

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 distinct 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 (Alunni et al., 2013; Basak et al., 2012; Ehm 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 homogenous (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.

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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

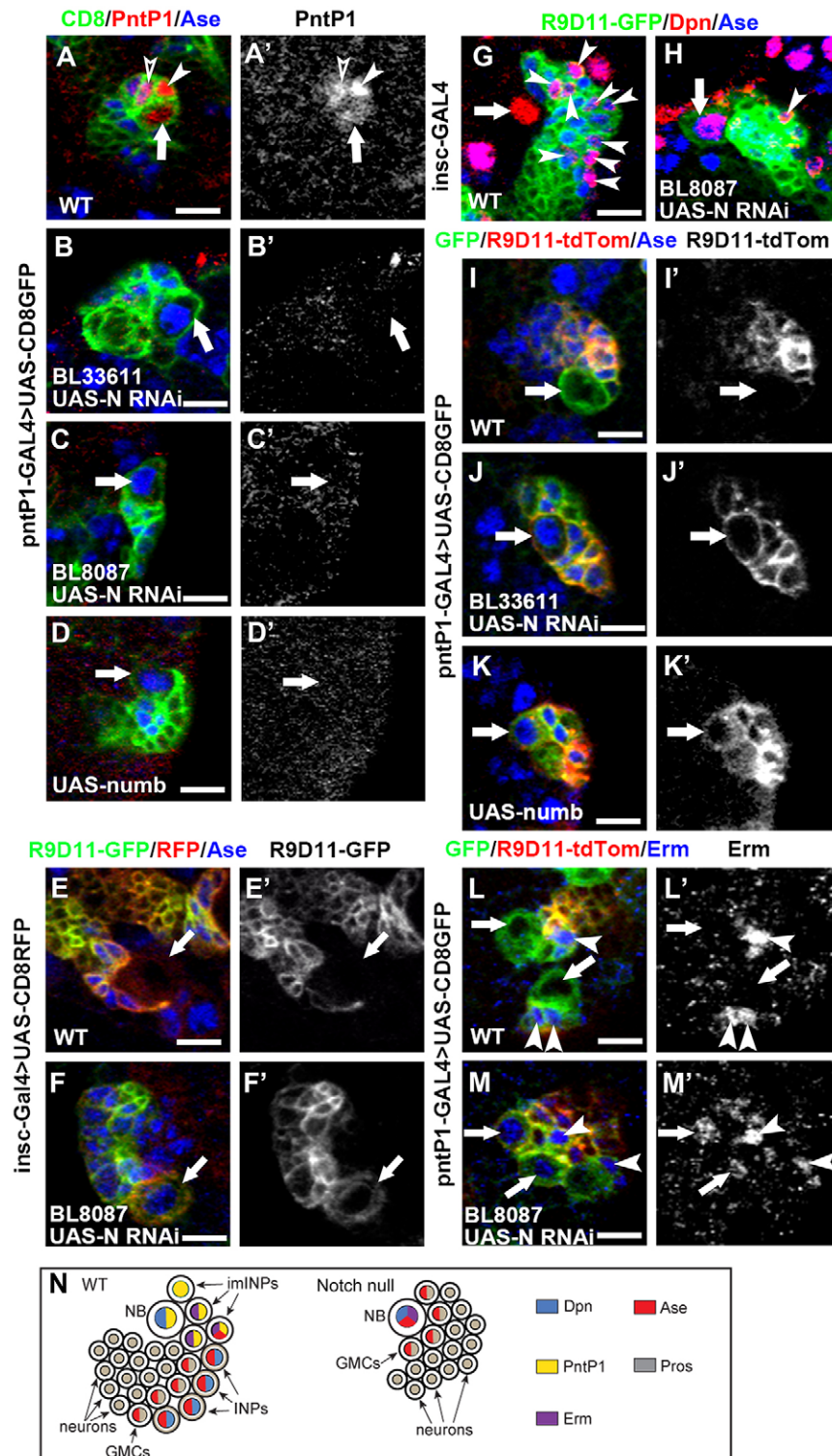


Fig. 1. Loss of Notch abolishes PntP1 expression and induces ectopic Erm expression in *Drosophila* type II NBs. (A–D') Loss of Notch (N) signaling leads to loss of PntP1 expression. (A,A') A wild-type type II NB lineage expresses PntP1 in the NB and in Ase⁺ (solid arrowheads) and Ase⁺ (open arrowheads) 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',L,L') or Erm proteins (L,L') are expressed in imINPs (arrowheads in L–M') but not 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/tdTom* (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 μ m.

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,I',L,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. 2J,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. 2I,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 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

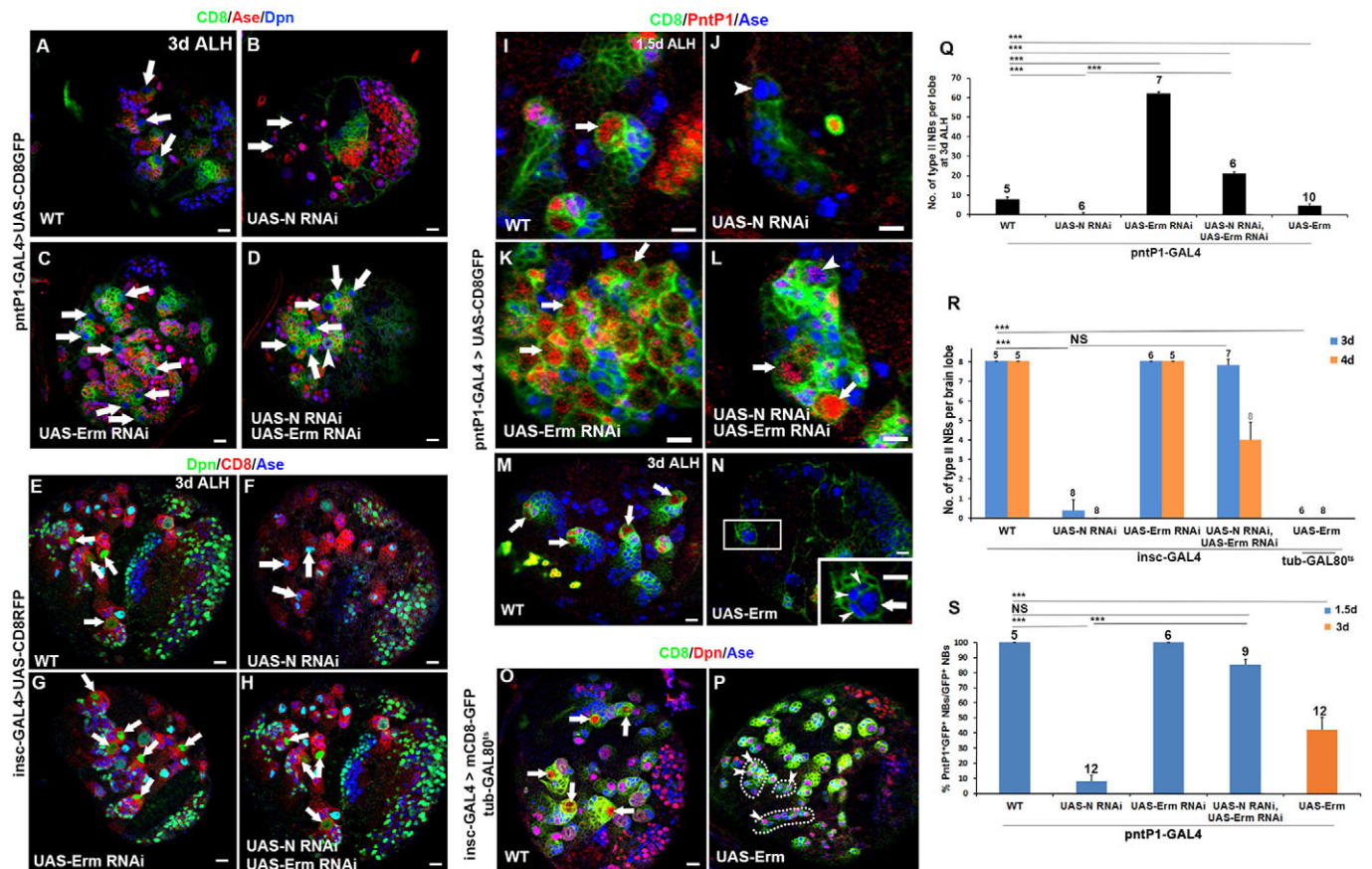


Fig. 2. Ectopic Erm expression is responsible for the transformation and premature loss of type II NBs resulting from Notch knockdown. (A–H) Erm knockdown blocks the Notch knockdown-induced transformation and premature loss of type II NBs. (A, B, E, F) Multiple type II NB lineages exist in wild-type brains (A, E) but are eliminated by Notch knockdown at 3 days after larval hatching (ALH) (B, F). Note that there are no GFP-labeled NBs in B (arrows) and all NBs are Ase⁺ in F (arrows). (C, G) The number of type II NBs is increased when Erm is knocked down by *pntP1-GAL4* (C) but not by *insc-GAL4* (G). (D, H) Multiple type II NBs still exist when Notch and Erm are simultaneously knocked down. Arrowhead (D) indicates Ase⁺ type II NBs. (I–L) Erm knockdown rescues the loss of PntP1. (I, J) PntP1 is expressed in normal type II NBs (I) but not in Notch-knockdown type II NBs with ectopic Ase expression (arrowhead, J). (K) Erm knockdown type II NBs express PntP1 but no Ase. (L) PntP1 remains expressed in the majority of Notch and Erm double-knockdown type II NBs. Arrowhead points to a type II NB with ectopic Ase expression and reduced PntP1 expression. (M, N) Erm misexpression driven by *pntP1-GAL4* reduces the number of type II NBs and abolishes PntP1 expression. Note the loss of PntP1 and ectopic Ase expression in the remaining NBs and direct generation of GMCs (arrowheads) from the NB in N. Inset (N) is an enlarged view of the highlighted (boxed) lineage. (O, P) Erm misexpression driven by *insc-GAL4* completely eliminates type II NBs. Dotted lines (P) indicate lineages with remaining INPs (arrowheads) but no Ase⁺ type II NBs. (Q–S) Quantification of the number of type II NBs (Q, R) or percentage of PntP1⁺ type II NBs (S) in larval brains. Numbers on each bar are sample sizes. Error bars indicate s.d. ****P* < 0.005 (Student's *t*-test); NS, not statistically significant. Scale bars: 10 μ m.

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 the loss of Notch 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, J). 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. 3G, H, J) or 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 INP-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

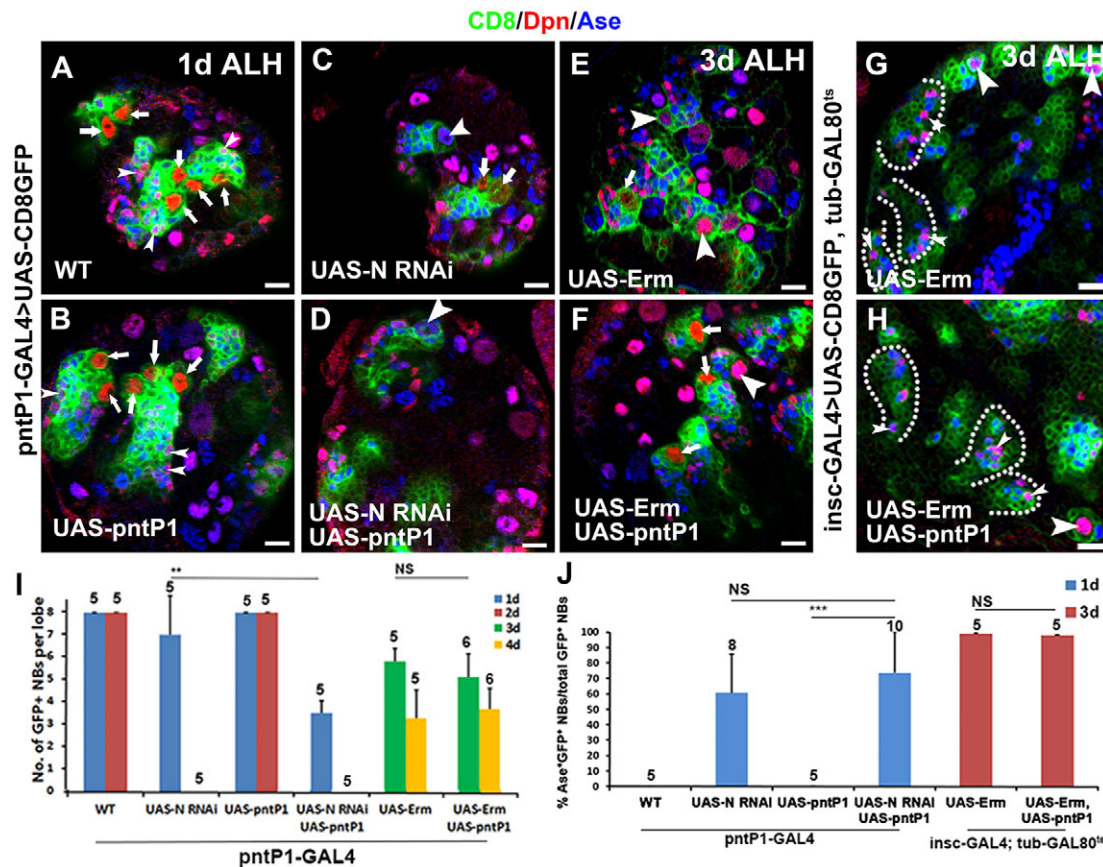


Fig. 3. Maintained PntP1 expression fails to prevent the transformation of type II NBs resulting from Notch knockdown or Erm misexpression.

(A–D) PntP1 expression driven by *pntP1-GAL4* fails to suppress the ectopic Ase expression in Notch-knockdown type II NBs and enhances the loss of type II NBs induced by Notch knockdown. Note that multiple type II NBs are still present in the Notch-knockdown brain lobe at 1 day ALH (C), but most type II NBs are lost in the concomitant Notch-knockdown and PntP1-overexpression brain lobe (D). Ase remains expressed in Notch-knockdown type II NBs when PntP1 is expressed (D). (E–H) PntP1 expression driven by *pntP1-GAL4* (E,F) or *insc-GAL4* (G,H) does not prevent the transformation of type II NBs into type I NBs induced by Erm overexpression. Arrowheads (E,F) point to type II NBs with ectopic Ase expression. Dotted lines mark type II NB lineages that have a few remaining INPs (arrowheads) but no NBs. Small arrowheads (A,B,G,H) indicate INPs; large arrowheads (C–H) indicate Ase⁺ type II NBs. (I,J) Quantification of the number of type II NBs (I) or percentage of Ase⁺ NBs (J). Error bars indicate s.d. ***P*<0.01, ****P*<0.005 (Student's *t*-test); NS, not statistically significant. Scale bars: 10 μm.

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 ~7 type II NBs at 1 day ALH (Fig. 3A,I). However, when PntP1 was overexpressed simultaneously, the loss of type II NBs was significantly enhanced and there were only 3–4 type II NBs at 1 day ALH (Fig. 3D,I). By contrast, PntP1 knockdown largely suppressed the loss of type II NBs caused by Notch knockdown. Knockdown of PntP1 alone doubled the number of type II NBs at 3–4 days ALH (Fig. 4A,C,E), which is likely to be due to reduced expression of Erm in imINPs and the subsequent dedifferentiation of INPs (Y.X., X.L., Xiaobing Deng, Yanjun Hou, Krysten O'Hara, Andreacarola Urso, Ying Peng and S.Z., unpublished). However, concomitant knockdown of Notch and PntP1 did not show an obvious loss of type II NBs at 3 days

ALH compared with PntP1 knockdown alone (Fig. 4D–E). Even at 4 days ALH, the number of type II NBs was reduced by only ~25% compared with PntP1 knockdown alone (Fig. 4E). Accordingly, PntP1 knockdown abolished the ectopic *R9D11-tdTom* expression in ~60% of Notch-knockdown type II NBs at 3 days ALH (Fig. 4B,B',D,D',F). These results demonstrate that PntP1 is the upstream activator of the ectopic Erm expression in type II NBs.

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

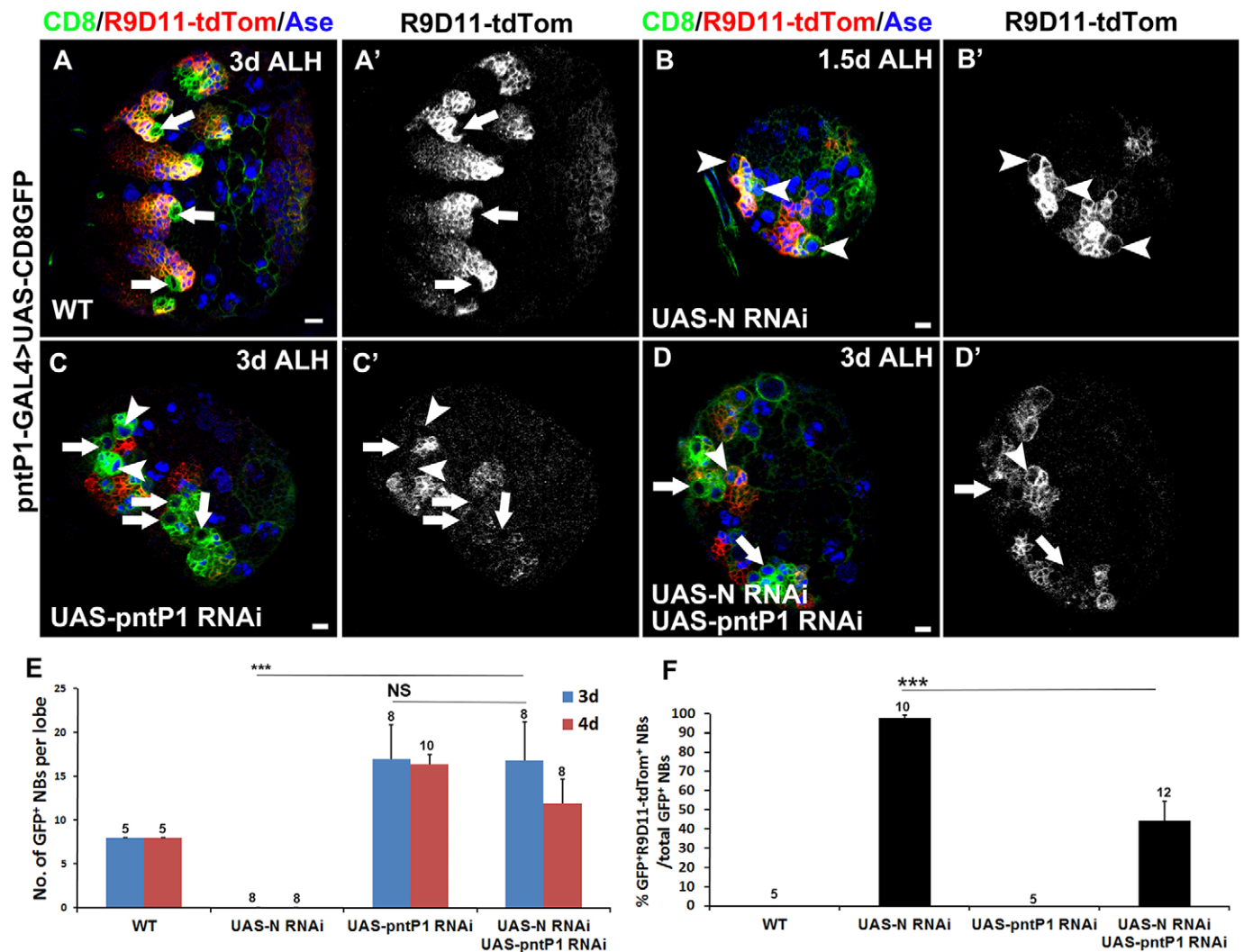


Fig. 4. Knocking down PntP1 suppresses the premature loss of type II NBs and inhibits ectopic Erm expression. (A,A') Wild-type type II NB lineages labeled with mCD8-GFP driven by *pntP1-GAL4* and *R9D11-tdTom*. (B,B') Notch knockdown reduced the number of type II NBs and induced ectopic *R9D11-tdTom* expression in all remaining type II NBs (arrowheads) at 1.5 days ALH. (C,C') PntP1 knockdown increases the number of type II NBs. Arrowheads indicate Ase⁺ type II NBs. (D,D') Type II NBs remain at 3 days ALH but most of them do not express *R9D11-tdTom* (arrows) when Notch and PntP1 are simultaneously knocked down. Arrowheads indicate *R9D11-tdTom*⁺ type II NBs. (E,F) Quantification of the number of type II NBs (E) and the percentage of type II NBs that express *R9D11-tdTom* (F) at 3 days or 4 days ALH. Error bars indicate s.d. ****P*<0.005 (Student's *t*-test); NS, not statistically significant. Scale bars: 10 μ m.

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.

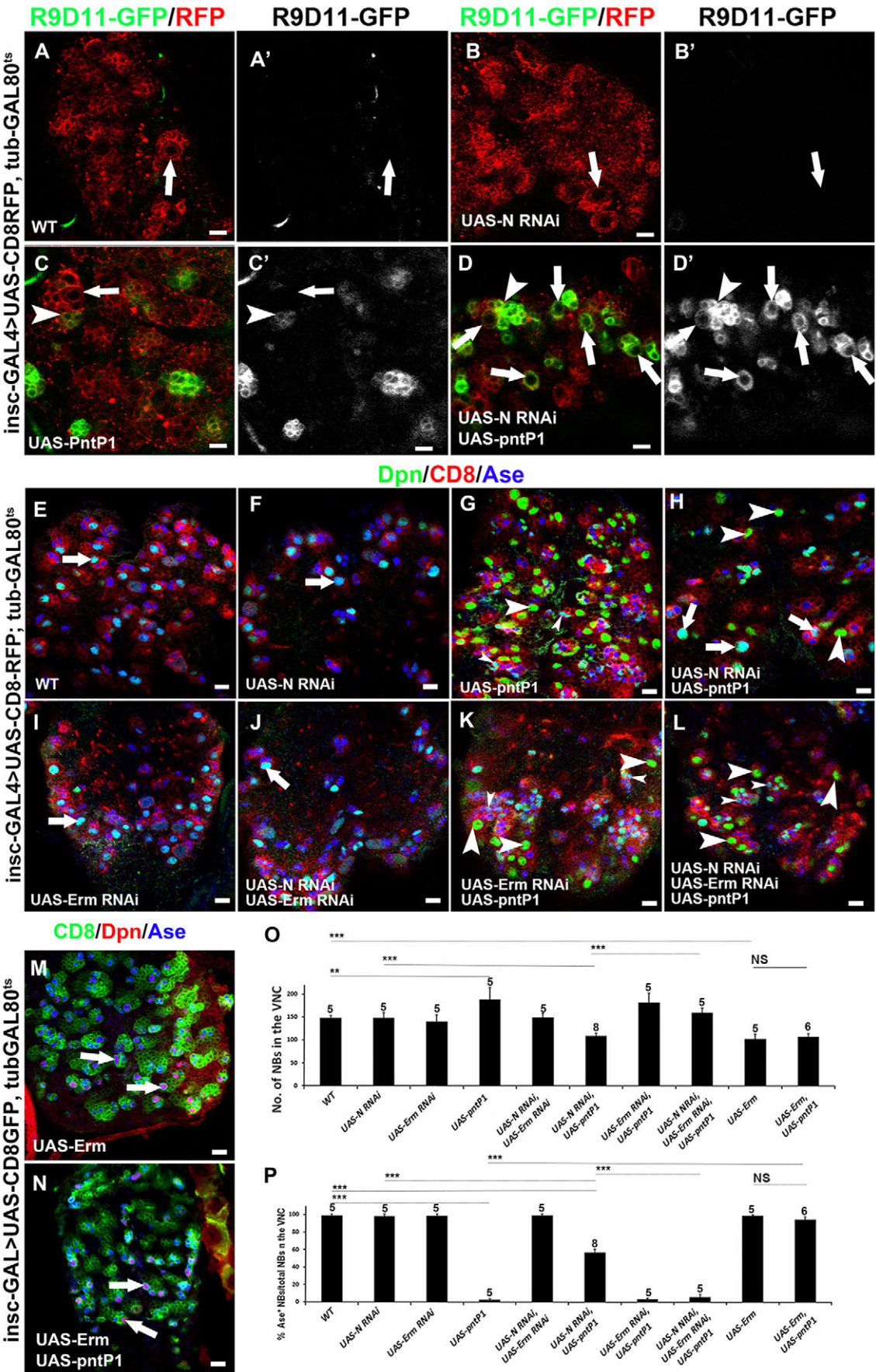


Fig. 5. See next page for legend.

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* misexpression reduces the number of type I NBs (M) and blocks the suppression of *Ase* and the generation of INP-like cells induced by PntP1 (N). Note that all NBs remain *Ase*⁺ and there are no INP-like cells in N. (E–N) Arrows indicate *Ase*⁺ NBs; large arrowheads indicate *Ase*[−] NBs; and small arrowheads indicate INP-like cells. (O,P) Quantification of the number of NBs (O) and the percentage of *Ase*⁺ NBs (P) in VNCs. Error bars indicate s.d. ***P*<0.01, ****P*<0.005 (Student's *t*-test); NS, not statistically significant. Scale bars: 10 μm.

Notch functions through the canonical pathway to suppress *Erm* expression in type II NBs

Activation of Notch signaling requires Spdo and Aph-1 (Hu and Fortini, 2003; Skeath and Doe, 1998). Upon activation, Notch could function through the canonical or the non-canonical pathway (Lecourtis and Schweisguth, 1995; Lee and Lundell, 2007). In the canonical pathway, the activated intracellular domain of Notch, together with Suppressor of Hairless [Su(H)], activates the expression of Enhancer of Split [E(Spl)] family proteins, such as E(Spl)^{my} and E(Spl)^{m8} [E(spl)^{my}-HLH and E(spl)^{m8}-HLH – FlyBase] (Zacharioudaki et al., 2012). To determine whether Notch functions through the canonical or the non-canonical pathway to suppress expression of *Erm* in type II NBs, we examined *Erm*

expression in *spdo*, *aph-1*, *Su(H)* or *Df(3R)E(spl)^{b32.2}* mutant type II NB clones. *Df(3R)E(spl)^{b32.2}* carries a deletion that covers the entire E(Spl) complex (Knust et al., 1992). We found that, similar to Notch knockdown or Numb overexpression, *spdo*, *aph-1*, *Su(H)* or *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.

DISCUSSION

In this study we demonstrate that Notch maintains type II NB self-renewal and identity by preventing *erm* from being activated by PntP1 in type II NBs. Knocking down Notch or overexpressing Numb 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.,

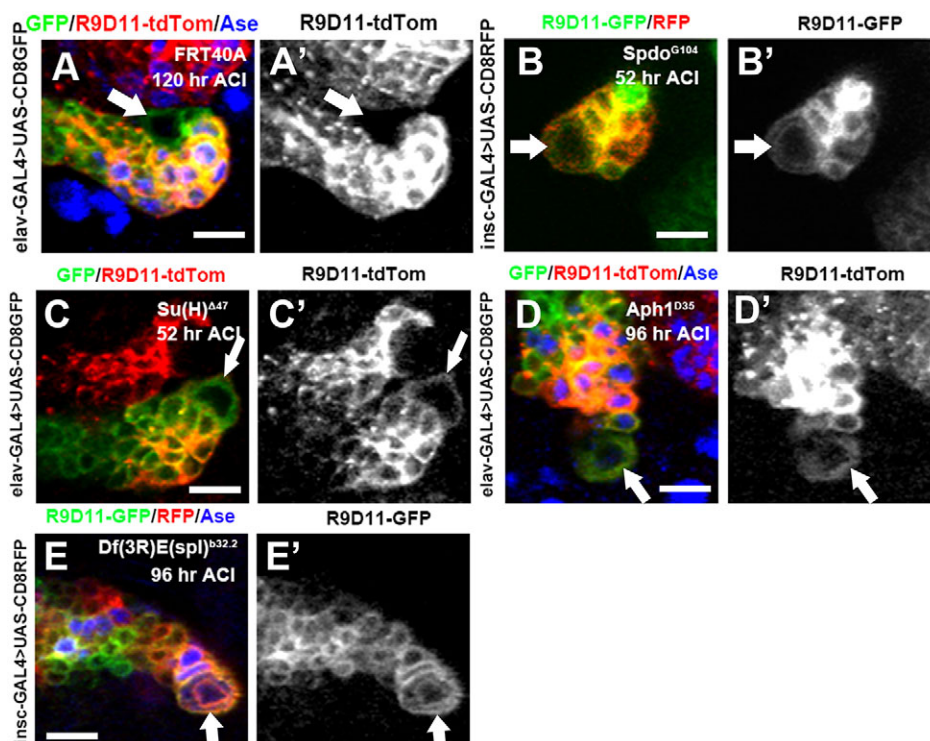


Fig. 6. Notch functions through the canonical pathway to repress *Erm* in type II NBs. (A,A') A wild-type type II NB clone at 120 h after clone induction (ACI) does not express *R9D11-tdTom* in the NB. (B–E') *spdo^{G104}* (B,B'), *Su(H)^{A47}* (C,C'), *aph-1^{D35}* (D,D') and *Df(3R)E(spl)^{b32.2}* (E,E') mutant clones show ectopic *R9D11-GFP* or *R9D11-tdTom* expression in type II NBs at 52 or 96 h ACI. Scale bars: 10 μm.

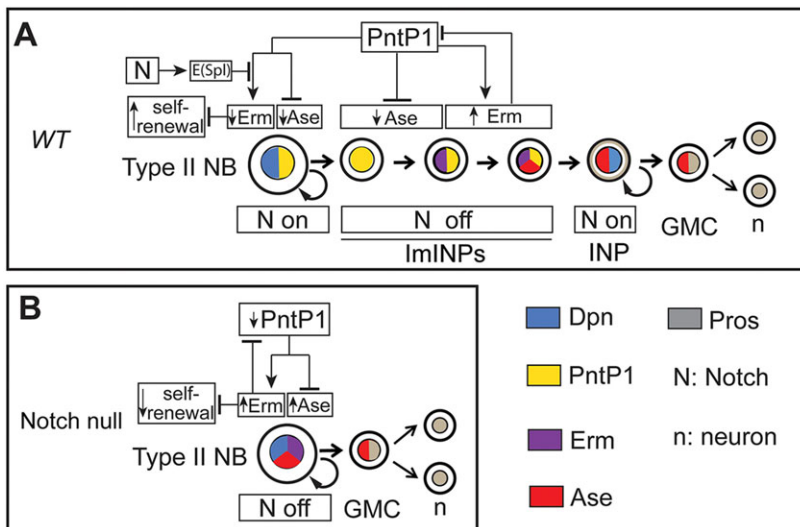


Fig. 7. Summary of type II NB lineage development in the wild type and in the absence of Notch. (A) In the wild type, Notch inhibits *erm* activation by PntP1 in the NB, allowing type II NBs to maintain self-renewal and identity. In imINPs, PntP1 activates *erm* due to a lack of Notch signaling. Erm in turn exerts a negative-feedback effect on PntP1, allowing imINPs to express Ase and mature. (B) In the absence of Notch, Erm is ectopically activated by PntP1 in the NB. Erm then inhibits PntP1 function and expression in the NB, which in turn leads to ectopic Ase expression in type II NBs and the elimination of INPs. Meanwhile, Erm promotes the premature termination of NB self-renewal.

2013). dMyc is activated by the canonical Notch pathway in brain tumors derived from type II NBs (Song and Lu, 2011), whereas Marf regulates mitochondrial fusion and mTORC2/AKT signaling, which is activated by the non-canonical Notch pathway through interaction with the mitochondrial serine/threonine kinase Pink1 (Lee et al., 2013). Although it remains to be seen if dMyc and Marf are involved in maintaining normal type II NBs, it would be interesting to investigate whether dMyc and mTORC2/AKT signaling have any functional relationship with the suppression of Erm in type II NBs.

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 (Klamt, 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/expression 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 act 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 *GAL4¹⁴⁻⁹⁴*) (Zhu et al., 2011) and *btd-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 #26778 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). *R9D11-mCD8-GFP* (on the second or the third chromosome) (Zhu et al., 2011) and *R9D11-CD4-tdTomato* (Han et al., 2011) were used as Erm reporters. *Su(H)^{Δ47}*, *spdo^{G104}*, *aph-1^{D35}* and *Df(3R)E(spl)^{b32.2}* mutant alleles (BDSC #51287, #9933, #63242, #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^{ts}* 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 (#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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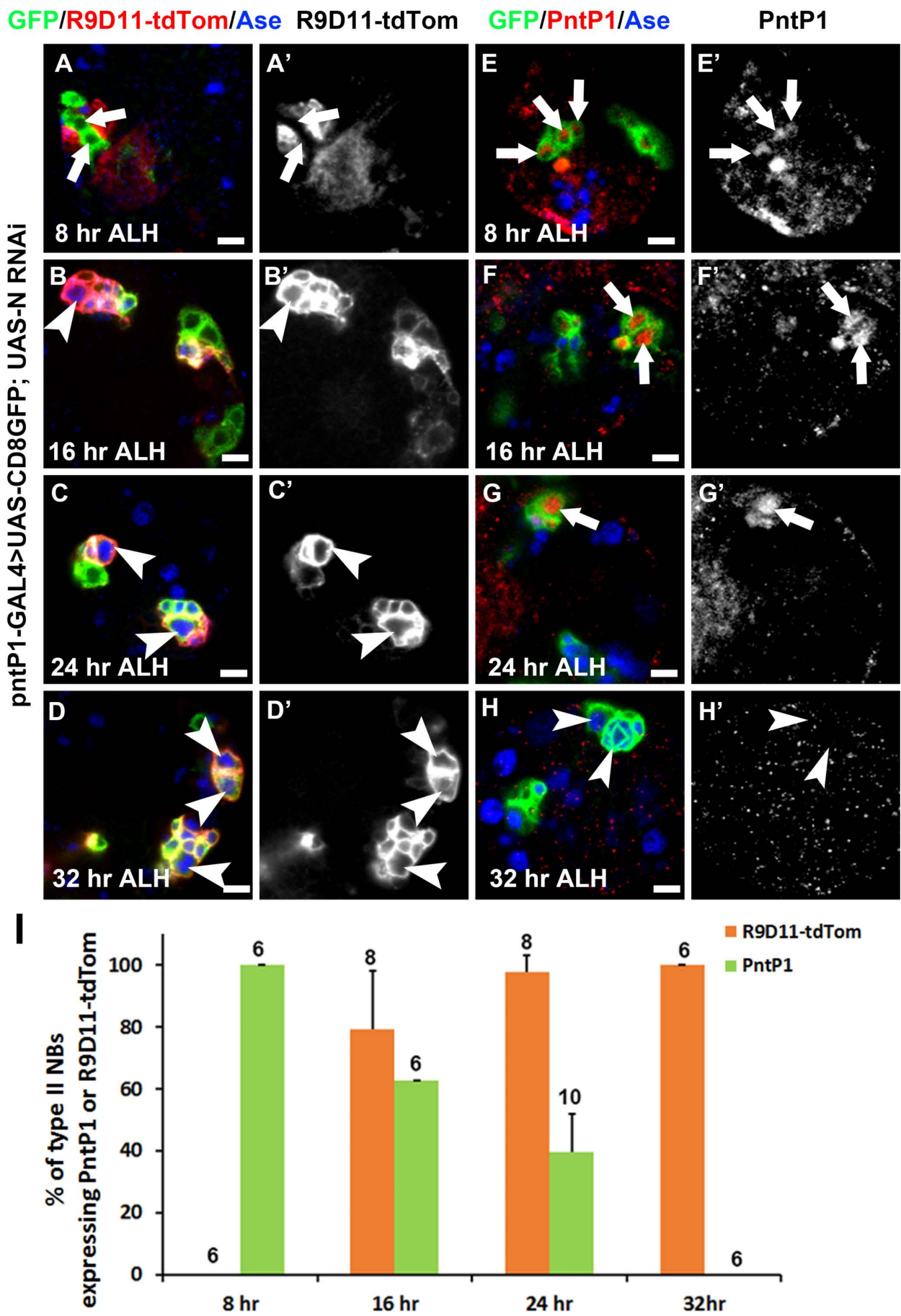


Figure S1: Dynamics of PntP1 and Erm expression in Notch knockdown type II NBs. Notch is knocked down by *pntP1-GAL4*, which also drives the expression of mCD8-GFP to label type II NB lineages. **(A-D'')** R9D11-tdTom expression in Notch knockdown type II NBs at 8 – 32 hours ALH. Arrows: type II NBs without R9D11-tdTom expression; arrowheads: type II NBs with the ectopic R9D11-tdTom expression. **(E-H)** PntP1 expression in Notch knockdown type II NBs at 8 - 32 hours ALH. Arrows: type II NBs with PntP1 expression; arrowheads: type II NBs without PntP1 expression. **(I)** Quantifications of the percentage of Notch knockdown type II NBs with PntP1 or R9D11-tdTom expression. Numbers on top of each bar represent the number of brain lobes examined. Error bars represent STDEV. Scale bars: 10 μ m