It Takes a "Village of Stem Cells and Progenitor Cells" to Build a Brain

by

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To

My husband Ji Zhang

My parents Chonggang Xiao and Li Chen

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ABSTRACT

A small pool of neural stem cells generates diverse differentiated cells that underpin a complex network of neuronal circuits and enable the brain of higher eukaryote to carry out sophisticated intellectual and cognitive tasks. Neural stem cells can generate differentiated cells directly or indirectly through producing intermediate progenitor cells (IPCs). The functional identity of IPCs must be precisely distinguished from neural stem cells, and defects in specifying their functional identity can result in the formation of aberrant neural stem cells at the expense of differentiated cells. My thesis work revealed a mechanism that regulates the competence of neural stem cells to generate IPCs and a mechanism that promotes precise specification of IPCs. These two mechanisms likely function cooperatively to ensure the proper IPCs production in the neural stem cell lineage.

The brain of a fruit fly larva possesses two populations of neural stem cells (type I and type II neuroblasts) that generate progeny with distinct functional characteristics. I identified a transcription factor called *buttonhead* that endows type II neuroblasts with the unique competence to generate intermediate neural progenitors (INPs), which undergo limited proliferation to generate differentiated cells. Type II neuroblasts lacking *buttonhead* function lose the capacity to generate INPs. By contrast, mis-expressing *buttonhead* enables type I neuroblasts to generate INPs which never exist in wild type type I neuroblast lineages. Thus, *buttonhead* plays a key role in regulating the neuroblast competence to generate INPs during fly larval brain neurogenesis.

Separately, I identified the *klumpfuss* gene that plays a key role in preserving a steady pool of neuroblasts. Type II neuroblasts lacking *klumpfuss* function prematurely differentiate. By contrast, mis-expressing *klumpfuss* in uncommitted INPs leads to the formation of supernumerary neuroblasts. Thus, rapid down-regulation of *klumpfuss* function in uncommitted INPs is essential for their

commitment to an INP functional identity. In summary, Klumpfuss functions as a transcriptional regulator to promote neuroblast self-renewal and prevent a precocious commitment to the INP identity. Since Buttonhead and Klumpfuss are highly conserved from flies to humans, their homologs might also regulate neural stem cells during vertebrate neurogenesis.

Chapter I

Introduction

1. Neural stem cell and transit amplifying cell lineages determine the development and evolution of mammalian brain

Neural stem cells in the mammalian brain generate transit amplifying cells through asymmetric division to fulfill the requirement of rapidly increasing brain size and surface area while maintaining the stem cell pool at a steady level. Transit amplifying cells, also known as intermediate progenitor cells (IPCs), usually undergo rapid proliferation to generate differentiated neurons and glia, which form complex neural circuits required for the intellectual and cognitive function in higher organisms (Englund et al., 2005; Kowalczyk et al., 2009; Noctor et al., 2008). Neural stem cells and their IPC progeny provide a good system for studying the regulation of stem cell self-renewal and differentiation, asymmetric cell division and cell-to-cell communication.

Rodent neural stem cells, also called radial glia cells, generate differentiated neural progenies directly or indirectly through producing IPCs. In the embryonic stage, neural stem cells, which are located in the ventricular zone (VZ), and contact with both pial and ventricular surfaces by radial fibers (Weissman et al., 2003), undergo repeated asymmetric divisions to self-renewal and to generate a neuron or an IPC (Kriegstein and Alvarez-Buylla, 2009). IPC are located in the subventricular zone (SVZ), and usually undergo limited rounds of cell division to generate neurons or glia, which migrate to the developing cortical layers through the radial fibers (Kriegstein and Alvarez-Buylla, 2009). Neurogenesis in the adult brain mainly occurs in the SVZ and subgranular zone (SGZ) of the hippocampus (Cameron et al., 1993; Kaplan and Hinds, 1977; Lois and Alvarez-Buylla, 1994). The adult SVZ

contains relatively quiescent neural stem cells (B cells), which give rise to actively proliferating transit amplifying cells (C cells) that are similar to IPCs. The C cells differentiate into immature neuroblasts (A cells), which can divide to generate more neuroblasts and migrate through the rostral migratory stream to the olfactory bulb, where they differentiate into mature interneurons (Carleton et al., 2003; Doetsch et al., 1999; Lois and Alvarez-Buylla, 1994). The adult SGZ also contains neural stem cells with radial glia nature and give rise to neurons indirectly by generating IPCs. These neurons are located in the adult dentate gyrus and have specific function in learning and memory. Recent studies based on the lineage clone analysis also indicated that individual neural stem cells which exclusively generate neurons or both neurons and astrocytes coexist in the SGZ of the adult mouse dentate gyrus (Bonaguidi et al., 2011).

The developing human brain contains a second type of neural stem cells that reside in the outer subventricular zone (OSVZ) and also display a radial glial cells morphology (Fietz et al., 2010; Hansen et al., 2010). OSVZ neural stem cells likely arise from the asymmetric division of neural stem cells in the VZ, and then migrate to the OSVZ where they undergo limited rounds of symmetric division to expand their numbers (Hansen et al., 2010). These OSVZ neural stem cells also produce IPCs through repeated rounds of asymmetric division. Compared with IPCs in the SVZ, these OSVZ IPCs undergo more symmetric divisions to amplify their number, which allows them to generate more neurons for the significant expansion of human neocortex (Fietz et al., 2010; Hansen et al., 2010; Lui et al., 2011). Because the radial fibers of OSVZ neural stem cells may not extend all the way to the pial surface, their neuronal progeny migrate to the cortical plate in a disperse way and contribute to the expansion of the neocortical surface (Lui et al., 2011).

Vertebrate studies strongly suggest that IPCs play a key role in generating the requisite number of diverse differentiated cells required for proper brain development and brain homeostasis. Thus, mechanistic insight into the generation and the specification of IPCs will significantly improve our understanding of

neurogenesis process. However, lack of sophisticated lineage tracing tools as well as the complex architectural organization of the developing cortex has hindered the investigation of the regulation of IPCs in a physiologically relevant environment.

2. Neural stem cells and progenitor cells in *Drosophila* larva brain

Neural stem cells (neuroblasts) in the fly larval brain provide an excellent *in vivo* genetic model to investigate various fundamental questions in stem cell biology. Similar to vertebrate neural stem cells, larval brain neuroblasts undergo repeated asymmetric stem cell divisions to self-renew and to generate differentiating cells directly or indirectly through intermediate progenitor cells (Figure 1.1) (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). Every asymmetric division of a type I neuroblast leads to the generation of a ganglion mother cell (GMC), which divides once to produce two differentiated cells. In contrast, each asymmetric division of a type II neuroblast leads to the generation of an uncommitted intermediate neural progenitors (immature INP). An immature INP is transiently arrested in the cell cycle and undergoes maturation to acquire the INP functional identity (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). An INP possesses limited self-renewal capacity and undergoes six-to-eight rounds of asymmetric division to self-renew and to generate a GMC each time (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). Similar to IPCs during vertebrate neurogenesis, INPs can also generate diverse differentiated cell types required for the development of an adult fly brain (Awasaki, et al., 2014; Bayraktar, et al., 2013; Wang, et al., 2014; Yang, et al., 2013). Thus, understanding the mechanisms that regulate the generation and the specification of INPs will provide critical insight into invertebrate and vertebrate neurogenesis.

The cortex of mitotic neuroblast is highly polarized with protein complexes assembling in the apical and the basal cortical domains (Doe, 2008; Knoblich, 2008; Wu et al., 2008). The apical complexes segregate into the self-renewing neuroblast

and function to target the basal protein complexs into the GMC or immature INP. The basal proteins include Brain tumor (Brat), Prospero (Pros) and Numb, and they function to down-regulate the activity of self-renewal factors or promoting differentiation in the GMC or immature INP (Betschinger, et al., 2006; Doe, 2008; Lee, et al., 2006; Neumuller, et al., 2009). Brat is the fly ortholog of mammalian TRIM32 protein, which functions to induce neuronal differentiation (Schwamborn et al., 2009). Brat is dispensable for GMC specification, but plays a key role in preventing the reversion of immature INPs into type II neuroblasts by antagonizing the function of self-renewal factors Deadpan (Dpn) (Janssens, et al., 2014). Pros is only expressed in type I neuroblasts, and encodes an evolutionarily conserved homeodomain transcription factor, and prevents the reversion of GMCs into supernumerary type I neuroblasts by up-regulating the expression of differentiation genes (Choksi et al., 2006). Numb is an evolutionarily conserved negative regulator of Notch signaling, and functions to promote the specification of GMCs and immature INPs by antagonizing Notch signaling, a central regulator of neuroblast self-renewal (Haenfler et al., 2012; Xiao et al., 2012). Thus, Brat and Numb asymmetrically extinguish the function of self-renewal factors in the GMC and the immature INP whereas Pros promotes the differentiation of GMCs (Figure 1.2).

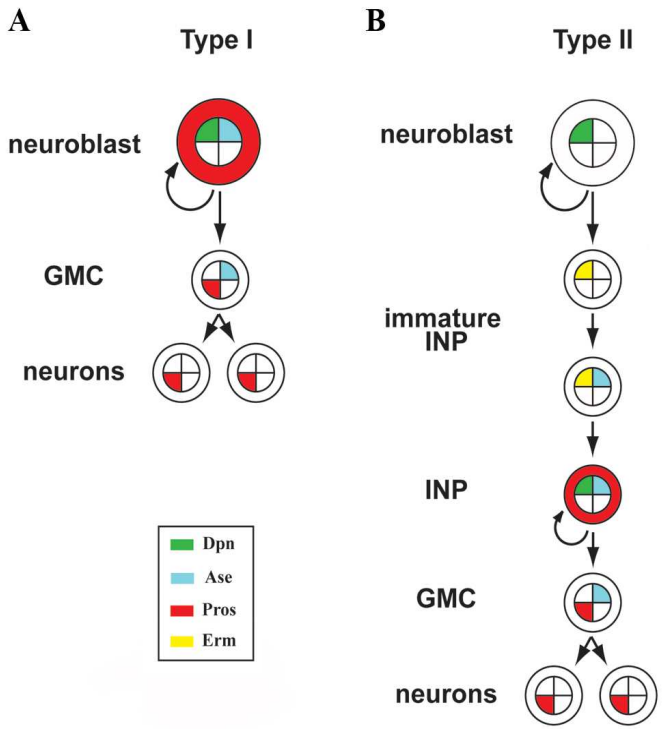

Figure 1.1 Neural stem cell (neuroblast) lineages in the *Drosophila* larval brain

The cell fate markers allow unambiguous identification of neuroblasts/progenitor cells in the type I and type II neuroblast lineages in the larval brain.

(**A**) A type I neuroblast expresses bHLH factors Deadpan (Dpn) and Asense (Ase) in the nucleus and Prospero (Pros) in the cytoplasm and always generates a GMC expressing Ase and Pros in the nucleus. GMCs cannot self-renew and divides once to produce two $Pros^{+}$ terminally differentiated neurons.

(**B**) A type II neuroblast expresses Dpn in the nucleus and always generates an immature INP expressing transcription factor Earmuff (Erm) in the nucleus. Along with the maturation process, the late stage of immature INP starts to express Ase. After the INP acquires the functional identity (maturation), it restarts the Dpn, Ase and Pros expression, and undergoes limited rounds of asymmetric division to self-renew and to generate a GMC each time.

3. What are the mechanisms that specify GMCs or INPs

During neuroblast asymmetric division, both the self-renewing neuroblast and its differentiating sibling inherit self-renewal factors through the cytoplasm of their parental neuroblast. Thus, efficient down-regulation of the activity of self-renewal factors in the differentiating progeny is pivotal for proper specification of the GMC or INP functional identity. Studies from several groups have collectively established a network of factors required for the self-renewal of larval brain neuroblasts (Berger et al., 2012; San-Juan and Baonza, 2011; Xiao et al., 2012; Zacharioudaki et al., 2012; Zhu et al., 2012). A central component of the self-renewal network is *Notch*, which is essential for the maintenance of type II neuroblasts but dispensable for the self-renewal of type I neuroblasts (Haenfler et al., 2012). *Notch* directly regulates the expression of a self-renewal factor *Enhancer of split mγ* (*E(spl)mγ)* (Zacharioudaki et al., 2012), which is a fly ortholog of the vertebrate Hes family of transcription factors (Zacharioudaki et al., 2012). E(spl)mγ acts redundantly with another self-renewal factor Dpn to maintain both type I and type II neuroblasts (Zacharioudaki et al., 2012). Interestingly, even though Dpn is also belong to the Hes family, it does not function downstream of Notch. All three self-renewal factors display a high expression level in the neuroblast, and down-regulation of these factors is essential for specification of the GMC or INP functional identity (Berger et al., 2012; San-Juan and Baonza, 2011; Xiao et al., 2012; Zacharioudaki et al., 2012; Zhu et al., 2012). This thesis will discuss the function of other self-renewal factor in the larval brain, and how it coordinates with the known self-renewal transcription network to specify the functional identity of GMC or INP.

3.1 What are the mechanisms that specify GMCs

In order to specify a GMC functional identity, Pros activates the transcription of genes essential for cell cycle exit and neuronal differentiation in the presumptive GMC and Numb down-regulates *Notch* signaling (Choksi et al., 2006). In the

absence of *pros* or *numb*, GMCs revert into supernumerary type I neuroblasts, contributing to brain tumor formation in flies (Bowman et al., 2008; Choksi et al., 2006). Consistent to the *numb* mutant, aberrant activation of *Notch* signaling in GMCs also triggers them reversion into type I neuroblasts (Zacharioudaki et al., 2012). Over-expression of *dpn* also induces the formation of supernumerary type I neuroblasts, but the mechanisms that down-regulate *dpn* function in GMCs in the type I neuroblast lineage remain unknown (Zacharioudaki et al., 2012). Thus, Pros and Numb appear to play prominent roles in functionally distinguishing a GMC from a type I neuroblast (Figure 1.2).

3.2 What are the mechanisms that specify the INP functional identity

During the asymmetric division of a type II neuroblast, the basal proteins Brat and Numb segregate into the future immature INP where they prevent the reversion into a supernumerary type II neuroblast by antagonizing the function of self-renewal factors (Bowman et al., 2008; Xiao et al., 2012). Consistently, mis-expression of *Notch*, *E(spl)mγ* or *dpn* potently induces the reversion of early stage immature INPs into supernumerary type II neuroblasts. Brat appears to uniquely antagonize the function of Dpn in the newly born immature INP because removing the function of *dpn* suppresses supernumerary neuroblast formation in the *brat* null genetic background (Janssens et al., 2014; Xiao et al., 2012). In parallel, Numb down-regulates the function of *Notch* in the newly born immature INP (Xiao et al., 2012). Thus, Brat and Numb asymmetrically extinguish the function of the self-renewal network in the newly born immature INP, allowing the specification of the INP functional identity (Figure 1.2).

During the INP maturation process, another key gene named Earmuff (Erm), which encodes an evolutionarily conserved C_2H_2 zinc-finger transcription factor, functions to specify the INP identity (Janssens et al., 2014; Weng et al., 2010). In the *erm* mutant brain, the transition from the early stage (Ase^-) to the late stage of

immature INP (Ase^{+}) is indistinguishable from that in the wild type brain. However, upon the completion of maturation, INPs in the *erm* mutant brain spontaneously revert into supernumerary type II neuroblasts in a *Notch*-dependent manner (Janssens et al., 2014; Weng et al., 2010). These data strongly suggest that Erm either functions to specify or to maintain the INP functional identity. Two recent studies independently show that endogenous Erm expression is detected in the early stage as well as the late stage immature INP but undetectable in the type II neuroblast and the INP (Janssens et al., 2014; Koe et al., 2014). The temporal expression of Erm directly correlates with the timing of the specification of the INP functional identity. Consistently, mis-expression of the self-renewal factor Dpn or E(spl)mγ is sufficient to induce the reversion of INPs into supernumerary neuroblasts in *erm* hypomorphic mutant brain but not in the wild-type brain under an identical experimental condition (Janssens et al., 2014). Thus, Erm functions to specify the INP functional identity in the immature INP by altering the competence to respond to the self-renewal transcription factors (Figure 1.2).

The SWI/SNF complex is an evolutionarily conserved mechanism that regulates the packaging of the nucleosome and can alter the global genomic response to transcription factors (Ho et al., 2009; Kidder et al., 2009; Lessard and Crabtree, 2010). Three recent studies reported that the SWI/SNF complex plays a critical role in the specification of the INP functional identity (Eroglu et al., 2014; Janssens et al., 2014; Koe et al., 2014). First, knocking down the function of the BAP (Brahma-associated proteins) complex, a sub-type of the SWI/SNF complex, leads to the reversion of INPs into supernumerary type II neuroblasts. Second, reducing the function of the BAP complex further exacerbates the reversion of early stage immature INPs into supernumerary neuroblasts in the *brat* or *numb* mutant brain (Janssens et al., 2014). Lastly, multiple components of the BAP complex physically interact with Erm (Koe et al., 2014). Thus, Erm specifies the INP functional identity by programming the genome in the immature INP through the BAP complex. Importantly, extending the self-renewal capability of an INP by removing the function of a transcription factor *hamlet* is not sufficient to induce the reversion into

a supernumerary neuroblast (Eroglu et al., 2014). Taken together, these studies strongly suggest that the Erm-dependent mechanism permanently alters the competence to respond to the self-renewal transcription factors during the specification of the INP functional identity and functionally distinguishes an INP from a type II neuroblast (Figure 1.2).

4. What are the mechanisms that regulate the competence of INP generation

Generation of intermediate progenitors helps neural stem cells produce more and diverse neurons. Thus the mechanisms that regulate neural stem cell's competence to produce intermediate progenitors are essential for neurogenesis. In fly larval brain, only type II neuroblasts generate INPs, thus the factors that regulates the competence of INP generation are most likely to express in type II neuroblasts uniquely (Komori et al., 2014; Zhu et al., 2011). One of these genes is *buttonhead* (*btd*) that functions to endow type II neuroblasts with the competence to generate INPs (Komori et al., 2014). *btd* mutant type II neuroblasts generate progenies that adopt a GMC identity instead of INP, and these progenies do not revert to type II neuroblasts in *brat* mutant background.. Most importantly, mis-expression of *btd* is sufficient to trigger a type I neuroblast to generate functional immature INPs which are sensitive to the loss of *brat* function (Komori et al., 2014). Thus, *btd* is a critical factor that endow type II neuroblasts with the competence to generate INPs. Considering that *btd* is the fly ortholog of mammalian gene *sp8*, the results in fly could shed light on the study of intermediate progenitor generation in the mammalian system.

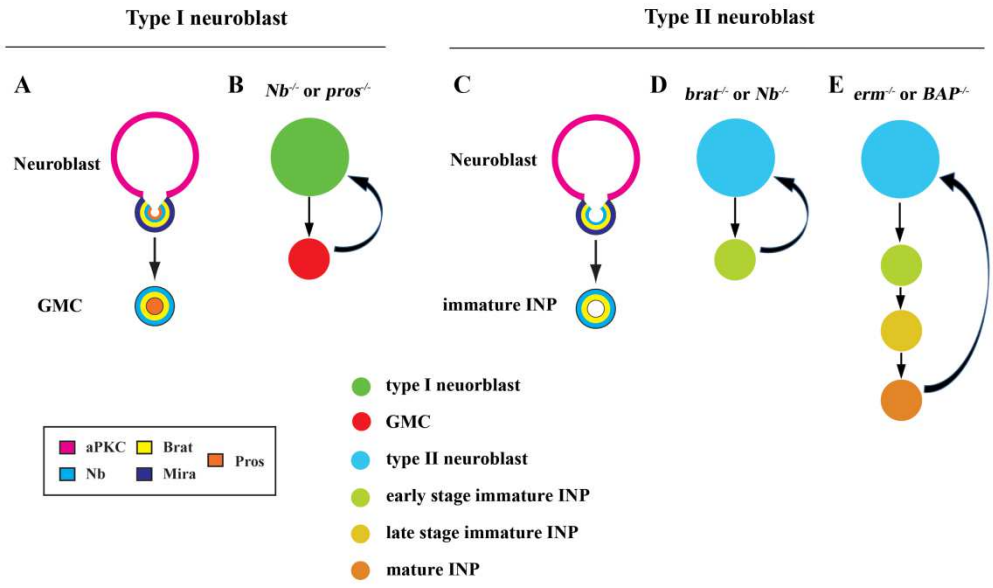

Figure 1.2 Summary of regulatory mechanisms that functionally distinguish neural stem cells to progenitor/precursor cells

(A) The apical and basal protein complexes unequally segregate during asymmetric divisions of neural stem cell/precursor in the type I neuroblast lineage.

(**B**) *numb* or *pros* mutant GMCs revert to type I neuroblasts. Nb: Numb

(**C**) The apical and basal protein complexes unequally segregate during asymmetric divisions of neural stem cell/progenitor in the type II neuroblast lineage.

(**D**) *brat* or *numb* mutant immature INPs revert to type II neuroblasts. Nb: Numb

(**E**) *erm* or *BAP* mutant INPs revert to type II neuroblasts.

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CHAPTER II

Trithorax maintains the functional heterogeneity of neural stem cells through the transcription factor Buttonhead

Summary

The mechanisms that maintain the functional heterogeneity of stem cells, which generates diverse differentiated cell types required for organogenesis, are not understood. In this study, we report that Trithorax (Trx) actively maintains the heterogeneity of neural stem cells (neuroblasts) in the developing *Drosophila* larval brain. *trx* mutant type II neuroblasts gradually adopt a type I neuroblast functional identity, losing the competence to generate intermediate neural progenitors (INPs) and directly generating differentiated cells. Trx regulates a type II neuroblast functional identity in part by maintaining chromatin in the *buttonhead* (*btd*) locus in an active state through the histone methyltransferase activity of the SET1/MLL complex. Consistently, *btd* is necessary and sufficient for eliciting a type II neuroblast functional identity. Furthermore, over-expression of *btd* restores the competence to generate INPs in *trx* mutant type II neuroblasts. Thus, Trx instructs a type II neuroblast functional identity by epigenetically promoting Btd expression, thereby maintaining neuroblast functional heterogeneity.

Introduction

Stem cells employ several strategies to generate the requisite number of diverse differentiated cell types required for organ development and organ homeostasis in higher eukaryotes (Franco and Müller, 2013; Kohwi and Doe, 2013). One such strategy involves stem cells changing their temporal identities. For example, neuroblasts sequentially express distinct temporal-identity transcription factors, allowing them to generate diverse differentiated cells in the fly embryonic ventral

nerve cord (Isshiki et al., 2001; Pearson and Doe, 2003). Another strategy involves maintaining a functionally heterogeneous pool of tissue-specific stem cells. Studies in flies and vertebrate systems show that functionally heterogeneous stem cells directly contribute to the generation of diverse cell types during hematopoiesis, gut homeostasis, and brain development (Barker et al., 2007; Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Graf and Stadtfeld, 2008; Copley et al., 2012; Franco et al., 2012; Marianes and Spradling, 2013). Numerous patterning mechanisms have been described to explain how the fates of distinct stem cells within a developing organ become specified, but how their functional heterogeneity is maintained throughout the lifespan of an organism remains completely unknown. The central complex of the insect brain is comprised of an intricate network of neurons and glia that process a vast number of environmental inputs essential for daily life (Boyan and Reichert, 2011; Boyan and Williams, 2011). All differentiated cell types in the central complex arise from repeated rounds of self-renewing asymmetric divisions of type I and type II neuroblasts, which are molecularly and functionally distinct (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008) (Figure 2.1—2.1S). In every asymmetric division, a type I neuroblast always generates a precursor cell (ganglion mother cell or GMC) that divides once to produce two differentiated cells. By contrast, every asymmetric division of a type II neuroblast invariably leads to the generation of an immature INP that acquires an INP functional identity during maturation. An INP undergoes 5–8 rounds of asymmetric division to regenerate and generate a GMC with each division (Homem et al., 2013). Thus, the ability to generate INPs functionally distinguishes these two types of neuroblasts. Type II neuroblasts uniquely express the ETS transcription factor Pointed P1 (PntP1) (Zhu et al., 2011; Xiao et al., 2012). Mis-expression of PntP1 can induce a type II neuroblast functional characteristic in a type I neuroblast (Zhu et al., 2011). However, the physiological function of PntP1 in the maintenance of a type II neuroblast functional identity remains unclear. The *pnt* locus encodes at least three distinct alternatively spliced transcripts. Thus, it is formally possible that multiple isoforms of Pnt or a yet unknown mechanism function to maintain a type II

neuroblast functional identity. Epigenetic mechanisms such as the methylation of histone H3 Lysine 4 (H3K4) play central roles in specifying cell type identities during development (Lim et al., 2009; Ang et al., 2011; Schuettengruber et al., 2011; Shilatifard, 2012; Yang et al., 2012). The evolutionarily conserved SET1/Mixed-lineage leukemia (MLL) complexes catalyze the methylation of H3K4 and maintain the target gene loci in a transcriptionally active state (Miller et al., 2001; Roguev et al., 2001; Krogan et al., 2002). The fly genome encodes three orthologs of the SET1/MLL protein, Trx, Trithorax-related (Trr), and dSet1. Similar to their mammalian counterparts, Trx, Trr, or dSet1 can each assemble functionally active complexes by binding to Absent, small, or homeotic discs 2 (Ash2), Retinoblastoma binding protein 5 (Rbbp5), and Will die slowly (Wds) (Wu et al., 2008; Ardehali et al., 2011; Mohan et al., 2011). Functionally, Trr or dSet1 regulates global mono- or tri-methylation of H3K4 respectively. In contrast, Trx appears to selectively regulate the expression of the Hox genes through the methylation of H3K4 (Breen and Harte, 1993; Yu et al., 1995). However, little is known about the targets of Trx beyond the Hox genes.

Here, we report that Trx maintains the type II neuroblast functional identity by regulating the transcription of *btd* during fly larval brain neurogenesis. Type II neuroblasts mutant for *trx* or genes encoding the core components of the SET1/MLL complex display a type I neuroblast marker expression profile and generate GMCs instead of INPs. These results indicate that Trx maintains a type II neuroblast functional identity by regulating the transcription of specific target genes. We identified a direct downstream target of Trx, Btd, that plays an important role in the maintenance of a type II neuroblast functional identity. *btd* mutant type II neuroblasts adopt a type I neuroblast functional identity and directly generate GMCs instead of INPs. Conversely, type I neuroblasts over-expressing *btd* assume a type II neuroblast functional identity and generate INP progeny. Most importantly, over-expression of *btd* restores the competence of *trx* mutant type II neuroblasts to generate INPs. Thus, we conclude that Trx functions to epigenetically maintain Btd

expression in type II neuroblasts, thereby maintaining neuroblast functional heterogeneity in the larval brain.

Results

trx regulates neuroblast heterogeneity by maintaining a type II neuroblast identity

Analyses of gene transcription in mutant larval brains enriched with type I or type II neuroblasts led us to hypothesize that differential regulation of gene expression contributes to neuroblast functional heterogeneity (Carney et al., 2012) (Komori and Lee, unpublished observation). Because the *trx* gene contributes to cell fate maintenance in a variety of developmental processes, we tested whether it is required for maintaining neuroblast heterogeneity. We induced GFP-marked mosaic clones derived from single wild-type or *trx* mutant type I or II neuroblasts and assessed the identities of cells in the clones by examining the expression of cell fate markers in a time-course study (Figure 2.1—2.1S). Identical to wild-type neuroblasts, *trx* mutant type I neuroblasts maintained the expression of Deadpan (Dpn) and Asense (Ase) and the cytoplasmic localization of Prospero (Pros), but lacked PntP1 expression ($Dpn^{+}Ase^{+}PntP1^{-}Pros^{cytoplasmic}$) (Table 2.1, data not presented). In addition, both wild-type and *trx* mutant type I neuroblasts were always surrounded by GMCs ($Dpn^{-}Ase^{+}Pros^{nuclear}$) (data not presented). Thus, Trx is dispensable for the maintenance of a type I neuroblast functional identity. While all wild-type type II neuroblasts displayed a $Dpn^{+}Ase^{-}PntP1^{+}Pros^{-}$ marker expression profile in all stages examined, *trx* mutant type II neuroblasts progressively altered their marker expression profile (Figure 2.1A–D, Table 2.1). Strikingly, almost all *trx* mutant type II neuroblasts in 72-hr clones displayed a type I neuroblast marker expression profile (Figure 2.1B–D; Table 2.1). These data strongly suggest that *trx* mutant type II neuroblasts adopt a type I neuroblast identity.

We extended our analyses to examine the identity of progeny directly derived from *trx* mutant type II neuroblasts. We observed a time-dependent reduction in INPs in

trx mutant type II neuroblast clones as compared to identically staged wild-type clones. At 72 hr after clone induction, a control type II neuroblast was surrounded by approximately 20 INPs and 12 INP-derived GMCs that can be unambiguously identified by the expression of an erm-lacZ reporter transgene (Figure 2.1C,E–F,H, Figure 2.1—2.1S). In contrast, an identically staged *trx* mutant neuroblast was directly surrounded by non-neuroblast progeny that displayed a $Dpn^{-}Ase^{+}Pros^{nuclear}erm\text{-}lacZ^{-}$ expression profile identical to GMCs derived from type I neuroblasts (Figure 2.1C,G, Figure 2.1—figure supplement 2.1). Although *trx* mutant clones also contained an average of 3 INPs and 4 INP-derived GMCs, these cells were located at the extreme distal end of the clone, consistent with *trx* mutant type II neuroblasts adopting a type I neuroblast identity following the clone induction (Figure 2.1C,E,G–H). These data strongly suggest that Trx regulates neuroblast heterogeneity by maintaining a type II neuroblast identity.

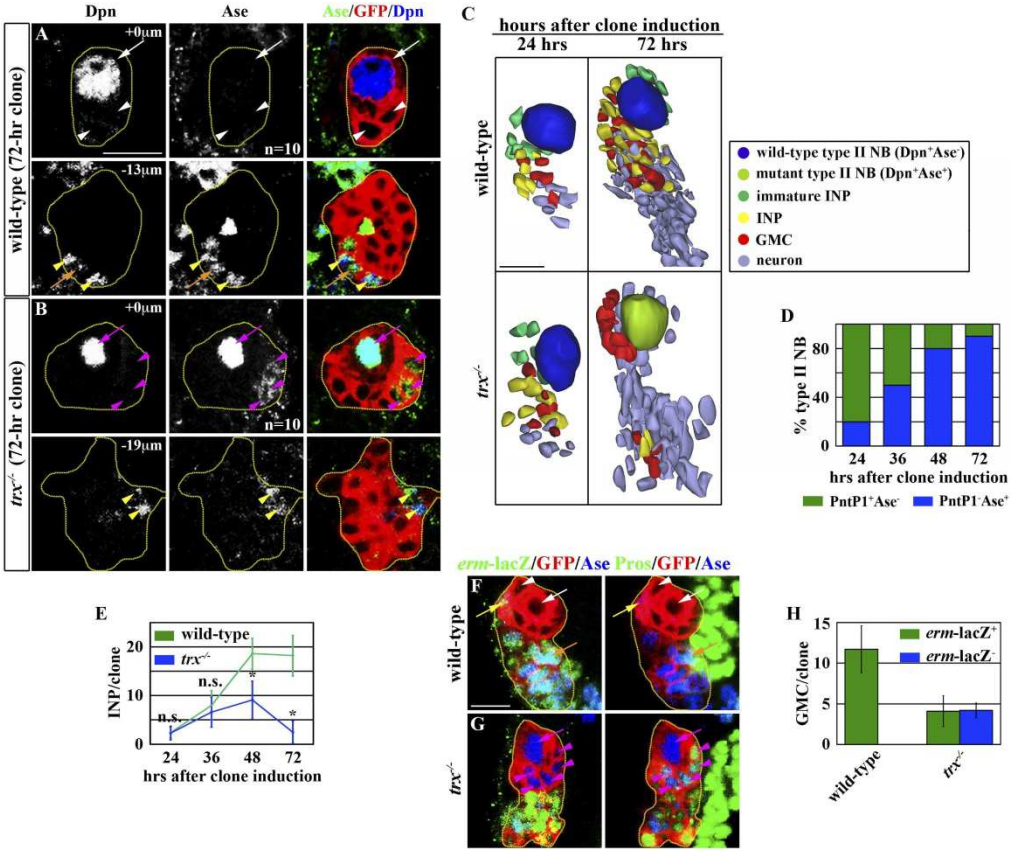

Figure 2.1 *trx* mutant type II neuroblasts adopt a type I neuroblast functional identity.

(**A-D**) *trx* mutant type II neuroblasts progressively acquire a type I neuroblast functional identity. (A-B) In the 72-hour GFP-marked clone, a wild-type type II neuroblast displays a $Dpn^{+}Ase^{-}$ marker expression profile whereas a *trx* mutant type II neuroblast displays a $Dpn^{+}Ase^{-}$ expression profile. Scale bar, 10 m. (C) Three-dimensionally reconstructed images of type II neuroblasts clones of the indicated genotypes. Scale bar, 10 m. (D) The frequency of *trx* mutant type II neuroblasts displaying a type I neuroblast maker expression profile ($PntP1^{-}Ase^{+}$). N=10 per time point.

(**E-H**) *trx* mutant type II neuroblasts directly generate GMCs. (E-F) In the 48-hour clones, a wild-type type II neuroblast shows undetectable expression of Pros in the telophase whereas a *trx* mutant type II neuroblast shows the basal cortical localization of Pros. Scale bar, 10 m. (G) The frequency of wild-type or *trx* mutant mitotic type II neuroblasts displaying the

basal localization of Pros. (H) The average number of type I neuroblasts per type II neuroblast clone of the indicated genotypes at 72 hours after clone induction.

(**I-L**) *trx* mutant type II neuroblasts lose the ability to generate INPs. (I) The average number of INPs per staged type II neuroblast clone of the indicated genotype. N=10 per time point. (J-K) In the 72-hour GFP-marked clones, a wild-type type II neuroblast is surrounded by INPs and their GMC progeny identified by *erm*-lacZ expression. In contrast, a *trx* mutant type II neuroblast is surrounded by GMCs that are directly derived from neuroblasts and lack *erm*-lacZ expression. (L) The average number of GMCs with or without *erm*-lacZ expression per type II neuroblast clone of the indicated genotypes.

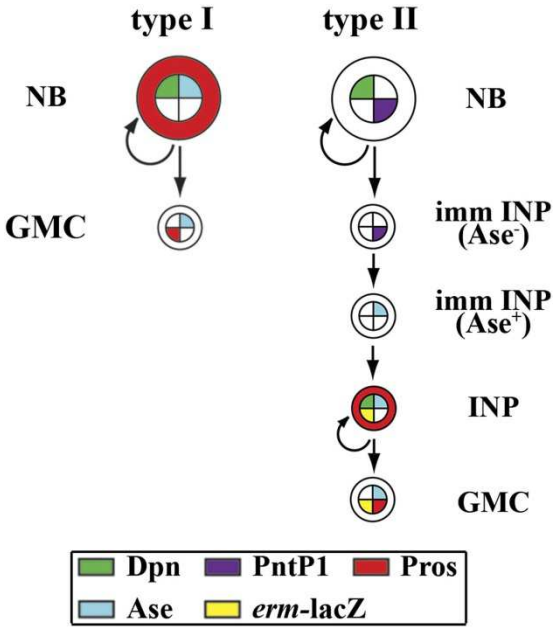

Figure 2.1S *trx* mutant type II neuroblasts adopt a type I neuroblast identity.

(A) A summary of the cell fate marker expression profile in type I and type II neuroblast lineage in the larval brain. NB: neuroblast; GMC: ganglion mother cell; INP: intermediate neural progenitor; imm INP: immature INP.

(B-C) *trx* mutant type II neuroblasts are surrounded by GMCs

Genotype	Neuroblast type	Dpn	Ase	Pros*	PntP1
wild-type	I	+	+	+	−
wild-type	II	+	−	−	+
Trx$^{-/-}$	I	+	+	+	−
Trx$^{-/-}$	II	+	+	+	−
Rbbp5$^{-/-}$	I	+	+	+	−
Rbbp5$^{-/-}$	II	+	+	+	−
btd$^{-/-}$	I	+	+	+	−
btd$^{-/-}$	II	+	−	−	+

Table 2.1 Summary of the marker expression profile in various genetic backgrounds

'+' indicates detected marker expression whereas '−' indicates lack of marker expression. '*' indicates basal asymmetric localization at the basal cortex in mitotic neuroblasts.

trx maintains the functional identity of type II neuroblasts

The competence to generate INPs is a main feature that distinguishes the functional identity of a type II neuroblast from that of a type I neuroblast (Weng and Lee, 2011; Homem and Knoblich, 2012; Janssens and Lee, 2014). *brain tumor* (*brat*) and *erm* function in the immature INP to promote INP identity specification in the type II neuroblast lineage, and the defective specification of an INP identity leads to the formation of supernumerary type II neuroblasts in the *brat* or *erm* mutant brain (Xiao et al., 2012; Eroglu et al., 2014; Janssens et al., 2014; Koe et al., 2014; Komori et al., 2014). If *trx* mutant type II neuroblasts indeed adopt a type I neuroblast functional identity, their progeny should be insensitive to the loss of *brat* or *erm* function and generate differentiated cells instead of reverting into supernumerary neuroblasts. A control type II neuroblast clone in the *brat* mutant brain contained more than 100 supernumerary type II neuroblasts and was devoid of GMCs and neurons (Figure 2.2A,E). By contrast, a *trx* mutant type II neuroblast clone in the *brat* mutant brain contained far fewer supernumerary type II neuroblasts and far more GMCs and neurons as compared to the control clone (Figure 2.2A–B,E). Similarly, a control type II neuroblast clone in the *erm* mutant brain contained more than 50 supernumerary type II neuroblasts and few GMCs and neurons (Figure 2.2C,E). In contrast, a *trx* mutant type II neuroblast clone in the *erm* mutant brain contained fewer supernumerary type II neuroblasts but more GMCs and neurons as compared to the control clone (Figure 2.2C–E). Together, these data strongly suggest that *trx* mutant type II neuroblasts lost the competence to generate immature INPs.

We directly tested whether *trx* mutant type II neuroblasts adopt a type I neuroblast functional identity and directly generate GMCs. Pros segregates exclusively into GMCs where it suppresses a type I neuroblast functional identity during asymmetric division of a type I neuroblast, but is undetectable in mitotic type II neuroblasts (Knoblich et al., 1995; Spana and Doe, 1995; Choksi et al., 2006; Bayraktar et al., 2010). In a telophase *trx* mutant type II neuroblast, however, Pros localized asymmetrically in the basal cortex and segregated uniquely into the cortex of the

future non-neuroblast progeny (Figure 2.2F–H). Most importantly, removing pros function in *trx* mutant type II neuroblasts leads to the formation of supernumerary type I neuroblasts (Figure 2.2I). These data confirm that *trx* mutant type II neuroblasts adopt a type I neuroblast functional identity and directly generate GMCs. Thus, we conclude that *trx* regulates neuroblast heterogeneity by maintaining a type II neuroblast functional identity.

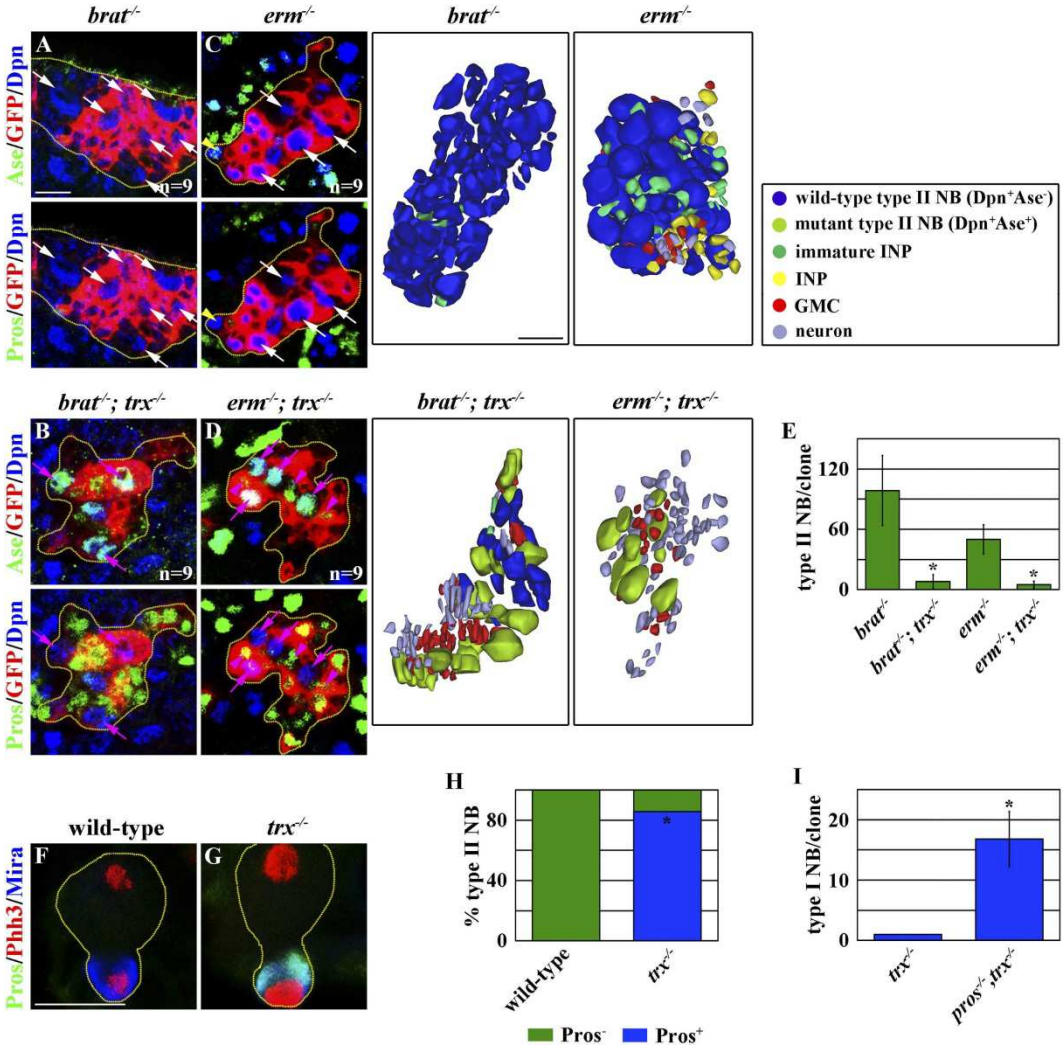

Figure 2.2 *trx* mutant type II neuroblast directly generates GMCs

(**A–E**) *trx* is required for the expansion of supernumerary type II neuroblasts in the *brat* or *erm* mutant. (A–D) Removing *trx* function suppresses the expansion of supernumerary type II neuroblasts and restores differentiation in the 96-hr *brat* or *erm* mutant type II neuroblast clones. Three-dimensionally reconstructed images of the clones are shown to the right. Scale bar, 10 μm. (E) The average number of type II neuroblasts per clone of the indicated genotypes.

(**F–I**) *trx* mutant type II neuroblasts exclusively distribute Pros to their progenies to specify GMC identity. (F–G) In the 48-hr clones, a wild-type type II neuroblast shows undetectable expression of Pros in telophase, whereas a *trx* mutant type II neuroblast shows the basal cortical localization of Pros. Scale bar, 10 μm. (H) The frequency of wild-type or *trx* mutant mitotic type II neuroblasts displaying the basal localization of Pros. (I) The average number of type I neuroblasts per type II neuroblast clone of the indicated genotypes at 72 hr after clone induction.

Trx maintains the type II neuroblast functional identity through the histone methyltransferase activity of the SET1/MLL complex

We assessed whether the histone methylation activity of Trx is required for maintaining a type II neuroblast functional identity. We induced mosaic clones derived from type II neuroblasts carrying the trx^{Z11} allele, which results in a missense mutation in the SET domain of Trx and reduces the histone methyltransferase activity of the Trx protein (Smith et al., 2004; Tie et al., 2014). Twenty-seven percent of trx^{Z11} type II neuroblasts assumed a type I neuroblast functional identity as determined by both the expression of a type I neuroblast marker expression profile and the generation of GMCs (Figure 2.3A–B). This result indicates that the histone methylation activity of Trx is essential for the maintenance of a type II neuroblast functional identity. Trx was co-purified with the core components of the SET1/MLL complex, Ash2, Rbbp5, and Wds, from the lysate extracted from S2 cells (Mohan et al., 2011). Thus, we tested whether the core components of the SET1/MLL complex are required for maintaining a type II neuroblast identity. Indeed, knocking down the function of *ash2*, *rbbp5*, or *wds* individually leads to fewer type II neuroblasts and INPs per brain lobe, identical to reducing *trx* function (Figure 2.3—2.3S1A–G). Together, these data strongly support our hypothesis that Trx maintains a type II neuroblast functional identity through the SET1/MLL complex via a mechanism dependent of the histone methyltransferase activity.

We focus on the Rbbp5 protein, which is essential for eliciting the histone methyltransferase activity of the SET1/MLL complex (Cao et al., 2010), to test whether Trx maintains a type II neuroblast functional identity through the SET1/MLL complex. We first generated a null allele of the *rbbp5* gene ($rbbp5^{null}$) by excising a transposable P-element inserted at the 5′ end from the transcription start site (Figure 2.3—2.3S2A). Mutant analyses confirmed that $rbbp5^{null}$ type II neuroblasts indeed adopt a type I neuroblast functional identity (Figure 2.3C–F, Table 2.1, Figure 2.3—2.3S2B). Thus, a $rbbp5^{null}$ type II neuroblast is phenotypically indistinguishable from a *trx* mutant type II neuroblast. We next

examined the H3K4 methylation pattern in the *rbbp5*^{null} type II neuroblast. All cells in the clones derived from single *rbbp5*^{null} type II neuroblast showed undetectable mono- and tri-methylation of H3K4 (Figure 2.3G, data not presented). This result is consistent with the SET1/MLL complex exerting its regulatory functions through the H3K4 methylation. Most importantly, over-expression of a UAS-*rbbp5*^{FL} transgene that encodes a full-length Rbbp5 completely restored a type II neuroblast functional identity and significantly restored both the H3K4 mono- and tri-methylation in *rbbp5*^{null} type II neuroblasts (Figure 2.3F,H–I, Figure 2.3—2.3S2B, data not presented). By contrast, over-expression of a UAS-*rbbp5*^{SG} transgene, which encodes a mutant Rbbp5 protein predicted to perturb the histone methyltransferase activity of the SET1/MLL complex (Figure 2.3—2.3S2C) (Cao et al., 2010), failed to restore a type II neuroblast functional identity and the methylation of H3K4 in *rbbp5*^{null} type II neuroblasts (Figure 2.3F,J–K, Figure 2.3—2.3S2B, data not presented). Similarly, type II neuroblasts bearing a strong *ash2* mutant allele also adopted a type I neuroblast functional identity and lost most H3K4 methylation based on the same criteria (data not presented). Thus, the histone methyltransferase activity of the SET1/MLL complex is required for the maintenance of a type II neuroblast identity. We conclude that Trx maintains a functional identity of type II neuroblasts through the histone methylation activity of the SET1/MLL complex.

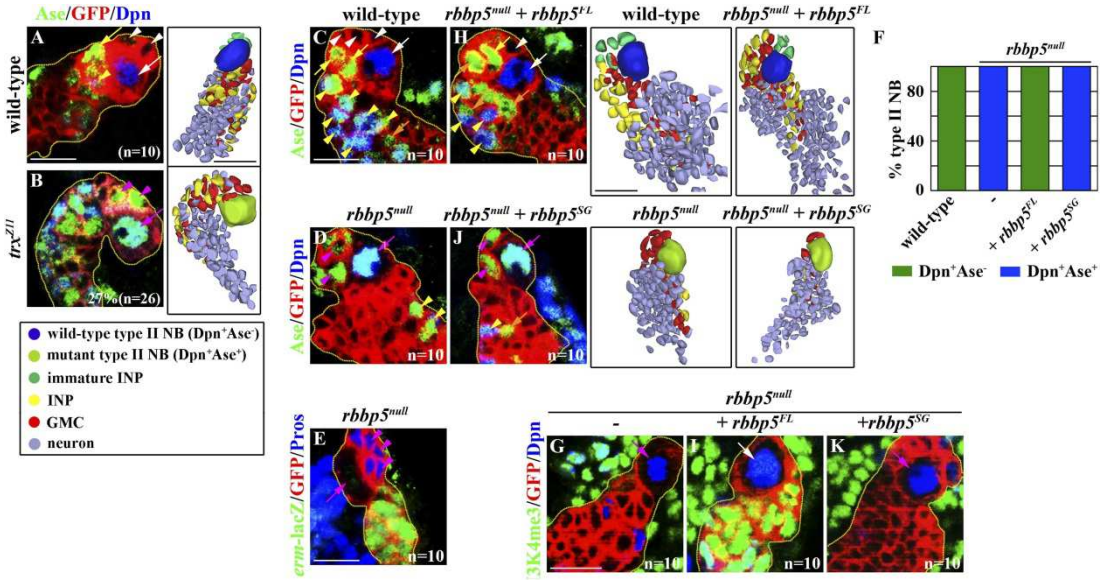

Figure 2.3 Trx and the core components of the SET/MLL complex maintain a type II neurobalst functional identity dependently on their catalytic activity for H3K4 methylaiton

(**A–B**) The function of *trx* for the H3K4 methylation is required for the maintenance of a type II neuroblast functional identity. In the 72-hr clones, a trx^{Z11} mutant type II neuroblast displays a type I neuroblast marker expression profile and directly generates GMCs. Scale bar, 10 μm. Three-dimensionally reconstructed images of the clones are shown to the right.

(**C–K**) The function of *rbbp5* for the H3K4 methylation is required for the maintenance of a type II neuroblast functional identity. (C–E, H, J) In the 96-hr clones, $rbbp5^{null}$ type II neuroblasts display a type I neuroblast marker expression profile and directly generate GMCs. Over-expression of $rbbp5^{FL}$ but not $rbbp5^{SG}$ restores a type II neuroblast functional identity in $rbbp5^{null}$ type II neuroblasts. Three-dimensionally reconstructed images of the clones are shown to the right. Scale bar, 10 μm. (F) The frequency of type II neuroblasts of the indicated genotypes displaying the type I or type II marker expression profiles. (G, I, K) *rbbp5* function is essential for the H3K4 methylation in fly larval brains. Scale bar, 10 μm.

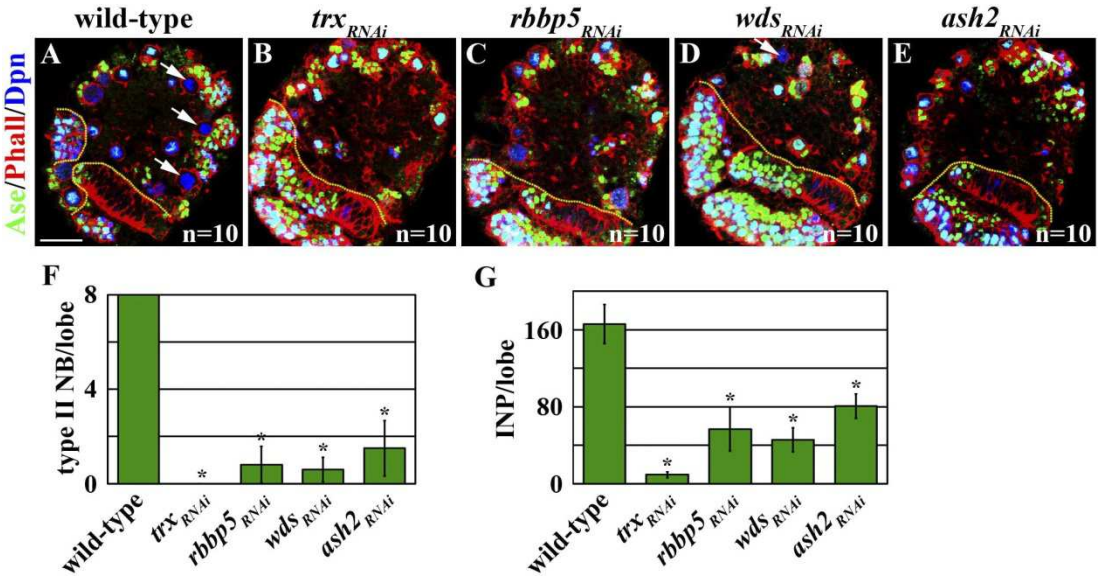

Figure 2.3S1 Decreasing the function of the core components of the SET1/MLL complex leads to a reduction in type II neuroblasts

(A–E) Knocking down the function of *trx*, *rbbp5*, *wds* or *ash2* specifically reduces the number of type II neuroblasts per brain lobe. Scale bar, 20 μm.

(F–G) The average number of type II neuroblasts or INPs per brain lobe of the indicated genotypes after knocking down the function of *trx*, *rbbp5*, *wds*, or *ash2* for 72 hr

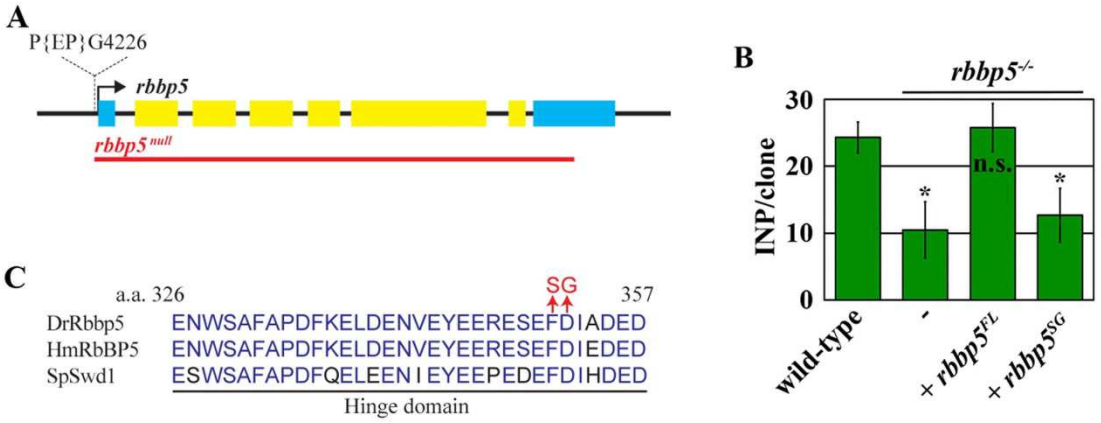

Figure 2.3S2 Generation of the *rbbp5*^{null} allele and the UAS-*rbbp5*^{SG} transgene

(A) The genomic organization of the *rbbp5* locus. The *rbbp5*^{null} allele was generated via imprecise excision of the P(EP)G4226 element, which removes the entire *rbbp5* coding region. Yellow squares indicate the coding exons of *rbbp5* while blue squares indicate the untranslated regions. The red line indicates the molecular lesion induced by the *rbbp5*^{null} allele.

(B) The average number of INPs per clone of the indicated genotypes at 96 hr after clone induction.

(C) An alignment of the hinge region of the yeast, fly, and human Rbbp5 protein. The amino acid substitutions in the Rbbp5^{SG} transgenic protein are indicated in red.

Trx regulates a type II neuroblast functional identity by maintaining an active chromatin state in the btd locus

Knocking down the function of *trr* or *dset1* drastically reduced the global H3K4 mono- or tri-methylation in type II neuroblasts but had no effects on the maintenance of their functional identity (Figure 2.4—2.4S1A–J). By contrast, removing *trx* function had no appreciable effects on the global H3K4 pattern in type II neuroblasts (Figure 2.4—2.4S1K–N). These data led us to hypothesize that Trx maintains the type II neuroblast functional identity by regulating a small number of genes that are specifically expressed in the type II neuroblast. We compared gene transcription profiles by using mRNAs isolated from dissected larval brains enriched with type I or II neuroblasts to identify the candidate Trx target genes (Bowman et al., 2008; Weng et al., 2010; Carney et al., 2012; Haenfler et al., 2012). *pnt* and *btd* were among a small number of genes that were dramatically up-regulated in the mRNAs isolated from larval brains enriched with type II neuroblasts as compared to the mRNAs isolated from larval brains enriched with type I neuroblasts. We confirmed that both *pntP1* and *btd* transcripts were indeed highly enriched in the brain lysate enriched with type II neuroblasts by qRT-PCR (Figure 2.4A). Furthermore, we detected the binding of Trx to the transcription start site for both the *pntP1* and *btd* transcription units (Figure 2.4B, Figure 2.4—2.4S2A). In addition, the promoter region of both the *pntP1* and *btd* transcription units also displayed a high level of H3K4 di-methylation, consistent with Trx-maintaining chromatin in an active state in these two loci through the H3K4 methylation (Figure 2.4B, Figure 2.4S2A). By contrast, we did not detect Trx binding to the negative control region located 7.5 kilobases 3′ from the *btd* transcription unit (Figure 2.4B; data not presented) (Petruk et al., 2012). Thus, both *pnt* and *btd* are the direct target genes of Trx.

We next tested whether either one of these two genes might regulate a functional identity of type II neuroblasts.

1. *pnt*: because the *pnt* locus encodes multiple alternatively spliced transcripts, we assessed the function of *pnt* in the type II neuroblast by over-expressing three independent *UAS-RNAi* transgenes targeting two different regions of the same exon shared by all *pnt* transcripts (Figure 2.4—2.4S2A). All three RNAi transgenes efficiently reduced *pnt* expression as indicated by a drastic reduction in the PntP1 protein (Figure 2.4—2.4S2B–C; data not presented). Unexpectedly, knocking down the function of *pnt* in type II neuroblasts led to the formation of supernumerary neuroblasts (Figure 2.4—2.4S2D–F). These results strongly suggest that *pnt* functions in the immature INP to promote INP identity specification similar to *brat* and *erm*. Consistently, heterozygosity of the *pnt* locus strongly enhanced the supernumerary neuroblast phenotype in the *brat* or *erm* hypomorphic brain (Figure 2.4—2.4S2G). In addition, overexpression of *pntP1* failed to restore a type II neuroblast functional identity in *trx* mutant type II neuroblasts (data not presented). Thus, we conclude that *pnt* functions downstream of *trx* to specify an INP identity in the immature INP rather than to maintain the type II neuroblast functional identity.
2. *btd*: a specific antibody against Btd is currently unavailable, and a genomic transgene that carries a BAC clone containing the entire *btd* locus led to embryonic lethality (Komori and Lee, unpublished). Thus, we determined the spatial expression pattern of the *btd* gene by examining the expression of a *btd-Gal4* transgene containing an enhancer element that was bound by Trx and displayed a high level of the di-methylation of H3K4 located 5 Kb upstream from the *btd* transcription start site (Figure 2.4B). The expression of a UAS reporter transgene driven by *btd-Gal4* was detected specifically in type II neuroblasts but was undetectable in type I neuroblasts in wild-type brains (Figure 2.4C). Importantly, the expression of *btd-Gal4* was drastically reduced in $rbbp5^{null}$ mutant brains (Figure 2.4D). Together, these data strongly support our hypothesis that *btd* is an excellent candidate for

functioning downstream of *trx* to maintain the type II neuroblast functional identity.

If Trx maintains a type II neuroblast functional identity by regulating *btd* transcription, removing *btd* function should trigger type II neuroblasts to adopt a type I neuroblast functional identity. We assessed the identities of cells in the clones derived from single *btd* mutant type II neuroblasts by examining cell fate marker expression. *btd* mutant type II neuroblasts maintained a type II neuroblast marker expression profile in all stages examined, but these clones displayed a time-dependent reduction in INPs (Figure 2.4F–G). Unlike the control clone, however, INPs in the 72-hr *btd* mutant clone were always located at the extreme distal end of the clone (data not presented). In these clones, *btd* mutant type II neuroblasts were surrounded by 1–2 progeny resembling Ase^{-} immature INPs but never Ase^{+} immature INPs (Figure 2.4F). Instead, the remaining cells directly adjacent to the *btd* mutant type II neuroblast displayed a marker expression profile indicative of GMCs and immature neurons that are normally found in the type I neuroblast lineage (Figure 2.4F,H). These observations prompted us to test whether the progeny of the *btd* mutant type II neuroblast resembling Ase^{-} immature INPs were indeed functional by examining their dependency on *brat* function. In the *brat* mutant type II neuroblast clone, Ase^{-} immature INPs rapidly reverted to supernumerary neuroblasts (Figure 2.4I) (Xiao et al., 2012; Komori et al., 2014). Most importantly, we never detected supernumerary neuroblast formation in the *btd*, *brat* double type II neuroblast clone, indicating that the direct progeny of the *btd* mutant type II neuroblast were insensitive to the loss of *brat* function (Figure 2.4J). These data led us to conclude that *btd* mutant type II neuroblasts generate non-functional Ase^{-} immature INPs that likely adopt an identity of GMCs normally found in the type I neuroblast lineage. Thus, we conclude that Trx most likely maintains the type II neuroblast functional identity through *btd*.

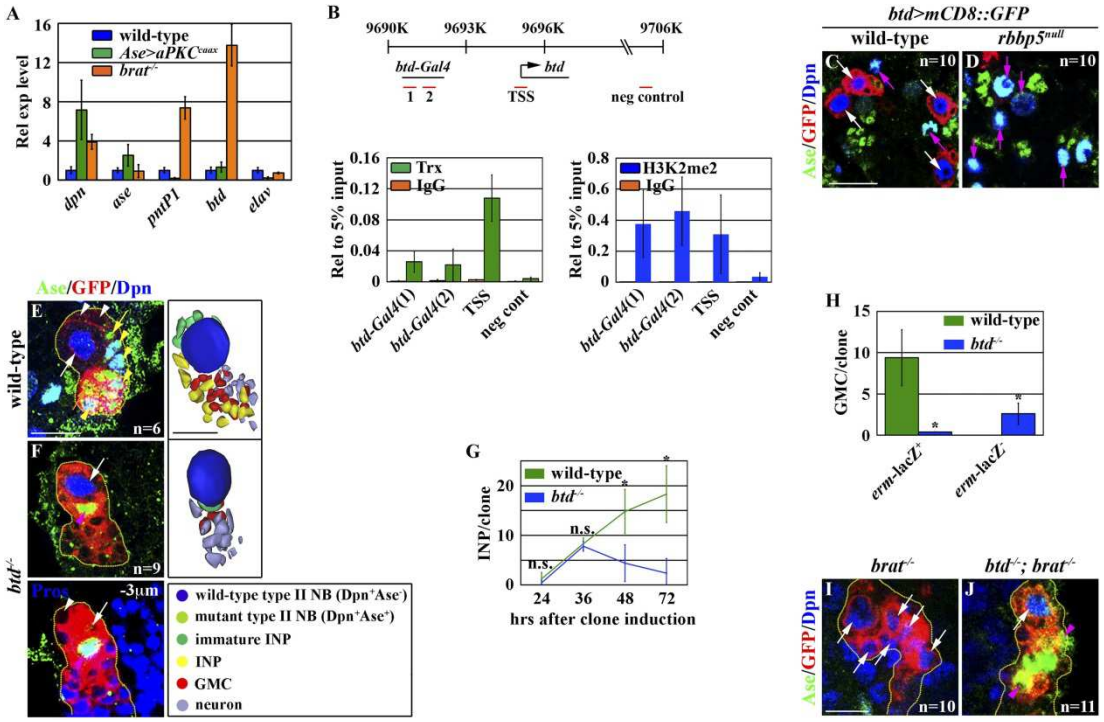

Figure 2.4 Btd likely acts downstream of Trx to maintain a type II neuroblast functional identity

(**A–D**) The *btd* gene is an excellent candidate target of Trx in the type II neuroblast. (A) The *btd* mRNA is highly enriched in the lysate extracted from larval brain enriched with type II neuroblasts. The *elav* transcript is highly enriched in differentiated neurons. The quantification represents the average of three biological replicates. (B) Trx directly binds to the type II neuroblast-specific enhancer element as well as the transcription start site (TSS) of the *btd* gene. The ChIP experiments were performed using the extract isolated from dissected *brat* mutant brains that are enriched with type II neuroblasts. Quantification of chromatin immunoprecipitated by the indicated antibodies relative to 5% of input. The quantification represents the average of three biological replicates. (C–D) An enhancer element from the *btd* gene is sufficient to induce type II neuroblast-specific expression of a UAS-mCD8::gfp reporter transgene in wild-type brain, while the enhancer activity of *btd*-Gal4 was reduced in *rbbp5*^{null} brain. Scale bar, 20 μm.

(**E–H**) *btd* is required for maintaining the functional identity but not the molecular signature of a type II neuroblast. (E–F) In the 72-hr clones, *btd* mutant type II neuroblasts maintain a type II neuroblast marker expression profile and are surrounded by 1–2 immature INP-like cells. Three-dimensionally reconstructed images of the clones are shown below. Scale bar, 10 μm. (G) The average number of INPs per clone of the indicated genotypes. (H) The average number of GMCs with or without *erm*-lacZ expression per type II neuroblast clones of the indicated genotypes at 72 hr after clone induction.

(**I–J**) The immature INP-like cells generated by *btd* mutant type II neuroblasts are insensitive to loss of *brat* function. Removing *brat* function does not lead to supernumerary neuroblast formation in the 72-hr *btd* mutant type II neuroblast clones. Scale bar, 10 μm.

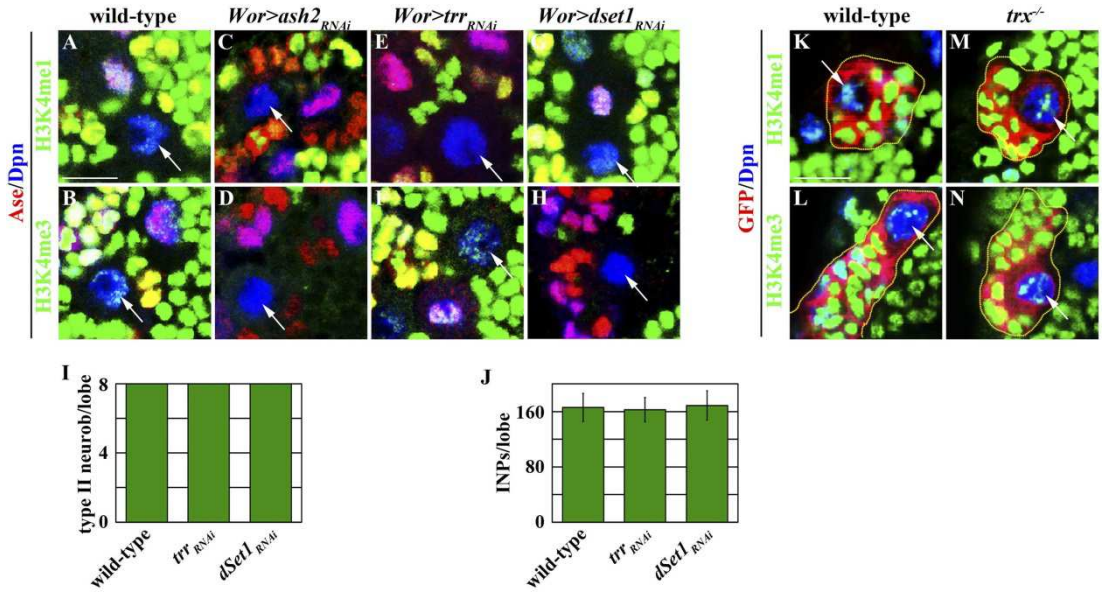

Figure 2.4S1 Global H3K4 mono- or tri-methylation is not required for maintenance of a type II neuroblast functional identity

(**A–H**) The core component of the SET1/MLL complex is required for the global methylation of H3K4. (A, C, E, G) Knocking down the function of *ash2* or *trr* leads to global loss of the H3K4 mono-methylation while knocking down the function of dSet1 does not. Scale bar, 10 μm. (B, D, F, H) Knocking down the function of *ash2* or dSet1 leads to global loss of the H3K4 mono-methylation while knocking down the function of *trr* does not.

(**I–J**) *trr* and dSet1 are dispensable for the maintenance of type II neuroblasts. The average number of type II neuroblasts or INPs per brain lobe of the indicated genotypes after knocking down the function of *trr* or dSet1 for 72 hr.

(**K–N**) *trx* mutant type II neuroblasts do not display appreciable reduction in the global methylation pattern. Scale bar, 10 μm

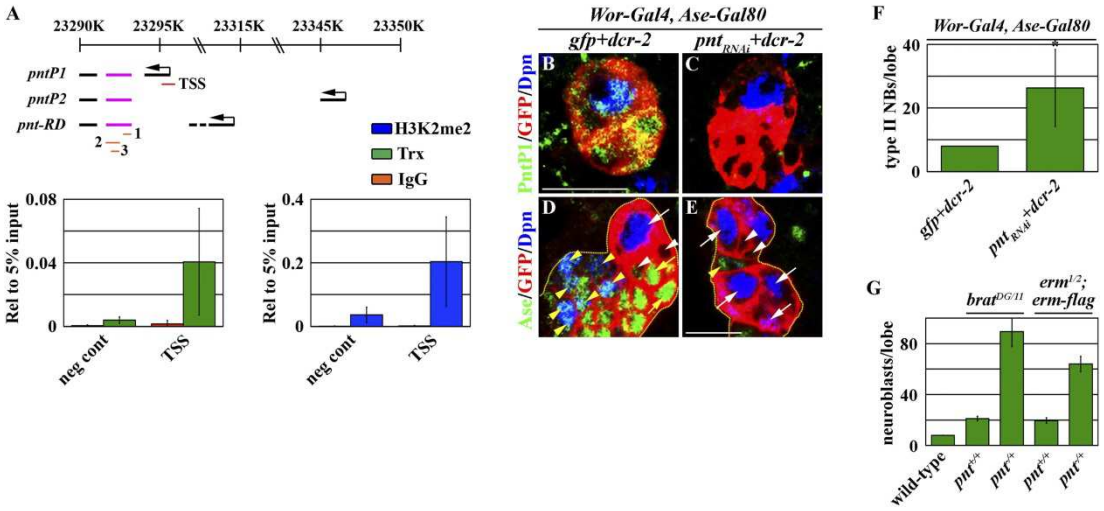

Figure 2.4S2 Pnt likely functions to specify an INP identity

(**A**) Trx directly binds to transcription start site (TSS) of the pntP1 transcript. Quantification of chromatin immunoprecipitated by the indicated antibodies relative to 5% of input. The quantification represents the average of three biological replicates. The black lines indicate three different *pn*t transcripts. The magenta lines indicate three *UAS-RNAi* used to target the common exon of *pnt* transcripts. (1) *UAS-pntRNAi* (7171), (2) *UAS-pntRNAi* (TRiP.JF02227), and (3) *UAS-pntRNAi* (TRiP.HMSO1452).

(**B–C**) Expression of the *UAS-pntRNAi* transgene efficiently reduces PntP1 protein expression throughout the type II neuroblast lineage.

(**D–E**) Knocking down the function of *pnt* induces supernumerary neuroblast formation. Scale bar, 10 μm.

(**F–G**) The average number of type II neuroblasts per clone of the indicated genotypes.

Over-expression of *btd* is sufficient to trigger a type I neuroblast to generate INPs

Because *btd* is necessary for the maintenance of a type II neuroblast functional identity, we tested whether over-expression of *btd* is sufficient to induce a type II neuroblast functional identity in a type I neuroblast. We induced GFP-marked lineage clones derived from single type I neuroblasts mis-expressing a *UAS-btd* transgene and assessed the identities of cells in the clones by examining the expression of cell fate markers. In the control clones, type I neuroblasts maintained Ase expression and generated GMCs (Figure 2.5A). Eighteen percent of type I neuroblasts mis-expressing *btd* lost Ase expression and generated progeny displaying a marker expression profile that is typically diagnostic of an immature INP or an INP (Figure 2.5B,D). Another 10% of type I neuroblasts mis-expressing *btd* generated progeny that resembled immature INPs or INPs by marker expression, but maintained Ase expression (Figure 2.5C). Thus, we conclude that mis-expression of *btd* is sufficient to trigger the characteristics that are specific for a type II neuroblast in a type I neuroblast.

We extended our analysis to assess whether mis-expression of *btd* might endow a type I neuroblast with the functional feature unique to a type II neuroblast—the competence to generate INPs. We reasoned that if a type I neuroblast mis-expressing *btd* indeed assumes a type II neuroblast functional identity, it should be able to generate immature INPs capable of maturing into an INP, a process critically dependent on the function of *brat* and *erm*. While removing *brat* function had no effects on the identities of progeny derived from control type I neuroblasts, it led to supernumerary type II neuroblast formation in the lineage clones derived from single type I neuroblasts mis-expressing *btd* (Figure 2.5E–F). Similarly, removing *erm* function also led to supernumerary type II neuroblast formation in the lineage clones derived from single type I neuroblast mis-expressing *btd* while not having any effects on the control type I neuroblast clones (Figure 2.5G–H). Since *brat* and *erm* function specifically in the immature INP to promote an INP identity (Xiao et al.,

2012; Janssens et al., 2014; Komori et al., 2014), these data strongly suggest that mis-expression of *btd* was sufficient to endow a type I neuroblast with the competence to generate INPs. Thus, we conclude that *btd* plays an important role in eliciting the functional identity of a type II neuroblast.

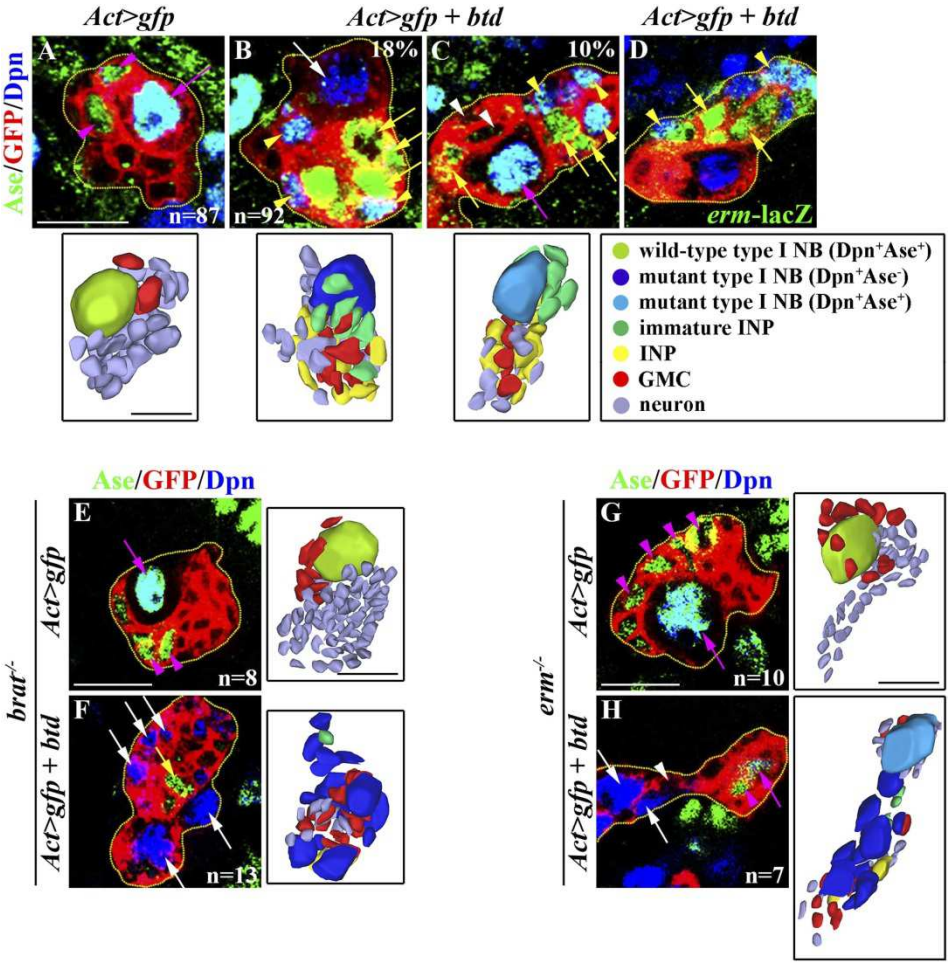

Figure 2.5 Over-expression of *btd* is sufficient to instruct a type II neuroblast functional identity in the type I neuroblast

(**A–D**) Over-expression of *btd* is sufficient to elicit a type II neuroblast functional identity. In the 72-hr clones, 18% of type I neuroblasts over-expressing *btd* lose Ase expression and are surrounded by INP-like cells. An additional 10% of these neuroblasts maintain Ase expression despite being surrounded by INP-like cells. Three-dimensionally reconstructed images of the clones are shown to the right. Scale bar, 10 μm.

(**E–H**) Progeny of type I neuroblasts over-expressing *btd* revert back to supernumerary neuroblast in the *brat* mutant or *erm* mutant. In the 72-hr clones, removing *brat* or *erm* function induces the formation of supernumerary type II neuroblasts derived from the progeny of type I neuroblasts over-expressing *btd*. Three-dimensionally reconstructed images of clones are shown to the right. Scale bar, 10 μm.

Btd mediates Trx-dependent maintenance of a type II neuroblast functional identity

Finally, we tested whether Trx maintains the type II neuroblast functional identity through *btd*. Consistent with our hypothesis, 40% of *trx* mutant type II over-expressing *btd* regained the characteristics that are specific for a type II neuroblast including loss of Ase expression and the generation of immature INPs and INPs (Figure 2.6A–C). Furthermore, over-expression of *btd* also significantly enabled *trx* mutant type II neuroblasts to generate INPs (Figure 2.6D). Thus, we conclude that *btd* is a key downstream target gene of Trx in the maintenance of the type II neuroblast functional identity.

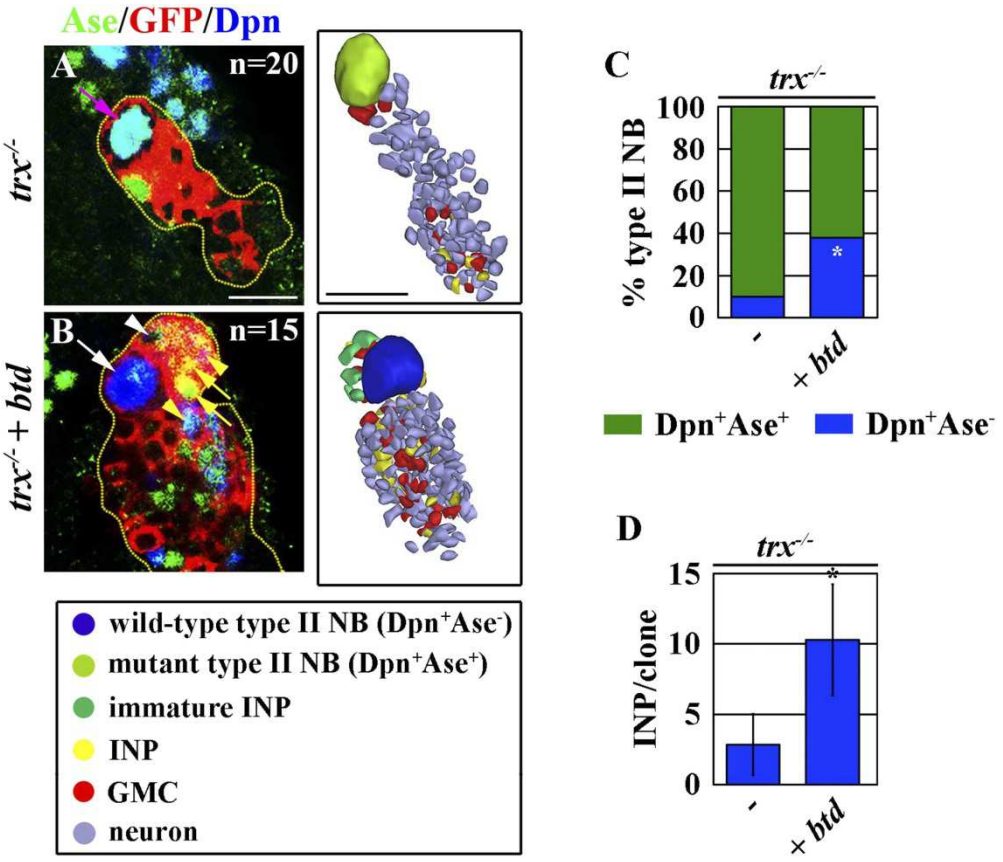

Figure 2.6 Over-expression of *btd* restores a type II neuroblast functional identity in *trx* mutant type II neuroblasts.

(**A–D**) Overexpression of *btd* reinstates the ability to generate INPs in *trx* mutant type II neuroblasts. (A–B) In the 72-hr clones, while the control *trx* mutant type II neuroblasts are surrounded by GMCs, *trx* mutant type II neuroblasts over-expressing *btd* are surrounded by INP progeny. Three-dimensionally reconstructed images of the clones are shown to the right. Scale bar, 10 μm. (C) The neuroblast marker expression profile displayed by type II neuroblasts of the indicated genotypes. (D) The average number of INPs per clone of the indicated genotypes.

Discussion

Maintaining functionally distinct stem cell populations allows higher organisms to generate the requisite number of diverse cell types required for organogenesis. For example, neural stem cells in the subventricular zone and in the outer subventricular zone collectively contribute to the generation of all the cell types required for the development of a human brain (Fietz et al., 2010; Hansen et al., 2010). Similarly, heterogeneous stem cell pools have also been reported in other organs including the blood and intestine (Barker et al., 2007; Graf and Stadtfeld, 2008; Copley et al., 2012; Marianes and Spradling, 2013). Although the mechanisms that specify the identity of distinct stem cell types within a given organ have been proposed, the mechanisms that maintain the functional heterogeneity of stem cells have never been reported. In this study, we used the two well defined and functionally distinct types of neuroblasts in the fly larval brain to investigate the mechanisms that maintain stem cell functional heterogeneity during neurogenesis. We discovered that Trx functions uniquely to maintain a type II neuroblast identity through the H3K4 methylation activity of the SET1/MLL complex, thereby contributing to neuroblast heterogeneity during larval brain neurogenesis. We identified the homeodomain transcription factor Btd as a direct downstream target of Trx in the maintenance of a type II neuroblast identity. To our knowledge, this Trx-Btd-dependent mechanism provides the first mechanistic insight into the maintenance of stem cell functional heterogeneity within an organ (Figure 2.7). The homologs of Trx and Btd have been shown to play critical roles in regulating vertebrate neural stem cell functions (Lim et al., 2009; MuhChyi et al., 2013). Our findings lead us to speculate that the SET1/MLL histone methyltransferase complex might also contribute to the maintenance of stem cell heterogeneity in other higher eukaryotes.

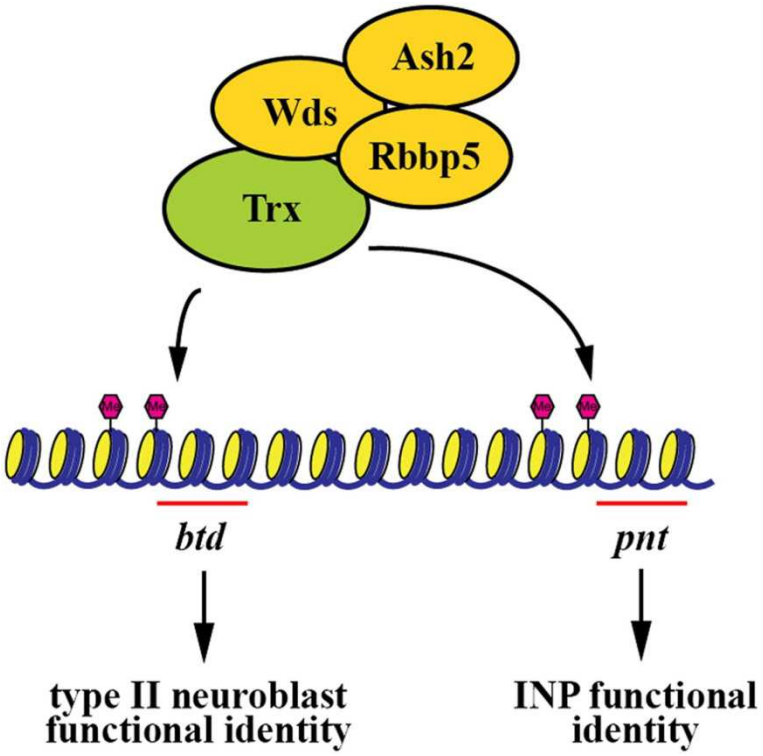

Figure 2.7 A summary model.

The Trx histone methyltransferase complex maintains the type II neuroblast functional identity through the *btd* gene whereas it promotes INP identity specification through the *pnt* gene.

Trx maintains the type II neuroblast functional identity through the H3K4 methylation activity of the SET1/MLL complex

The SET1/MLL complex elicits biological responses by maintaining its target genes in an active state through the methylation of H3K4 (Shilatifard, 2012). Our data showed that the core components of the SET1/MLL complex is required for the maintenance of the H3K4 methylation in a type II neuroblast and the maintenance of a type II neuroblast functional identity (Figure 2.3C–D,F, Figure 2.3S1). Most importantly, over-expression of *rbbp5*^{FL}, but not *rbbp5*^{SG}, which encodes a mutant Rbbp5 protein that partially compromises the H3K4 methylation activity of the SET1/MLL complex (Cao et al., 2010), restored both H3K4 methylation and a type II neuroblast functional identity in *rbbp5* null type II neuroblasts (Figure 3C–K). These results indicate that the H3K4 methylation activity of the SET1/MLL complex is required for maintaining the functional identity of a type II neuroblast. In the fly genome, Trx, Trr, and dSet1 can each bind to the core components of the SET1/MLL complex (Wu et al., 2008; Ardehali et al., 2011; Mohan et al., 2011). Although the methylation activity of Trx was required for maintaining the type II neuroblast functional identity, removing *trx* function did not alter the global H3K4 methylation (Figure 3A–B, Figure 4—figure supplement 1K–N). In contrast, knocking down the function of *trr* or *dset1* did not affect the maintenance of a type II neuroblast functional identity despite resulting in the global loss of H3K4 mono- or tri-methylation (Figure 4—figure supplement 1A–J). These data strongly suggest that Trx maintains a type II neuroblast functional identity by regulating H3K4 methylation in specific downstream target loci.

The Trx-Btd mechanism regulates the functional identity of a type II neuroblast

The functional identity of a type II neuroblast is defined by the competence of a neuroblast to generate INPs (Weng and Lee, 2011; Homem and Knoblich, 2012; Janssens and Lee, 2014). Our data indicate Trx plays a central role in maintaining the functional identity of a type II neuroblast by promoting the expression of a small

number of genes (Figures 2.1 and 2.4A). We identified the *btd* gene as a critical downstream target of Trx that is both necessary and sufficient for the regulation of the type II neuroblast functional identity (Figures 2.4–2.7). *btd* encodes a C_2H_2 zinc finger transcription factor required for proper patterning of the head segment during fly embryogenesis and likely functions as a transcription activator (Wimmer et al., 1993; Schöck et al., 1999). However, the role of Btd in regulating neuroblasts has never been established, and the mechanisms by which Btd elicits biological responses remain unclear. Several possible reasons exist to explain the relatively inefficient nature of eliciting the type II neuroblast functional identity in a type I neuroblast by the mis-expression of *btd* (Figure 2.5). First, certain co-factors might be required for Btd to efficiently activate its target gene transcription, and a lower abundance of these co-factors in type I neuroblasts hinders the functional output of mis-expressed Btd. Second, the epigenetic landscape might be vastly different between the two types of neuroblasts such that mis-expressed Btd may not have access to all of its target genes required to elicit the type II neuroblast functional identity in a type I neuroblast. Lastly, additional transcription factors might function in parallel with Btd to regulate the functional identity of a type II neuroblast. Btd is a highly conserved transcription factor (Estella and Mann, 2010; MuhChyi et al., 2013). Future studies to elucidate the mechanisms by which Btd regulates the functional identity of a type II neuroblast will provide critical insight in the regulation of neural stem cell heterogeneity during both invertebrate as well as vertebrate neurogenesis.

The Trx-Pnt mechanism specifies an INP identity in the type II neuroblast lineage

We identified the *pnt* gene as another direct downstream target of Trx (Figure 2.4A, Figure 2.4S2A). We initially hypothesized that Pnt might function in parallel with Btd to maintain the functional identity of a type II neuroblast. This hypothesis was extremely appealing in light of a previous study demonstrating mis-expression of PntP1 can transform a type I neuroblast into a type II neuroblast (Zhu et al., 2011).

Unexpectedly, knocking down the function of the *pnt* gene, which encodes at least three alternatively spliced transcripts, had no effect on the maintenance of the type II neuroblast functional identity, and instead, resulted in the formation of supernumerary type II neuroblasts (Figure 2.4—2.4S2). This result led us to revise our hypothesis and propose that Pnt functions in the immature INP to specify an INP identity. Consistently, heterozygosity of the *pnt* locus dominantly enhanced the supernumerary neuroblast in the *brat* or *erm* hypomorphic genetic background (Figure 2.4—2.4S2G). These two genetic backgrounds have been used extensively for elucidating the mechanisms that regulate the specification of an INP identity in the immature INP (Xiao et al., 2012; Janssens et al., 2014; Komori et al., 2014). Furthermore, over-expression of pntP1 failed to restore the functional identity of a type II neuroblast in *trx* mutant type II neuroblasts (data not presented). Together, these data strongly suggest that *pnt* mainly functions to specify an INP identity rather than to maintain the type II neuroblast functional identity. Thus, we propose that in addition to maintaining the type II neuroblast functional identity, Trx also functions to promote INP identity specification through pnt (Figure 2.7).

Attenuation of the competence to generate intermediate progenitor cells might provide a novel strategy to thwart the expansion of cancer stem cells

Strategies that uniquely target the functional properties of cancer stem cells will revolutionize cancer treatments. Cancer stem cells generate a hierarchy of progeny that include cell types directly contributing to the exponential expansion of cancer stem cells (Magee et al., 2012). Thus, reprogramming their functional identity to bypass the cell types that directly contribute to the exponential expansion of cancer stem cells should halt further tumor growth. In our study, removing *trx* function efficiently reduced the number of supernumerary type II neuroblasts, which are proposed to serve as cancer stem cells in several Drosophila brain tumor models (Caussinus and Gonzalez, 2005; Xiao et al., 2012; Eroglu et al., 2014; Janssens et al., 2014; Koe et al., 2014; Komori et al., 2014), and increased the number of differentiated cells in the *brat* or *erm* mutant brain (Figure 2.2). Similarly,

attenuating the competence of type II neuroblasts to generate INPs by removing *btd* function also efficiently halted the expansion of *brat* or *erm* mutant brain tumors (Figure 2.4I–J, data not presented). Our results strongly support the hypothesis that reprogramming the functional identity of putative cancer stem cells can significantly alter the course of tumorigenesis. As such, understanding the mechanisms that maintain stem cell heterogeneity during normal development might provide novel insight into designing rational therapies to promote switching of cancer stem cells to an alternative, non-cancerous stem cell type.

Materials and methods

Fly genetics and transgenes

Fly strains used in this study include Oregon R, Ase-Gal4 (Zhu et al., 2006), Ase-Gal80 (Neumüller et al., 2011), bratDG19310, bratk06028 and brat11 (Komori et al., 2014), erm1 and erm2 (Weng et al., 2010), erm-flag (Janssens et al., 2014), erm-lacZ and UAS-aPKCCAAX (Haenfler et al., 2012), pntΔ88 (Morimoto et al., 1996), trxZ11 (Tie et al., 2014), and Wor-Gal4 (Lee et al., 2006). The following stocks were obtained from the Bloomington Drosophila Stock Center: Elav-GAL4, Act-FRT-Stop-FRT-GAL4, ash21, btdXA, FRT19A, FRT2A, FRT82B, GMR85C07-GAL4 (Btd-GAL4), hs-flp, P(EP)G4226, pros17, UAS-pntRNAi (TRiP.JF02227), UAS-pntRNAi (TRiP.HMS01452), trxE2, tubP-Gal80, tubP-Gal80 ts, UAS-Dcr-2.D, UAS-mCD8-GFP, and UAS-trrRNAi (TRiP.JF03242). We obtained the following stocks from the Vienna Drosophila RNAi Center UAS-ash2RNAi (100718), UAS-dSet1RNAi (40683), UAS-pntRNAi (7171), UAS-rbbp5RNAi (106139), UAS-trxRNAi (108122), and UAS-wdsRNAi (105371). UAS-HA-btd, UAS-HA-pntP1, UAS-rbbp5FL-myc, and UAS-rbbp5SG-myc were generated in this study by cloning the cDNA cloned into p{UAST}attB vector. The transgenic fly lines were generated

via ϕC31 integrase-mediated transgenesis (Bischof and Basler, 2008). The rbbp5 null allele was generated by imprecisely excising the P(EP)G4226 element.

Clonal analyses

Clones were induced following previously published methods (Janssens et al., 2014). Three-dimensional model of clones was generated using the Mimics software from Materialize, Leuven, Belgium. Confocal images were acquired using a Z-step size of 1.5 μm, and the identity of every cell within a clone was determined individually.

Immunofluorescent staining and antibodies

Larvae brains were dissected in Schneider's medium (Sigma, St. Louis, MO) and fixed in 100 mM Pipes (pH 6.9), 1 mM EGTA, 0.3% Triton X-100, and 1 mM MgSO4 containing 4% formaldehyde for 23 min. Larval brains were processed for immunofluorescent staining according to a previously published protocol (Weng et al., 2012). Antibodies used in this study include chicken anti-GFP (1:2000; Aves Labs, Tigard, OR), guinea pig anti-Ase (1:1000; Wang H), mouse anti-cMyc (1:100 Roche, Basel, Switzerland), mouse anti-Pros (MR1A; 1:500; DSHB, Iowa city, IA), rabbit anti-Ase (1:400), rabbit anti-β-gal (1:1000; MP Biomedicals, Santa Ana, CA), rabbit anti-H3K4me1 (1:500; Abcam, Cambridge, United Kingdom), rabbit anti-H3K4me3 (1:500; Active motif, Carlsbad, CA), rabbit anti-Phospho-Histone-H3(Ser10) (1:1000; EMD Millipore, Billerica, MA), rabbit anti-PntP1 (1:600; Skeath JB), rat anti-Dpn (1:2), rat anti-Mira (1:500). Secondary antibodies were from Jackson ImmunoResearch Inc., West Grove, PA. The confocal images were acquired on a Leica SP5 scanning confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL).

Chromatin immunoprecipitation

To obtain more than 2 × 106 supernumerary type II neuroblasts, we dissected 100 brains from brat mutant larvae aged for 4 days at 33°C in Schneider's medium (Sigma, St. Louis, MO) and fixed in 1.8% formaldehyde solution for 20 min. We

stopped fixation by incubating the lysate with Glycine (0.25 M) at room temperature for 4 min and on ice for 10 min. Following fixation, samples were washed with wash buffer (1xPBS, 5 mM Tris–HCl pH7.5, 1 mM EDTA) containing proteinase inhibitors (Roche, Basel, Switzerland) and 1 mM PMSF for three times and homogenized in SDS lysis buffer (1% SDS, 50 mM Tris–HCl pH8.1, 10 mM EDTA) to obtain nuclear extracts. The nuclear extracts were disrupted by using a sonicator (18 cycles of sonicating for 30 s and interval for 30 s). Five percent of the sonicated sample was stored for INPUT. The rest of the sonicated chromatin was incubated with antibodies in ChIP dilution buffer (0.01% SDS, 1.1% Trition X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH8.1, 167 mM NaCl) at 4°C overnight. Samples were incubated with Dynal beads (Life technologies, Grand Island, NY) at 4°C overnight, washed twice with low salt immune complex wash buffer (0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris–HCl pH8.1, 150 mM NaCl), twice with high salt immune complex wash buffer (0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris–HCl pH8.1, 500 mM NaCl), three times with LiCl immune complex wash buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl pH8.1), twice with TE buffer, and then were eluted from beads. Cross-linking of chromatin–protein complex was reverted at 65°C overnight. Samples were treated with RNase A at 55°C for 2 hr and incubated with 2 μg of proteinase K at 45°C for 1 hr. Samples were cleaned up by phenol:chloroform and precipitated by EtOH precipitation. Samples were resuspended in 100 μl of water. 5 μl were used in each qPCR reaction. Antibodies used in this experiment were anti-Trx antibody (Mazo A), anti-H3K4me2 (07–030; Millipre, Billerica, MA), and rabbit IgG (ab46540; Abcam). The following individual specific primer sets were used for quantitative PCR: btd-E1, 5′-gttggccattgcgtgtcctgtttc-3′ and 5′-gccccgctgcgctctatcca-3′, btd-E2, 5′-ggattaccgcagacgat-3′ and 5′-ggttggccggtggttgagt-3′, btd-TSS, 5′-cagcagcagcagcagcaacagt-3′ and 5′-gtcggcccgggtccaagtaa-3′, negative control, 5′-cagcagcagcagcagcaacagt-3′ and 5′-gtcggcccgggtccaagtaa-3′, pntP1-TSS, 5′-tttggtgttgttgtttttcttctt,-3′ and 5′-acgcgttctgttctgtttt-3′. Another negative control primer set was used in previously published paper (Petruk et al., 2012).

qRT-PCR

Total RNA was extracted following the standard Trizol RNA isolation protocol (Life technologies, Grand Island, NY) and cleaned by the RNeasy kit (Qiagen, Venlo, Netherlands). First strand cDNA was synthesized from the extracted total RNA using First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche, Basel, Switzerland). qPCR was performed using ABsolute QPCR SYBR Green ROX Mix (Thermo Fisher Scientific Inc., Waltham, MA). Data were analyzed by the comparative CT method, and the relative mRNA expression is presented. The following individual specific primer sets were used for quantitative PCR: ase, 5′-agcccgtgagcttctacgac-3′ and 5′-gcatcgatcatgctctcgtc-3′, btd, 5′-gcacggacgtacgcacaccaat-3′ and 5′-cctcggcggccaataccttct-3′, dpn, 5′-catcatgccgaacacaggtt-3′ and 5′-gaagattggccggaactgag-3′, elav, 5′-gcggcgcgtatcccattttcatct-3′ and 5′-tggccgcctcatcgtagttggtca-3′, pntP1, 5′-ggcagtacgggcagcaccac-3′ and 5′-ctcaacgcccccaccagatt-3′.

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CHAPTER III

klumpfuss distinguishes stem cells from progenitor cells during asymmetric neuroblast division

Summary

Asymmetric stem cell division balances maintenance of the stem cell pool and generation of diverse cell types by simultaneously allowing one daughter progeny to maintain a stem cell fate and its sibling to acquire a progenitor cell identity. A progenitor cell possesses restricted developmental potential, and defects in the regulation of progenitor cell potential can directly impinge on the maintenance of homeostasis and contribute to tumor initiation. Despite their importance, the molecular mechanisms underlying the precise regulation of restricted developmental potential in progenitor cells remain largely unknown. We used the type II neural stem cell (neuroblast) lineage in *Drosophila* larval brain as a genetic model system to investigate how an intermediate neural progenitor (INP) cell acquires restricted developmental potential. We identify the transcription factor Klumpfuss (Klu) as distinguishing a type II neuroblast from an INP in larval brains. *klu* functions to maintain the identity of type II neuroblasts, and *klu* mutant larval brains show progressive loss of type II neuroblasts due to premature differentiation. Consistently, Klu protein is detected in type II neuroblasts but is undetectable in immature INPs. Misexpression of *klu* triggers immature INPs to revert to type II neuroblasts. In larval brains lacking *brain tumor* function or exhibiting constitutively activated Notch signaling, removal of *klu* function prevents the reversion of immature INPs. These results led us to propose that multiple mechanisms converge to exert precise control of *klu* and distinguish a progenitor cell from its sibling stem cell during asymmetric neuroblast division.

Introduction

Asymmetric stem cell divisions provide an efficient mechanism for maintaining a steady stem cell pool while generating progenitor cells that give rise to differentiated progeny within the tissue where the stem cells reside (Morrison and Kimble, 2006; Pontious et al., 2008; Kriegstein and Alvarez-Buylla, 2009; Knoblich, 2010; Weng and Lee, 2011). Progenitor cells possess restricted developmental potential and function to protect the genomic integrity of stem cells by minimizing their proliferation. Since both daughter cells inherit the cellular content from their parental stem cell during asymmetric division, proper specification of sibling cell identity requires precise control of stem cell determinants. Failure to properly downregulate stem cell determinants in presumptive progenitor cells might allow them to acquire stem cell-like functional properties, and can perturb tissue homeostasis and contribute to tumor formation (Krivtsov et al., 2006; Wei et al., 2008). Thus, mechanistic insight into how the sibling cells assume distinct identities during asymmetric stem cell division is likely to advance our knowledge in stem cell biology, developmental biology and tumor biology.

In fly larval brains, two classes of neuroblast lineage can be unambiguously identified based on the expression of cell fate markers and the properties of their progeny (Chia et al., 2008; Doe, 2008; Egger et al., 2008; Knoblich, 2010; Weng and Lee, 2011). A type I neuroblast expresses Deadpan (Dpn) and Asense (Ase) and divides asymmetrically to self-renew and to generate a progenitor cell called a ganglion mother cell (GMC). By contrast, a type II neuroblast (Dpn^+ Ase^-) divides asymmetrically to self-renew and to generate an immature intermediate neural progenitor (INP) that lacks the expression of Dpn and Ase and undergoes maturation during which it acquires an INP identity (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). Following maturation, an INP (Dpn^+ Ase^+) undergoes limited rounds of asymmetric division to regenerate and to produce GMCs. A key functional property that distinguishes these two neuroblast lineages rests on their dependence on Notch signaling for the maintenance of their identity (Bowman et al., 2008; Song

and Lu, 2011; Weng et al., 2011). Although dispensable for the maintenance of a type I neuroblast, Notch signaling is crucial for the maintenance of type II neuroblasts (Haenfler et al., 2012).

In mitotic type II neuroblasts, polarization of the cell cortex allows the basal proteins, including Brain tumor (Brat) and Numb, to segregate into the cortex of the presumptive immature INP and promote the formation of INPs (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006a; Lee et al., 2006c; Wang et al., 2006; Bowman et al., 2008; Wirtz-Peitz et al., 2008; Prehoda, 2009). Whereas a wild-type type II neuroblast is surrounded by three to five immature INPs and twenty to thirty INPs, a *brat* or *numb* mutant type II neuroblast is always surrounded by supernumerary neuroblasts at the expense of INPs. Thus, previous studies have proposed that *brat* and *numb* function in immature INPs, where these proteins promote the specification of an INP identity. However, the mechanisms by which *brat* and *numb* trigger an immature INP to assume the identity of an INP remain unknown.

In this study, we show that precise regulation of *klu* function is pivotal for distinguishing the self-renewing neuroblast from its sibling progenitor cell during asymmetric neuroblast division. Klu is necessary for the maintenance of type I and II brain neuroblasts, as *klu* mutant larvae showed progressive loss of both types of neuroblast. Klu is detected in all neuroblasts but is absent from their immediate daughter progenitor progeny. Misexpression of *klu* in immature INPs led to the formation of supernumerary type II neuroblasts. Importantly, removal of *klu* function prevented the reversion of immature INPs to type II neuroblasts triggered by the loss of *brat* function or constitutive activation of Notch signaling. Furthermore, overexpression of *klu* also exacerbated the reversion of GMCs to type I neuroblasts as triggered by the aberrant activation of Notch signaling. Together, we conclude that precise control of *klu* function by multiple signaling mechanisms distinguishes a neuroblast from a progenitor cell during asymmetric division of fly larval brain neuroblasts.

Results

klu functions to maintain the identity of larval brain neuroblasts

Brat is required cell-autonomously for the formation of INPs in larval brains (Betschinger et al., 2006; Lee et al., 2006c; Bowman et al., 2008). Thus, understanding how *brat* regulates the maturation of immature INPs will provide crucial insight into the mechanisms that distinguish the fates of sibling cells following the asymmetric division of type II neuroblasts. We assessed the identity of cells in the GFP-marked mosaic clones derived from a single wild-type or *brat* null mutant type II neuroblast using the onset of Ase expression as a marker for an intermediate stage during maturation (Figure 3.1S1A-B; see Discussion for more details). Each wild-type clone always contained one neuroblast surrounded by two to three Ase^{-} immature INPs, two to three Ase^{+} immature INPs, INPs and GMCs (Figure 3.1S1C-D″,H; n=7 per stage). By contrast, a similarly staged *brat* mutant clone consisted of mostly neuroblasts, with very few Ase^{-} immature INPs and never any Ase^{+} immature INPs or INPs (Figure 3.1 S1E-I; n=7 per stage). These results led us to conclude that Brat functions during maturation to prevent an immature INP from acquiring a neuroblast fate while promoting it to assume an INP identity.

To elucidate the mechanisms by which Brat regulates the maturation of immature INPs, we screened for haploinsufficient loci in the fly genome that modify the supernumerary type II neuroblast phenotype in a sensitized $brat^{DG19310/11}$ mutant genetic background (H.K. and C.-Y.L., unpublished). We identified *klu* as a genetic suppressor of *brat*, as heterozygosity of the *klu* locus strongly suppressed the formation of supernumerary neuroblasts in the *brat*-sensitized genetic background (Figure 3.1S1J-L; n=18 per genotype). Thus, we propose that Brat regulates the maturation of immature INPs by antagonizing *klu*.

To test whether Brat functions to prevent an immature INP from reacquiring a neuroblast fate or by promoting it to assume an INP identity, we first analyzed the expression of cell fate and cell proliferation markers in wild-type and *klu* mutant

larval brains (Figure 3.1A). In wild-type larvae, the total number of neuroblasts reached the plateau of almost 100 per brain hemisphere 72 hours after larval hatching (ALH) and remained at 100 per lobe at 96 hours ALH (Figure 3.1B-B″,F; n=10 brains per stage). In similarly staged *klu* mutant larvae, total neuroblasts plateaued at ~80 per brain hemisphere at 72 hours ALH and decreased to less than 60 per lobe at 96 hours ALH (Figure 3.1C-C″,F; n=10 brains per stage). Importantly, brain neuroblasts in wild-type or *klu* mutant larvae displayed similar proliferation profiles as indicated by the expression of Cyclin E (CycE) and EdU pulse-chase labeling (Figure 3.1D,E; 100% of neuroblasts in the brain, n=10; data not shown). These results strongly suggest that *klu* is required for the maintenance of brain neuroblasts.

We next tested whether *klu* functions cell-autonomously to maintain brain neuroblasts by inducing GFP-marked mosaic clones derived from a single wild-type or *klu* mutant neuroblast. Although both wild-type and *klu* mutant type I neuroblast clones maintained a single neuroblast per clone, 36.7% of the *klu* mutant clones contained neuroblasts of reduced cell diameter (≤10 μm) (Figure 3.1G-H‴,L; n=30 clones per genotype). Similarly, half of the *klu* mutant type II neuroblast clones also contained neuroblasts of reduced cell diameter (≤10 μm) (Figure 3.1I-J‴,L; n=8 clones per genotype). Reduction in neuroblast diameter was previously shown to correlate with the onset of premature differentiation (Lee et al., 2006b; Song and Lu, 2011). Consistently, 12.5% of the *klu* mutant clones contained multiple INPs, GMCs and their progeny (Figure 3.1K-L; n=8 clones). Together, these results led us to conclude that *klu* functions to maintain the identity of neuroblasts in larval brains and to propose that Brat is likely to prevent an immature INP from reacquiring a neuroblast fate by antagonizing Klu.

Defects in cell polarity or aberrant activation of cell death can lead to premature neuroblast loss in larval brain (Lee et al., 2006b; Bello et al., 2007), so we tested whether *klu* maintains neuroblast identity by regulating cell polarity or cell survival. To assess whether *klu* is required for polarization of the neuroblast cortex, we examined the localization of atypical Protein kinase C (aPKC), Miranda (Mira) and

Numb (Albertson and Doe, 2003; Rolls et al., 2003; Lee et al., 2006a; Lee et al., 2006c) in telophase neuroblasts in *klu* mutant brains. We detected aPKC segregated exclusively into the cortex of the future neuroblast and Mira and Numb localized asymmetrically in the cortex of the future progenitor cell in *klu* mutant brains (Figure 3.1S2A,B). Thus, since mitotic *klu* mutant neuroblasts displayed asymmetric localization of the apical and basal proteins, it is unlikely that *klu* maintains the identity of neuroblasts by regulating polarization of the neuroblast cortex. To determine if *klu* is required for the maintenance of neuroblast survival, we examined whether blocking activation of apoptosis would prevent the premature loss of neuroblasts in *klu* mutant brains. We generated mosaic clones derived from a single type I or II neuroblast lacking *klu* alone or *klu* and the *Df(3R)H99* locus. The *H99* locus contains three crucial activators of apoptosis in the fly genome (White et al., 1994; Grether et al., 1995; Chen et al., 1996; White et al., 1996). However, removal of the *H99* locus did not significantly decrease the occurrence of neuroblasts of reduced cell diameter (≤10 μm) or revert the absence of type II neuroblasts in *klu* mutant clones (Figure 3.1S2C-D'''; n=14 per genotype). Furthermore, we failed to detect aberrant activation of caspases in *klu* mutant brains, and blocking caspase activity did not prevent premature neuroblast loss in *klu* mutant brains (Figure 3.1S2E-I; n=15 per genotype). Thus, we conclude that Klu does not maintain the identity of neuroblasts by regulating cell polarity or cell survival.

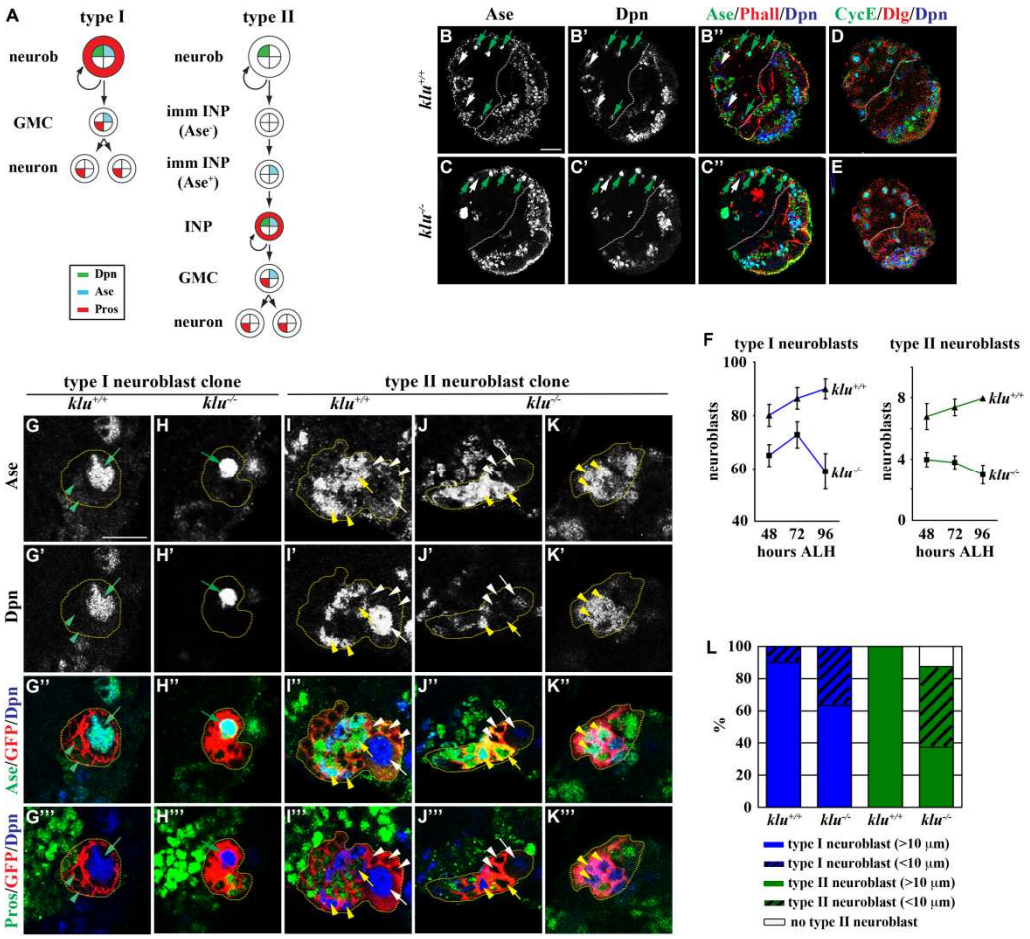

Figure 3.1 Neuroblasts prematurely differentiate in *klu* mutant brains.

(**A**) Summary of the cell fate marker expression pattern in type I and II neuroblast lineages in *Drosophila* larval brains. GMC, ganglion mother cell; INP, intermediate neural progenitor; imm INP, immature INP; neurob, neuroblast; Pros, Prospero.

(**B-F**) *klu* mutant brains show progressive loss of neuroblasts. (B-E) Brains were dissected from wild-type or $klu^{R51/09036}$ mutant larvae at 96 hours ALH and stained for the markers indicated. The white dotted line separates the central brain (left) from the optic lobe (right). Discs large (Dlg) marks the cell cortex. (F) Average type I and II neuroblasts per brain lobe in larvae of genotypes and stages indicated. Error bars indicate s.e.m.

(**G-L**) Neuroblasts show reduced cell diameter and are likely to prematurely differentiate in *klu* mutant brains. Larvae carrying GFP-marked $klu^{+/+}$ or $klu^{-/-}$ mosaic neuroblast clones (outlined by the yellow dotted line) were aged for 110 hours after clone induction and larval brains were stained for the markers indicated. (G-H‴) Type I neuroblast clones. (I-K‴) Type II neuroblast clones. (L) The frequency of $klu^{+/+}$ or $klu^{-/-}$ clones containing neuroblasts of the cell diameter indicated. The following are indicated: type I neuroblast (Dpn^{+} Ase^{+}), green arrow; GMC (Dpn^{-} Ase^{+}), green arrowhead; type II neuroblast (Dpn^{+} Ase^{-}), white arrow; Ase^{-} immature INP (Dpn^{-} Ase^{-}), white arrowhead; Ase^{+} immature INP (Dpn^{-} Ase^{+}), yellow arrow; INP (Dpn^{+} Ase^{+}), yellow arrowhead. Scale bars: 20 μm in B-E; 10 μm in G-K.

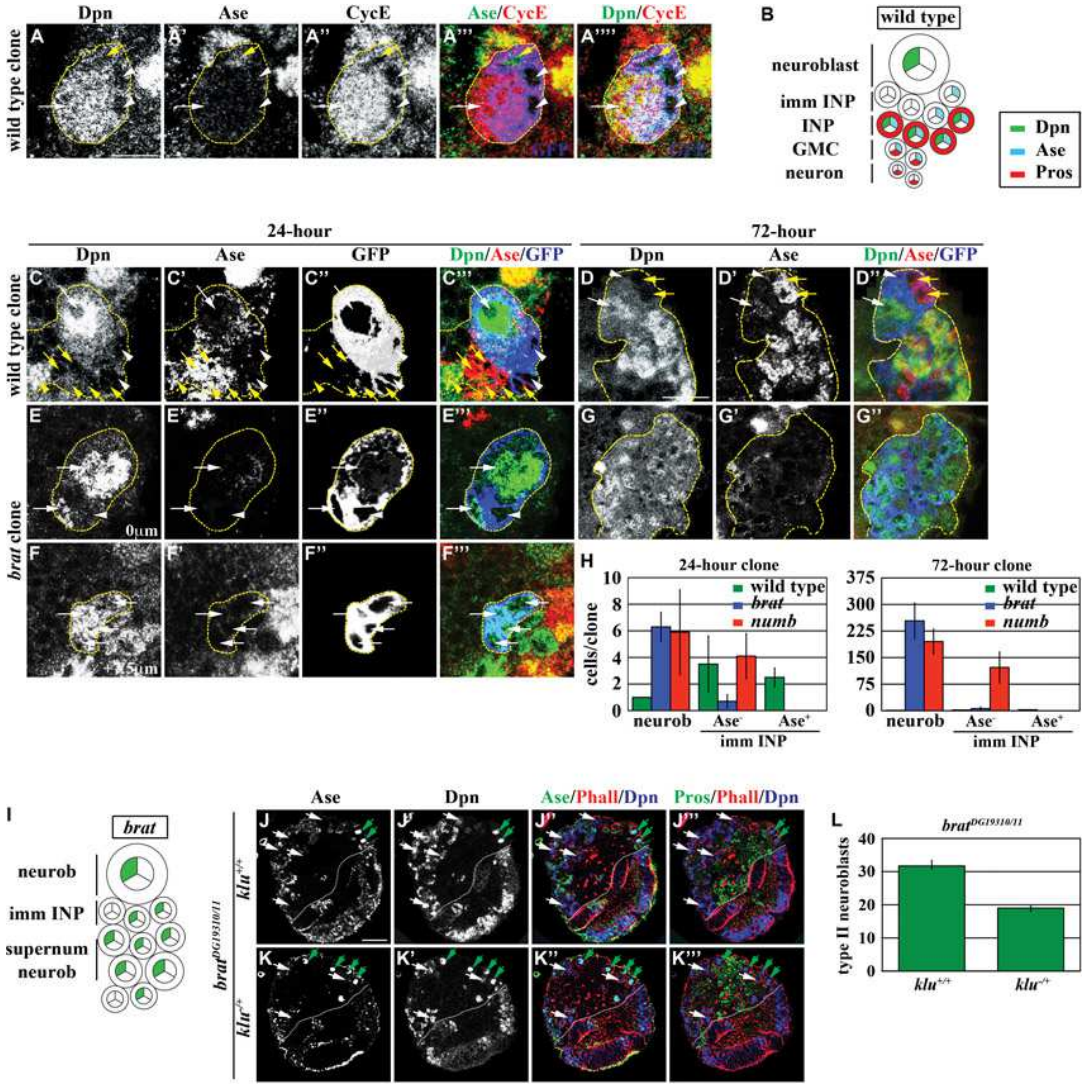

Figure 3.1S1 Heterozygosity of *klu* suppresses supernumerary type II neuroblasts in *brat* $^{11/DG19310}$ mutant brains.

(A-B) Ase serves as a marker for an intermediate stage of maturation. (A-A′′′′) Larvae carrying GFP-marked wild-type type II neuroblast lineage clones (outlined by the yellow dotted line) were aged for 16 hours after clone induction, and brains were stained for the markers indicated. Scale bar: 10 μm. (B) Summary of the cell fate marker expression pattern in the type II neuroblast lineage.

(**C-I**) *brat* functions in immature INPs to suppress reversion into type II neuroblasts and to initiate the specification of INP identity. (C-G″) Larvae carrying GFP-marked wild-type or *brat* mutant type II neuroblast mosaic clones (outlined by the yellow dotted line) were aged for 24 or 72 hours after clone induction, and brains were stained for the markers indicated. (H) Quantification of various cell types in the wild-type or *brat* mutant type II neuroblast clone. (I) Summary of the identity of cells in the *brat* mutant type II neuroblast clone. supernum neurob, supernumerary neuroblast.

(**J-L**) Heterozygosity of *klu* suppresses supernumerary type II neuroblasts in sensitized *brat* mutant brains. (J-K‴) *brat* $^{DG19310/11}$*; klu*$^{+/+}$ or *brat* $^{DG19310/11}$*; klu*$^{-/+}$ mutant larvae were aged for 96 hours ALH, and brains were stained for the markers indicated. The white dotted line separates the central brain (left) from the optic lobe (right). (L) Average type II neuroblasts per brain lobe in larvae of the genotype indicated. Scale bar: 20 μm. Type II neuroblast (Dpn^+ Ase^-, white arrow); Ase^- immature INP (Dpn^- Ase^-, white arrowhead); Ase^+ immature INP (Dpn^- Ase^+, yellow arrow); INP (Dpn^+ Ase^+, yellow arrowhead).

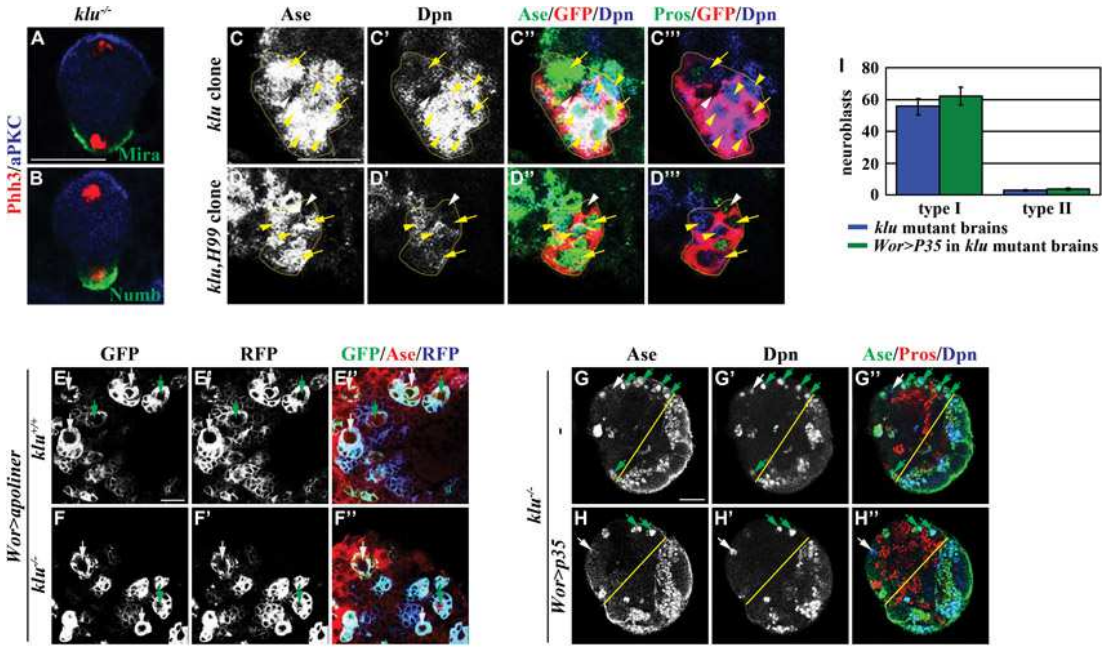

Figure 3.1S2 *klu* mutant neuroblasts show asymmetric localization of apical and basal proteins and do not display aberrant activation of caspases.

(**A**,**B**) Telophase *klu* mutant neuroblasts show asymmetric localization of aPKC, Miranda and Numb. Scale bar: 5 µm.

(**C-D**) Removal of the *Df(3L)H99* locus does not block premature loss of neuroblasts in *klu* mutant type II neuroblast clones. Larvae carrying GFP-marked *klu* single-mutant or *klu,H99* double-mutant type II neuroblast mosaic clones (outlined by the yellow dotted line) were aged for 72 hours after clone induction, and brains were stained for the markers indicated. Scale bar: 10 µm.

(**E-F**) *klu* mutant neuroblasts do not show aberrant activation of caspases. Wild-type or *klu* mutant brains overexpressing the *UAS-apoliner* transgene were stained for the markers indicated. Scale bar: 10 µm.

(**G-I**) Overexpression of the caspase inhibitor protein p35 does not suppress premature loss of neuroblasts in *klu* mutant brains. *klu* mutant brains alone or overexpressing the *UAS-p35* transgene were stained for the markers indicated. The yellow line separates the central brain (left) from the optic lobe (right). Scale bar: 20 μm. Type I neuroblast (Dpn^+ Ase^+, green arrow); GMC (Dpn^- Ase^+, green arrowhead); type II neuroblast (Dpn^+ Ase^-, white arrow); Ase^- immature INP (Dpn^- Ase^-, white arrowhead); Ase^+ immature INP (Dpn^- Ase^+, yellow arrow); INP (Dpn^+ Ase^+, yellow arrowhead).

Overexpression of *klu* induces massive expansion of type II neuroblasts

Phenotypic analyses of *klu* mutant brains led us to conclude that klu functions to maintain the identity of neuroblasts in larval brains, so we hypothesized that *klu* should be expressed in both type I and II neuroblasts. We first assessed the spatial expression pattern of the *klu*-lacZ enhancer trap line in larval brains. We detected lacZ expression in both type I and II neuroblasts as well as in their immediate progenitor progeny in larval brain (Figure 3.2S; n=10). Since the half-life of the β-gal protein might be longer than that of endogenous Klu protein, we stained larval brains carrying GFP-marked lineage clones derived from a single wild-type type I or II neuroblast with an antibody specific for Klu protein. In the type I neuroblast lineage, Klu was detected in the neuroblast but undetectable in GMCs and their progeny (Figure 3.2A-A″,C; n=9 clones). In the type II neuroblast lineage, Klu was present in the neuroblast and INPs but absent from immature INPs and GMCs (Figure 3.2B-C; n=5 clones). Thus, we conclude that Klu is expressed in both types of neuroblast but is absent from their immediate progenitor progeny.

The spatial expression pattern of Klu is consistent with its proposed function in the maintenance of neuroblast identity, so we tested whether increased function of *klu* can trigger the formation of supernumerary neuroblasts. We first overexpressed a *UAS-klu* transgene under the control of a pan-neuroblast *wor-GAL4* driver in larval brains. Unexpectedly, we observed massive expansion of type II neuroblasts but did not detect any increase in type I neuroblasts (Figure 3.2D-E″; n=7 per genotype). Similarly, lineage clones derived from a single type I neuroblast overexpressing *klu* driven by a constitutively active Actin-GAL4 driver reproducibly contained one neuroblast per clone (Figure 3.2F-F’’’; 100%, n=10 clones). By contrast, type II neuroblast clones overexpressing *klu* contained mostly neuroblasts (Figure 3.2G-G’’’; 100%, n=10 clones). Together, these results indicate that increased function of *klu* specifically leads to the expansion of type II neuroblasts.

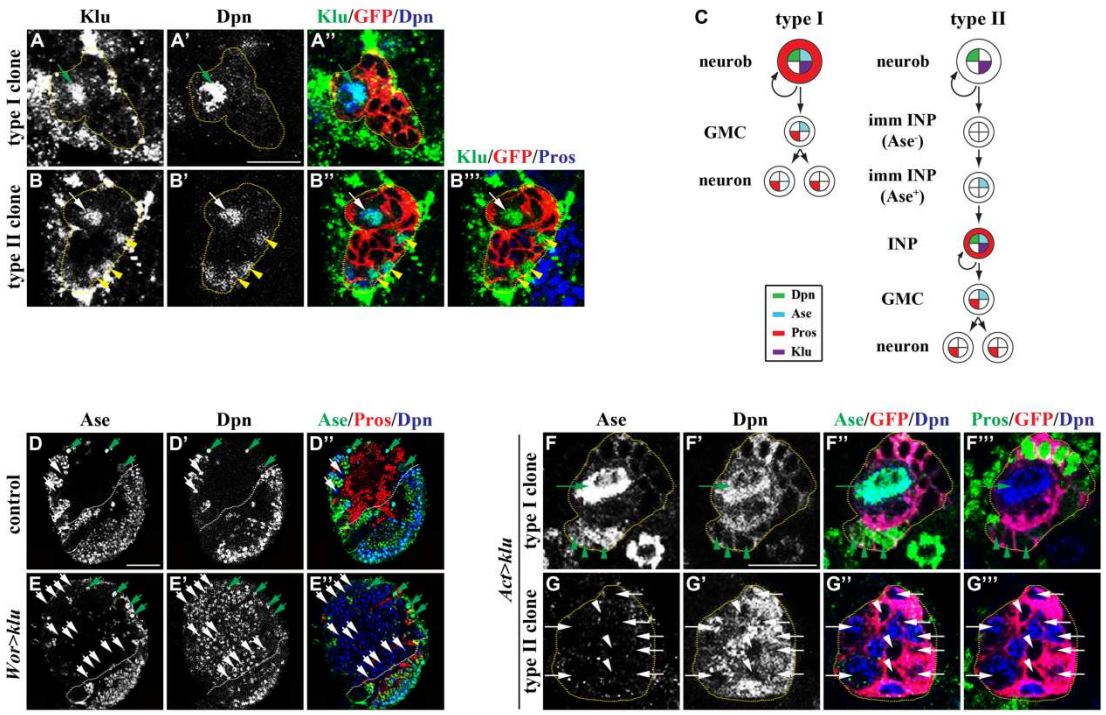

Figure 3.2 Overexpression of *klu* induces supernumerary type II neuroblasts.

(**A-C**) Klu is detected in neuroblasts but is undetectable in their immediate progenitor progeny. (A-B$^{'''}$) *Drosophila* larvae carrying GFP-marked wild-type type I or II neuroblast lineage clones (outlined by the yellow dotted line) were aged for 72 hours after clone induction and larval brains were stained for the markers indicated. (C) Summary of the Klu expression pattern in type I and II neuroblast lineages.

(**D-E**) Overexpression of *klu* induces excess type II neuroblasts. Larvae were raised at 31°C for 72 hours ALH and larval brains were stained for the markers indicated. The white dotted line separates the central brain (left) from the optic lobe (right).

(**F-G**) Overexpression of *klu* specifically induces supernumerary neuroblasts in type II neuroblast lineage clones. Larvae carrying GFP-marked type I or II lineage clones (outlined by the yellow dotted line) overexpressing *klu* were aged for 24 hours after clone induction and brains were stained for the markers indicated. Abbreviations and arrows/arrowheads as in Figure 3.1. Scale bars: 10 μm in A-B$^{'''}$,F-G$^{'''}$; 20 μm in D-E

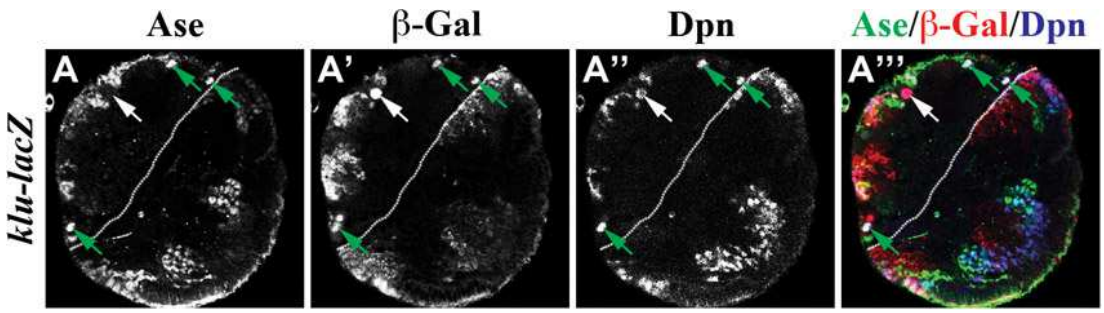

Figure 3.2S *klu-lacZ* is detectable in both type I and II neuroblasts and their progenitor progeny in larval brains.

(**A-A**) Larvae carrying a *klu-lacZ* enhancer trap transgene were aged for 96 hours after larval hatching, and larval brains were stained for the markers indicated. The white line separates the central brain (left) from the optic lobe (right). Type I neuroblast (Dpn^+ Ase^+, green arrow); type II neuroblast (Dpn^+ Ase^-, white arrow).

Misexpression of *klu* in immature INPs leads to supernumerary type II neuroblasts

We next examined the cell type from which supernumerary neuroblasts arise in the type II neuroblast clones overexpressing Klu. We tested whether type II neuroblasts overexpressing Klu undergo symmetric division in telophase to generate supernumerary neuroblasts by analyzing the localization of aPKC, Mira and Numb. We observed that aPKC segregates into the cortex of the future neuroblast and Mira and Numb partition into the cortex of the future immature INP (Figure 3.2B,C; n=15 per genotype). This result strongly suggests that a type II neuroblast overexpressing *klu* divides asymmetrically to generate a neuroblast and an immature INP. We reproducibly observed Ase^{-} immature INPs in all type II neuroblast clones overexpressing *klu* (Figure 3.2G-G'''). Thus, it is unlikely that type II neuroblasts overexpressing Klu undergo symmetric division to generate supernumerary neuroblasts. We next tested whether supernumerary neuroblasts arise from de-differentiation of INPs in type II neuroblast clones overexpressing Klu. The lineage clones derived from INPs overexpressing *klu* maintained a single INP per clone and contained GMCs and their progeny but never type II neuroblasts, indicating that overexpression of *klu* is not sufficient to trigger INPs to de-differentiate back into type II neuroblasts (Figure 3.3A-A'''; 100%, n=8). Thus, it is unlikely that supernumerary neuroblasts in type II neuroblast clones overexpressing Klu originate from symmetric neuroblast division or de-differentiation of INPs.

As an alternative, we tested whether overexpression of *klu* in neuroblasts indirectly leads to increased function of Klu in immature INPs, triggering them to acquire a neuroblast fate. We searched for GAL4 lines that can drive expression of the UAS transgene in immature INPs. The *erm-GAL4* transgene inserted on the third chromosome (III) in the fly genome is sufficient to induce UAS transgene expression in INPs but not in type II neuroblasts (Pfeiffer et al., 2008; Weng et al., 2010). The identical *erm-GAL4* transgene inserted on the second chromosome (II) (kindly provided by Dr G. Rubin, HHMI) showed a similar spatial expression pattern in

larval brain, as ectopic expression of a *UAS-prospero* transgene driven by *erm-GAL4* (II) induced premature loss of immature INPs and INPs without affecting the maintenance of type II neuroblasts (Figure 3.3S; n=8). We next tested whether onset of the *erm-GAL4* (II) and (III) activity occurs in immature INPs by colocalizing the expression of a *UAS-GFP* reporter transgene with Ase and PointedP1 (PntP1). We reproducibly detected GFP expression driven by *erm-GAL4* (II) in both Ase^{-} and Ase^{+} immature INPs (Figure 3.3D-D''',F; n=8). By contrast, the reporter expression driven by *erm-GAL4* (III) was only first detected specifically in Ase^{+} immature INPs (Figure 3.3E-F; n=8). We then tested whether increased function of *klu* in Ase^{-} or Ase^{+} immature INPs can lead to the formation of supernumerary neuroblasts. Indeed, misexpression of *klu* driven by *erm-GAL4* (II) led to a greater than 10-fold increase in type II neuroblasts per brain lobe compared with a similarly staged wild-type brain lobe (Figure 3.3G,J and Figure 3.1F; n=8). Although misexpression of one copy of *UAS-klu* driven by one copy of *erm-GAL4* (III) failed to induce supernumerary type II neuroblasts, doubling the number of *UAS-klu* and *erm-GAL4* (III) transgenes led to modest expansion of type II neuroblasts (Figure 3.3H-J; n=12 per genotype). Together, these data strongly suggest that immature INPs can indeed revert to type II neuroblasts in response to misexpression of *klu*.

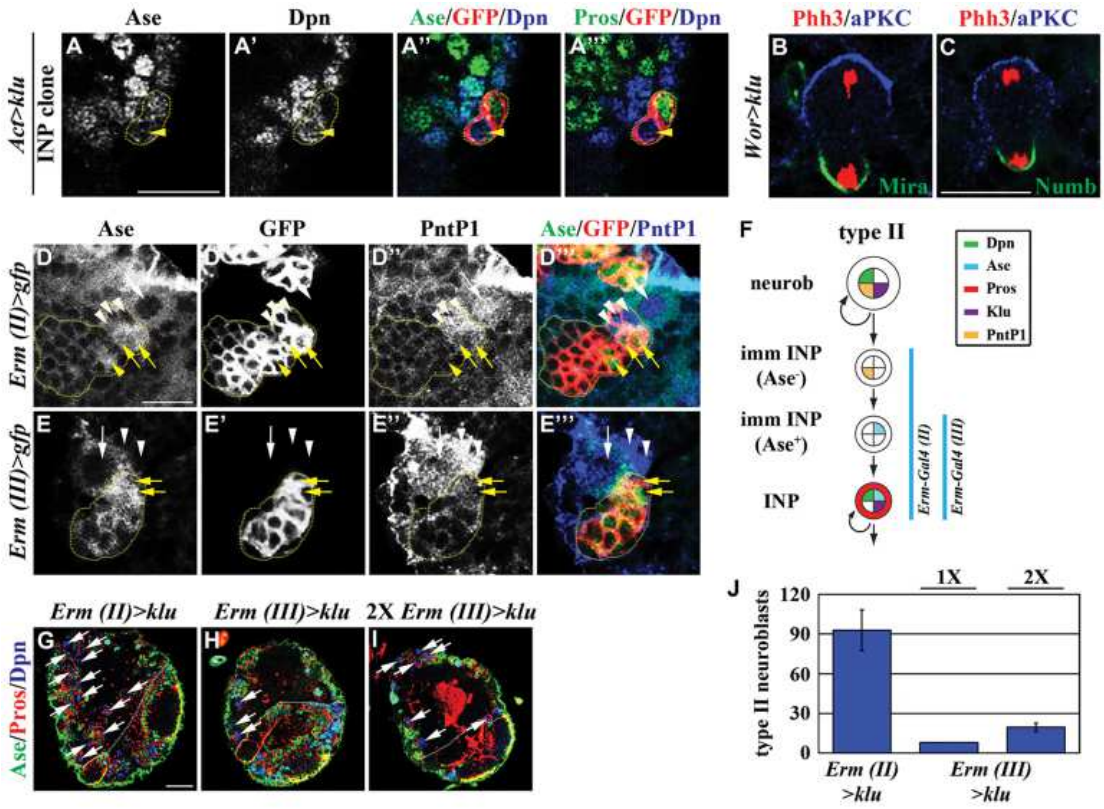

Figure 3.3 Misexpression of *klu* triggers the reversion of immature INPs to type II neuroblasts.

(**A-A‴**) Overexpression of *klu* is not sufficient to trigger de-differentiation of INPs. *Drosophila* larvae carrying GFP-marked INP lineage clones (outlined by the yellow dotted line) overexpressing *klu* were aged for 24 hours after clone induction and brains were stained for the markers indicated.

(**B**,**C**) Telophase neuroblasts overexpressing *klu* show asymmetric localization of apical and basal proteins. Phh3, phosphorylated histone H3.

(**D-F**) The activity of *erm-GAL4* is first detected in immature INPs. (D-E‴) Larvae expressing GFP driven by *erm-GAL4* (II) or *erm-GAL4* (III) were aged for 72 hours and brains were stained for the markers indicated. PointedP1 (PntP1) marks type II neuroblasts and Ase^{-} immature INPs. (F) Summary of the *erm-GAL4* expression pattern in the type II neuroblast lineage.

(**G-J**) Overexpression of *klu* in immature INPs leads to supernumerary type II neuroblasts. (G-I) Larvae overexpressing *klu* driven by *erm-GAL4* were raised at 31°C for 72 hours ALH and brains were stained for the markers indicated. The white dotted line separates the central brain (left) from the optic lobe (right). (J) Average type II neuroblasts per brain lobe in larvae of the genotype indicated. 1×, 2× indicate the copy number of *UAS-klu* and *erm-GAL4 (III)* transgenes. Error bars indicate s.e.m. Abbreviations and arrows/arrowheads as Figure 3.1. Scale bars: 10 μm in A-A‴,D-E‴; 5 μm in B,C; 20 μm in G-I.

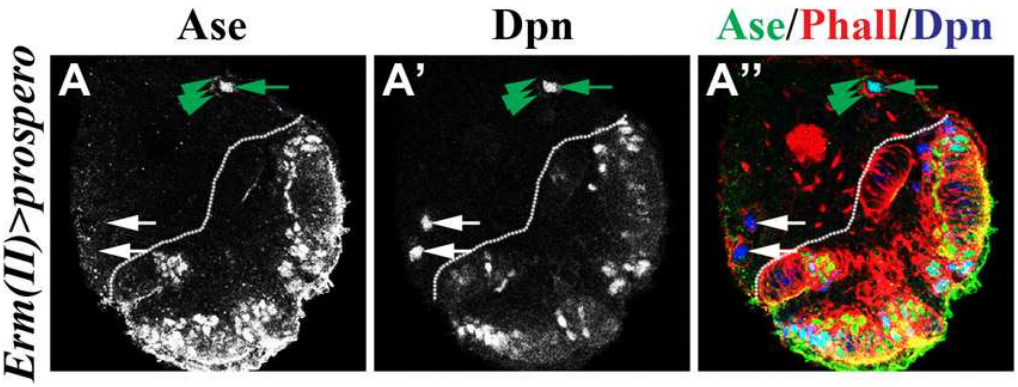

Figure 3.3 S *Erm-GAL4* is not expressed in type II neuroblasts.

(**A-A″**) Larvae carrying an *Erm-GAL4* and an *UAS-prospero* transgene were raised at 31.5°C to induce the expression of Prospero for 96 hours after larval hatching, and larval brains were stained for the markers indicated. The white line separates the central brain (left) from the optic lobe (right). Type I neuroblast (Dpn^+ Ase^+, green arrow); GMC (Dpn^- Ase^+, green arrowhead); type II neuroblast (Dpn^+ Ase^-, white arrow).

Promotion by Klu of supernumerary type II neuroblast formation is dependent on the zinc-finger motifs

klu, the fly ortholog of the mammalian Wilms tumor 1 (WT1) gene, encodes a putative transcriptional regulator characterized by four C2H2 zinc-finger motifs in the C-terminus (Klein and Campos-Ortega, 1997; Yang et al., 1997). Vertebrate studies have shown that WT1 requires its zinc-finger motifs to regulate transcription of its target genes (Roberts, 2005). To test whether Klu triggers supernumerary neuroblasts by acting as a transcriptional regulator, we ectopically expressed a series of *UAS-klu* transgenes in neuroblasts (Figure 3.4A). We focused our analyses on the type II lineage as overexpression of the full-length Klu transgenic protein specifically led to the expansion of type II neuroblasts (Figure 3.2E-E″).

Expression of the Klu1-583 transgenic protein (which lacks all four zinc-finger motifs) failed to induce supernumerary neuroblasts, indicating that the zinc-finger motifs are indispensable for Klu to promote the identity of type II neuroblasts (Figure 3.4B,E; 100%, n=10 per genotype). Although expression of the KluΔzf1 transgenic protein (which lacks zinc-finger 1) was sufficient to induce supernumerary neuroblasts, it appeared to be less potent than expression of full-length Klu (Figure 3.2E-E″ and Figure 3.4C,E; 100%, n=10 per genotype). This result strongly suggests that zinc-finger 1 is necessary for the optimal function of Klu in promoting the formation of supernumerary neuroblasts. Significantly, expression of the KluΔzf4 transgenic protein (which lacks zinc-finger 4) completely failed to induce supernumerary neuroblasts, strongly suggesting that zinc-finger 4 is essential for Klu function (Figure 3.4D,E; 100%, n=10).

Finally, we confirmed that expression levels of the various truncated Klu transgenic proteins under the above experimental conditions were indistinguishable from each other (Figure 3.4S). Our data correlate well with a previously published domain analysis of the Klu protein in the developing sensory organ precursor cell (Kaspar et

al., 2008). Thus, we propose that Klu promotes the identity of type II neuroblasts by regulating gene transcription.

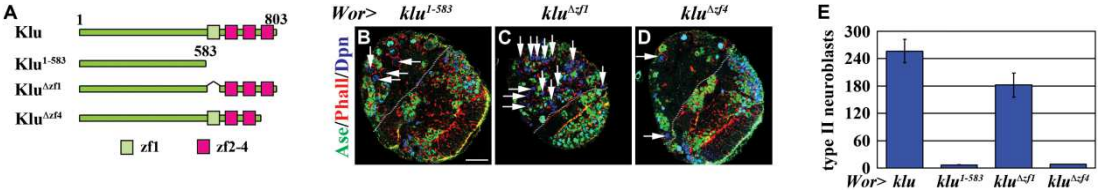

Figure 3.4 Induction of supernumerary type II neuroblasts by Klu is dependent on the zinc-finger motifs.

(**A**) The *klu* transgenes used in this study.

(**B-D**) *Drosophila* larvae overexpressing various *klu* transgenes were raised at 31°C for 72 hours ALH and brains were stained for the markers indicated. The white dotted line separates the central brain (left) from the optic lobe (right). Phalloidin (Phall) marks the cell cortex. Type II neuroblasts (Dpn^+ Ase^-) are indicated (arrows). Scale bar: 20 μm.

(**E**) Average type II neuroblasts per brain lobe in larvae of the genotype indicated. Error bars indicate s.e.m.

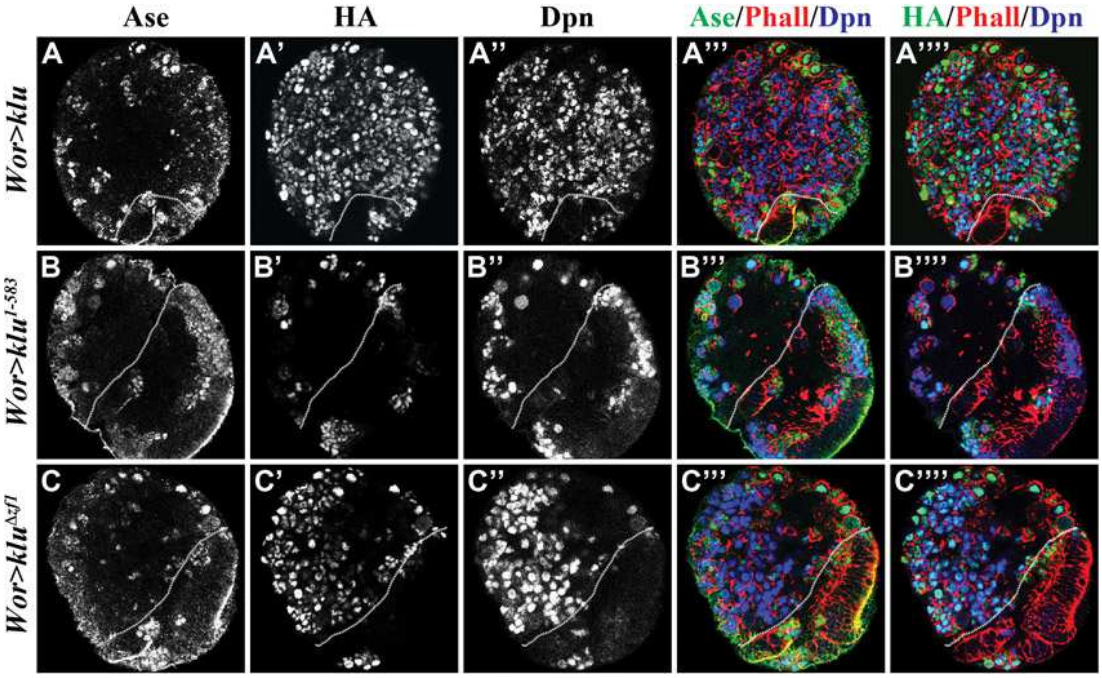

Figure 3.4S Overexpression of various truncated Klu transgenic proteins in larval brains.

(**A-C′′′′**) Larvae carrying *Wor-GAL4* in combination with one of several *UAS-klu* transgenes were raised at 31.5°C to induce the expression of Klu for 96 hours after larval hatching, and larval brains were stained for the markers indicated. The white line separates the central brain (left) from the optic lobe (right). Type I neuroblast (Dpn^+ Ase); type II neuroblast (Dpn^+ Ase^-).

Brat prevents the reversion of immature INPs to type II neuroblasts by antagonizing Klu

Our data thus far are consistent with our hypothesis that Brat distinguishes an immature INP from its sibling type II neuroblast in part by antagonizing the function of Klu. We directly tested whether removal of *klu* function can suppress the formation of supernumerary neuroblasts and restore INPs in $brat^{11/k06028}$ strong hypomorphic mutant brains. The control type II neuroblast clones carrying both copies of the wild-type *klu* gene in $brat^{11/k06028}$ mutant brains contained mostly neuroblasts and very few INPs (Figure 3.5A-A‴,C; 100%, n=10 clones). By contrast, *klu* mutant type II neuroblast clones in $brat^{11/k06028}$ mutant brains contained a single neuroblast per clone and possessed INPs and GMCs (Figure 3.5B-C; 92%, n=12 clones). These data strongly support our hypothesis that Brat distinguishes an immature INP from its sibling type II neuroblast by antagonizing Klu.

To confirm that Brat can indeed antagonize Klu in immature INPs, we induced genetic clones derived from a single type II neuroblast overexpressing *klu* alone or *klu* and *brat* simultaneously. The control clones overexpressing *klu* consisted of virtually all neuroblasts with very few Ase^{-} immature INPs (Figure 3.5D-D’’’’,F; 62.5%, *n*=16 clones). Co-expression of *brat* but not an unrelated *UAS* transgene significantly suppressed the supernumerary neuroblast phenotype and restored the formation of Ase^{-} and Ase^{+} immature INPs, INPs and GMCs in the type II neuroblast clones overexpressing *klu* (Figure 3.5E-F; 100%, *n*=10 clones; data not shown). Finally, overexpression of *brat* alone did not alter cell fate specification in the type II neuroblast clones (data not shown). Together, these data led us to conclude that Brat antagonizes Klu in the immature INP, distinguishing it from its sibling type II neuroblast (Figure 3.6H).

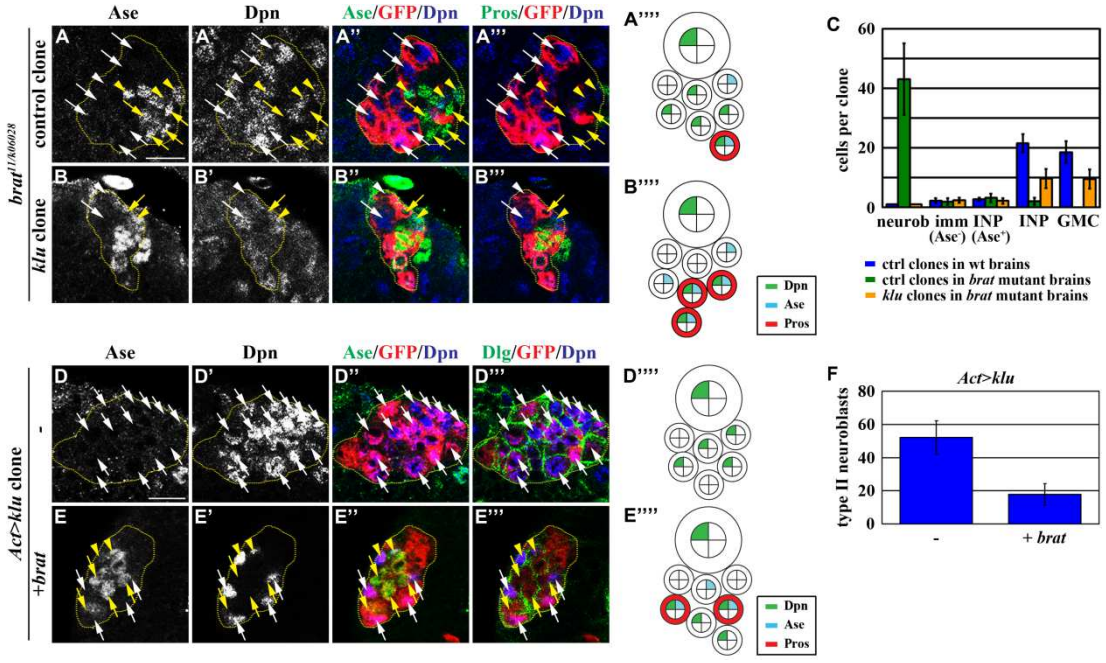

Figure 3.5 Brat suppresses reversion of immature INPs by antagonizing Klu.

(**A-C**) Removal of *klu* function suppresses supernumerary type II neuroblasts and restores the formation of INPs and GMCs in *brat* strong hypomorphic mutant brains. (A-B′′′′) *brat*$^{11/k06028}$ mutant *Drosophila* larvae carrying GFP-marked control (*klu*$^{+/+}$) and *klu* mutant type II neuroblast mosaic clones (outlined by the yellow dotted line) were aged for 72 hours after clone induction and brains were stained for the markers indicated. (C) Quantification of various cell types in the control and *klu* mutant clones in *brat*$^{11/k06028}$ mutant brains.

(**D-F**) Co-expression of Brat suppresses Klu-induced supernumerary type II neuroblasts. (D-E′′′′) Larvae carrying GFP-marked type II neuroblast lineage clones (outlined by the yellow dotted line) overexpressing *klu* or *klu* and *brat* were aged for 72 hours after clone induction and brains were stained for the markers indicated. (F) Average type II neuroblasts per brain lobe in larvae of the genotype indicated. Error bars indicate s.e.m. Arrows/arrowheads as Figure 3.1 Scale bars: 10 μm.

Aberrant activation of Notch signaling promotes the reversion of immature INPs through *klu*

The basal protein Numb, which is an evolutionarily conserved inhibitor of Notch signaling, is also necessary for the formation of INPs in larval brain, but how Numb regulates maturation of immature INPs has never been characterized (Rhyu et al., 1994; Guo et al., 1996; Bowman et al., 2008). We first investigated the role of Numb during maturation by assessing the identity of cells in the GFP-marked clones derived from a single *numb* null mutant type II neuroblast. Whereas a 24-hour *numb* mutant clone contained 5.9±3.2 neuroblasts, 4.7±1.7 Ase^- immature INPs and no Ase^+ immature INPs, a 72-hour *numb* clone possessed 195.4±35.4 neuroblasts, 122.2±43.6 Ase^- immature INPs and no Ase^+ immature INPs (Figure 3.1S1H and Figure 3.6S; *n*=9 per stage). Indistinguishable from the *numb* mutant clones, the type II neuroblast clones expressing a constitutively activated form of Notch ($Notch_{intra}$) also contained neuroblasts and Ase^- immature INPs but never Ase^+ immature INPs and INPs (Figure 3.6A-A‴,D; 100%, *n*=15). Numb thereby functions to prevent an immature INP from acquiring a neuroblast fate and instead promotes it to assume an INP identity most likely through inhibition of Notch signaling.

We next tested whether aberrant activation of Notch signaling induces the reversion of immature INPs to type II neuroblasts via a Klu-dependent mechanism. Removal of *klu* function significantly reduced supernumerary neuroblasts and restored INPs and GMCs in half of the clones derived from type II neuroblasts overexpressing $Notch_{intra}$ (Figure 3.6B-B‴,D; *n*=18 clones). Most significantly, 33.3% of these clones possessed a single neuroblast per clone (Figure 3.6C-D; *n*=18 clones). Thus, aberrant activation of Notch signaling in immature INPs leads to the formation of supernumerary neuroblasts via a Klu-dependent mechanism.

We directly tested whether *klu* acts downstream of Notch signaling to maintain type II neuroblasts by assessing the identity of cells in the mosaic clones derived from *Notch* mutant type II neuroblasts and those overexpressing *klu*. Whereas most *Notch*

mutant clones did not contain neuroblasts, overexpression of *klu* completely suppressed the premature loss of type II neuroblasts in the *Notch* mutant clones (Figure 3.6E-G; 100%, *n*=8 clones). This result strongly suggests that Notch signaling maintains the identity of type II neuroblasts via a *klu*-dependent mechanism. Interestingly, overexpression of *klu* in *Notch* mutant type II neuroblast clones failed to induce the formation of supernumerary neuroblasts (Figure 3.6F-G; 100%, *n*=8 clones). Thus, we propose that aberrant activation of Notch signaling induces the reversion of immature INPs to type II neuroblasts by activating multiple downstream genes including *klu* (Figure 3.6H).

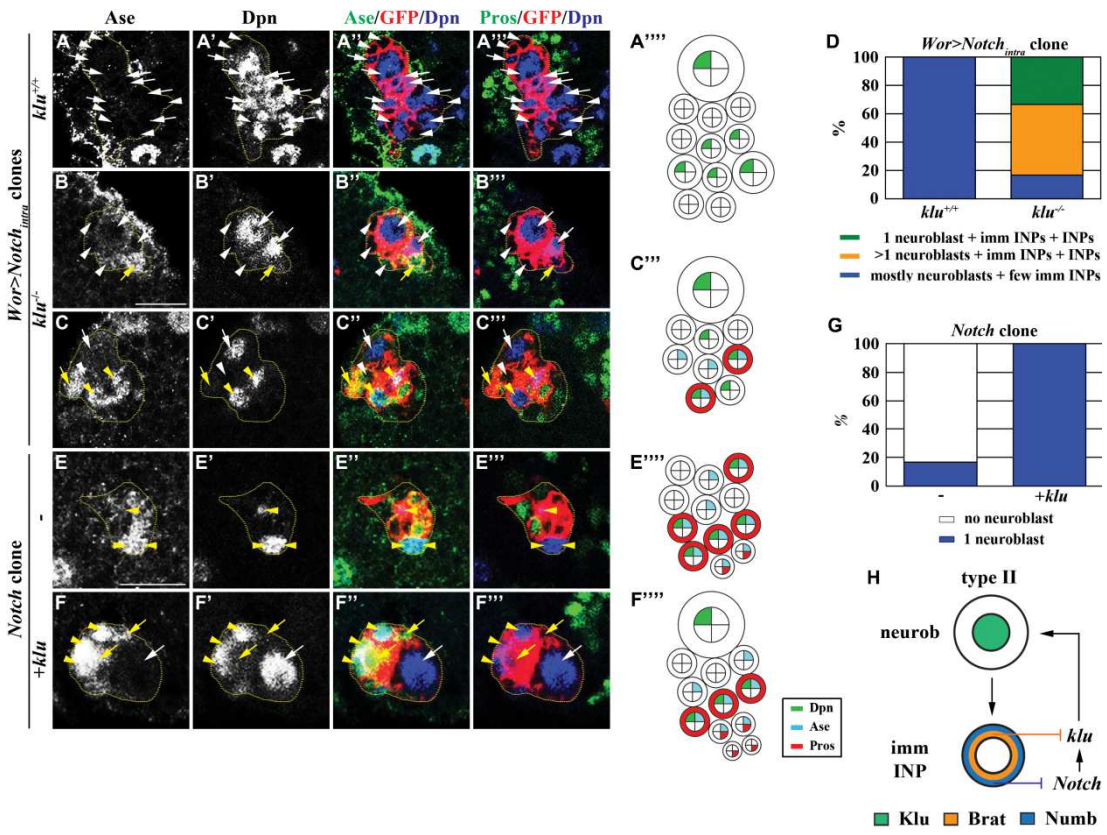

Figure 3.6 Aberrant activation of Notch signaling induces reversion of immature INPs through *klu*.

(**A-D**) Removal of *klu* function suppresses supernumerary type II neuroblasts induced by constitutively activated Notch signaling. (A-C'''') *Drosophila* larvae carrying GFP-marked wild-type ($klu^{+/+}$) or $klu^{-/-}$ type II neuroblast mosaic clones (outlined by the yellow dotted line) overexpressing $Notch_{intra}$ were aged for 72 hours after clone induction and brains were stained for the markers indicated. (D) The frequency of clones containing one or more type II neuroblasts in larvae of the genotype indicated.

(**E-G**) Overexpression of *klu* prevents *Notch* mutant type II neuroblasts from premature differentiation. (E-F'''') Larvae carrying GFP-marked *Notch* mutant type II neuroblast mosaic clones (outlined by the yellow dotted line) alone or

overexpressing *klu* were aged for 72 hours after clone induction and brains were stained for the markers indicated. (G) The frequency of clones containing one or no type II neuroblasts in larvae of the genotype indicated.

(**H**) Model: Brat or Numb prevent the reversion of immature INPs to type II neuroblasts by antagonizing Klu. Abbreviations and arrows/arrowheads as Figure 3.1. Scale bars: 10 μm.

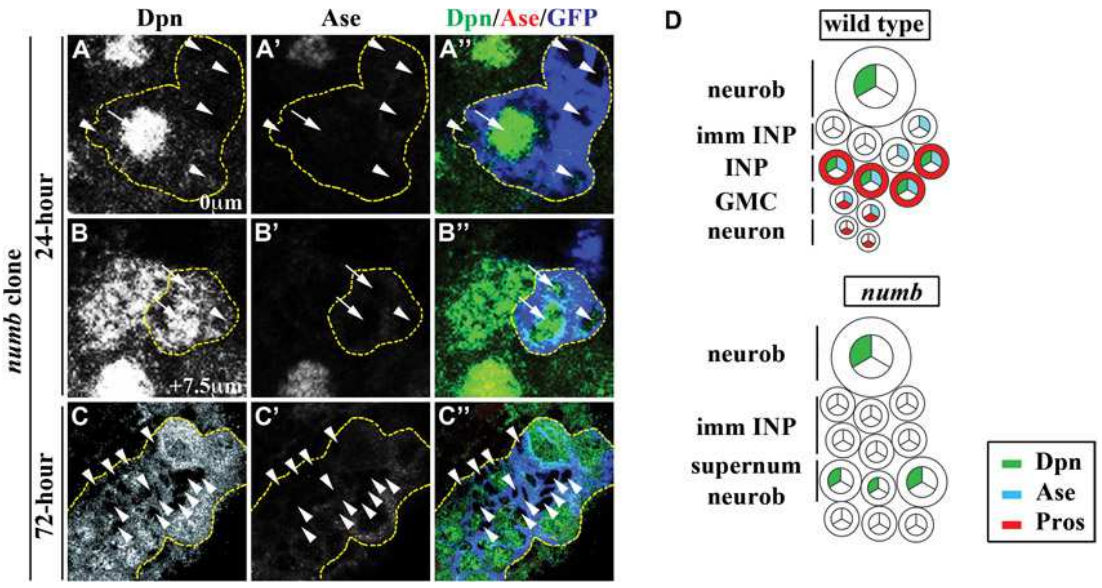

Figure 3.6S *numb* functions in immature INPs to suppress reversion into type II neuroblasts and to initiate specification of INP identity.

(**A-C′′**) Larvae carrying GFP-marked wild-type or *numb* mutant type II neuroblast mosaic clones (outlined by the yellow dotted line) were aged for 24 or 72 hours after clone induction, and brains were stained for the markers indicated.

(**D**) Summary of the identity of cells in the *numb* mutant type II neuroblast clone. supernum neurob, supernumerary neuroblast. Type II neuroblast (Dpn^+ Ase^-, white arrow); Ase^- immature INP (Dpn^- Ase^-, white arrowhead).

Aberrant activation of Notch signaling promotes reversion of GMCs through *klu*

Although *klu* is necessary for the maintenance of type I neuroblasts, overexpression of *klu* did not lead to an increase in type I neuroblasts. One plausible reason is that additional fate determinants might function redundantly in the specification of GMC identity, leading us to identify Notch signaling as an excellent candidate (Bowman et al., 2008; Wirtz-Peitz et al., 2008; Kaspar et al., 2008). We tested this hypothesis by first overexpressing *klu* in the numb mutant clones. Whereas the numb mutant clones possessed an average of three neuroblasts per clone, overexpression of *klu* tripled the number of neuroblasts in the same genetic background (Figure 3.7S; n=10 per genotype). This indicates that increased function of *klu* can trigger a further increase in supernumerary type I neuroblasts in the absence of Numb.

We next tested whether Klu can exacerbate the formation of supernumerary type I neuroblasts induced by activated Notch signaling by examining the identity of cells in the clones derived from a single type I neuroblast ectopically expressing Notchintra alone or Notchintra and *klu* simultaneously. Although the type I neuroblast clones overexpressing Notchintra contained an average of six neuroblasts per clone, only 60% of these clones contained more than one neuroblast per clone (Figure 3.7B-B‴″,D; n=10 per genotype). By contrast, the type I neuroblast clones co-expressing Notchintra and *klu* contained an average of 18 neuroblasts per clone, and 100% of the clones displayed the supernumerary neuroblast phenotype (Figure 3.7C-D; n=10 per genotype). Since the clones derived from neuroblasts overexpressing Notchintra alone or Notchintra and *klu* contained GMCs and their progeny, it is unlikely that the supernumerary neuroblasts arose from symmetric neuroblast division. Instead, increased function of *klu* most likely further enhances the reversion of GMCs to type I neuroblasts induced by aberrant activation of Notch signaling. To test whether activated Notch signaling promotes the reversion of GMCs to type I neuroblasts via a *klu*-dependent mechanism, we induced type I neuroblast clones overexpressing Notchintra with or without *klu* function. Removal

of *klu* function significantly reduced the average number of supernumerary neuroblasts per clone as well as the frequency of clones containing greater than one neuroblast compared with the control clones (Figure 3.7E-G; n=20 clones per genotype). Thus, we propose that aberrant activation of Notch signaling induces the reversion of GMCs to type I neuroblasts by activating multiple downstream genes including *klu* (Figure 3.7H).

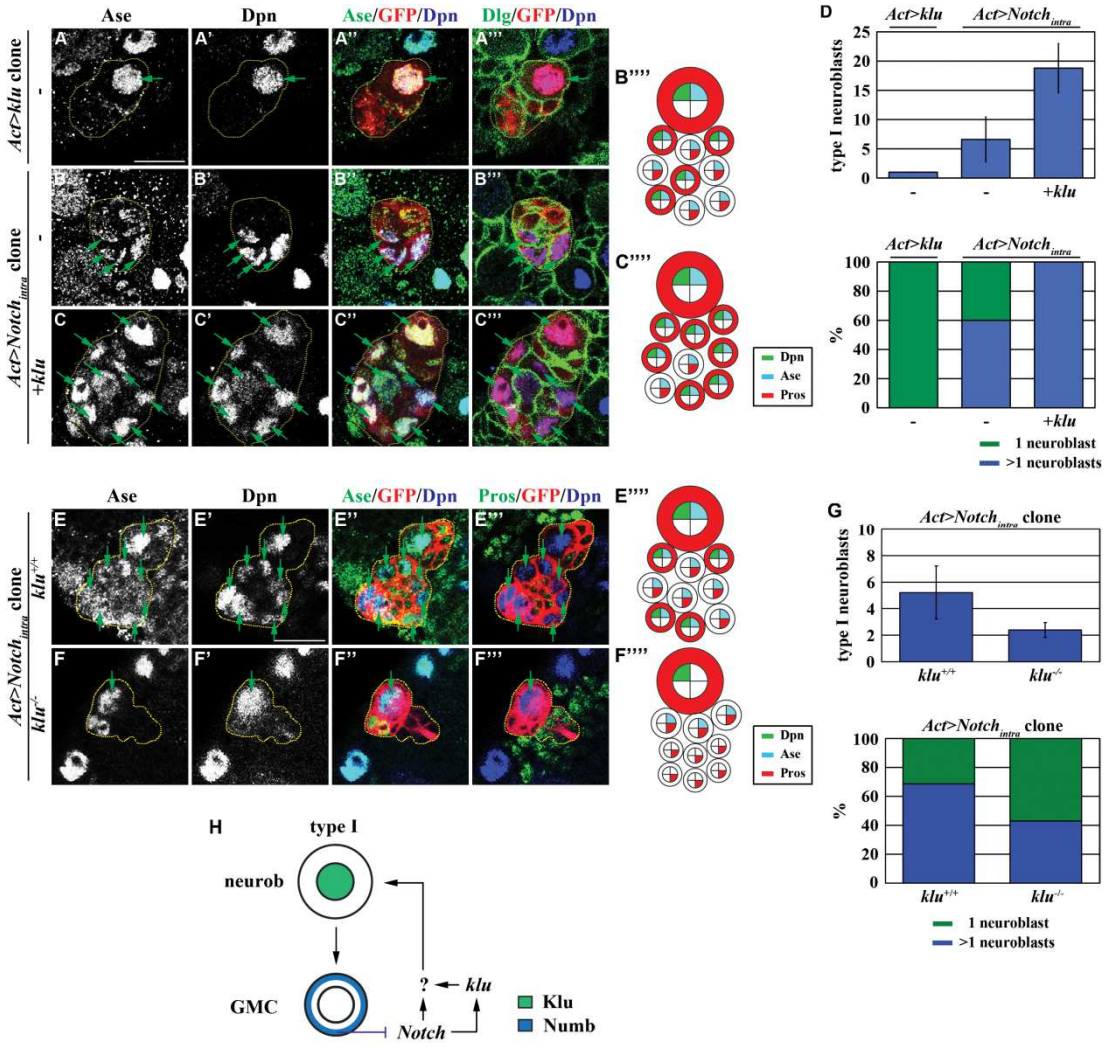

Figure 3.7 Aberrant activation of Notch signaling induces reversion of GMCs in part through *klu*.

(**A-D**) Co-expression of *klu* further exacerbates the formation of supernumerary type I neuroblasts induced by constitutively activated Notch signaling. (A-C'''') *Drosophila* larvae carrying GFP-marked type I neuroblast lineage clones (outlined by the yellow dotted line) overexpressing *klu, Notch*$_{intra}$ or *klu* and *Notch*$_{intra}$ were aged for 48 hours after clone induction and brains were stained for the markers

indicated. (D) Average type I neuroblasts per clone and the frequency of clones containing one or more type I neuroblasts in larvae of the genotype indicated.

(**E-G**) Removal of *klu* function suppresses supernumerary type I neuroblasts induced by constitutively activated Notch signaling. (E-F‴) Larvae carrying GFP-marked $klu^{+/+}$ or $klu^{-/-}$ type I neuroblast mosaic clones (outlined by the yellow dotted line) overexpressing $Notch_{intra}$ were aged for 72 hours after clone induction and brains were stained for the markers indicated. (G) Average type I neuroblasts per clone and the frequency of clones containing one or more type I neuroblasts in larvae of the genotype indicated.

(**H**) Model: Numb prevents the reversion of GMCs to type I neuroblasts by antagonizing Klu. Abbreviations and arrows/arrowheads as Figure 3.1. Scale bars: 10 μm.

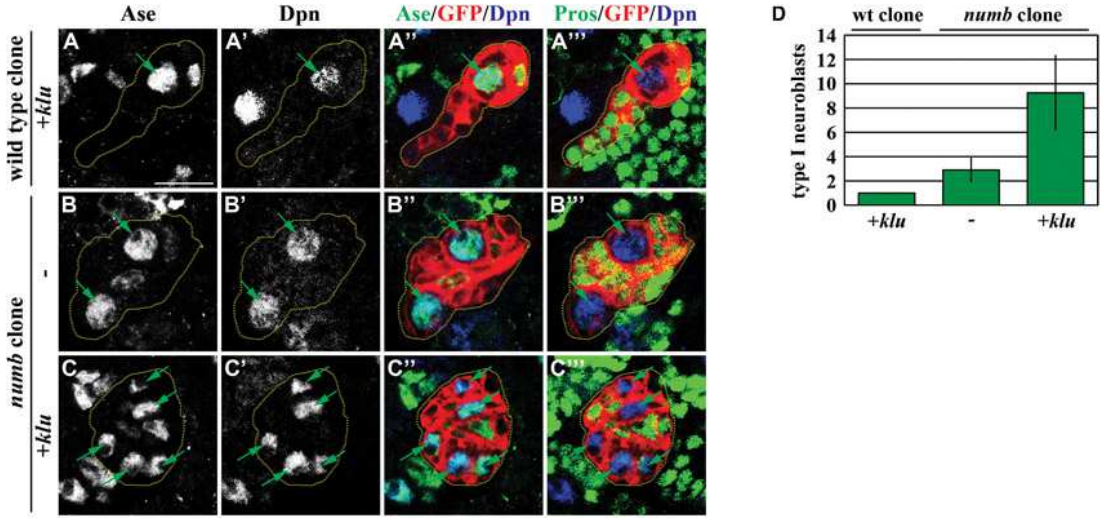

Figure 3.7S Overexpression of *klu* enhances the reversion of GMCs into neuroblasts in *numb* mutant type I neuroblast clones.

(**A-C**) Larvae carrying GFP-marked wild-type or *numb* mutant type I neuroblast mosaic clones (outlined by the yellow dotted line) alone or overexpressing *klu* were aged for 48 hours after clone induction, and brains were stained for the markers indicated. Scale bar: 10 μm.

(**D**) Average type I neuroblasts per brain lobe in larvae of the genotype indicated. Type I neuroblast (Dpn^+ Ase^+, green arrow); GMC (Dpn^- Ase^+, green arrowhead).

Discussion

Asymmetric stem cell division provides an efficient mechanism to preserve a steady stem cell pool while generating differentiated progeny within the tissue where the stem cells reside. Precise spatial control of the stem cell determinants inherited by both sibling cells in every asymmetric cell division ensures that a daughter cell maintains the stem cell characteristics while the sibling progeny acquires the progenitor cell identity. In mitotic type II neuroblasts, the basal proteins Brat and Numb segregate into immature INPs and are required for the formation of INPs (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006a; Lee et al., 2006c; Wang et al., 2006; Bowman et al., 2008; Wirtz-Peitz et al., 2008). Our study significantly extends the findings from previous studies and showed that Brat and Numb function in immature INPs to prevent them from acquiring a neuroblast fate while promoting the INP identity (Figure 3.1S1, 3.6S). Identification and characterization of the *klu* gene led us to propose that Brat and Numb converge to exert precise control of Klu to distinguish an immature INP from its sibling type II neuroblast (Figure 3.6H). Numb also prevents a GMC from reverting to a type I neuroblast by inhibiting Notch signaling in the type I neuroblast lineage (Figure 3.7 and supplementary material Figure 3.7S). Interestingly, although overexpression of *klu* was insufficient to induce supernumerary type I neuroblasts, increased function of *klu* can drastically enhance the reversion of GMCs to type I neuroblasts in the presence of activated Notch signaling (Figure 3.7). Thus, we propose that aberrant activation of Notch signaling induces reversion of GMCs by activating multiple downstream genes including *klu*. Together, our data led us to conclude that precise regulation of *klu* by multiple signaling mechanisms distinguishes a progenitor cell from its sibling stem cell during asymmetric stem cell division.

Regulation of INP maturation

The essential role of Brat and Numb in regulating the formation of INPs is well established, but lack of insight into maturation has hindered investigation into the

mechanisms by which these two proteins distinguish an immature INP from its sibling type II neuroblast (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006a; Lee et al., 2006c; Wang et al., 2006; Bowman et al., 2008; Wirtz-Peitz et al., 2008). A previous study defined immature INPs by the following criteria: (1) being immediately adjacent to the parental type II neuroblast, (2) lacking Dpn expression and (3) displaying a very low level of CycE expression (Bowman et al., 2008). Based on these criteria, analyses of the spatial expression pattern of various cell fate markers in the type II neuroblast lineage clones in wild-type brains revealed that onset of Ase expression correlates with an intermediate stage of maturation (Figure 3.1S1A-A‴). In the 16-hour clones, we reproducibly observed one type II neuroblast (Dpn^+ Ase^- $CycE^+$), two to three Ase^- immature INPs (Dpn^- Ase^- $CycE^-$), two to three Ase^+ immature INPs (Dpn^- Ase^+ $CycE^-$) and INPs (Dpn^+ Ase^+ $CycE^+$) (Figure 3.1S1A-B). Furthermore, we showed that Ase^- immature INPs maintain expression of the type II neuroblast-specific marker PntP1, whereas Ase^+ immature INPs showed virtually undetectable PntP1 expression (Figure 3.3F-H). Thus, onset of Ase expression should serve as a useful marker for an intermediate stage during maturation.

Our data led us to propose that Brat distinguishes an immature INP from its sibling type II neuroblast by indirectly antagonizing the function of Klu based on the following evidence. First, Klu was undetectable in Ase^- immature INPs in the *brat* single-mutant or *brat* and *numb* double-mutant type II neuroblast clones (data not shown). Thus, a Brat-independent mechanism must exist to downregulate Klu in immature INPs. Second, overexpression of a truncated Brat transgenic protein lacking the NHL domain, which is required for repression of mRNA translation (Sonoda and Wharton, 2001), completely suppresses the formation of supernumerary neuroblasts (H.K. and C.-Y.L., unpublished). Thus, it is unlikely that downregulation of Klu in immature INPs occurs via a Brat-dependent translational repression of *klu* mRNA. We propose that Brat might suppress the expression of a co-factor necessary for the function of Klu, just as WT1 requires co-factors in order to regulate the

expression of its target genes in vertebrates (Roberts, 2005). Further investigation will be necessary to discern how Brat establishes restricted developmental potential in immature INPs by antagonizing the function of Klu.

The role of Klu in promoting neuroblast identity

WT1 requires its zinc-finger motifs to regulate transcription of its target genes and can function as an activator or a repressor of transcription in a context-dependent manner (Roberts, 2005). A previous study showed that overexpression of Klu can partially suppress the expression of a lacZ reporter transgene containing the cis-regulatory elements from the even-skipped gene, a putative direct target of Klu, in the fly embryonic central nervous system (McDonald et al., 2003). Since Klu and WT1 display extensive homology in zinc-fingers 2-4, Klu is likely to recognize a similar DNA binding sequence as WT1 (Klein and Campos-Ortega, 1997; Yang et al., 1997; McDonald et al., 2003). The even-skipped cis-regulatory element contains three putative WT1 binding sites, but nucleotide substitutions in these sites that were predicted to abolish Klu binding failed to render the lacZ reporter transgene unresponsive to overexpression of *klu* (McDonald et al., 2003). These data led us to speculate that Klu might recognize a distinct consensus DNA binding sequence to WT1. To test this hypothesis, we generated two UAS-WT1 transgenes that encode the two most prevalent isoforms of the WT1 protein, WT1 −KTS and WT1 +KTS. Interestingly, neither WT1 transgene, when overexpressed by *wor-GAL4*, triggered the formation of supernumerary type II neuroblasts in larval brain (data not shown). This is consistent with Klu recognizing a distinct consensus DNA binding sequence to WT1. However, we cannot rule out the possibility that the inability of the WT1 transgenic protein to induce supernumerary type II neuroblasts is simply due to the absence of necessary co-factors in the fly, as repression of target gene transcription by WT1 requires additional co-factors in vertebrates (Shervington et al., 2006). More studies will be necessary to elucidate the molecular function of Klu in promoting type II neuroblast identity.

Progressive restriction of developmental potential during maturation of immature INPs

Restricted developmental potential functionally defines progenitor cells and allows them to generate differentiated progeny through limited rounds of cell division without impinging on the homeostatic state of the stem cell pool (Zon, 2008; Knoblich, 2010; Weng and Lee, 2011). Despite their importance, the molecular mechanisms by which progenitor cells acquire restricted developmental potential remain experimentally inaccessible in most stem cell lineages. However, studies from various groups have paved the way for using fly larval brain neuroblast lineages as an in vivo model system for investigating how progenitor cells acquire restricted developmental potential (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Bayraktar et al., 2010; Weng et al., 2010).

In this study, we describe the expression pattern of additional molecular markers that allow us to unambiguously identify two distinct populations of immature INPs. Furthermore, we provide experimental evidence strongly suggesting that these two groups of immature INPs possess distinct functional properties. More specifically, Ase^{-} immature INPs readily revert to type II neuroblasts in response to misexpression of Klu, whereas Ase+ immature INPs appear much less responsive to Klu. These data led us to propose that the genome in immature INPs becomes reprogrammed during maturation such that these cells become progressively less responsive to neuroblast fate determinants such as Klu. As a consequence, an INP becomes completely unresponsive to Klu following maturation. Further experiments will be required to validate this model in the future.

Materials and Methods

Fly strains

Mutant and transgenic flies used include brat150 (Betschinger et al., 2006), numb2 (Skeath and Doe, 1998), kluR51 (Kaspar et al., 2008), erm-GAL4 (III) (Pfeiffer et al., 2008), wor-GAL4 (Lee et al., 2006b), UAS-klu-HA, UAS-klu1-583-HA and UAS-kluΔzf1-HA (Kaspar et al., 2008), UAS-Notchintra (Chung and Struhl, 2001) and UAS-cMyc (Benassayag et al., 2005). erm-GAL4 (II) was generously provided by Dr G. Rubin (HHMI). The following stocks were obtained from the Bloomington Drosophila Stock Center: Oregon R, bratDG19310, bratk06028 (Arama et al., 2000), brat11 (Arama et al., 2000), Notch55e11 (Artavanis-Tsakonas et al., 1984), klu09036, Df(H99) (White et al., 1994), UAS-mCD8-GFP, UAS-apoliner (Bardet et al., 2008), UAS-p35, UAS-GFP, FRT19A (Lee and Luo, 2001), FRT2A, hs-flp (Lee and Luo, 2001), Act-FRT-Stop-FRT-GAL4 (Pignoni and Zipursky, 1997), tub-GAL80 (Lee and Luo, 2001) and tub-GAL80ts (Bloomington Drosophila Stock Center). Transgenic fly lines UAS-brat-myc, UAS-HA-klu, UAS-HA-klu1-583, UAS-HA-kluΔzf1 and UAS-HA-kluΔzf4 were generated using the pUAST-attB vector for insertion into an identical docking site in the fly genome via ϕC31 integrase-mediated transgenesis (Bischof and Basler, 2008).

Immunofluorescent staining and antibodies

Larval brains were dissected in Schneider's medium (Sigma), fixed in 4% formaldehyde for 23 minutes and washed twice for 20 minutes each in 1× PBS containing 0.3% Triton X-100 (PBST). After washing, brains were incubated with primary antibodies in PBST for 3 hours at room temperature. Antibodies used include rat anti-Dpn (1:1000; this study), rabbit anti-Ase (1:400) (Weng et al., 2010), guinea pig anti-Ase (1:50; this study), mouse anti-Prospero (MR1A, 1:100) (Lee et al., 2006a), guinea pig anti-CycE (1:1000; T. Orr-Weaver, Massachusetts Institute of Technology, MA, USA), mouse anti-Dlg (1:50; Developmental Studies Hybridoma Bank), chicken anti-GFP (1:2000; Aves Labs), rabbit anti-Klu (1:200) (Yang et al.,

1997), rat anti-Mira (1:100) (Lee et al., 2006a), guinea pig anti-Numb (1:1000; J. Skeath, Washington University, WA, USA), rabbit anti-aPKC (1:1000; Sigma), mouse anti-phosphohistone H3 (1:2000; Upstate Biotechnology), rabbit anti-PntP1 (1:600; J. Skeath) and rabbit anti-RFP (1:100; Rockland). Secondary antibodies were from Molecular Probes and Jackson Labs. We used Rhodamine phalloidin (1:100; Invitrogen) to visualize cortical actin. The confocal images were acquired on a Leica SP5 scanning confocal microscope.

Clonal analyses

Lineage clones were induced following the previously published method (Lee and Luo, 2001; Weng et al., 2010).

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CHAPTER IV

Conclusions and Perspectives

Neural stem cells employ several strategies to generate the requisite number of diverse differentiated cell types required for proper brain development, but the precise mechanisms underlying these strategies are not understood. My thesis work focuses on elucidating the mechanistic insight into two of the strategies-maintenance of functionally heterogeneous neural stem cells and precise specification of the intermediate progenitor cell functional identity. I used neural stem cells in the fly larval brain as a model system for my study because they provide an excellent *in vivo* genetic model for investigating various fundamental questions in neural stem cell biology (Bello, 2008; Boone, 2008; Bowman, 2008). The outcome of my thesis work has significantly advanced our understanding in the regulation of neural stem cell functional heterogeneity and the specification of intermediate progenitor cell functional identity during fly larval neurogenesis. Given that these signaling mechanisms are highly conserved, the findings from my thesis will likely have direct relevance in the regulation of neural stem cells during vertebrate brain development.

Chapter II of my thesis elucidates an epigenetic regulatory mechanism that maintains the heterogeneity of neural stem cells (neuroblasts). In collaboration with a post-doctoral fellow in the lab, this series of experiments shows that the functional identities of neuroblasts are specified at birth but require the maintenance of an epigenetic memory to continuously preserve their identities afterbirth. This study identified the Trithorax (Trx) histone methyltransferase complex, the fly homolog of the vertebrate the SET1/MLL complex, as the key regulator that maintains the epigenetic memory required to preserve the functional heterogeneity of larval brain neuroblasts. By combining biochemical and genetic approaches, this study identifies that the *buttonhead* (*btd*) gene, which encodes a highly conserved C_2H_2 zinc-finger

transcription factor, elicits the Trx-regulated epigenetic memory to maintain neuroblast heterogeneity during fly larval brain neurogenesis.

Trx and Polycomb-group (PcG) proteins frequently act antagonistically to regulate target gene transcription: Trx activates gene transcription whereas PcG proteins repress gene transcription (Schuettengruber, 2011). Thus, it is tempting to speculate that the PcG proteins might also contribute to the maintenance of fly larval neuroblasts. However, a previously published study as well as our own study strongly suggests that the PcG proteins mainly function to maintain the viability of larval brain neuroblasts. Thus, it is likely that Trx maintains neuroblast heterogeneity via a PcG-independent mechanism. Furthermore, immunofluorescent staining using an antibody specifically against Trx revealed that the endogenous Trx protein is expressed ubiquitously in all cells in the fly larval brain. As such, two key questions derived from this series of results await future investigations. First, how is the histone methyltransferase activity of the Trx complex specifically conferred to the promoter region of the *btd* gene that functions exclusively to maintain the functional identity of type II neuroblasts? One possible mechanism might be that the Trx complex maintains interacts with other sequence-specific transcription factors that are uniquely expressed in type II neuroblast. Thus, identifying additional proteins that interact with the Trx complex will be a critical first step toward elucidating the mechanisms that confer the specificity of the histone methyltransferase activity of the Trx complex to maintain the chromatin in the *btd* locus in an open state. Second, are there additional downstream targets of Trx that also function to maintain neuroblast heterogeneity? The combination of genomic and genetic approaches will most certainly lead to the identification of additional candidate genes that might act in parallel or downstream of *btd* to maintain neuroblast heterogeneity during larval brain neurogenesis.

Chapter III of my thesis investigates the mechanisms underlying specification of the intermediate progenitor cell (INP) functional identity. I found that the *klumpfuss* (*klu*) gene, the fly homolog of the vertebrate *Wilm's tumor 1* tumor suppressor gene,

functions as a key regulator for neuroblast self-renewal. Removing *klu* function leads to loss of both type I and type II neuroblasts, whereas mis-expression of *klu* induces the reversion of an immature INP into a supernumerary type II neuroblast. Importantly, reducing *klu* function significantly reduces the formation of supernumerary type II neuroblasts in the larval brain mutant for the *brain tumor* gene, which asymmetrically extinguishes the function of self-renewal factors in the immature INP. Thus, rapid down-regulation of *klu* is essential for proper specification of the INP functional identity.

Klu is predicted to function as a transcriptional repressor protein, and a key future question is how a transcription repressor protein might function to regulate neuroblast self-renewal. To begin to address this question, I first tested whether Klu indeed regulates neuroblast self-renewal by transcriptional repressing gene expression. Consistently, over-expression of a Klu chimeric transgenic protein that acts solely as a transcriptional repressor induces supernumerary type II neuroblast formation whereas over-expression of a second Klu chimeric transgenic protein that acts solely as a transcriptional activator has no effects (unpublished data). In addition, I identified that the *rpd3* gene, which encodes a class I histone deacetylase (Yang, 2008), is required for the formation of supernumerary type II neuroblasts induced by over-expression of *klu* (data not presented). These data strongly suggest that Klu promotes neuroblast self-renewal by transcriptionally repressing gene expression. Interestingly, Rpd3 is also required for the formation of supernumerary type II neuroblasts induced by over-expression of two other neuroblast self-renewal transcription factors Deadpan (Dpn) and Enhancer of splits mγ (E(spl)mγ) that are also predicted to function as transcriptional repressor proteins. These data strongly suggest that components of a stem cell self-renewal network most likely maintain the neuroblast identity by preventing differentiation. These results provide a powerful platform for future experiments to investigate how transcriptional repression of the differentiation program contributes to the self-renewal of neuroblasts and the specification of INPs. Given that Klu, Dpn and E(spl)mγ are highly conserved from

flies to humans, the mechanisms by which these transcriptional repressor proteins regulate neural stem cell self-renewal and intermediate progenitor cell specification during fly larval brain neurogenesis might be directly relevant to the regulation of neural stem cells during vertebrate neurogenesis.

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