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# bHLH-O proteins are crucial for *Drosophila* neuroblast self-renewal and mediate Notch-induced overproliferation

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## SUMMARY

*Drosophila* larval neurogenesis is an excellent system for studying the balance between self-renewal and differentiation of a somatic stem cell (neuroblast). Neuroblasts (NBs) give rise to differentiated neurons and glia via intermediate precursors called GMCs or INPs. We show that E(spl)mγ, E(spl)mβ, E(spl)m8 and Deadpan (Dpn), members of the basic helix-loop-helix-Orange protein family, are expressed in NBs but not in differentiated cells. Double mutation for the *E(spl)* complex and *dpn* severely affects the ability of NBs to self-renew, causing premature termination of proliferation. Single mutations produce only minor defects, which points to functional redundancy between E(spl) proteins and Dpn. Expression of *E(spl)mγ* and *m8*, but not of *dpn*, depends on Notch signalling from the GMC/INP daughter to the NB. When Notch is abnormally activated in NB progeny cells, overproliferation defects are seen. We show that this depends on the abnormal induction of *E(spl)* genes. In fact *E(spl)* overexpression can partly mimic Notch-induced overproliferation. Therefore, E(spl) and Dpn act together to maintain the NB in a self-renewing state, a process in which they are assisted by Notch, which sustains expression of the E(spl) subset.

**KEY WORDS: *Drosophila*, E(spl), Notch, bHLH-Orange, Dpn, Neuroblast**

## INTRODUCTION

Although the anatomy of the invertebrate versus vertebrate developing nervous system is very different, it is becoming appreciated that neural stem cells in these phyla are similar in terms of cell division patterns and molecular signatures (Brand and Livesey, 2011). *Drosophila* neuroblasts (NBs) are easy to identify, making them a favourable system for studying the balance between differentiation and self-renewal in neural stem cells. After delaminating from the ventral ectoderm (Hartenstein and Campos-Ortega, 1984), neuroblasts undergo multiple rounds of asymmetric divisions, each producing a renewed NB and a ganglion mother cell (GMC). GMCs divide once to produce two neurons (or glia). Upon division, embryonic NBs shrink and, by the end of embryogenesis, stop dividing and die or enter quiescence (Wu et al., 2008). In early larval life, feeding activates the InR and TOR pathways in the dormant NBs (Chell and Brand, 2011; Sousa-Nunes et al., 2011), which triggers new rounds of cell division. Two types of NBs have been described in the larval central brain and ventral nerve cord (VNC). Type I NBs, the majority, divide in the same mode as embryonic NBs. The only difference is that they regrow and sustain divisions for a longer time. Type II neuroblasts, of which there are only eight per brain lobe, are located dorsomedially and follow a more complex lineage. They divide asymmetrically to self-renew and produce immature intermediate progenitors (iINPs). Upon maturation, the latter divide asymmetrically a few times to self-renew and generate a GMC. The GMC divides a final time into neurons or glia (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Izergina et al., 2009; Knoblich, 2008). The existence of two transit amplifying progenitors (INPs and GMCs) ensures that type II NBs produce a large number of differentiated progeny.

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In many invertebrate and vertebrate species, bHLH-O repressors (Fischer and Gessler, 2007; Iso et al., 2003; Kageyama et al., 2007) are implicated in inhibition of neural differentiation. These proteins constitute a subclass of the basic helix-loop-helix (bHLH) transcriptional regulators containing a characteristic 'Orange' dimerization domain (Taelman et al., 2004). In *Drosophila*, seven genes of the *E(spl)* locus, encoding paralogous bHLH-O proteins, are expressed transiently in the undifferentiated neuroectoderm and inhibit NB formation; when NBs arise, they downregulate *E(spl)* expression (Delidakis and Artavanis-Tsakonas, 1992; Jennings et al., 1994; Knust et al., 1992; Nakao and Campos-Ortega, 1996; Wech et al., 1999). At the same time, however, nascent NBs turn on another bHLH-O gene, *deadpan* (*dpn*) which persists in NBs throughout embryonic and larval stages (Bier et al., 1992). Loss of *dpn* causes subtle defects in the neuronal circuitry of the animal, leading to its death in pupal stages. Loss of *E(spl)*, however, is embryonic lethal with severe NB/neural hyperplasia at the expense of epidermis, also a derivative of the neuroectoderm (Lehman et al., 1983). In vertebrates a handful of bHLH-O proteins, namely Hes1, Hes3 and Hes5, are also expressed in the neuroectoderm, accumulating within neural stem cells and downregulated in neuronal progeny (Kageyama et al., 2008). Triple *Hes1*, *Hes3* and *Hes5* knockout mice display premature neural differentiation, disruption of the neuroectoderm and a hypoplastic nervous system owing to loss of neural stem cells (Hatakeyama et al., 2004).

bHLH-O genes are transcriptional targets of Notch signalling in many contexts (Kageyama et al., 2007; Krejci et al., 2009). For example, *E(spl)* expression in the *Drosophila* neuroectoderm and *Hes1/Hes5* expression in mammalian neural stem cells are known to require Notch input. Despite their complementary expression patterns in the embryonic neuroectoderm, *E(spl)mγ*, one of the seven *E(spl)* genes, and *dpn* are later co-expressed within larval NBs. Both have been reported to be targets of Notch signalling at this stage (Almeida and Bray, 2005; San-Juán and Baonza, 2011). The role of Notch signalling (and bHLH-O proteins) in post-embryonic neurogenesis in *Drosophila* is not well understood. Null clones for the pathway seem not to affect the proliferation of most

NBs (Almeida and Bray, 2005; Monastirioti et al., 2010), whereas a more global knockdown reduces NB numbers (Wang et al., 2006) and eliminates type II lineages (Bowman et al., 2008). Conversely, overactivation of the pathway causes significant NB hyperplasia, accompanied by loss of differentiated cells (Bowman et al., 2008; Wang et al., 2006; Weng et al., 2010), although it is not clear whether this hyperplasia arises from all NBs or only from the more sensitive type II variety. The mild phenotypes resulting from *Notch* loss of function in *Drosophila* contrast with the neural hypoplasia and stem cell loss observed upon Notch knockout in mice (Imayoshi et al., 2010; Yoon and Gaiano, 2005). The paradox of the dispensability of Notch signalling in *Drosophila* is accentuated, considering the fact that, during mitosis, NBs have developed an elaborate mechanism to segregate asymmetrically Numb, an inhibitor of Notch signalling, to the GMC (Babaoglan et al., 2009; Guo et al., 1996; Rhyu et al., 1994; Spana and Doe, 1996; Wang et al., 2006). Mutations in *numb*, which are expected to result in Notch hyperactivation, lead to fully penetrant type II NB overproliferation, but only partially penetrant overproliferation in type I (Bowman et al., 2008; Lin et al., 2010; Truman et al., 2010).

In this work, we address the role of E(spl) and Dpn bHLH-O proteins in NB maintenance and proliferation, both in normal development and upon Notch-induced overproliferation. We found that both are expressed in NBs from embryogenesis onwards and have redundant functions in NB maintenance during normal development. We further showed that *E(spl)mγ* is a target of Notch, whereas *dpn* is not. Accordingly, for Notch overactivation to induce overproliferation, only *E(spl)* genes, not *dpn*, are needed.

## MATERIALS AND METHODS

### Fly strains

*Drosophila* stocks were obtained from the Bloomington Stock Center or individual laboratories.

For mosaic generation:

*y w$^{a}$ N$^{5419}$ FRT19A/ FM7*;

*w; FRT82B Dl$^{rev10}$/ TM6B*;

*w; FRT82B Ser$^{RX106}$/ TM6B*;

*w; FRT82B Dl$^{rev10}$ e Ser$^{RX106}$/ TM6B*;

*w; Su(H)$^{Δ47}$ FRT40A/ CyO*;

*h th st FRT82B neur$^{1}$ cu e/ TM6B*;

*w; mib$^{EY9780}$ FRT2A/ TM6B*;

*w; FRT82B e$^{s}$ spdo$^{G104}$/ TM3*;

*FRT82B P[gro$^{+}$] Df(3R)E(spl)$^{b32.2}$* [deficiency of the whole E(spl) locus];

*FRTG13 dpn$^{7}$/ CyO*;

*FRTG13 Df(2R)dpn$^{2}$/ CyO*;

*FRTG13 mam$^{8}$/ CyO*; and

appropriate *FRT aTub-Gal80* counter-chromosomes combined with *hs-FLP, aTub-Gal4, UAS-GFP*.

For *dpn; E(spl)* double mutant clones, we constructed *yw hs-FLP aTub-GAL4 UAS-nlsGFP; FRTG13 dpn$^{7}$/ FRTG13 aTub-GAL80; FRT82B P[gro$^{+}$]Df(3R)E(spl)b32.2/ FRT82B aTub-GAL80* larvae. In these, GFP is expressed only upon recombination at both FRTG13 and FRT82B sites.

For ectopic expression: *grh-Gal4*, *UAS-E(spl)mβ*, *UAS-E(spl)mγ*, *UAS-E(spl)mδ*, *UAS-E(spl)m3*, *UAS-E(spl)m5*, *UAS-E(spl)m7*, *UAS-E(spl)m8*, *UAS-E(spl)mγKNEQ*, *UAS-dpn*, *UAS-NΔecd*, *UAS-Nicd*, *UAS-CD8-GFP* and *aTub-Gal80ts*.

For mosaic analysis coupled with hyperactivation of Notch: *yw hs-FLP aTub-GAL4 UAS-nlsGFP; UAS-NΔecd/ +; FRT82B P[gro$^{+}$] Df(3R)E(spl)b32.2/ FRT82B aTub-GAL80* and *yw hs-FLP aTub-GAL4 UAS-nlsGFP; FRTG13 dpn$^{7}$ / FRTG13 aTub-GAL80; UAS-Nicd/+*.

Corresponding control clones were obtained by substituting the mutant chromosome with *FRT82B lacZ* or *FRTG13*, respectively.

For mosaic analysis coupled with overexpression of *UAS-E(spl)mγ*: *y w hs-FLP aTub-GAL4 UAS-nlsGFP; FRT40A Su(H)$^{Δ47}$/ FRT40A aTub-Gal80; UAS-mγ* and *y w hs-FLP aTub-GAL4 UAS-nlsGFP; FRT40A πMyc/ FRT40A aTub-Gal80; UAS-mγ*.

### Immunohistochemistry

Fixation and immunohistochemistry of embryos and larval tissues was performed according to standard protocols. Primary antibodies were rabbit-anti-β-gal (Cappel); rat-anti-Elav 7E8A10 (DSHB); rabbit-anti-GFP (Minotech); mouse-anti-GFP (Molecular Probes); guinea-pig-anti-Hey (Monastirioti et al., 2010); rat-anti-Dpn (Boone and Doe, 2008); rabbit-anti-Ase (a gift from A. Jarman, University of Edinburgh, UK); mouse-anti-Pros MR1A (DSHB); and mouse-anti-CycE (a gift from H. Richardson, Peter MacCallum Cancer Centre, Melbourne, Australia).

To monitor E(spl) expression patterns we used the *E(spl)mγGFP* genomic transgene (Almeida and Bray, 2005). Owing to its weak expression [probably reflecting inherently low expression of the *E(spl)mγ* gene], a line carrying four transgenic copies was used for embryos; a single copy was sufficient for larval experiments. In all cases, anti-GFP Ab staining was performed, as native GFP fluorescence was too weak to detect. *E(spl)m8lacZ* (Lecourtois and Schweisguth, 1995), *E(spl)mβ-0.5-lacZ* (Cooper et al., 2000) and an *E(spl)m7* genomic transgene bearing a Myc-tag (P. Piwko and C.D., unpublished) were also used.

Secondary antibodies were conjugated to Alexa488, 555, 568, 633 or 647 (Molecular Probes), or Cy3 (Jackson ImmunoResearch). Samples were imaged on Leica SP2 confocal microscope (University of Crete confocal facility).

### Quantitative PCR (qPCR)

Larval brains were dissected and RNA was extracted using the RNeasy mini kit (Qiagen). cDNA was generated by RT-PCR with ImProm-II Reverse Transcription System (Promega). All qPCR reactions were performed in triplicates with QuantiTect SYBR Green PCR kit (Qiagen). Generation of specific PCR products was confirmed by melting-curve analysis. The calibration curve was constructed from serial dilutions of genomic DNA, and values for all genes were normalized to the levels of *rp49*. For data analysis, the second-derivative maximum method was applied and fold-induction of target cDNA was calculated. Primer sequences are available upon request.

### Electrophoretic mobility shift assay (EMSA)

Full-length CDSs of *dpn*, *m8* and *mγ* were cloned in pRSET-A. Proteins were produced using the T7 polymerase TnT in vitro coupled transcription-translation kit (Promega).

The $E_B$-box primers 5′ TGTCTGTGG**CACGTG**CTAATG 3′ and 5′ TGGCATTAG**CACGTG**CCACAG 3′ were annealed and radiolabelled using Klenow *E. coli* DNA polymerase. Binding was performed on ice for 20 minutes with 10 ng/μl probe, 5% glycerol, 20 mM Hepes (pH 7.9), 100 mM KCl, 2 mM $MgCl_2$, 2 mM spermidine, 1 mM DTT, 1 mM PMSF and 10 ng/μl poly-dI-dC. The reaction was electrophoresed on a non-denaturing polyacrylamide gel at 4°C. The gel was imaged using Molecular Dynamics Storm 840.

## RESULTS

### *E(spl)mγ* is expressed in both neuroectoderm and neuroblasts of the embryonic CNS

We revisited the embryonic expression of E(spl)mγ to determine whether it becomes activated in the NBs prior to larval stages. We used an *E(spl)mγGFP* transgenic line (Almeida and Bray, 2005) to detect E(spl)mγ via a GFP antibody, as the available E(spl) antibodies are not as sensitive. At stage 8, before NB segregation, E(spl)mγ is expressed in the neuroectoderm while Dpn is off. By stage 9 (Fig. 1A), the first neuroblasts delaminate from the neuroectoderm and are Dpn positive and E(spl)mγ negative, as previously described (Jennings et al., 1994). E(spl)mγ expression is first weakly detected in NBs at stage 10 (Fig. 1B, arrows) and by stage 11 all NBs express both Deadpan and E(spl)mγ (Fig. 1C). By

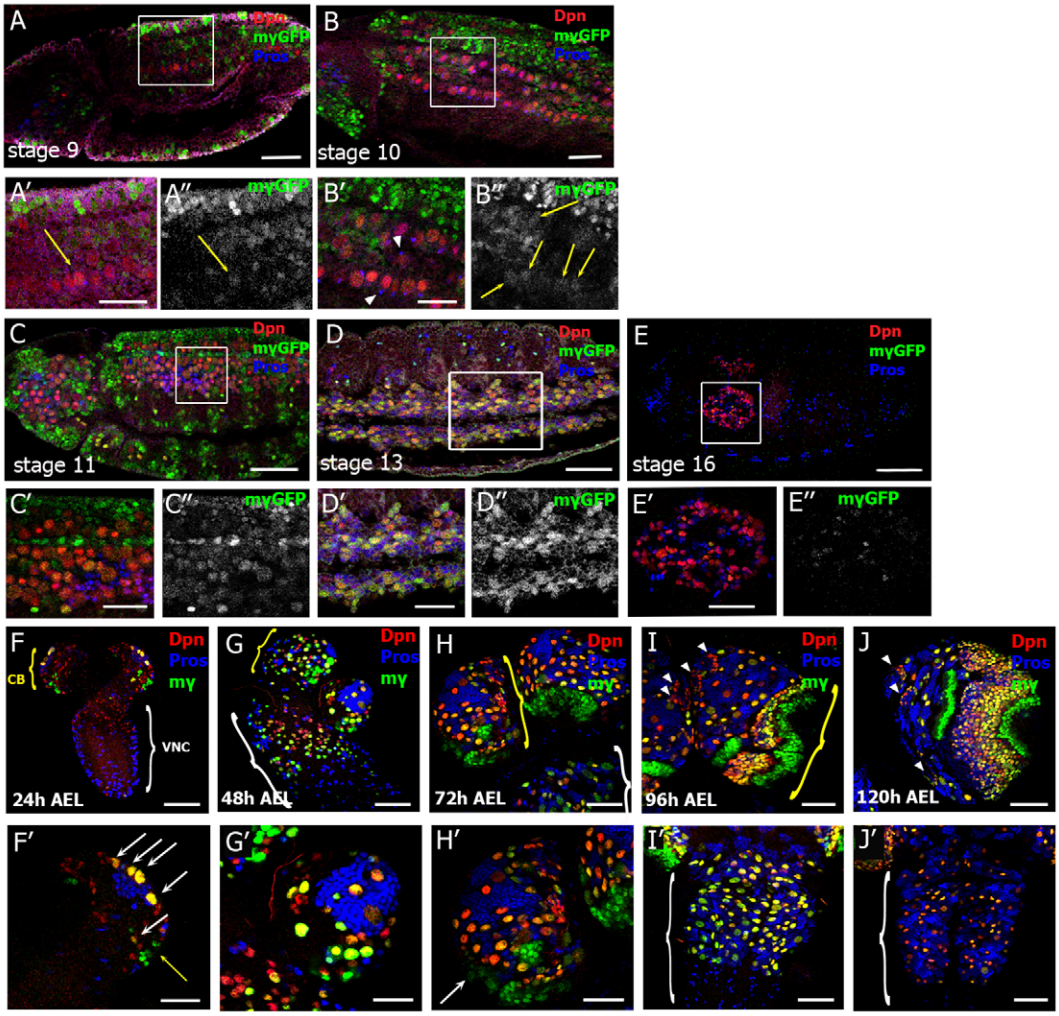


**Fig. 1. Expression of E(spl)mγ and Dpn in the embryonic and larval CNS.** (A-E) Confocal projections of *E(spl)mγ-GFP* embryos stained for GFP (green), Dpn (red) and Pros (blue). Anterior is towards the left. (A′-E′) Higher magnifications of boxed areas; (**A-A″**) Stage 9 embryo. Dpn is expressed in NBs, whereas E(spl)mγ is expressed in the neurectoderm (top of panels, superficial layer) and in a few neuroblasts (arrow). (**B-B″**) Stage 10 embryo. E(spl)mγ is now expressed in more neuroblasts (arrows). Arrowheads indicate Pros-positive GMCs. (**C-C″**) Stage 11 embryo. All neuroblasts are both Dpn positive and E(spl)mγ positive. (**D-D″**) The same is observed in a stage 13 embryo. (**E-E″**) In a stage 16 embryo, neuroblasts are small in size. E(spl)mγ is only barely detected. (F-J) Confocal projections of larval CNSs. A brain lobe is marked with a yellow bracket, the VNC is marked with a white bracket. (**F**,**F′**) Freshly hatched larva, 24 hours after-egg-laying (AEL). E(spl)mγ is expressed in only four large proliferating NBs, the mushroom body NBs, as well as a small lateral NB (white arrows in F′). E(spl)mγ-positive/ Dpn-negative cells are the optic lobe primordium (yellow arrow). (**G,G′**) 48h AEL: E(spl)mγ is co-expressed with Dpn in NBs that have enlarged and exited quiescence. (**H**,**H′**) 72h AEL: White arrow marks the optic lobe. (**I**,**I′**) 96h AEL. (**J**,**J′**) 120h AEL. Arrowheads show Type II NBs. Scale bars: 75 μm in A,C,E; 73 μm in B; 24 μm in B′,B″,C′,C″,D′,D″; 48 μm in A′,A″,D,F,G,H,I-J′; 32 μm in E′,E″,H′; 19 μm in F′,G′.

stage 13, when NBs delamination is complete, E(spl)mγ is lost from the neuroectoderm, but persists in the NBs (Fig. 1D). At the end of the embryonic proliferative period (stage 16) most neuroblasts decrease in size and enter quiescence. These neuroblasts remain Dpn positive but turn E(spl)mγ off, with only a few displaying weak levels (Fig. 1E). We conclude that, after an initial downregulation at the time of NB birth, *E(spl)mγ* is re-expressed in the embryonic neuroblasts in addition to the overlying neuroectoderm. NB expression is correlated with mitotic activity. It begins when the first Pros-positive cells (GMCs) are detected (Fig. 1B′, arrowheads) and is downregulated at the time NBs enter quiescence (Fig. 1E). Dpn, however, is expressed before embryonic neuroblasts start dividing and remains on at the onset of quiescence; therefore, it does not correlate with mitotic activity.

## bHLH-O genes are expressed in the larval CNS

We also monitored the expression of E(spl)mγ and Dpn throughout larval life. Dpn is detected in neuroblasts of newly hatched larvae [24 hours after egg laying (AEL)] indicating that it remains active throughout quiescence (Fig. 1F). These early larval Dpn-positive NBs are small and E(spl)mγ-negative, with the exception of five neuroblasts, one lateral and the four anterodorsal mushroom body NBs (Fig. 1F, white arrows). These four are the only NBs that never cease proliferating in the embryo-to-larva transition (Ito and Hotta, 1992; Sousa-Nunes et al., 2011). E(spl)mγ is also found in a few Dpn-negative cells, which will give rise to the optic lobe (Fig. 1F, yellow arrow). By early L2 stage (48 hours AEL, Fig. 1G), neuroblasts increase in size, become E(spl)mγ-positive and

start dividing, as evidenced by the appearance of neighbouring Prospero-positive GMCs. This continues till the end of larval life (Fig. 1H-J).

We further tested whether other members of the *E(spl)* Complex are also activated in larval NBs. As no specific antibodies were available, we used a Myc-tagged genomic construct for *E(spl)m7* (P. Piwko and C.D., unpublished) and several *lacZ* reporters (supplementary material Fig. S1). We found that *E(spl)m8* and *E(spl)mβ* are expressed in larval neuroblasts, whereas *E(spl)m7* is mostly expressed in the optic lobe and weakly in the neuroblasts and some surrounding cells. We did not test *E(spl)m3*, *E(spl)m5* and *E(spl)mδ* owing to lack of availability of reporter lines in our lab**.** We conclude that, besides *mγ*, additional *E(spl)* genes are expressed in post-embryonic NBs.

## The function of bHLH-O proteins in larval neuroblasts

To test the role of Dpn and E(spl) in proliferation, we performed mosaic genetic analysis in L2-L3 CNSs, where NB proliferation rate is at its highest. Type I lineages deficient for all *E(spl)* displayed no defects in the type of progeny they generated compared with wild type. Each mutant lineage contained a large Dpn/Ase-positive NB, 3-5 Ase/Pros-positive (Dpn-negative) GMCs and many Pros-positive (Dpn/Ase-negative) neurons (Fig. 2A-C). Type II lineages, however, displayed a slight decrease in the number of INPs compared with wild type. Immature INPs are characterized by the lack of staining for all Dpn, Ase and Pros and their proximity to the parent type II NB (Dpn positive, Ase negative). Mature INPs are slightly further from the NB and express both Ase and Dpn (Boone and Doe, 2008; Bowman et al., 2008). *E(spl)*$^{-}$ type II clones contained a single Dpn-positive/Ase-negative NB, a few adjacent Dpn/Ase-negative iINPs and ~10 Dpn/Ase-positive mINPs, compared with ~25 in wild type (Fig. 2E,F). Singly mutant clones for *dpn* also exhibited normal type I lineages and a similar decrease in the number of INPs in type II lineages (Fig. 2D,G).

In contrast to the virtual absence of defects observed in single *dpn* or *E(spl)* clones, double *dpn; E(spl)* mutants displayed a dramatic phenotype. In 48/64 (75%) of type I mutant lineages, a NB could not be detected and there were fewer neurons per lineage (5.5 cells/lineage; Fig. 2H,J) compared with wild-type clones of the same age (23 cells/lineage). The remaining double mutant lineages appeared normal (22 cells/lineage; Fig. 2I,K,L). A likely scenario is that NB loss probably took place gradually over the 3 days between clone induction and fixation; up until their loss, the mutant NBs kept dividing, accounting for the (small) number of mutant neurons detected in the NB-less clones. The one-quarter of the lineages that still had not lost their NB displayed a normal size, suggesting that proliferation rate was not altered by *dpn; E(spl)* loss. Therefore Dpn and E(spl) possess a redundant function that is needed for NB long-term maintenance rather than for ongoing proliferation. Type II mutant clones were never recovered, suggesting that *dpn; E(spl)* loss of function has a more severe effect in these lineages, perhaps immediate cessation of proliferation.

We also addressed whether bHLH-O proteins are needed for NB maintenance during quiescence. For this reason, we carefully monitored NB number in larvae immediately after hatching and for the whole of larval life at 24-hour intervals. We expected that *dpn* loss of function might prevent NB reactivation, as it is strongly expressed during the late embryonic stages of NB quiescence. Although a reduction in larval NB numbers was found, it was rather moderate. By late third-instar, $dpn^{2}/dpn^{7}$ had an average of 52 NBs (wild type=95) per brain lobe, as seen also by San-Juán and Baonza (San-Juán and Baonza, 2011), and 100 NBs (wild type=150) per VNC (supplementary material Fig. S2), suggesting that a large number of NBs can exit quiescence. Indeed almost all wild-type and $dpn^{2}/dpn^{7}$ NBs had turned on Ase expression and were surrounded by Pros-positive GMCs/neurons, indicating mitotic activity, by 48 hours after hatching (supplementary material Fig. S2H). We therefore entertained the possibility that another bHLH-O factor may act redundantly with Dpn even during quiescence. Indeed, the fact that E(spl)mγ is barely detectable in late embryos does not preclude the possibility that another E(spl) family member is expressed in NBs at that time, so we asked whether loss of the *E(spl)* locus might enhance the *dpn* phenotype As *E(spl)*$^{-}$ homozygous embryos do not hatch, owing to earlier neuroectoderm defects (Lehman et al., 1983), we could not test the complete null state for *E(spl)*. However, upon halving the *E(spl)* dose in a $dpn^{2}/dpn^{7}$ background, we observed a dramatic phenotypic enhancement in three respects. First, embryonic lethality was increased: $dpn^{2}/dpn^{7}$*; E(spl)*$^{b32.2}$/+ larvae hatched at 31% of expected frequency, whereas the control and $dpn^{2}/dpn^{7}$ genotypes hatched at 80% of expected. Second, total Dpn-positive NB numbers were strongly reduced (supplementary material Fig. S2M,N), suggesting premature NB loss around the time of quiescence. Finally, among the remaining NBs, only a few were active (Ase-positive) 24 hours after hatching, a time when most control [$dpn^{2}$/+*; E(spl)*$^{b32.2}$/+] and $dpn^{2}/dpn^{7}$ NBs had already re-entered the cycle (supplementary material Fig. S2O). Inability to reactivate NBs after hatching was accompanied by fully penetrant first-instar lethality, precluding analysis at later stages. Therefore, *E(spl)* cooperates with *dpn* to maintain the NB fate both during proliferative and during quiescent phases.

Motivated by this genetic redundancy, we hypothesized that the cohort of bHLH-O proteins expressed in NBs (at least Dpn, mγ, mβ, m8) could have the same effects on NB chromatin. As a preliminary test, we asked whether they can form DNA-binding competent heterodimers, using the electrophoretic mobility shift assay (EMSA). We tested an $E_B$-box oligo [a high-affinity binding site for bHLH-O proteins (Jennings et al., 1999)] with various combinations of in vitro translated Dpn, mγ and m8 proteins. We readily detected homodimers for all proteins. Upon mixing two bHLH-O proteins, we also observed new shifted complexes that point to the existence of all possible heterodimers (supplementary material Fig. S3). These biochemical results are consistent with a redundant role between Dpn and the E(spl) bHLH-O proteins in larval NB maintenance.

## Notch signalling activates *E(spl)mγ* and *m8*, but not *dpn*, expression in neuroblasts

*E(spl)* genes are common transcriptional targets of Notch signalling (Bray and Bernard, 2010). We tested Notch dependence for *E(spl)m8*, *E(spl)mγ* and *dpn*; there are reports of the latter two being targets of Notch (Almeida and Bray, 2005; San-Juán and Baonza, 2011). We made mosaics for null alleles of various components of the pathway and assayed *lacZ*-reporters of *E(spl)mγ* and *E(spl)m8*, as well as Dpn protein. A cohort of mutations were studied that affect Notch signal reception [*N, Su(H), mam, spdo*] or emission [*Dl, Ser, neur, mib1*]. With the exception of *Ser* and *mib1*, which showed no defects, disruption of the Notch pathway affected *E(spl)* but not *dpn* expression (Fig. 3; supplementary material Fig. S4; Table 1). Specifically, *E(spl)mγ-lacZ* was completely abolished, whereas *E(spl)m8-lacZ* was either abolished or strongly downregulated.

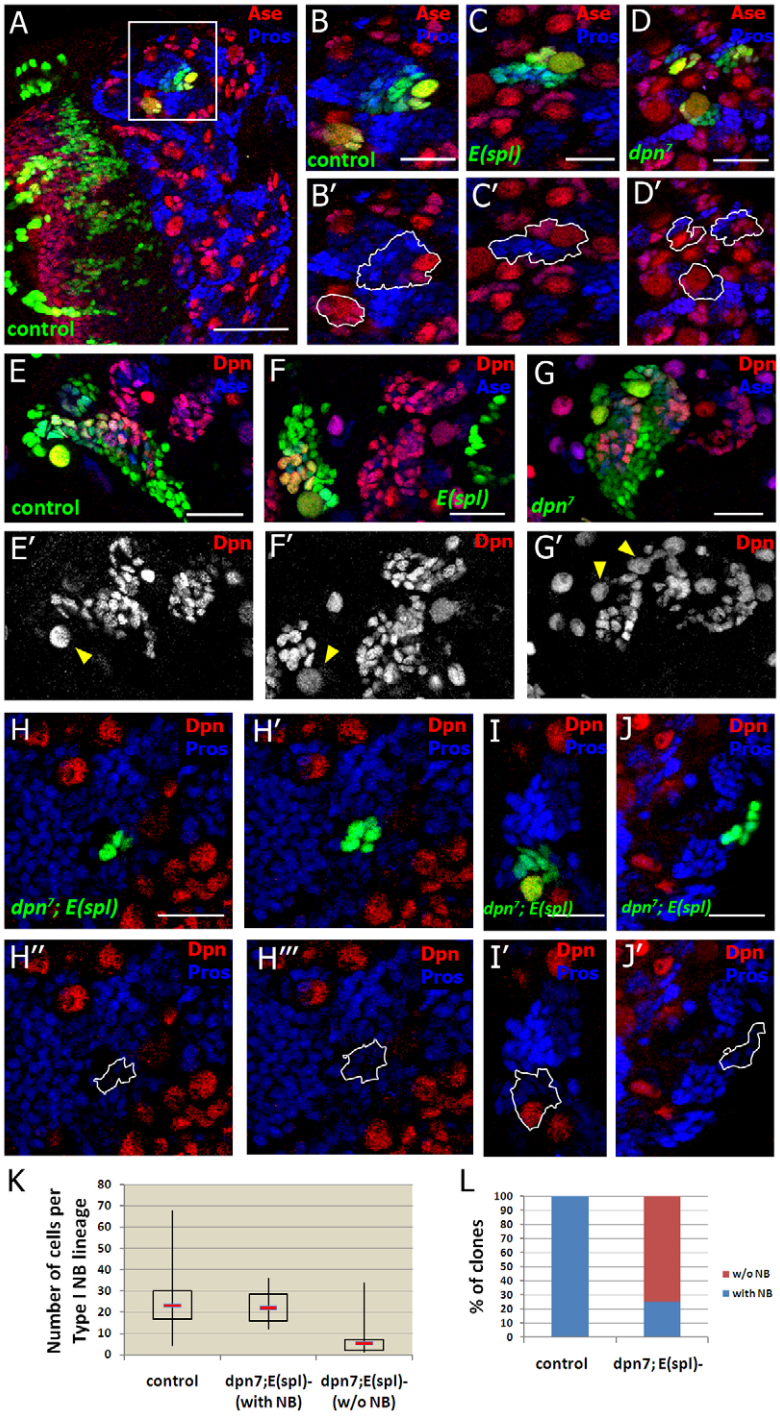


**Fig. 2. The function of Dpn and E(spl)s in larval neuroblasts.** GFP marked larval CNS clones. (**A**) Ventral brain-lobe and (**B**) enlargement of boxed area show control 3-day-old type I clones stained for Ase (red) and Pros (blue). (**C**,**D**) Clones deficient for *E(spl)* (C) or *dpn* (D) appear wild type. (**B′-D′**) Ase/Pros channels only; clones are outlined. (**E-G′**) Type II clones stained for Dpn (red) and Ase (blue). (E) Wild-type lineage. (F) *E(spl)* mutant lineage contains fewer INPs. The same holds for the two $dpn^{7}$ clones in G. Arrowheads indicate GFP-marked type II NBs. (**H-J′**) *dpn; E(spl)* type I lineages stained for Dpn (red) and Pros (blue). (H,H″) Superficial view of a dorsal brain clone; no NB is detected. (H′,H‴) Deeper layer from the same clone. (I,I′) A *dpn; E(spl)* VNC clone appears wild type. (J,J′) Another *dpn; E(spl)* clone lacking the NB. Scale bars: 48 μm in A; 24 μm in B-F′,I-J′; 19 μm in G-H‴. (**K**) Box-plot diagram depicting the total number of cells per type I lineage in 104 control versus 64 *dpn; E(spl)* clones, of which 16 contained a NB and 48 did not. The box encloses the central 50% of the distribution of values; the pink line indicates the median. (**L**) Clone frequencies.

While this work was in progress, San-Juán and Baonza (San-Juán and Baonza, 2011) reported that *dpn* contains a NB-specific enhancer that is regulated by Notch signalling. Although seemingly contradictory to our results, they also observed that Dpn protein expression is not affected in a *Su(H)* background; therefore, the Notch-responsive enhancer must play only a minor role in *dpn* expression. We also examined Dpn expression in embryos mutant for Notch pathway components. In these genotypes, the hyperplastic NBs were strongly Dpn positive (supplementary material Fig. S5), even at late embryonic stages, consistent with the fact that *dpn* expression is independent of Notch signalling in embryos, as in larvae.

Despite the near extinction of *E(spl)mγ* and *m8* expression, no obvious effect was seen in Notch pathway mutant type I lineages in the larva: they consisted of one Dpn-positive NB, 2-5 GMCs and several neurons, as noted before (Almeida and Bray, 2005; Monastirioti et al., 2010; San-Juán and Baonza, 2011). Instead, type II clones were recovered less efficiently and showed reduced proliferation (supplementary material Fig. S6). The ‘resilience’ of type I clones to Notch disruption may be due to the persistence of Dpn (and partially m8) expression, as we showed above that Dpn and E(spl) have redundant functions. To test this hypothesis, we generated doubly mutant clones for null *mam* and *dpn* alleles, as *mam* mutation severely affects *E(spl)* expression (Fig. 3;

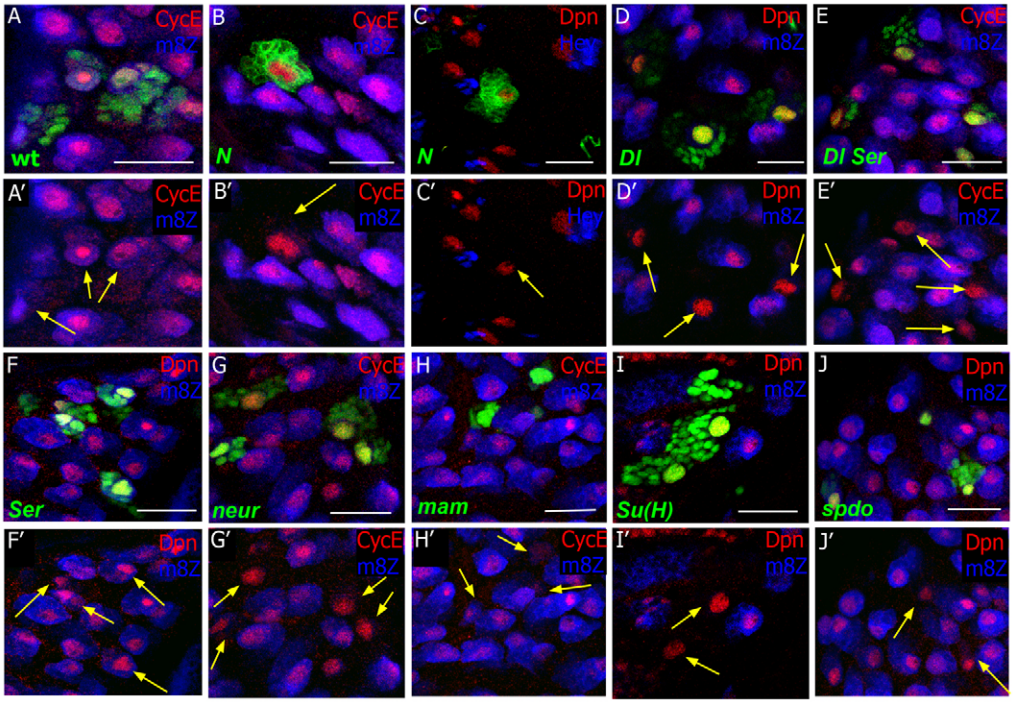


**Fig. 3. Notch pathway disruption affects *E(spl)m8* but not *dpn* or *cycE* expression in the larval CNS.** GFP-marked clones (green) stained for β-galactosidase (*E(spl)m8-lacZ*) or Hey (blue) and Dpn or CyclinE (red), as indicated. Hey is a Notch target gene expressed in secondary neurons (Monastirioti et al, 2010). GFP is nuclear in all panels except in Notch clones (B,C) where a membrane-targeted GFP was used. In A′-J′ the GFP channel has been removed. Yellow arrows indicate marked NBs. (**A**,**A′**) neutral (wild-type) clones. (**B-C′**) $N^{5419}$ mutant clones display marked reduction in *E(spl)m8-lacZ* expression levels, whereas CyclinE and Dpn are unaffected. (**D**,**E**) The same holds for $Dl^{rev10}$ and $Dl^{rev10}$ $Ser^{RX106}$ clones. (**F**,**F′**) $Ser^{RX106}$ clones appear wild type. (**G**,**G′**) $neur^{1}$, (**H**,**H′**) $mam^{8}$, (**I**,**I′**) $Su(H)^{\Delta 47}$ and (**J**,**J′**) $spdo^{G104}$ clones show reduction or elimination of E(spl)m8-lacZ, but not Dpn. Scale bars: 24 μm in A-B′, D-J′; 20 μm in C,C′.

supplementary material Fig. S4). We observed frequent NB loss in $dpn^{7}$ $mam^{8}$ clones (60%), much more than observed in single clones (0-25%; supplementary material Fig. S7), suggesting that *dpn* removal aggravates the effects of Notch pathway disruption, as predicted.

A useful corollary from our mosaic analysis regards the cells involved in this Notch signalling event. There were no instances of 'exceptional' NBs expressing *E(spl)mγ* in mutants known to act non-cell-autonomously (*Dl*, *Dl Ser* and *neur*; collectively, 151 type I lineages scored). We therefore conclude that the signal responsible for *mγ* expression emanates from cells within the lineage of the mutant NB, otherwise the mutant NB would be able to respond to signal from an adjacent lineally unrelated $Dl^{+}$ cell. The cells that signal to the NB are most likely to be the adjacent GMCs, which stay in close contact with their parent NB. This interpretation is also consistent with the fact that embryonic NB *mγ* expression starts at the same time as GMCs are first detected (Fig. 1).

## Hyperactivation of Notch increases bHLH-O expression levels and causes overproliferation of larval neuroblasts

As our results suggested a role for E(spl) proteins and Dpn in maintaining the NB and opposing premature differentiation, we examined how these proteins behave upon overactivation of Notch and also whether they contribute to the hyperactive Notch phenotype of NB overproliferation. We ectopically expressed a UAS-NΔecd transgene, a constitutively active Notch deleted for its extracellular domain (Rebay et al., 1993; Fuerstenberg and Giniger, 1998), under the *grh-Gal4* driver (Prokop et al., 1998). *grainyhead* is expressed in most post-embryonic neuroblasts and their GMCs, outside the optic lobe (supplementary material Fig. S8). As overexpression of NΔecd throughout development is lethal, we added temporal control in the system using the $Gal80^{ts}$ inhibitor of Gal4, which blocks UAS transgene expression at the permissive temperature of 18°C. UAS-NΔecd was induced by placing larvae at the restrictive temperature (30°C) for 24 hours before dissection.

**Table 1. Mosaic analysis of E(spl)mγ and E(spl)m8 expression in the larval CNS**

| | NB mγ+* | NB mγ−‡ | % NB mγ− | NB high m8§ | NB low m8¶ | NB no m8# | % NB low m8 | % NB no m8 |
|---|---|---|---|---|---|---|---|---|
| $N^{5419}$ | 0 | 17 | 100 | 0 | 23 | 3 | 88.5 | 11.5 |
| $Su(H)^{\Delta 47}$ | 0 | 69 | 100 | 4 | 75 | 18 | 77.3 | 18.6 |
| $Dl^{rev10}$ $Ser^{RX106}$ | 0 | 39 | 100 | 3 | 153 | 1 | 97.5 | 0.6 |
| $Dl^{rev10}$ | 0 | 67 | 100 | 0 | 38 | 3 | 92.7 | 7.3 |
| $Ser^{RX106}$ | 70 | 0 | 0 | 116 | 0 | 3 | 0 | 2.5 |
| $neur^{1}$ | 0 | 45 | 100 | 3 | 96 | 25 | 77.4 | 20.2 |
| $mib1^{EY9780}$ | 57 | 1 | 1.7 | 37 | 0 | 0 | 0 | 0 |
| $spdo^{G104}$ | 0 | 44 | 100 | 2 | 37 | 9 | 77.1 | 18.8 |
| $mam^{8}$ | 0 | 72 | 100 | 1 | 32 | 24 | 56.1 | 42.1 |
| Wild type | 132 | 1 | 0.7 | 33 | 0 | 0 | 0 | 0 |

Clones mutant for the indicated allele were scored in third instar larval CNSs. The number of clones from all regions of the CNS are shown, with the exception of the mushroom body NBs, where *mγ* and *m8* are weakly expressed.
*Lineages with an *mγ-lacZ* positive NB.
‡Lineages with an *mγ-lacZ* negative NB.
§Lineages with normal levels of *m8-lacZ* expression in the NB.
¶Lineages with reduced but detectable *m8-lacZ* expression in the NB.
#Lineages with undetectable *m8-lacZ* expression in the NB.

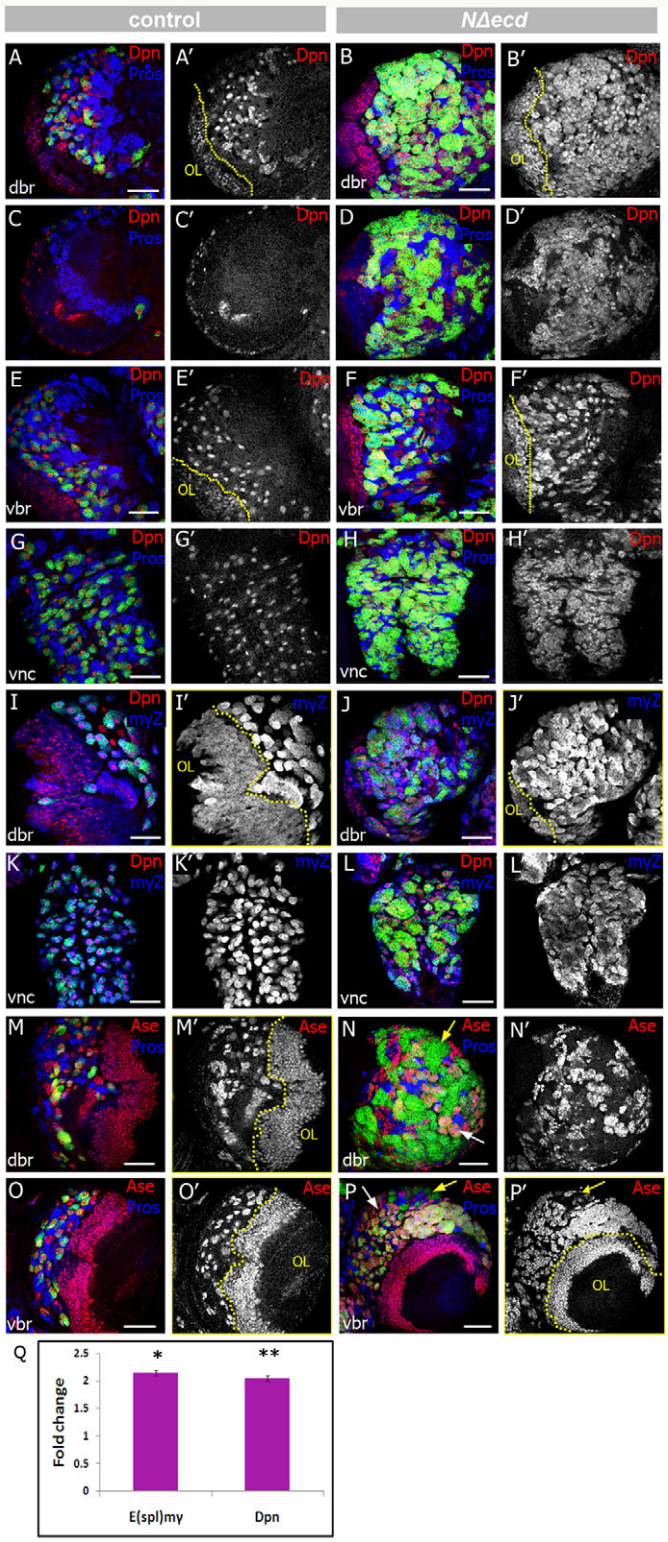


**Fig. 4. Notch hyperactivation causes overproliferation in the larval CNS.** A constitutively active Notch (NΔecd) was co-expressed with CD8-GFP in neuroblasts and GMCs under the *grh-Gal4* driver (B,B′,D,D′,F,F′,H,H′,J,J′,L,L′,N,N′,P,P′). Expression was induced for 24 hours. (A,A′,C,C′,E,E′,G,G′,I,I′,K,K′,M,M′,O,O′) Identically treated control CNSs, where *grh-Gal4* drives only CD8-GFP. GFP is green in all panels. Dpn or Ase (red) and Pros or E(spl)mγ-lacZ (blue) are imaged. Anterior is at the top. vbr, ventral brain; dbr, dorsal brain; VNC, ventral nerve cord; OL, optic lobe. (**A**,**A′**) Control brain lobe. (**B**,**B′**) In a *grh>NΔecd* brain, supernumerary NBs (Dpn-positive) arise at the expense of neurons (Pros positive). (**C-D′**) Views of deeper layers. (**E-H′**) Ventral views of a control (E,E′) versus a *grh>NΔecd* brain; (F,F′) and a control (G,G′) versus a *grh>NΔecd* VNC (H,H′). (**I-L′**) Control versus NΔecd-overexpressing animals that carry *E(spl)mγ-lacZ*. All supernumerary Dpn-positive NBs are also E(spl)mγ-positive. (**M-P′**) Comparison of wild type versus NΔecd-overexpressing brains stained for Ase and Pros. NΔecd-expressing cells are partly Ase negative (yellow arrows) and partly Ase positive (white arrows). Scale bars: 48 μm in A-M′,O,O′; 75 μm in N,N′,P,P′. (**Q**) Q-RT-PCR results showing the fold-increase in *E(spl)mγ* and *dpn* RNA levels in CNSs overexpressing NΔecd for 24 hours versus control CNSs. Mean ± s.e.m. of three repeats are shown. Statistically significant differences from the controls at $*P<0.5$ and $**P<0.01$ are shown using a two-tailed *t*-test.

Overexpression of NΔecd for 24 hours led to massive overproliferation of NBs throughout the CNS (Fig. 4B,D,F,H). Consistent with previous reports using different Gal4 drivers (Bowman et al., 2008; Wang et al., 2006; Weng et al., 2010), NB overproliferation was at the expense of neuronal differentiation (fewer Pros-positive cells; Fig. 4B,D,F,H). The supernumerary Dpn-positive NB-like cells had a smaller size than normal NBs and were not only found superficially, where NB are normally located, but also migrated into deep layers, invading neuronal territories (Fig. 4C,D). Overproliferating neuroblasts of the dorsal brain lacked Ase expression (Fig. 4N), suggesting a type II origin, and were more invasive, as they also migrated superficially around the anterior of the brain lobe, towards the ventral brain (Fig. 4P, yellow arrow). Overproliferating NBs in the ventral brain and VNC were both Dpn (Fig. 4B,D,F,H) and Ase-positive (Fig. 4P, white arrow) (type I). Besides expressing Dpn, all supernumerary type I or II NBs were also *E(spl)mγ-lacZ*-positive (Fig. 4I-L). We found an increase of 2.14 and 2.04 fold, respectively, of *E(spl)mγ* and *dpn* RNA expression levels by Q-PCR on whole dissected larval CNSs (Fig. 4Q).

## E(spl) proteins play an important role in the Notch gain-of-function phenotype, but Dpn has only a minor role

If bHLH-O proteins mediate the overproliferation effect of Notch, we expect this effect to disappear if we compromise bHLH-O protein activity. We therefore generated clones deficient for either the whole *E(spl)* locus or *dpn*, where at the same time we overexpressed an activated form of Notch. We assessed both type I and type II GFP-marked lineages for hyperplastic phenotypes. We scored type I lineages as overproliferating, when they contained two or more Dpn/Ase-positive cells. Type II lineages were scored as overproliferating when they contained more than 28 Dpn-positive cells. A further category of 'highly overproliferating' type II NB lineages were seen containing more than 80, and as many as 400, Dpn-positive cells.

Consistent with the grh>NΔecd results, clonal overexpression of NΔecd in a wild-type background produced a high percentage of overproliferating lineages; 30-50% of type I and 100% of type II clones (Fig. 5A,C,E; Table 2). Overproliferating type I clones contained 3-63 Dpn/Ase-positive NB-like cells, usually intermediate in size between a normal NB and a GMC (supplementary material Fig. S9A). In type II lineages 38-430 Dpn-positive/Ase-negative

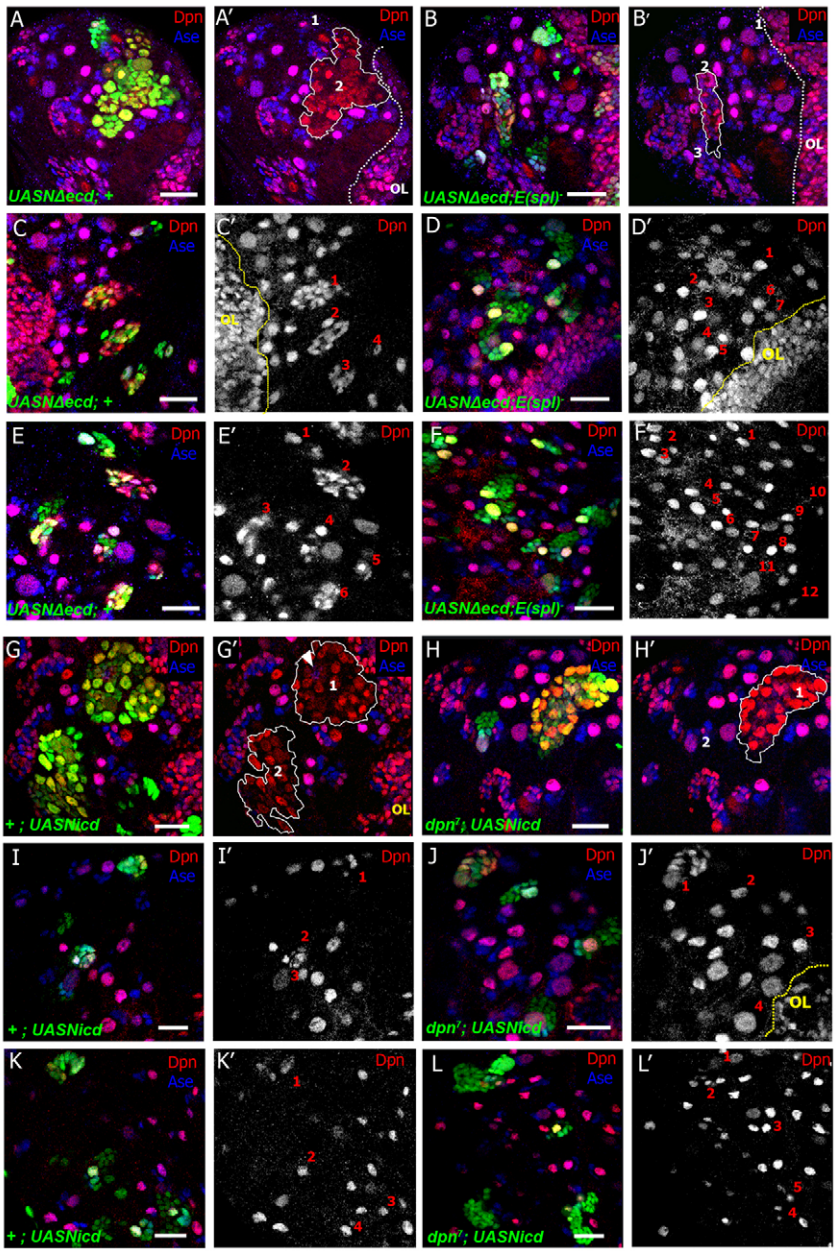


**Fig. 5. The role of *E(spl)* and *dpn* in Notch-induced NB overproliferation.** (A-F′) CNS clones (green) overexpressing NΔecd in wild type (A,A′,C,C′,E,E′) or *E(spl)* mutant background (B,B′,D,D′,F,F′). Anterior is at the top. Ase (blue) and Dpn (red) were imaged. (**A**,**A′**) A type II clone (#2) with supernumerary Dpn-positive/Ase-negative NBs and a normal type I clone (#1) are shown. (**B**,**B′**) A *UAS-NΔecd; E(spl)*$^{-}$ type II clone (#2) contains normal-looking INPs, in size, number and markers (Dpn/Ase positive). Type I clones also appear normal (#1,3). (**C**,**C′**) Ventral brain clones either possess supernumerary Dpn/Ase-positive cells (#1,2,3) or appear normal (#4). (**D**,**D′**) In the absence of *E(spl)* ventral brain, clones appear normal (#1-7). (**E**,**E′**) NΔecd-overexpressing lineages in the VNC have more than one Dpn/Ase-positive cell (#1-6). (**F**,**F′**) NΔecd-overexpressing/*E(spl)* VNC clones revert to normal (#1-12). (G-L′) CNS clones expressing a weaker *UAS-Nicd* transgene either in wild type (G,G′,K,K′,I,I′) or in *dpn* mutant background (H,H′,J,J′,L,L′). (**G**,**G′**) Dorsal brain clones showing supernumerary type II neuroblasts (#1,2). Rare Ase-positive cells are seen (arrowhead). (**H**,**H′**) Nicd-overexpressing/ *dpn*$^{7}$ mutant type II lineages display a similar strong overproliferation phenotype (#1). The nearby type I clone (#2) looks normal. (**I**,**I′**) Ventral brain clones appear either normal (#3) or have supernumerary Dpn/Ase-positive cells (#1,2). (**J**,**J′**) Ventral brain Nicd overexpressing/*dpn*$^{7}$ mutant lineages behave similarly to I,I′, having either one (#2-4) or more than two Dpn/Ase-positive cells (#1). (**K**,**K′**) Nicd overexpressing lineages in the VNC have either one (#2-4) or several Dpn/Ase-positive cells (#1). (**L**,**L′**) Nicd overexpressing/*dpn*$^{7}$ mutant lineages in the VNC are either normal (#3,5) or have additional Dpn/Ase-positive cells (#1,2,4). Scale bars: 30 μm in A,A′; 24 μm in B-E′,G,G′,I-L′; 26 μm in F,F′,H,H′.

cells were scored, most of which had the size of an INP, whereas up to 36 had the size of a NB. Ase-positive cells were hardly ever seen in these clones (supplementary material Fig. S9B).

Upon removal of the *E(spl)* genes, overproliferation by Nicd expression was drastically reduced (Table 2). Only 5% of type I clones in the brain lobes displayed mild overproliferation. These contained a smaller number of Dpn-positive cells per clone (2-15 cells) (Fig. 5C,D). No overproliferation was detected in the VNC (Fig. 5F). The majority of type II clones (15/19 clones) also reverted to a wild-type phenotype upon E(spl) removal (Fig. 5B). They contained a single Ase-negative NB and 25-28 mature INPs (Dpn and Ase positive). Three clones with mild overproliferation (35-43 Dpn-positive cells) and one clone with 161 Dpn-positive cells were recovered. Even in these clones most of the supernumerary Dpn-positive cells were also Ase positive in sharp contrast to the *E(spl)*$^{+}$ control clones. As noted before, loss of *E(spl)* function in a wild-type background has a mild effect in type II lineages, reducing INP number to about 10 (Fig. 2E) – and has no effect on type I lineages. So the wild-type appearance of type II *UAS-NΔecd; E(spl)*$^{-}$ clones is an intermediate phenotype between NΔecd overexpression and *E(spl)* loss of function. The conclusion is that E(spl) function seems to be more important in the pathological NB overproliferation obtained upon Notch hyperactivation than it is in the background of normal Notch activity.

Analogous experiments to address the role of *dpn* in Notch-induced overproliferation used a different activated Notch (*UAS-Nicd*) (Seugnet et al., 1997) for technical reasons (see Materials and methods). This transgene elicited weaker overproliferation upon clonal expression (with the same *α-tub-Gal4*) than did *UAS-Δecd*. Ten to 13% of Type I and 100% of type II lineages produced overproliferating clones (Table 2): type I clones had 2-15 Dpn-positive cells (Fig. 5I,K; supplementary material Fig. S9C), whereas Type II had 44-269 Dpn-positive cells (Fig. 5G; supplementary material Fig. S9D). With the weaker Nicd, all Type II clones, despite their hyperproliferation, contained a small number of Ase-positive cells (Fig. 5G′, white arrowhead). Upon

**Table 2. E(spl) proteins are necessary for Notch-mediated overproliferation of NBs in the larval brain, whereas Dpn is dispensable**

| | Normal NBs | Overproliferating NBs | Highly overproliferating NBs | % Normal | % Overproliferating | % Highly overproliferating |
|---|---|---|---|---|---|---|
| ***UASNΔE$^{B7D}$; FRT82B +*** | | | | | | |
| CB type II NBs | 0 | 5 | 25 | 0 | 16.7 | 83.3 |
| CB type I NBs | 106 | 60 | - | 63.8 | 36.2 | - |
| VNC type I NBs | 82 | 97 | - | 45.8 | 54.2 | - |
| ***UASNΔE$^{B7D}$; FRT82B E(spl)-*** | | | | | | |
| CB type II NBs | 15 | 3 | 1 | 79.0 | 15.8 | 5.2 |
| CB type I NBs | 250 | 14 | - | 94.7 | 5.3 | - |
| VNC type I NBs | 242 | 0 | - | 100.0 | 0 | - |
| ***FRTG13 +; UASNicd*** | | | | | | |
| CB type II NBs | 0 | 4 | 10 | 0 | 28.6 | 71.4 |
| CB type I NBs | 141 | 21 | - | 87.0 | 13.0 | - |
| VNC type I NBs | 118 | 13 | - | 90.1 | 9.9 | - |
| ***FRTG13 dpn$^{7}$; UASNicd*** | | | | | | |
| CB type II NBs | 0 | 5 | 3 | 0 | 62.5 | 37.5 |
| CB type I NBs | 82 | 32 | - | 72.0 | 28.0 | - |
| VNC type I NBs | 61 | 14 | - | 81.3 | 18.7 | - |

GFP-marked lineages that overexpress activated Notch in two different wild-type (*FRT82B* or *FRTG13*), *E(spl)*$^{-}$ or *dpn*$^{-}$ backgrounds were scored. Two different activated Notch transgenes were used, one inserted in the second chromosome (*UAS-NΔecd*$^{B7D}$) and one on the third (*UAS-Nicd*). The central brain (CB) and VNC were scored separately. The CB was further subdivided into Type I and Type II lineages. Overproliferating lineages were so called if they contained more than one Dpn-positive NB. Type II lineages containing more than 80 NBs/INPs (Dpn positive, Ase negative or positive) were classified as highly overproliferating. The distributions of Dpn-positive cell numbers in the various categories are shown as box-plots in supplementary material Fig. S9.

removal of *dpn*, the percentage of Type I overproliferating clones rose to 19-28% (Table 2), which we consider insignificant, as the vast majority contained only two Dpn-positive cells (Fig. 5J,L). Type II clones continued to overproliferate with full penetrance and only had a moderate decrease in Dpn-positive cell number (Table 2, Fig. 5H). This analysis was performed with two null/antimorphic *dpn* alleles, *dpn*$^{7}$ and *dpn*$^{2}$ (Barbash and Cline, 1995), yielding the same results. Therefore despite the fact that abnormal Notch activity induces both *dpn* and *E(spl)*, NB hyperproliferation can still occur in the absence of *dpn*.

## E(spl) overexpression mimics the Notch hyperactivation phenotype

We next examined whether *E(spl)* overexpression might be sufficient to bypass Notch and induce hyperplasia in the larval CNS. We used *grh-Gal4* to drive the expression of various *UAS-E(spl)*, as well as *UAS-dpn* transgenes in neuroblasts. Overexpression of *E(spl)mβ*, *E(spl)mγ*, *E(spl)mδ*, *E(spl)m3* or *dpn* caused overproliferation, more severe in the cases of *m3*, *mγ* and *mδ* (supplementary material Fig. S10B). *E(spl)m5*, *E(spl)m7* or *E(spl)m8* did not produce any NB hyperplasia. The effect of *E(spl)mγ* (and others) was limited to dorsal brain lineages, including type II NBs, in contrast to the more global *UAS-NΔecd* effects. Ectopic NBs were a mix of Ase-positive and Ase-negative ones. Overexpression of *UAS-E(spl)mγKNEQ* (supplementary material Fig. S10D), a mutant that lacks the ability to bind DNA (Giagtzoglou et al., 2003), caused no overproliferation.

For further analysis, we selected one of our most potent transgenes, *UAS-E(spl)mγ*, and asked whether it is sufficient to cause overproliferation even in the absence of Notch signalling. To that end, we compared *E(spl)mγ* overexpressing clones in a wild-type versus *Su(H)* mutant background. In a wild-type background, *mγ* overexpression produced a moderate level of overproliferation in type I NBs (16% of clones in the brain and 4.5% in the VNC; see Table 3) and fully penetrant (96%) overproliferation in Type II clones (Fig. 6A). The latter contained 28-487 Dpn-positive cells (supplementary material Fig. S11). The effect of E(spl)mγ overexpression was similar to the activity of the weaker *UAS-Nicd* transgene described above. In *E(spl)mγ*-overexpressing *Su(H)* mutant clones, overproliferation could still be observed in both type II and

**Table 3. E(spl)mγ expression bypasses Notch to induce a hyperproliferation phenotype**

| | Normal NBs | Overproliferating NBs | Highly overproliferating NBs | % Normal | % Overproliferating | % Highly overproliferating |
|---|---|---|---|---|---|---|
| ***FRT40A: UAS-E(spl)mγ*** | | | | | | |
| CB type II NBs | 1 | 4 | 21 | 3.8 | 15.4 | 80.8 |
| CB type I NBs | 104 | 23 | - | 81.9 | 18.1 | - |
| VNC type I NBs | 105 | 5 | - | 95.5 | 4.5 | - |
| ***Su(H)$^{\Delta 47}$ FRT40A; UAS-E(spl)mγ*** | | | | | | |
| CB type II NBs | 2 | 16 | 6 | 8.3 | 66.7 | 25 |
| CB type I NBs | 125 | 18 | - | 87.4 | 12.6 | - |
| VNC type I NBs | 106 | 4 | - | 96.4 | 3.6 | - |

GFP-marked lineages that overexpress *E(spl)mγ* in a wild-type (*FRT40A*) or a *Su(H)* mutant background were scored. The central brain (CB) and VNC were scored separately. The CB was further subdivided into type I and type II lineages. Overproliferating lineages were so called if they contained more than one Dpn-positive NB. Type II lineages containing more than 80 NBs/INPs (Dpn positive, Ase negative or positive) were classified as highly overproliferating. The distributions of Dpn-positive cell numbers in the various categories are shown as box-plots in supplementary material Fig. S11.

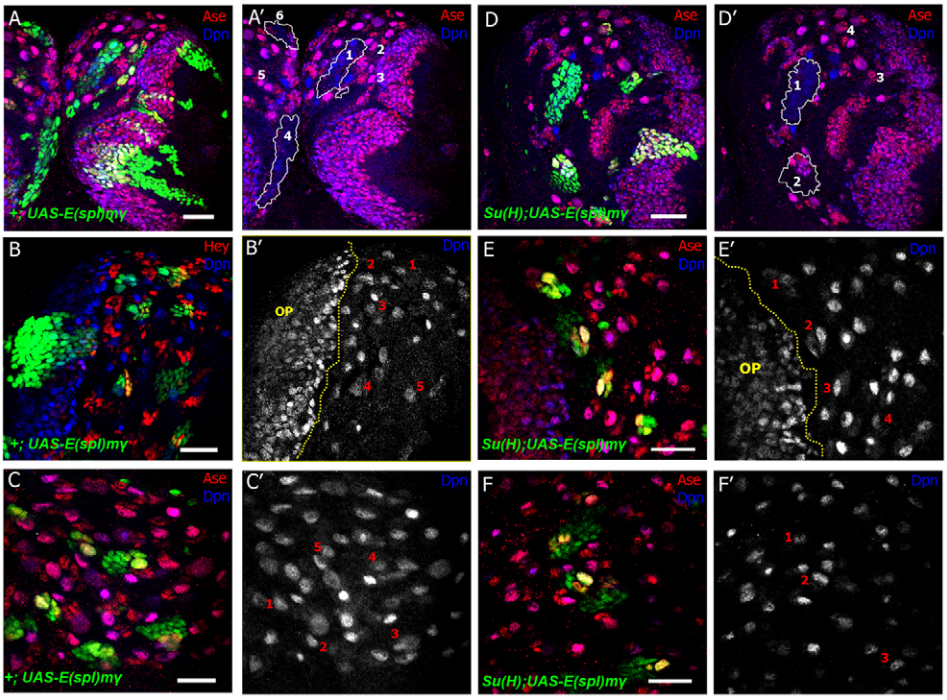


**Fig. 6. E(spl)mγ is sufficient to cause overproliferation of larval CNS neuroblasts.** CNS where *E(spl)mγ* is overexpressed in wild-type (A-C′) or *Su(H)* (D-F′) clones. Ase (red) and Dpn (blue) are shown, except B, where Hey is in red. (**A**,**A′**) Dorsal brain where supernumerary Dpn-positive/Ase-negative neuroblasts are seen in type II clones (#1,4,6), whereas type I lineages appear normal (#2,3,5). (**B**,**B′**) In the ventral brain, rare mild overproliferation (clones #2,4) is seen; clones 1,3,5 are normal. (**C**,**C′**) In the VNC, *E(spl)mγ* overexpression has no effect on proliferation (#1-5). (**D**,**D′**) Overexpression of *E(spl)mγ* in *Su(H)* mutant clones in the dorsal brain still results in overproliferation in most type II lineages (#1), whereas a few remain unaffected (#2). Type I lineages in this example are normal (#3,4). (**E**,**E′**) In the ventral brain, rare mild overproliferation is still seen (#2), whereas most lineages appear normal (#1,3,4). (**F**,**F′**) In the VNC, almost all *E(spl)mγ* overexpressing/*Su(H)* mutant lineages appear normal (#1-3). Scale bars: 48 μm in A,A′,D,D′; 75 μm in B,B′; 24 μm C,C′,E-F′.

type I clones (Fig. 6D-F) at the same frequencies (Table 3). Although type I clones were essentially indistinguishable from those generated in a wild-type background, type II clones contained significantly fewer Dpn-positive cells (28-158; supplementary material Fig. S11). We conclude that E(spl)mγ is sufficient to cause NB overproliferation in the absence of Notch. In type II lineages, Notch signalling can boost this activity, indicating some degree of interdependence/synergy between E(spl)mγ and other Notch downstream effectors.

## DISCUSSION

We have presented an analysis of the expression and function of two types of bHLH-O proteins expressed in *Drosophila* neuroblasts, Dpn and the E(spl) family. Our main conclusions are that (1) these two types of factors have distinct expression modalities – E(spl)mγ and m8 are targets of Notch signalling, whereas Dpn is not; (2) these factors have redundant functions to maintain NBs in a self-renewing state in normal development, yet (3) in a pathological NB hyperproliferation context, Dpn and E(spl) have distinct functions.

### Different modes of bHLH-O expression in neuroblasts

It was heretofore thought that embryonic NBs are cells that escape from Notch signalling, which is only perceived in the surrounding neuroectoderm. Only in the later post-embryonic period, were the NBs thought to respond to Notch (Almeida and Bray, 2005; San-Juán and Baonza, 2011). We have shown that this happens much earlier, already in the embryonic NBs. Soon after the NB delaminates, a time when it sends, but does not receive, a Notch signal, it starts asymmetrically dividing. Our results are consistent with the daughter GMCs sending a Delta signal back to their sister NBs, thereby initiating *E(spl)* expression. *E(spl)mγ* expression ceases when the NB enters quiescence, only to restart when proliferation resumes.

Dpn, another bHLH-O protein, is also expressed in NBs, but much less dynamically. Its expression initiates upon NB delamination from the neuroepithelium and persists throughout its life. *dpn* does display some degree of dynamic expression, as it is rapidly turned off in the iINPs, only to be reactivated upon maturation. Our loss-of-function data (Fig. 3; supplementary material Fig. S4) clearly indicate that it is not a target of Notch in the NB, in contrast to *E(spl)*. Paradoxically, *dpn* is induced upon Notch hyperactivation (Fig. 4). This could be an indirect effect mediated through E(spl). Indeed, E(spl)mγ overexpression can induce ectopic *dpn* expression (Fig. 6). Still, *dpn* does harbour a Notch-responsive enhancer that drives expression in larval NBs (San-Juán and Baonza, 2011). This same region scored positive for Su(H) binding in a ChIP-chip approach in a cell line of mesodermal origin (Krejci et al., 2009). How this enhancer contributes to the overall expression pattern of *dpn* will be a matter of future analysis.

### The function of bHLH-O proteins: proliferation or anti-differentiation?

Despite their different expression modalities, Dpn and E(spl) have redundant functions in the larval NBs, as only double mutant clones show proliferation defects. These mutant NBs do not stop

proliferating immediately, rather gradually terminate their cycling within a few days following homozygosing of the mutant alleles (Fig. 2). We propose that Dpn/E(spl) keep the NB in an undifferentiated state and proliferation is a consequence of the ability of these cells to respond to mitogens. Upon Dpn/E(spl) loss, this state becomes unstable and prone to switch to a terminally differentiated state. This transition takes a few days, probably reflecting the time needed to accumulate pro-differentiation factors. A redundant role of Dpn/E(spl) in maintaining the undifferentiated state also during quiescence transpired from our genetic analysis of NB re-activation after embryogenesis. Whereas $dpn^{-/-}$ NBs quite successfully re-entered the cell cycle, $dpn^{-/-}$;$E(spl)^{+/-}$ NBs were unable to do so, despite trophic growth factor stimulation.

*E(spl)* expression has been associated with the less differentiated of two alternative outcomes in other instances. For example, during NB formation, *E(spl)* genes are expressed in the undifferentiated embryonic neuroectoderm and not in the NBs. The same happens in the optic lobe neuroepithelium (Egger et al., 2010; Wang et al., 2011). In this work, we have presented evidence for a similar role for Dpn/E(spl) in the NB. Excessive Dpn/E(spl) activity in GMCs/INPs can revert these partially differentiated cells back to a NB-like fate. For this reason, NB asymmetric divisions must ensure that Dpn and E(spl) are never expressed in the GMC or iINP. Regarding E(spl), we propose that this is ensured by the directionality of Notch signalling (GMC to NB). Dpn is also never seen to accumulate in the GMCs/iINPs, suggesting a repression mechanism at work in these cells, e.g. via Pros (Choksi et al., 2006; Southall and Brand, 2009). These modes of transcriptional control are probably combined with active protein clearance by degradation.

An anti-differentiation role has also been proposed for vertebrate homologues of Dpn/E(spl), the Hes proteins. Hes1, Hes5 and Hes3 are all expressed in proliferating neural stem cells of the embryonic CNS. Upon *Hes* knockout, neural stem cells prematurely differentiate resulting in a hypoplastic nervous system, with increasing severity as more Hes genes are lost (Hatakeyama et al., 2004). In an interesting analogy, only *Hes1* and *Hes5* are direct targets of Notch signalling (Nishimura et al., 1998). Another example where anti-differentiation during quiescence is mediated by high *Hes1* expression are cultured fibroblasts and rhabdomyosarcoma cells (Sang et al., 2008). Similar to what we observed with Dpn/E(spl)-mutant embryos, a quiescence trigger, like serum depletion, can result in irreversible cell-cycle withdrawal, if *Hes1* activity is compromised.

## The Notch/bHLH-O axis in normal and pathological neurogenesis

Our results have shed light on the paradox of why Notch loss of function has only minor effects in larval neurogenesis, whereas its hyperactivation causes significant overproliferation (Bowman et al., 2008; Lin et al., 2010; San-Juán and Baonza, 2011; Truman et al., 2010; Wang et al., 2006; Weng et al., 2010). Notch loss of function decreases *E(spl)* expression, leaving Dpn levels unaffected. Furthermore, Notch pathway disruption does not seem to directly affect NB proliferation, as Cyclin E expression is not eliminated (Fig. 3). Therefore, Notch signalling from the GMC/iINP to the NB acts to ensure robustness in NB maintenance, in collaboration with Dpn.

When Notch signalling is aberrantly activated in the GMCs/iINPs, both type I and type II lineages overproliferate, although the former do so with lower penetrance (fewer lineages) and expressivity (smaller clones) (Table 2). Yet, for both types of lineages, *E(spl)* genes are necessary (Fig. 5) and sufficient (Fig. 6) to implement overproliferation. This is consistent with our hypothesis that ectopic E(spl)/Dpn activity in the GMCs/iINPs inhibits their differentiation and makes them competent to respond to mitogenic stimuli.

Why are Type II lineages more sensitive than type I lineages to Notch gain of function? A crucial difference between these NBs is the lack of expression of Ase in type II, as its artificial reinstatement can revert the latter to type I-like behaviour (Bowman et al., 2008). It was recently demonstrated that Ase downregulates *E(spl)* expression (Southall and Brand, 2009). It is even possible that Ase antagonizes E(spl) proteins post-transcriptionally, as the two can interact (Alifragis et al., 1997) and we have documented extensive antagonistic interactions between E(spl) proteins and Sc, a protein related to Ase, in different contexts (Giagtzoglou et al., 2003; Giagtzoglou et al., 2005). Thus, N hyperactivation will probably cause a smaller increase in E(spl) levels/activity in type I cells, compared with type II. If resistance to differentiation stimuli depends on the level of E(spl)/Dpn activity, this would account for the relative resilience of type I lineages to Notch-induced overproliferation.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material available online at
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