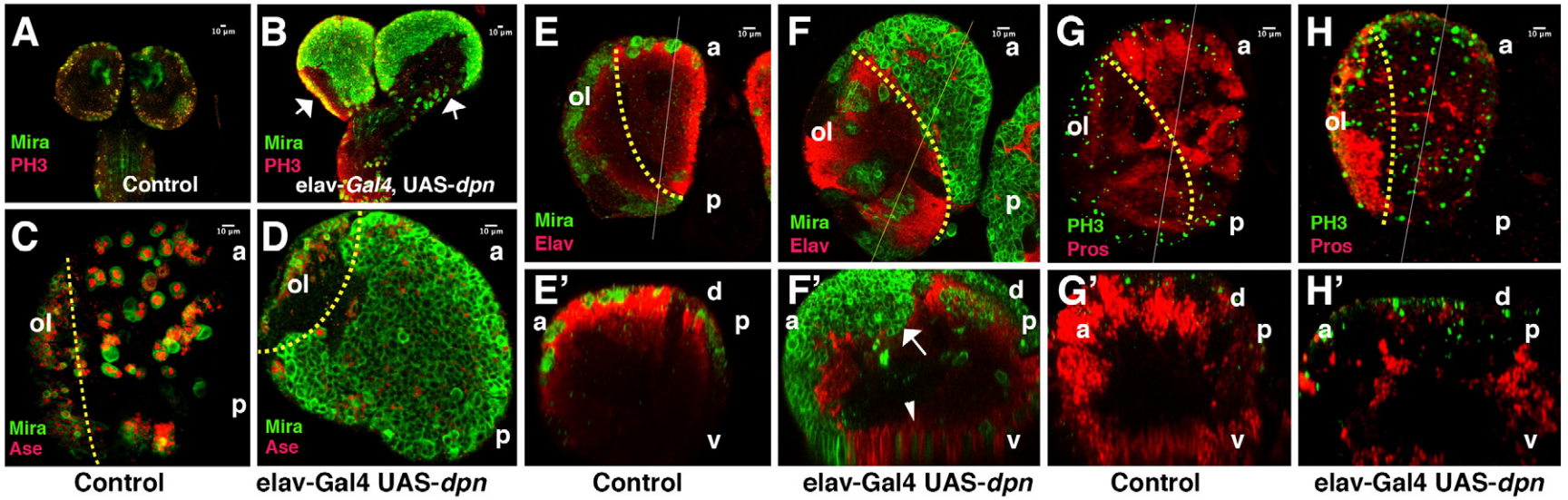


Fig. 2. Ectopic expression of *dpn* induces an excess of type II neuroblast-like cells. (A, C, E and G) Single focal planes of wild-type or (B, D, F and H) *elav-Gal4;*UAS-*dpn* third instar brains. (E', F', G' and H') Optical Z-sections throughout the entire brains of the panels E, F, G and H, respectively; the position of the cross-section is indicated by white lines. Dorsal (d) is up, ventral (v) down, anterior (a) to the left and posterior (p) to the right. (B) *elav-Gal4;*UAS-*dpn* brains are dramatically enlarged compared to control brains. These mutant brains contain an excess of NBs, marked by Mira in green, except in ventral regions (white arrows). (C) In control brains, most of NBs (marks with anti-Mira in green) are type I, since they express Ase (in red). (D) *elav-Gal4*;UAS-*dpn* brains are mostly constitute by type II NBs, as they express Mira (green), but not Ase (red). (F–F') NBs over-growth (marked by Mira in green) in *elav-Gal4;* UAS-*dpn* brains occurs at the expense of Elav-expressing neurons in red. NBs are found deep inside these brains (arrow in F'). Note that the effects are weaker in the ventral regions (arrowhead in F'). (G–G') Control and (H–H') *elav-Gal4;*UAS-*dpn* brains stained for Prospero in red and phospho-Histone 3 (PH3) in green. Note that when *dpn* is over-expressed there is a strong reduction in the number of Prospero-positive cells and an increase the number of PH3-positive.

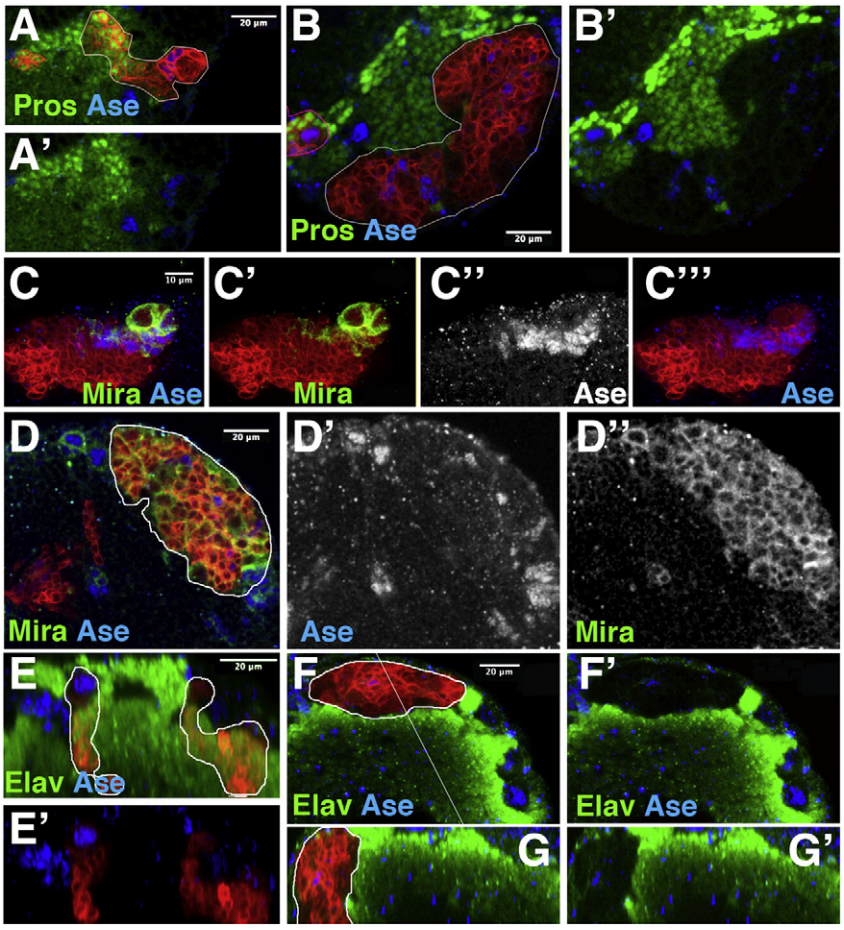


Fig. 3. Ectopic expression of *dpn* induces an excess of type II NBs. Third instar larval brains containing control (A–A', C–C''' and E–E') or MARCM UAS-*dpn* clones (B–B', D–D'', F–F' and G–G'). All clones were positively marked with GFP in red. (A–A', C–C''' and E–E') Control clones derived from type II NB (Ase$^-$ in blue in all panels) always contain a single NB that is positively marked with anti-Mira (green in C and C') and numerous smaller cells that express Prospero (green in A and A') and Elav (green in E). In panel E is also shown a clone generated in a type I NB (Ase$^+$). (B–B', D–D'', F–F' and G–G') In contrast, most *dpn*-expressing cells developed from type II NB (Ase$^-$ in blue) fail to express Prospero (green in B–B') and Elav (green in F–G') and stain positively for anti-Mira (green in D and gray in D''). Clones derived from Ase$^+$ NBs do not cause these effects, see in panels B and B' a clone generated in an Ase$^+$ NB (out line in red). (G–G') Longitudinal cross-section at the position of the white line of panel E.

affects two other genes located 5' upstream of *dpn* (see Materials and methods), whereas dpn^2 is a large deficiency that removes several genes 3' downstream of *dpn* (Barbash and Cline, 1995). Because the molecular details of the genes affected in this last deficiency are unknown, the function of additional genes might be compromised in dpn^2/dpn^{Def3D5} mutants. Therefore, we also examined larval brains of the point allele dpn^7 over dpn^2. The adult phenotypes displayed by dpn^2/dpn^{Def3D5} and dpn^2/dpn^7 mutants were very similar. In both combinations, we found few adult escapers, which were uncoordinated and flightless, suggesting that the effects observed in dpn^{Def3D5}/dpn^2 brains are caused by the depletion of *dpn*.

The total number of NBs in wild-type larvae increased during development from 17 ± 2 NBs at 0–24 h ALH to 88 ± 10 NBs at 120–148 h ALH. Of all these NBs 4 ± 0.5 at 0–24 h ALH correspond to type II NBs, which steadily increased during larval stages to reach the final number of 8 NBs at 120 h ALH (Fig. 4A and Movie 1). In both dpn^2/dpn^{Def3D5} (data not shown) and dpn^2/dpn^7 larval brains, we found that the total number of NBs was smaller during larval stages (Fig. 4). Interestingly, none of the type II NBs was observed in dpn^2/dpn^7 brains at late stages of larval development (Fig. 4 and Movie 2), and only occasionally we found one type II NBs per brain lobe in early larval brains (Fig. 4). The strong reduction of type II NBs at early stages of larval development suggests a possible function of *dpn* during embryonic neurogenesis.

To test further this hypothesis, we have analyzed the pattern of expression of Miranda and Ase during embryonic brain development. In early control embryos (12/13 stage), we observed a large number of positively staining Mira$^+$ Ase$^+$ cells (182 ± 11 $n=6$) (Fig. 4C). Some of these cells are very small, because it has been proposed that the total number of NBs at this stage is around of 100 (Urbach and Technau, 2004), likely these small cells are post-mitotic cells with a remnant of Mira. Interestingly, we found 23 ± 3 ($n=6$) large cells that do not express Ase at detectable levels. These cells are preferentially localized in the medial and posterior regions of the brain lobes (Fig. 4C). At later stages (16), the total number of Mira$^+$ Ase$^+$ cells, as well as Mira$^+$ Ase$^-$ cells are strongly reduced (Fig. 4E) (133 ± 4 $n=9$ and 6 ± 1 $n=9$, respectively), indicating that during embryogenesis some of these cells disappear. The existence of Mira$^+$ Ase$^-$ during early embryonic brain development suggests the possibility that at least part of the larval type II NBs might be originated from these cells during embryogenesis. A detail analysis of the fate of these cells could help us to understand the origin of type II NBs.

The brains of dpn^2/dpn^7 mutant embryos (stage 12/13) have a reduced number of Mira$^+$ Ase$^+$ positive cells (168 ± 20 $n=9$) compared to control brains. This reduction is significantly stronger when we only compare the number of Mira$^+$ Ase$^-$ cells (6 ± 2 $n=9$ in mutant embryos compared to 23 ± 3 in controls) (Fig. 4D). At stage 16 the total number of Mira$^+$ Ase$^+$ 113 ± 5 ($n=8$) and Mira$^+$ Ase$^-$ 5 ± 1 ($n=8$) was still reduced compared with controls (Fig. 4F), but the difference was less severe. All together, these results suggest that the function of *dpn* may be required for the specification of NBs during embryonic neurogenesis, and more specific for Mira$^+$ Ase$^-$ NBs. Further

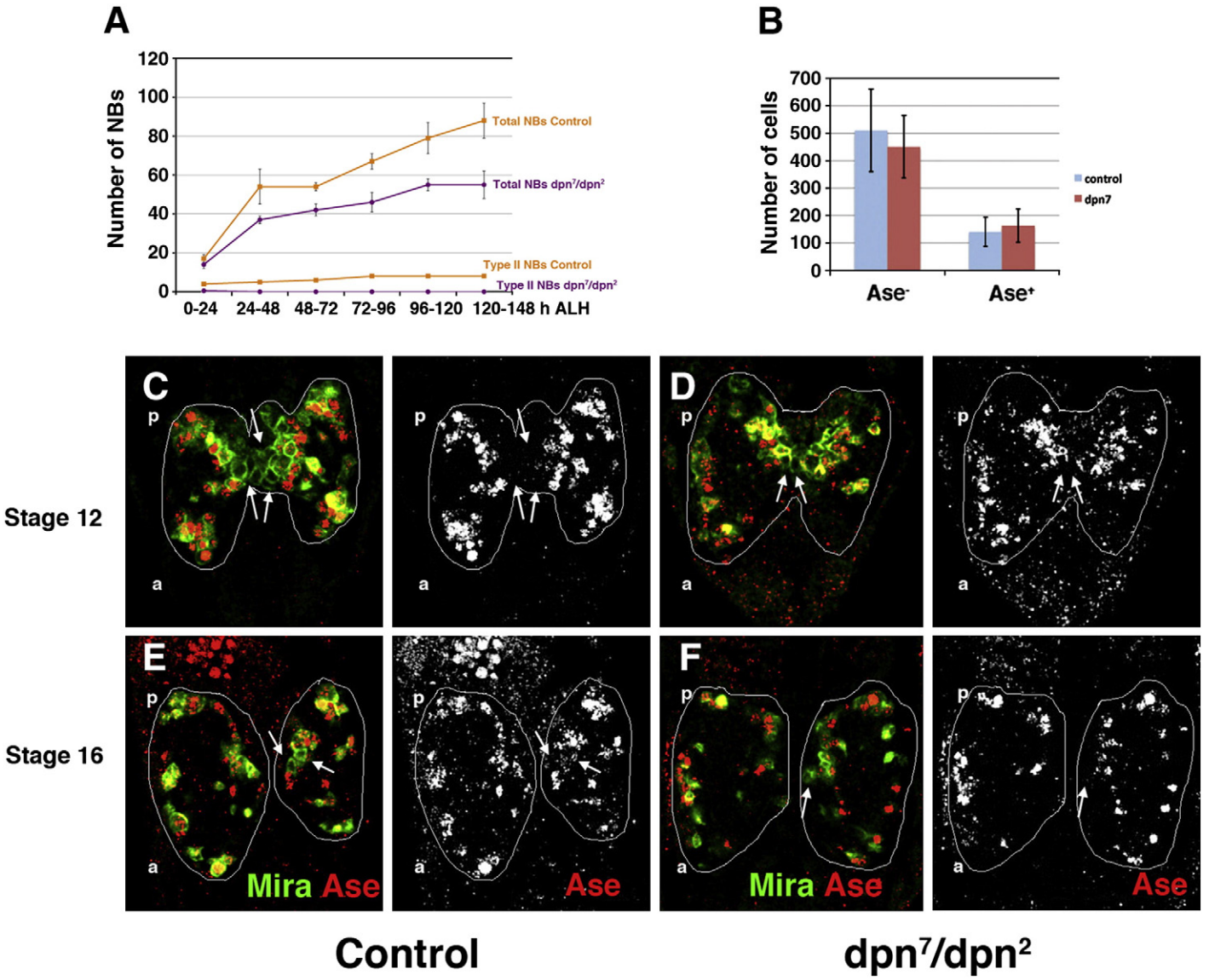


Fig. 4. (A) Quantification of the number of type I and type II NBs in wild-type and *dpn* mutant brains from 0–24 h to 122–148 h after larval hatching (ALH). Wild-type: 0–24 h $n=7$, 24–48 h $n=9$, 48–72 h $n=10$, 72–96 h $n=11$, 96–120 h $n=8$, 120–124 h $n=20$ ALH; dpn^2/dpn^7: 0–24 h $n=6$, 24–48 h $n=7$ 48–72 h $n=3$, 72–96 h $n=5$, 96–120 h $n=6$, 120–124 h $n=18$ ALH. (B) Quantitative analysis of the size of the dpn^7 clones. Clone sizes were analyzed by counting the number of cells in the clones induced at 24–48 h AEL and analyzed 96 h later, in type I (Ase^+) or type II (Ase^-) NBs. (C–F) Embryonic brains stained for Anti-Mira (green) and Anti-Ase (red). The approximately region occupied by the brain lobes is out lined in white. (C) Brain of wild-type embryo stage 12. We found multiples $Mira^+$ Ase^- cells (arrows). (D) In dpn^2/dpn^7 mutant brains stage 12, we found a reduced number of $Mira^+$ Ase^- cells compare to control brains (arrows). (E) Brain of control embryo stage 16. We still found multiples $Mira^+$ Ase^- cells (arrows). (F) dpn^2/dpn^7 brain stage 16. The arrow indicates a $Mira^+$ Ase^- cell.

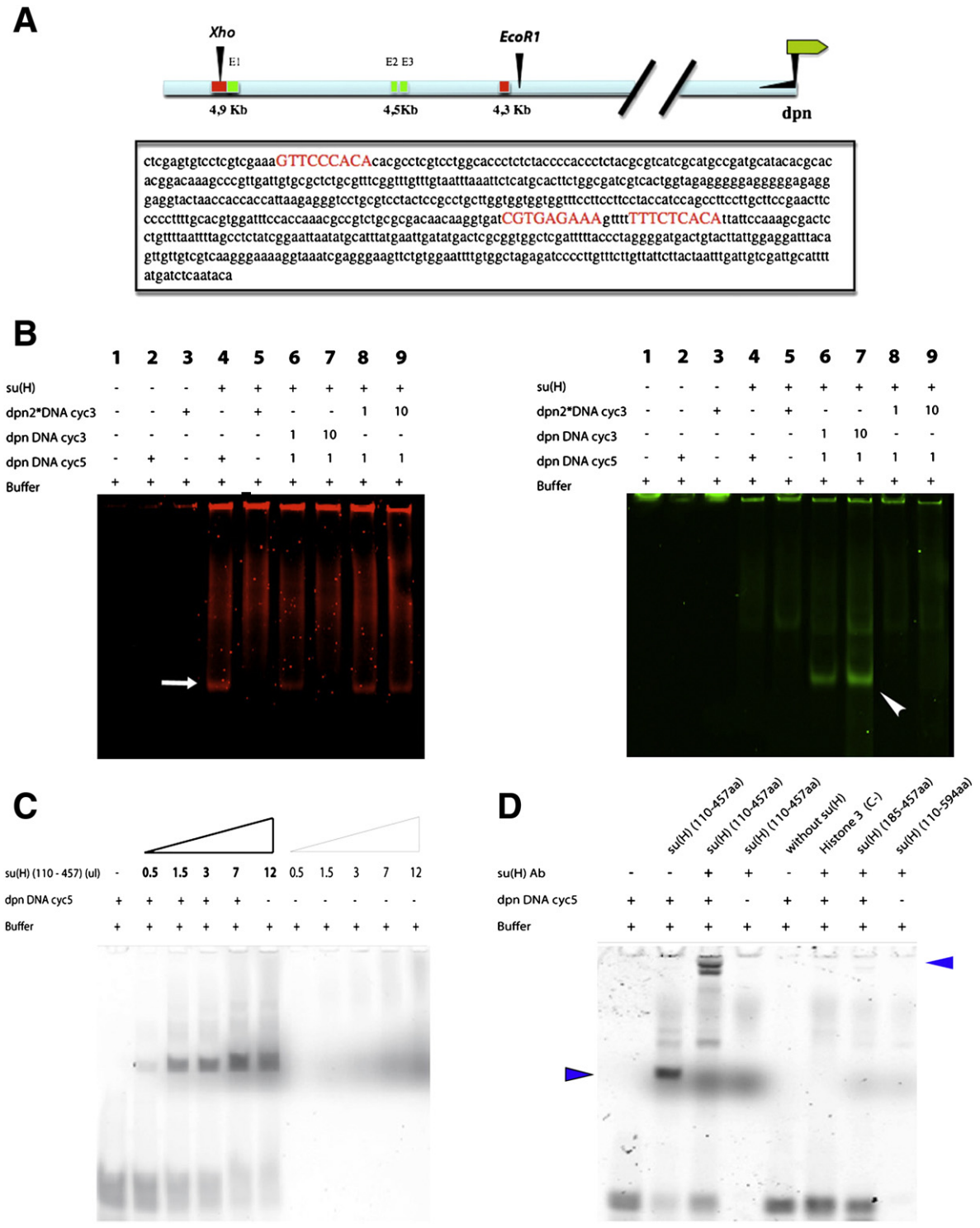


Fig. 5. (A) Schematic representation of the *dpn* promoter region. Red squares delimit an evolutionarily conserved region. Green squares mark three putative binding sites for Su(H). The specific sequence of this region is displayed under the figure, with the three consensus binding sites highlighted in red. (B) Band-shift assay. The DNA-binding properties of Su(H) were tested using cyc5dpn (red), cyc3dpn (green) and cyc3dpn2* (green) probes. In this latter probe, the putative Su(H) binding sites were mutated (see Materials and methods). The gel was cut off to show only the specific band formed by DNA–protein complex. Not band is found when Su(H) is not present (lanes 1–3). Addition of Su(H) resulted in the formation of complexes with reduced mobility (arrow in lane 4, red chanel). No band is present when the mutated probe cyc3dpn2* is added (lane 5 in green channel). (Lanes 6–9) Competition assay. The addition of equal amounts of cyc5dpn (red channel) and cyc3dpn (green channel) resulted in a band in both panels (lanes 6 in both channels). When the amount of cyc3dpn was increased, the band disappeared from the red channel and it was only observed in the green channel (arrowhead in lane 7 green channel). The addition of an equivalent amount of mutated cyc3dpn2* probe failed to compete (lanes 8–9). (C) Saturation assay. Increasing amount of in vitro-translated Su(H) (110–457 aa) was pre-incubated with a cyc5dpn probe (lanes 2, 3, 4, 5 and 6). As expected, the intensity of the band signal increased until it reached saturation. The cyc5dpn probe was also pre-incubated without Su (H) (lanes 7, 8, 9, 10 and 11). (D) Anti-Su(H) super-shift assay. The mobility of the Su(H) [110–457 aa]-cyc5dpn probe complex shifted when anti-Su(H) antibody was added. The smear observed in the lanes that contain protein is caused by the unspecific signal produced by the radiolabeled protein used in this assay.

investigation will be required to determine the details of this possible function of *dpn*.

To determine whether *dpn* mutants also affects NB lineage, we have induced clones of mutant cells for dpn^{7} and dpn^{Def1D6}. The expression of Dpn is completely eliminated in these clones (data not shown). Surprisingly, we found that these clones can be generated in both type I and type II NBs and that they always contained a single NB (data not shown). Under our experimental conditions, dpn^{7} mutant clones generated in type I NBs have a similar size to control clones. However, although the average size of dpn^{7} clones developed from type II NBs were only slightly smaller than control clones (510 ± 150 control vs. 450 ± 110 in dpn^{7}) (Fig. 4 B), we never found mutant clones as large as control clones (the largest dpn^{7} clones found was 600 cells, whereas 30% of control clones were larger than that).

The reduced number of NBs in dpn^{2}/dpn^{7} larval brains cannot be merely explained by the diminution of the number of type II NBs, as there are only eight in each brain lobe. This result suggests that *dpn* might be required during brain development for the specification and/or maintenance of type I NBs. However, we did not find that clones of loss or ectopic expression of *dpn* have any effect on type I NBs. This paradox could be explained if some of the precursors of type I NBs are misspecified during embryogenesis in *dpn* mutants.

dpn acts redundantly with other signals

Our data suggest that *dpn* might be a target gene of Notch signaling at least in INPs. Accordingly, we expected that a reduction of *Notch* function should not rescue the effects caused by clones of *dpn*-expressing cells. We have generated clones of *dpn*-expressing cells in a *Notch* temperature-sensitive mutant ($Notch^{ts}$). When mutant larvae for this allele are shifted to the restrictive temperature from the first instar larval stage onward, the number of NBs in larval brains is reduced (Wang et al., 2006) (75 ± 4 NBs compare to 88 ± 10 NBs in control at 120 ± 12 h ($n = 20$)). Clones of *dpn*-expressing cells in N^{ts} mutant background are undistinguishable from clones of *dpn*-expressing cells (Supplementary Fig. 4), suggesting that *dpn* functions downstream of Notch pathway. We also performed the reciprocal experiment by inducing clones of *dpn* mutant cells that simultaneously expressed N^{intra}. Surprisingly, we found

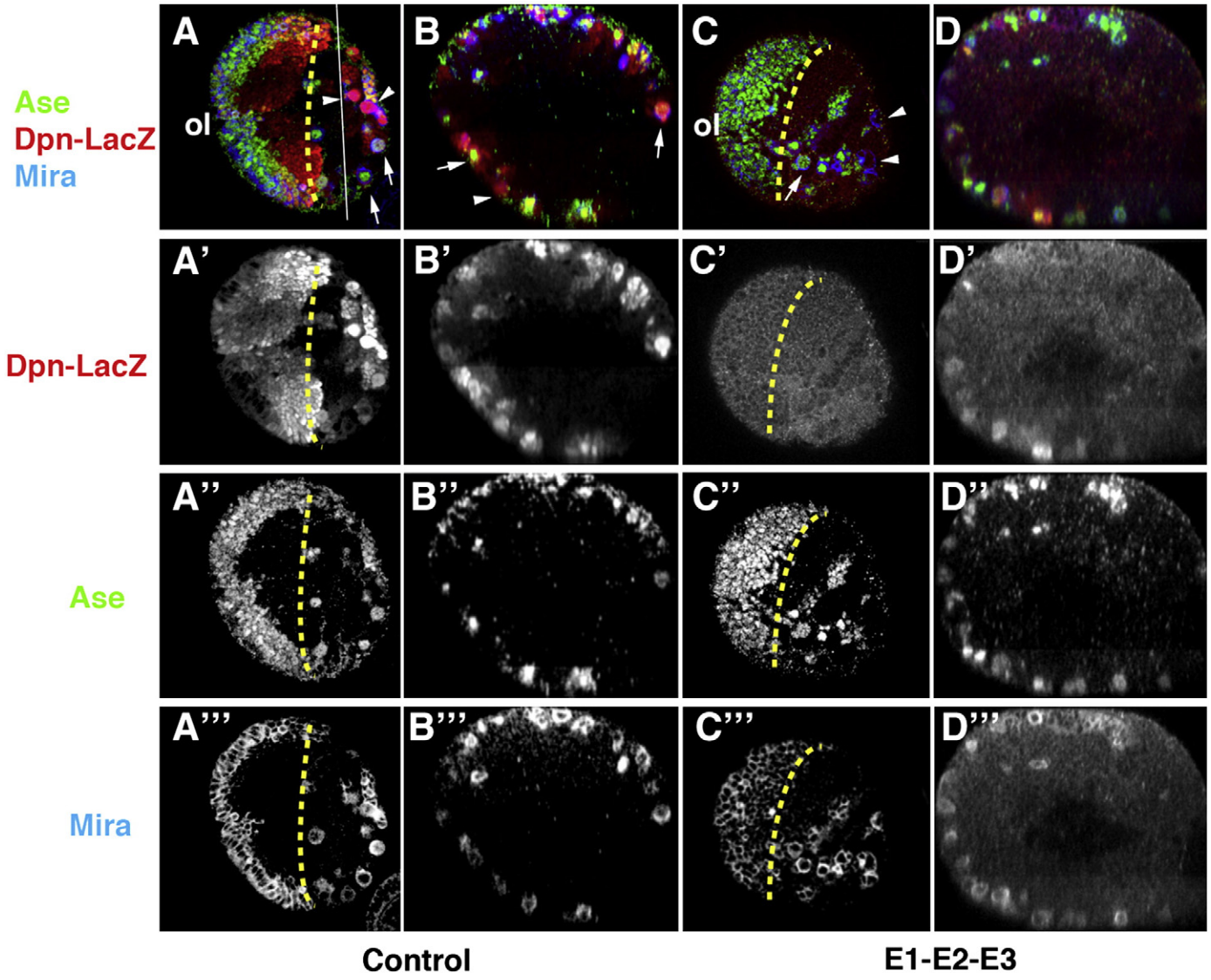
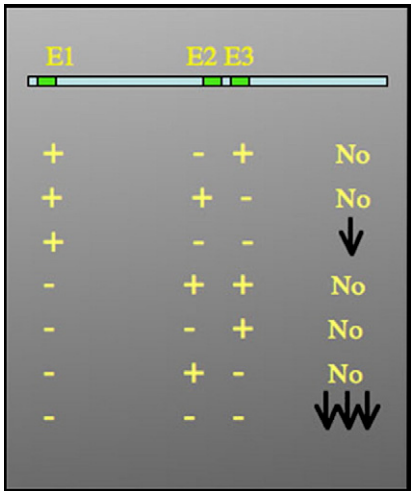


Fig. 6. *dpn*-reporter is expressed in all central brain NBs. (A–D''') Single focal planes of third instar larval brains stained with anti-β-galactosidase (red in A, B, C and D, and gray in A', B', C' and D') to reveal the pattern of expression of control *dpn*-reporter in (A, A', B and B') or a reporter with deletions on E1, E2 and E3 (C, C', D and D'). (B–B''' and D–D''') Longitudinal cross-section at the position of the white line of panels A–A''' and C–C''', respectively. Ase is shown in green in A, B, C and D and gray in A'', B'', C'' and D'', and Mira in blue in A, B, C and D and gray in A''', B''', C''' and D'''. (A–B''') Under the regulation of a *dpn*-reporter Lac-Z is expressed in both type I (Ase^{+} $Mira^{+}$, arrows in A and B) and type II (Ase^{-} $Mira^{+}$, arrowheads in A and B) NBs. (C–D''') Reporters with deletions on E1, E2 and E3 sites do not express Lac-Z in type II (Ase^{-} $Mira^{+}$, arrowheads in C) and in most of type I (Ase^{+} $Mira^{+}$, arrow in C) NBs.

Table 2
Summary of the results obtained with the different reporters used in our analysis. The number of lines analyzed for each genotype is as follows: Control *dpn*-reporter (6); E1–E2–E3 (12); E1–E2 (12); E1–E3 (9); E1 (8); E2–E3 (3); E2 (3); E3 (3).



that the lack of *dpn* did not suppress the effects caused by the ectopic activation of *Notch* signaling (Supplementary Fig. 4). This result implies the existence of at least one other, currently unidentified, target gene by which Notch maintains type II NB self-renewal and/or promotes maturation of immature INPs. This is consistent with the mild clones phenotype displayed by mutant clones of *dpn*.

Su(H) directly binds to the dpn promoter region

We have shown that Su(H) is the transducer of the function of Notch signaling during type II NBs development. Moreover, our data suggest that *dpn* might be a Notch target gene during this process (see above). Thus, the simplest molecular explanation for these results is that Su(H) acts directly on the *dpn* promoter. To test this idea, we scanned the *dpn* promoter region for putative Su(H)-binding sites. Although there are various putative Su(H) binding sites throughout the promoter region of *dpn,* the region between nucleotides 4940 and 4320 upstream of the first transcriptional initiation site, is especially interesting, as contains three putative Su(H)-binding sites within this short region (we named these sites E1, E2 and E3) (Fig. 5A). Two of these sites, E2 and E3, are adjacent, whereas E1 is only 300 nucleotides upstream. Interestingly, it has been reported that an important regulatory element is contained in this region (Emery and Bier, 1995). We found that this region is remarkably conserved among different *Drosophila* species (Supplementary Fig. 5). To test the function of this regulatory region *in vivo*, we cloned it in front of a minimal promoter driving *Lac-Z* expression. This reporter was sufficient to drive Lac-Z expression in both type I and type II NBs and in part of their progeny in a pattern resembling that obtained with Dpn antibodies (Fig. 6A–B''' and Supplementary Fig. 6). As endogenous Dpn is found specifically in NB and INP cells, the expression of the *dpn*-reporter in part of NB progeny may be due to the persistence of the Lac-Z protein or to the lack of regulatory sequences necessary to repress *dpn*. We next explored whether the putative binding sites for Su(H) contained in the *dpn*-reporter were required to drive the expression of Lac-Z in NBs and INPs. To this end, we generated several reporters in which the different Su(H)-binding sites were deleted, either singly or in combination (Fig. 6C–D''', Supplementary Fig. 7 and Table 2). Reporters containing single deletions (E1 or E2 or E3) or combinations of two deletions (E1 E2 or E1 E3) of Su(H)-binding sites reproduced the pattern of Lac-Z expression found in the control *dpn*-reporter (data not shown). However, when the two adjacent binding sites E2 and E3 were simultaneously eliminated, the level of Lac-Z expression was reduced (Supplementary Fig. 7). Reporters with deletions of all three sites (E1, E2 and E3) could not drive the expression of Lac-Z in type II NBs and in most type I neuroblasts, we only found few isolated type I NBs in the ventral region (Fig. 6C–D'''). Accordingly, we found that β-Gal expression driven by *dpn*-reporter was strongly reduced in *Su(H)* clones (Supplementary Fig. 8). These results indicate that our reporter is specifically regulated by Notch signaling. However, these results contradict our previous data, as we have found that in *Su(H)* clones Dpn expression is not affected in NBs. Although we do not fully understand the basis for this discrepancy, a plausible explanation is that the expression of *dpn* in NBs might be redundantly regulated by several other pathways. The existence of multiple enhancers in the regulatory region of *dpn* could partially replace the function of Notch signaling in NBs and it would ensure that neurons would be generated in appropriate numbers even in the absence of one or more signaling pathways. Our reporter would be specifically regulated by Notch because it only contained the Notch-responsive enhancer.

Su(H) binds to sequences of the dpn promoter region (E1–E2 and E3)

To test whether Su(H) binds to the E1, E2 and E3 sequences in the *dpn* promoter, we performed gel-shift experiments. We found that only the forms of Su(H) that contained the DNA-binding motif strongly bound to the *dpn* promoter probes (Supplementary Fig. 7D). The specificity of Su(H) for binding to the promoter region of *dpn* was tested in more detail in a competition assay (Fig. 5B and C) and with a super-shift assay (Fig. 5D). With the competition assay we also demonstrated that Su(H) only binds to probes that contain Su(H)-binding sites but fails to bind mutant *dpn* probes without these sites (Fig. 5B). These mutant probes contain the same deletions that were used in the mutant *dpn*-reporters used in our "in vivo" assay. Altogether, these results indicate that Su(H) specifically binds to the putative E1, E2 and E3 binding sites present in the *dpn* promoter.

dpn negatively regulates Prospero expression

Our data suggest that the down-regulation of *dpn* is necessary to permit differentiation of neurons during larval brain development. An opposite function has been proposed for Prospero (Choksi et al., 2006; Betschinger et al., 2006). In clones of *dpn*-expressing cells generated in type II NBs, the expression of Prospero was never found in the nucleus, and it was strongly reduced overall (Fig. 3B–B'). Thus, it is possible that *dpn* may regulate *prospero* expression. Accordingly, in clones of *dpn*-expressing cells, *prospero* is transcriptionally down-regulated in type II NBs, as assayed using a *prospero*-Lac-Z reporter (Fig. 7A–C'). However, in loss of function clones of *dpn* Pros is not up-regulated in NBs or INPs (data not shown), suggesting either that *dpn* indirectly regulates the expression of Pros or that *dpn* functions redundantly with other signals.

To further investigate whether *prospero* might function downstream of *dpn*, we generated clones that ectopically co-express *dpn* and *prospero*. Because these genes have antagonistic effects, we expected that if *prospero* functions downstream of *dpn,* its expression would suppress the phenotype induced by the ectopic expression of *dpn*. In contrast to clones of *dpn*-expressing cells, UAS-*dpn* UAS-*pros* clones were very small (1–4 cells). They did not contain Mira-positive cells, and all cells expressed the neuronal marker Elav (Fig. 7E–E'''). These effects are similar to those displayed by clones of *pros*-expressing cells (data not shown), suggesting that *prospero* might act downstream of *dpn*.

Discussion

Identifying the genetic network and intercellular signaling pathways that are involved in the control of NB self-renewal is essential in order to understand how stem cells regulate the balance between self-renewal and differentiation. Notch signaling has been proposed to be involved in

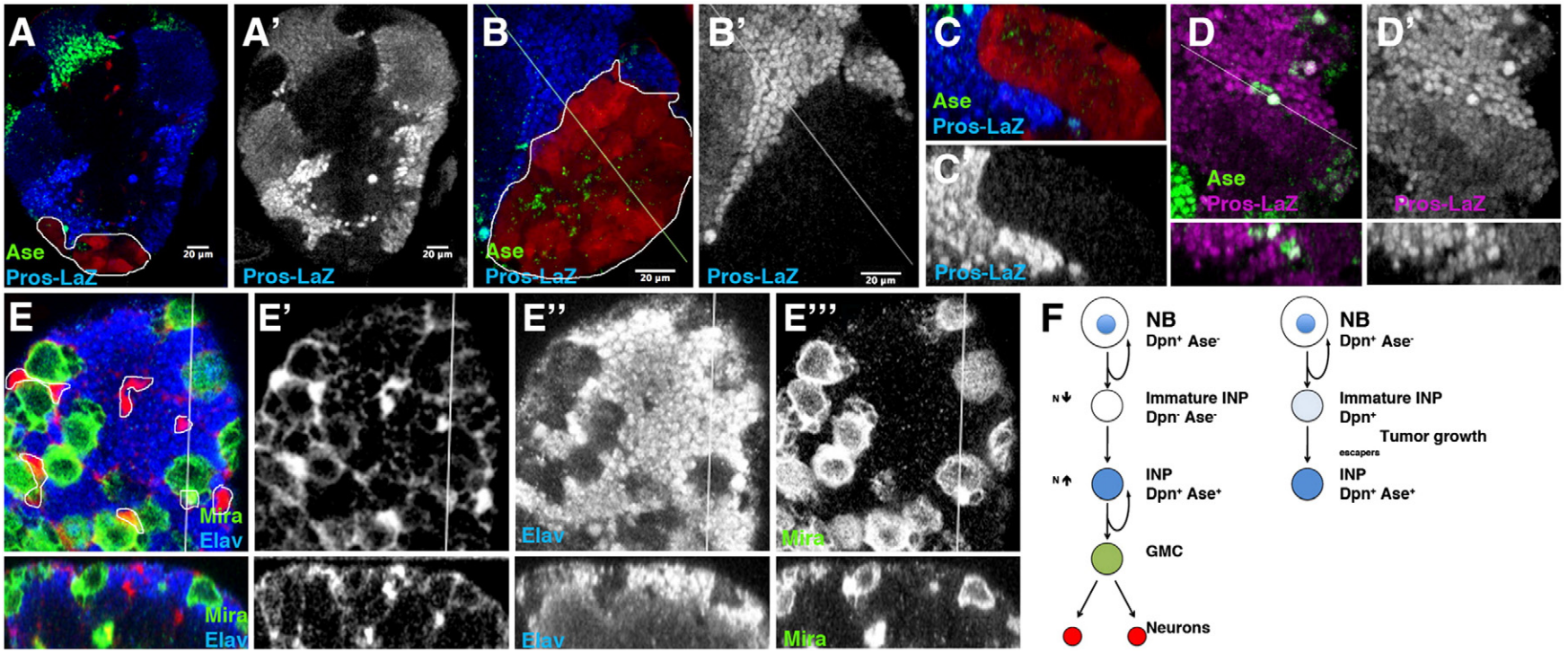


Fig. 7. *dpn* regulates the expression of *prospero* in type II NBs. (A–C') Single focal planes of third instar *FLP1.22;Act5C <FRTyellow^{+}FRT> Gal4* UAS-*GFP*/*P{PZ}pros^{10419}*; UAS-*dpn*/+ larval brains containing clones of *dpn*-expressing cells, which were positively marked with GFP in red (A, B and C), with anti-Ase (green in A, B and C) and anti-β-galactosidase (blue in A, B and C and gray in A', B' and C'). The expression of the reporter is strongly reduced in all mutant cells. These clones are mainly constituted by Ase^{-} cells. (C–C') Z-section of panel B. (D–D') Control *P{PZ}pros^{10419}* brain marked with anti-Ase (green in D) and anti-β-galactosidase to reveal the expression pattern of *pros* (violet in D and gray in D'). Note that this reporter is expressed in NBs. Longitudinal cross-section at the position of the white lines of panels D and D' are shown in lower panels. (E–E''') A brain containing clones of cells that co-express *UAS-dpn* and *UAS-prospero* (red in E and gray in E'). These clones are smaller than UAS-*dpn* clones (compare with Fig. 3), and they do not modify the expression of Mira (green in E and gray in E''') of Elav (blue in E and gray in E''). Z-section of panel E–E''' are shown in lower panels. (F) Model of type II NB lineage. During normal development type II NBs generate Ase^{-} Dpn^{-} progeny (immature INP) that mature into self-renewing Ase^{+} Dpn^{+} (INP). In this latter cells Notch signaling is required to transcriptional activates Dpn. INPs produce GMCs, which divide terminally to produce neurons. When *dpn* is ectopically expressed in immature INPs, these cells are unable to become Ase^{+} (INP) and they adopt a NB-like fate causing tumors growth.

this process (Wang et al., 2006; Bowman et al., 2008; Weng et al., 2010). During the division of type II NBs the asymmetric sequestration of Numb into one daughter cell ensures that the activity of Notch signaling is restricted to the NBs, whereas it is blocked in the other daughter cell, the immature INP cell. The down-regulation of Notch signaling in this latter cell prevents it being transformed into NBs. When Notch signaling is ectopically expressed in the immature INP, it cannot mature into an INP and it then over-proliferates as NB-like cells (Bowman et al., 2008; Weng et al., 2010). Our results indicate that this function of Notch signaling is through Su(H). Here we present evidences that suggest that the bHLH factor *dpn* is one direct target of Notch signaling during this process. Alterations in the activity of this gene reproduce the effects found when Notch signaling is ectopically activated. In addition, we have identified a regulatory region upstream of the transcriptional initiation site that drives the expression of *dpn* in NBs and INPs, which is directly regulated by Su(H). Altogether, these results lead us to propose the following model: After the asymmetric division of NBs, the down-regulation of Notch signaling in the immature INP prevents the activation of *dpn* in this cell. This process, which likely occurs in conjunction with other mechanisms that promote Dpn degradation, rapidly eliminates Dpn in immature INP. The loss of Dpn permits the maturation of the immature INP into INP cell. When Dpn is continuously expressed during asymmetric NB division, either by the activation of Notch signaling or by ectopic expression of Dpn, both recently born cells will express high levels of Dpn. This event can cause the prevention of maturation of immature INP into INP that would cause this cell to adopt its parental NB fate, entering mitosis and initiating over-growth of NB-like cells (Fig. 7F). Although, these over-growths are mostly constituted by NB-like cells, we occasionally found INPs (Ase^{+}) and also few Elav positive cells. We think that the diversity of cell types within clones of *dpn*-expressing cells is likely due to differentiation of some of the immature INPs contained in these clones. These few escaper cells can give rise to all cell types found in a type II NB lineage.

How does Notch signaling regulate dpn expression?

We have identified a Notch-responsive enhancer contained in a regulatory region upstream of the transcriptional initiation site of *dpn*. This enhancer drives the expression of *dpn* in all NBs as well as in INPs, reproducing the pattern of expression of the endogenous Dpn. These data suggest that Notch regulates the expression of *dpn* in all these cells. However, we have found that in clones of a null allele of *Su(H)*, *dpn* expression is not altered in NB and is only eliminated in the INPs. This clonal phenotype suggests that Notch signaling might function redundantly with other signals in NB. Thus, it is possible that in NBs multiples enhancers act redundantly to regulate the expression of *dpn*, and therefore its regulation depends on different signals. For instance, *numb* and *brain tumor* seem to function cooperatively to ensure the maturation of immature INP cells (Boone and Doe, 2008; Bowman et al., 2008). Brat function appears independent of Notch signaling, suggesting that additional signals are required to promote the progression of recently born cells to INPs. Thus, it is possible that several signals function redundantly to ensure that NBs would be generated in appropriate numbers even in the absence of one or more genes.

Functional redundancy?

According to our model, if *dpn* were the only target of Notch signaling during NBs proliferation, we would expect that the loss of *dpn* would be sufficient to suppress the effects caused by the ectopic activation of the pathway. However, although we find that in *dpn* mutant brains the total number of NBs is reduced and we do not find type II NBs, clones of *dpn* mutant cells always contained a single neuroblast. In addition, the loss of *dpn* is not sufficient to suppress the effects caused by the ectopic activation of Notch. Although we do not fully understand the reasons for these relatively mild clonal phenotypes, one possibility is that the system ensures its robustness by the existence of genetic redundancy. This redundancy may occur with other bHLH genes. We have tested only some members of the E(spl) complex, and therefore we cannot rule out a possible requirement of other members of this complex. This redundancy could ensure that neurons would be generated in appropriate numbers.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.01.019.

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References

Almeida, M.S., Bray, S.J., 2005. Regulation of post-embryonic neuroblasts by *Drosophila* Grainyhead. Mech. Dev. 122, 1282–1293.
Bailey, A.M., Posakony, J.W., 1995. Supressor of Hairless directly activates transcription of Enhacer of split Complex genes in response to Notch receptor activity. Gen. Dev. 2609–2622.
Barbash, D.A., Cline, T.W., 1995. Genetic and molecular analysis of the autosomal component of the primary sex determination signal of *Drosophila melanogaster*. Genetics 141, 1451–1471.
Bello, B.C., Izergina, N., Caussinus, E., Reichert, H., 2008. Amplification of neural stem cell proliferation by intermediate progenitor cells in *Drosophila* brain development. Neural Dev. 19 (3), 5.
Betschinger, J., Knoblich, J.A., 2004. Dare to be different: asymmetric cell division in *Drosophila*, *C. elegans* and vertebrates. Curr. Biol. 14, R674–R685.
Betschinger, J., Mechtler, K., Knoblich, J.A., 2006. Asymmetric segregation of the tumor suppressor brat regulates self-renewal in *Drosophila* neural stem cells. Cell 124, 1241–1253.
Bier, E., Vaessin, H., Younger-Shepherd, S., Jan, L.Y., Jan, Y.N., 1992. deadpan, an essential pan-neural gene in *Drosophila*, encodes a helix-loop-helix protein similar to the hairy gene product. Genes Dev. 6, 2137–2151.
Boone, J.Q., Doe, C.Q., 2008. Identification of *Drosophila* type II neuroblast lineages containing transit amplifying ganglion mother cells. Dev. Neurobiol. 68, 1185–1195.
Bossing, T., Udolph, G., Doe, C.Q., Technau, G.M., 1996. The embryonic central nervous system lineages of *Drosophila melanogaster* I. Neuroblast lineages derived from the ventral half of the neuroectoderm. Dev. Biol. 179, 41–64.
Bowman, S.K., Rolland, V., Betschinger, J., Kinsey, K.A., Emery, G., Knoblich, J.A., 2008. The tumor suppressors Brat and Numb regulate transit-amplifying neuroblast lineages in *Drosophila*. Dev. Cell 14, 535–546.
Choksi, S.P., Southall, T.D., Bossing, T., Edoff, K., de Wit, E., Fischer, B.E., van Steensel, B., Micklem, G., Brand, A.H., 2006. Prospero acts as a binary switch between self-renewal and differentiation in *Drosophila* neural stem cells. Dev. Cell 11, 775–789.
Datta, S., 1995. Control of proliferation activation in quiescent neuroblasts of the *Drosophila* central nervous system. Development 121, 1173–1182.
de Celis, J.F., de Celis, J., Ligoxygakis, P., Preiss, A., Delidakis, C., Bray, S., 1996. Functional relationship between Nocth, Su(H) and the bHLH genes of the E(spl) complex: the E(spl) genes mediate only a subset of Notch activities during imaginal developmet. Development 122, 2719–2728.
Delidakis, C., Artavanis-Tsakonas, S., 1992. The *Enhancer of split* locus of *Drosophila* encodes seven independent helix-loop-helix proteins. Proc. Natl Acad. Sci. USA 89, 8731–8735.
Doe, C.Q., 2008. Neural stem cells: balancing self-renewal with differentiation. Development 135, 1575–1587.
Egger, B., Gold, K.S., Brand, A.H., 2010. Notch regulates the switch from symmetric to asymmetric neural stem cell division in the *Drosophila* optic lobe. Development 18, 2981–2987.
Emery, J.F., Bier, E., 1995. Specificity of CNS and PNS regulatory subelements comprising pan-neural enhancers of the deadpan and scratch genes is achieved by repression. Development 121, 3549–3560.
Gonczy, P., 2008. Mechanisms of asymmetric cell division: flies and worms pave the way. Nat. Rev. Mol. Cell Biol. 9, 355–366.
Ito, K., Awano, W., Suzuki, K., Hiromi, Y., Yamamoto, D., 1997. The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. Development 124, 761–771.

Jan, Y.N., Jan, L.Y., 2001. Asymmetric cell division in the *Drosophila* nervous system. Nat. Rev. Neurosci. 2, 772–779.

Knoblich, J.A., 2008. Mechanisms of asymmetric stem cell division. Cell 132, 583–597.

Knust, E., Schrons, H., Grawe, F., Campos-Ortega, J.A., 1992. Seven genes of the *Enhancer of split* complex of *Drosophila melanogaster* encode Helix-loop-Helix protein. Genetics 132, 505–518.

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

Ligoxygakis, P., Yu, S.Y., Delidakis, C., Baker, N.E., 1998. A subset of Notch functions during Drosophila eye development require Su(H) an the E(spl) gene complex. Development 125, 2893–2900.

Ohsako, S., Hyer, J., Panganiban, G., Oliver, I., Caudy, M., 1994. Hairy function as a DNA-binding helix-loop-helix repressor of *Drosophila* sensory organ formation. Genes Dev. 8, 2743–2755.

Quinn, L.M., Herr, A., McGarry, T.J., Richardson, H., 2001. The *Drosophila* Geminin homolog: roles for Geminin in limiting DNA replication, in anaphase and in neurogenesis. Genes Dev. 15, 2741–2754.

Southall, T.D., Brand, A.H., 2009. Neural stem cell transcriptional networks highlight genes essential for nervous system development. EMBO J. 16, 3799–3807.

Urbach, R., Technau, G.M., 2004. Neuroblast formation and patterning during early brain development in *Drosophila*. Bioessays 26, 739–751.

Wallace, K., Liu, T., Vaessin, H., 2000. The pan-neural bHLH proteins DEADPAN and ASENSE regulate mitotic activity and cdk inhibitor dacapo expression in the *Drosophila* larval optic lobes. Genesis 26, 77–85.

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

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

Xu, T., Rubin, G.M., 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development 117, 1223–1237.

Younger-Shepherd, S., Vaessin, H., Bier, E., Jan, L.Y., Jan, Y.N., 1992. deadpan, an essential pan-neural gene encoding an HLH protein, acts as a denominator in *Drosophila* sex determination. Cell 70, 911–922.