

**The role of Lgl, aPKC, and Numb in distinguishing neural stem cells from
progenitor cells in *Drosophila***

by

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To my husband Adam and son Kurt,
who sustain my drive to explore
and make everyday a new adventure

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ABSTRACT

Asymmetric cell division is a conserved mechanism for distinguishing cells following mitosis in order to produce two daughter cells with unique functions and characteristics. Significant progress has been made in understanding how proteins at the cell cortex become asymmetrically localized and how determinants function in establishing distinct cell fates. Also, recent studies have shown that cells actively maintain the fate which they initially acquired. My studies utilize clonal analysis and genetic techniques to investigate how this cell polarity and maintenance of cell fate are linked in order to distinguish neural stem cells from progenitor cells in *Drosophila*. In mitotic neural stem cells (neuroblasts) in fly larval brains, the antagonistic interaction between the polarity proteins Lethal (2) giant larvae (Lgl) and atypical Protein Kinase C (aPKC) ensures self-renewal of a daughter neuroblast and generation of a progenitor cell by regulating asymmetric segregation of fate determinants. In the absence of *lgl*, increased cortical aPKC activity triggers progenitor cells to revert back into neuroblasts. Additionally, I found that together Lgl and aPKC ensure asymmetric localization and segregation of Numb in the cortex of the mitotic neuroblast. Specifically, aPKC regulates asymmetric localization of Numb via phosphorylation of the previously undefined serines 48 and 52 sites in the novel ACBD3 binding region. However, the phosphorylation status at these two sites does not affect the function of Numb in the progenitor cells. Finally, the ACBD3

binding region exerts neuroblast-specific suppression of *Notch* signaling by Numb independently of phosphorylation by aPKC. Taken together, my work suggests that mutual antagonism between Lgl and aPKC ensures that Numb properly segregates exclusively into the progenitor cells where Numb maintains limited potential irrespective of the phosphorylation by aPKC.

Chapter 1

Introduction

For more than a century, developmental biologists have aimed to understand how multicellular organisms arise from a single cell. During development, both cell proliferation and cell fate specification occur in order to increase the number of cells and to generate cells with unique functions, respectively. Studies of the leech cell lineage revealed that distinct cytoplasmic regions of the leech egg are differentially inherited by its descendants. This led to the hypothesis that the two daughter cells of a single division can be different from one another from the time they are produced (Whitman, 1878). By the early Twentieth century, it became clear that certain tissues or organs originate from specific cell lineages due to determinants that are localized to particular regions of the zygote (Conklin, 1905; Wilson, 1925). It was proposed that these determinants act by controlling distinct patterns of gene expression in each cell lineage (Morgan, 1934). Together with many experimental advances over the last hundred years, these studies have led to the identification of several mechanisms for generating cellular diversity in developing organisms, including the identification of embryonic and tissue-specific stem cells.

Stem cells are the foundation of tissues and organs during development and maintain tissue homeostasis in adults. Importantly, stem cells maintain their pool while producing distinct progeny through asymmetric cell division.

Asymmetric stem cell divisions result in self-renewal of a stem cell and differentiation of the other daughter cell. Defects in asymmetric division potentially lead to depletion or expansion of the stem cell pool (Figure 1.1). Such defects can perturb homeostasis and contribute to tumor initiation. Thus, insight into the mechanisms regulating asymmetric cell division will likely improve our understanding of normal developmental and aberrant processes from developmental defects to tumorigenesis. Recently, we have learned that not only do these cells need to initially commit to their distinct cell fates, but they must also employ active mechanisms to maintain their identities (Weng et al., 2010). However, the interplay between the mechanisms that establish cortical cell polarity and the mechanisms that maintain distinct cell fates are not well understood. In chapter 2, I present my work on the role of cell polarity components in both distinguishing daughter cells and maintaining cell fates in the *Drosophila* neural stem cell lineages.

Overview of Asymmetric Cell Division

Asymmetric cell division of both stem cells and other somatic cells contributes significantly to generating cellular diversity during development. Both extrinsic and intrinsic mechanisms can direct two daughter cells to adopt distinct fates (Horvitz and Herskowitz, 1992). The extrinsic mechanism relies on cell-cell communication and disparate localization of the daughter cells with respect to the external environment. Here, regulated spindle alignment asymmetrically positions the two identical daughter cells and causes them to receive distinct cues from their sibling cell and/or neighboring cells, which ultimately leads to

unique signaling events and fate specification in each daughter cell. In contrast, the intrinsic mechanism depends on proper spindle alignment and asymmetric localization of cell fate determinants for unequal partitioning immediately following mitosis. In this case, each daughter cell inherits a unique set of factors that distinguishes them from birth. In various contexts, extrinsic and intrinsic mechanisms of asymmetry are utilized both separately and in conjunction with one another.

Asymmetric Cell Divisions in the *Drosophila* Nervous System

In both vertebrates and *Drosophila*, asymmetric cell division is an essential method for generating the diverse cells of both the peripheral and central nervous system (PNS and CNS, respectively). In the *Drosophila* PNS, the cells that form each external sensory organ derive from characteristic sequential divisions of a single sensory organ precursor (SOP) cell (Hartenstein and Posakony, 1989). Additionally, neural stem cells, called neuroblasts, in the *Drosophila* CNS divide asymmetrically at each division to self-renew and generate a daughter cell destined for differentiation. Though similar in many respects, neurogenesis in the PNS and CNS also has some distinguishing characteristics.

To generate the external sensory organ, each SOP cell is specified from a pool of neural competent cells by lateral inhibition. The SOP then divides asymmetrically to generate a posterior pIIa and an anterior pIIb cell. The pIIb cell divides to give rise to a glial cell and a pIIIb cell, which subsequently divides to produce a sheath cell and a neuron. The glial cell migrates away from the

sensory organ (Gho et al., 1999) and, at least in some lineages, undergoes apoptosis (Fichelson and Gho, 2003). Asymmetric division of the pIIa cell generates a socket cell and a hair cell. Each division in the SOP lineage is governed by both cell-extrinsic cues, such as differential Notch signaling between the daughter cells, and cell-intrinsic cues, such as asymmetric localization of cell fate determinants. All of these divisions are asymmetric and each results in two daughter cells with distinct fates, ultimately producing the four cells that make up each external sensory organ.

In the *Drosophila* CNS, neuroblasts are also specified by lateral inhibition during embryogenesis. Once specified, embryonic neuroblasts delaminate from the neuroectoderm and enter the cell cycle to undergo up to 20 rounds of asymmetric cell division. During mitosis, the spindle aligns along the apical-basal axis of the overlying epithelia and cytokinesis results in two daughter cells with distinct sizes and fates. The larger, apical daughter cell retains the neuroblast identity, while the smaller, basal daughter cell differentiates into a ganglion mother cell (GMC), which divides to produce two terminally differentiated post-mitotic neurons or glia. Here, the asymmetry of size and fate is governed cell autonomously, while extrinsic signaling determines the apical-basal orientation of the daughter progeny relative to the epithelia and organismal axis.

Following a brief quiescent period during the embryo-to-larval transition, the reactivated neuroblasts once again divide asymmetrically in the larval stage. In the larval central brain, there are at least two types of neuroblast lineages with unique cellular compositions and molecular characteristics (Fig. 1.2) (Bello et al.,

2008; Boone et al., 2008; Bowman et al., 2008). Type I neuroblasts are marked by the presence of the transcription factors Deadpan (Dpn) and Asense (Ase) and divide asymmetrically to regenerate a neuroblast and produce a GMC, which undergoes a terminal division to generate two post-mitotic cells (neurons and/or glia). On the other hand, type II neuroblasts are unambiguously identified by the presence of Dpn and lack of Ase expression and divide asymmetrically to produce a neuroblast and a progenitor cell, called an intermediate neural progenitor (INP). Immature INPs are derived directly from the asymmetric type II neuroblast division, lack expression of both Dpn and Ase, and are arrested in the cell cycle. Upon maturation, INPs re-acquire proliferation potential and undergo a limited number of asymmetric divisions to self-renew an INP and produce a GMC. In addition to this limited ability to self-renew, INPs are smaller than neuroblasts and express a distinct set of markers, namely Dpn, Ase, and Earmuff (Erm) (Weng et al., 2010). Asymmetric divisions of the neuroblasts and INPs depend solely on the intrinsic establishment of cortical cell polarity and asymmetric segregation of cell fate determinants.

Mechanisms for Asymmetric Localization in the *Drosophila* Nervous System

Many molecular components have been identified and characterized to regulate asymmetric cell division in multiple contexts. Asymmetric segregation of cell fate determinants is established by cortical polarity cues during *Drosophila* neurogenesis. While the SOP and neuroblast lineages employ common molecular components and mechanisms during asymmetric division, many

differences have emerged. In the dividing SOP, the polarization of the cell cortex orients the mitotic spindle in respect to the planar polarity cue (Gho et al., 1998; Bellaiche et al., 2001a; Roegiers et al., 2001) by forming an anterior crescent of Dlg, Pins, and Gai (Bellaiche et al., 2001b; Schaefer et al., 2001). In turn, Dlg/Pins/Gai directs the posterior positioning of the Par complex components, including Bazooka/Par3 (Baz), Par6, and atypical Protein Kinase C (aPKC) (Bellaiche et al., 2001b). The conserved Par complex is required to promote anterior localization of cell fate determinants, including Numb (Bellaiche et al., 2001b; Roegiers et al., 2001; Rolls et al., 2003). aPKC is a critical component of the Par complex that localizes opposite to Numb during SOP mitosis. The Par complex functions, at least in part, through aPKC-mediated phosphorylation of the cortical protein Lethal(2) giant larvae (Lgl) at the posterior cortex, which in turn regulates polarized localization of cell fate determinants (Betschinger et al., 2003). However, in the complete absence of *lgl* activity, asymmetric localization of Numb can be observed in the dividing SOP (Langevin et al., 2005; Justice et al., 2003). This observation led to the finding that aPKC directly phosphorylates Numb and these phosphorylation sites are critical for regulating asymmetric membrane localization of Numb during SOP divisions (Smith et al., 2007).

The basic mechanisms that govern asymmetric protein localization are conserved between embryonic and larval brain neuroblasts. One notable exception is that the G-protein coupled receptor *Tre1* links the orientation of division for embryonic neuroblasts to the overlying epithelia (Yoshiura et al., 2012), while larval brain neuroblasts orient division independently of an extrinsic

cue. The initial establishment of the apical domain in embryonic neuroblasts results from the inheritance of the apical Par complex from the epithelial cells of the neuroectoderm as the neuroblasts delaminate (Wodarz et al., 1999; Schober et al., 1999; Wodarz et al., 2000; Rolls et al., 2003). The presence of Inscuteable (Insc) in neuroblasts links the Pins/Gai complex to the Par complex and results in co-localization of these two complexes in the apical cortex (Parmentier et al., 2000; Schaefer et al., 2000; Yu et al., 2000), unlike the localization of Dlg/Pins/Gai and the Par complex to opposite poles of the dividing SOP. The Insc/Pins/Gai complex also interacts with the microtubule binding protein Mud to orient the mitotic spindle along the apical-basal polarity axis (Siller et al., 2006; Izumi et al., 2006; Bowman et al., 2006). Localization of the Par complex to the apical cortex directs segregation of the cell fate determinants and their adapter proteins to the basal side of the neuroblast.

Similarly to the SOP lineage, Lgl is a substrate of aPKC in neuroblasts (Betschinger et al., 2003). In embryonic neuroblasts, Lgl is necessary for proper localization of the basal determinants; however, loss of *lgl* does not affect spindle orientation or the apical localization of the Par complex (Peng et al., 2000; Ohshiro et al., 2000). In larval neuroblasts of *lgl* mutants, aPKC also becomes mislocalized and hyperactivated (Alberson and Doe, 2003; Lee et al., 2006b). In addition, phosphorylation of Lgl by aPKC induces an intramolecular interaction that prevents Lgl function (Betschinger et al., 2005), while non-phosphorylatable Lgl inhibits asymmetric protein localization (Betschinger et al., 2003). A more recent study found that the presence of Lgl in a complex with aPKC and Par6

prevents phosphorylation of Numb by aPKC. Upon phosphorylation by aPKC, Lgl is exchanged for Baz/Par3 and the complex, now containing aPKC, Baz/Par3, and Par6, is capable of phosphorylating Numb and directing its asymmetric localization (Wirtz-Peitz et al., 2008). Therefore, Lgl regulates the substrate specificity and possibly the activity of aPKC. aPKC also phosphorylates and regulates the cortical localization of another basal determinant, Miranda (Mira) (Atwood and Prehoda, 2009). Together, the antagonistic interaction between Lgl and aPKC directs asymmetric localization of the basal protein complexes (Fig. 1.3). The data presented in chapter 2 builds upon this knowledge and clarifies how aPKC regulates the localization and function of Numb during asymmetric cell division in the *Drosophila* nervous system.

Specification of Distinct Cell Fates in the *Drosophila* Nervous System

Segregating determinants are partitioned unequally into the daughter cells and promote or suppress specific cell fate decisions during asymmetric cell division. Numb was the first factor to be identified as both segregating asymmetrically and determining the differences in cell fate (Rhyu, et al 1994). During SOP and neuroblast mitosis, Numb localizes as a cortical crescent opposite of the Par complex and is exclusively inherited by one of the two daughter cells, where it inhibits Notch signaling (Uemura et al., 1989; Rhyu et al., 1994). In the dividing SOP, loss of *numb* causes both daughter cells to acquire the cell fate normally associated with the cell that does not inherit the Numb protein, similar to overactivation of Notch (Rhyu et al., 1994). On the other hand,

Numb overexpression prior to SOP division results in the opposite cell fate conversion and loss of bristles, which phenocopies the loss of *Notch* (Frise et al., 1996; Rhyu et al., 1994). Further genetic and biochemical analyses demonstrate that asymmetrically inherited Numb inhibits Notch signaling in one daughter cell to establish a binary cell fate decision (Guo et al., 1996; Hartenstein and Posakony, 1990). However, the mechanism by which Numb inhibits Notch signaling remains controversial, possibly due to the existence of context specific mechanisms.

The transmembrane Notch receptor is activated by ligands, such as Delta, followed by multiple cleavage events that ultimately release the intracellular domain of Notch from the membrane. Subsequently, the intracellular domain of Notch translocates into the nucleus (Couturier et al., 2012) and triggers binary cell fate decisions in the SOP lineage through the downstream targets, Suppressor of Hairless (Su(H)) (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995) and Tramtrack (Guo et al., 1996). Epistatic analysis reveals that *numb* functions upstream of *Notch* during formation of the sensory organs (Guo et al., 1996). Numb inhibits Delta-dependent Notch signaling in cultured *Drosophila* S2 cells and ectopically expressed Numb inhibits Notch function during wing development (Frise et al., 1996). Additionally, the phospho-tyrosine binding (PTB) domain of Numb, which is functionally critical, interacts directly with the Notch intracellular domain (Guo et al., 1996; Yaich et al., 1998), an interaction that is conserved amongst the mammalian homologs (Zhong et al.,

1996). This direct binding is potentially implicated in Numb-mediated endocytosis of the Notch receptor (Couturier et al., 2012).

During neuroblast divisions, improper specification of cell fate leads to cell fate transformations characterized by either the expansion or loss of the self-renewing neuroblast population. The daughter cell that inherits aPKC remains a neuroblast and continues to proliferate, while the daughter cell lacking aPKC undergoes differentiation to become a progenitor cell. Consistently, unrestrained cortical aPKC activity results in supernumerary neuroblasts whereas removal of aPKC leads to a reduction in neuroblasts (Lee et al., 2006b; Rolls et al., 2003). Concurrently, segregation of the basal complexes, consisting of Mira-Pros-Brat and Pon-Numb, into the progenitor cells (GMCs and/or INPs) promotes differentiation of these cells. While *pros* is necessary during asymmetric type I neuroblast and INP divisions (Choksi et al., 2006; Weng et al., 2010) and *brat* is primarily required for fate specification in the type II neuroblast lineage (Lee et al., 2006c), *numb* distinguishes progenitor cells from neuroblasts in both type I and type II neuroblast lineages (Bowman et al., 2008).

In the CNS, like in the PNS, Numb mediates binary cell fates by antagonizing Notch. In type I neuroblasts, the loss of *numb* or failure to asymmetrically segregate Numb results in supernumerary neuroblasts at the expense of differentiated neurons (Lee et al., 2006a; Wang et al., 2007; Bowman et al., 2008; Wirtz-Peitz et al., 2008; Haenfler et al., 2012). Mutation of *numb* in the type II neuroblast lineage results in expansion of neuroblasts due to rapid reversion of immature INPs (Bowman et al., 2008). In agreement with the role of

Numb to inhibit Notch signaling, ectopic expression of constitutively active Notch (Notch_{intra}) disrupts asymmetric neuroblast divisions and leads to supernumerary neuroblasts in both type I and II lineages. Interestingly, reductions in Notch signaling cause a complete loss of type II neuroblasts, but have no effect on the type I neuroblast number (Bowman et al., 2008; Haenfler et al., 2012). Thus, Numb establishes differential Notch signaling in daughter cells following asymmetric division to regulate cell fate determination. In chapter 2, I address whether Numb functions by the same mechanism during both SOP and neuroblast asymmetric divisions.

Maintenance of Cell Fate after Asymmetric Division of *Drosophila*

Neuroblasts

A recent study found that, following specification and maturation, the progenitor cells (INPs) in the type II neuroblast lineage require an active mechanism, mediated by the transcription factor Erm, to maintain their cell fate (Weng et al., 2010). In *erm* mutant larval brains, the type II neuroblasts divide asymmetrically to produce correctly specified INPs; however, without *erm* activity, these INPs can dedifferentiate back into the type II neuroblasts, which are indistinguishable from the normal parental neuroblasts. Furthermore, constitutive activity of Notch also transforms INPs into neuroblasts as demonstrated by expansion of the type II neuroblast pool following ectopic expression of Notch_{intra}. Finally, Erm prevents dedifferentiation by antagonizing Notch signaling (Weng et al., 2010). In chapter 2, I show that *lgf* antagonizes *aPKC* to maintain the asymmetric cell fates in both type I and type II neuroblasts.

No such mechanism has been described in the *Drosophila* PNS and perhaps it is uniquely necessary for progenitor cells that retain a more extensive potential to proliferate. Taken all together, this suggests that proper regulation of Notch signaling is critical not only for the establishment but also for the maintenance of asymmetric cell fates.

Concluding Remarks

Distinguishing neural stem cells from progenitor cells involves several key processes, including asymmetric protein segregation, cell fate specification, and maintenance of cell identity. The study of asymmetric cell division in *Drosophila* reveals that the functions of several core signaling pathways are conserved. In particular, the Par complex regulates the asymmetric localization of cell fate determinants, while the asymmetric Notch signaling distinguishes daughter cell fates in both SOP and neuroblast lineages. Therefore, understanding the molecular pathways involved in asymmetric division in the *Drosophila* nervous system will likely provide valuable knowledge for other cellular asymmetries. For example, Numb plays a role in directional cell migration, which is an asymmetric process and is important for development, immune response, and cancer metastasis (Nishimura et al., 2007). Both Numb and Notch also play roles in mammalian neurogenesis (Zhong et al., 1996; Lowell et al., 2006). Further investigations analyzing the commonalities and eccentricities of these model systems will identify the overarching themes and mechanisms employed during asymmetric cell division. Since asymmetric cell division contributes to normal

development, tissue homeostasis, and perhaps tumorigenesis, future studies are likely to unveil critical insights for both invertebrate and vertebrate biology.

Figures

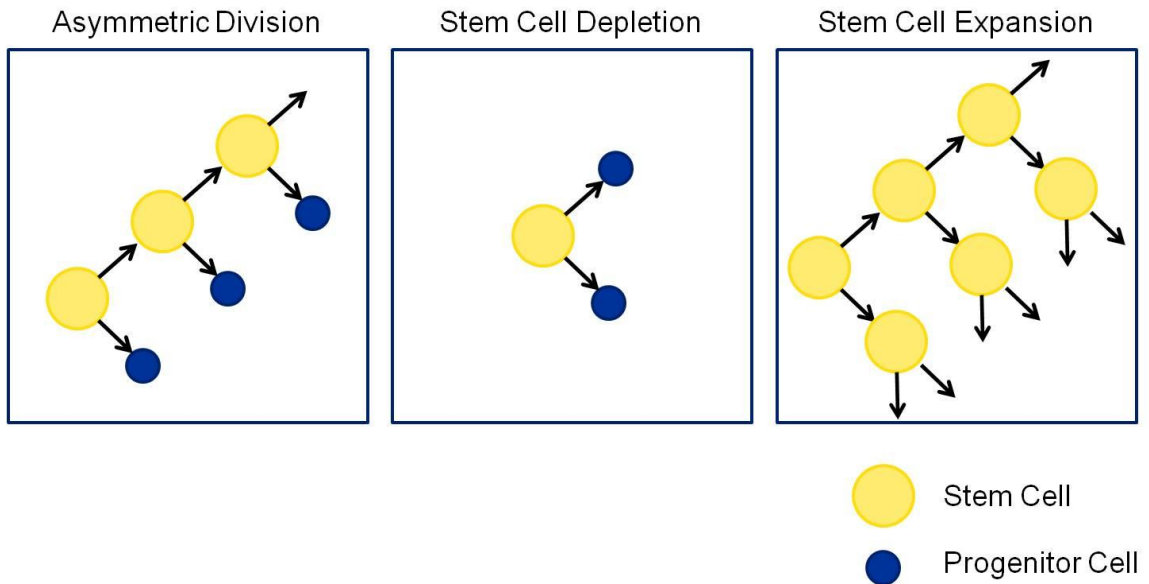


Figure 1.1 Defects in asymmetric division disrupt the stem cell pool

The schematic diagram shows normal asymmetric division (left panel) of a stem cell (large yellow circle) to self-renew and produce differentiating progeny (small blue circle). Each subsequent division results in a similar outcome. Disrupting the asymmetric division of a stem cell can result in depletion (middle panel) or expansion (right panel) of the stem cell pool.

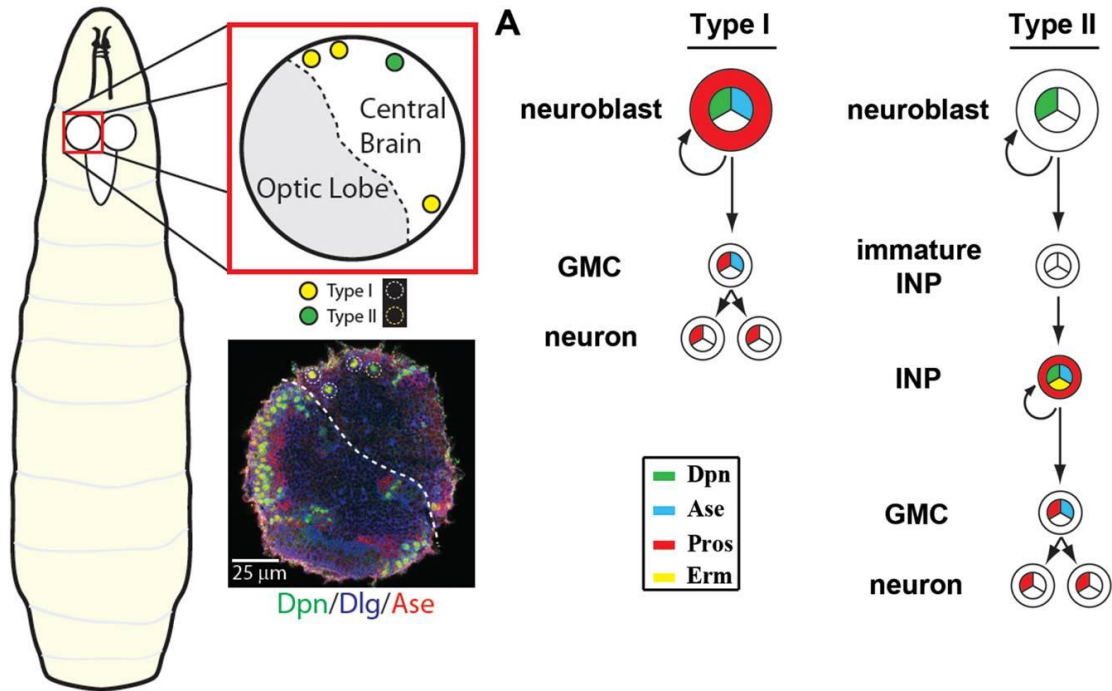


Figure 1.2 *Drosophila* neuroblasts divide asymmetrically

Type I and type II neuroblasts in the *Drosophila* larval central brain can be identified by specific markers (left) and produce distinct lineages (right).

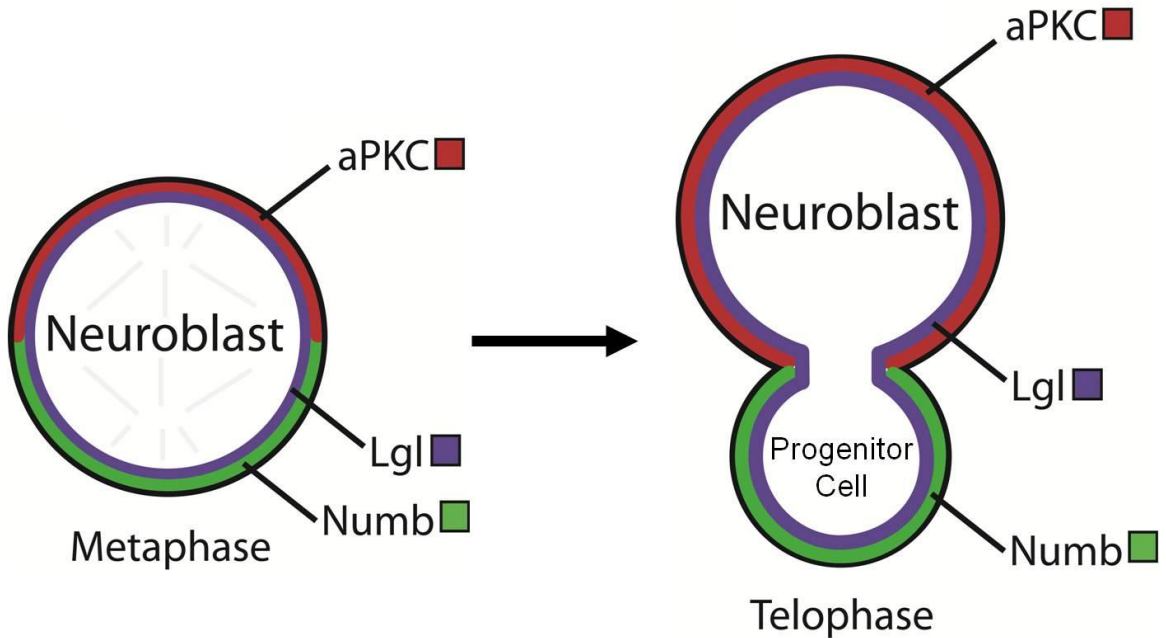


Figure 1.3 Cell fate determinants localize and segregate asymmetrically in mitotic neuroblasts

Lgl localizes throughout the cortex of mitotic neuroblasts and regulates asymmetric segregation. aPKC localizes to the apical cortex during metaphase and is inherited by the future neuroblast during telophase. Together, aPKC and Lgl regulate the localization of Numb to the basal cortex and subsequent segregation of Numb into the future progenitor cell at telophase.

Chapter 2

Cortical aPKC kinase activity distinguishes neural stem cells from progenitor cells by ensuring asymmetric segregation of Numb

Summary

During asymmetric stem cell division, polarization of the cell cortex targets fate determinants unequally into the sibling daughters, leading to regeneration of a stem cell and production of a progenitor cell with restricted developmental potential. In mitotic neural stem cells (neuroblasts) in fly larval brains, the antagonistic interaction between the polarity proteins Lethal (2) giant larvae (Lgl) and atypical Protein Kinase C (aPKC) ensures self-renewal of a daughter neuroblast and generation of a progenitor cell by regulating asymmetric segregation of fate determinants. In the absence of Lgl function, elevated cortical aPKC kinase activity perturbs unequal partitioning of the fate determinants including Numb and induces supernumerary neuroblasts in larval brains.

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However, whether increased aPKC function triggers formation of excess neuroblasts by inactivating Numb remains controversial. To investigate how increased cortical aPKC function induces formation of excess neuroblasts, we analyzed the fate of cells in neuroblast lineage clones in *Igl* mutant brains. Surprisingly, our analyses revealed that neuroblasts in *Igl* mutant brains undergo asymmetric division to produce progenitor cells, which then revert back into neuroblasts. In *Igl* mutant brains, Numb remained localized in the cortex of mitotic neuroblasts and failed to segregate exclusively into the progenitor cell following completion of asymmetric division. These results led us to propose that elevated aPKC function in the cortex of mitotic neuroblasts reduces the function of Numb in the future progenitor cells. We identified that the acyl-CoA binding domain containing 3 protein (ACBD3) binding region is essential for asymmetric segregation of Numb in mitotic neuroblasts and suppression of the supernumerary neuroblast phenotype induced by increased aPKC function. The ACBD3 binding region of Numb harbors two aPKC phosphorylation sites, serines 48 and 52. Surprisingly, while the phosphorylation status at these two sites directly impinged on asymmetric segregation of Numb in mitotic neuroblasts, both the phosphomimetic and non-phosphorylatable form of Numb suppressed formation of excess neuroblasts triggered by increased cortical aPKC function. Thus, we propose that precise regulation of cortical aPKC kinase activity distinguishes the sibling cell identity in part by ensuring asymmetric partitioning of Numb into the future progenitor cell where Numb maintains restricted potential independently of regulation by aPKC.

Introduction

During asymmetric stem cell divisions, polarization of the cell cortex allows unequal partitioning of the cell fate determinants that instruct the daughter progeny to either self-renew as a stem cell or adopt the progenitor cell identity (Neumüller and Knoblich, 2009; Prehoda, 2009). Progenitor cells possess restricted developmental potential and undergo limited rounds of cell division that give rise to differentiated progeny. Mis-regulation of cortical polarity in asymmetrically dividing stem cells can impinge on the accumulation and/or function of fate determinants in the intended recipient cell. Such defects can lead to generation of progenitor cells that possess stem cell-like properties, perturbing homeostasis and contributing to tumor initiation. Thus, insight into the mechanisms that distinguish sibling cell identity during normal tissue development will likely improve our understanding of aberrant processes from congenital birth defects to tumorigenesis.

In fly larval brains, two classes of neuroblast lineages can be unambiguously identified based on expression of the cell fate markers and properties of their daughter progeny (Chia et al., 2008; Doe, 2008; Egger et al., 2008; Knoblich, 2008; Knoblich, 2010; Weng and Lee, 2011) (Figure 2.7S A). A type I neuroblast expresses Deadpan (Dpn) and Asense (Ase) and divides asymmetrically to self-renew a neuroblast and to generate a progenitor cell called a ganglion mother cell (GMC). In contrast, a type II neuroblast (Dpn^+Ase^-) divides asymmetrically to self-renew and to generate an immature intermediate neural progenitor cell (INP) that lacks the expression of Dpn and Ase and is transiently

arrested in the cell cycle while acquiring 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 divisions to regenerate and to produce GMCs. A key functional property that distinguishes these two neuroblast lineages rests on their dependence on *Notch* signaling for maintenance of their identity (Bowman et al., 2008; Song and Lu, 2011; Weng et al., 2011). While dispensable for maintenance of a type I neuroblast, *Notch* signaling is crucial in maintaining type II neuroblasts (Figs. 2.7S B-E).

The mutually antagonistic interaction between Lgl and aPKC in mitotic neuroblasts ensures that Numb segregates exclusively into the cortex of the presumptive progenitor cell where Numb functions to specify progenitor cell identity (Lee et al., 2006b; Rolls et al., 2003; Wang et al., 2006). In *lgl* mutant brains, increased cortical aPKC function disrupts asymmetric segregation of Numb in mitotic neuroblasts and triggers formation of supernumerary neuroblasts. Consistent with Numb acting as a conserved inhibitor of *Notch* signaling, neuroblasts lacking *numb* function or expressing constitutively active *Notch* generate supernumerary neuroblasts at the expense of progenitor cells (Bowman et al., 2008; Frise et al., 1996; Guo et al., 1996; Lee et al., 2006b; Rhyu et al., 1994; San-Juán and Baonza, 2011; Wang et al., 2006; Zhong et al., 1997). Thus, elevated cortical aPKC kinase activity induces supernumerary neuroblast formation likely by attenuating Numb-dependent regulation of *Notch* signaling. The fly Numb protein contains five evolutionarily conserved aPKC phosphorylation sites, and the non-phosphorylatable form of the Numb

transgenic protein at these sites (Numb^{5A}) fails to segregate asymmetrically in mitotic sensory organ precursor cells (Dho et al., 2006; Nishimura and Kaibuchi, 2007; Smith et al., 2007). aPKC can indeed directly phosphorylate Numb through these sites and render Numb non-functional (Dho et al., 2006; Nishimura and Kaibuchi, 2007; Smith et al., 2007; Wirtz-Peitz et al., 2008). Together, these results led to the hypothesis that increased cortical aPKC kinase activity induces supernumerary neuroblasts by perturbing the localization and the function of Numb. Thus far, evidence supporting this proposed mechanism appears largely correlative. First, direct evidence linking aPKC kinase activity to the de-localization of Numb from the cortex of mitotic neuroblasts is absent. Second, whether phosphorylation by aPKC indeed renders Numb inactive in progenitor cells has never been tested. Finally, type II neuroblasts require *Notch* signaling for maintenance of their identity; therefore, over-expression of Numb or Numb^{5A} most likely induces supernumerary type II neuroblasts in *lgl* mutant brains to undergo premature differentiation rather than restoring proper specification of INP identity (Wirtz-Peitz et al., 2008) (Figs. 2.7S B-G). As such, whether increased cortical aPKC kinase activity induces supernumerary neuroblasts by impinging on the localization and the function of Numb remains an open question

In this study, we show that despite failing to segregate Numb asymmetrically, neuroblasts in *lgl* mutant brains reproducibly undergo asymmetric division to generate progenitor cells. This result suggests that increased cortical aPKC kinase activity impinged on the segregation but not the function of Numb. Surprisingly, the non-phosphorylatable Numb^{5A} at the five

conserved aPKC phosphorylation sites exclusively partitioned in mitotic neuroblasts, indicating that Numb contains additional aPKC phosphorylation sites required for asymmetric segregation. Indeed, the two aPKC phosphorylation sites, serines 48 and 52, in the ACBD3 binding region played a pivotal role in asymmetrically segregating Numb into the cortex of the future progenitor cell. Most unexpectedly, Numb suppressed supernumerary neuroblasts induced by increased cortical aPKC function regardless of the phosphorylation status at serines 48 and 52. Thus, we propose that the antagonistic interaction between Lgl and aPKC ensures that sufficient Numb reaches the future progenitor cells where Numb maintains their limited potential irrespective of its phosphorylation by aPKC.

Materials and Methods

Fly Stocks and Transgenes

The novel *lgl* mutants were generated by EMS mutagenesis following standard procedures. The *numb* deletion constructs were generated by site-directed mutagenesis of the *numb* cDNA, sequenced, and cloned in the *pUAST-HA* vector for germline transformation. The *UAS-numb^{S2A}* and *UAS-numb^{S2D}* flies were generated using the *pUAST-attB-HA* vector for insertion into an identical docking site in the fly genome via the ϕ C31 integrase-mediated transgenesis (Bischof and Basler, 2008). *Erm-lacZ* flies were generated by cloning the *R9D11* enhancer element upstream of a minimal promoter and the *lacZ* gene followed by ϕ C31 integrase-mediated transgenesis. *Drosophila* cultures were maintained at

25°C under standard conditions. Other mutant and transgenic flies used in the study include *lgl*³³⁴ (Peng et al., 2000), *aPKC*⁰⁶⁴⁰³ (Rolls et al., 2003), *UAS-aPKC*^{caax} (Sotillos et al., 2004), *Erm-GAL4* (Pfeiffer et al., 2008), *Wor-GAL4*, *Ase-GAL4* (Zhu et al., 2006), *UAS-numb* (Knoblich et al., 1997), *UAS-numb*^{5A} (Smith et al., 2007), *UAS-numb*^{ΔN} (Knoblich et al., 1997), *UAS-Notch*_{intra} (Chung and Struhl, 2001), and *aph-1*⁵⁰⁷² (Weng et al., 2011). The *UAS-Notch*_{RNAi} and *UAS-spdo*_{RNAi} lines were obtained from the Vienna Drosophila Resource Center. *Oregon R*, *Sca-GAL4*, *UAS-dcr2*, *aph-1*^{D35}, *hs-flp*, *Act-FRT-Stop-FRT-GAL4*, *UAS-GFP*, *UAS-flp*, *Act-FRT-Stop-FRT-lacZ*, and *tubGal80*^{ts} flies were obtained from the Bloomington Drosophila Stock Center.

Immunofluorescent Staining and Antibodies

Antibody staining was performed as previously described. Antibodies used in this study include rat anti-Dpn (1:1), rabbit anti-Ase (1:400), mouse anti-Pros (1:100), sheep anti-Lgl (1:1000, S. Goode), guinea pig anti-Numb (1:2500, J. Skeath), mouse anti-Dlg (1:50, DSHB), mouse anti-β-gal (1:100, Sigma), chicken anti-GFP (1:2000, Aves Labs), rat anti-α-tubulin (1:100, Serotec), rabbit anti-aPKC (1:1000, Sigma), rabbit anti-phospho-HistoneH3 (1:1000, Upstate), mouse anti-HA (1:1000, Covance), and mouse anti-c-Myc (1:50, DSHB). Secondary antibodies were from Invitrogen and Jackson ImmunoResearch (details are available upon request). Fluorescent conjugates of phalloidin (Invitrogen), which stain F-actin, were used to mark the cell cortex. All images are single confocal sections acquired on a Leica SP5 scanning confocal microscope.

Lineage Clone Induction

For neuroblast clone induction, wild-type or *lgl* mutant larvae containing *hs-flp* were heat-shocked as follows to induce recombination and marking by the *Act-FRT-STOP-FRT-GAL4* driving *UAS-GFP* expression. After hatching, larvae were cultured for 24 hours at 25°C, subjected to a 1 hour heat-shock at 37°C, and returned to 25°C for 24 or 48 hours as indicated. For INP clone induction, wild-type or *lgl* mutant larvae containing *erm-GAL4*, *tubGAL80^{ts}*, and *UAS-flp* were cultured at 33°C for 72 hours to induce recombination and marking by the *Act-FRT-STOP-FRT-lacZ* reporter. For clones over-expressing *Notch_{intra}*, larvae containing *hs-flp* were cultured at 25°C and heat-shocked at 24 hours after hatching for 2 hours at 37°C to induce *Act-FRT-STOP-FRT-GAL4* recombination and expression of *UAS-Notch_{intra}* and *UAS-GFP*. Larval brains were then dissected and stained as described previously.

Over-expression Experiments

For expression of transgenes using *wor-GAL4* alone or in the *lgl* rescue experiments using *ase-GAL4*, larvae were cultured at 32.5°C for 72 hours. For *aPKC^{caax}* overexpression studies, larvae were cultured at 31°C for 72 hours and at 33°C for 96 hours when using *Erm-GAL4*. Larval brains were then dissected and stained as described previously.

Results

Supernumerary neuroblasts in *lgl* mutant brains most likely arise from progenitor cells

Increased cortical aPKC kinase activity phosphorylates Numb, an evolutionarily conserved protein instrumental for specification of the daughter sibling cell fate during asymmetric cell division, possibly rendering it inactive in *lgl* mutant brains (Guo et al., 1996; Nishimura and Kaibuchi, 2007; Rhyu et al., 1994; Smith et al., 2007; Wirtz-Peitz et al., 2008; Zhong et al., 1997). In addition, analyses of various *lgl* mutant alleles showed that both type I and II neuroblasts become aberrantly expanded in *lgl* mutant brains (Figs. 2.1E, J and 2.8S). These data led us to hypothesize that supernumerary neuroblasts in *lgl* mutant brains arise from symmetric neuroblast divisions. We tested this hypothesis by assessing the identity of cells in the lineage clones derived from single type I neuroblasts in wild-type or *lgl* mutant brains at 24 or 48 hours after clone induction. In wild-type brains, we detected a single neuroblast (Dpn^+Ase^-) per type I neuroblast clone, and the neuroblast was always surrounded by GMCs (Dpn^-Ase^+) and their daughter progeny (Dpn^-Ase^-) (Figs. 2.1A, C, E, K and 2.7S A). Unexpectedly, all 24-hour type I neuroblast clones in *lgl* mutant brains also contained a single neuroblast surrounded by GMCs and their daughter progeny (Figs. 2.1B, E and K). However, 25% of the 48-hour type I neuroblast clones in *lgl* mutant brains contained more than one neuroblast per clone, and we frequently observed supernumerary neuroblasts formed basally in the clones (Figs. 2.1D, E and K; $n = 91$). Thus, we propose that in *lgl* mutant brains, type I

neuroblasts divide asymmetrically to self-renew and to generate GMCs, which revert back into type I neuroblasts.

The 24-hour or 48-hour type II neuroblast lineage clones in wild-type brains contain a single neuroblast (Dpn^+Ase^-) per clone, and the neuroblast was always directly surrounded by immature INPs (Dpn^-Ase^- or Dpn^-Ase^+) while INPs (Dpn^+Ase^+), GMCs and their daughter progeny were typically one or more cells away (Figs. 2.1F, H, J-K and 2.7S A). Surprisingly, all 24-hour type II neuroblast clones in *lgl* mutant brains also contained a single neuroblast surrounded by immature INPs, INPs and their daughter progeny (Figs. 2.1G, J and K). Most importantly, 47% of the 48-hour clones in *lgl* mutant brains contained more than one neuroblast per clone, and we reproducibly observed supernumerary neuroblasts formed basally in the clones (Figs. 2.1I, J and K; n = 144). These results led us to conclude that in *lgl* mutant brains, type II neuroblasts also divide asymmetrically to self-renew and to produce immature INPs that mature into INPs, but INPs revert back into type II neuroblasts. Based on these data, we propose that Lgl functions to maintain restricted potential in progenitor cells including INPs and GMCs.

Increased cortical aPKC kinase activity triggers reversion of INPs back into neuroblasts

If Lgl indeed functions to maintain restricted potential in progenitor cells, we predict that the genetic clones derived from INPs in *lgl* mutant brains should contain supernumerary type II neuroblasts. In order to induce the INP lineage

clones, we first examined whether INPs in *lgl* mutant brains show expression of the INP-specific *earmuff-lacZ* reporter transgene (Weng et al., 2010) (this study). *earmuff-LacZ* was detected in small Dpn⁺Ase⁺ cells surrounding type II neuroblasts but was undetectable in both type I and II neuroblasts in wild-type and *lgl* mutant brains (Figs. 2.2A-B). Thus, the INP-specific enhancer element in the *earmuff* gene remains active in *lgl* mutant brains, allowing us to induce lineage clones derived from INPs in wild-type or *lgl* mutant brains by expressing flipase driven by the *earmuff-Gal4* (Weng et al., 2010). All INP clones in wild-type brains (n = 31) contained only progeny that lack Dpn and Ase expression but never type II neuroblasts (Fig. 2.2C). In contrast, *lgl* mutant brains (86%, n = 21) contained INP clones with one or more type II neuroblasts (Fig. 2.2D). These aberrant neuroblasts can indeed undergo asymmetric division to self-renew and to produce progenitor cells as indicated by the presence of immature INPs within the clones. Thus, INPs can indeed revert back into type II neuroblasts in *lgl* mutant brains.

Since Lgl functions with aPKC in mitotic neuroblasts, we examined if reversion of INPs back into type II neuroblasts in *lgl* mutant brains occurs due to increased cortical aPKC kinase activity. We first tested if reduced function of *aPKC* can suppress supernumerary type II neuroblasts and INPs in *lgl* mutant brains. While a wild-type brain lobe contained 8 type II neuroblasts and 58 ± 8 INPs (Dpn⁺Ase⁺*earmuff-LacZ*⁺), an *lgl* mutant brain lobe possessed 36 ± 9 type II neuroblasts and 131 ± 25 INPs (Fig. 2.9S B). Consistent with our hypothesis, an *lgl* mutant brain lobe heterozygous for *aPKC* contained 13 ± 5 type II neuroblasts

and 66 ± 19 INPs (Fig. 2.9S B). We next directly assessed if unrestrained cortical aPKC kinase activity is sufficient to trigger reversion of INPs back into type II neuroblasts. Indeed, INPs ectopically expressing constitutively membrane localized aPKC^{caax} under the control of *earmuff-Gal4* generated supernumerary type II neuroblasts (Figs. 2.2E-F and 2.9S A). Thus, precise regulation of aPKC kinase activity plays a critical role in maintaining restricted potential in INPs.

Numb requires the ACBD3 binding region for its localization and function in neuroblasts

Numb, which localized in the basal cortex of mitotic INPs and type I neuroblasts, is an excellent candidate for acting downstream of Lgl to maintain restricted potential in progenitor cells (Figs. 2.3A, C-D). In *lgl* mutant brains, Numb localized uniformly in the cortex of metaphase INPs (100%, n = 9) and type I neuroblasts (100%, n = 46) and became enriched in the cortex of the future GMC in telophase neuroblasts (76%, n = 13) (Figs. 2.3B, E and F). Furthermore, heterozygosity of *aPKC* restored asymmetric localization and segregation of Numb in mitotic INPs and type I neuroblasts in *lgl* mutant brains (Figs. 2.3G-H; metaphase = 78%, n = 9; telophase = 89%, n = 9). Moreover, neuroblasts ectopically expressing aPKC^{caax} showed uniform cortical localization of Numb in both metaphase and telophase (Fig. 2.10S) (Wang et al., 2006). These data led us to conclude that increased cortical aPKC kinase activity perturbs asymmetric localization of Numb in the cortex of mitotic neuroblasts and likely reduces the function of Numb in the cortex of the future progenitor cell in *lgl* mutant brains.

We tested if aPKC regulates asymmetric localization of Numb in mitotic neuroblasts via the five conserved aPKC phosphorylation sites proposed by a previous study (Wirtz-Peitz et al., 2008). Surprisingly, ectopic expression of the non-phosphorylatable Numb^{5A} transgenic protein at these sites in the presence of the endogenous Numb segregated exclusively into the cortex of the future progenitor cell in telophase neuroblasts (Fig. 2.3J; 100%, n = 21). This result strongly suggested that aPKC regulates Numb via alternative phosphorylation sites prompting us to first identify the domain(s) required for asymmetrically localizing Numb in mitotic brain neuroblasts. We ectopically expressed the *UAS-numb* transgenes that encode various truncated forms of Numb in type I neuroblasts in the presence of endogenous Numb and examined their localization pattern (Fig. 2.3I). Identical to the full-length Numb transgenic protein, Numb^{ΔN}, Numb^{ΔPTB} and Numb^{ΔC} segregated exclusively into the cortex of the future GMC in the telophase neuroblasts (Figs. 2.3K-L and N; data not presented; 100%, n = 10, 15, and 27, respectively). In contrast, the Numb^{ΔAB} transgenic protein failed to segregate exclusively into the cortex of the future GMC in the telophase neuroblasts (Fig. 2.3M; 61%, n = 57). Thus, we conclude that the ACBD3 binding region is necessary for asymmetric segregation of Numb.

We next ectopically expressed this series of the *UAS-numb* transgenes in type I neuroblasts, where *Notch* signaling is dispensable for maintenance of their identity, to determine which domains mediate the function of Numb in suppressing reversion of progenitor cells in *lgl* mutant brains. While

ectopic expression of Numb, Numb^{ΔN} and Numb^{ΔC} efficiently suppressed supernumerary neuroblasts in *lgl* mutant brains, expression of Numb^{ΔPTB} did not have any effects on the supernumerary neuroblast phenotype (Figs. 2.3O and Q-S). The PTB domain mediates Numb binding to the Notch receptor protein and is essential for Numb suppression of *Notch* signaling (Frise et al., 1996; Yaich et al., 1998). Additionally, ectopic expression of Numb in type I neuroblasts of wild-type brains using *ase-GAL4* had no effect on neuroblast number (data not presented). These data strongly suggest that aberrant activation of *Notch* signaling leads to supernumerary type I neuroblasts in *lgl* mutant brains. Most importantly, the Numb^{ΔAB} transgenic protein also failed to suppress supernumerary neuroblasts in *lgl* mutant brains (Figs. 2.3P and S). We independently tested whether the ACBD3 binding region is indeed necessary for suppressing supernumerary neuroblasts induced by unrestrained cortical aPKC kinase activity. While ectopic expression of Numb or Numb^{ΔC} efficiently suppressed massive supernumerary neuroblasts induced by aPKC^{caax}, expression of Numb^{ΔAB} or Numb^{ΔPTB} did not have any effects on the supernumerary neuroblast phenotype (Figs. 2.3T-V; data not presented). Thus, Numb requires the ACBD3 binding region to suppress reversion of progenitor cells in *lgl* mutant brains. Together, we conclude that the ACBD3 binding region is necessary for the localization and the function of Numb during asymmetric division of brain neuroblasts.

Serines 48 and 52 are required for asymmetric localization of Numb but likely dispensable for regulating specification of progenitor cells

Our data showed that the ACBD3 binding region is indispensable for the localization and the function of Numb during neuroblast asymmetric division; therefore, we investigated if aPKC might regulate Numb through the phosphorylation sites in this domain. The ACBD3 binding region of Numb harbors two aPKC phosphorylation sites, serines 48 and 52 (Nishimura and Kaibuchi, 2007; Smith et al., 2007). If aPKC indeed regulates the localization of Numb through the ACBD3 binding region, the phosphorylation status at serines 48 and 52 should directly impinge on the distribution of the Numb protein in mitotic neuroblasts. Consistently, the non-phosphorylatable Numb^{S2A} transgenic protein localized throughout the cortex of the telophase neuroblasts (Figs. 2.3I and 2.4A; 100%, n = 17). In contrast, the phosphomimetic Numb^{S2D} transgenic protein became basally enriched in the telophase neuroblasts (Figs. 2.3I and 2.4B; 74%, n = 19). Together, these data strongly suggest that aPKC excludes Numb from the apical cortex of mitotic neuroblasts by phosphorylating serines 48 and 52.

We next tested if increased cortical aPKC kinase activity inactivates the function of Numb by phosphorylating serines 48 and 52 in the ACBD3 binding region during neuroblast asymmetric division. We ectopically expressed Numb^{S2A} or Numb^{S2D} in type I neuroblasts in *lgl* mutant brains. Surprisingly, either Numb^{S2A} or Numb^{S2D} efficiently suppressed supernumerary type I neuroblasts in *lgl* mutant brains (Figs. 2.4C-E). In addition, while Numb^{ΔAB} failed to suppress

supernumerary neuroblasts induced by $aPKC^{caax}$ in larval brains, $Numb^{S2A}$ or $Numb^{S2D}$ completely suppressed the supernumerary neuroblast phenotype in the same genetic background (Figs. 2.4F-I). Thus, the phosphorylation status of serine 48 and 52 has no effects on the ability of the Numb transgenic protein to restore restricted potential in progenitor cells in *Igl* mutant brains. We propose that serine 48 and 52 play a critical role in asymmetric localization of Numb but are likely dispensable for regulation of progenitor cell potential.

The ACBD3 binding region mediates Numb-dependent suppression of *Notch* signaling specifically in brain neuroblasts

The ACBD3 binding region is necessary for the function of the mouse Numb protein, but how this domain mediates the function of the fly Numb protein has never been investigated (Zhou et al., 2007). The ACBD3 binding region is necessary for Numb to suppress supernumerary type I neuroblasts in larval brains lacking *Igl* function or ectopically expressing $aPKC^{caax}$, phenotypes that required activation of *Notch* signaling (Figs. 2.3 and 2.11S). Thus, we hypothesize that the ACBD3 binding region mediates Numb suppression of *Notch* signaling. We tested this hypothesis by ectopically expressing the *UAS-numb^{ΔAB}* transgene under the control of a pan-neuroblast *Wor-Gal4* driver in the larval brain. While increased function of *numb* or decreased function of *Notch* led to premature differentiation of type II neuroblasts, expression of $Numb^{ΔAB}$ did not have any effects on maintenance of the type II neuroblast identity (Figs. 2.7S C-E, G and 2.5A). Importantly, expression of $Numb^{S2A}$ or $Numb^{S2D}$ led to complete

loss of type II neuroblasts prematurely in larval brains, indicating that the phosphorylation status at serines 48 and 52 does not affect the function of Numb to antagonize *Notch* signaling (Fig. 2.5A).

Asymmetric divisions of sensory organ precursors give rise to the bristles on the scutellum of the adult fly and are highly sensitive to changes in *Notch* signaling (Frise et al., 1996; Knoblich et al., 1997; Rhyu et al., 1994; Yaich et al., 1998). Similar to over-expression of Numb^{ΔC}, unexpectedly, ectopic expression of Numb^{ΔAB}, Numb^{S2A} or Numb^{S2D} all led to cell fate transformation in the sensory organ precursor lineage and resulted in decreased bristles on the scutellum (Figs. 2.5B-G). This result indicates that the ACBD3 binding region is dispensable for Numb-mediated suppression of *Notch* signaling in sensory organ precursor cells in the peripheral nervous system. Together, we conclude that the ACBD3 binding region specifically mediates the function of Numb in suppressing *Notch* signaling in the brain regardless of the phosphorylation by aPKC.

Discussion

The antagonistic interaction between the polarity proteins Lgl and aPKC provides an evolutionarily conserved mechanism for regulating the cell fate determinants inherited into the daughter siblings during asymmetric cell division (Beatty et al., 2010; Betschinger et al., 2003; Hoege et al., 2010; Lee et al., 2006b). However, regulation of the localization and the function of fate determinants by the polarity proteins can be uncoupled in a context-dependent manner. Our study led us to conclude that the antagonistic interaction between

Lgl and aPKC maintains limited potential in progenitor cells at least in part by ensuring asymmetric partitioning of Numb into the future progenitor cells, where Numb acts irrespective of its phosphorylation by aPKC (Fig. 2.6). We showed that neuroblasts in *lgl* mutant brains undergo asymmetric division to self-renew and to generate progenitor cells, which can produce post-mitotic progeny but can also revert back into neuroblasts (Figs. 2.1 and 2.2). This indicates that although progenitor cells initially establish the proper identity these cells fail to maintain their limited potential. Additionally, Numb remained localized in the cortex of telophase neuroblasts lacking *lgl* function or ectopically expressing *aPKC^{caax}* (Fig. 2.3F and data not presented). The phosphomimetic Numb^{2D} transgenic protein at serines 48 and 52, which are essential for asymmetric cortical localization of Numb, also remained localized in the cell cortex of telophase neuroblasts (Fig. 2.4B). Finally, increased function of Numb efficiently suppressed supernumerary neuroblasts in *lgl* mutant brains irrespective of the phosphorylation status at serines 48 and 52 (Fig. 2.4C-D). Thus, we propose that Lgl antagonizes aPKC to ensure a necessary threshold of Numb in the progenitor cells where Numb maintains limited potential regardless of phosphorylation by aPKC (Fig. 2.6).

Lgl maintains limited potential in progenitor cells

How Lgl suppresses formation of supernumerary neuroblasts in larval brains has remained a mystery largely due to the existence of a phenomenon called “telophase rescue” (Albertson and Doe, 2003; Cai et al., 2001). In *lgl*

mutant brains, the basal proteins including Miranda and Numb fail to localize to the basal crescent in metaphase neuroblasts but by and large re-localize asymmetrically in telophase neuroblasts (Lee et al., 2006b; Rolls et al., 2003; Wirtz-Peitz et al., 2008). Furthermore, Miranda and Numb appear to localize independently of each other in mitotic neuroblasts (Lu et al., 1998; Shen et al., 1997). Thus, the transcription factor Dpn whose expression and localization pattern is not impinged upon by defective cortical cell polarity provides an excellent cell identity marker to investigate the cellular origin of supernumerary neuroblasts in *lgl* mutant brains (Fig. 2.7S A; Komori and Lee, unpublished). Surprisingly, only the 48-hour, but not the 24-hour, type I and II neuroblast lineage clones in *lgl* mutant brains contained supernumerary neuroblasts, which frequently localized basally from the parental neuroblasts (Fig. 2.1). The most recently born daughter always remains immediately adjacent to the parental neuroblasts while the earlier born progeny gradually becomes displaced away from the parental neuroblasts (Bayraktar et al., 2010; Bowman et al., 2008; Weng et al., 2010). Thus, supernumerary neuroblasts in *lgl* mutant brains most likely originated from the progenitor cells rather than symmetric neuroblast division. We propose that in the type I neuroblast lineage Lgl prevents aPKC kinase activity in the basal cortex to ensure that GMCs maintain limited potential and generate only post-mitotic progeny. In the type II neuroblast lineage, Lgl prevents aPKC kinase activity in the basal cortex to ensure that after maturation the INP can maintain limited potential and generate only GMCs during limited rounds of asymmetric divisions.

Although these results do not exclude the possibility that GMCs in the type II neuroblast lineages in *lgl* mutant brains can revert back into neuroblasts, we believe that reacquisition of the type II neuroblast fate by GMCs might be less likely. First, the basal protein Prospero plays a critical role in regulating the function of GMCs, and mosaic clones derived from *prospero* mutant INPs contained massive supernumerary INPs but never supernumerary type II neuroblasts (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995; Weng et al., 2010). Second, mosaic clones derived from *numb* mutant INPs contained supernumerary INPs but never supernumerary type II neuroblasts (Komori and Lee, unpublished). Thus, blocking differentiation in GMCs allows them to retain the identity of their immediate parental cell type, which is INP in the type II neuroblast lineage. Until the enhancer elements that exhibit GMC-specific expression become available, we cannot conclusively rule out the possibility that GMCs can re-acquire the type II neuroblast fate in *lgl* mutant brains.

aPKC regulates asymmetric localization but not the function of Numb in neuroblasts

A previous study strongly suggested that increased cortical aPKC kinase activity induces supernumerary neuroblasts in *lgl* mutant brains by phosphorylating Numb, therefore, displacing it from the neuroblast cortex and inactivating its function (Wirtz-Peitz et al., 2008). This proposed mechanism was in part based on studies in vertebrates showing that phosphorylation by aPKC

perturbs cortical localization and the function of Numb (Nishimura and Kaibuchi, 2007; Smith et al., 2007). Inconsistent with this proposed mechanism, we reproducibly detected disruption in asymmetric distribution of Numb in the cortex of mitotic neuroblasts in *lgl* mutant brains (Fig. 2.3E-H). Failure to displace Numb from the cortex of mitotic neuroblasts in *lgl* mutant brains was unlikely due to insufficient aPKC kinase activity as Numb remained localized uniformly in the cortex of neuroblasts over-expressing aPKC^{caax} (Fig. 2.10S). Additionally, the phosphomimetic Numb^{2D} transgenic protein at serines 48 and 52, two residues required for asymmetric segregation of Numb in mitotic neuroblasts, remained localized in the neuroblast cortex (Fig. 2.4B). Thus, increased cortical aPKC kinase activity most likely disperses Numb in the cortex of mitotic neuroblasts and reduces accumulation of Numb in the cortex of the future progenitor cell in *lgl* mutant brains. Most importantly, over-expression of Numb^{2D} suppressed supernumerary neuroblasts in *lgl* mutant brains as efficiently as Numb^{2A}, strongly suggesting that phosphorylation by aPKC does not inactivate the function of Numb (Fig. 2.4). Together, these data led us to propose that increased cortical aPKC kinase activity induces supernumerary neuroblasts in *lgl* mutant brains by reducing accumulation of Numb rather than inactivation of Numb in the progenitor cells (Fig. 2.6).

Studies in vertebrates identified five conserved aPKC phosphorylation sites in the fly Numb, and the non-phosphorylatable Numb^{5A} transgenic protein at these sites localized uniformly cortical in mitotic sensory organ precursors (Nishimura and Kaibuchi, 2007; Smith et al., 2007).

Surprisingly, Numb^{5A} localized asymmetrically in mitotic brain neuroblasts, indicating that these sites are dispensable for exclusion of Numb from the apical cortex by aPKC (Fig. 2.3J). Thus, many important questions regarding the significance of these five conserved aPKC phosphorylation sites on the localization and the function of Numb remain to be tested. For example, does the phosphomimetic Numb^{5D} transgenic protein indeed fail to localize to the cell cortex of mitotic sensory organ precursors? Furthermore, is Numb^{5D} indeed non-functional? Does over-expression of Numb^{5D} have any effects on cell fate determination in the sensory organ precursor cell lineage? Are the non-conserved aPKC phosphorylation sites dispensable for the function of Numb in the sensory organ precursor cell lineage? Most importantly, the extent to which these conserved and non-conserved aPKC phosphorylation sites might impinge on other biological processes regulated by Numb requires additional direct and rigorous assessment.

The ACBD3 binding region mediates Numb suppression of *Notch* signaling specifically in the brain

Numb is a highly conserved protein and exerts its antagonistic effect on *Notch* signaling via the PTB domain, which mediates direct binding to the Notch receptor protein (Frise et al., 1996; Knoblich et al., 1997; Yaich et al., 1998; Zhong et al., 1997). A previous study identified that the ACBD3 binding region is required for asymmetric localization of the Numb protein and Numb-dependent suppression of *Notch* signaling (Zhou et al., 2007). Our study extended this result

and showed that the ACBD3 binding region mediates asymmetric cortical localization of the fly Numb protein in mitotic neuroblasts through an aPKC-regulated mechanism (Fig. 2.3). Interestingly, the ACBD3 binding region regulates tissue-specific suppression of *Notch* signaling by Numb despite the presence of the PTB domain (Fig. 2.5). Since the ACBD3 binding region appears to mediate direct protein-protein interactions, we propose that this domain serves as a platform in which tissue-specific regulators can exert precise control of the Numb function in antagonizing *Notch* signaling. Identification and functional characterization of proteins that interact with Numb through the ACBD3 binding region will provide novel mechanistic insight into how the evolutionarily conserved Numb-dependent suppression of *Notch* signaling can be precisely regulated in a tissue-specific manner.

Figures

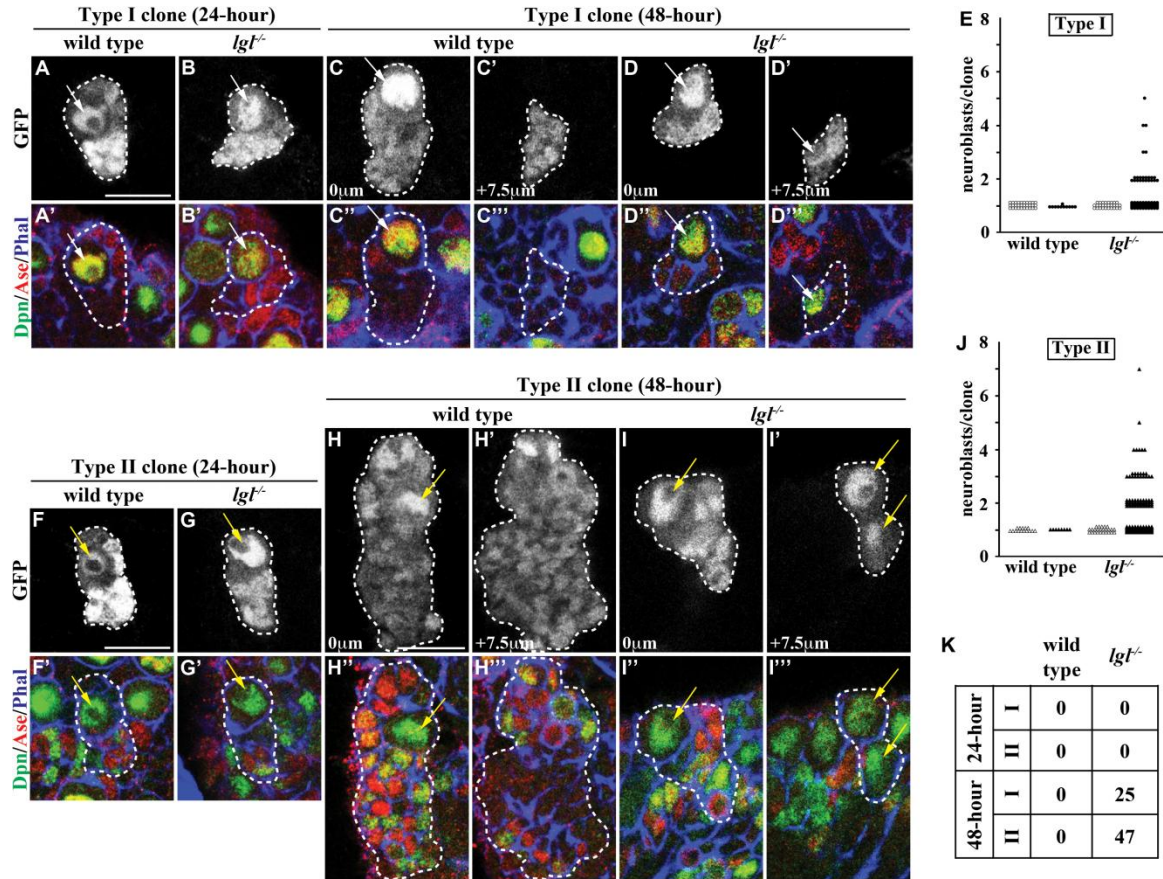


Figure 2.1 *Igf* is required for the maintenance, but not specification, of progenitor cells

(A-B) At 24 hours after clone induction, type I neuroblast clones in both wild-type and *Igf*^{334/3644} mutant brains contained a single neuroblast surrounded by GMCs and neurons. (n = 30 and 28 clones, respectively) **(C-D)** At 48 hours after clone induction, type I neuroblast clones in wild-type brains still contained a single neuroblast surrounded by GMCs and neurons; however, type I neuroblast clones in *Igf*^{334/3644} mutant brains contained a parental neuroblast immediately adjacent to GMCs and neurons with supernumerary neuroblasts located in the basal portion of the clone. (n = 11 and 91 clones, respectively) **(E)** Quantification of the number of neuroblasts per type I neuroblast clone is shown for wild-type and *Igf* mutant clones at 24 (open) and 48 (filled) hours after clone induction. **(F-G)** At 24 hours after clone induction, type II neuroblast clones in both wild-type and *Igf*^{334/3644} mutant brains contained a single neuroblast surrounded by immature INPs, INPs, GMCs and neurons. (n = 15 and 25 clones, respectively) **(H-I)** At 48 hours after clone induction, type II neuroblast clones in wild-type brains still contained a single neuroblast surrounded by immature INPs, INPs, GMCs and neurons; however, type II neuroblast clones in *Igf*^{334/3644} mutant brains contained a parental neuroblast isolated from the supernumerary neuroblasts by many

immature INPs, INPs, GMCs and neurons. (n = 8 and 144 clones, respectively)
(J) Quantification of the number of neuroblasts per type II neuroblast clone is shown for wild-type and *lgl* mutant clones at 24 (open) and 48 (filled) hours after clone induction. **(K)** The table shows the frequency of clones containing supernumerary neuroblasts. Brains were stained with the indicated markers. Single neuroblast clones marked by GFP are circled by the dotted line. Arrows indicate the neuroblasts (white, Type I; yellow, Type II). Single confocal planes of the same clone are shown at 0 mm and +7.5 mm (C-D and H-I). All scale bars are 10 μ m.

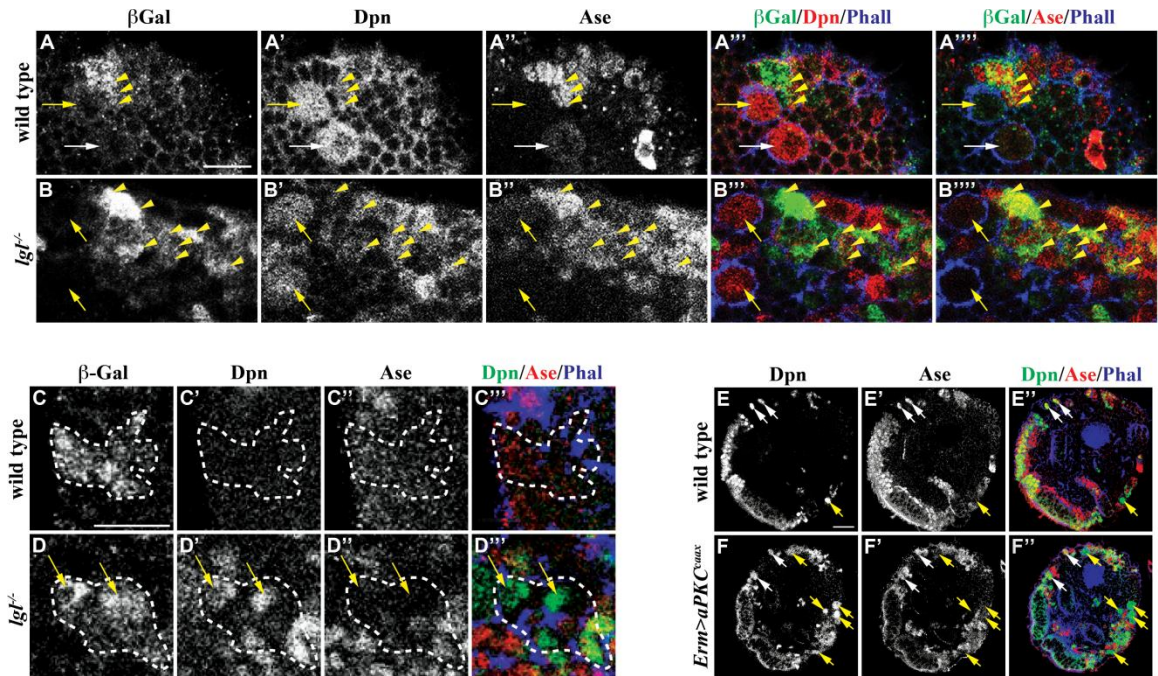


Figure 2.2 *Igl* mutant and *aPKC^{caax}* overexpressing INPs revert back to type II neuroblasts

(A-B) Wild-type and *Igl*^{334/3644} mutant brains expressed *erm-lacZ* specifically in INPs, but not in neuroblasts. **(C-D)** While an INP-derived clone in a wild-type brain only contained neurons, an INP-derived clone in an *Igl*^{334/3644} mutant brain contained multiple supernumerary type II neuroblasts. (n = 31 and 21 brains, respectively) **(E-F)** Overexpression of *aPKC^{caax}* driven by *Erm-GAL4* leads to supernumerary type II neuroblasts in comparison to a wild-type brain. (n = 8 and 9 brains, respectively) Brains were stained with the indicated markers. Clones marked by β -galactosidase are circled by the dotted line. Arrows indicate the neuroblasts (white, Type I; yellow, Type II) and yellow arrowheads indicate the INPs. All scale bars are 10 μ m.

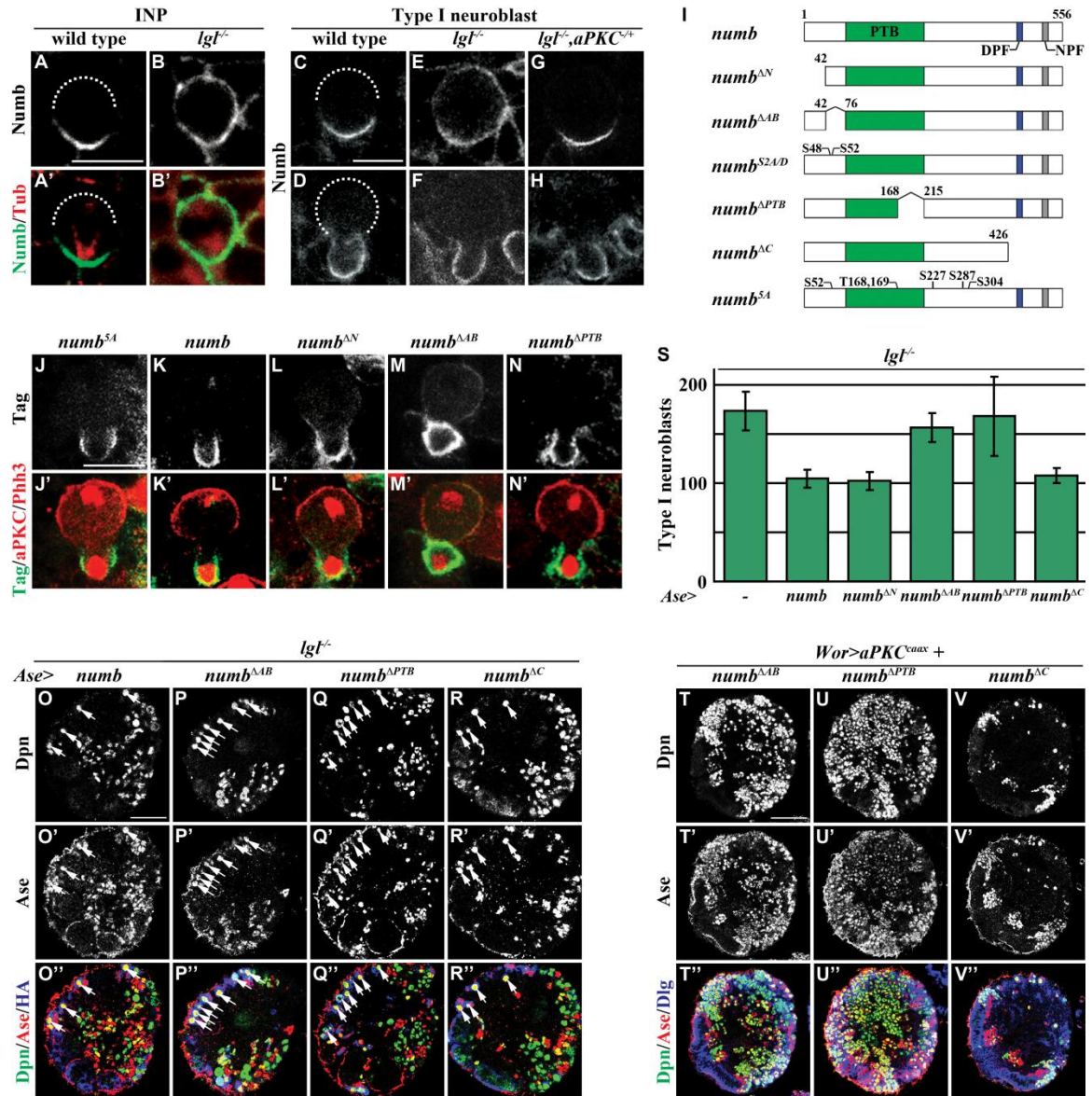


Figure 2.3 The localization and function of Numb in neuroblasts requires the ACBD3 binding region

(A-B) Numb localized to the basal cortex of a wild-type INP, but distributed uniformly throughout the cortex of an *lgt*^{334/3644} mutant INP. (n = 8 and 9, respectively) **(C-H)** Wild-type neuroblasts showed asymmetric localization of Numb at metaphase and telophase, while *lgt*^{334/3644} mutant neuroblasts showed uniform cortical Numb localization at metaphase and basal enrichment of Numb at telophase. Additionally, the heterozygous mutant *aPKC*^{06403/+} restored the asymmetric localization of Numb in *lgt*^{334/3644} mutant neuroblasts at metaphase and telophase. (n = 30, 23, 46, 13, 9 and 9, respectively) **(I)** The diagram shows an illustration of the Numb protein as well as the deletion and mutant constructs used. **(J-N)** Similar to the full-length Numb transgenic protein, Numb^{5A}, Numb^{ΔN},

and Numb^{ΔPTB} localized exclusively in the basal cortex of telophase neuroblasts; however, Numb^{ΔAB} did not localize exclusively to the basal cortex. (Tag indicates the myc epitope tag in J-L and the HA epitope tag in M-N) (n = 21, 10, 15, 57, and 27, respectively) **(O-R)** Supernumerary expression of either full-length Numb or Numb^{ΔC} using *Ase-GAL4* suppressed the supernumerary type I neuroblasts in *Igf*^{334/3644} mutant brains, but expression of Numb^{ΔAB} or Numb^{ΔPTB} failed to rescue the supernumerary neuroblast phenotype. (n = 7, 10, 6, and 5, respectively) **(S)** Quantification of the number of type I neuroblasts per brain lobe is shown for expression of each transgenic Numb protein by *Ase-GAL4* in *Igf*^{334/3644} mutant brains. **(T-V)** Expression of Numb^{ΔC}, but not Numb^{ΔAB} or Numb^{ΔPTB}, suppressed the supernumerary neuroblasts induced by expression of *aPKC*^{caax} driven by *Wor-GAL4*. (n = 6 per genotype) Brains were stained with the indicated markers. Dotted lines mark the location of the apical cortex. White arrows indicate the type I neuroblasts. Scale bars are 5 μm (A-B), 10 μm (C-N), and 25 μm (O-V).

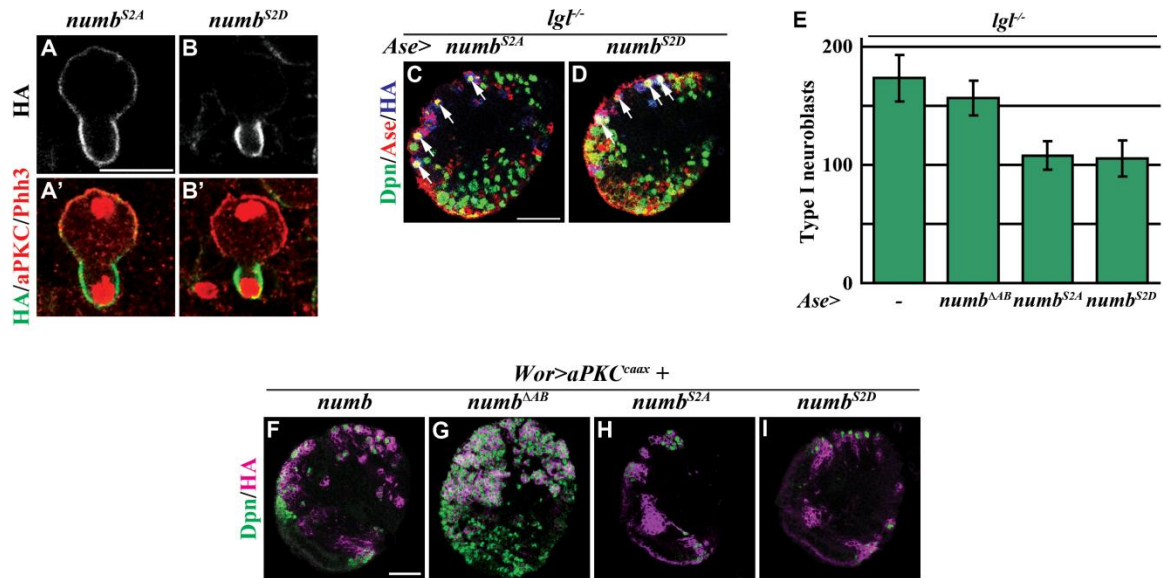


Figure 2.4 Phosphorylation of Numb at serines 48 and 52 regulates its localization, but not function

(A-B) Numb^{S2A} localized uniformly throughout the entire cortex of the telophase neuroblast, while Numb^{S2D} was enriched in the basal cortex. (n = 17 and 19, respectively) (C-D) Expression of Numb^{S2A} or Numb^{S2D} driven by Ase-GAL4 suppressed the supernumerary neuroblasts in *lgt*^{334/3644} mutant brains. (n = 8 and 9, respectively) (E) Quantification of the number of type I neuroblasts per brain lobe is shown for expression of the transgenic Numb proteins by Ase-GAL4 in *lgt*^{334/3644} mutant brains. (F-I) Similar to full-length Numb, expression of Numb^{S2A} or Numb^{S2D} suppressed the supernumerary neuroblasts induced by expression of *aPKC*^{caax} driven by *Wor*-GAL4, while expression of Numb^{ΔAB} did not have an effect on the supernumerary neuroblast phenotype. (n = 8 per genotype) Brains were stained with the indicated markers. White arrows indicate the type I neuroblasts. Scale bars are 10 μm (A-B) and 25 μm (C-I).

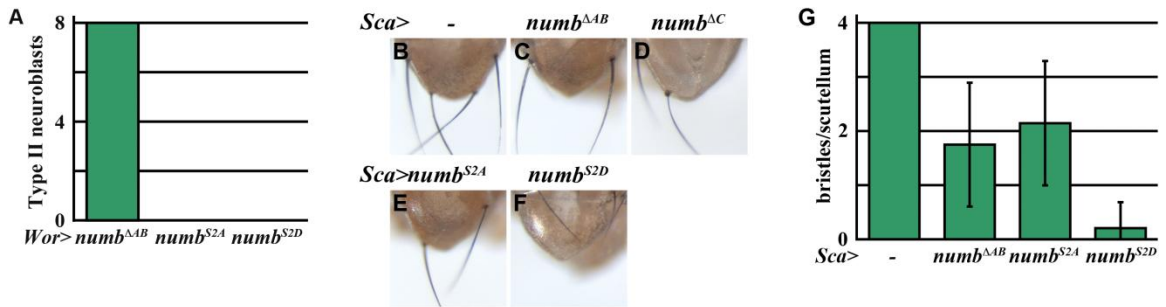


Figure 2.5 The ACBD3 binding region of Numb specifically mediates the inhibition of *Notch* signaling in larval neuroblasts

(A) Quantification of type II neuroblasts per brain lobe is shown for *Wor-GAL4* driving expression of Numb^{ΔAB}, Numb^{S2A}, or Numb^{S2D}. (n = 5 per genotype) **(B-F)** Compared to wild-type flies, overexpression of Numb^{ΔAB}, Numb^{S2A}, Numb^{S2D}, or Numb^{ΔC} driven by *Sca-GAL4* leads to a loss of scutellar bristles. (n = 259, 187, 83, and 34, respectively) **(G)** Quantification of the number of bristles per scutellum is shown for *Sca-GAL4* driving expression of Numb^{ΔAB}, Numb^{S2A}, or Numb^{S2D}.

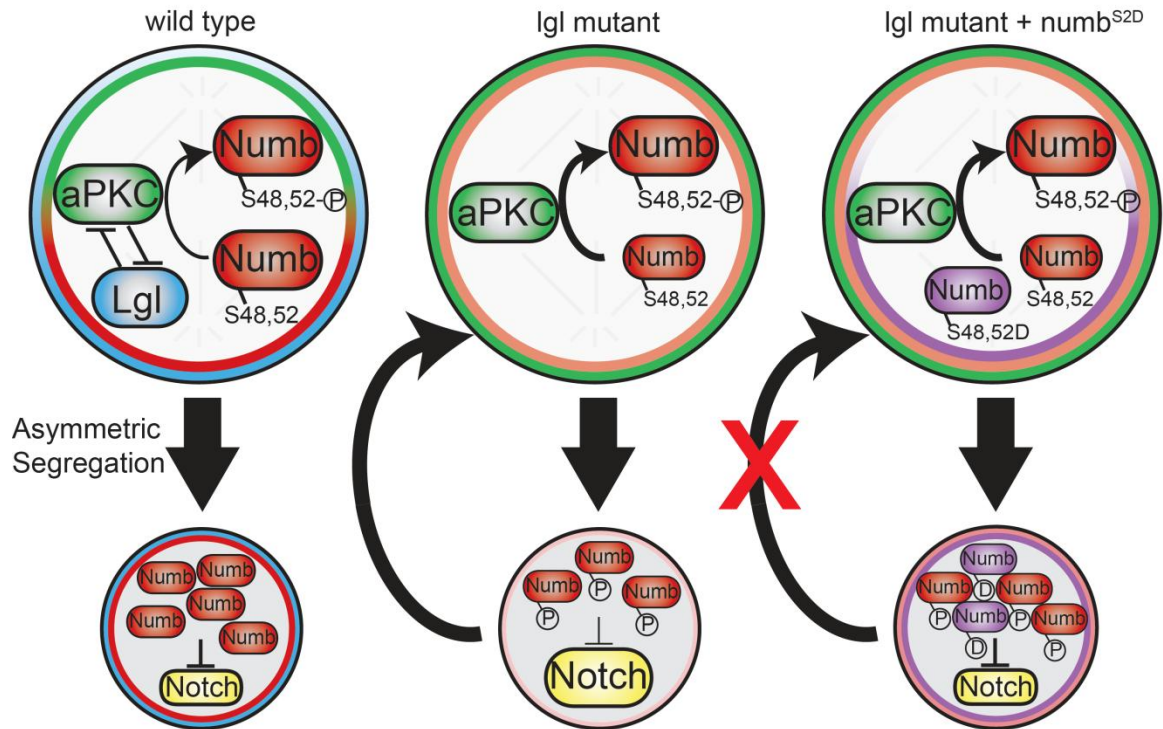


Figure 2.6 Diagram of Proposed Model

In wild-type neuroblasts, the mutual antagonism between Lgl and aPKC ensures asymmetric segregation of Numb into the progenitor cell, where Numb antagonizes *Notch* signaling via its PTB and ACDB3 protein binding domains. In the absence of *lgl*, increased cortical aPKC kinase activity redistributes Numb in the cortex of the dividing neuroblast, potentially leading to insufficient Numb to inhibit Notch in the progenitor cell. This increases the *Notch* activity in the progenitor cells and drives the progenitor cell to revert back into a neuroblast. Furthermore, expression of the phosphomimetic Numb^{S2D} rescues the *lgl* mutant supernumerary neuroblast phenotype by restoring Numb and the inhibition of *Notch* activity in the progenitor cell.

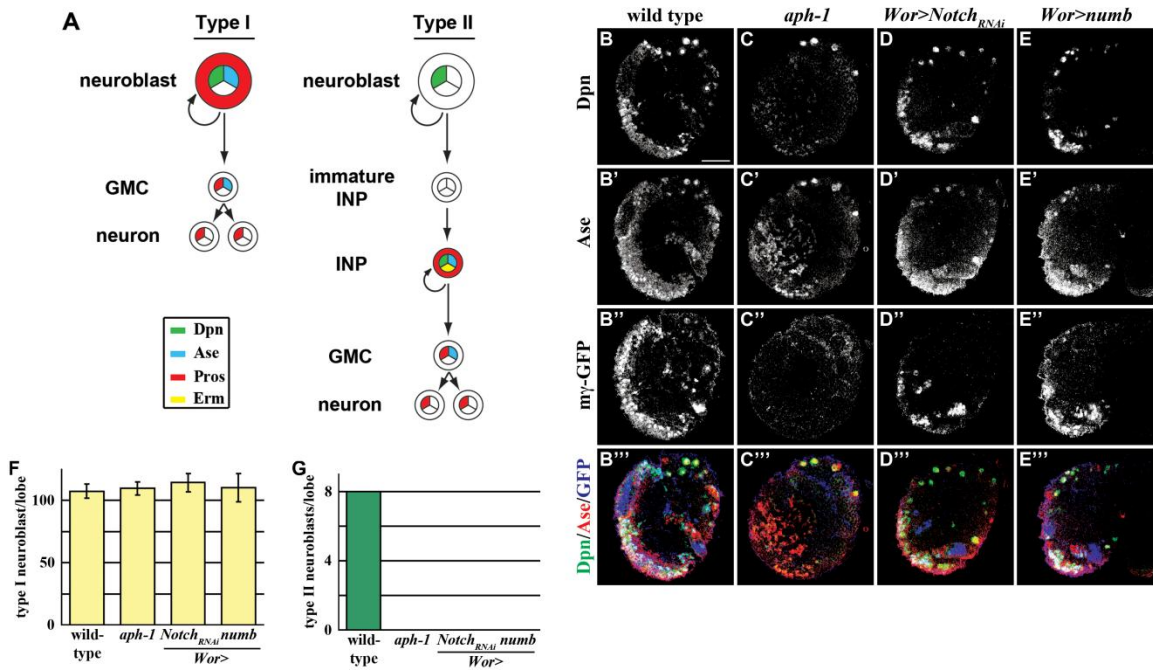


Figure 2.7S Neuroblast lineages and Notch signaling

(A) A diagram of the type I and type II neuroblast lineages is shown. **(B-E)** Reductions in Notch signaling by *aph-1* mutation, RNAi knock-down of the Notch receptor, or ectopic expression of Numb leads to a loss of Notch reporter (mγ-GFP) expression and premature loss of type II neuroblasts. (n = 5 per genotype) **(F-G)** Quantification of the number of type I and type II neuroblasts per brain lobe corresponding to the genotypes in B-E is shown.

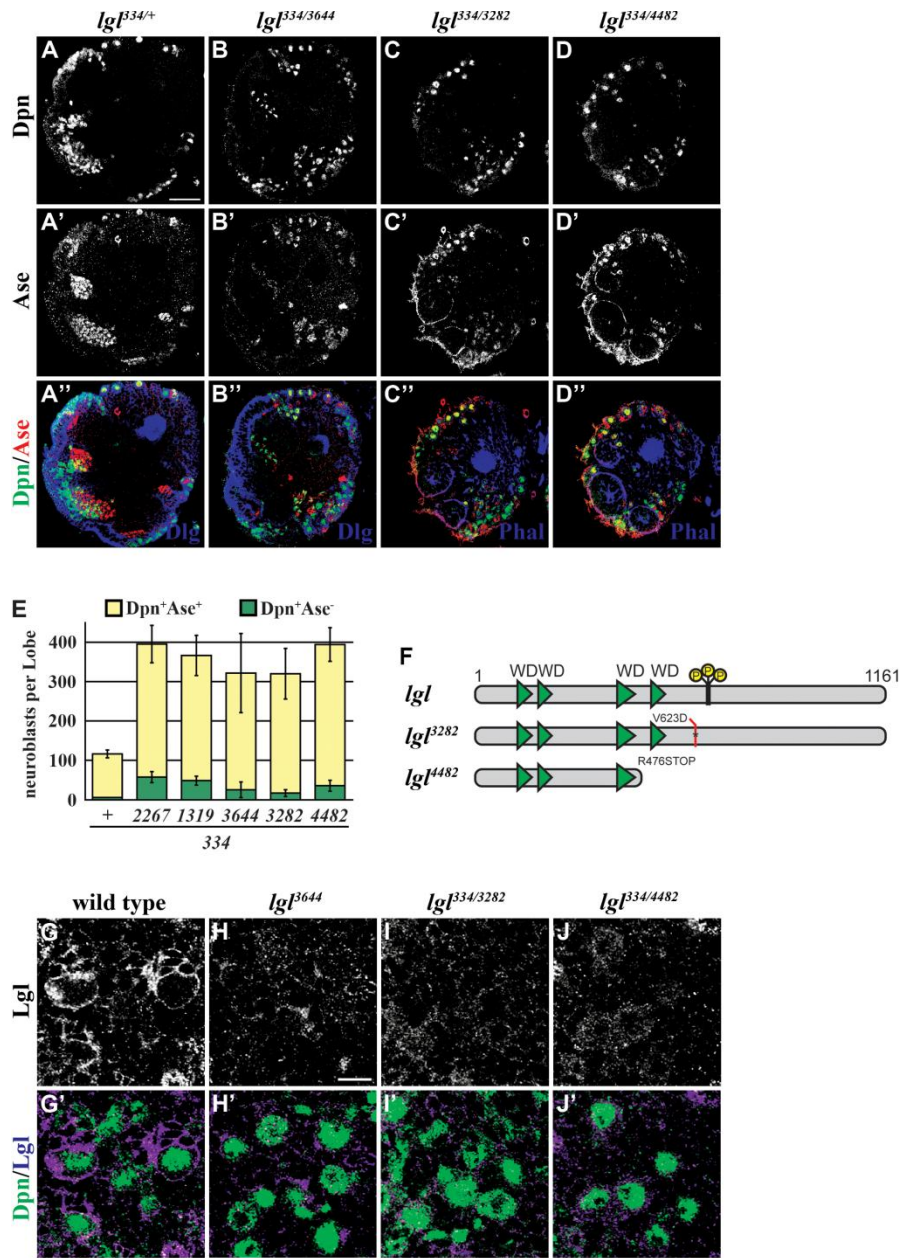


Figure 2.8S *Igf* mutant alleles cause increased type I and II neuroblasts

(A-E) Various *Igf* mutant alleles show supernumerary type I and type II neuroblasts. **(F)** Illustrations of the isolated mutations in *Igf* are shown. **(G-J)** The isolated mutations in *Igf* show a loss of Lgl protein compared to wild-type brains.

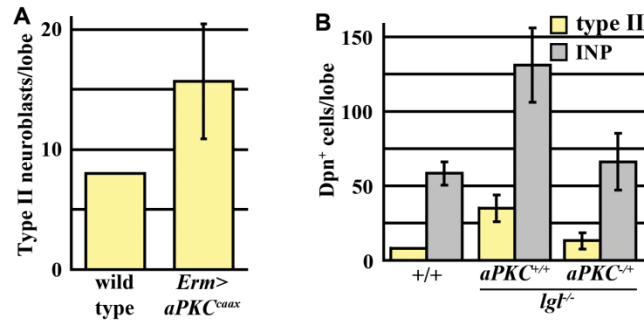


Figure 2.9S *aPKC* is sufficient and necessary for INPs to revert back to Type II neuroblasts

(A) Quantification of the number of type II neuroblasts per lobe is shown for wild-type brains and brains ectopically expressing *aPKC^{Caax}* by *Erm-GAL4*. **(B)** Quantification of the number of type II neuroblasts and INPs per brain lobe is shown for wild-type, *lgl^{334/3644}* mutant, and *lgl^{334/3644}, aPKC^{06403/+}* mutant brains. (n = 13, 17, and 14 brains, respectively)

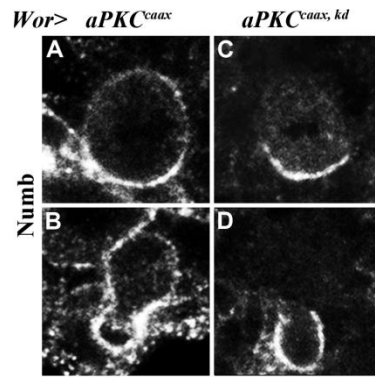
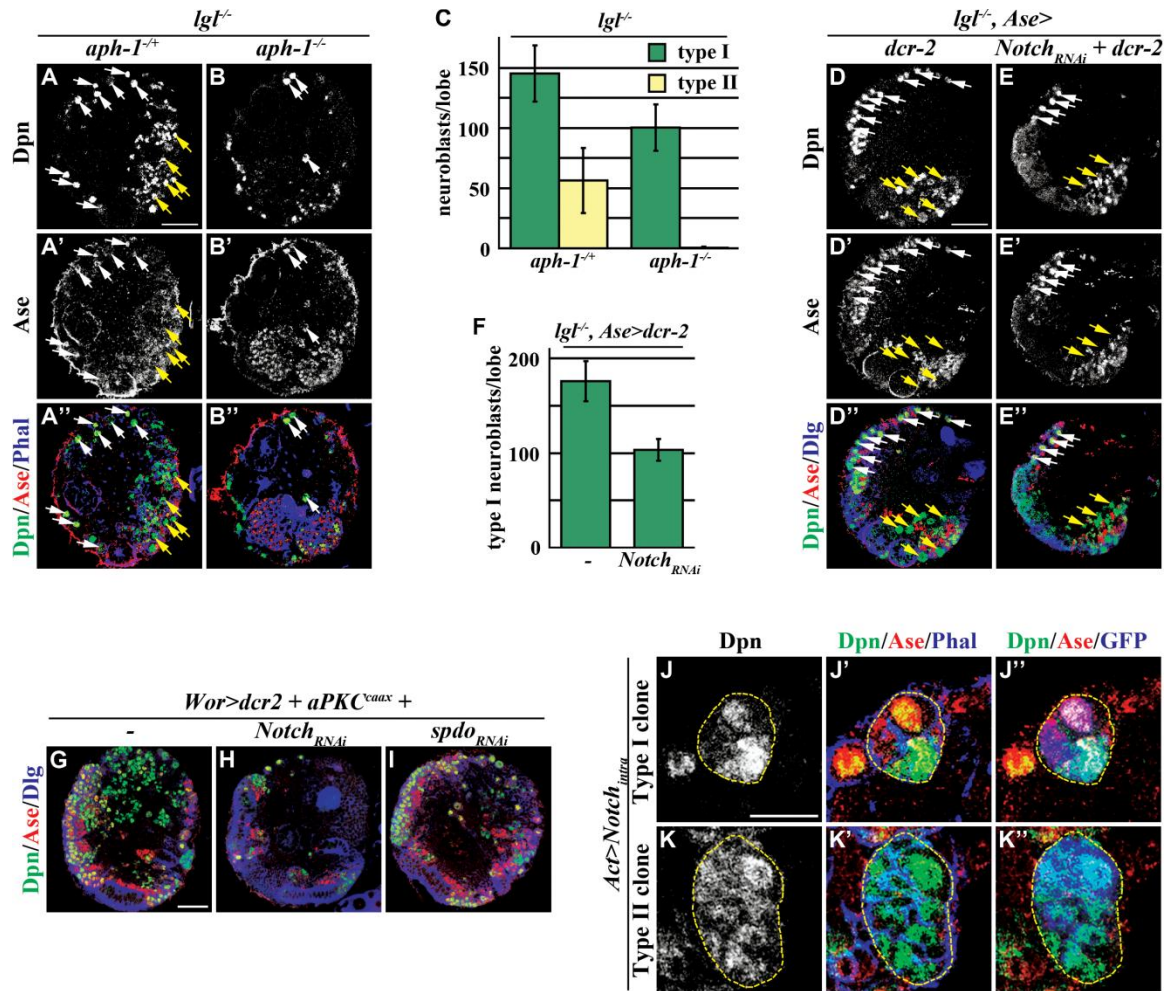


Figure 2.10S Cortical aPKC kinase activity disrupts asymmetric localization of Numb

(A-B) Neuroblasts ectopically expressing aPKC^{caax} showed uniform cortical Numb localization at metaphase and telophase. **(C-D)** Ectopic expression of a kinase-dead aPKC^{caax} showed asymmetric localization of Numb in neuroblasts at both metaphase and telophase.



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Chapter 3

Perspectives and Future Directions

During asymmetric division of fly larval brain neuroblasts, the polarity proteins Lgl and aPKC distinguish neural stem cells from progenitor cells by ensuring that Numb segregates exclusively into the cortex of the future progenitor cell. The work here shows that: (1) Lgl and aPKC antagonize one another to ensure asymmetric localization and segregation of Numb in the cortex of the mitotic neuroblast; (2) aPKC regulates asymmetric localization of Numb via the previously undefined serines 48 and 52 sites in the novel ACBD3 binding region; (3) the phosphorylation status at these two sites, however, does not affect the function of Numb in the restricted progenitor cells; and, (4) the ACBD3 binding region exerts neuroblast-specific suppression of *Notch* signaling by Numb independently of phosphorylation by aPKC. These results led us to propose a mechanism whereby mutual antagonism between Lgl and aPKC ensures that Numb properly segregates exclusively into the progenitor cells where Numb maintains limited potential irrespective of the phosphorylation by aPKC (Haenfler et al., 2012). While the data presented in chapter 2 significantly increases our understanding, several questions remain unanswered.

Regulation of Numb Localization

My research indicates that the control of Numb asymmetry during mitosis is achieved through disparate mechanisms in the SOP and neuroblasts. In the SOP, mutation of the five conserved aPKC phosphorylation sites of Numb mislocalizes Numb throughout the cortex of the dividing SOP cell (Smith et al., 2007). However, my studies show that mutation of these same sites has no effect on the localization of Numb in mitotic neuroblasts. Rather, two phosphorylation sites (serines 48 and 52) in the ACBD3 binding region are responsible for mediating asymmetric localization of Numb. I propose that the SOP and neuroblasts employ unique molecules for asymmetrically segregating Numb and that these molecules interact specifically with unique phosphorylation site on Numb.

Additionally, I found that increased aPKC activity mislocalizes Numb throughout the cortex of mitotic INPs and neuroblasts, but does not release Numb from the cortex into the cytoplasm as previously hypothesized (Wirtz-Peitz et al., 2008). Therefore, it is unclear how phosphorylation of Numb by aPKC partitions Numb exclusively into the basal cortex. Further studies should seek to determine the effect of aPKC phosphorylation on Numb trafficking and dynamics in neuroblasts. Does aPKC phosphorylation regulate the lateral movement of Numb at the cortex? Or are phosphorylated and unphosphorylated pools of Numb sequestered into separate microdomains? Also, the adapter protein, Partner of Numb (Pon), also localizes asymmetrically in mitotic neuroblasts (Wang et al., 2007). Therefore, how does Pon-mediated Numb localization

coordinate with aPKC phosphorylation of Numb? Answers to these questions and more will be critical for our complete understanding of how Numb is asymmetrically localized.

Extending the Functional Studies of Numb

The previously mentioned model suggests that phosphorylation of Numb not only regulates its localization, but also controls the activity of Numb, such that phosphorylation of Numb inactivates it while unphosphorylated Numb is active (Wirtz-Peitz et al., 2008). The data in chapter 2 directly challenges this aspect of the model as well. While the phosphorylation status of Numb at serines 48 and 52 has a dramatic effect on localization, both non-phosphorylatable and phospho-mimetic versions of Numb are able to rescue the *lgl* mutant and *aPKC^{caax}* overexpression supernumerary neuroblast phenotypes. Therefore, it remains unclear how Numb activity is regulated in neuroblasts.

While my studies analyze the significance of various deletions and mutations of Numb in rescuing the *lgl* mutant phenotype, our knowledge of Numb function could benefit from further investigations. First, understanding whether expression of these constructs at endogenous levels rescues the *lgl* mutant could be informative. In addition, rescue experiments for *numb* loss of function should be performed to determine whether the deleted or mutated forms of Numb are capable of substituting for the endogenous Numb. Evaluating the degree of rescue for *numb* mutant embryonic lethality, SOP clones and neuroblast clones could provide novel mechanistic insights into the function of Numb in these diverse contexts.

Context Specific Mechanisms of Numb Inhibition of Notch

Despite the long-standing knowledge that Numb is a potent inhibitor of Notch signaling (Guo et al, 1996), it remains unclear how Numb achieves its function. Previous studies have suggested differing mechanisms for Numb inhibition of Notch, including endocytosis of the Notch receptor (Couturier et al., 2012), an endocytosis- and proteasome-independent pathway (Tang et al., 2005), ubiquitination and degradation of the Notch receptor (McGill and McGlade, 2003), or endocytosis of the Notch signaling component Sanpodo (spdo) (Hutterer and Knoblich, 2005; O'Connor-Giles and Skeath, 2003). However, since all of these studies were conducted in other systems, their results may not be relevant to the inhibition of Notch signaling by Numb in neuroblasts.

By examining the role of the ACBD3 binding region of Numb in both the SOP and neuroblasts, I found that that this region exerts context dependent inhibition of Notch signaling (Haenfler et al., 2012). Previously, a study found that the C-terminal region of Numb interacts with the EAR domain of α -adaptin and this interaction is essential for asymmetric cell fates in the SOP lineage (Berdnik et al., 2002). My studies suggest that the C-terminus of Numb may be dispensable for the function of Numb in neuroblasts (Haenfler et al., 2012). Consistent with this, it was recently reported that neither the C-terminal domain of Numb nor the EAR domain of α -adaptin are crucial for regulating neuroblast asymmetric division. Instead, the authors found that a novel interaction between the N-terminal half of Numb and the trunk domain of α -adaptin mediates their

functions in neuroblasts (Song and Lu, 2012). These findings highlight a few of the differences in how Numb regulates Notch signaling between the SOP and neuroblasts and underscores the need to carefully examine the detailed mechanism in neuroblasts, even though much is already understood in other model systems. I propose that the mechanism by which Numb inhibits Notch signaling differs such that the ACBD3 binding region performs a function in neuroblasts that is either unnecessary or carried out by a different domain of Numb in the SOP.

Comparison of Type I Neuroblasts, Type II Neuroblasts, and INPs

While INPs are restricted in their ability to self-renew, they share more features with type I neuroblasts than with their parental type II neuroblasts, and the similarities between INPs and type I neuroblasts appear more extensive than the similarities between type I and type II neuroblasts. Both type I neuroblasts and INPs are marked by both Dpn and Ase, whereas type II neuroblasts express Dpn, but not Ase. Additionally, asymmetric division of both type I neuroblasts and INPs directly gives rise to GMCs, while type II neuroblast division produces immature INPs (Bowman et al., 2008). The transcription factor Pros localizes to the cytoplasm during interphase and becomes restricted to the basal cortex during mitosis in both type I neuroblasts and INPs, but not type II neuroblasts. Other polarized proteins, such as aPKC, Mira, and Numb, segregate similarly in INPs as in type I and type II neuroblasts (Weng et al., 2010; Haenfler et al., 2012).

More extensive studies comparing and contrasting type I neuroblasts, type II neuroblasts, and INPs could reveal key molecular differences that account for the functional disparities between these populations. One major difference between type I neuroblasts and type II neuroblasts is their requirement of Notch signaling for their maintenance (Bowman et al., 2008; Haenfler et al., 2012). Therefore, it would be interesting to determine whether INPs require Notch signaling for their maintenance. It is possible that the similar molecular profiles of INPs and type I neuroblasts could signify that Notch signaling is correspondingly dispensable for the maintenance of both of these cell types. Alternatively, the requirement for Notch signaling, seen in type II neuroblasts, could be retained throughout the type II lineage, including INPs. In order to answer these questions, new tools for manipulating Notch signaling specifically in INPs will need to be developed.

INP-Specific Rescue Experiments

My studies show that the increased neuroblasts in *lgl* mutant brains result from reversion of progenitor cells back into the parental neuroblast (Haenfler et al. 2012). However, it is unclear whether the function of *lgl* is restricted to the neuroblast or whether *lgl* is also required in the progenitor cells. Particularly, it would be important to determine whether expression of Lgl specifically in INPs or GMCs could rescue the reversion of these progenitor cells observed in the *lgl* mutant. Additionally, examining to what extent ectopic expression of Numb in progenitor cells rescues the *lgl* mutant supernumerary neuroblast phenotype could reveal the contribution of progenitor cell reversion to the overall phenotype.

Finally, the expansion of type II neuroblasts induced by INP-specific expression of aPKC^{caax} is unlikely due to effects on Numb since Numb functions independently of its phosphorylation status in progenitor cells (Haenfler et al., 2012). To address this directly, co-overexpression studies of aPKC^{caax} and Numb in INPs should be performed. If aPKC^{caax} does not induce progenitor cell to neuroblast reversion via Numb, what other factors or mechanisms are implicated?

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