

# The Ets protein Pointed prevents both premature differentiation and dedifferentiation of *Drosophila* intermediate neural progenitors

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## Summary Statement

In *Drosophila* type II neuroblast lineages, the Ets transcription factor Pointed maintains the balance between differentiation and dedifferentiation of intermediate neural progenitors by acting through two distinct pathways.

## ABSTRACT

Intermediate neural progenitor cells (INPs) need to avoid both dedifferentiation and differentiation during neurogenesis, but the mechanisms are not well understood. In *Drosophila*, the Ets protein Pointed P1 (PntP1) is required to generate INPs from type II neuroblasts. Here, we investigated how PntP1 promotes INP generation. By generating *pntP1*-specific mutants and using RNAi knockdown, we show that the loss of PntP1 leads to both an increase in the type II neuroblast number and the elimination of INPs. We show that the elimination of INPs results from premature differentiation of INPs due to the ectopic Prospero expression in newly generated immature INPs (imINP), whereas the increase in the type II neuroblast number results from the dedifferentiation of imINPs due to a loss of Earmuff at later stages of imINP development. Furthermore, reducing Buttonhead enhances the loss of INPs in *pntP1* mutants, suggesting that PntP1 and Buttonhead act cooperatively to prevent premature INP differentiation. Our results demonstrate that PntP1 prevents both the premature differentiation and dedifferentiation of INPs by regulating the expression of distinct target genes at different stages of imINP development.

## INTRODUCTION

The generation of brain complexity in higher order animals involves the production of intermediate neural progenitor cells (INPs) from neural stem cells (NSCs) (Lui et al., 2011; Pontious et al., 2008). INPs transiently proliferate to amplify the NSC output. However, INPs have very limited developmental potential and can generate only fate-restricted progeny. Defects in maintaining INP proliferation owing to precocious differentiation and cell cycle exit could lead to reduced brain complexity and brain malformation (Colasante et al., 2015; Quinn et al., 2007; Reillo et al., 2011), whereas the aberrant dedifferentiation of INPs may result in tumorigenic overgrowth (Liu et al., 2011; Walton et al., 2009). Therefore, INPs need to avoid not only differentiation but also dedifferentiation in order to produce a precise number of progeny with specific cell fates. It is critical to decipher the mechanisms that prevent INP differentiation and dedifferentiation in order to understand the generation of brain complexity and the formation of brain tumors.

In *Drosophila* larval brains, INPs have similar roles to mammalian INPs in amplifying neuronal output from type II neuroblasts (NBs). Unlike ganglion mother cells (GMCs) generated from type I NBs, which divide only once and produce two neurons (Hartenstein et al., 2008), each INP generated from type II NBs produces approximately 10 neurons by dividing in multiple rounds and thereby generating several GMCs (Bello et al., 2008; Boone and Doe, 2008;

Bowman et al., 2008; Wang et al., 2014). INPs must maintain their self-renewal and avoid differentiation while dividing to produce neurons. However, newly generated INPs are immature and are prone to dedifferentiate into NBs if they fail to differentiate into mature INPs (Bowman et al., 2008; Eroglu et al., 2014; Koe et al., 2014). Thus, *Drosophila* INPs provide an excellent model for studying how INPs avoid dedifferentiation and premature differentiation.

Over the past several years, studies have begun to identify key genetic programs that prevent the dedifferentiation or premature differentiation of INPs. For example, preventing the dedifferentiation of immature INPs (imINPs) requires cell fate determinants Brain tumor (Brat) and Numb, the Fez family transcription factor Earmuff (Erm), as well as SWI/SNF and Histone deacetylase 3 (HDAC3) chromatin remodeling complexes (Bowman et al., 2008; Eroglu et al., 2014; Koe et al., 2014; Weng et al., 2010). The loss of any of these factors could result in imINP dedifferentiation of and supernumerary type II NBs. In contrast, preventing the premature differentiation of INPs requires the suppression of the homeodomain protein Prospero (Pros) in INPs. Our recent studies show that the Sp8 family transcription factor Buttonhead (Btd) is required to suppress Pros in imINPs (Xie et al., 2014). The loss of Btd results in ectopic Pros expression in imINPs and the premature differentiation of INPs into GMCs. However, our understanding of the mechanisms that prevent the dedifferentiation and premature differentiation of INPs is still incomplete.

Our previous studies revealed that the Ets family transcription factor Pointed

P1 (PntP1) was specifically expressed in type II NBs and imINPs (Zhu et al., 2011). Functional analyses show that inhibiting PntP1 activity by its antagonist Yan transforms type II NBs into type I NBs and eliminates INPs, whereas PntP1 misexpression transforms type I NBs into type II NBs and promotes INP generation, suggesting that PntP1 is required for type II NB specification and INP generation (Zhu et al., 2011). However, it is still unknown exactly how PntP1 promotes of INP generation. In this study, we investigated PntP1's function in imINPs by taking advantage of our novel *pntP1*-specific mutant alleles and using RNAi knockdown. We show that PntP1 prevents both dedifferentiation and premature differentiation of INPs by regulating the expression of distinct target genes at different stages of imINP development. Our work reveals the mechanistic details of PntP1-mediated generation of INPs in type II NB lineages and provides novel insights into the mechanisms that maintain the balance between INP dedifferentiation and differentiation .

## RESULTS

### ***Partial loss of PntP1 results in the elimination of INPs and an increase in the number of type II NBs***

The only currently available *pntP1*-specific allele is a null allele, *pnt*<sup>A33</sup>, in which the *pntP1*-specific exon 1, including part of the coding region, is deleted (O'Neill et al., 1994). However, *pnt*<sup>A33</sup> is not ideal for loss-of-function phenotypic analyses in *Drosophila* larval type II NB lineages for two reasons. First, PntP1 proteins perdure in *pnt*<sup>A33</sup> mutant type II NB clones even at late 3<sup>rd</sup> instar larval stages (Zhu et al., 2011). Second, the embryonic lethality of *pnt*<sup>A33</sup> makes it impossible to examine type II NB lineage development in *pnt*<sup>A33</sup> homozygous mutant larvae. Therefore, we decided to generate novel *pntP1*-specific mutant alleles using the CRISPR/Cas9 technology (Jinek et al., 2012; Port et al., 2014; Sebo et al., 2014) to investigate how PntP1 regulates INP generation. We chose two 20-nucleotide sequences that are 249 and 536 base pairs downstream of the translation start codon as gRNA targets (Figure 1Aa-b). We generated two mutant lines, named *pntP1*<sup>82</sup> and *pntP1*<sup>90</sup>, which carry small indels. These indels cause frame shifts and premature stop codons, resulting in deletions of 535 and 445 amino acids at the C-terminus, including the Ets DNA-binding domain, in the *pntP1*<sup>82</sup> and *pntP1*<sup>90</sup> mutants, respectively (Figure 1Ab-c). Both *pntP1*<sup>90</sup> and *pntP1*<sup>82</sup> homozygotes are late 3<sup>rd</sup> instar larval lethal, indicating that they are likely hypomorphic alleles.

We then examined type II NB lineages in *pntP1<sup>90</sup>* and *pntP1<sup>82</sup>* homozygous mutant larvae in order to investigate how the loss of PntP1 would affect type II NB lineage development. In wild type larval brains, there are only 8 type II NBs/lobe, which can be distinguished from type I NBs by the absence of the proneural protein Asense (Ase). Each type II NB lineage contains approximately 20-25 mature INPs, which express both Ase and the bHLH protein Deadpan (Dpn) (Figure 1B-B', I-K, P) (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). We found that in *pntP1<sup>90</sup>* mutant brains, the number of Ase<sup>-</sup> type II NBs increased one-fold. However, the number of INPs was reduced by 50% and approximately 25% of Ase<sup>-</sup> type II NBs did not produce mature INPs. Instead, only a few Ase<sup>+</sup> Dpn<sup>-</sup> cells that resembled GMCs were generated (Figure 1C-C', I-K, P). In *pntP1<sup>82</sup>* mutants, the loss of INPs was even more severe. There were only 2-3 INPs per Ase<sup>-</sup> NB on average and approximately 75% of the Ase<sup>-</sup> type II NBs did not produce INPs (Figure 1D-D', I-K, P). However, in contrast to the increase in the number of Ase<sup>-</sup> type II NBs in the *pntP1<sup>90</sup>* mutants, the number of Ase<sup>-</sup> type II NBs was reduced to approximately 6/lobe in the *pntP1<sup>82</sup>* mutants. Because PntP1 is required to suppress Ase expression in type II NBs (Zhu et al., 2011), it is possible that some type II NBs might have become Ase<sup>+</sup> in the *pntP1<sup>82</sup>* mutants (and possibly in *pntP1<sup>90</sup>* mutants as well) as observed in Pnt knockdown type II NBs (see below).

To verify whether the phenotypes observed in the *pntP1<sup>90</sup>* and *pntP1<sup>82</sup>* mutants were indeed caused by mutations of *pntP1*, we examined phenotypes in *pntP1<sup>90</sup>/pnt<sup>Δ88</sup>* or *pntP1<sup>82</sup>/pnt<sup>Δ88</sup>* transheterozygotes and compared them with phenotypes in Pnt knockdown type II NB lineages. *pnt<sup>Δ88</sup>* carries a deletion that covers the entire *pntP1* transcript, including the 3' exons shared by other *pnt* transcripts (Brunner et al., 1994). We found that *pntP1<sup>90</sup>/pnt<sup>Δ88</sup>* mutants had similar but more severe phenotypes compared to the *pntP1<sup>90</sup>* mutants (Figure 1E-E', I-K, P). In the *pntP1<sup>90</sup>/pnt<sup>Δ88</sup>* mutants, the number of Ase<sup>-</sup> type II NBs was further increased to approximately 25/lobe and the number of INPs was reduced by nearly 70%. In the *pntP1<sup>82</sup>/pnt<sup>Δ88</sup>* mutants, we observed a similar loss of INPs as in the *pntP1<sup>82</sup>* mutants, but a slight increase in the number of Ase<sup>-</sup> type II NBs (Figure 1F-F', I-K, P). Consistently, we observed 0.5-1-fold increase in the total number of type II NBs and complete elimination of INPs in over 90% of type II NB lineages when Pnt was knocked down by two independent *UAS-pnt RNAi* lines driven by type II NB lineage-specific *pntP1-GAL4*. Furthermore, Ase was ectopically expressed in 60-80% of Pnt knockdown type II NBs (Figure 1G-H', L-O, P), which is in accord with our previous results showing that the Yan-mediated inhibition of Pnt activity leads to ectopic *ase* activation in type II NBs. These data show that the loss of PntP1 leads to not only the transformation of type II NBs into type I NBs as we previously reported (Zhu et al., 2011) but also the elimination of INPs and the generation of extra type II NBs (Figure 1P).



***PntP1 suppresses the expression of Pros in Ase<sup>-</sup> imINPs and prevents the premature differentiation of INPs into GMCs***

We then examined the expression of Pros in imINPs and the identity of the Ase<sup>+</sup> progeny in *pnt* mutant or Pnt knockdown type II NB lineages without INPs to investigate why the loss of PntP1 led to the elimination of mature INPs. During INP maturation, newly generated imINPs first differentiate from an Ase<sup>-</sup> state to an Ase<sup>+</sup> state before they fully mature. Pros is not normally expressed in type II NBs or imINPs (Figure 2A-A'') (Bowman et al., 2008). Our recent studies show that in the absence of Btd, Ase<sup>-</sup> imINPs ectopically express nuclear Pros, which promotes premature differentiation of INPs into GMCs and eliminate mature INPs (Xie et al., 2014). Therefore, we wondered whether the elimination of INPs resulting from the loss of PntP1 was also due to the Pros-mediated premature differentiation of INPs into GMCs, which express both Ase and nuclear Pros. We examined Pros expression in the Pnt knockdown and *pntP1<sup>82</sup>* mutant type II NB lineages that showed a more consistent loss of INPs. Indeed, unlike in normal type II NB lineages, nuclear Pros was consistently expressed in newly generated Ase<sup>-</sup> imINPs, which can be identified as Ase<sup>-</sup> cells adjacent to the NBs, in the Pnt knockdown and *pntP1<sup>82</sup>* mutant type II NB lineages. Furthermore, the Ase<sup>+</sup> progeny that were generated in lineages without mature INPs also expressed nuclear Pros, indicating that they were GMCs (Figure 2B-D''). These results suggest that PntP1 is required to suppress Pros expression in newly generated imINPs and prevent imINPs from prematurely differentiating into GMCs.

### ***Ectopic Pros expression in imINPs is responsible for the loss of INPs***

Given that the main function of Pros is to promote cell cycle exit and differentiation (Choksi et al., 2006; Maurange et al., 2008), it is very likely that the loss of INPs resulting from the loss of PntP1 is due to the ectopic expression of nuclear Pros in imINPs. To test this idea, we then examined whether reducing nuclear Pros in imINPs. To test this idea, we then examined whether reducing Pros expression could rescue the loss of INPs in Pnt knockdown type II NB lineages. Therefore, we knocked down Pnt in *pros*<sup>17</sup> heterozygous background. In the *pros*<sup>17</sup> heterozygous mutants, the type II NB lineages produced similar numbers of INPs as in the wild type (Figure 3A-B", E-F). However, unlike the Pnt knockdown in the wild type background, which eliminated INPs in nearly all type II NB lineages, Pnt knockdown in *pros*<sup>17</sup> heterozygotes only eliminated INPs in approximately 50% of lineages and there were still an average of approximately 6 INPs/lineage (Figure 3C-F), suggesting that reducing Pros expression partially rescued the loss of INPs in the Pnt knockdown type II NB lineages. These results indicate that the ectopic nuclear Pros expression in imINPs is responsible for the loss of INPs resulting from the PntP1 loss.

In addition to the rescue of INPs, we found that reducing Pros expression partially restored the suppression of Ase in Pnt knockdown type II NBs. When Pnt was knocked down in the wild type background, only 25% of type II NBs remained Ase<sup>-</sup>. However, when Pnt was knocked down in the *pros*<sup>17</sup> heterozygous background, which still increased the total number of type II NBs to

15.8 ± 3.1/lobe (mean ± Stdev, n=13), approximately 70% of type II NBs were Ase<sup>-</sup> (Figure 3C-D", G). Because nuclear Pros is not detected in the Pnt knockdown type II NBs, the restoration of Ase suppression in Pnt knockdown type II NBs is likely an indirect effect. We previously reported that maintaining PntP1 expression in type II NBs may require a feedback signal from INPs and the loss INPs could lead to a loss/reduction of PntP1 expression in type II NBs (Xie et al., 2014). Therefore, one possibility could be that the rescue of INPs by the reduction of Pros may help restore the feedback signal, which may in turn partially restore the expression of PntP1 and the suppression of Ase in the NBs, as occurs in *btd* mutant clones (Xie et al., 2014). However, we could not detect PntP1 proteins after knocking down PntP1 in either the wild type or *pros*<sup>17</sup> heterozygous background, suggesting that the partially restored PntP1 expression could still be below the detection limit (data not shown)

***PntP1 and Btd genetically interact to inhibit premature INP differentiation and suppress Ase expression in type II NBs***

Because the loss of PntP1 or Btd leads to similar Pros-mediated premature differentiation of INPs, next we investigated whether PntP1 and Btd functioned in the same pathway by performing genetic interaction tests. We examined if reducing the expression of Btd would enhance the loss of INPs in *pntP1*<sup>190</sup> homozygous mutants, which have a less severe loss of INPs and might be more sensitive to the reduction of Btd expression. We used *btd*<sup>XG81</sup> or *btd-GAL4*

heterozygous mutants to reduce Btd expression. *btd*<sup>XG81</sup> is a missense loss-of-function allele, and *btd-GAL4* is a lethal p{GAL4} insertion allele, in which the *GAL4* is integrated in the *btd* promoter (Estella and Mann, 2010; Wimmer et al., 1993). We labeled type II NBs lineages with mCD8-GFP driven by *btd-GAL4*, which is only expressed in type II NB lineages on the dorsal side of larval brains (Xie et al., 2014), to quantify the number of INPs in *btd-GAL4/+; pntP1<sup>90</sup>* mutants. For the *pntP1<sup>90</sup>* or *btd*<sup>XG81</sup>/*+*; *pntP1<sup>90</sup>* mutants, we focused on Ase<sup>-</sup> type II NBs for the quantification because we could not identify type II NBs if they became Ase<sup>+</sup>. In the *pntP1<sup>90</sup>* homozygous mutants, the average number of INPs per Ase<sup>-</sup> NB was reduced by approximately 50% and approximately 25% of the Ase<sup>-</sup> NBs did not have associated INPs (Figure 4A-A', C-C', G-H), whereas *btd*<sup>XG81</sup> or *btd-GAL4* heterozygous mutants did not exhibit an obvious loss of INPs (Figure 4B-B", E-E", G-H). However, the loss of INPs in the *pntP1<sup>90</sup>* mutant type II NB lineages was dramatically enhanced in the *btd*<sup>XG81</sup> or *btd-GAL4* heterozygous mutant background. In the *btd-GAL4/+; pntP1<sup>90</sup>* or *btd*<sup>XG81</sup>/*+*; *pntP1<sup>90</sup>* mutants, the number of INPs was reduced by more than 80% and approximately 90% of NBs did not have associated INPs (Figure 4D-D', F-F', G-H). The enhancement of the loss of INPs in the *pntP1<sup>90</sup>* mutants by the reduction of Btd suggests that Btd and PntP1 genetically interact to prevent the premature differentiation of INPs and that Btd and PntP1 likely function in the same pathway.

Interestingly, in addition to enhancing the loss of INPs, reducing Btd expression also promoted ectopic activation of *ase* in *pntP1<sup>90</sup>* mutant type II NBs.

The *pntP1<sup>90</sup>* mutants usually had about 13 Ase<sup>-</sup> NBs/lobe. However, the number of Ase<sup>-</sup> type II NBs was reduced to 4-6 /lobe in the *btd<sup>XG81/+</sup>; pntP1<sup>90</sup>* or *btd-GAL4/+; pntP1<sup>90</sup>* mutants, although the *btd<sup>XG81</sup>* and *btd-GAL4* heterozygotes had the same number of Ase<sup>-</sup> type II NBs as the wild type (Figure 4C-F', I-J). By labeling type II NB lineages with mCD8-GFP driven by *btd-GAL4*, we found that the total number of type II NBs in the *btd-GAL4/+; pntP1<sup>90</sup>* mutants was still significantly increased compared with the wild type, similar to the *pntP1<sup>90</sup>* mutants, but over 60% of the type II NBs were Ase<sup>+</sup> (Figure 4F-F', I-J), indicating that the reduction of Ase<sup>-</sup> type II NBs in the *btd-GAL4/+; pntP1<sup>90</sup>* mutants (and likely in the *btd<sup>XG81/+</sup>; pntP1<sup>90</sup>* mutants as well) was due to the ectopic activation of *ase* in the NBs. This ectopic *ase* activation likely occurred throughout type II NB lineage development because the ectopic Ase expression could already be observed at 1 day ALH and the number of Ase<sup>+</sup> type II NBs continued to increase as the total number of type II NBs increased from early to late larval stages (Supplementary Figure S1). The ectopic *ase* activation in the *pntP1<sup>90</sup>* mutant type II NBs resulting from the reduction of Btd expression suggests that PntP1 and Btd function cooperatively to suppress Ase expression in type II NBs.

### ***Pnt knockdown in Ase<sup>-</sup> imINPs leads to the generation of extra type II NBs***

In addition to the elimination of INPs, the loss of PntP1 also increased the number of type II NBs. We next investigated the cellular origin of the extra type II NBs. Several previous studies have shown that imINPs are not fully committed to

their cell fate and can revert back to the NB fate in the absence of tumor suppressors such as Brat, Numb, or Erm (Bowman et al., 2008; Weng et al., 2010). Because PntP1 is highly expressed in imINPs (Zhu et al., 2011), we thus wondered whether the generation of ectopic type II NBs resulting from the loss of PntP1 was also due to imINP dedifferentiation. To test this idea, we tried to knockdown Pnt in imINPs using *erm-GAL4 (III)* or *erm-GAL4 (II)* (Xiao et al., 2012). *erm-GAL4 (III)* is mainly expressed in Ase<sup>+</sup> imINPs, whereas *erm-GAL4 (II)* is expressed in both Ase<sup>-</sup> and Ase<sup>+</sup> imINPs, except for the newly generated Ase<sup>-</sup> imINPs. Our results showed that Pnt knockdown by *erm-GAL4 (III)* did not produce any extra type II NBs in 3<sup>rd</sup> instar larval brains (Figure 5A-B", E), whereas Pnt knockdown by *erm-GAL4 (II)* significantly increased the number of type II NBs to approximately 16/brain lobe (Figure 5C-D'", E). These results suggest that the generation of extra type II NBs resulting from the loss of PntP1 is likely due to dedifferentiation of Ase<sup>-</sup> imINPs into type II NBs. The dedifferentiation of imINPs likely occurs at a low frequency because the total number of type II NBs was only increased 1-2-fold at the 3<sup>rd</sup> instar larval stages when *pntp1* was mutated or knocked down and individual ectopic type II NB lineages were often well separated (e.g. Figure 1G-H, 4F). However, the dedifferentiation could occur at any stage during development, as indicated by the existence of lineages with multiple NBs at early and late larval stages (e.g., the second GFP-labeled lineage from the left in Figure 4F and the second GFP-labeled lineage from the right in Supplementary Figure S1A)

### ***Erm expression is lost/reduced in Pnt knockdown type II NB lineages***

Why does the loss of PntP1 lead to dedifferentiation of imINPs into type II NBs? Our previous studies show that the misexpression of PntP1 in type I NB lineages is sufficient to induce the expression of Erm in INP-like cells but that inhibiting PntP1 activity with Yan abolishes the expression of Erm in type II NB lineages (Zhu et al., 2011). Furthermore, Erm and PntP1 are co-expressed in imINPs (Janssens et al., 2014; Zhu et al., 2011), and loss of Erm similarly leads to an increase in the number of type II NBs. Therefore, Erm could be a potential PntP1 target, and the dedifferentiation of imINPs resulting from the loss of PntP1 could be due to the loss of Erm in imINPs. We examined Erm expression in Pnt knockdown type II NB lineages by immunostaining to determine whether this hypothesis is true. In normal type II NB lineages, Erm is expressed in Ase<sup>-</sup> and Ase<sup>+</sup> imINPs (Figure 6A-A'') (Janssens et al., 2014). However, we did not observe obvious Erm staining in Pnt knockdown type II NB lineages even if Ase was not ectopically expressed in the NBs (Figure 6B-D), indicating that the expression of Erm was largely abolished by Pnt knockdown and the loss of Erm expression was not due to the transformation of type II NBs into type I-like NBs. The loss of Erm expression in Pnt knockdown type II NB lineages provides evidence to support the hypothesis that Erm could be a PntP1 target.

### ***The loss of Erm accounts for the generation of extra type II NBs resulting from the loss of PntP1***

To determine whether the loss of Erm expression was indeed responsible for the dedifferentiation of imINPs resulting from the loss of PntP1, we then tested genetic interactions between *erm* and *pntP1* by examining whether reducing Erm expression would further increase the generation of extra type II NBs resulting from the partial loss of PntP1 and if restoring Erm expression would suppress the generation of extra type II NBs. If the dedifferentiation of imINPs resulting from the loss of PntP1 is indeed due to the loss or reduction of Erm expression, then removing one wild type copy of the *erm* gene would further increase the number of type II NBs. In contrast, restoring Erm expression in imINPs should suppress the dedifferentiation of imINPs and thus reduce the number of type II NBs. Indeed, we found that although *erm*<sup>2</sup> heterozygous mutants did not have any extra type II NBs (Figure 7A-B, K), knockdown of Pnt in *erm*<sup>2</sup> heterozygous mutants led to a significant increase in the total number of type II NBs (including both Ase<sup>-</sup> and Ase<sup>+</sup> type II NBs) compared with Pnt knockdown in the wild type background. Similar increases were observed when two independent *UAS-pnt RNAi* lines were used (Figure 7C-F, K). Consistently, the removal of one wild type copy of *erm* increased the number of Ase<sup>-</sup> type II NBs one-fold in *pntP1*<sup>90</sup> mutant larvae (Figure 7G-H, L). In contrast, when *erm-GAL4 (II)* drove the expression of *UAS-erm* in imINPs, the generation of extra type II NBs in the *pntP1*<sup>90</sup> mutants was significantly suppressed (Figure 7I-J, L). The total number



of Ase<sup>-</sup> type II NBs in the *pntP1<sup>90</sup>* mutants was reduced to 10/lobe when *UAS-erm* was expressed in imINPs, suggesting that restoring Erm expression largely prevents the dedifferentiation of imINPs. Taken together, the enhancement and the suppression of the generation of ectopic type II NBs by reducing or maintaining Erm expression, respectively, indicate that the loss of Erm expression accounts for the dedifferentiation of imINPs into type II NBs resulting from the loss of PntP1.

## DISCUSSION

We have previously shown that PntP1 is required for the generation of INPs in type II NB lineages (Zhu et al., 2011), but the underlying mechanisms have not been elucidated. We show in this study that PntP1 has at least two distinct roles in INPs: preventing the premature differentiation of INPs into GMCs by acting together with Btd to suppress Pros expression in newly generated imINPs, and preventing the dedifferentiation of INPs into type II NBs by activating Erm expression late in imINP development. Therefore, PntP1 is able to maintain the balance between INP differentiation and dedifferentiation by functioning through two distinct pathways (Figure 7M).

Our results show that a reduction in PntP1 function leads to both a loss of INPs and an increase in the number of type II NBs without completely transforming all type II NBs into type I-like NBs. The loss of INPs is particularly

obvious in the *pntP1<sup>82</sup>* mutant and Pnt knockdown type II NB lineages possibly because there is more severe reduction of PntP1 function in the *pntP1<sup>82</sup>* mutants or after Pnt knockdown. We provide several lines of evidence to demonstrate that the loss of INPs is due to the Pros-mediated premature differentiation of INPs. First, the loss of INPs is not due to the transformation of type II NBs into type I NBs because the loss of INPs occurs independently of the ectopic Ase expression in the NBs. Second, when INPs are lost, the type II NBs generate GMCs instead. Third, nuclear Pros is ectopically activated in the newly generated imINPs when Pnt is lost. Fourth, the loss of INPs can be almost fully rescued by reducing Pros expression. Therefore, PntP1 normally inhibits Pros expression in the newly generated imINPs so that the imINPs can differentiate into mature INPs and undergo self-renewing divisions instead of becoming GMCs and exiting the cell cycle.

Although it remains to be investigated exactly how PntP1 suppresses Pros expression, our work suggests that PntP1 suppresses Pros by functioning together with Btd, which is also required to suppress Pros in imINPs (Xie et al., 2014). We show that reducing Btd expression enhances the loss of INPs in *pntP1<sup>90</sup>* mutants. Although reducing Btd expression also enhances the ectopic activation of Ase in type II NBs, our data do not support the hypothesis that the enhancement of the loss of INPs is due to the ectopic activation of Ase because the enhancement of the loss of INPs also occurs in lineages without the ectopic Ase expression. The genetic interaction between Btd and PntP1 suggests that

they function in the same pathway to suppress Pros expression. However, it is unlikely there is a direct regulatory relationship between Btd and PntP1 for several reasons. First, although Btd and PntP1 show similar expression patterns in type II NB lineages, Btd, but not PntP1, is also expressed in a subset of type I NB lineages. Second, we did not observe obvious changes in the expression of mCD8-GFP driven by *btd-GAL4* in the *pntP1<sup>90</sup>* mutant or Pnt knockdown type II NB lineages (data not shown). Third, PntP1 expression is maintained in the majority of *btd* mutant type II NB clones (Xie et al., 2014). Fourth, PntP1 promotes the generation of INPs from type I NBs only when Btd is coexpressed, but not when PntP1 is expressed alone (Xie et al., 2014). It is well documented that Ets family proteins could bind to other transcription factors and that this partnership could enhance the binding to the promoters of target genes and contribute to the functional specificity of Ets proteins (Hollenhorst et al., 2011). Therefore, one interesting possibility could be that PntP1 and Btd physically interact and bind cooperatively to the promoters of their target genes.

In addition to preventing premature differentiation of INPs, our phenotypic analyses in the *pntP1* mutants and Pnt knockdown type II NB lineages suggest that PntP1 also prevents imINP dedifferentiation. We show that there is a significant increase in the number of type II NBs in *pntP1<sup>90</sup>* homozygous or *pntP1<sup>90</sup>/pnt<sup>Δ88</sup>* transheterozygous mutants or after knocking down Pnt. The extra type II NBs are likely derived from dedifferentiation of Ase<sup>-</sup> imINPs, as knocking down Pnt by *erm-GAL4 (II)* but not *erm-GAL4 (III)* leads to an increased number

of type II NBs. We also provide several lines of evidence to show that the dedifferentiation of imINPs is at least in part due to the loss of Erm in imINPs. First, Erm expression in imINPs is lost after Pnt knockdown and the loss of Erm occurs independently of the transformation of type II NBs into type I NBs. Second, reducing the expression of Erm significantly enhances the generation of extra type II NBs resulting from the loss of PntP1, whereas maintaining Erm expression in *Ase<sup>-</sup>* imINPs significantly suppresses the generation of extra type II NBs in the *pntP1<sup>90</sup>* mutants. Our results are consistent with a recent report showing that knockdown of Pnt by the type II NB driver *wor-GAL4 ase-GAL80* increases the number of type II NBs, and reducing Pnt expression enhances the generation of ectopic type II NBs in *erm* mutants (Komori et al., 2014). However, our work identifies additional cellular and molecular mechanisms by which extra type II NBs are generated following the loss of PntP1.

One of the defining features of type II NBs is the lack of *Ase* expression. We previously reported that PntP1 was responsible for the suppression of *Ase* in type II NBs (Zhu et al., 2011). However, a recent study argues that PntP1 is only required for INP specification, but not type II NB specification, by showing that knockdown of Pnt by *wor-GAL4 ase-GAL80* only increases the number of type II NBs but does not ectopically activate *Ase* in type II NBs (Komori et al., 2014). Here, we show that Pnt knockdown by *pntP1-GAL4* is sufficient to ectopically activate *Ase* in approximately 80% of type II NBs, confirming that PntP1 indeed specifies type II NB identity. The discrepancy could be due to differences in the

efficiency of Pnt RNAi knockdown involving different GAL4 drivers. However, our results show that reducing Pros expression not only rescues the loss of INPs but also restores the suppression of Ase expression in Pnt knockdown type II NBs, raising a question whether PntP1 specifies type II NBs by directly acting in the NBs or by indirectly promoting INP generation. Our previous results show that PntP1 misexpression is able to suppress Ase expression in type I NBs, even if INPs are not generated, suggesting that PntP1 likely acts in the NBs to suppress Ase expression. However, INPs are also likely involved in maintaining type II NB identity by providing a feedback signal to maintain PntP1 expression in the NB as we proposed in a previous study (Xie et al., 2014). One candidate feedback signal could be the Notch ligand. Our recent studies suggest that Notch signaling is required to maintain PntP1 expression and type II NB identity (Li et al., 2016; Zhu et al., 2012). INPs could provide the ligand to activate Notch in the NBs, as suggested in a recent study (Song and Lu, 2011). This Notch-mediated feedback mechanism for maintaining neural progenitor cells is also conserved in mammals (Campos et al., 2001; Lui et al., 2011; Yoon et al., 2008).

However, it is surprising that Ase remains suppressed at least in a subset of *pntP1<sup>90</sup>* or *pntP1<sup>82</sup>* mutant type II NBs. Given that PntP1 is a transcription factor, one might expect that the deletion of the Ets DNA-binding domain in the *pntP1<sup>90</sup>* and *pntP1<sup>82</sup>* mutants would lead to a complete loss of PntP1 function and ectopic activation of Ase in all type II NBs. However, our genetic data suggest they are likely hypomorphic alleles because the phenotypes in the *pntP1<sup>90</sup>* and *pntP1<sup>82</sup>*

mutants are weaker than in the *pntP1<sup>90</sup>/pnt<sup>Δ88</sup>* transheterozygotes. Furthermore, the *pntP1<sup>90</sup>* and *pntP1<sup>82</sup>* mutants show different degrees of severity in the phenotype, which also indicates that deletion of the Ets DNA-binding domain does not completely abolish PntP1 function. Therefore, the truncated PntP1 protein generated in the *pntP1<sup>90</sup>* or *pntP1<sup>82</sup>* mutants could be still partially functional and the DNA-binding domain of PntP1 might be dispensable for function. Our results show that reducing Btd expression significantly enhances the ectopic activation of Ase in *pntP1<sup>90</sup>* mutant type II NBs. Although we do not rule out the possibility that the enhanced ectopic Ase activation could be secondary to the enhanced loss of INPs and their feedback signal(s), Btd and PntP1 may actually function together to suppress Ase. In support of this notion, we previously showed that Btd overexpression was able to partially suppress Ase expression in a subset of type I NBs in larval brains (Xie et al., 2014). The truncated PntP1 proteins generated from the *pntP1<sup>90</sup>* or *pntP1<sup>82</sup>* alleles might still be able to interact with Btd to regulate target gene expression.

In summary, our studies shed new light on the mechanistic details of PntP1-mediated generation of INPs as well as type II NB specification. However, it remains to be investigated how PntP1 and Btd act together to specify type II NBs and inhibit Pros expression in INPs. Understanding this may rely on identification of the direct targets of PntP1 and Btd in the future.

## MATERIALS AND METHODS

### Fly stocks

*UAS-pnt RNAi* lines (#31936 and #35038, Bloomington *Drosophila* stock Center, Bloomington, Indiana) were used for Pnt knockdown. Type II NB lineage-specific *pntP1-GAL4* (named as *GAL4<sup>14-94</sup>* previously) (Zhu et al., 2011) and *erm-GAL4* (II) or (III) (Pfeiffer et al., 2008; Xiao et al., 2012) were used to drive the expression of *UAS-transgenes* in type II NB lineages or in immature INPs, respectively. Other fly lines include: *pnt<sup>Δ88</sup>/Tm6Tb* (Brunner et al., 1994) was used for *pntP1* loss of function phenotypic analyses; *pros<sup>17</sup>/TM6Tb* (Doe et al., 1991); *btd<sup>XG81</sup>, FRT19A/FM7c, Kr-GFP*; *btd-GAL4, FRT19A/FM7c, Kr-GFP* (Estella and Mann, 2010; Wimmer et al., 1993); and *erm<sup>2</sup>/Cyo, act-GFP* (Weng et al., 2010) for reducing the expression of Pros, Btd, and Erm, respectively.

### Generation of *pntP1* mutants using the CRISPR/Cas9 system

gRNA targets were selected using an online design tool (<http://tools.flycrispr.molbio.wisc.edu/targetFinder/>). The gRNA-expressing vectors were generated by a series of PCR reactions (Supplementary Materials and Methods) (Sebo et al., 2014) and injected into *y<sup>1</sup>, P[vas-Cas9.S]ZH-2A, w<sup>1118</sup>* (BL #52669) at 1ug/ml (Rainbow Transgenic Flies, Inc., Camarillo, California) for generating mutant lines. Mutations were detected by PCR amplification of

genomic DNAs followed by sequencing using *pntP1*-specific primers.

### **UAS-transgene expression**

For RNAi knockdown or misexpression of transgenes, larvae were raised at 30°C after hatching. *UAS-dcr2* was co-expressed with *UAS-RNAi* transgenes to enhance the efficiency of RNAi knockdown. Phenotypes were examined at third instar larval stages. For examining if reducing Pros expression could rescue the loss of INPs resulting from Pnt RNAi knockdown, *UAS-pnt RNAi* was first recombined with *pntP1-GAL4*. Then the *UAS-pnt RNAi pntP1-GAL4* recombinant flies were crossed with either wild type or *pros<sup>17</sup>/Tm6,Tb* and their progenies were raised at 25°C.

### **Immunostaining, confocal microscopy, and statistical analyses**

Dissection, fixation, and immunostaining of larval brains were performed as described (Lee and Luo, 1999). Primary antibodies used for immunostaining include: guinea pig anti-Ase (1:5000) (Brand et al., 1993), rabbit anti-Dpn (1:500) (Bier et al., 1992) (gifts from Y.N. Jan), rat anti-mCD8 (Life Technologies, Grand Island, New York, 1:100), mouse anti-Pros (Developmental Studies Hybridoma Bank, Iowa City, Iowa, 1:20), rabbit anti-Erm (a gift from H.Y. Wang, 1:50) (Janssens et al., 2014). Secondary antibodies conjugated to Cy2, Cy3, Cy5, or DyLight 647 (Jackson ImmunoResearch, West Grove, Pennsylvania) were used



at 1:100, 1:500, or 1:500, respectively. A Zeiss LSM510 confocal microscopy was used for acquiring images, which were processed with Adobe Photoshop. Two-tailed student *t*-tests were used for statistical analyses.

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### **Competing interests:**

The authors declare that no competing interests exist.

### **Author Contributions**

S.Z. and Y.X. designed the project and approaches, interpreted data, and wrote the paper. Y. X., X.L., Y.H., and X.D. conducted experiments and analyzed data. K.O., A.U., and L.C. provided technical support. Y.P. designed the strategy for generating pntP1 mutants. Y.H., X.D., K.O., Y.P., and L.C. contributed to manuscript editing.

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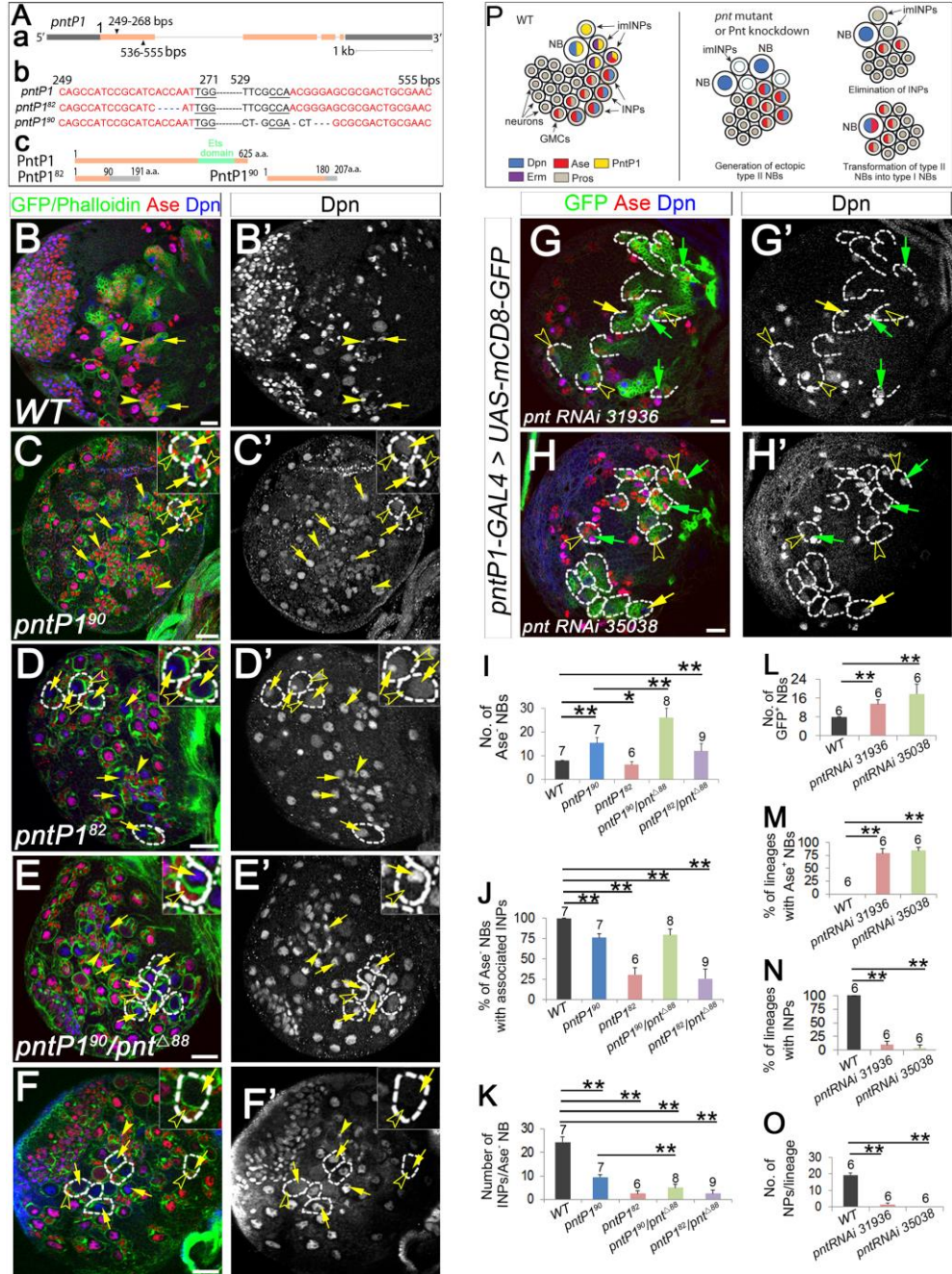
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# Figures





**Figure 1.** Partial loss of PntP1 increases the number of type II NBs and eliminates INPs.

**(A)** A diagram of the generation of *pntP1*-specific mutants. (a) The gene structure of *pntP1* and gRNA target sites (arrowheads). Gray boxes: untranslated regions; orange boxes: coding regions. (b) Sequences of gRNA target sites (red) and indels in *pntP1<sup>82</sup>* and *pntP1<sup>90</sup>* mutants. (c) The protein structure of PntP1 and truncated PntP1 proteins generated from *pntP1<sup>82</sup>* and *pntP1<sup>90</sup>* mutants. Gray areas: non-specific sequences caused by frame shifts.

**(B-B')** A wild-type (*WT*) brain lobe. Type II NB lineages are labeled with mCD8-GFP driven by *pntP1-GAL4*. Arrows: Ase<sup>-</sup> type II NBs; arrowheads: Dpn<sup>+</sup> Ase<sup>+</sup> INPs.

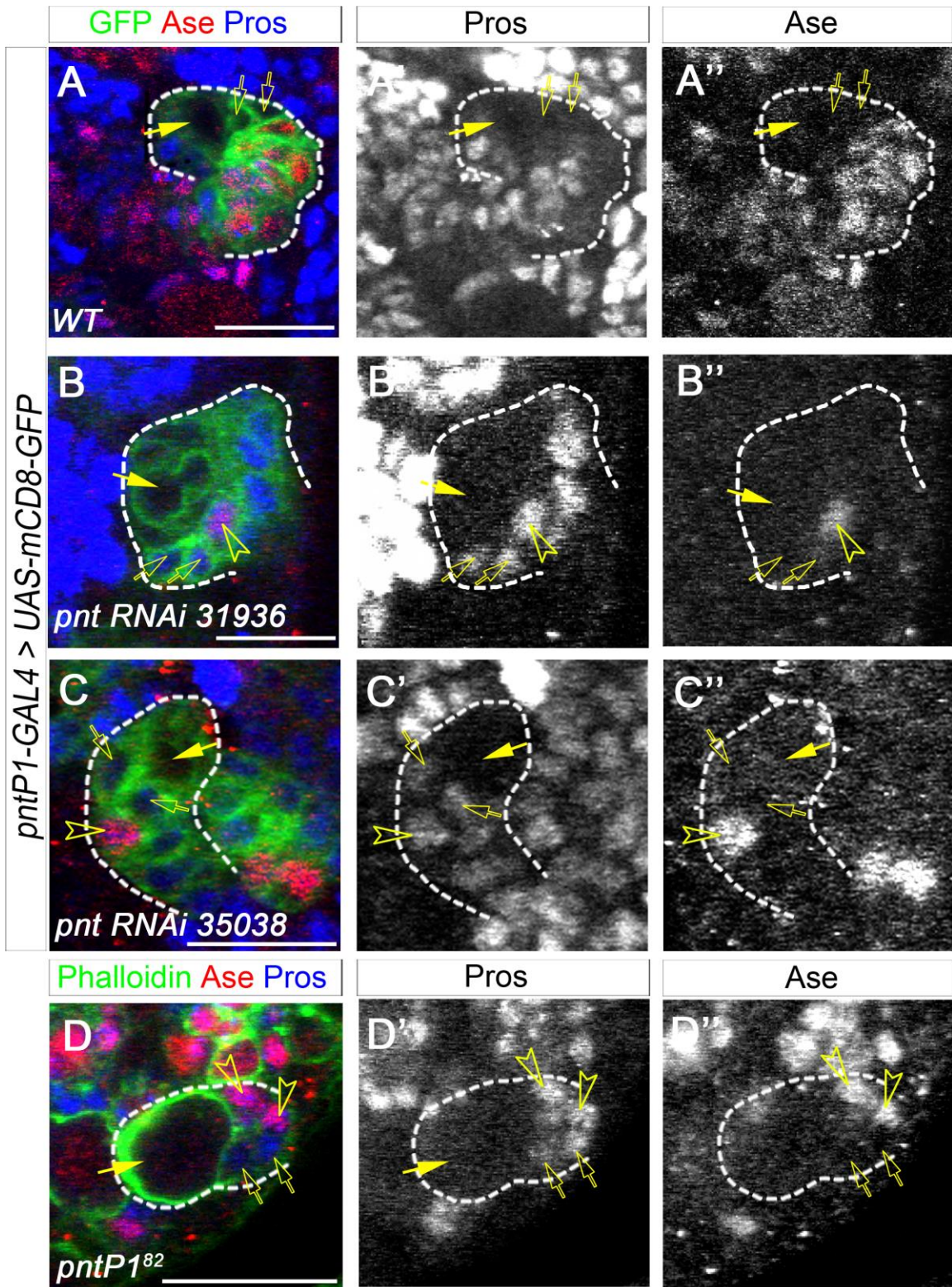
**(C-F')** *pntP1* mutant brains with indicated genotypes. Brains are stained with phalloidin to outline NBs. The number of Ase<sup>-</sup> type II NBs (arrows) is significantly increased in *pntP1<sup>90</sup>* (C-C'), *pntP1<sup>90</sup>/pnt<sup>Δ88</sup>* (E-E'), *pntP1<sup>82</sup>/pnt<sup>Δ88</sup>* (F-F') mutants but not in *pntP1<sup>82</sup>* (D-D'). Dashed circles outline type II NB lineages that generated GMCs (open arrowheads) but not INPs. Insets: enlarged views of lineages without INPs.

**(G-H')** Pnt knockdown leads to ectopic Ase expression in type II NBs (green arrows) and elimination of INPs in most lineages (dashed lines). Yellow arrows: Ase<sup>-</sup> NBs; open arrowheads: GMCs.

**(I-K)** Quantifications of the number of Ase<sup>-</sup> type II NBs (I), percentage of Ase<sup>-</sup> NBs with INPs (J), number of INPs (K) in *pnt* mutants.

**(L-O)** Quantifications of the total number of type II NBs (L), percentage of lineages with Ase<sup>+</sup> NBs (M), percentage of lineages with INPs (N), and number of INP (O) in Pnt knockdown brains. Numbers on top of each bar represent sample sizes; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; scale bars: 20  $\mu\text{m}$ .

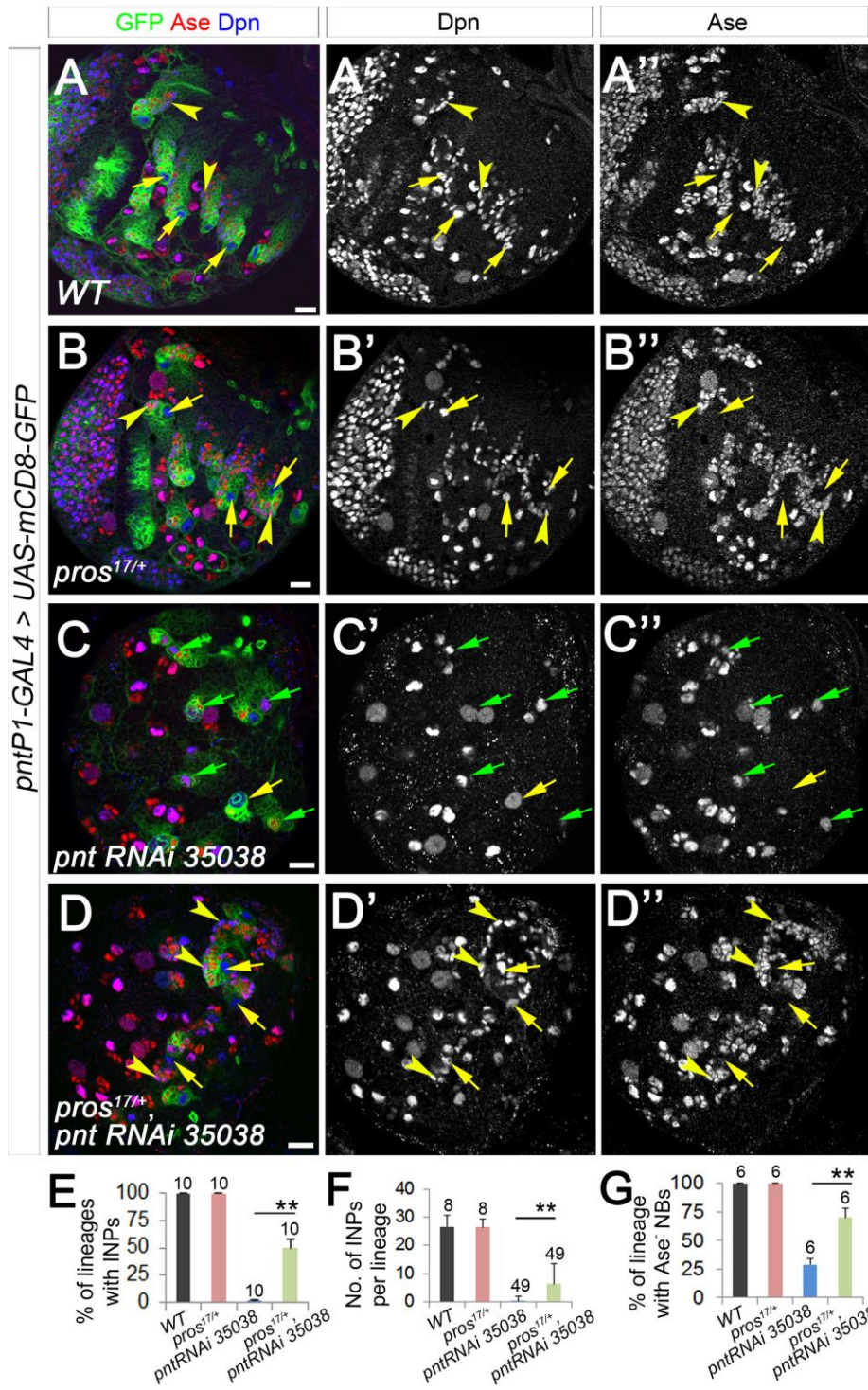
**(P)** Schematic diagrams of wild type type II NB lineages and phenotypes in *pnt* mutant or Pnt knockdown type II NB lineages.



**Figure 2.** Loss of PntP1 results in ectopic nuclear Pros expression in imINPs.

**(A-A'')** Pros is not expressed in  $Ase^-$  imINPs (open arrows) in a WT GFP-labeled type II NB lineage. Arrows: type II NBs.

**(B-D'')** Nuclear Pros is detected in  $Ase^-$  imINPs (open arrows) and  $Ase^+$  progeny (open arrowheads) in Pnt knockdown (B-C'') and *pntP1<sup>82</sup>* mutant (D-D'') type II NB lineages. Scale bars: 20  $\mu$ m.



**Figure 3.** Reducing Pros rescues the loss of INPs resulting from Pnt knockdown.

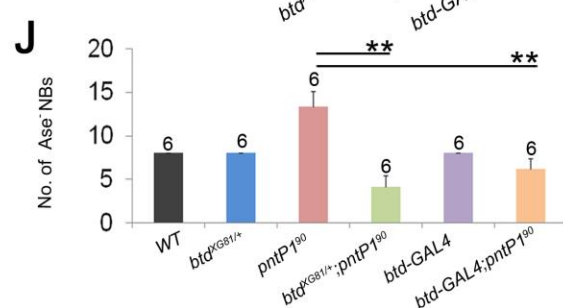
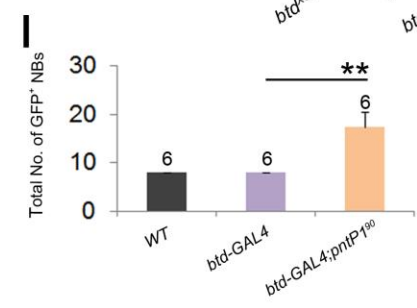
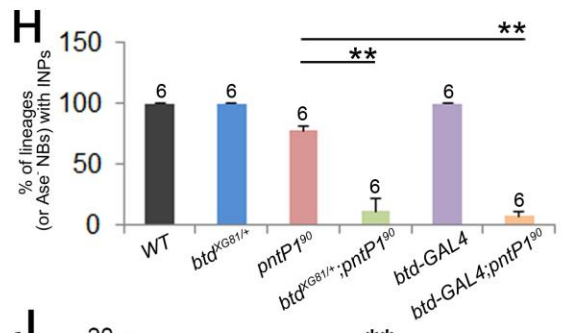
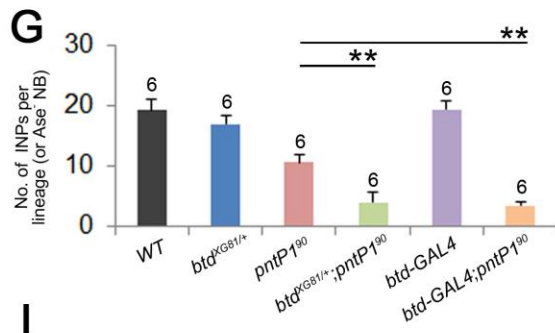
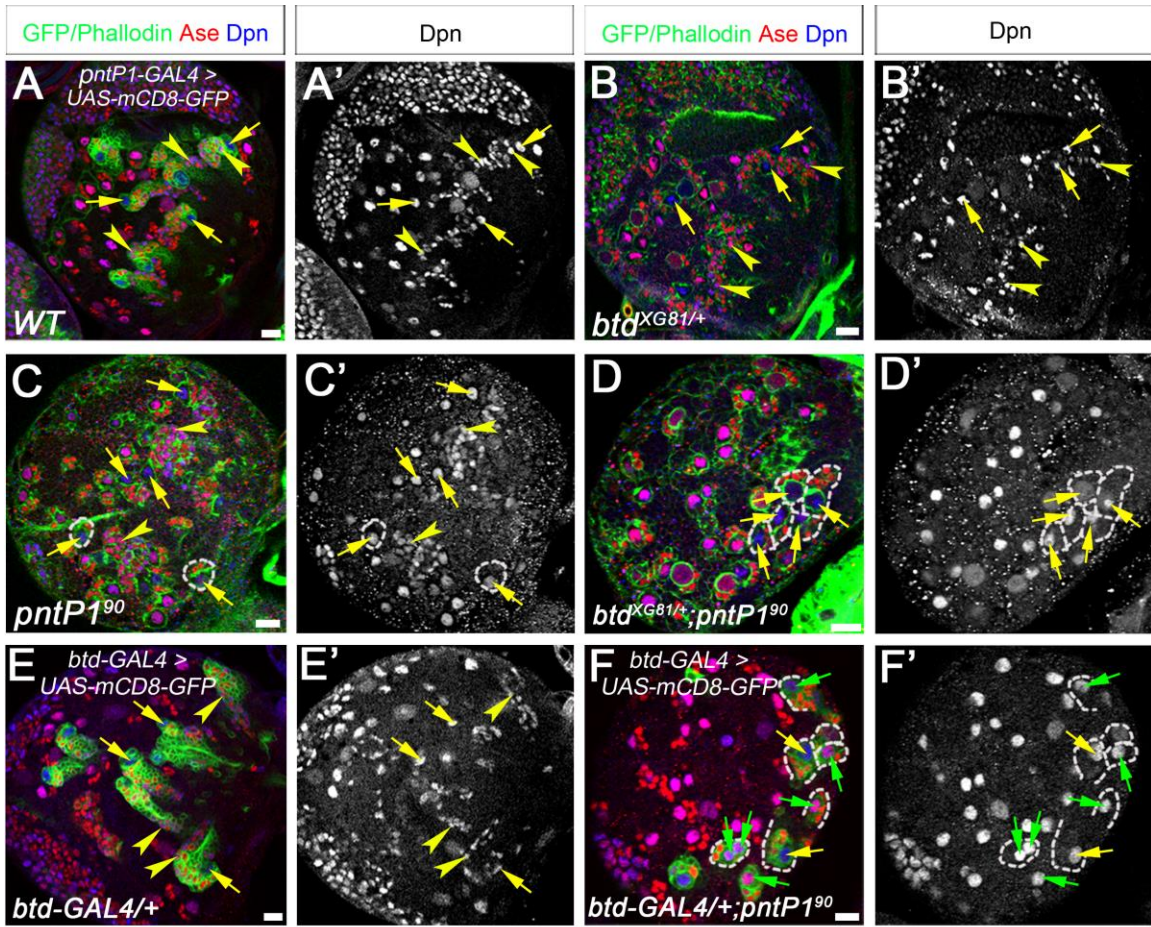
Type II NB lineages are labeled with mCD8-GFP driven by *pntP1-GAL4*. Arrows: type II NBs; arrowheads: INPs.

**(A-B'')** WT (A-A'') or *pros*<sup>17</sup> heterozygous (B-B'') brains. Each type II NB lineage has multiple mature INPs.

**(C-C'')** INPs are largely eliminated in Pnt knockdown type II NB lineages. Note that most type II NBs become Ase<sup>+</sup> (green arrows).

**(D-D'')** INPs are produced in a subset of type II NB lineages and Ase remains suppressed in most type II NBs (arrows) when Pnt is knocked down in *pros*<sup>17/+</sup> heterozygotes.

**(E-G)** Quantifications of the percentage of lineages with INPs (E), the number of INPs (F), and the percentage of lineages with Ase<sup>-</sup> NBs (G). \*\**p* < 0.01. Scale bars: 20 μm.



**Figure 4.** PntP1 and Btd genetically interact to suppress premature differentiation of INPs and Ase expression in type II NBs.

Type II NB lineages are labeled with either mCD8-GFP driven by *pntP1-GAL4* (A) or *btd-GAL4* (E-F) or phalloidin (C,B,D). Arrows: type II NBs; arrowheads: INPs.

**(A-B', E-E')** WT (A-A'), *btd<sup>XG81/+</sup>* (B-B'), and *btd-GAL4/+* (E-E') larval brains.

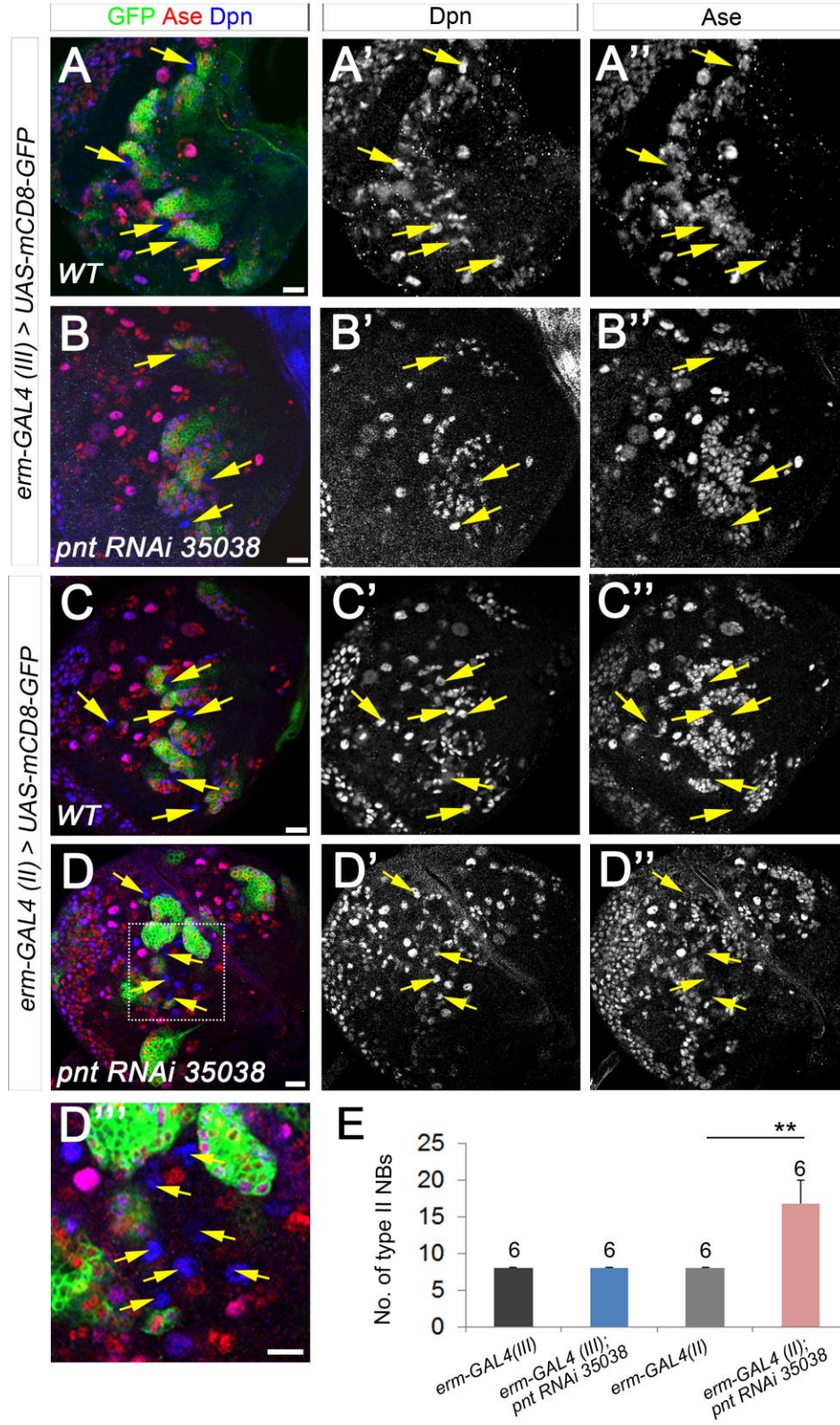
Each type II NBs is associated with multiple INPs. .

**(C-C')** A *pntP1<sup>90</sup>* mutant brain shows an increased number of Ase<sup>-</sup> type II NBs and loss of INPs in a small subset of type II NB lineages (e.g. dashed circles).

**(D-D', F-F')** *btd<sup>XG81/+</sup> pntP1<sup>90</sup>* (D-D') or *btd-GAL4/+ pntP1<sup>90</sup>* (F-F') mutants have decreased numbers of Ase<sup>-</sup> type II NBs and no INPs in most type II NB lineages (dashed lines). However, the total number of GFP-labeled NBs is increased in *btd-GAL4/+ pntP1<sup>90</sup>* mutants but majority of them become Ase<sup>+</sup> (green arrows) (F-F').

**(G-J)** Quantifications of the number of INPs per lineage (for WT, *btd<sup>XG81/+</sup>*, and *btd-GAL4/+*, and *btd-GAL4/+ pntP1<sup>90</sup>*) or per Ase<sup>-</sup> NB (for *pntP1<sup>90</sup>* or *btd<sup>XG81/+</sup> pntP1<sup>90</sup>*) (G), percentage of lineages (or Ase<sup>-</sup> NBs) with INPs (H), and the total number of type II NBs (I) or the number of Ase<sup>-</sup> type II NBs (J). \*\**p* < 0.01. Scale bars: 20 μm.





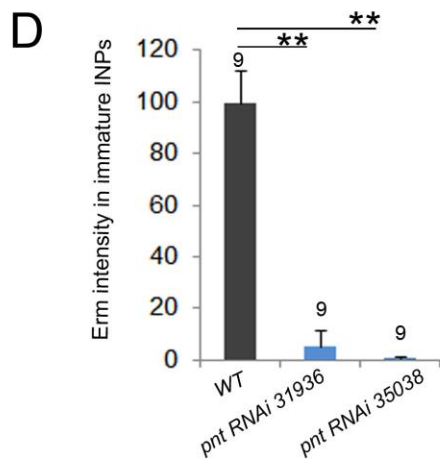
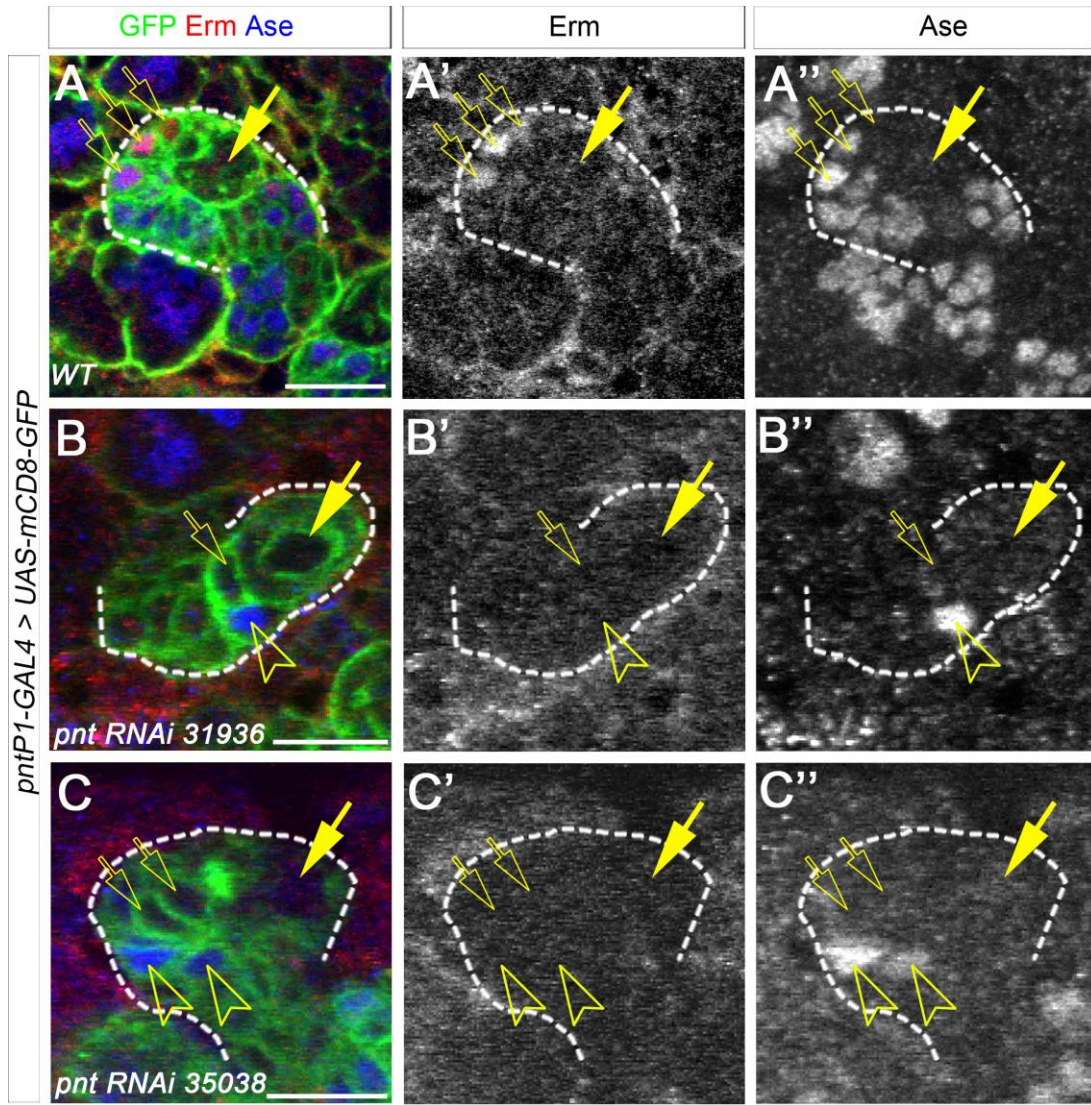
**Figure 5.** Knockdown of Pnt in Ase<sup>-</sup> imINPs increases the number of type II NBs.

**(A-A'')**, **(C-C'')** *WT* type II NB lineages labeled with mCD8-GFP driven by *erm-GAL4 (III)* (A-A'') or *erm-GAL4 (II)* (C-C''). Arrows: type II NBs.

**(B-B'')** Knockdown of Pnt by *erm-GAL4 (III)* does not lead to generation of extra type II NBs (arrows).

**(D-D''')** Knockdown of Pnt by *erm-GAL4 (II)* results in an increased number of type II NBs (arrows). (D''') An enlarged view of the highlighted area in (D).

**(E)** Quantifications of total numbers of type II NBs. \*\* $p < 0.01$ . Scale bars: 20  $\mu\text{m}$ .

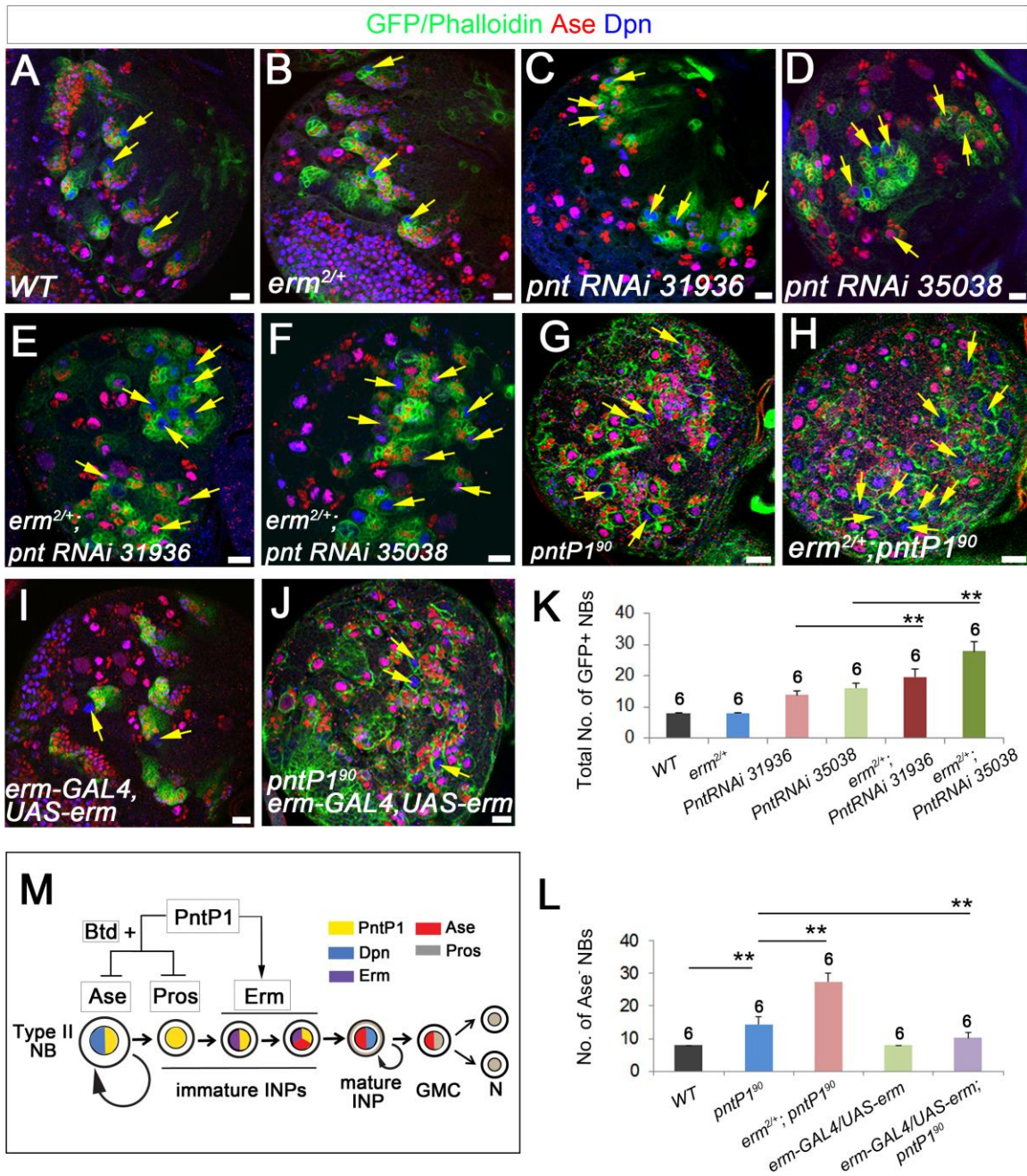


**Figure 6.** Erm is largely abolished in Pnt knockdown type II NB lineages.

**(A-A'')** Erm is expressed in Ase<sup>-</sup> and Ase<sup>+</sup> imINPs (open arrows) in a *WT* type II NB lineage labeled by GFP. Arrows: type II NBs.

**(B-C'')** Erm is not detected in Ase<sup>-</sup> imINPs (open arrows) or Ase<sup>+</sup> progeny (open arrowheads) when Pnt is knocked down.

**(D)** Quantifications of Erm staining intensities.  $**p < 0.01$ . Scale bars: 20  $\mu\text{m}$ .



**Figure 7.** Loss of *Erm* is responsible for the generation of ectopic type II NBs resulting from the loss of *PntP1*.

Type II NB lineages are labeled with mCD8-GFP driven by either *pntP1-GAL4* (A-F) or *erm-GAL4* (I) or phalloidin staining (G-H, J). Arrows: type II NBs.

**(A-B)** *WT* (A) and *erm*<sup>2</sup> heterozygous mutant (B) brains have 8 type II NBs (only 7 and 4 are shown, respectively).

**(C-F)** Knockdown of Pnt in *erm*<sup>2</sup> heterozygotes (E-F) leads to generation of more type II NBs than in the wild type (C-D).

**(G-H)** The generation of ectopic Ase<sup>-</sup> type II NBs in *pntP1*<sup>90</sup> homozygous brains is enhanced in *erm*<sup>2</sup> heterozygous background.

**(I-J)** Expression of Erm driven by *erm-GAL4* (*II*) reduces the number of Ase<sup>-</sup> type II NBs in *pntP1*<sup>90</sup> mutant brains (J-J) but not in the wild type (I-I).

**(K-L)** Quantifications of the total number of type II NBs (K) and the number of Ase<sup>-</sup> type II NBs (L). \*\**p* < 0.01.

**(M)** A working model of PntP1. PntP1 functions together with Btd to suppress Ase and Pros in the NB and newly generated imINPs, respectively, to specify type II NBs and prevent premature differentiation of INPs. At later stages of imINP development, PntP1 activates *erm* to promote INP maturation. N, neuron.

## SUPPLEMENTARY MATERIALS AND METHODS

### Construction of gRNA-expressing vectors

We chose two 20-nt sequences within the *pntP1*-specific exon 1, CAGCCATCCGCATCACCAAT (+) and ACGGGAGCGCGACTGCGAAC (-), which are 249 and 532 base pairs downstream of the start codon, as gRNA targets. For constructing gRNA-expressing vectors, we first amplified the U6 promoter from genomic DNA by PCR reactions using primers 5'-TTTTTTGCTCACCTGTGATT-3' (forward1) and 5'-CGACGTAAATTGAAAATAGGTC-3' (Reverse1). Then target-specific 20-nt spacer sequences followed by the first 24-nt of the common tracrRNA sequence were added downstream of U6 promoter by PCR reactions using primers 5'-TTTTTTGCTCACCTGTGATTGCTCCTACTCAAATACA-3'(forward2) and 5'-CTTGCTATTTCTAGCTCTAAAACATTGGTGATGCGGATGGCTGCGACGTTAAATTGAAAATAGGTC-3' (reverse2-1) or 5'-CTTGCTATTTCTAGCTCTAAAACACGGGAGCGCGACTGCGAACCGACGTTAAATTGAAAATAGGTC-3' (reverse2-2). Finally, the remaining 56-nt tracrRNA sequence was added to the previous PCR products by PCR reactions using primers forward2 and primer 5'-AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAACTTGCTATTTCTAGCTCTAAAAC-3' (reverse3). All PCR reactions were carried out using CloneAmp™ HiFi PCR Premix (Clontech, Mountain View, California ). Final PCR products were cloned into pJET1.2/blunt vector (ThermoFisher scientific, Pittsburgh, Pennsylvania). For detecting mutations in *pntP1* mutants, following primers were used for PCR amplification of genomic DNAs isolated from homozygous mutants and sequencing (Genewiz, South Plainfield, New Jersey): forward: 5'-ATGCCGCCCTCTGCGTTTTTA-3'; reverse: 5'-CTGTACCTGAACTAAAGAAGTTGACATCC-3'.

*btd-GAL4/+; UAS-mCD8-GFP/+; pntP1<sup>90</sup>*

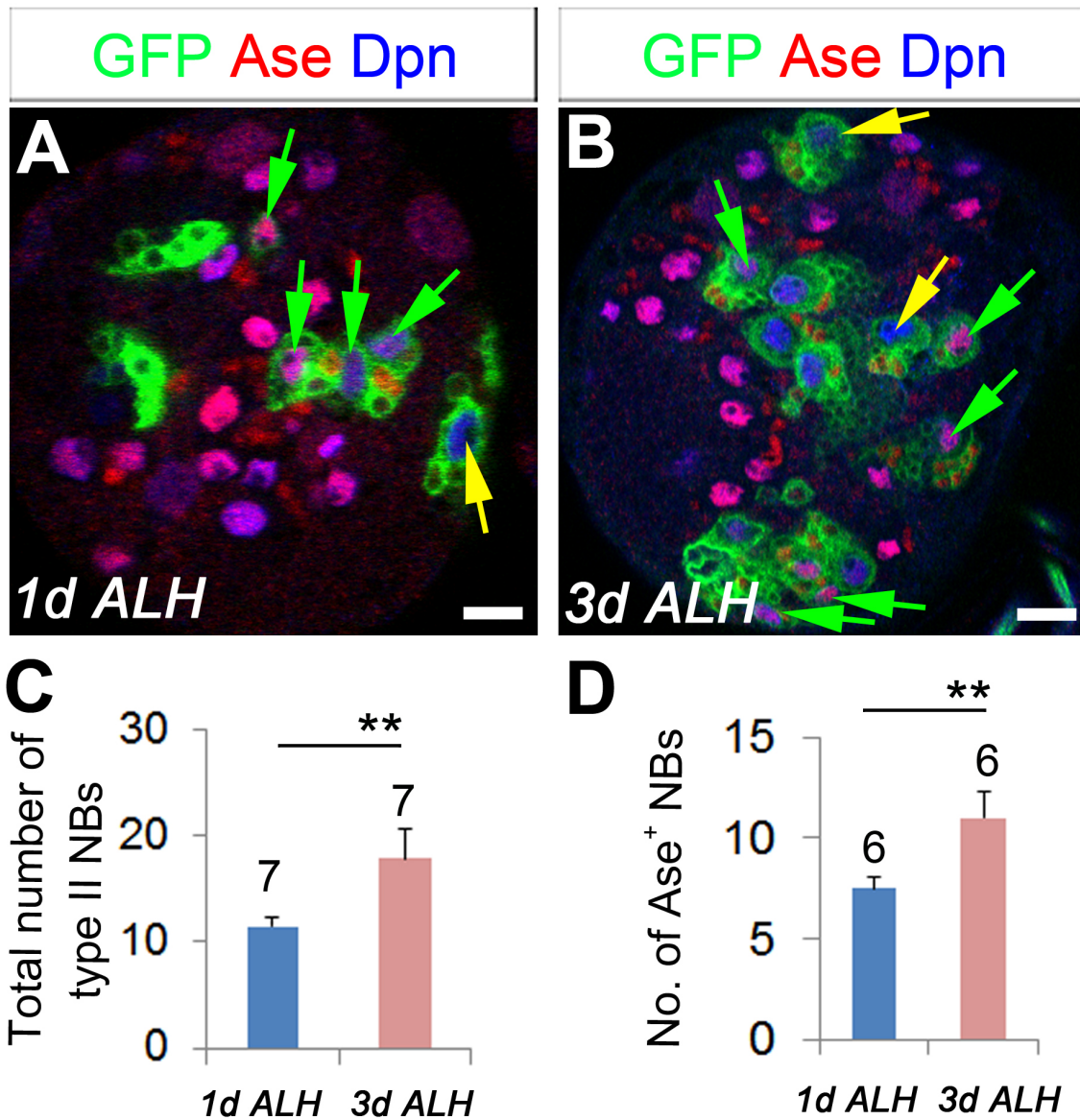


Figure S1. Reduction of Btd promotes ectopic Ase expression in *pntP1* mutant type II NBs. (A-B) *btd-GAL4/+; pntP1<sup>90</sup>* mutant larval brains at 1 day (A) and 3 days (B) ALH. Type II NB lineages are labeled with mCD8-GFP driven by *btd-GAL4*. Green arrows point to Ase<sup>+</sup> type II NBs and yellow arrows point to Ase<sup>-</sup> type II NBs. (C-D) Quantifications of the total number of type II NBs (C) and the Ase<sup>+</sup> type II NB number (D) in *btd-GAL4/+; pntP1<sup>90</sup>* mutant larval brains at 1 day and 3 days ALH. Numbers on top of each bar are sample sizes and individual bars represent mean  $\pm$  Stdev. \*\*,  $p < 0.01$  (student *t*-test). Scal bars: 20 μm.