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bHLH-O proteins balance the self-renewal and differentiation of *Drosophila* neural stem cells by regulating Earmuff expression

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Abstract

Balancing self-renewal and differentiation of stem cells requires differential expression of self-renewing factors in two daughter cells generated from the asymmetric division of the stem cells. In *Drosophila* type II neural stem cell (or neuroblast, NB) lineages, the expression of the basic helix-loop-helix-Orange (bHLH-O) family proteins, including Deadpan (Dpn) and E(spl) proteins, is required for maintaining the self-renewal and identity of type II NBs, whereas the absence of these self-renewing factors is essential for the differentiation of intermediate neural progenitors (INPs) generated from type II NBs. Here, we demonstrate that Dpn maintains type II NBs by suppressing the expression of Earmuff (Erm). We provide evidence that Dpn and E(spl) proteins suppress Erm by directly binding to C-sites and N-boxes in the cis-regulatory region of *erm*. Conversely, the absence of bHLH-O proteins in INPs allows activation of *erm* and Erm-mediated maturation of INPs. Our results further suggest that Pointed P1 (PntP1) mediates the dedifferentiation of INPs resulting from the loss of Erm or overexpression of Dpn or E(spl) proteins. Taken together, these findings reveal mechanisms underlying the regulation of the maintenance of type II NBs and differentiation of INPs through the differential expression of bHLH-O family proteins.

Keywords

Drosophila; neuroblast; intermediate neural progenitor; b-HLH; Deadpan; E(spl)

Introduction

During the development of the nervous system, neural stem cells (NSCs) need to maintain their own population to generate a large number of various types of neurons and glia. To this end, NSCs undergo asymmetric self-renewing divisions, enabling the generation of new

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X.L. and S.Z. conceived the idea, analyzed the data, and wrote the manuscript. X.L. and R.C. carried out the experiments and collected the data.

Competing financial interests

The authors declare no competing financial interests.

NSCs and production of fate-restricted progenitor cells or post-mitotic neurons/glia. The acquisition of distinct cell fates of the two daughter cells generated from the asymmetric division requires the unequal inheritance of cell fate determinants and differential expression of self-renewing factors. Such asymmetric expression of cell fate determinants and self-renewing factors is critical not only for maintaining the NSC population but also for avoiding tumorigenesis (Neumuller and Knoblich, 2009; Paridaen and Huttner, 2014).

In both vertebrates and invertebrates, basic helix-loop-helix-Orange (bHLH-O) family proteins play important roles in maintaining NSC self-renewal (Imayoshi and Kageyama, 2014; Zacharioudaki et al., 2012; Zhu et al., 2012). The bHLH-O proteins are transcriptional repressors that contain a bHLH DNA-binding domain and a distinctive “Orange” dimerization domain (Davis and Turner, 2001; Fisher and Caudy, 1998). In vertebrates, a subfamily of bHLH-O proteins called Hes proteins, which are homologs of *Drosophila* Hairy and Enhancer of split [E(spl)] proteins, are highly expressed in neural epithelial cells and radial glia cells. In the absence of Hes proteins, radial glial cells prematurely differentiate into neurons, resulting in the premature depletion of radial glial cells and a hypoplastic nervous system. Conversely, the misexpression of Hes proteins inhibits neuronal differentiation (Hatakeyama et al., 2004; Ishibashi et al., 1995; Ohtsuka et al., 1999). Hes proteins maintain radial glial cells by inhibiting the expression of proneural genes such as *Mash1* and *Neurogenin 2* (*Ngn2*), which promote neuronal differentiation (Imayoshi and Kageyama, 2014; Ishibashi et al., 1995).

In *Drosophila*, there are total 13 members of the bHLH-O protein family. Among these proteins, Deadpan (Dpn) and at least three members of the E(spl) complex, including E(spl)mγ, E(spl)mβ, and E(spl)m8, are expressed in neuroblasts (NBs, the *Drosophila* NSCs) but not in their differentiating daughter cells generated from asymmetric division (Bier et al., 1992; Zacharioudaki et al., 2012). E(spl)mγ and m8 are activated through Notch signaling, but Dpn functions independently (Zacharioudaki et al., 2012; Zhu et al., 2012). Functional analyses revealed that Dpn and Notch signaling are crucial for maintaining NBs. In *Drosophila* larval brains, there are two different types of NBs, type I and type II. Type I NBs produce terminally dividing ganglion mother cells (GMCs) and also divide to self-renew, whereas type II NBs produce intermediate neural progenitor cells (INPs) (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). INPs undergo several rounds of asymmetric self-renewing divisions, similar to type I NBs, to produce GMCs after these cells differentiate from an immature status to a mature status. Interestingly, type II NB lineages are more sensitive to the changes of Dpn or Notch signaling. In the absence of Dpn or Notch signaling, type II NBs are completely eliminated, but type I NBs are not (Bowman et al., 2008; San-Juan and Baonza, 2011; Zacharioudaki et al., 2012; Zhu et al., 2012). Furthermore, type II NBs are transformed into type I-like NBs in the absence of Dpn or Notch signaling before these cells are lost (Zhu et al., 2012). When Dpn is overexpressed or Notch signaling is overactivated by expressing the intracellular domain of Notch (N^{intra}) or its target E(spl) proteins, type II NBs also show much more severe overproliferation than type I NBs due to dedifferentiation of immature INPs (imINPs) (Bowman et al., 2008; Zacharioudaki et al., 2012; Zhu et al., 2012).

We recently demonstrated that Notch maintains type II NBs at least in party by activating the canonical pathway for the expression of E(spl) proteins, which in turn suppresses the activation of *earmuff* (*erm*) by Pointed P1 (PntP1) (Li et al., 2016). PntP1 is expressed in type II NBs and imINPs to specify type II NBs and promote the generation of INPs by preventing both the dedifferentiation and premature differentiation of INPs (Xie et al., 2016; Zhu et al., 2011). Erm is normally activated by PntP1 only in imINPs, in which Notch is turned off (Koe et al., 2014; Xie et al., 2016). In the absence of Notch or its downstream E(spl) proteins, PntP1 activates *erm* in type II NBs. The ectopically expressed Erm exerts a negative feedback on PntP1 by inhibiting its function and expression, resulting in transformation of type II NBs into type I-like NBs. Additionally, the ectopically expressed Erm promotes the termination of type II NB self-renewal through an unknown mechanism (Li et al., 2016). However, it remains unclear how Dpn maintains type II NBs and how Dpn overexpression or Notch overactivation leads to the tumorigenic overproliferation of type II NBs.

Similar to E(spl) proteins, Dpn functions as a transcriptional repressor by binding to similar DNA sequences (Fisher and Caudy, 1998; Paroush et al., 1994). Given that Dpn and Notch show similar loss-of-function and gain-of-function phenotypes in type II NB lineages, we hypothesize that Dpn maintains type II NBs by similarly suppressing *erm* expression in type II NBs and the absence of Dpn as well as E(spl) proteins in imINPs allows activation of *erm* and Erm-mediated maturation of INPs. Here, we test this hypothesis by examining how the manipulation of Dpn and E(spl) protein expression would affect Erm expression and how changes in Erm expression contribute to the defects of type II NB lineage development that result from changes in Dpn expression or Notch signaling.

MATERIALS AND METHODS

Fly stocks

GAL4 lines used for transgene expression include *pntP1-GAL4* (or $GAL4^{14-94}$) (Zhu et al., 2011), *erm-GAL4 (II)* (Xiao et al., 2012), and *insc-GAL4* (Betschinger et al., 2006). UAS transgenes for RNAi knockdown or overexpression include *UAS-erm RNAi* (#26778, Bloomington Drosophila Stock Center, Bloomington, IN, USA [BDSC]), *UAS-dpn RNAi* (BDSC, #51440), UAS-rCD2 RNAi (BBSC, #56169) (Yu et al., 2009), *UAS-numb RNAi* (BDSC, #35045), *UAS-E(spl)m5* (BDSC, #26680), *UAS- E(spl)m7* (BDSC, #26681), *UAS-E(spl)m8* (BDSC, #26872 and #26873), *UAS-E(spl)mβ* (BDSC, #26675), *UAS-E(spl)mδ* (BDSC, #26677), $UAS\text{-}N^{intra}$ (BDSC, #52008), *UAS-dpn* (Wallace et al., 2000), and *UAS-erm* (Weng et al., 2010). *R9D11-CD4-tdTomato* (Han et al., 2011) was used as an *erm* reporter. dpn^{1} (Younger-Shepherd et al., 1992), dpn^{7} (Barbash and Cline, 1995), *erm*, and erm^{2} mutant alleles (Weng et al., 2010) were used for mutant phenotypic analyses.

RNAi knockdown and transgene expression

RNAi knockdown experiments were carried out at 29°C in order to maximize the efficiency. Misexpression or overexpression of UAS-transgenes was conducted at 29°C. $tub\text{-}GAL80^{ts}$ (McGuire et al., 2003) was used in combination with specific GAL4 lines for temporal

control of RNAi knockdown or misexpression/overexpression. Animals were shifted from 18°C to 29°C for inactivating *GAL80^{ts}* at specific temporal windows.

Electrophoretic mobility shift assays (EMSA)

Coding regions of seven *E(spl)* genes and *dpn* were amplified by using Q5 Hot Start High-Fidelity DNA Polymerase (Catalog #M0491, New England BioLabs Inc., Ipswich, MA). The amplified products were cloned into pcDNA™3.1/His expression vectors (Catalog #V38520, Life Technologies Co., Grand Island, NY). All proteins are expressed from the pcDNA™3.1/His construct using the TNT® T7 Quick Coupled Transcription/Translation kit (Catalog #L1170, Promega Co., Madison, WI) according to manufacturer's instructions. A 20-nt sequence flanking the bHLH-O3 was chosen as a probe, which was labeled with Cy5 at the 5'-end of both strands. The binding reaction was performed by incubating 0.05 pmol of cy5-bHLH-O3 with 2μl TNT-T7 expressing product. To determine the binding specificity, the same unlabeled bHLH-O3 (bHLH-O3S) was used as a specific competitor and the unlabeled bHLH-O3 containing mutations of two nucleotides in the C-site (bHLH-O3NS) as a non-specific competitor. To investigate if the other two sites can bind to E(spl) or Dpn proteins, unlabeled probes containing 20-nt sequences from bHLH-O1 or bHLH-O2 were used to compete with Cy5-bHLH-O3 for the binding. Competitions were performed with 2 pmol of unlabeled bHLH-O3S, bHLH-O3NS, bHLH-O1, or bHLH-O2 probes. Proteins were preincubated with competitors for 10 minutes at room temperature followed by 20-minute incubation with Cy5-bHLH-O3. 10μl of EMSA reactions were analyzed on 5% nondenaturing polyacrylamide Mini-PROTEAN® TBE gels, which were scanned with ChemiDoc™ XRS. Sequences of the oligonucleotide probes used in the EMSA are listed below. All probes were synthesized by Integrated DNA Technologies (Carolville, IA) and annealed to form double-strand DNAs.

Cy5-bHLH-O3: Cy5-CCGGTGGCACGCGCCTTTAT

bHLH-O3S: CCGGTGGCACGCGCCTTTAT

bHLH-O3NS: CCGGTGGtAtGCGCCTTTAT

bHLH-O1: AGCTGGCACGAGCCAATT

bHLH-O2: CTTGAGCGCGTGCCGTGC

Construction of plasmids and generation of transgenic lines for *erm* reporters

The gateway cloning system was used for cloning *erm* enhancer fragments listed in Figure 4 (including R9D11, B, R1, and R2) into pDEST-HemmarR vector (Han et al., 2011), which allows these enhancer fragments to drive the expression of *CD4-tdTomato*. Briefly, *erm* enhancer fragments were amplified by PCR from genomic DNAs and cloned into the donor vector pDONR221 first by BP reactions to make entry clones pENTR-R9D11, B, R1, and R2. LR reactions were then carried out between the entry clones and the pDEST-HemmarR destination vectors to make pHemmarR- R9D11, B, R1, and R2. To clone *erm* enhancer fragments with deletion of specific binding sites (R9D11ΔbHLH-O3, R9D11ΔbHLH-O123) into the pDEST-HemmarR vector, overlapping PCR was used to remove the E(spl)/Dpn binding sites from the pENTR- R9D11 vector. *R9D11* enhancer fragments with deletion of specific bHLH-O binding sites were then cloned into pDEST-HemmarR by LR reactions.

All constructs were integrated into the attP2 site on the third chromosome by injecting constructs into P{y[+t7.7]=CaryP} attP2 embryos. Primers used for making these reporter lines are listed below with the 5'-end on the left.

R9D11-forward: GAAGTCCAACGCGCTATC

R9D11-reverse: GCTGCGGTTTGCTATGAT

B-forward: CGAAATCAAATGTCAGGCCAGT

B-reverse: GTCAATTTCTGCGGCTAACCAA

R1-forward: GAAGTCCAACGCGCTATC

R1-reverse: ACCGGAAGTAAGTGGAAGCCAA

R2-forward: GAAGTCCAACGCGCTATC

R2-reverse: CTGGAAAGAAAAGGGGGAGGTG

R9D11ΔbHLH-O-1-forward: ACAAGCAAGCATTTAGAATGCCAAATTCG

R9D11ΔbHLH-O-1-reverse: CATTCTAAATGCTTGCTTGTAATGCGAG

R9D11ΔbHLH-O-2-forward: GCATGTCCTTTGCGTCCTTAGGAGACCT

R9D11ΔbHLH-O-2-reverse: TAAGGACGCAAAGGACATGCGAGCAGTA

R9D11ΔbHLH-O-3-forward:
AAATTGAACGCCGCCGGTTTATTGGCTCATCTTCC

R9D11ΔbHLH-O-3-reverse:
GGAAGATGAGCCAATAAACCGGCGGCGTTCAATTT

Immunostaining and confocal microscopy

Larval brains were dissected and immunostained as described (Lee and Luo, 1999). Primary antibodies include rabbit anti-Dpn (1:500) (Bier et al., 1992), guinea pig anti-Ase (1:5000) (Brand et al., 1993) (gifts from Y. N. Jan), rat anti-Erm (gift from C. Desplan, 1:100), rat antimCD8 (clone #5H10, Life Technologies Co., Grand Island, NY; 1:100), rabbit anti-dsRed (Catalog #632392, Clontech; 1:500), and chicken anti-GFP (catalog #GFP-1020, Aves Labs, Tigard, OR; 1:1000). Secondary antibodies conjugated to Dylight 488, Cy3 or Dylight 647 (Jackson ImmunoResearch) were used at 1:100, 1:500, or 1:500, respectively. Images were taken with a Zeiss 780 confocal microscope and processed with Adobe Photoshop. Two-tailed *t*-tests were used for statistical analyses.

RESULTS

Loss of Dpn leads to ectopic activation of erm in type II NBs

To determine whether Dpn maintains type II NBs by suppressing Erm expression, we first examined Erm expression in Dpn knockdown or dpn^{1}/dpn^{7} trans-heterozygous mutant type II NBs using *R9D11-CD4-tdTomato* (abbreviated as *R9D11-tdTom*) as a reporter. *R9D11-tdTom* utilizes a 3.9kb DNA enhancer fragment *R9D11* from the *erm* to drive the expression of *CD4-tdTomato* and shows a similar expression pattern as endogenous Erm proteins (Han

et al., 2011; Pfeiffer et al., 2010). In wild-type larvae, there are 8 type II NBs/brain lobe, which can be distinguished from type I NBs by the lack of the proneural protein Asense (Ase) (Figure 1A–A", D) (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). We previously showed that in *dpn* mutants, the majority of type II NBs were lost by the mid-1st instar larval stage and the remaining type II NBs were transformed into type I-like NBs as indicated by ectopic Ase expression in the NBs (Figure 1C–C", D) (Zhu et al., 2012). Knocking down Dpn using the type II NB-specific driver *pntP1-GAL4* (Zhu et al., 2011) led to a similar loss of type II NBs and transformation of type II NBs into type I-like NB as in *dpn* mutant animals. By 20 hours after larval hatching (ALH), only approximately 5 type II NBs per brain lobe were observed when Dpn was knocked down, and 81% of the remaining type II NBs ectopically expressed Ase (Figure 1B–B", D). Interestingly, *R9D11-tdTom*, which is only expressed in imINPs but not in type II NBs in wild-type larvae (Figure 1A–A"), was ectopically expressed in both Dpn knockdown and *dpn* mutant type II NBs at the 1st instar larval stage (Figure 1B–C"). Consistently, endogenous Erm protein was also ectopically expressed in Dpn knockdown type II NBs (Supplementary Figure S1). These results demonstrate that the *erm* is ectopically activated in type II NBs in the absence of Dpn.

Our previous studies, among others have shown that the loss of type II NBs resulting from the loss of Dpn only occurs during the 1st instar larval stage but not at later larval stages. In *dpn* mutant type II NB clones induced after reactivation of NBs, type II NBs are neither lost nor transformed into type I-like NBs, even at late 3rd instar larval stages (Zhu et al., 2012). One proposed reason is that Notch signaling, which is activated in reactivated NBs, functions redundantly in maintaining type II NBs (Zacharioudaki et al., 2012). Thus, we examined whether the ectopic activation of *erm* resulting from the loss of Dpn only occurred in quiescent type II NBs but not in proliferating cells. To address this question, we knocked down Dpn at 1 day ALH when all type II NBs were reactivated. We used the temperature-sensitive GAL80 under the control of the *tubulin* promoter (*tub-GAL80^{ts}*) (McGuire et al., 2003) for the temporal control of the expression of *UAS-dpn RNAi*. Using this approach, we abolished Dpn expression at 4 days ALH (Figure 1E–E'). Surprisingly, we still observed the ectopic expression of *R9D11-tdTom* in type II NBs at 4 days ALH (Figure 1F–F'). However, the expression level was much weaker than that in Dpn knockdown or *dpn* mutant type II NBs at 1st instar larval stages. Furthermore, we did not observe any loss of type II NBs or ectopic Ase expression in the NBs (Figure 1F), indicating that the expression level of the ectopically expressed Erm may not be sufficient to induce the transformation of type II NBs into type I-like NBs or premature loss of type II NBs. These results suggest that both Dpn and Notch signaling are required to completely suppress Erm expression in type II NBs and these molecules may not be totally functionally redundant.

Removing Erm prevents the transformation and premature loss of type II NBs resulting from the loss of Dpn

Misexpression of Erm in type II NBs is detrimental. It not only terminates NB self-renewal prematurely through a yet-to-be-identified mechanism but also transforms type II NBs into type I-like NBs by inhibiting PntP1 activity and expression (Li et al., 2016; Weng et al., 2010). Therefore, we next examined whether the transformation and premature loss of type

II NBs resulting from the loss of Dpn was due to the ectopic activation of *erm* in type II NBs. We addressed this question by generating *dpn erm* double mutants or simultaneously knocking down Dpn and Erm in type II NBs using *pntP1-GAL4* as a driver. We first examined type II NBs in *dpn erm* double mutants at 20–24 hours ALH. At this stage, nearly all type II NBs were lost in *dpn* mutants (Figure 2A–B, M). However, in *erm dpn* double mutants, we could still observe 7–8 Ase^{-} type II NBs in each brain lobe (Figure 2D, M), suggesting that in the absence of Erm, *dpn* mutant type II NBs maintained their self-renewal and cellular identities. Similarly, simultaneous knockdown of Erm almost completely recovered the loss of type II NBs resulting from Dpn knockdown. There were similar numbers of Ase^{-} type II NBs in Erm Dpn double knockdown brains and Erm knockdown brains at 4 days ALH, which is in stark contrast to the complete loss of Ase^{-} type II NBs in Dpn knockdown brains at the same stage (Figure 2E–H, N).

Since increased numbers of type II NBs in *erm* mutant or Erm knockdown brains were observed (Figure 2C, G, M, N), one might argue that the recovery of type II NBs could be due to dedifferentiation of INPs resulting from the loss of Erm. Therefore, we also examined type II NBs in *erm dpn* double mutant brains immediately after larval hatching when type II NBs are still quiescent. We reasoned that if type II NBs were not actively dividing to produce INPs, then the loss of Erm would not lead to the dedifferentiation of INPs or an increased number of type II NBs. Since quiescent type I NBs in newly hatched larvae do not express Ase either, we used mCD8-GFP driven by *pntP1-GAL4* to label type II NBs. We observed that similar to wild-type, there were still only 8 type II NBs in *erm* mutants at 0 – 4 hours ALH, but the number of type II NBs had reduced to 5/brain lobe in *dpn* mutants at the same stage (Figure 2I–K, M). These remaining type II NBs in *dpn* mutants ectopically expressed *R9D11-tdTom* and showed markedly reduced expression of mCD8-GFP driven by *pntP1-GAL4* (Figure 2J) likely due to the inhibitory effect of ectopically expressed Erm on *pntP1* expression (Li et al., 2016). However, in *erm dpn* double mutants, the number of type II NBs remained the same as that in wild-type and the expression level of mCD8-GFP driven by *pntP1-GAL4* was also restored (Figure 2L–M), suggesting that the rescue of type II NBs in *dpn erm* double mutant or Dpn Erm double knockdown brains observed at later developmental stages is not due to dedifferentiation of INPs. These results demonstrate that the transformation and premature loss of type II NBs resulting from the loss of Dpn is at least in part due to the ectopic activation of *erm* in type II NBs.

Dpn and E(spl) proteins directly bind to the cis-regulatory region of the erm gene

Dpn and Notch downstream targets, E(spl) proteins, are Hes family proteins that function as transcriptional repressors and preferentially bind to DNA sequences containing the N-box (CACNAG) or the class C-site [CACG(C/A)G] (Kageyama et al., 2007; Oellers et al., 1994; Winston et al., 1999) {Fisher, 1998 #379;Winston, 1999 #416;Winston, 1999 #416}. To investigate how Dpn and E(spl) proteins suppress Erm expression in type II NBs and determine which E(spl) proteins might mediate the suppression of *erm* by the Notch signaling, we next examined whether Dpn and E(spl) proteins directly bind to the *erm* regulatory region and whether different E(spl) proteins show distinct binding affinities. We first searched for potential Dpn/E(spl) binding sites in the *R9D11* enhancer region using the FlyFactorSurvey (http://mccb.umassmed.edu/ffs/) and the Target Explorer (http://

te.cryst.bbk.ac.uk/). We detected three putative Dpn/E(spl) binding sites located at −9,463, −8,424, and −7,456 bps from the transcription start site, which contained consensus sequences of the N-box or C-site and were named as bHLH-O1, bHLH-O2, and bHLH-O3, respectively (Figure 3B, Figure 4A). These putative binding sites are well conserved among different *Drosophila* species (data not shown), suggesting that they could function as bona fide Dpn/E(spl) binding sites. We then performed gel mobility shift assays to determine whether Dpn or any E(spl) proteins could directly bind to these putative sites. We first used a Cy5-labeled 20 bp DNA fragment containing the bHLH-O3 as a probe (Cy5-bHLH-O3) (Figure 3B). We observed that Dpn as well as five E(spl) proteins, including E(spl)m3, E(spl)m7, E(spl)mβ, E(spl)mδ, and E(spl)mγ, could bind to the probe, as indicated by the presence of retarded Cy5-labeled bands (Figure 3A, C). The observed binding appeared to be specific, as non-labeled probes with the same sequence (bHLH-O3-S) could compete for the binding ("S" in Figure 3C) but probes with mutations in the C-site (bHLH-O3-NS) could not ("NS" in Figure 3C). However, different from other E(spl) proteins, E(spl)m5 and E(spl)m8 did not bind to Cy5-bHLH-O3 (Figure 3A, C). Comparison of amino acid sequences of the bHLH domain of Dpn and E(spl) proteins revealed that E(spl)m5 and E(spl)m8 are more distantly related to other E(spl) proteins or Dpn (Figure 3A), suggesting that E(spl)m5 and E(spl)m8 might bind to different DNA sequences.

To determine whether Dpn or E(spl) proteins could also bind to other two putative binding sites, we used non-labeled 20bp DNA fragments containing either bHLH-O1 or bHLH-O2 (Figure 3B) to compete with Cy5-bHLH-O3 for the binding. We observed that the probe containing bHLH-O1 could fully compete with the Cy5-bHLH-O3 for the binding with Dpn, E(spl)m3, E(spl)m7, or E(spl)mβ (Figure 3D). However, the bHLH-O2 probe could only partially compete with the Cy5-bHLH-O3 for the binding, particularly for E(spl)m3, m7, and mβ (Figure 3D). These data suggest that bHLH-O3 and bHLH-O1 have higher affinity for Dpn and E(spl) proteins than bHLH-O2.

Taken together, the results of the gel mobility shift assays indicate that Dpn and a subset of E(spl) proteins can directly bind to the cis-regulatory region of the *erm* gene. However, not all E(spl) proteins share the same core DNA binding sequences as previously suggested (Jennings et al., 1999).

bHLH-O binding sites mediate the suppression of erm in type II NBs by Dpn/E(spl) proteins

Having demonstrated that the putative binding sites can bind to Dpn and E(spl) proteins *in vitro*, we next wanted to determine whether these binding sites, particularly bHLH-O1 and bHLH-O3, could indeed mediate the suppression of *erm* by Dpn or E(spl) proteins *in vivo*. To this end, we examined whether the deletion of any of these putative bindings sites would lead to ectopic *erm* expression in type II NBs. We first utilized the existing *R9D10-lexA* line to examine how deleting bHLH-O1 would affect *erm* expression. *R9D10* is a 3.6kb DNA fragment from the *erm* regulatory region containing bHLH-O2 and bHLH-O3 but not bHLH-O1 (Figure 4A) (Pfeiffer et al., 2008). We observed that, similar to *R9D11-tdTom*, *lexAop-rCD2-GFP* driven by *R9D10-LexA* is only expressed in INPs but not in type II NBs (Figure 4B–C, I), indicating that deleting bHLH-O1 is not sufficient to induce the ectopic activation of *erm* in type II NBs and that bHLH-O3 and/or bHLH-O2 might function

redundantly with bHLH-O1. Therefore, we further deleted bHLH-O3 by generating a series of 3' deletions of the overlapping sequence between *R9D11* and *R9D10* or specifically deleting 12bp sequences containing the C-site (Figure 4A). The truncated or deletion mutant sequences were fused with the reporter gene *CD4-tdTom* to examine their expression in type II NBs.

Our results showed that the expression of CD4-tdTom remained suppressed in over 80% of type II NBs when it was driven by the entire overlapping sequence (B) between R9D11 and R9D10 or the overlapping sequence with the deletion of a 350bp fragment at the 3'-end (R1) (Figure 4D–E, I). However, deletion of an additional 384bp sequence (R2), including the bHLH-O3, led to ectopic expression of CD4-tdTom in all type II NBs (Figure 4F, I). Consistently, deletion of all three putative binding sites from *R9D11* (R9D11-ΔbHLH-O123) led to similar ectopic activation of the reporter in all type II NBs (Figure 4H–I). In contrast, deletion of the bHLH-O3 alone from *R9D11* (R9D11-ΔbHLH-O3) did not cause ectopic expression of the reporter in type II NBs (Figure 4G, I). These results demonstrate that bHLH-O1 and bHLH-O3 might function redundantly to mediate the suppression of *erm* by Dpn or E(spl) proteins. In support of this notion, we showed that the expression of R1 and R9D11-ΔbHLH-O3 in imINPs could still be dramatically suppressed when Dpn was overexpressed in type II NB lineages (Supplementary Figure S2). However, bHLH-O2 may not be critical for the suppression of *erm* in type II NBs, consistent with its relative low binding affinity for Dpn and E(spl) proteins.

Ectopic activation of dpn or Notch signaling inhibits erm activation in imINPs

In normal type II NB lineages, Erm is only expressed in imINPs, where *dpn* and Notch signaling are not activated (Janssens et al., 2014). Our previous studies, among others, have shown that Dpn overexpression or Notch overactivation leads to tumorigenic overproliferation of type II NBs due to the dedifferentiation of imINP (Bowman et al., 2008; Zacharioudaki et al., 2012; Zhu et al., 2012). Our findings that Dpn and Notch signaling suppress *erm* activation in type II NBs prompted us to examine whether the absence of Dpn or Notch signaling is essential for the activation of *erm* and whether the overexpression of Dpn or overactivation of Notch signaling suppresses Erm expression in imINPs, which in turn contributes to the dedifferentiation of imINPs and subsequent overproliferation of type II NBs. Thus, we next examined how the ectopic activation of *dpn* or Notch signaling in imINPs would affect Erm expression. In addition, we examined whether misexpression of different E(spl) proteins with distinct binding affinities for the bHLH-O binding sites would affect *erm* expression in imINPs differently. We used *pntP1-GAL4*, which is highly expressed in imINPs in addition to type II NBs (Zhu et al., 2011), as a driver for manipulating the expression of these genes in imINPs.

The results showed that the overexpression of Dpn in type II NB lineages resulted in massive overproliferation of type II NBs and imINPs, but a majority of imINPs did not express the *erm* reporter *R9D11-tdTom* (Figure 5B), which is typically expressed in imINPs except newly generated imINPs next to the NB in wild-type larval brains (Figure 5A). Similar results were also observed when N^{intra} or E(spl) proteins, such as E(spl)mβ and E(spl)mδ, which can bind to bHLH-O binding sites, were overexpressed or Numb was knocked down

(Figure 5C–F). However, the overexpression of E(spl)m8 or m5, which do not bind to the identified bHLH-O binding sites, did not affect either type II NB lineage development or *R9D11-tdTom* expression (Figure 5G–H). These results suggest that Dpn overexpression or Notch overactivation suppress the expression of *erm* in imINPs, but only the E(spl) proteins that bind to the bHLH-O binding sites could function downstream of Notch to suppress *erm*. Therefore, the absence of Dpn or Notch signaling in imINPs is essential for the activation of *erm* in imINPs.

Erm suppression largely accounts for the dedifferentiation of imINPs resulting from ectopic activation of dpn or Notch signaling in imINPs

The activation of *erm* in imINPs is required for the maturation of INPs. In the absence of Erm, INPs fail to mature and dedifferentiate into type II NBs (Weng et al., 2010). Therefore, the suppression of *erm* in imINPs likely contributes to the overproliferation of type II NBs resulting from Notch or Dpn ectopic activation in imINPs. However, in addition to functioning through the canonical pathway to activate the expression of *E(spl)* genes, Notch could act through noncanonical pathways. Similarly, Dpn may also regulate the expression of other target genes in NBs/INPs, as Dpn is also required for maintaining the self-renewal of type I NBs, in which Erm is not expressed (Janssens et al., 2014; Zhu et al., 2011). Therefore, we examined whether the suppression of *erm* in imINPs was fully responsible for the overproliferation of type II NBs resulting from the ectopic activation of Dpn or Notch signaling.

To answer this question, we examined how maintaining Erm expression in imINPs would affect the type II NB overproliferation resulting from ectopic activation of Dpn, Notch, or E(spl) proteins. To maintain Erm expression in imINPs, we used *erm-GAL4 (II)* (Xiao et al., 2012) to drive the expression of *UAS-erm* while we used the same GAL4 to drive the expression of Dpn, N^{intra}, E(spl)mβ, or mδ, or knock down Numb in imINPs. Ectopic expression of Dpn, N^{intra}, E(spl)mβ, or E(spl)mδ, or knockdown of Numb in imINPs all increased the numbers of type II NBs (Figure 6A, C, E, G, I, K, M). However, when *erm-GAL4 (II)* was used to drive the expression of Erm, the overproliferation phenotype caused by E(spl)mβ or mδ misexpression was completely suppressed, resulting in only 8 type II NBs/brain lobe (Figure 6B, H, J, M). Similarly, the overproliferation phenotype resulting from Dpn misexpression was also suppressed by over 98% (Figure 6L–M). These results indicate that the suppression of Erm is largely responsible for the overproliferation of type II NBs resulting from the ectopic activation of the canonical Notch pathway or Dpn misexpression. However, the overproliferation of type II NBs resulting from misexpression of N^{intra} or Numb knockdown was only suppressed by 80% or 50% (Figure 6D, F, M), respectively, although their phenotypes were comparable to those caused by the misexpression of Dpn or E(spl) proteins (Figure 6C, E, G, I, K, M), suggesting that Notch also likely acts through a non-canonical pathway to promote the overproliferation of type II NBs.

PntP1 mediates the dedifferentiation of imINPs resulting from the loss of Erm or misexpression of Dpn or E(spl) proteins

Why does the suppression of Erm in imINPs lead to the dedifferentiation of imINPs and subsequent overproliferation of type II NBs? Recently studies have shown that Erm could inhibit the expression of PntP1 and antagonize the function of PntP1 (Janssens et al., 2017; Li et al., 2016). Therefore, we wondered whether Erm promotes INP maturation by similarly inhibiting the expression and/or function of PntP1 in imINPs, in which both PntP1 and Erm are expressed (Janssens et al., 2014; Zhu et al., 2011), and whether the de-repression of PntP1's expression and/or function in imINPs contribute to the dedifferentiation of imINPs resulting from the loss of Erm and misexpression of Dpn or E(spl) proteins. To test this idea, we then tried to knockdown PntP1 while simultaneously knocking down Erm or ectopically expressed Dpn or E(spl)mδ in imINPs. Consistent with a previous report (Koe et al., 2014), knocking down Erm in imINPs using *erm-GAL4 (II)* led to an increase in the number of type II NBs from 8/brain lobe to approximately 18/brain lobe (Figure 7A, D, M). However, when Erm and PntP1 were simultaneously knocked down in imINP, only approximately 9 type II NBs per brain lobe were observed (Figure 7B–C, E–F, M), suggesting that the overproliferation of type II NBs resulting from Erm knockdown was largely suppressed by Pnt knockdown. Similarly, the overproliferation of type II NBs resulting from the misexpression of Dpn or E(spl)mδ was also reduced by 90% or 65%, respectively, when PntP1 was simultaneously knocked down in imINPs (Figure 7G–M). These results provide evidence to support that the dedifferentiation of imINPs resulting from the loss of Erm or the misexpression of Dpn or E(spl) proteins is in part due to de-repression of PntP1 expression and/or function in imINPs.

DISCUSSION

In the present study, we demonstrate that similar to the canonical Notch signaling, Dpn maintains the identity and self-renewal of type II NBs at least in part by inhibiting Erm expression. We show that the loss of Dpn leads to the ectopic activation of *erm* in type II NBs and that removing Erm not only prevents the transformation of *dpn* mutant or Dpn knockdown type II NBs into type I-like NBs but also largely inhibits their premature termination of self-renewal. The results from our gel-shift assays and reporter assays provide evidence to support that Dpn and E(spl) proteins suppress Erm expression by directly binding to at least two of the three putative bHLH-O binding sites in the *erm* enhancer.

Although Dpn and canonical Notch signaling could function through a similar mechanism, these factors do not appear to be completely functionally redundant as previously suggested (Zacharioudaki et al., 2012). First, during early 1st instar larval stages when type II NBs are still quiescent, the maintenance of type II NBs may mainly rely on Dpn in that Notch is not activated in quiescent type II NBs (Zacharioudaki et al., 2012), as evidenced through our findings showing that the loss of Dpn at early 1st instar larval stages leads to ectopic Erm-mediated transformation and the premature loss of type II NBs. Second, after reactivation of type II NBs, both Dpn and Notch signaling are required to suppress the ectopic Erm expression in type II NBs because both the loss of Dpn and the components of the canonical Notch signaling pathways alone (Li et al., 2016) lead to ectopic Erm expression in type II

NBs. However, the Notch signaling likely plays a dominant role in suppressing ectopic Erm expression and maintaining type II NBs. We have previously shown that the loss of components of the canonical Notch pathway, including E(spl) proteins, leads to ectopic Erm expression and the transformation and premature loss of type II NBs, despite the presence of Dpn in the NBs, whereas the knockdown of Dpn after the reactivation of NBs only results in the weak ectopic activation of *erm* but not transformation or premature loss of type II NBs (Li et al., 2016; Zhu et al., 2012). Therefore, Dpn and Notch signaling may not be completely functionally redundant in suppressing the ectopic Erm expression or maintaining type II NBs, and their functions might be dependent on developmental stages. Furthermore, a recent study reported that Klu could also bind to the *R9D11* enhancer to repress the expression of Erm (Janssens et al., 2017). Thus, type II NBs likely utilize multiple mechanisms to ensure that *erm* will not be prematurely activated.

Previous studies suggested that all E(spl) proteins share similar DNA sequences (Jennings et al., 1999). However, results from the present study suggest that this similarity may not always be the case. Our gel-shift assays show that only 5 members of the E(spl) family, including E(spl)mγ, mβ, mδ, m3, and m7, can bind to the bHLH-O binding sites in the *erm* regulatory region, whereas the other two, E(spl)m5 and m8, cannot. The difference in their DNA binding specificity is consistent with differences in the amino acid sequences of their bHLH domains and their overexpression phenotypes in type II NB lineages (Zacharioudaki et al., 2012). Therefore, although multiple E(spl) proteins have been shown to be expressed in larval NBs and at least two of them, E(spl)mγ and m8, are activated by Notch (Zacharioudaki et al., 2012), these E(spl) proteins may bind to different DNA sequences and regulate the expression of different target genes, which may in turn determine their functional specificity.

In contrast to the maintenance of type II NBs, the maturation of imINPs requires the activation of *erm* by PntP1 and shutdown of Dpn expression and Notch signaling. It has previously been shown that the loss of Erm or aberrant activation of *dpn* or Notch signaling in imINPs both lead to the dedifferentiation of imINPs and overproliferation of type II NBs (Bowman et al., 2008; Weng et al., 2010; Zacharioudaki et al., 2012; Zhu et al., 2012). However, the functional relationship between the activation of *erm* and the absence of Dpn or Notch signaling in imINPs has never been established. Here, we demonstrate here that the absence of Dpn and Notch signaling is essential for the activation of *erm* and subsequent Erm-mediated maturation of INPs. First, the results show that aberrant activation of *dpn* or Notch signaling inhibits the activation of *erm* in imINPs. Second, maintaining Erm expression in imINPs largely blocks the overproliferation of type II NBs resulting from the misexpression of E(spl) or Dpn proteins, suggesting that one main reason for the dedifferentiation of imINPs caused by Dpn or E(spl) overexpression is the suppression of Erm. However, the overproliferation of type II NBs resulting from the overexpression of N^{intra} or Numb knockdown can only be partially suppressed by concomitant Erm expression. Therefore, in addition to functioning through the canonical pathway to activate E(spl) expression, Notch may also act through noncanonical pathways, such as the mTORC2/Akt pathway (Lee et al., 2013), to regulate type II NB proliferation.

How does Erm promotes INP maturation and prevents the dedifferentiation of imINP? It has previously been suggested that Erm prevents the dedifferentiation of INPs by activating *pros* expression and attenuating the response of INPs to self-renewing factors such as Dpn and E(spl) proteins (Janssens et al., 2014; Weng et al., 2010). However, two pieces of evidence argue against this notion. First, the loss of Pros only induces the overproliferation of INPs but not the dedifferentiation of imINPs into type II NBs (Bowman et al., 2008). Second, Erm is only expressed in imINPs, which do not express Dpn or E(spl) proteins (Boone and Doe, 2008; Janssens et al., 2014; Song and Lu, 2011). In the present study, we provide evidence demonstrating that Erm likely promotes INP maturation in part by inhibiting the expression and/or function of PntP1. These results show that the overproliferation of type II NBs resulting from the loss of Erm or overexpression of Dpn or E(spl) proteins, which leads to suppression of Erm expression, could be significantly inhibited by knocking down PntP1. These data strongly argue that the dedifferentiation of imINPs and generation of extra type II NBs resulting from the loss of Erm is in part due to de-repression of PntP1 expression and/or function in imINPs, which is consistent with the PntP1 function in specifying type II NBs and suppressing the activation of *ase* (Zhu et al., 2011). Similar to other Ets family proteins that are commonly involved in tumorigenesis (Kar and Gutierrez-Hartmann, 2013; Seth and Watson, 2005), PntP1 may also activate the expression of cell cycle regulators that promote nonproliferative imINPs to enter the cell cycle and initiate unrestricted tumorigenic overproliferation. However, PntP1 may not be the only target of Erm in imINPs. As shown in a recent study, in addition to PntP1, Erm also directly inhibits the expression of Grh-O in imINPs (Janssens et al., 2017). Therefore, Erm likely promotes the maturation of INPs by regulating the expression/function of multiple target genes.

In conclusion, we demonstrate here that similar to Notch signaling, Dpn maintains the identity and self-renewal of type II NBs in part by inhibiting Erm expression. Whereas in imINPs, the absence of Dpn and E(spl) proteins allows PntP1-mediated activation of *erm*, which in turn promotes INP maturation by inhibiting the expression and/or function of PntP1 and Grh-O in imINPs. Thus, the present study elucidates the mechanistic details of the maintenance of type II NBs and maturation of INPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Deadpan maintains *Drosophila* type II neuroblasts by suppressing Earmuff (Erm)
- bHLH-O proteins bind directly to the *erm* promoter
- Absence of bHLH-O proteins in imINPs allows *erm* activation and INP maturation
- Erm promotes INP maturation by antagonizing the function of Pointed P1

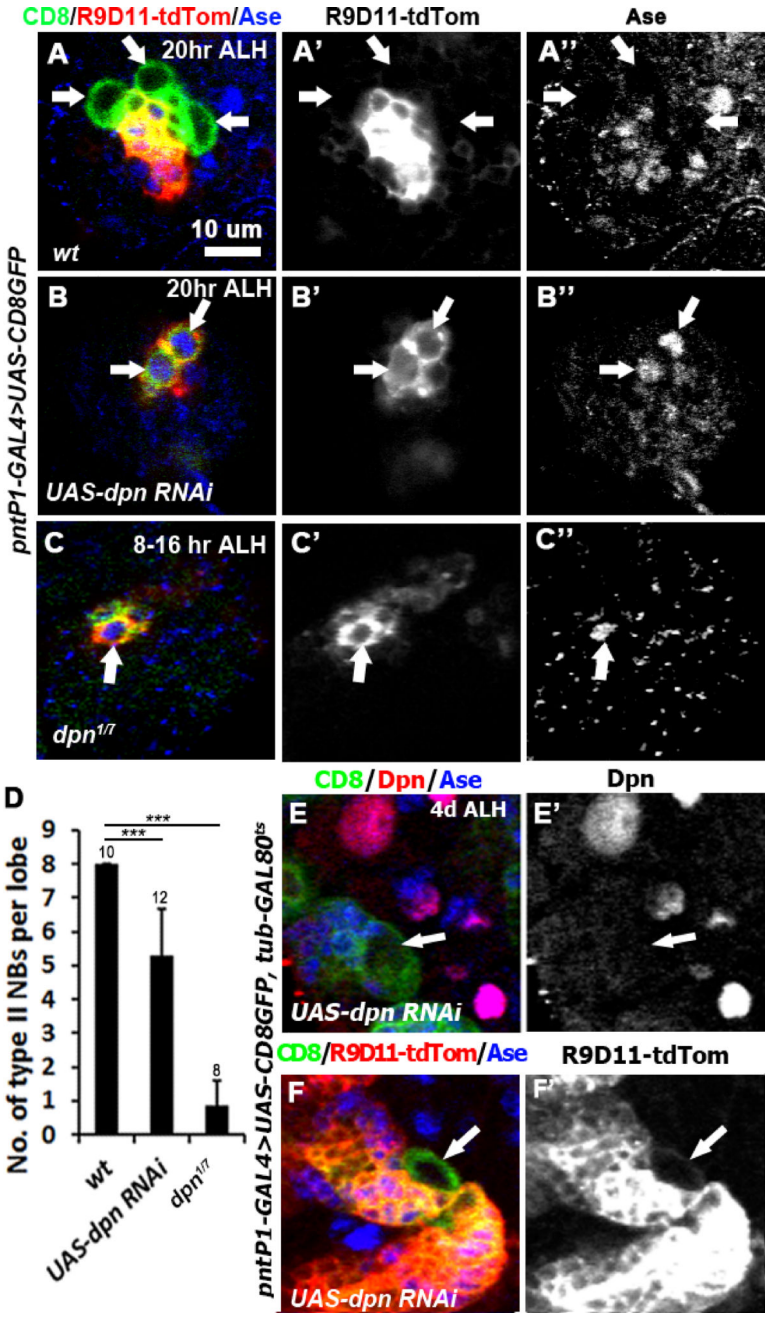

Figure 1. Loss of Dpn leads to ectopic Erm expression in type II NBs

Type II NB lineages are labeled with mCD8-GFP driven by *PntP1-GAL4* in all panels. Arrows point to type II NBs. (A–B") R9D11-tdTom and Ase are ectopically expressed in Dpn knockdown (B–B") but not wild-type (A–A") type II NBs at 20 hours ALH. (C–C") Most type II NBs are lost in $dpn^{1/7}$ mutant larval brains at 8–16 hours ALH and the remaining type II NBs express R9D11-tdTom and Ase. (D) Quantifications of the number of type II NBs in wild-type and Dpn knockdown brains at 20 hours ALH and *dpn* mutant brains at 8–16 hours ALH. Numbers on top of each bar in this and all other bar graphs represent sample sizes. ***, $P < 0.01$. (E–F') Knocking down Dpn after the 1st instar larval stage

abolishes Dpn expression (E–E') and ectopically activates R9D11-tdTom in type II NBs (F–F') at 4 days ALH.

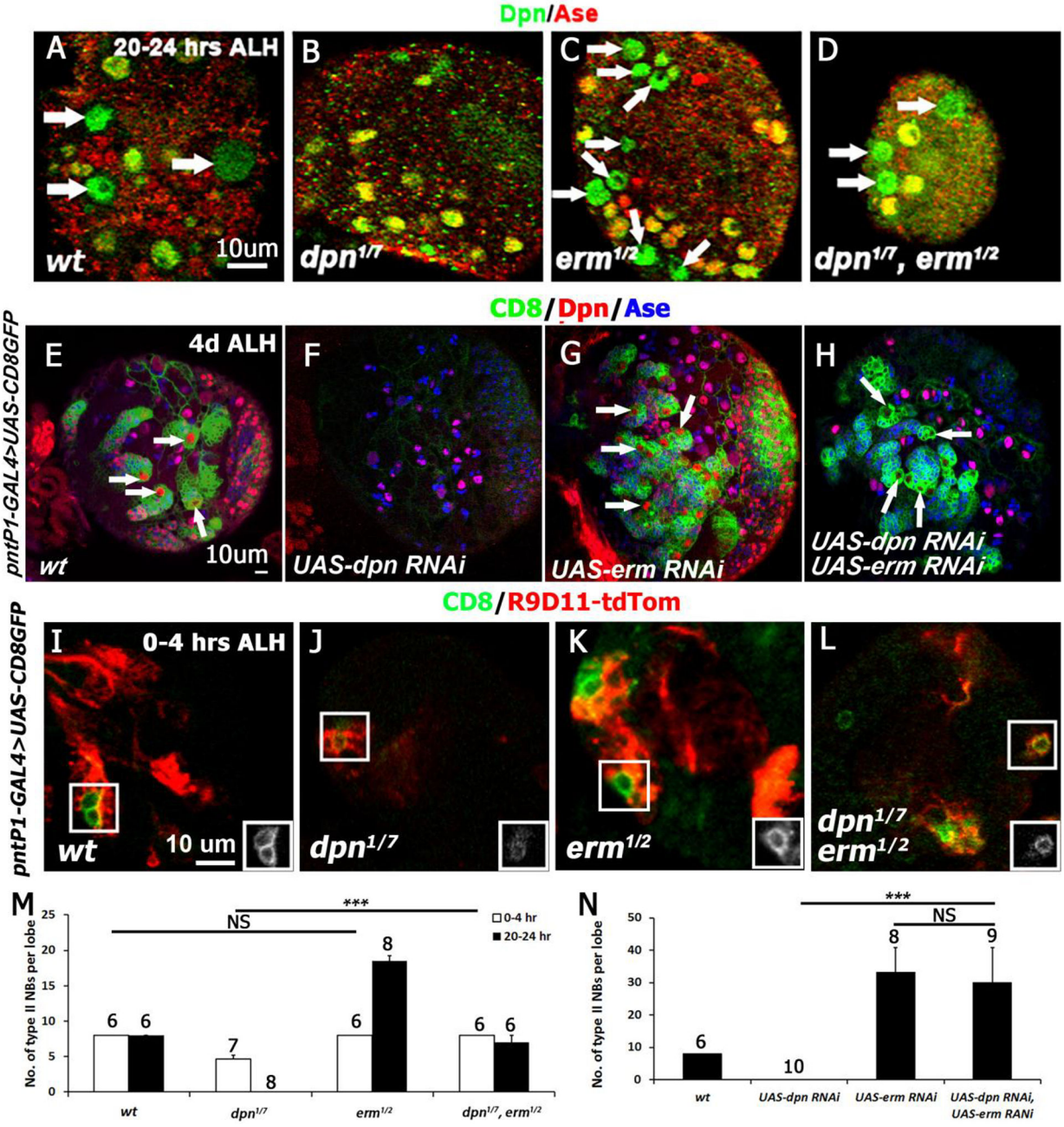

Figure 2. Ectopic Erm expression is responsible for the premature loss of type II NBs resulting from loss of Dpn

(A–D) *dpn* mutant type II NBs are maintained in *erm* mutant background. Type II NBs, which are identified as Dpn^{+} Ase^{-} cells (arrows), are completely lost in *dpn* mutant brains (B) but are still present in *erm dpn* double mutants (D) as in the wild-type (A) or *erm* mutant (C) brains at 20–24 hours ALH. Note that there is an increased number of type II NBs in *erm* mutant brains (C). (E–H) Knockdown of Erm rescues the loss of type II NBs resulting from Dpn knockdown. Type II NBs labeled by mCD8-GFP are present in wild-type (E) but not in Dpn knockdown brains (F) at 4 days ALH. However, the number of type II NBs in Erm Dpn double knockdown brains (H) is comparable to that in Erm knockdown brains (G)

at 4 days ALH. (I–L) Removing Erm restores the number of type II NBs and *pntP1-GAL4* expression in newly hatched $dpn^{1/7}$ mutant larval brains. Type II NBs are largely lost in *dpn* mutant brains (J) at 0–4 hours ALH and the remaining type II NBs ectopically express R9D11-tdTom and have reduced expression levels of mCD8-GFP driven by *pntP1-GAL4* compared with that in wild-type brains (I). In *erm dpn* double mutants (L), type II NBs and their expression of mCD8-GFP expression are still maintained as in the wild-type (I) or *erm* mutant brains (K) but R9D11-tdTom is still ectopically expressed in the NBs. Insets show mCD8-GFP expression in highlighted type II NBs. (M–N) Quantifications of the number of type II NBs in larval brains with indicated genotypes at 0–4 hours or 20–24 hours ALH (M) or 4 days ALH (N). ***, $P < 0.01$.

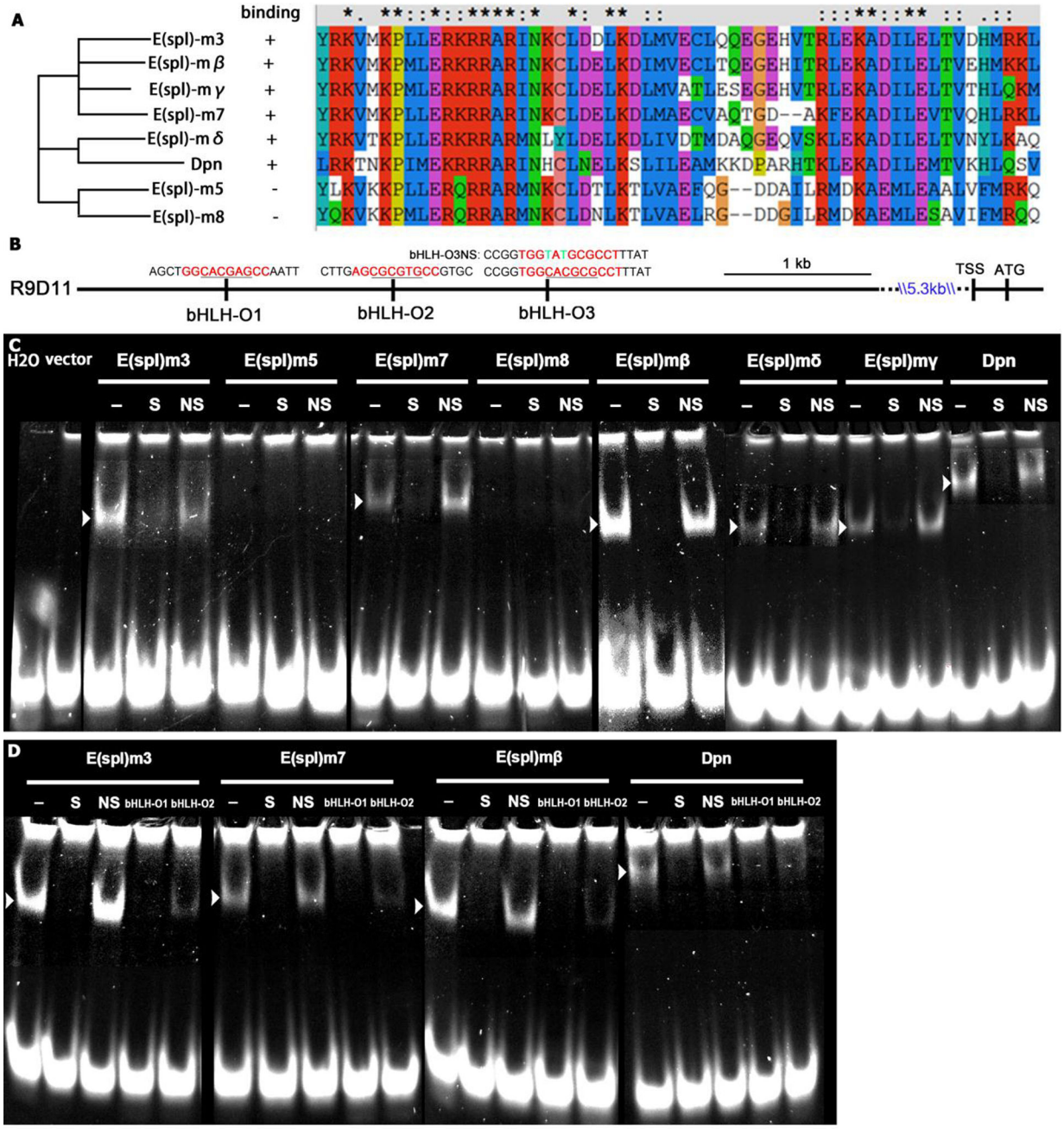

Figure 3. Dpn and E(spl) proteins directly bind to the *erm* regulatory region

(A) Dpn and seven E(spl) proteins can be classified into three subfamilies (left) based on the amino acid sequence similarity of their bHLH domains, which were aligned using ClustalW on the right. "+" and "−" indicate their binding to the bHLH-O3 in the *R9D11* region. (B) Three predicted Dpn/E(spl) binding sites (bHLH-O1, bHLH-O2, and bHLH-O3) in the R9D11 region. Probe sequences used for the gel-shift assays are shown on top of individual binding sites with the core putative Dpn/E(spl) binding sequences indicated in red. Sequences of the N-box in bHLH-O1 and the C-sites in bHLH-O2 and bHLH-O3 are underlined. Note that the C-site sequence in bHLH-O2 is in a reverse orientation. The two

mutated nucleotides in the probe bHLH-O3NS are shown in green. TSS: transcription start site. (C) Gel-shift assays show E(spl)m3, m7, mβ, mδ, mγ, and Dpn, but not E(spl)m5 or mδ, bind to the cy5-labeled bHLH-O3 probe. The Non-labeled wild-type probe bHLHL-O3S (S) is used as a specific competitor and the non-labeled mutant probe bHLH-O3NS (NS) as a non-specific competitor. (D) The binding of E(spl) and Dpn proteins to the probe Cy5-bHLH-O3 is completely abolished in the presence of the non-labeled probe bHLH-O1 but is only reduced in the presence of bHLH-O2. Arrowheads in (C) and (D) indicate locations of retarded bands.

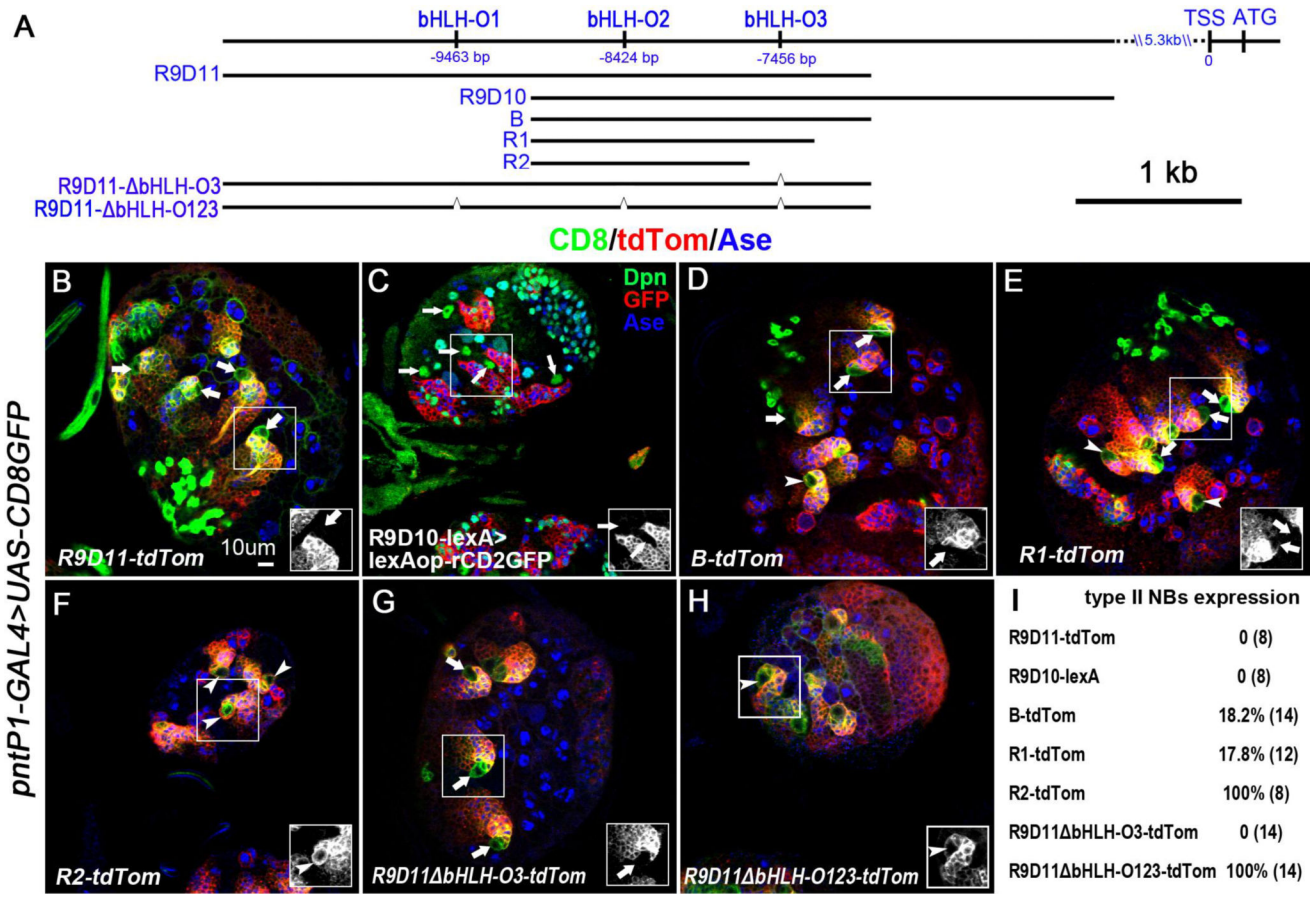

Figure 4. bHLH-O binding sites mediate the suppression of *erm* in type II NBs

(A) A schematic diagram shows DNA fragments from the *erm* enhancer region that were used to drive the expression of CD4-tdTom. TSS: transcription start site. (B–H) Expression of the CD4-tdTom reporter driven by individual *erm* enhancer fragments in type II NB lineages labeled with mCD8-GFP. Arrowheads and arrows point to type II NBs with or without CD4-tdTom expression, respectively. (I) Percentages of type II NBs with the expression of CD4-tdTom driven by individual *erm* enhancer fragments. Numbers in parenthesis are the number of brain lobes examined.

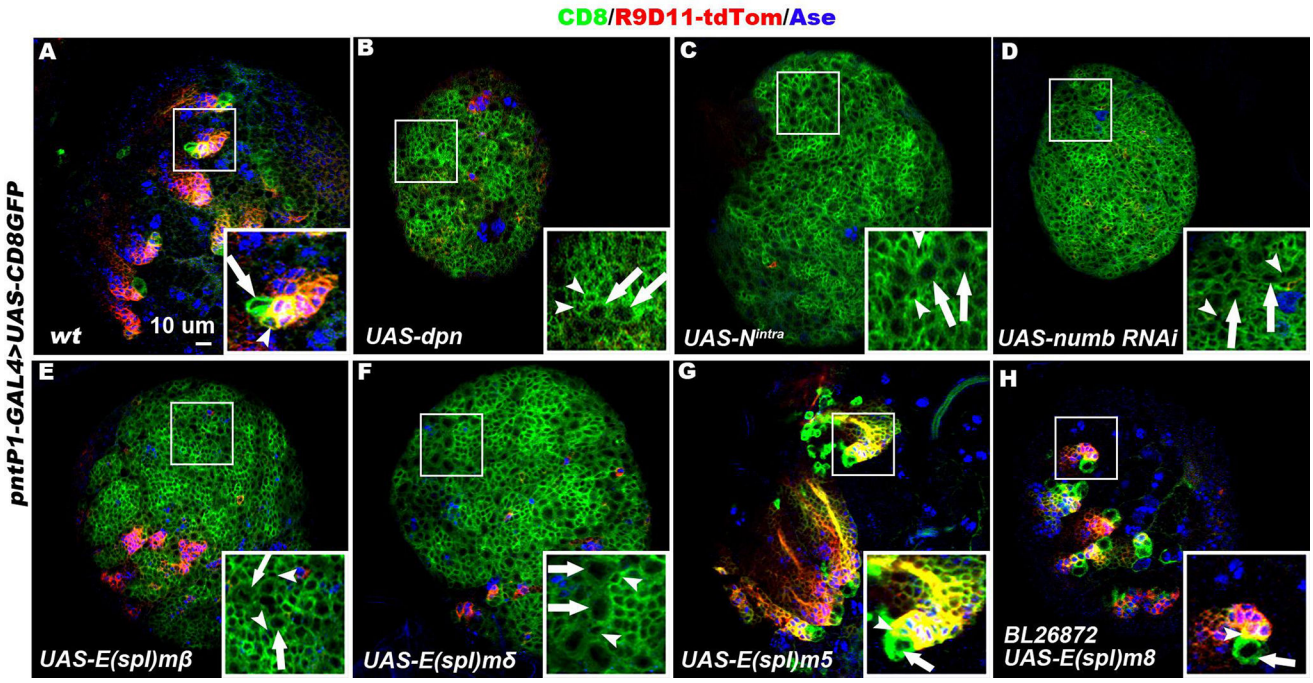

Figure 5. Manipulating the expression of Dpn or components of the Notch signaling pathway differentially affects Erm expression and type II NB lineage development

Type II NB lineages are labeled with mCD8-GFP driven by *pntP1-GAL4*. Arrows: type II NBs; arrowheads: imINPs. (A) R9D11-tdTom is expressed in imINPs but not the NBs in wild-type type II NB lineages. (B–F) Overexpression of Dpn (B), N^{intra} (C), E(spl)mβ (E), or E(spl)mδ (F), or knockdown of Numb (D) leads to overproliferation of imINPs and type II NBs and suppression of R9D11-tdTom in most imINPs. (G–H) Overexpression of E(spl)m5 (G) or E(spl)m8 (H) neither causes overproliferation of type II NBs nor suppresses the expression of R9D11-tdTom in imINPs.

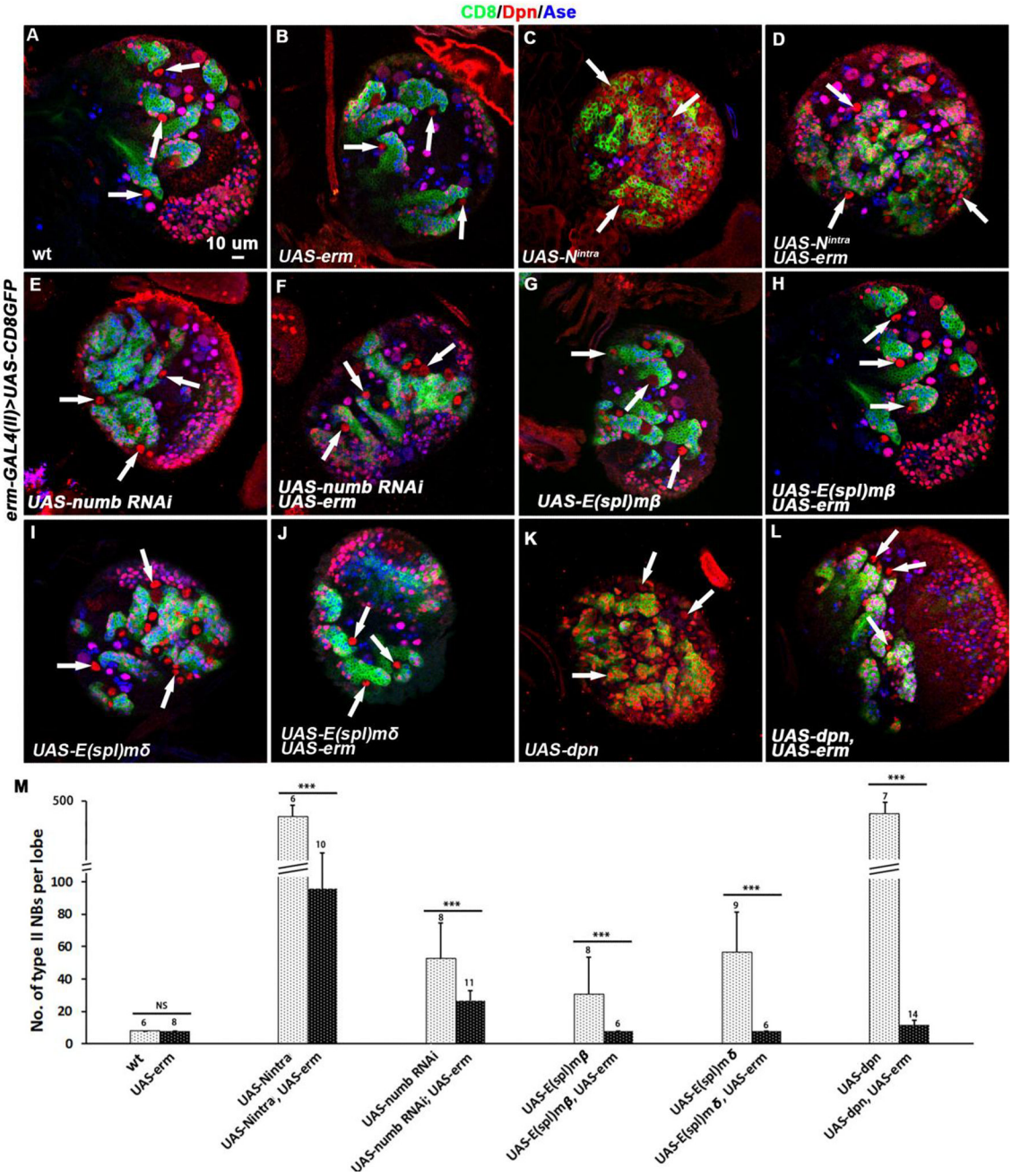

Figure 6. Maintaining Erm expression in imINPs largely suppresses the overproliferation of type II NBs induced by Dpn overexpression or Notch overactivation

Type II NB linages are labeled with mCD8-GFP driven by *erm-GAL4 (II)* and arrows point to type II NBs. (A) A wild type brain lobe has only 8 type II NBs. (B) Overexpression of Erm in imINPs does not affect type II NB lineage development. (C, E, G, I, K) Overexpression of N^{intra} (C), E(spl)mβ (G), E(spl)mδ (I), Dpn (K), or knockdown of Numb (E) in imINPs leads to overproliferation of type II NBs. (D, F, H, J, L) Expression of Erm driven by *erm-GAL4 (II)* completely or near completely inhibits the generation of extra type II NBs resulting from the overexpression of E(spl)mβ (H), E(spl)mδ (J), or Dpn (L), but only partially suppresses the type II NB overproliferation induced by N^{intra} overexpression

(D) or Numb knockdown (F). (M) Quantifications of the number of type II NBs in brains with indicated genotypes. ***, $P < 0.01$; NS: not significant.

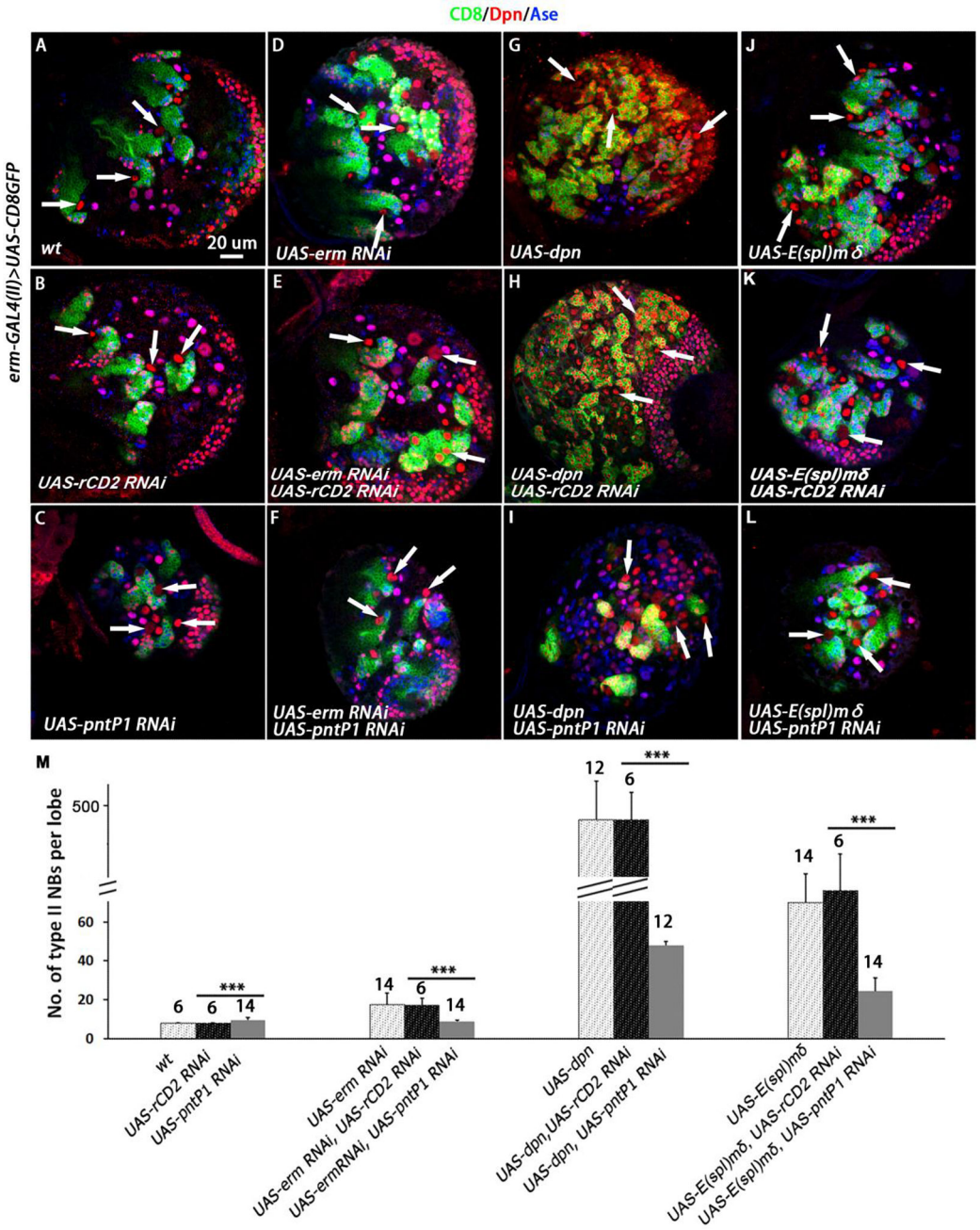

Figure 7. Knocking down PntP1 greatly inhibits the dedifferentiation of imINPs resulting from Erm knockdown or overexpression of Dpn or E(spl)mδ

Type II NB lineages are labeled with mCD8-GFP driven by *erm-GAL4 (II).* Arrows point to type II NBs. *UAS-rCD2 RNAi* serves as a control *UAS- RNAi.* (A) A wild-type brain lobe has only 8 type II NB lineages. (B) Expressing *UAS-rCD2* RNAi does not affect type II NB lineage development. (C) Knocking down PntP1 in imINPs by *erm-GAL4 (II)* slightly increases the number of type II NBs. (D, G, J) Knocking down Erm (D) or overexpressing Dpn (G) or E(spl)mδ (J) in imINPs induces overproliferation of type II NBs. (E, H, K) Knockdown of rCD2 does not affect type II NB overproliferation resulting from Erm knockdown (E) or overexpression of Dpn (H) or E(spl)mδ (K). (F, I, L) Knockdown of

PntP1 suppresses the type II NB overproliferation resulting from Erm knockdown (F) or overexpression of Dpn (I) or E(spl)mδ (L). (M) Quantifications of the number of type II NBs in brains with indicated genotypes. ***, $P < 0.01$.