

## RESEARCH ARTICLE

## STEM CELLS AND REGENERATION

# Earmuff restricts progenitor cell potential by attenuating the competence to respond to self-renewal factors

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## ABSTRACT

Despite expressing stem cell self-renewal factors, intermediate progenitor cells possess restricted developmental potential, which allows them to give rise exclusively to differentiated progeny rather than stem cell progeny. Failure to restrict the developmental potential can allow intermediate progenitor cells to revert into aberrant stem cells that might contribute to tumorigenesis. Insight into stable restriction of the developmental potential in intermediate progenitor cells could improve our understanding of the development and growth of tumors, but the mechanisms involved remain largely unknown. Intermediate neural progenitors (INPs), generated by type II neural stem cells (neuroblasts) in fly larval brains, provide an *in vivo* model for investigating the mechanisms that stably restrict the developmental potential of intermediate progenitor cells. Here, we report that the transcriptional repressor protein Earmuff (Erm) functions temporally after Brain tumor (Brat) and Numb to restrict the developmental potential of uncommitted (immature) INPs. Consistently, endogenous Erm is detected in immature INPs but undetectable in INPs. Erm-dependent restriction of the developmental potential in immature INPs leads to attenuated competence to respond to all known neuroblast self-renewal factors in INPs. We also identified that the BAP chromatin-remodeling complex probably functions cooperatively with Erm to restrict the developmental potential of immature INPs. Together, these data led us to conclude that the Erm-BAP-dependent mechanism stably restricts the developmental potential of immature INPs by attenuating their genomic responses to stem cell self-renewal factors. We propose that restriction of developmental potential by the Erm-BAP-dependent mechanism functionally distinguishes intermediate progenitor cells from stem cells, ensuring the generation of differentiated cells and preventing the formation of progenitor cell-derived tumor-initiating stem cells.

**KEY WORDS:** Earmuff, Brm, Developmental potential, Intermediate neural progenitor, Neuroblast, Self-renewal factors

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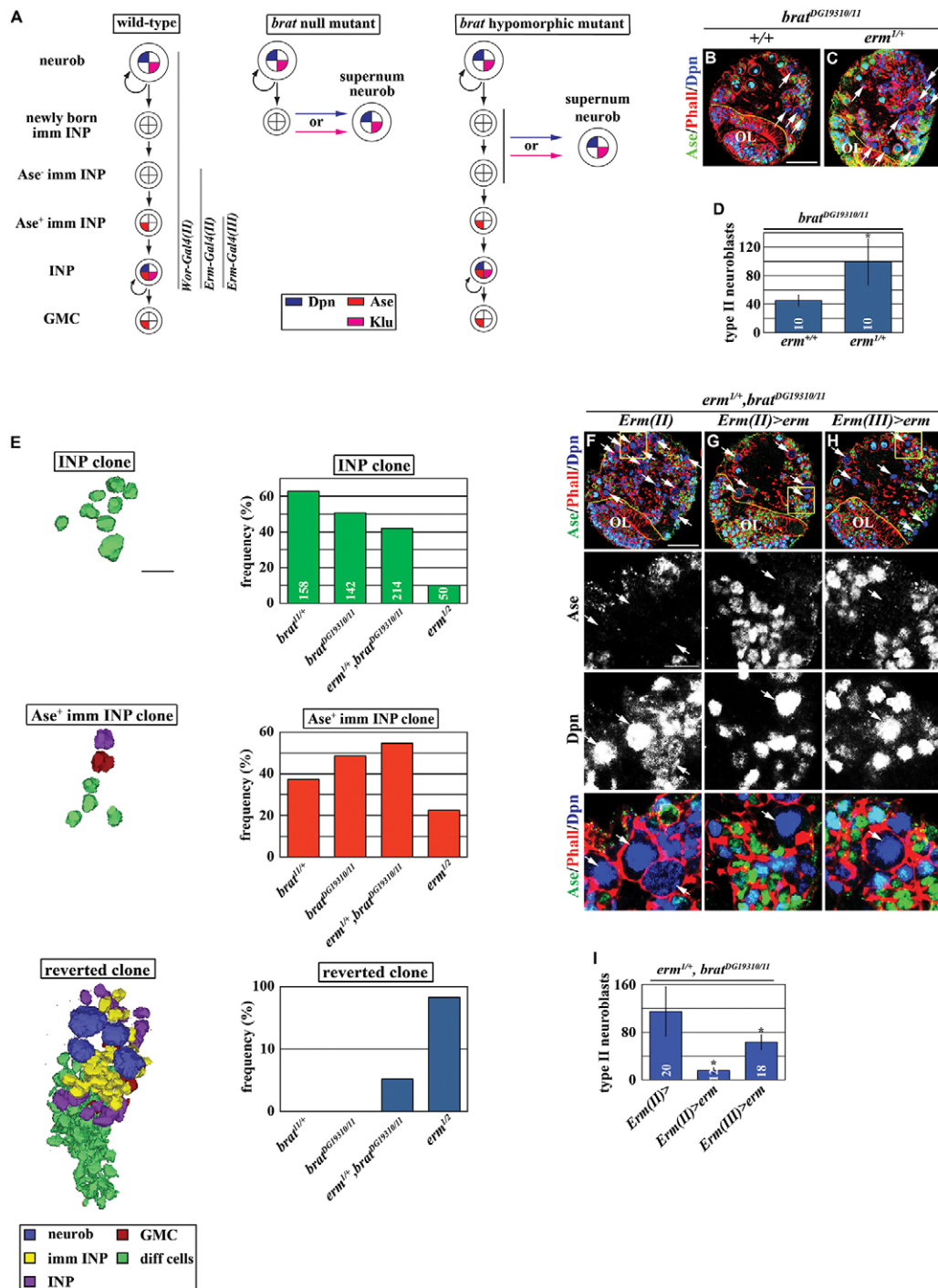
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## INTRODUCTION

Tissue-specific stem cells often generate differentiated cell types indirectly through intermediate progenitor cells during normal development and the maintenance of homeostasis (Lui et al., 2011; Ming and Song, 2011; Weng and Lee, 2011; Chang et al., 2012; Homem and Knoblich, 2012; Franco and Müller, 2013). Intermediate progenitor cells possess restricted developmental potential, which allows them to give rise to exclusively differentiated progeny, thereby amplifying the output of stem cells. Accumulating evidence suggests that the acquisition of aberrant stem cell properties by intermediate progenitor cells might be an underlying mechanism that leads to the initiation of tumorigenesis (Weng et al., 2010; Liu et al., 2011; Haenfler et al., 2012; Xiao et al., 2012; Schwitalla et al., 2013; Komori et al., 2014). Thus, understanding the mechanisms that restrict the developmental potential of intermediate progenitor cells might lead to the discovery of novel strategies to attenuate tumor growth.

The type II neuroblast lineage in the fly larval brain provides an excellent genetic model in which to investigate the mechanisms that restrict the developmental potential of intermediate progenitor cells *in vivo* (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Weng et al., 2010; Xiao et al., 2012; Komori et al., 2014). A type II neuroblast can be unambiguously identified by the expression of Deadpan (Dpn<sup>+</sup>) and lack of Asense (Ase<sup>-</sup>), and divides asymmetrically to self-renew and to generate a newly born immature intermediate neural progenitor (INP) (Fig. 1A). Although the expression of self-renewal factors is maintained in the type II neuroblast, their expression becomes rapidly extinguished in the newly born immature INP (Xiao et al., 2012). This newly born INP undergoes a stereotypical maturation process during which its developmental potential becomes stably restricted and the expression of Ase is activated. Upon completing maturation, an INP divides only five or six times to generate exclusively differentiated progeny despite reactivating the expression of all known neuroblast self-renewal factors. Thus, it is likely that the restriction of developmental potential during the maturation of an immature INP results in attenuated competence to respond to the neuroblast self-renewal factors in an INP, but the mechanisms are not understood.

The neuroblast self-renewal factors include Dpn, Klumpfuss (Klu), Enhancer of split my [E(spl)my] and Notch (Weng et al., 2010; San-Juán and Baonza, 2011; Xiao et al., 2012; Zacharioudaki et al., 2012; Zhu et al., 2012). Removal of *Notch* function alone or *dpm* and *E(spl)my* function simultaneously leads to premature neuroblast differentiation, whereas overexpression of any of the neuroblast self-renewal factors in type II neuroblasts leads to massive formation of supernumerary neuroblasts. Unexpectedly, whereas overexpression of *klu* in Ase<sup>-</sup> immature INPs driven by the *Erm-Gal4(II)* driver induces a robust supernumerary neuroblast phenotype, overexpression of *klu* in Ase<sup>+</sup> immature INPs driven by the *Erm-Gal4(III)* failed to induce supernumerary neuroblast



**Fig. 1. Erm functions in immature INPs to suppress supernumerary type II neuroblast formation.** (A) A summary of the *brat* mutant phenotype and the expression patterns of the *Gal4* drivers used in this study. Neurob, neuroblast; imm INP, immature INP; GMC, ganglion mother cell. (B-D) The heterozygosity of *erm* enhances the supernumerary neuroblast phenotype in *brat*<sup>DG19310/11</sup> brains (as seen in B,C). Phalloidin (Phall) marks the cell cortex. Scale bar: 40  $\mu$ m. (D) Quantification of the average number of type II neuroblasts (Dpn<sup>+</sup>Ase<sup>-</sup>) per brain lobe in larvae of the indicated genotypes. Error bars indicate s.d. (E) Reduction in *erm* function increases the frequency of formation of supernumerary neuroblasts originating from the Ase<sup>-</sup> immature INPs or INPs. Lineage clones marked with  $\beta$ -gal were induced and analyzed following the scheme shown in supplementary material Fig. S2. INP clone: a clone derived from a single INP; Ase<sup>+</sup> imm INP clone: a clone derived from a single Ase<sup>+</sup> immature INP; reverted clone: a clone containing supernumerary neuroblasts. The bar graphs show the frequency of clones observed in larval brains of the indicated genotype, and the total number of clones used to determine the frequency of the clones is shown in the bar graph for the INP clone. (F-I) Restoring *erm* function in the Ase<sup>-</sup> immature INPs or Ase<sup>+</sup> immature INPs rescues the enhancement of the supernumerary neuroblast phenotype in *brat*<sup>DG19310/11</sup> brains induced by the heterozygosity of *erm*. High magnification images of the boxed areas in the low magnification image are shown below. Scale bars: 40  $\mu$ m (low magnification images); 10  $\mu$ m (high magnification images). (I) The quantification of the average number of type II neuroblasts per brain lobe of the indicated genotypes. Key for this and all subsequent figures: dotted yellow line separates the brain from the optic lobe (OL); white arrow, type II neuroblast; white arrowhead, newly born immature INP and Ase<sup>-</sup> immature INP; yellow arrow, Ase<sup>+</sup> immature INP; yellow arrowhead, INP. \**P*<0.05 between the marked genotype and the control genotype in the same bar graph, as determined by the Student's *t*-test. n.s., not statistically significant.

formation (Xiao et al., 2012). The expression level of *Erm-Gal4(III)* is ~50% of *Erm-Gal4(II)* (D.H.J. and C.-Y.L., unpublished observation). However, overexpression of two copies of the *UAS-klu* transgenes driven by two copies of the *Erm-Gal4(III)* driver was not sufficient to induce a supernumerary neuroblast phenotype remotely comparable to overexpression of one copy of the *UAS-klu* transgene driven by one copy of the *Erm-Gal4(II)* driver (Xiao et al., 2012). Although we cannot quantitatively control the exact expression level of the *UAS-klu* transgenes driven by *Erm-Gal4(II)* versus *Erm-Gal4(III)* in these experiments, these results suggest that *Ase*<sup>+</sup> immature INPs are significantly less responsive to the expression of neuroblast self-renewal factors than *Ase*<sup>-</sup> immature INPs. Understanding the mechanisms that alter the responsiveness to neuroblast self-renewal factors in *Ase*<sup>+</sup> immature INPs will provide important insight into the restriction of developmental potential.

The transcription factor Erm (also known as dFefz) functionally distinguishes an INP from a neuroblast (Weng et al., 2010). *erm* encodes an evolutionarily conserved C<sub>2</sub>H<sub>2</sub> zinc-finger transcription factor, and the vertebrate orthologs of Erm can activate or repress gene expression in a context-dependent manner (Hirata et al., 2006; Weng et al., 2010; Yang et al., 2012). Erm is dispensable for the formation of INPs, but INPs in *erm*-null brains spontaneously revert into supernumerary type II neuroblasts. Importantly, restoring *erm* function by overexpressing *erm* or the vertebrate ortholog of *erm* (*fez* or *fezl*) rescued the supernumerary neuroblast phenotype, strongly suggesting that the function of Erm is evolutionarily conserved. Erm suppresses the reversion of INPs by antagonizing Notch signaling (Weng et al., 2010). However, the mechanisms by which Erm restricts the functional output of Notch signaling in INPs are completely unknown. In addition, understanding whether Erm exerts a similar regulatory effect on other neuroblast self-renewal factors will provide important insight into the mechanisms that functionally distinguish an INP from a neuroblast.

In this study, we show that Erm functions as a transcriptional repressor to stably restrict the developmental potential in immature INPs. Erm functions temporally after Brat and Numb in immature INPs to suppress the formation of supernumerary neuroblasts, and endogenous Erm protein is exclusively expressed in immature INPs. Erm-dependent restriction of the developmental potential in immature INPs leads to attenuated competence to respond to all known neuroblast self-renewal factors in INPs. Thus, the Erm-dependent mechanism stably and globally restricts the genomic response to neuroblast self-renewal factors. We identified that the BAP chromatin-remodeling complex also functions temporally after Brat and Numb to restrict the developmental potential of immature INPs. Importantly, overexpression of a dominant-negative form of Brm strongly enhanced the supernumerary neuroblast phenotype in *erm* hypomorphic brains. Thus, we propose that Erm and the BAP complex function cooperatively to stably restrict the developmental potential of immature INPs and to functionally distinguish an INP from a neuroblast.

## RESULTS

### Erm functions in immature INPs to suppress the formation of supernumerary type II neuroblasts

We hypothesized that restriction of the developmental potential in immature INPs alters the responsiveness to neuroblast self-renewal factors, preventing INPs from aberrantly reverting into supernumerary neuroblasts in response to the re-activation of neuroblast self-renewal factors. We used the *brat*<sup>DG19310/11</sup> hypomorphic genetic background to investigate the mechanisms that

restrict the developmental potential of immature INPs (Xiao et al., 2012; Komori et al., 2014). Briefly, Brat prevents the formation of supernumerary neuroblasts by acting at two temporally distinct stages during maturation (Fig. 1A). First, Brat prevents a newly born immature INP, which lacks the expression of both *Erm-Gal4(II)* and *Ase* (Fig. 1A; supplementary material Fig. S1D), from reverting into a supernumerary neuroblast by rapidly extinguishing the function of neuroblast self-renewal factors. Newly born immature INPs mutant for *brat* rapidly revert into supernumerary neuroblasts instead of progressing through maturation, and removing the function of the neuroblast self-renewal gene *klu* or *dpn* suppressed the supernumerary neuroblast phenotype (supplementary material Fig. S1A-C) (Xiao et al., 2012). Second, Brat continues to play an important role in the *Ase*<sup>-</sup> immature INP, which shows the expression of *Erm-Gal4(II)* (Fig. 1A), to promote the maturation of immature INPs. We confirmed that the temporal expression pattern of *Erm-Gal4(II)* and *Erm-Gal4(III)* is not altered in *brat*<sup>DG19310/11</sup> brains (supplementary material Fig. S1D-G). Restoring *brat* function in *Ase*<sup>-</sup> immature INPs suppressed the supernumerary neuroblast phenotype in *brat*<sup>DG19310/11</sup> brains, whereas restoring *brat* function in *Ase*<sup>+</sup> immature INPs had no effects (supplementary material Fig. S1H-L). Thus, Brat functions in the newly born immature INP and the *Ase*<sup>-</sup> immature INP to prevent supernumerary neuroblast formation.

The unstable nature of *Ase*<sup>-</sup> immature INPs provides an excellent *in vivo* system to elucidate the mechanisms that restrict developmental potential during the maturation of immature INPs. We identified the *erm* gene as a genetic enhancer of *brat* by screening for haploinsufficient loci that further exacerbate the supernumerary neuroblast phenotype in *brat*<sup>DG19310/11</sup> brains. Although the heterozygosity of *erm* alone did not lead to a supernumerary neuroblast phenotype, it doubled the number of supernumerary neuroblasts in *brat*<sup>DG19310/11</sup> brains (Fig. 1B,C). To examine whether the enhancement of the supernumerary neuroblast phenotype in *brat*<sup>DG19310/11</sup> brains by the heterozygosity of *erm* originated from *Ase*<sup>-</sup> or *Ase*<sup>+</sup> immature INPs, we induced  $\beta$ -gal-marked lineage clones derived from either a single *Ase*<sup>+</sup> immature INP or an INP by FRT-mediated recombination (supplementary material Fig. S2A). Briefly, first instar larvae carrying a *UAS-flipase* transgene and a flip-out reporter transgene under the control of *Erm-Gal4(III)* and *Tub-Gal80<sup>ts</sup>* were heat-shocked at 30°C for 0–12 hours (supplementary material Fig. S2B–D). We determined that in the absence of heat shocking, the leaky basal level of *Erm-Gal4(III)* expression was sufficient to induce an average of three clones per lobe in the control brain (supplementary material Fig. S2E). The low frequency of clone induction under this condition is ideal for analyzing the identity of cells in an individual clone. We only recovered *Ase*<sup>+</sup> immature INP clones, which contain one INP per clone, GMCs and differentiated cells, and INP clones, which contain only differentiated cells, in control *brat*<sup>DG19310/+</sup> or *erm*<sup>1/+</sup> brains (Fig. 1E). This is consistent with a wild-type INP maintaining restricted developmental potential. In *brat*<sup>DG19310/11</sup> brains, more than 99% of the clones were either *Ase*<sup>+</sup> immature INP clones or INP clones, and only 0.7% of the clones contained supernumerary neuroblasts (the reverted clone) (Fig. 1E). This result is consistent with Brat mainly functioning in the newly born immature INP and the *Ase*<sup>-</sup> immature INP to prevent the formation of supernumerary neuroblasts. Importantly, 3.5% of the clones in *brat*<sup>DG19310/11</sup> brains heterozygous for *erm* were the reverted clones, and these clones consistently possessed more supernumerary neuroblasts than the reverted clones in *brat*<sup>DG19310/11</sup> brains (Fig. 1E; supplementary



material Fig. S2F). Thus, heterozygosity of *erm* increases the frequency of *Ase*<sup>+</sup> immature INPs or INPs reverting to neuroblasts in *brat*<sup>DG19310/11</sup> brains. The occurrence of the reverted clones increased dramatically in *erm*-null brains, consistent with *erm* playing a crucial role suppressing supernumerary neuroblast formation (Fig. 1E). These data strongly suggest that *erm* functions temporally after *brat* in immature INPs to prevent supernumerary neuroblast formation.

We tested whether *erm* indeed functions temporally after *brat* in immature INPs by restoring *erm* function in either *Ase*<sup>-</sup> or *Ase*<sup>+</sup> immature INPs in *brat*<sup>DG19310/11</sup> brains heterozygous for *erm*. Consistent with our hypothesis, restoring *erm* function in either *Ase*<sup>-</sup> or *Ase*<sup>+</sup> immature INPs rescued the enhancement of the supernumerary neuroblast phenotype in *brat*<sup>DG19310/11</sup> brains induced by the heterozygosity of *erm* (Fig. 1F-I). Thus, we conclude that *erm* functions temporally after *brat* in immature INPs to suppress the formation of supernumerary neuroblasts.

### Erm functions temporally after Brat and Numb in immature INPs

We assessed the expression pattern of endogenous Erm to confirm that *erm* indeed functions in *Ase*<sup>-</sup> and *Ase*<sup>+</sup> immature INPs. We generated a transgenic fly line carrying a BAC clone containing the entire *erm* genomic locus fused in frame to a RFP epitope (*erm-rfp*). In the type II neuroblast lineage, the expression of *erm*-RFP was detectable in immature INPs located immediately adjacent to the type II neuroblast but became rapidly downregulated in INPs (Fig. 2A). The relative position of these Erm-expressing immature INPs to the type II neuroblast strongly suggests that endogenous Erm is expressed in *Ase*<sup>-</sup> immature INPs. To unambiguously verify the identity of cells in which endogenous Erm is expressed, we generated a specific antibody against the Erm protein. Co-immunolocalization using specific antibodies against *Ase* and Erm confirmed that endogenous Erm was absent from the newly born immature INP but became detectable in both *Ase*<sup>-</sup> and *Ase*<sup>+</sup>

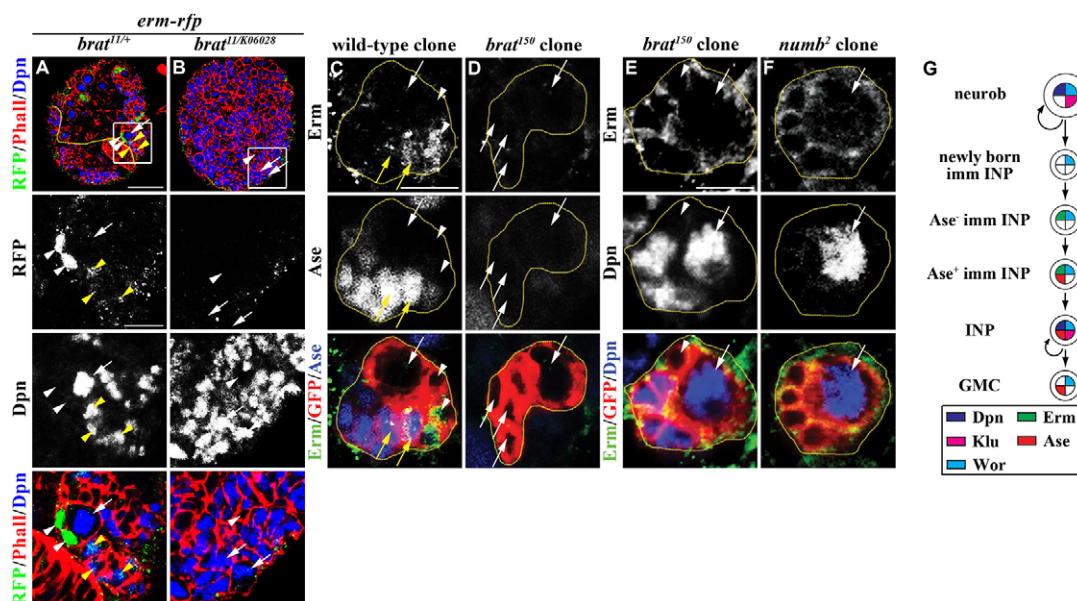
immature INPs (Fig. 2C). These data are consistent with *erm* functioning in *Ase*<sup>-</sup> and *Ase*<sup>+</sup> immature INPs to restrict developmental potential.

We next examined the expression pattern of endogenous Erm in *brat*-null brains to confirm that *erm* indeed functions temporally after *brat* in immature INPs. Indeed, the expression of *erm*-RFP was completely absent from the *brat*-null brain, and endogenous Erm was undetectable in the newly born immature INP in *brat*-null type II neuroblast clones (Fig. 2B,D,E). Thus, these results confirm that Erm functions temporally after Brat in immature INPs.

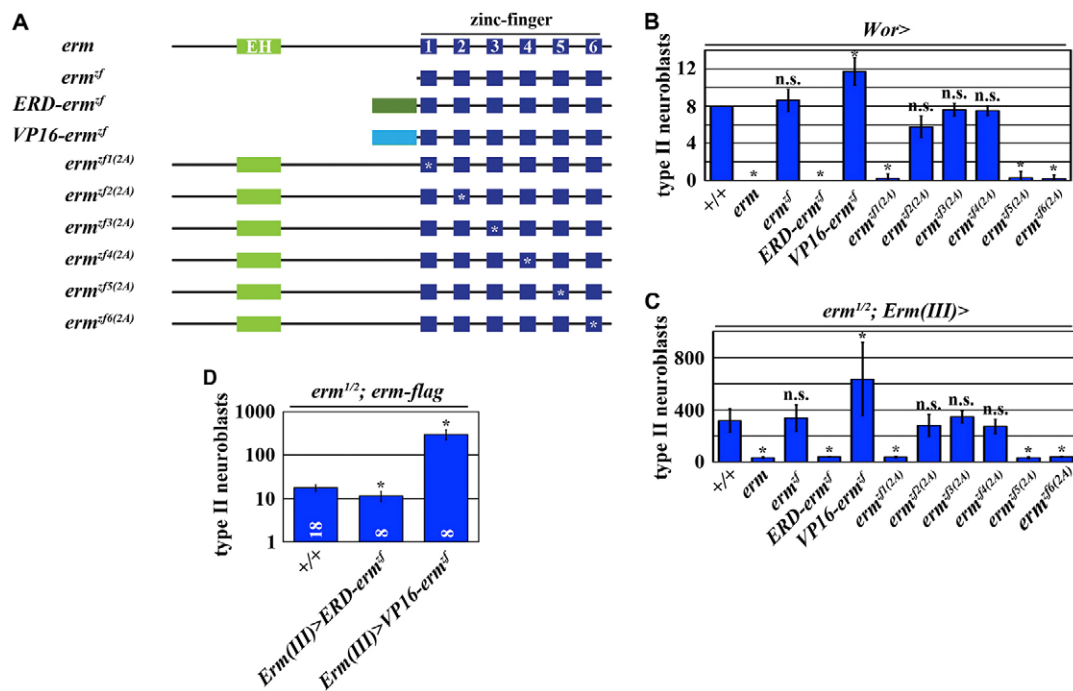
Numb functions in parallel to Brat to prevent the formation of supernumerary neuroblasts (Xiao et al., 2012). A *numb*-null type II neuroblast clone contained many supernumerary neuroblasts and showed an aberrant accumulation of immature INPs lacking *Ase* expression. Importantly, we never detected the expression of Erm in immature INPs lacking *Ase* expression in *numb*-null clones, indicating that these cells are newly born immature INPs (Fig. 2F). Thus, a *numb*-null clone aberrantly accumulates newly born immature INPs. Taken together, these data indicate that Erm functions temporally after Brat and Numb to restrict the developmental potential of immature INPs.

### Erm restricts the developmental potential of immature INPs by repressing gene transcription

Because the vertebrate orthologs of Erm can activate or repress gene expression in a context-dependent manner (Hirata et al., 2006; Yang et al., 2012), we investigated whether Erm restricts developmental potential by activating or repressing gene expression. Although mis-expression of wild-type Erm in neuroblasts induced premature differentiation of all eight type II neuroblasts in a larval brain lobe, mis-expression of *Erm*<sup>zf</sup> (containing only the zinc-fingers) had no effect (Fig. 3A,B). Thus, the N-terminus of the Erm protein is essential for its function in restricting developmental potential. We fused the Engrailed repressor domain (ERD) to *Erm*<sup>zf</sup>, converting it to act solely as a transcriptional repressor (ERD-



**Fig. 2. Erm is exclusively expressed in *Ase*<sup>-</sup> and *Ase*<sup>+</sup> immature INPs.** (A,B) The expression of *erm-rfp* is detected in immature INPs in wild-type brains but undetectable in *brat*-null brains. High magnification images of the boxed area are shown below. Scale bars: 40  $\mu$ m (low magnification images); 10  $\mu$ m (high magnification images). (C,D) Endogenous Erm is undetectable in the newly born immature INP. The GFP-marked lineage clones are outlined in yellow. Scale bar: 10  $\mu$ m. (E,F) Erm is undetectable in *brat*- or *numb*-null mutant clones. The GFP-marked lineage clones are outlined in yellow. Scale bar: 10  $\mu$ m. (G) A cartoon summarizing the expression pattern of Erm in the type II neuroblast lineage.



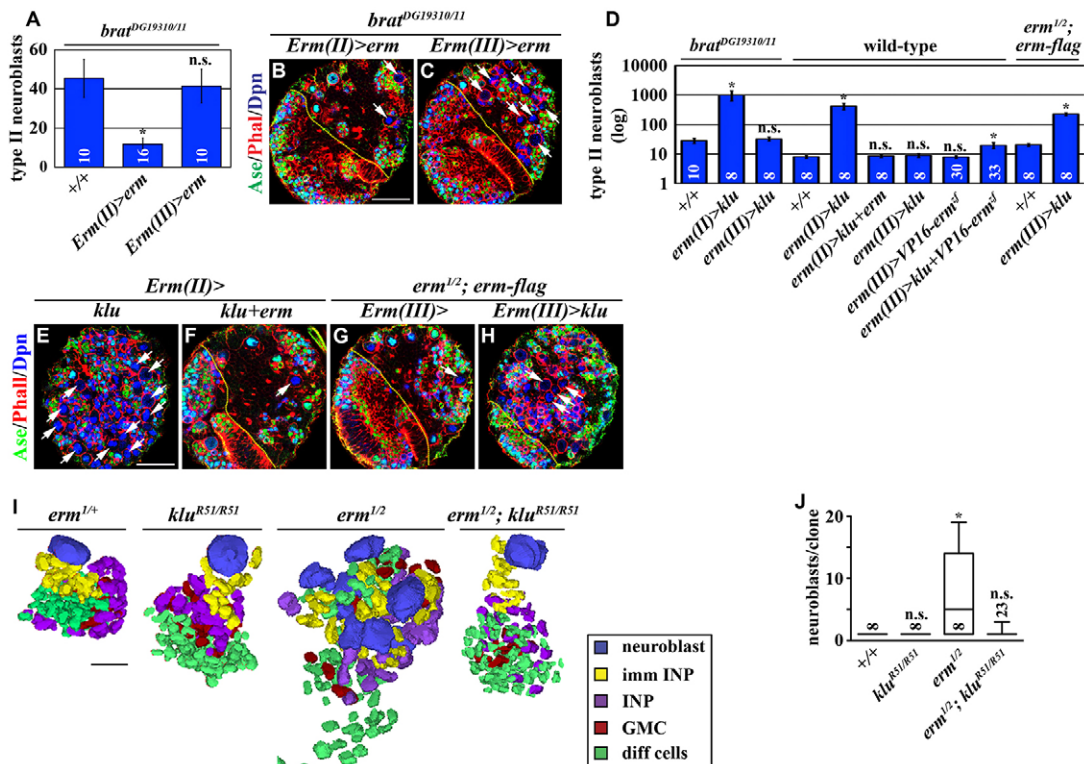
**Fig. 3. Erm restricts the developmental potential in immature INPs by repressing gene transcription.** (A) Schematics of the *UAS-erm* transgenes used in this series of experiments. ERD, engrailed repressor domain; VP16, transactivation domain. (B) Overexpression of *erm* induces premature neuroblast differentiation by repressing gene transcription. Third instar larval brains of the indicated genotype were stained for Dpn, Ase and Phalloidin, and the total number of type II neuroblasts (Dpn<sup>+</sup>Ase<sup>+</sup>) per brain lobe was quantified. (C) Restoring *erm* expression rescues the supernumerary neuroblast phenotype in *erm*-null brains by repressing gene transcription. Third instar larval brains of the indicated genotypes were treated and analyzed as in B. (D) Overexpression of VP16-*erm<sup>zf</sup>* exerts a dominant-negative effect and further exacerbates the supernumerary neuroblast phenotype in *erm* hypomorphic brains. Third instar larval brains of the indicated genotypes were treated and analyzed as in B.

*Erm<sup>zf</sup>*), or the VP16 transactivation domain to *Erm<sup>zf</sup>*, converting it to act solely as a transcriptional activator (VP16-*Erm<sup>zf</sup>*) (Fig. 3A). Mis-expression of ERD-*Erm<sup>zf</sup>* in neuroblasts also led to premature differentiation of type II neuroblasts whereas mis-expression of VP16-*Erm<sup>zf</sup>* resulted in a mild increase in type II neuroblasts (Fig. 3B). These data strongly suggest that Erm restricts developmental potential by acting as a transcriptional repressor. We tested this hypothesis by taking two complementary approaches. First, we tested whether overexpression of the *erm* transgene can rescue the supernumerary neuroblast phenotype in *erm*-null brains. Restoring wild-type Erm function driven by the *Erm-Gal4(III)* driver rescued the supernumerary neuroblast phenotype in *erm*-null brains (Fig. 3C) (Weng et al., 2010). Importantly, overexpression of ERD-*Erm<sup>zf</sup>* under the identical conditions also rescued the supernumerary neuroblast phenotype in *erm*-null brains, but overexpression of VP16-*Erm<sup>zf</sup>* enhanced the supernumerary neuroblast phenotype (Fig. 3C). Second, we tested whether overexpression of the *erm* transgene can rescue the supernumerary neuroblast phenotype in an *erm* hypomorphic genetic background (*erm<sup>1/2</sup>; erm-flag*) (Fig. 3D). We confirmed that the temporal expression pattern of *Erm-Gal4(III)* is not altered in *erm* hypomorphic brains (supplementary material Fig. S3). Consistently, overexpression of ERD-*Erm<sup>zf</sup>* driven by the *Erm-Gal4(III)* driver rescued the supernumerary neuroblast phenotype in *erm* hypomorphic brains, but overexpression of VP16-*Erm<sup>zf</sup>* enhanced the supernumerary neuroblast phenotype (Fig. 3D). Together, these data led us to conclude that Erm restricts the developmental potential in immature INPs by acting as a transcriptional repressor, and that VP16-*Erm<sup>zf</sup>* can exert a dominant-negative effect on the restriction of developmental potential.

We next examined which C<sub>2</sub>H<sub>2</sub> zinc-finger elicits the function of Erm in restricting developmental potential. We generated *UAS-erm<sup>zf(2A)</sup>* transgenes that encode Erm transgenic proteins containing substitutions of alanine for cysteine in individual zinc-fingers (Fig. 3A). Mis-expression of *erm<sup>zf2(2A)</sup>*, *erm<sup>zf3(2A)</sup>* or *erm<sup>zf4(2A)</sup>* failed to induce premature differentiation of type II neuroblasts in wild-type brains and failed to rescue the supernumerary neuroblast phenotype in *erm*-null brains (Fig. 3B,C). By contrast, mis-expression of *erm<sup>zf1(2A)</sup>*, *erm<sup>zf5(2A)</sup>* or *erm<sup>zf6(2A)</sup>* induced premature differentiation of type II neuroblasts and rescued the supernumerary neuroblast phenotype in *erm* null brains (Fig. 3B,C). Thus, the zinc-finger 2-4 are essential to confer Erm function. Together, we conclude that Erm restricts the developmental potential in immature INPs by repressing gene transcription through the zinc-finger 2-4.

#### Erm-dependent restriction of the developmental potential in immature INPs leads to attenuated competence to respond to Klu in INPs

To begin investigating the mechanisms by which Erm restricts the developmental potential of immature INPs, we examined whether overexpression of *erm* can substitute for the function of *brat* and suppress the supernumerary neuroblast phenotype in *brat<sup>DG19310/11</sup>* brains. Indeed, overexpression of *erm* in Ase<sup>+</sup> immature INPs efficiently suppressed the supernumerary neuroblast phenotype in *brat<sup>DG19310/11</sup>* brains, but overexpression of *erm* in Ase<sup>+</sup> immature INPs could not (Fig. 4A-C). These results strongly suggest that *brat* and *erm* suppress supernumerary neuroblast formation by regulating similar downstream mechanisms. *Brat* suppresses supernumerary neuroblast formation by antagonizing Klu (Xiao et al., 2012). Similar to overexpression of *klu* in wild-type brains, mis-expression



**Fig. 4. Erm-dependent restriction of developmental potential in immature INPs leads to attenuated competence to respond to Klu in INPs.**

(A-C) Overexpression of *erm* in Ase<sup>-</sup> immature INPs can suppress the supernumerary neuroblast phenotype in *brat* hypomorphic brains as seen in B and C. Scale bar: 40  $\mu$ m. (A) Quantification of total type II neuroblasts (Dpn<sup>+</sup>Ase<sup>-</sup>) per brain lobe of the indicated genotypes. (D-H) Co-expression of *erm* can suppress the supernumerary neuroblast phenotype induced by mis-expression of *klu*, as seen in E-H. Scale bar: 40  $\mu$ m. (D) The quantification of total type II neuroblasts (Dpn<sup>+</sup>Ase<sup>-</sup>) per brain lobe of the indicated genotypes. (I, J) Removing *klu* function suppresses supernumerary neuroblast formation in *erm*-null brains. (I) Three-dimensional reconstructed images of clones of the genotype indicated. Third instar larval brains carrying GFP-marked mosaic clones derived from single neuroblasts of the genotype indicated were stained for GFP, Dpn, Ase, Pros and Elav. Scale bar: 10  $\mu$ m. (J) Quantification of total type II neuroblasts (Dpn<sup>+</sup>Ase<sup>-</sup>) per clone for the indicated genotypes.

of *klu* in Ase<sup>-</sup> immature INPs in *brat*<sup>DG19310/11</sup> brains led to supernumerary neuroblast formation, but mis-expression of *klu* in Ase<sup>+</sup> immature INPs had no effect (Fig. 4D). The inefficiency in inducing supernumerary neuroblasts by mis-expression of *klu* in Ase<sup>+</sup> immature INPs correlates with Erm mainly functioning in Ase<sup>+</sup> immature INPs to restrict developmental potential. Thus, we hypothesized that Erm restricts developmental potential by antagonizing Klu function. Consistently, co-expression of *erm* completely suppressed supernumerary neuroblast formation induced by mis-expression of *klu* in Ase<sup>-</sup> immature INPs in wild-type brains (Fig. 4D-F). Furthermore, although mis-expression of *VP16-erm<sup>f</sup>* or *klu* alone in Ase<sup>+</sup> immature INPs did not have any effect, co-expression of *VP16-erm<sup>f</sup>* and *klu* induced supernumerary neuroblast formation (Fig. 4D). This result indicates that the downstream mechanisms regulated by Erm can act cooperatively with Klu to induce supernumerary neuroblast formation. Finally, mis-expression of *klu* in Ase<sup>+</sup> immature INPs also enhanced supernumerary neuroblast formation in *erm* hypomorphic brains (Fig. 4D, G, H). Together, these data strongly suggest that Erm restricts developmental potential by antagonizing Klu function.

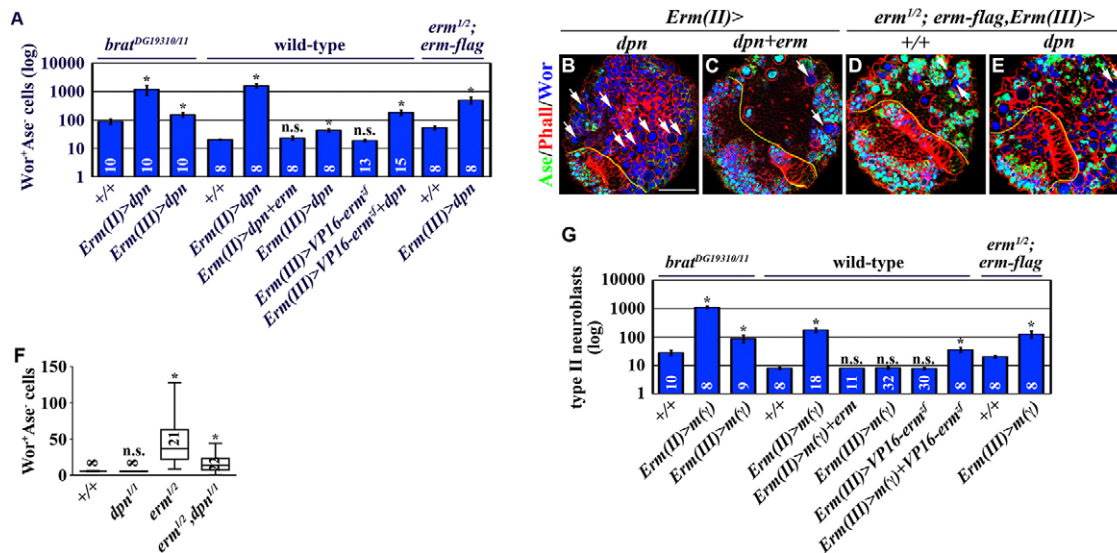
We directly tested whether removing *klu* function can suppress supernumerary neuroblast formation in *erm*-null brains. In *erm*-null brains, GFP-marked mosaic clones derived from single type II neuroblasts contained multiple neuroblasts per clone (Fig. 4I, J). Clonally removing *klu* function for 72 hours was not sufficient to induce premature differentiation of type II neuroblasts (Berger et al.,

2012; Xiao et al., 2012). By contrast, clonally removing *klu* function strongly suppressed supernumerary neuroblasts in *erm*-null brains (Fig. 4I, J). Thus, *klu* is required for supernumerary neuroblast formation in *erm*-null brains. Because Klu expression is extinguished in immature INPs but becomes re-activated in INPs (Xiao et al., 2012), INPs are most likely the cells of origin of supernumerary neuroblasts in *erm*-null brains. Therefore, Erm probably restricts developmental potential by indirectly antagonizing Klu function. We conclude that Erm-dependent restriction of the developmental potential in immature INPs leads to attenuated competence to respond to Klu in INPs.

#### Erm-dependent restriction of the developmental potential in immature INPs leads to attenuated competence to respond to Dpn and E(spl)<sup>my</sup>

Similar to *klu*, *dpn* is also required for supernumerary neuroblast formation in *brat*-null brains (supplementary material Fig. S1A-C). In addition, mis-expression of *dpn* in Ase<sup>+</sup> immature INPs also led to a significantly milder supernumerary neuroblast phenotype in wild-type as well as in *brat*<sup>DG19310/11</sup> brains compared with mis-expression in Ase<sup>-</sup> immature INPs (Fig. 5A). These results prompted us to test whether Erm restricts developmental potential by antagonizing Dpn function. Consistent with our hypothesis, co-expression of *erm* completely suppressed supernumerary formation induced by mis-expression of *dpn* in Ase<sup>-</sup> immature INPs in wild-type brains (Fig. 5A-C). Furthermore, co-expression of *VP16-erm<sup>f</sup>*





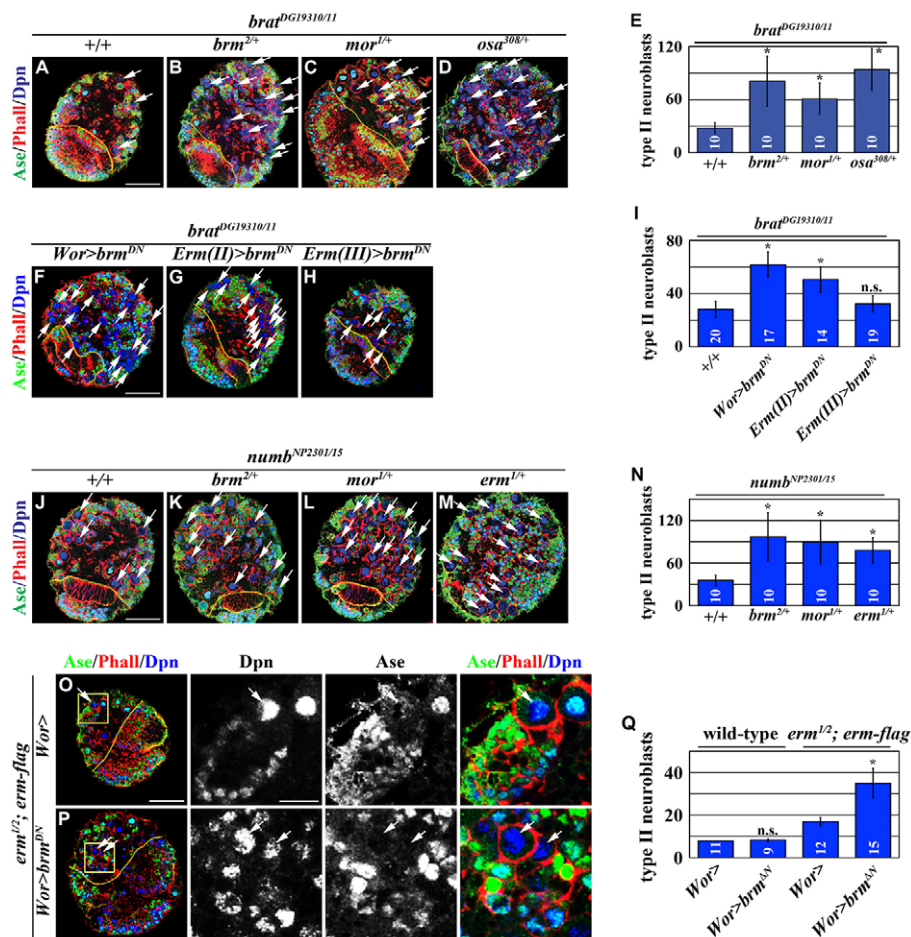
**Fig. 5. Erm-dependent restriction of developmental potential in immature INPs leads to attenuated competence to respond to Dpn and E(spl)my in INPs.** (A-E) Co-expression of *erm* can suppress the supernumerary neuroblast phenotype induced by mis-expression of *dpn*, as seen in B-E. Scale bar: 40  $\mu$ m. (A) Quantification of total Wor<sup>+</sup>Asc<sup>-</sup> cells (including type II neuroblasts and Ase<sup>-</sup> immature INPs) per brain lobe of the indicated genotypes. (F) Removing *dpn* function suppresses supernumerary neuroblast formation in *erm*-null brains. Quantification of total Wor<sup>+</sup>Asc<sup>-</sup> cells (including type II neuroblasts and Ase<sup>-</sup> immature INPs) per clone for the indicated genotypes. (F) Third instar larval brains carrying GFP-marked mosaic clones derived from single neuroblasts of the indicated genotypes were stained for GFP, Wor, Ase, Pros and Elav. (G) Co-expression of *erm* can suppress the supernumerary neuroblast phenotype induced by mis-expression of *E(spl)my*. Quantification of total type II neuroblasts (Dpn<sup>+</sup>Asc<sup>-</sup>) per clone for the indicated genotypes.

and *dpn* in Ase<sup>+</sup> immature INPs led to a significant increase in supernumerary neuroblasts compared with mis-expression of *dpn* alone under the identical conditions (Fig. 5A). Finally, mis-expression of *dpn* in Ase<sup>+</sup> immature INPs enhanced supernumerary neuroblast formation in *erm* hypomorphic brains (Fig. 5A,D,E). Together, these data strongly suggest that Erm restricts developmental potential by antagonizing Dpn function. Importantly, although not affecting the maintenance of type II neuroblasts (Zacharioudaki et al., 2012; Zhu et al., 2012) (Fig. 5F), clonally removing the function of *dpn* strongly suppressed the supernumerary neuroblast phenotype in *erm*-null brains (Fig. 5F). Because Dpn expression is extinguished in immature INPs but becomes re-activated in INPs (Xiao et al., 2012), INPs are most likely the cells of origin for supernumerary neuroblasts in *erm*-null brains. Thus, Erm-dependent restriction of the developmental potential in immature INPs leads to attenuated competence to respond to Dpn in INPs.

A recent study showed that E(spl)my also functions as a neuroblast self-renewal factor (Zacharioudaki et al., 2012). Similar to *klu* and *dpn*, co-expression of *erm* strongly suppressed supernumerary formation induced by mis-expression of *E(spl)my* in Ase<sup>-</sup> immature INPs in wild-type brains (Fig. 5G). In addition, co-expression of *VP16-erm<sup>Δ</sup>* and *E(spl)my* in Ase<sup>+</sup> immature INPs induced supernumerary neuroblasts, and mis-expression of *E(spl)my* in Ase<sup>+</sup> immature INPs enhanced supernumerary neuroblast formation in *erm* hypomorphic brains (Fig. 5G). Because E(spl)my expression is extinguished in immature INPs but becomes re-activated in INPs (L. Anhezini and C.-Y.L., unpublished observation), E(spl)my also probably contributes to the reversion of INPs into supernumerary neuroblasts in *erm*-null brains. These results show that Erm-dependent restriction of the developmental potential in immature INPs leads to attenuated competence to respond to all known neuroblast self-renewal factors in INPs.

### The BAP complex suppresses supernumerary neuroblast formation by restricting the developmental potential of immature INPs

To elucidate the mechanisms by which Erm restricts the developmental potential of immature INPs, we characterized additional haploinsufficient loci that enhanced the supernumerary neuroblast phenotype in *brat<sup>DG19310/11</sup>* brains. We identified that the *brm*, *mor* and *osa* genes act as genetic enhancers of *brat*. Specifically, although the heterozygosity of *brm*, *mor* or *osa* did not have effects on the type II neuroblast lineage in wild-type brains, the heterozygosity of any of these three genes enhanced the supernumerary neuroblast phenotype in *brat<sup>DG19310/11</sup>* brains (Fig. 6A-E). Because *brm*, *mor* and *osa* encode the core components of the BAP chromatin-remodeling complex (Mohrman et al., 2004; Carrera et al., 2008), we hypothesize that the BAP complex functions in immature INPs to suppress supernumerary neuroblast formation. Consistently, overexpression of a *UAS-brm<sup>DN</sup>* transgene, which encodes a dominant-negative form of Brm, specifically in Ase<sup>-</sup> immature INPs enhanced the supernumerary neuroblast phenotype in *brat<sup>DG19310/11</sup>* brains (Fig. 6F-I). These data support our hypothesis that the BAP complex functions temporally after Brat in immature INPs to suppress supernumerary neuroblast formation. Consistent with the observations from a previous study (Neumüller et al., 2011), we confirmed that knocking down or removing the function of *brm*, *osa* or *mor* led to supernumerary neuroblast formation (data not shown). We ruled out the possibility that supernumerary neuroblasts induced by the loss of the BAP complex function arise from symmetric neuroblast division because a mitotic *osa* mutant type II neuroblast displayed normal establishment and maintenance of the apical-basal cortical polarity (data not presented). Together, these results strongly suggest that the BAP complex functions temporally after Brat in immature INPs to prevent supernumerary neuroblast formation.



**Fig. 6. The BAP complex functions cooperatively with Erm to restrict the developmental potential in immature INPs.**

(A-E) Reduced function of the BAP complex enhances the supernumerary neuroblast phenotype in *brat* hypomorphic brains, as seen in A-D. Scale bar: 40  $\mu$ m. (E) Quantification of total type II neuroblasts per lobe for the indicated genotypes. (F-I) Reducing *brm* function in *Ase*<sup>-</sup> immature INPs enhances the supernumerary neuroblast phenotype in *brat* hypomorphic brains (F-H). Scale bar: 40  $\mu$ m. (I) The quantification of total type II neuroblasts per lobe for the indicated genotypes. (J-N) Reducing the function of the BAP complex or *erm* enhances the supernumerary neuroblast phenotype in *numb* hypomorphic brains (J-M). Scale bar: 40  $\mu$ m. (N) Quantification of total type II neuroblasts per lobe for the indicated genotypes. (O-Q) Reducing *brm* function enhances the supernumerary neuroblast phenotype in *erm* hypomorphic brains (O,P). High magnification images of the boxed areas are shown on the right. Scale bars: 40  $\mu$ m in the low magnification images; 10  $\mu$ m in the high magnification images. (Q) Quantification of total type II neuroblasts per lobe for the indicated genotypes.

We next tested whether the BAP complex also functions temporally after Numb to suppress supernumerary neuroblast formation. Similar to *Brat*, *Numb* also functions to prevent the newly born immature INP from aberrantly reverting into a supernumerary neuroblast (Fig. 2F). However, the supernumerary neuroblast phenotype displayed by a *numb*-null type II neuroblast clone is too severe to test gene function in immature INPs (Xiao et al., 2012). By contrast, a *numb*<sup>NP2301/15</sup> hypomorphic brain lobe, which contained 35.7 $\pm$ 7.5 type II neuroblasts and many INPs, provides a sensitized genetic background for testing gene function in immature INPs (Fig. 6J,N). Consistent with our hypothesis, the heterozygosity of *brm* or *mor* enhanced the supernumerary neuroblast phenotype in *numb*<sup>NP2301/15</sup> brains (Fig. 6K,L). These results strongly suggest that the BAP complex also functions temporally after Numb in immature INPs to suppress supernumerary neuroblast formation.

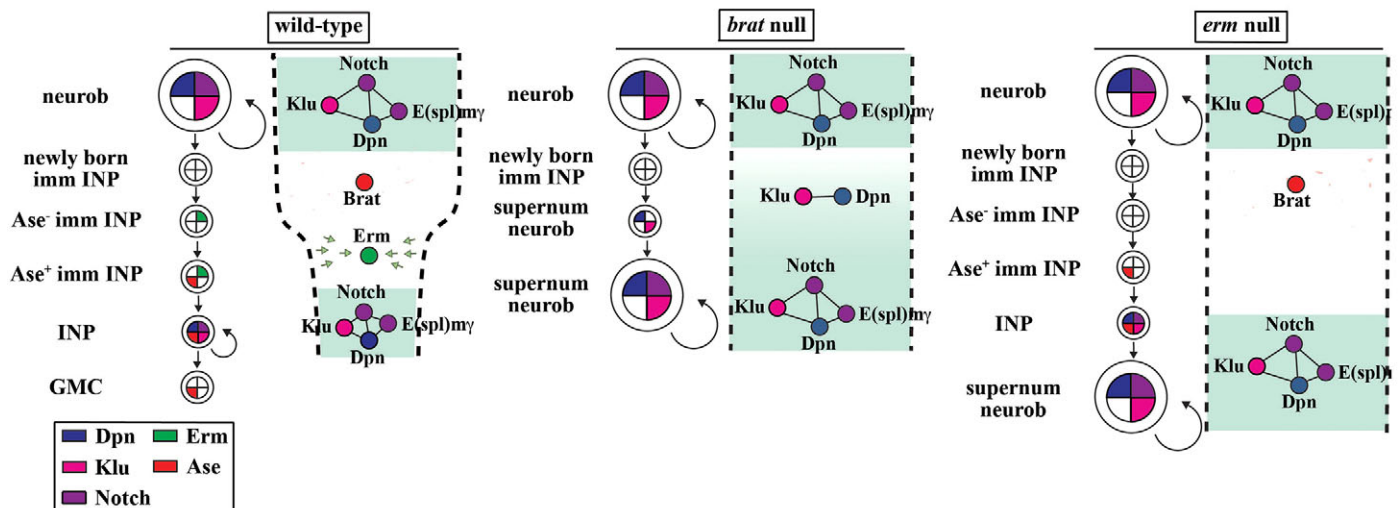
Both *Erm* and the BAP complex function temporally after *Brat* and *Numb* in immature INPs to suppress supernumerary neuroblast formation. Thus, we tested whether *Erm* and the BAP complex might function cooperatively to restrict the developmental potential of immature INPs. Similar to the BAP complex, the heterozygosity of *erm* also enhanced the supernumerary neuroblast phenotype in *numb*<sup>NP2301/15</sup> brains (Fig. 6M,N). Most importantly, although overexpression of the *UAS-brm*<sup>DN</sup> transgene alone did not have any effect on the type II neuroblast lineage, overexpression of *brm*<sup>DN</sup> significantly increased the formation of supernumerary neuroblasts in *erm* hypomorphic brains (Fig. 6O-Q). Taken together, these data strongly suggest that *Erm* and the BAP complex function

cooperatively to restrict the developmental potential of immature INPs.

## DISCUSSION

Understanding the molecular mechanisms that stably restrict the developmental potential of progenitor cells may lead to the identification of novel therapeutic targets to selectively target progenitor cell-derived tumor-initiating stem cells. However, restriction of developmental potential may occur while intermediate progenitor cells acquire their functional identity. Thus, well-established stem cell lineage information is essential for investigating the molecular mechanisms that restrict developmental potential. The type II neuroblast lineage in fly larval brains offers a unique model system to investigate the restriction of the developmental potential in uncommitted intermediate progenitor cells (Weng et al., 2010; Xiao et al., 2012; Komori et al., 2014). In this study, we show that stable restriction of developmental potential in immature INPs required a temporally coordinated effort of the asymmetrically inherited proteins *Brat* and *Numb* and the transcriptional repressor protein *Erm*. *Brat* and *Numb* function in the newly born immature INP where they prevent the reversion into a supernumerary neuroblast induced by the activities of self-renewal factors (Fig. 7). *Erm* functions downstream of *Brat* and *Numb* in the *Ase*<sup>-</sup> and *Ase*<sup>+</sup> immature INPs to restrict their genomic response to neuroblast self-renewal factors, leading to permanently attenuated competence to respond to these factors in INPs (Fig. 7). Furthermore, we also identified that the BAP chromatin-remodeling complex functions synergistically with *Erm* in immature INPs to





**Fig. 7. Schematic models depicting the role of Brat and Erm in the regulation of immature INPs.** In wild-type brains, Erm functions temporally after Brat to restrict the competence of immature INPs to respond to the self-renewal network (indicated by the width between dotted lines). The self-renewal network is expressed in type II neuroblasts (light cyan area), but is deactivated during maturation by Brat (white area). An Erm-dependent restriction of developmental potential (green arrows) in immature INPs, leads to an attenuated competence to respond to the re-activation of neuroblast self-renewal factors in INPs (light cyan area). In *brat* amorphic brains, the newly born immature INPs fail to undergo maturation and rapidly revert into supernumerary neuroblasts. In *erm* amorphic brains, immature INPs undergo successful maturation, but their competence to respond to neuroblast self-renewal factors is not attenuated. Thus, upon re-expression of neuroblast self-renewal factors, INPs revert to form supernumerary neuroblasts.

suppress the formation of supernumerary neuroblasts. Taken together, we propose that Erm functions cooperatively with the BAP complex to implement a stable restriction of the developmental potential in immature INPs by modifying their genome, which leads to an attenuated response to all neuroblast self-renewal factors in INPs (Fig. 7).

#### Erm-dependent restriction of the developmental potential in immature INPs leads to an attenuated competence to respond to all self-renewal transcription factors in INPs

Several pieces of evidence led us to conclude that Erm mainly restricts the developmental potential in the Ase<sup>+</sup> immature INPs. Erm is primarily detected in the Ase<sup>-</sup> and Ase<sup>+</sup> immature INPs but becomes undetectable in the INP, strongly suggesting that Erm functions in the Ase<sup>-</sup> and Ase<sup>+</sup> immature INPs (Fig. 2). Consistently, heterozygosity of *erm* further exacerbated supernumerary neuroblast formation from the Ase<sup>+</sup> immature INPs in *brat* hypomorphic brains, and restoring *erm* function in the Ase<sup>+</sup> immature INPs rescued the enhancement of the supernumerary neuroblast phenotype (Fig. 1E-I). Furthermore, restoring *erm* function in the Ase<sup>+</sup> immature INPs also rescued the supernumerary neuroblast phenotype in *erm*-null brains (Weng et al., 2010). Finally, overexpression of VP16-Erm<sup>zf</sup>, a dominant-negative form of Erm, in the Ase<sup>+</sup> immature INPs significantly increased supernumerary neuroblasts in various genetic backgrounds (Fig. 3). Although these data do not exclude the possibility that *erm* might still function in the INPs, *erm* most likely functions mainly in immature INPs to restrict developmental potential and suppress supernumerary neuroblast formation.

Erm restricts the developmental potential of immature INPs by acting as a transcriptional repressor, and removing the function of *klu* or *dpn* completely suppressed the supernumerary neuroblast phenotype in *erm*-null brains (Figs 3-5). Thus, Erm might restrict the developmental potential of immature INPs by directly repressing the transcription of the genes encoding neuroblast self-renewal factors or indirectly attenuating the competence to respond

to these factors. However, Ase<sup>+</sup> immature INPs in *erm*-null type II neuroblast clones never showed a premature onset of Dpn expression, and overexpression of *erm* in neuroblasts did not affect Dpn expression (Weng et al., 2010). In addition, transcriptome analyses indicated that *dpn* and *klu* are upregulated to a similar level in *brat*- and *erm*-null brains as in control brains (H.K. and C.-Y.L., unpublished data). Thus, it is unlikely that Erm directly regulates the transcription of neuroblast self-renewal genes. We favor the mechanism that Erm indirectly attenuates the competence to respond to these neuroblast self-renewal factors in INPs by restricting the developmental potential in immature INPs. Consistently, although overexpression of the dominant-negative VP16-Erm<sup>zf</sup> or a single neuroblast self-renewal factor alone induced a very weak supernumerary neuroblast phenotype, co-expression of VP16-Erm<sup>zf</sup> and a single neuroblast self-renewal factor led to a very robust supernumerary neuroblast phenotype (Figs 4, 5). Taken together, these data strongly suggest that stable restriction of developmental potential by the Erm-dependent mechanism in immature INPs leads to attenuated competence to respond to the re-activation of neuroblast self-renewal factors in INPs.

#### Erm restricts the developmental potential in immature INPs by repressing gene transcription

The vertebrate orthologs of Erm, Fezf1 and Fezf2, regulate cortical development either by activating or repressing gene expression (Hirata et al., 2006; Yang et al., 2012). As overexpression of either *fezf1* or *fezf2* can functionally substitute for the role of Erm in the Ase<sup>+</sup> immature INPs (Weng et al., 2010), the results from the vertebrate studies prompted us to investigate the molecular mechanism by which Erm restricts the developmental potential in immature INPs. Overexpression of ERD-Erm<sup>zf</sup>, which functions solely as a transcriptional repressor protein, efficiently rescued the supernumerary neuroblast phenotype in the Ase<sup>+</sup> immature INPs (Fig. 3). By contrast, overexpression of VP16-Erm<sup>zf</sup> exerted a dominant-negative effect and led to a further increase in

supernumerary neuroblasts in *brat* or *erm* hypomorphic brains (Fig. 3). Taken together, these data strongly suggest that Erm restricts developmental potential in immature INPs by repressing gene transcription.

We previously mapped the molecular lesion induced by the *erm*<sup>1</sup>-null allele to a single amino acid substitution in the third C<sub>2</sub>H<sub>2</sub> zinc finger of Erm, indicating that the third zinc finger is essential for the function of Erm. The C<sub>2</sub>H<sub>2</sub> zinc finger transcription factor typically binds to DNA with two or three zinc fingers (Brayer and Segal, 2008). Consistently, perturbing the folding of zinc-finger 2 (Erm<sup>zf2(2A)</sup>) or 4 (Erm<sup>zf4(2A)</sup>) also renders the transgenic protein unable to restrict the developmental potential in the Ase<sup>+</sup> immature INPs whereas perturbing the folding of zinc-finger 1 (Erm<sup>zf1(2A)</sup>), 5 (Erm<sup>zf5(2A)</sup>) or 6 (Erm<sup>zf6(2A)</sup>) had no effects (Fig. 3). These data indicate that the zinc-finger 2, 3 and 4 most likely mediate the binding of Erm to DNA.

### Erm might function cooperatively with the BAP chromatin-remodeling complex to modify the genomic response to neuroblast self-renewal factors

A genome-wide RNAi study showed that knocking down the function of several subunits in the BAP complex results in supernumerary neuroblast formation in fly larval brains (Neumüller et al., 2011). We independently identified that *brm*, *mor* and *osa*, which encode the core components of the BAP complex, probably function temporally after Brat and Numb to restrict the developmental potential in the Ase<sup>+</sup> immature INPs (Fig. 6). Because Brm and Osa are expressed ubiquitously in all cells in larval brains (H.K. and C.-Y.L., unpublished data), the BAP complex probably functions cooperatively with a transcription factor that is uniquely expressed in the immature INPs to restrict developmental potential. Erm is the only known transcriptional factor that is uniquely expressed in the immature INPs, and is an excellent candidate for functioning cooperatively with the BAP complex to restrict the developmental potential (Fig. 2G). Consistently, reducing the function of Brm enhanced the supernumerary neuroblast phenotype in *erm* hypomorphic brains (Fig. 6O–Q). Thus, we propose that Erm restricts the developmental potential in the immature INPs by recruiting the BAP complex to specific genomic loci where the BAP complex alters the nucleosome structures, leading to attenuated competence to respond to the reactivation of neuroblast self-renewal factors. Additional functional and biochemical experiments in the future will be required to validate this hypothesis.

## MATERIALS AND METHODS

### Fly strains

Mutant and transgenic fly strains used include *erm*<sup>1</sup>, *erm*<sup>2</sup> (Weng et al., 2010), *klu*<sup>R51</sup> (Kaspar et al., 2008), *dpm*<sup>1</sup> (Younger-Shepherd et al., 1992), *brat*<sup>150</sup> (Betschinger et al., 2006), *numb*<sup>15</sup> (Berdnik et al., 2002), *Erm-GAL4(II)* and *Erm-GAL4(III)* (Pfeiffer et al., 2008; Weng et al., 2010), *Wor-GAL4* (Lee et al., 2006a), *UAS-erm-HA* (Weng et al., 2010), *UAS-klu-HA* (Xiao et al., 2012), *UAS-brat-myc* (Xiao et al., 2012), *UAS-dpm* (Wallace et al., 2000), *UAS-E(spl)my* (Ligoxygakis et al., 1998) and *UAS-brm*<sup>DN</sup> (Herr et al., 2010). The following fly stocks were obtained from the Bloomington *Drosophila* Stock Center: Oregon R, *brat*<sup>DG19310</sup>, *brat*<sup>k06028</sup>, *brat*<sup>11</sup>, *brm*<sup>2</sup> and *mor*<sup>1</sup>, *osa*<sup>308</sup>, *Act-FRT-stop-FRT-GAL4*, *tub-GAL80*, *UAS-mCD8-GFP*, *FRTG13*, *FRT2A* and *hs-flp*, *tub-GAL80<sup>ts</sup>*, *Elav-GAL4*, *Act-FRT-stop-FRT-lacZ(nls)*, *UAS-GFP(nls)* and *UAS-flp*. *numb*<sup>NP2301</sup> was obtained from the Kyoto Stock Center.

The P[acman] BAC CH321-65B19 construct was used to generate *erm-flag* and *erm-rfp* transgenic fly lines following previously established protocol (Venken et al., 2006; Bischof et al., 2007; Venken et al., 2009).

### Immunofluorescence staining and antibodies

Larval brains were dissected in PBS. Larval brains were fixed in 100 mM PIPES (pH 6.9), 1 mM EGTA, 0.3% Triton X-100, 1 mM MgSO<sub>4</sub> containing 4% formaldehyde for 23 minutes and processed for immunofluorescence staining according to a previously published protocol (Weng et al., 2012). Antibodies used in this study include rabbit anti-Erm (1:100; this study), guinea pig anti-Ase (1:1000; this study), rat anti-Dpm (1:2) (Xiao et al., 2012), rat anti-Wor (1:2) (Lee et al., 2006a), rabbit anti-Ase (1:400) (Weng et al., 2010), mouse anti-Pros (MR1A, 1:100) (Lee et al., 2006b), mouse anti-Elav [1:100; 9F8A9, Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Dlg (1:50; 4F3E3E9, DSHB), mouse anti-Osa (1:2) (Treisman et al., 1997), rabbit anti-Brm (1:2000) (Nakayama et al., 2012), chicken anti-GFP (1:2000; cat. no. 1020, Aves Labs), chicken anti-β-gal (1:2000; cat. no. 1040, Aves Labs) and rabbit anti-RFP (1:100; cat. no. 600-401-379, lot 25003, Rockland). Species-specific fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch, 703-545-155, 112-605-167; Life Technologies, A-11034, A-11035, A-11074, A31553, A-31556) were used at 1:500. We used Rhodamine phalloidin (1:100; Invitrogen) to visualize cortical actin. The confocal images were acquired on a Leica SP5 scanning confocal microscope.

### Generation of a polyclonal antibody against Erm

The cDNA region encoding the C-terminal 332–611 amino acids of Earmuff (Erm-C) was amplified by PCR and subsequently cloned into *EcoRI* and *SalI* sites of pGEX-4T-1, using the In-Fusion HD Cloning Kit (Clontech, cat. no. 639649). The primers used were: 5'-TGGATCCCCGGAATTCCTCACCCGCCACATGCC-3' (forward) and 5'-GGCCGCTCGAGTCGACCTAAACACCTTGGCTATGA-3' (reverse). The expression of GST-Erm-C was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified using glutathione-Sepharose (GE Healthcare, cat. no. 71-5027-54) and eluted with glutathione. GST-Erm-C was injected into one rabbit and purified by GenScript (Hong Kong).

### Clonal analysis

To induce the lineage clone derived from a single Ase<sup>+</sup> immature INP or INP: *brat*<sup>DG19310/+</sup>, *brat*<sup>DG19310/11</sup>, *erm*<sup>1/+</sup>, *brat*<sup>DG19310/11</sup> or *erm*<sup>1/2</sup> larvae carrying the *UAS-flp*, *Erm-Gal4(III)*, *Tub-gal80<sup>ts</sup>* and *Act-FRT-stop-FRT-lacZ(nls)(III)* transgenes were genotyped at hatching, raised at 25°C and dissected at 96 hours after larval hatching. We empirically determined the experimental condition to obtain a small number of clones per brain lobe (supplementary material Fig. S2).

The protocol to examine the expression pattern of *Erm-Gal4(II)* or *Erm-Gal4(III)* in *brat* or *erm* hypomorphic brains is described in the legend for supplementary material Figs S1 and S3.

GFP-marked mosaic clones derived from single mutant neuroblasts in the various genetic background was induced following a standard protocol (Lee and Luo, 2001).

### Overexpression of UAS transgene

Mutant larvae carrying the *Wor-Gal4* and *Tub-Gal80<sup>ts</sup>* in combination with the *UAS* transgene were genotyped at hatching, and raised at 31°C for 72 hours after larval hatching. Larvae were dissected and processed for immunofluorescence staining.

### Three-dimensional modeling of clones

The model was generated using the *Mimics* software from *Materialize*. Confocal images were acquired using a z-step size of 1 μm and the identity of each cell within a clone was determined.

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## Competing interests

The authors declare no competing financial interests.

## Author contributions

D.H.J., H. K. and C.-Y.L. designed experiments. D.H.J., H.K. and D.G. performed experiments. K.C., C.T.K. and H.W. generated guinea pig Ase and Erm antibodies. D.H.J., H.K., D.G. and C.-Y.L. analyzed and interpreted data. D.H.J., H.K. and C.-Y.L. wrote the manuscript.

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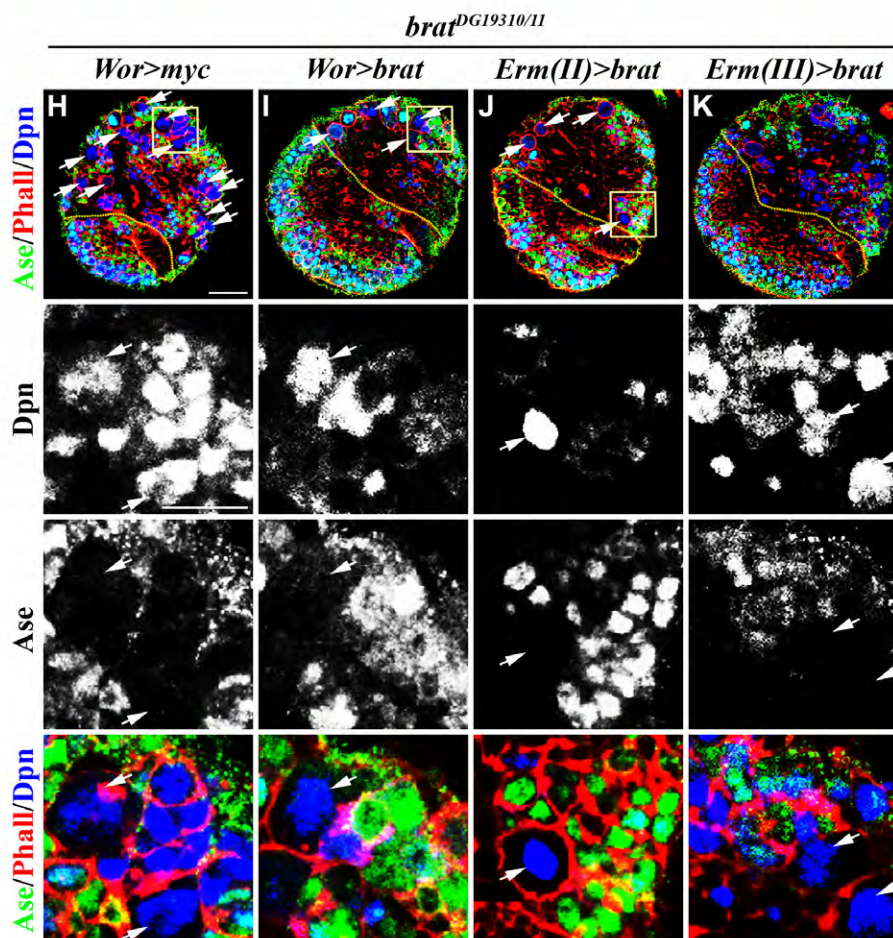
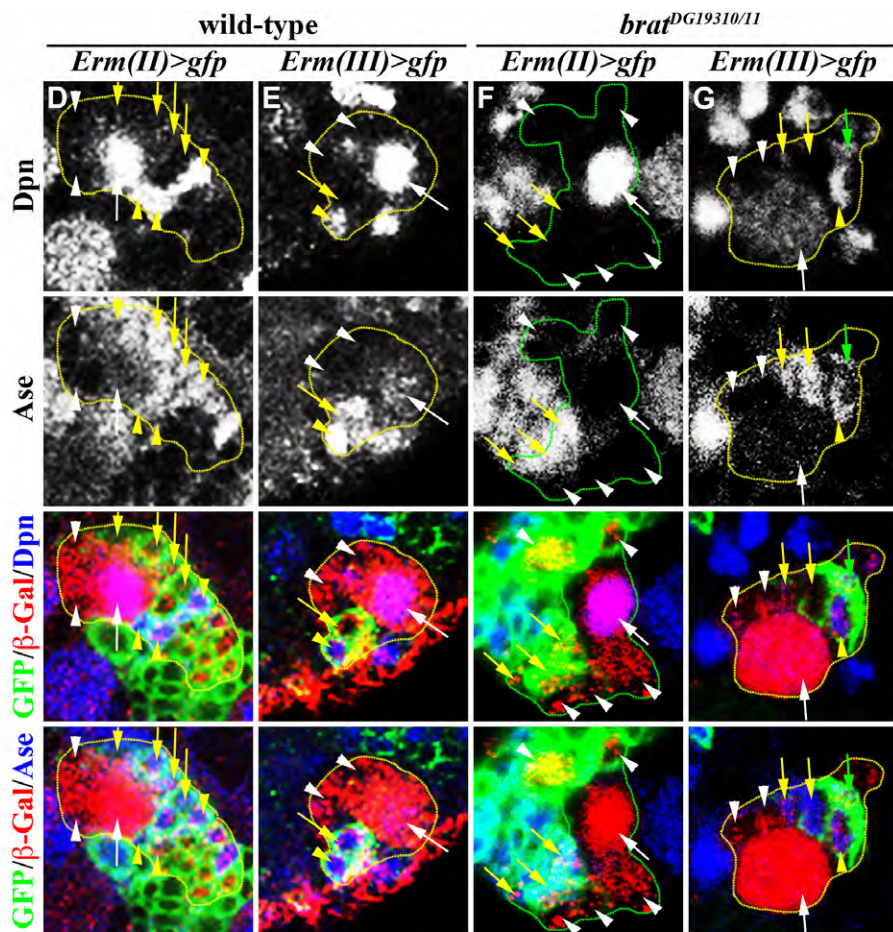
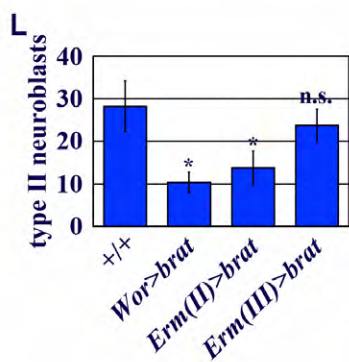
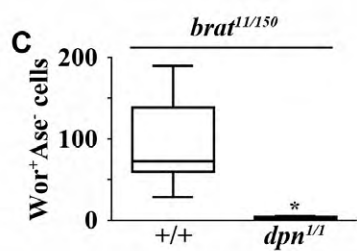
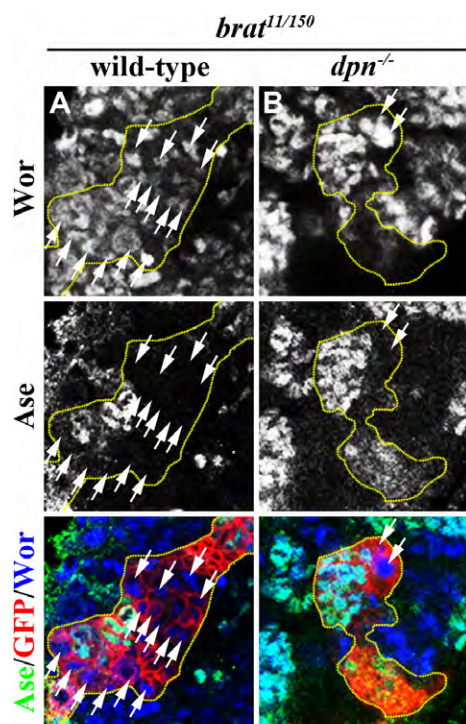
## Supplementary material

Supplementary material available online at  
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.106534/-/DC1

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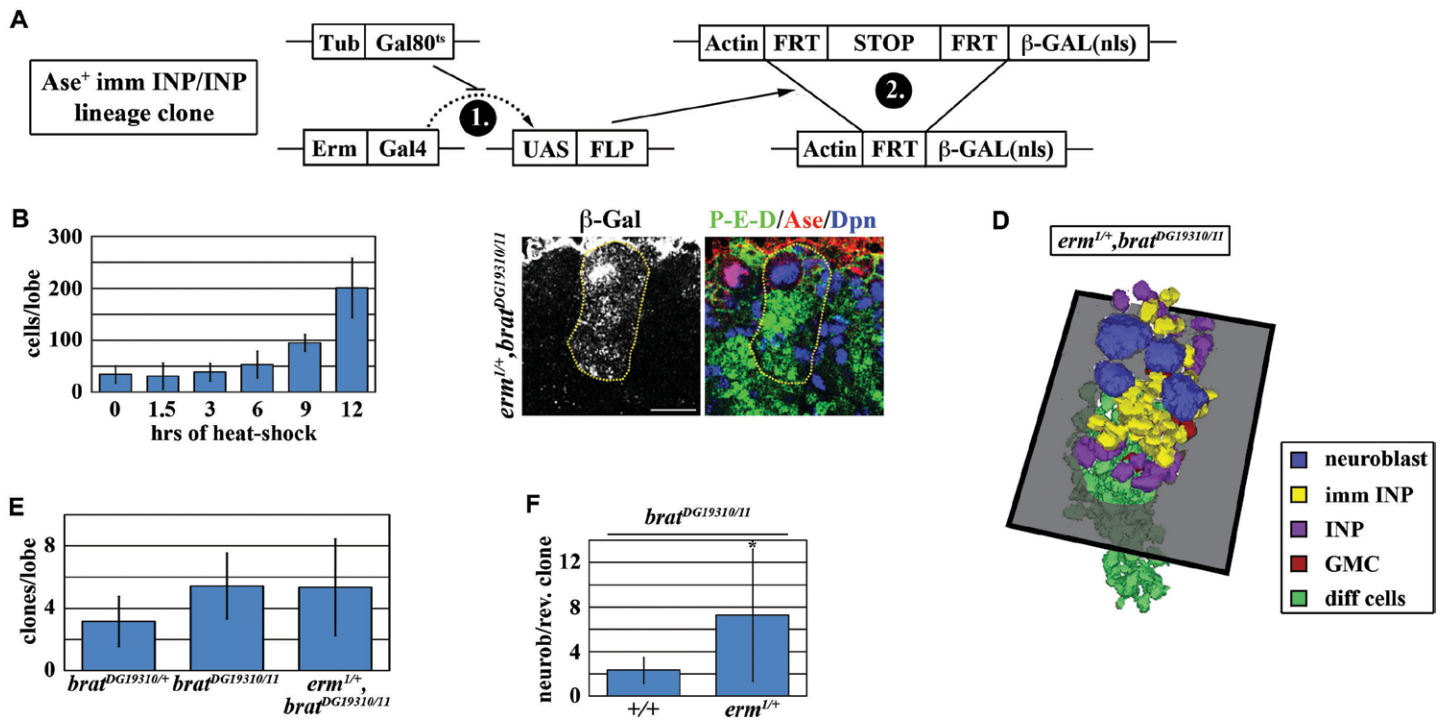
**Fig. S1** Brat functions in the newly born immature INP or Ase<sup>-</sup> immature INP to suppress the formation of supernumerary neuroblasts.

(A-C) Removal of *dpn* function suppressed the supernumerary neuroblast phenotype in *brat* null brains. (C) The quantification of supernumerary neuroblasts in wild-type or *dpn* mutant type II neuroblast clones in *brat* null brains.

(D-G) The expression pattern of *Erm-Gal4(II)* and *Erm-Gal4(III)* appeared indistinguishable between wild-type and *brat* hypomorphic brains. wild-type or *brat*<sup>DG19310/11</sup> larvae carrying the *UAS-mCD8-GFP*, *hs-flp*, Act-FRT-FRT-lacZ, *Erm-Gal4 (II)* or *Erm-Gal4(III)* transgenes were genotyped at hatching, and heat-shocked at 37°C for 90 minutes at 24 hours after hatching to induce the lineage clones. Larvae were dissected and processed for immunofluorescent staining at 96 hours after hatching. The specificity of *Erm-Gal4(II)* or *Erm-Gal4(III)* expression was examined in the β-Gal-marked lineage clones (outlined in yellow) derived from single type II neuroblasts in wild-type or *brat*<sup>DG19310/11</sup> brains.

(H-L) Over-expression of *brat* in neuroblasts or in Ase<sup>-</sup> immature INPs suppressed the supernumerary neuroblast phenotype in *brat* hypomorphic brains, but over-expression of *brat* in Ase<sup>+</sup> immature INPs had no effect. The high magnification image of the boxed area in the low magnification image is shown below. Scale bars, 40 μm in the low magnification image and 10 μm in the high magnification image. (L) The quantification of the average number of type II neuroblasts per brain lobe of the indicated genotypes.

**Key:** White arrow: type II neuroblast. White arrowhead: newly born immature INP and Ase<sup>-</sup> immature INP. Yellow arrow: Ase<sup>+</sup> immature INP. Yellow arrowhead: INP. The dotted yellow line separates the brain from the optic lobe (OL). Single asterisks indicate a statistically significant (p-value <0.05) difference between the marked genotype and the control genotype in the same bar graph as determined by the Student's t-test. n.s. indicates that the difference is statistically significant.



**Fig. S2** Empirical determination of the condition required for inducing a low number of clones derived from single *Ase*<sup>+</sup> immature INPs or INPs per brain lobe.

(A) The scheme used to induce clones derived from single *Ase*<sup>+</sup> immature INPs or INPs.

(B) The total number of  $\beta$ -Gal-marked cells per brain lobe when larvae of the genotype in A. are heat-shocked at 30°C for the indicated number of hrs.

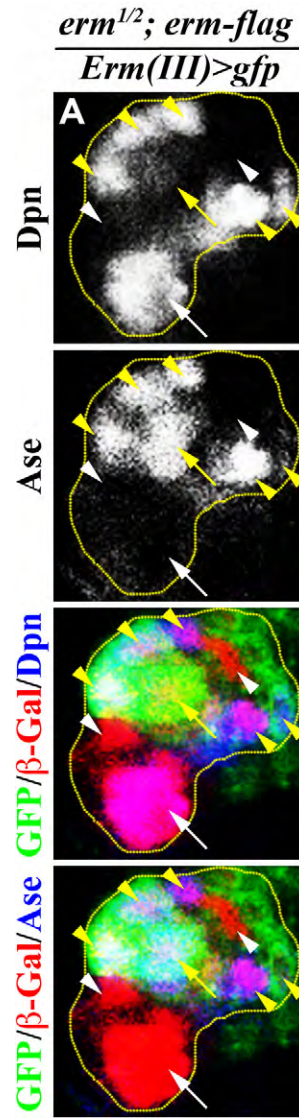
(C) The image of a reverted clone derived from a single *Ase*<sup>+</sup> immature INP or an INP in *brat* hypomorphic brains heterozygous for *erm*.

(D) A three-dimensional reconstruction of a reverted clone shown in C.

(E) The total number of clones per brain lobe induced by the basal leaky expression of *Erm-Gal4(III)* at 25°C in the genotype indicated.

(F) The average number of supernumerary neuroblasts in the reverted clones in *brat*<sup>DG19310/11</sup> brains or *brat*<sup>DG19310/11</sup> brains heterozygous for *erm*.





**Fig. S3** The expression pattern of *Erm-Gal4(III)* was not affected in *erm* hypomorphic brains. *erm*<sup>1/2</sup>; *erm-flag4C/+* larvae carrying the *UAS-mCD8-GFP*, *hs-flp*, *Act-FRT-FRT-lacZ*, *Erm-Gal4(III)* transgenes were genotyped at hatching, and heat-shocked at 37°C for 90 minutes at 24 hours after hatching to induce the lineage clones. Larvae were dissected and processed for immunofluorescent staining at 96 hours after hatching. The specificity of *Erm-Gal4(III)* expression was examined in the  $\beta$ -Gal-marked lineage clones (outlined in yellow) derived from single type II neuroblasts in *erm*<sup>1/2</sup> brains.