Notch maintains Drosophila type II neuroblasts by suppressing expression of the Fez transcription factor Earmuff

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ABSTRACT
Notch signaling is crucial for maintaining neural stem cell (NSC) self-renewal and heterogeneity; however, the underlying mechanism is not well understood. In Drosophila, loss of Notch prematurely terminates the self-renewal of larval type II neuroblasts (NBs, the Drosophila NSCs) and transforms type II NBs into type I NBs. Here, we demonstrate that Notch maintains type II NBs by suppressing the activation of earmuff (erm) by Pointed P1 (PntP1). We show that loss of Notch or components of its canonical pathway leads to PntP1-dependent ectopic Erm expression in type II NBs. Knockdown of Erm significantly rescues the loss-of-Notch phenotypes, and misexpression of Erm phenocopies the loss of Notch. Ectopically expressed Erm promotes the transformation of type II NBs into type I NBs by inhibiting PntP1 function and expression in type II NBs. Our work not only elucidates a key mechanism of Notch-mediated maintenance of type II NB self-renewal and identity, but also reveals a novel function of Erm.

KEY WORDS: Notch, Drosophila, Neuroblasts, Earmuff, Pointed P1

INTRODUCTION
Producing a large number of diverse types of neurons in the brain requires the generation of a heterogeneous population of neural stem cells (NSCs) that differ in their identities, developmental potential and proliferative capacity, as well as the maintenance of NSC self-renewal. Their heterogeneity allows different NSCs to generate different neuronal lineages, whereas self-renewal maintains the NSC pool. In both developing and adult brains, Notch signaling plays crucial roles in maintaining NSC self-renewal and heterogeneity (Ables et al., 2011; Giachino and Taylor, 2014; Pierfelice et al., 2011; Yoon and Gaiano, 2005). Loss of Notch or its downstream components can lead to depletion of the NSC pool and precocious neuronal differentiation (de la Pompa et al., 1997; Grandbarbe et al., 2003; Hatakeyama et al., 2004; Ishibashi et al., 1995; Ohtsuka et al., 1999; Yun et al., 2002). Differences in Notch receptors, signaling strength and dynamics, and pathway components may contribute to NSC heterogeneity (Alumni et al., 2013; Basak et al., 2012; Ehlm et al., 2010; Giachino and Taylor, 2014). However, the detailed mechanism of Notch-mediated maintenance of NSC self-renewal and heterogeneity is still not fully understood. This is mainly because the Notch downstream effectors that maintain NSC self-renewal and heterogeneity in distinct NSC populations remain largely elusive.

Drosophila larval neuroblasts (NBs, Drosophila NSCs) have recently emerged as an excellent NSC model. NBs in the larval central brain are heterogeneous and can be classified into type I and type II NBs, based on the expression of specific molecular markers and their neurogenesis patterns (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). Unlike type I NBs, which produce terminal dividing ganglion mother cells (GMCs), type II NBs produce intermediate neural progenitor cells (INPs) like mammalian NSCs (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). INPs divide asymmetrically like type I NBs to produce GMCs but have limited self-renewal capacity. Type II NBs lack the proneural protein Asense (Ase) and the homeodomain protein Prospero (Pros) but express the Ets family transcription factor Pointed (PntP1), which is required for type II NB specification and generation of INPs (Zhu et al., 2011). Newly generated INPs are immature and need to differentiate to become mature INPs before they divide (Fig. 1N; see also below, Fig. 7A). INP maturation requires the tumor suppressors Numb and Brain tumor (Brat), as well as the transcription factor Earmuff (Erm), a homolog of the vertebrate Forebrain embryonic zinc-finger (Fez) family (Bowman et al., 2008; Weng et al., 2010). Numb inhibits Notch signaling in immature INPs (imINPs), whereas Brat reduces the activity of Armadillo (Arm; β-catenin) in imINPs (Bowman et al., 2008; Komori et al., 2014b). Erm functions together with the SWI/SNF chromatin-remodeling complex in imINPs to restrict the developmental potential of INPs (Eroglu et al., 2014; Farnsworth et al., 2015; Janssens et al., 2014; Koe et al., 2014). Meanwhile, INPs need to avoid differentiating into GMCs prematurely and exiting the cell cycle. Preventing the premature differentiation of INPs requires the suppression of Pros in newly generated imINPs by the Sp family transcription factor Buttonhead (Btd) (Komori et al., 2014a; Xie et al., 2014).

Like mammalian NSCs, maintaining the self-renewal and heterogeneity of Drosophila larval NBs requires Notch signaling. Loss of Notch or components of the Notch pathway, such as Sanpodo (Spdo) and Anterior pharynx defective 1 (Aph-1), leads to a premature and complete loss of type II NBs (Bowman et al., 2008; Song and Lu, 2011; Zhu et al., 2012). Furthermore, loss of Notch also results in a transformation of type II NBs into type I-like NBs, which makes NBs in the central brain appear homogeneous (Zhu et al., 2012). Interestingly, loss of Notch alone largely spares type I NBs, even though Notch is similarly activated in type I and type II NBs (Zacharioudaki et al., 2012). However, it is still unclear why loss of Notch specifically affects the identity and self-renewal of type II but not type I NBs.

In this study, we investigate how Notch maintains type II NB identity and self-renewal. We present data to demonstrate that Notch maintains type II NB self-renewal and identity by functioning through the canonical pathway to inhibit PntP1-mediated activation of erm in type II NBs. Our work also reveals that Erm has an inhibitory role in controlling PntP1 function/expression. Our work not only provides mechanistic insights into Notch-mediated maintenance of type II NBs but also reveals a novel function of Erm in type II NB lineages.
RESULTS
Loss of Notch abolishes PntP1 expression and activates ectopic Erm expression in type II NBs

As a first step to investigate why loss of Notch signaling transforms type II NBs into type I-like NBs and prematurely eliminates type II NBs but spares type I NBs, we examined how the expression of the type II NB lineage-specific genes pntP1 and erm would be affected by the loss of Notch. We hypothesized that genes specifically expressed in type II NB lineages most likely contribute to the sensitivity of type II NBs to the loss of Notch signaling. pntP1 and erm are the only genes known to be specifically expressed in type II NB lineages (Weng et al., 2010; Zhu et al., 2011). Furthermore, PntP1 is required for type II NB specification and erm has been shown to promote premature loss of NBs when misexpressed. Therefore, they were likely candidates in investigating the underlying mechanism of Notch-mediated maintenance of type II NBs. We examined PntP1 and Erm expression at 1-2 days after larval hatching (ALH), when type II

Fig. 1. Loss of Notch abolishes PntP1 expression and induces ectopic Erm expression in Drosophila type II NBs. (A-D') Loss of Notch (N) signaling leads to loss of PntP1 expression. (A,A') A wild-type type II NB lineage expresses PntP1 in the NB and in Ase− (solid arrowheads) and Ase+ (open arrowheads) imINPs. (B-D') Notch knockdown (B-C') or Numb overexpression (D,D') abolishes PntP1 expression and ectopically activates Ase in type II NBs. (E-M') Erm is ectopically activated in type II NBs in the absence of Notch. (E,E',G,I,I',L,L') R9D11-GFP (E,E',G), R9D11-tdTom (I,I') or Erm proteins (L,L') are expressed in imINPs but are not normally expressed in normal type II NBs. (F,F',H,J,K,M,M') Notch knockdown (F,F',H,J,J',M,M') or Numb overexpression (K,K') results in ectopic expression of R9D11-GFP/td-Tom (F,F',H,J,K') or Erm proteins (M,M') in type II NBs. Note that Dpn is expressed in the R9D11-GFP-expressing NB (H). Arrowheads (G,H) indicate mature INPs. Arrows point to type II NBs in this and all following figures, except Fig. 5, if not otherwise described. (N) Diagrams of wild-type and Notch-knockdown type II NB lineages. GMC, ganglion mother cell; INP, intermediate neural progenitor; imINP, immature INP; NB, neuroblast. Scale bars: 10 μm.
NB lineages were not yet completely eliminated by Notch RNAi knockdown.

We first examined PntP1 expression by antibody staining. PntP1 is normally expressed in type II NBs and imINPs (Fig. 1A,A′,N) (Zhu et al., 2011). Knocking down Notch using two independent UAS-Notch RNAi lines driven by type II NB lineage-specific pntP1-GAL4 (Zhu et al., 2011) resulted in transformation of more than 80% of type II NBs into type I-like NBs at 36-44 h ALH, as indicated by ectopic Ase expression in the NBs and the elimination of INPs, which can be identified based on their expression of both Ase and the bHLH protein Deadpan (Dpn) and their smaller size compared with NBs (Fig. 1B-C,G,N; also see below, Fig. 3A,B) (Zhu et al., 2012). We found that in those transformed lineages PntP1 expression was largely abolished (Fig. 1B-C,N). Similar loss of PntP1 expression was observed when Notch was inhibited by Numb overexpression (Fig. 1D,D′). These results suggest that knockdown of Notch abolishes PntP1 expression, which is correlated with the transformation of type II NB lineages into type I-like NB lineages (Fig. 1N).

Next, we examined Erm expression using R9D11-CD4-tdTomato (abbreviated as R9D11-tdTom) (Han et al., 2011) or R9D11-mCD8-GFP (abbreviated as R9D11-GFP) (Zhu et al., 2011) as a reporter, or by Erm immunostaining. R9D11 is a DNA fragment from the erm promoter (Pfeiffer et al., 2010). R9D11-tdTom and R9D11-GFP show similar expression patterns to endogenous Erm proteins, which are detected only in imINPs (except the newly generated imINPs) but not in type II NBs (Fig. 1E,E′,G,I,L,N) (Janssens et al., 2014). Interestingly, when Notch was knocked down or inhibited by Numb, R9D11-tdTom or R9D11-GFP was ectopically expressed in type II NBs (Fig. 1F,F′,H,J-K′) at 1.5 days ALH. Consistently, endogenous Erm proteins were also detected in type II NBs by immunostaining when Notch was knocked down (Fig. 1M-N). We ruled out the possibility that the ectopic Erm expression was due to a transformation of type II NBs into imINPs in that these NBs were of similar size to typical NBs and expressed the NB marker Dpn (Fig. 1H). Erm+ imINPs are much smaller than NBs and do not express Dpn (Fig. 1L,L′) (Janssens et al., 2014). These data suggest that, in the absence of Notch, Erm is ectopically activated in type II NBs. Therefore, Notch normally suppresses erm expression in type II NBs.

Knockdown of Erm largely rescues the loss of type II NBs and PntP1 expression resulting from Notch knockdown

Previous studies have shown that misexpression of Erm promotes premature loss of type II NBs (Weng et al., 2010) and that inhibiting PntP1 activity by Yan (Anterior open – FlyBase) only transforms type II NB lineages into type I-like NB lineages but does not affect the self-renewal of type II NBs (Zhu et al., 2011). Therefore, the ectopic Erm expression is likely to mediate the premature loss of type II NBs, whereas the loss of PntP1 might contribute to the transformation of type II NBs. The ectopic Erm expression and the loss of PntP1 could either be independent of each other or have a causal relationship.

To distinguish between these possibilities and determine how ectopic Erm expression and loss of PntP1 contribute to the Notch loss-of-function phenotypes, we first examined whether knocking down Erm would rescue the loss of type II NBs. We used pntP1-GAL4 to simultaneously knock down Notch and Erm in type II NB lineages. Knockdown of Notch alone led to elimination of all type II NBs at 60-72 h ALH (Fig. 2A,B,Q), whereas knockdown of Erm resulted in an 8-fold increase in the number of type II NBs (Fig. 2C,Q) due to dedifferentiation of INPs, as reported before (Weng et al., 2010). However, simultaneous knockdown of Notch and Erm only resulted in less than a 60% reduction in the number of type II NBs at 60-72 h ALH, as compared with Erm knockdown alone (Fig. 2D,Q), indicating that Notch-knockdown type II NBs were maintained much longer when Erm was knocked down simultaneously. Interestingly, knockdown of Erm also largely prevented the loss of PntP1 and the transformation of type II NBs into type I-like NBs. Unlike Notch knockdown alone, which led to loss of PntP1 and ectopic Ase expression in ~80% of type II NBs at 1.5-2 days ALH (Fig. 1B-C′, Fig. 23,S), more than 85% of type II NBs remained Ase− when Notch and Erm were simultaneously knocked down (Fig. 2D). Consistently, PntP1 expression was also maintained in these type II NBs (Fig. 2L,K,L,S). These results demonstrate that knockdown of Erm not only rescues the loss of type II NBs but also prevents the loss of PntP1 expression and the transformation of type II NBs resulting from the loss of Notch.

Since pntP1-GAL4 is more strongly expressed in imINPs than in NBs (Zhu et al., 2011) and knockdown of Erm by pntP1-GAL4 increased the number of type II NBs, one might argue that the rescue of type II NBs could be due to dedifferentiation of INPs resulting from Erm knockdown. To rule out this possibility, we used the pan-NB driver inscutable-GAL4 (insc-GAL4) (Luo et al., 1994), which is mainly expressed in NBs, to knock down Erm. Knockdown of Erm by insc-GAL4 did not produce any extra type II NBs, even at third instar larval stages (Fig. 2E,G,R), whereas knockdown of Notch by insc-GAL4 was still sufficient to eliminate ~95% of type II NBs at 3 days ALH (Fig. 2F,R). However, when Notch and Erm were simultaneously knocked down by insc-GAL4, nearly all type II NBs still remained at 3 days ALH (Fig. 2H,R). Even at 4 days ALH, ~4-5 type II NBs were still maintained (Fig. 2R). These remaining type II NBs were Ase−, indicating that they were not transformed into type I-like NBs (Fig. 2H). These results provide strong evidence to support the notion that knockdown of Erm indeed largely rescues the loss of type II NBs and prevents the transformation of type II NBs. Taken together, we argue that the premature loss of type II NBs and the transformation of type II NBs resulting from the loss of Notch are primarily due to the ectopic activation of Erm in type II NBs and that the loss of PntP1 expression is likely to be a secondary effect.

Erm misexpression is sufficient to phenocopy the loss of Notch function in type II NBs

To further determine if ectopic Erm expression is the primary reason for the premature loss of type II NBs and the transformation of type II NBs resulting from the loss of Notch, we examined whether misexpression of Erm in type II NBs was sufficient to not only eliminate type II NBs as reported before (Weng et al., 2010) but also to abolish PntP1 expression and transform type II NBs into type I-like NBs. We first used pntP1-GAL to drive UAS-erm specifically in type II NB lineages. Consistent with the previous report (Weng et al., 2010), Erm misexpression in type II NBs led to a gradual loss of type II NBs (Fig. 2M,N,Q; see also below, Fig. 3I). Interestingly, similar to the loss of Notch, Erm misexpression transformed ~30% of type II NB lineages into type I-like NB lineages at 3-4 days ALH, as indicated by ectopic Ase expression in NBs and the loss of INPs (Fig. 2N). Accordingly, PntP1 expression was abolished in these NBs (Fig. 2N,S). However, compared with Notch knockdown, Erm misexpression phenotypes were relatively weak, possibly owing to the relatively weak expression of pntP1-GAL4 in the NB. Therefore, we then used insc-GAL4 to drive UAS-erm. We found no Ase− type II NBs at 3 days ALH, although a few INPs still remained in ~2-3 lineages per brain lobe (Fig. 2O,P). The premature loss of type II
NBs and the transformation of type II NB lineages into type I-like NB lineages caused by Erm misexpression indicate that Erm misexpression can phenocopy the loss of Notch. These results, together with the rescue of Notch loss-of-function phenotypes by Erm knockdown, provide compelling evidence to support the contention that the ectopic Erm expression in type II NBs primarily accounts for the loss-of-Notch phenotypes.

Maintaining PntP1 expression fails to prevent the transformation of type II NBs resulting from Notch knockdown or from Erm misexpression

Since PntP1 expression was largely abolished when type II NBs were transformed into type I-like NBs, we next aimed to determine if the loss of PntP1 expression accounted for the transformation of type II NBs resulting from Notch knockdown or Erm misexpression. We used pntP1-GAL4 or insc-GAL4 to drive the expression of UAS-pntP1 in Notch-knockdown or Erm-misexpression type II NBs. Surprisingly, maintaining PntP1 could not suppress the ectopic Ase expression in Notch-knockdown or Erm-misexpression type II NBs (Fig. 3A-F). In fact, when insc-GAL4 was used to drive PntP1 and Erm expression simultaneously, Ase could not be suppressed in any NBs in the brain (Fig. 3A-F) and in the ventral nerve cord (VNC), which is in stark contrast to the suppression of Ase in nearly all type I NBs when PntP1 is expressed alone (see below, Fig. 5G,M,N) (Zhu et al., 2011). These results suggest that Erm not only suppresses PntP1 expression in type II NBs but antagonizes PntP1 function.

PntP1 activates the ectopic Erm expression that results from the loss of Notch before it is inhibited by negative feedback of Erm

What is the upstream activator of the ectopic Erm expression in type II NBs? Our previous studies show that inhibiting PntP1 by Yan abolishes the expression of the Erm reporter R9D11-GFP in INPs, whereas PntP1 misexpression in type I NBs is sufficient to activate R9D11-GFP in IN-like cells (e.g. Fig. 5A,A′,C,C′) (Zhu et al., 2011), indicating that Erm expression in INPs is activated by PntP1 either directly or indirectly. Therefore, we investigated whether ectopic Erm expression in type II NBs was also activated by PntP1 before it was inhibited by negative feedback of PntP1.
feedback of Erm. We first examined if PntP1 was expressed in Notch-knockdown type II NBs before Erm was activated. Indeed, PntP1 was expressed in all type II NBs at 8 h ALH but was lost gradually thereafter, whereas $R9D11-tdTom$ was not activated until 16 h ALH (Fig. S1).

To determine if PntP1 activates the ectopic Erm expression, we then examined (1) if the loss of type II NBs resulting from Notch knockdown would be enhanced or suppressed by overexpressing or knocking down PntP1, respectively; and (2) if the ectopic Erm expression would be reduced by PntP1 knockdown. Our results showed that when Notch was knocked down alone, there were still $\sim 7$ type II NBs at 1 day ALH but was lost gradually thereafter, whereas $R9D11-tdTom$ was not activated until 16 h ALH (Fig. S1).

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**PntP1 misexpression makes type I NBs sensitive to the loss of Notch**

After having demonstrated that the ectopic activation of $erm$ by PntP1 is responsible for the transformation and premature loss of type II NBs resulting from the loss of Notch, we next asked if PntP1 misexpression would be sufficient to activate $erm$ in Notch-knockdown type I NBs and promote premature termination of their self-renewal. We first examined whether PntP1 was able to similarly activate $erm$ in type I NBs when Notch was knocked down. We focused on type I NBs in VNCs because VNCs contain only type I NBs and PntP1 misexpression can transform type I NBs in the VNC into type II-like NBs more efficiently (Zhu et al., 2011). Indeed, PntP1 misexpression similarly activated $R9D11-GFP$ in type I NBs when Notch was simultaneously knocked down (Fig. 5B,B',D,D'), whereas PntP1 misexpression alone...
activated R9D11-GFP expression only in INP-like cells (Fig. 5A,A′,C,C′).

To determine if the ectopically expressed Erm in type I NBs similarly promotes premature termination of self-renewal and antagonizes the activity of PntP1 as in type II NBs, we then examined the total number of type I NBs in the VNC and the suppression of Ase expression by PntP1 in NBs. By comparing the total number of NBs in the VNC regardless of their identity, we could also determine whether the expression of Erm in the NB truly mediates the premature termination of NB self-renewal. Our results showed that concomitant Notch knockdown and PntP1 misexpression significantly reduced the number of NBs compared with the wild type or PntP1 misexpression alone, the latter of which slightly increased the number of type I NBs at 3 days ALH (Fig. 5E-H,O). Furthermore, the suppression of Ase by PntP1 misexpression was also largely blocked in more than 50% of type I NBs when Notch was knocked down (Fig. 5E-H,P). However, the reduction in the number of type I NBs was significantly rescued and the suppression of Ase by PntP1 misexpression was almost fully restored when Erm was knocked down simultaneously (Fig. 5I-L,O,P). Since Erm knockdown together with PntP1 misexpression did not increase the total number of NBs compared with PntP1 misexpression alone (Fig. 4G,K,O), the rescue of NBs by Erm knockdown was unlikely to be contributed by the dedifferentiation of INP-like cells. Consistent with these results, we found that Erm misexpression was also sufficient to significantly reduce the total number of type I NBs in the VNC and block the suppression of Ase and the generation of INP-like cells in type I NB lineages induced by PntP1 misexpression (Fig. 5G,M-P).

Taken together, these results demonstrate that, like in type II NBs, PntP1 misexpression can make type I NBs similarly sensitive to the loss of Notch by activating erm expression in type I NBs. These results also provide compelling evidence to further support that PntP1 is the upstream activator of the ectopic Erm expression in the NB and that the ectopically expressed Erm is sufficient to promote premature termination of NB self-renewal and has a negative-feedback effect on PntP1 function.
Fig. 5. See next page for legend.
Fig. 5. PntP1 misexpression activates Erm expression in type I NBs and promotes the premature loss of type I NBs in the absence of Notch.

(A-D) PntP1 misexpression activates Erm in Notch-knockdown type I NBs in the ventral nerve cord (VNC). (A-B) R9D11-GFP is not expressed in wild-type (A,A) or Notch-knockdown (B,B) type I NBs (arrows). (C,C) PntP1 misexpression activates R9D11-GFP expression in INP-like cells (arrowheads) but not in NBs (arrows). (D,D) Simultaneous PntP1 misexpression and Notch knockdown activates R9D11-GFP in both INP-like cells (arrowheads) and NBs (arrows). (E-H) PntP1 misexpression promotes premature loss of type I NBs in the VNC and fails to promote the transformation of type I NBs into type II-like NBs when Notch is knocked down. Note that PntP1 misexpression alone inhibits Ase in most type I NBs and induces INP-like cells (G) but not when Notch is simultaneously knocked down (H). (I-L) Erm knockdown rescues the loss of NBs resulting from concomitant PntP1 misexpression and Notch knockdown and restores the suppression of Ase by PntP1. Note that Ase is suppressed in most type I NBs, and INP-like cells are still generated in some lineages in L. (M,N) Erm knockdown rescues the loss of NBs resulting from concomitant PntP1 misexpression and Notch knockdown and restores the suppression of Ase by PntP1. Note that all NBs remain Ase+ and there are no INP-like cells in N. (E-F) Arrows indicate Ase+ NBs; large arrowheads indicate Ase− NBs; and small arrowheads indicate INP-like cells.

DISCUSSION

In this study we demonstrate that Notch maintains type II NB self-renewal and identity by preventing erm expression in type II NBs. Knocking down Notch or overexpressing erm results in ectopic Erm expression in type II NBs. Knockdown of Erm largely suppresses the transformation and the premature loss of type II NBs resulting from the loss of Notch, whereas Erm misexpression phenocopies the loss of Notch. We also provide several lines of evidence to prove that the ectopic Erm expression in type II NBs is activated by PntP1. Therefore, one major function of Notch signaling is to prevent erm activation by PntP1 in type II NBs so that the self-renewal and identity of type II NBs can be properly maintained (Fig. 7A,B).

Notch could function through the canonical or non-canonical pathway. Our results suggest that Notch mainly acts through the canonical pathway to inhibit Erm expression. We show that loss of components of the canonical pathway, such as Su(H) or its downstream target E(Spl) proteins, lead to ectopic Erm expression in type II NBs. It is likely that E(Spl) proteins, which function as transcriptional repressors (Fisher and Caudy, 1998), directly bind to the erm promoter to suppress its expression. However, we do not completely rule out involvement of other mechanisms of the canonical pathway or the non-canonical pathway in maintaining type II NBs. A recent study reported that simultaneous Drosophila Myc (dMyc) overexpression and Marf knockdown can fully rescue the loss of type II NBs resulting from Notch knockdown (Lee et al.,

Fig. 6. Notch functions through the canonical pathway to repress Erm expression in type II NBs. (A,A) A wild-type type II NB clone at 120 h after clone induction (ACI) does not express R9D11-GFP expression in the NB. (B,B) Df(3R)E(spl)B32.2 mutant type II NBs all showed ectopic activation of R9D11-GFP or R9D11-tdTom at 52-96 h after clone induction (Fig. 6A-E), demonstrating that Notch functions through the canonical pathway to inhibit Erm expression in type II NBs.
that Erm is never turned on in imINPs, which is consistent with a previous report (Janssens et al., 2014). The absence of Erm in imINPs will in turn promote imINPs to dedifferentiate into type II NBs as observed in erm mutants.

Our findings concerning the inhibitory role of Notch signaling in PntP1-mediated activation of erm provide insight into why Erm expression is normally restricted in imINPs (Janssens et al., 2014) and why loss of Numb or Notch overactivation leads to overproliferation of type II NBs (Bowman et al., 2008). When type II NBs divide, Numb is asymmetrically segregated into the future imINPs to inhibit Notch activity. The inhibition of Notch in imINPs thus allows activation of erm by PntP1, which is expressed in type II NBs and imINPs (Zhu et al., 2011). The restricted expression of Erm in imINPs allows Erm to promote INP differentiation/maturation and causes the two daughter cells generated from the NB division to acquire distinct fates (Fig. 7A). However, when Numb is lost or Notch is overactivated, Notch will remain active in imINPs. The active Notch will then continue to suppress Erm in imINPs, which is consistent with a previous report that Erm is never turned on in numb mutant type II NB clones (Janssens et al., 2014). The absence of Erm in imINPs will in turn result in defects in INP maturation and cause subsequent dedifferentiation of INPs into type II NBs, as reported before (Weng et al., 2010).

How does the ectopically expressed Erm promote the transformation of type II NBs into type I-like NBs? Several lines of evidence suggest that Erm is likely to promote the transformation by inhibiting the function and/or expression of PntP1. First, Erm misexpression inhibits PntP1 expression and promotes the transformation of type II NBs, whereas Erm knockdown rescues the loss of PntP1 and suppresses the transformation of type II NBs resulting from the loss of Notch. Second, maintaining PntP1 expression could not prevent the Erm misexpression-induced transformation of type II NBs, nor could misexpression of PntP1 suppress Ase expression or induce the generation of INP-like cells when Erm is coexpressed in type I NBs. Erm functions as a transcriptional repressor in imINPs (Janssens et al., 2014). Erm might inhibit the expression of PntP1 target genes, as well as inhibiting PntP1 itself, either by binding to the promoter of pntP1 and PntP1 target genes or by physically interacting with PntP1 to inhibit its transcriptional activity. Alternatively, the loss of PntP1 expression in Notch-knockdown or Erm-misexpression type II NBs could be an indirect effect of the loss of INPs, which may in turn lead to a loss of potential feedback signals from INPs that are required for maintaining PntP1 expression in type II NBs, as we proposed recently (Xie et al., 2014).

Our findings that Erm has inhibitory roles in PntP1 function/expression could have important implications for elucidating how Erm promotes INP maturation and prevents the dedifferentiation of INPs. The first step of INP maturation is activation of Ase (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008), yet Ase and PntP1 are coexpressed in imINPs (Zhu et al., 2011), which seems contradictory to the inhibitory role of PntP1 in Ase expression. Interestingly, Erm is co-expressed with PntP1 in Ase+ imINPs. Therefore, Erm might antagonize the function of PntP1 in imINPs as well. The inhibition of PntP1 function then would allow the activation of Ase, as well as of other differentiation programs that promote INP maturation. Meanwhile, Erm might also turn off the expression of PntP1 when INPs mature (Fig. 7A). In the absence of Erm, PntP1 function/expression will not be inhibited anymore in INPs. PntP1 belongs to the Ets family of transcription factors (Klambt, 1993; Scholz et al., 1993), which have well-established roles in mediating mitogenic signaling, regulating the expression of cell cycle modulators, and tumorigenesis (Kar and Gutierrez-Hartmann, 2013; Oikawa and Yamada, 2003; Seth and Watson, 2005; Sharrocks, 2001). The derepression of PntP1 function/expression might lead to the activation of cell cycle regulators and inhibition of Ase and other differentiation programs in imINPs, which will in turn promote imINPs to dedifferentiate into NBs and initiate unrestricted proliferation, as observed in erm mutant type II NB lineages. In support of this idea, we showed in this study that PntP1 misexpression also slightly increases the number of NBs, which could result from the dedifferentiation of imINP-like cells. In future work it would be important to test whether PntP1 function or expression is elevated in erm mutants and if PntP1 indeed contributes to the dedifferentiation of imINPs in erm mutants.
Although the inhibition of PntP1 function/expresssion could contribute to the transformation of type II NBs, it is unlikely to be responsible for the premature loss of type II NBs resulting from the loss of Notch or Erm misexpression. In fact, we show that PntP1 overexpression enhances the loss of type II NBs resulting from Notch knockdown and that PntP1 knockdown suppresses the Notch knockdown-induced premature loss of type II NBs. It has been shown previously that loss of Notch signaling or misexpression of Erm both lead to ectopic Pros expression in type II NBs (Song and Lu, 2011; Weng et al., 2010). However, the ectopic Pros expression is unlikely to account for the premature loss of type II NBs. First, spdo pros double-mutant type II NBs are still lost prematurely (Song and Lu, 2011). Second, Notch knockdown or Erm misexpression in pros mutant clones still eliminates type II NBs (data not shown). Therefore, Erm must acts through a Pros- and PntP1-independent mechanism to promote the premature loss of type II NBs.

In summary, our findings not only elucidate important mechanisms of Notch-mediated maintenance of type II NB self-renewal and identity, but also provide important clues for investigating how loss of Numb or Erm leads to the tumorigenic overproliferation of type II NBs. Given that Notch has conserved roles in regulating mammalian NSC self-renewal and heterogeneity and that Fezf1/2, which are homologs of Erm, are also expressed in mammalian NSCs and regulate neurogenesis and NSC activation/quiescence (Berberoglu et al., 2014; Eckler and Chen, 2014; Guo et al., 2013), it would be of great interest to investigate if similar mechanisms are conserved in mammalian NSCs.

MATERIALS AND METHODS

Fly stocks

GAL4 lines used for transgene expression include insc-GAL4 (Luo et al., 1994), pntP1-GAL4 (or GAL4\textsuperscript{104}) (Zhu et al., 2011) and bd\textsuperscript{+}GAL4 (Estella et al., 2003). UAS transgenes for RNAi knockdown or overexpression include UAS-Notch RNAi \#7078 and #33611, Bloomington Drosophila Stock Center, Bloomington, IN, USA (BDSC); unless indicated otherwise in figures, the line #7078 was used for Notch knockdown. UAS-erm RNAi (BDSC 362778 and #50661), UAS-pnt RNAi (BDSC #31936 and #35038), UAS-numb (Wang et al., 1997), UAS-pntP1 (Zhu et al., 2011) and UAS-erm (Weng et al., 2010). RY911-mCD8-GFP (on the second or the third chromosome) (Zhu et al., 2011) and RY911-CD4-tTomato (Han et al., 2011) were used as Erm reporters. Spa(\textsuperscript{104}), spdo\textsuperscript{104}, aph\textsuperscript{104} and Dj(3R)E(sp\textsuperscript{12}) mutant alleles (BDSC #51287, #9933, #63324, #52011) were used for generating mutant clones.

Mosaic analyses and UAS transgene expression

Wild-type or mutant clones were induced by a 1 h heat shock at 0-24 h ALH as described (Lee and Luo, 1999). Larval brains were dissected at 1-5 days after clone induction. For RNAi knockdown, animals were raised at 29°C after larval hatching to boost the efficiency. Misexpression of UAS-pntP1 or UAS-erm was restricted to larval stages in order to avoid embryonic lethality by employing tub-GAL80\textsuperscript{P} and by shifting animals from 18°C to 29°C immediately after larval hatching. Unless for particular reasons explained in the text (e.g. insc-GAL4 for driving transgene expression in type I NBs), we used pntP1-GAL4 to drive UAS transgene expression in most experiments so that we could manipulate gene expression and examine phenotypes specifically in type II NB lineages. Although manipulating the expression of Notch or Erm could have impacts on PntP1 expression, pntP1-GAL4 was still effective in driving transgene expression, possibly because it took some time for these manipulations to take effect and GAL4 proteins perdured.

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) and guinea pig anti-Ase (1:5000) (Brand et al., 1993) (gifts from Y. N. Jan), rat anti-mCD8 (clone \#5H10, Life Technologies; 1:100), rabbit anti-PntP1 (Alvarez et al., 2003) (1:500; a gift from J. B. Skeath), rabbit anti-Erm (1:50; a gift from H. Y. Wang) (Janssens et al., 2014), rabbit anti-dsRed (BDSC #632392, Clontech; 1:500) and chicken anti-GFP (#GFP-1020, Aves Labs; 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 510 confocal microscope and processed with Adobe Photoshop. Two-tailed t-tests were used for statistical analyses.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.Z. and X.L. designed the project and approaches, interpreted data and wrote the paper. X.L. conducted experiments and generated data presented in Figs 1-6, Y.X. conducted experiments and generated data presented in Fig. 6 and contributed to manuscript editing.

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Supplementary information

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