

DISSECTING STEM CELL SELF-RENEWAL: THE ROLES OF MITOTIC KINASES

IN *DROSOPHILA* NEUROBLAST ASYMMETRIC CELL DIVISION

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DISSERTATION ABSTRACT

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Title: Dissecting Stem Cell Self-Renewal: The Roles of Mitotic Kinases in *Drosophila* Neuroblast Asymmetric Cell Division

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Regulation of stem cell self-renewal versus differentiation is critical for embryonic development and adult tissue homeostasis. *Drosophila* larval neuroblasts divide asymmetrically to self-renew and are a model system for studying stem cell self-renewal. Here, we identify two proteins involved in distinct steps of the cell cycle that regulate neuroblast self-renewal. We first describe three mutations showing increased brain neuroblast numbers that map to the *aurora-A* gene, which encodes a conserved kinase implicated in human cancer. Clonal analysis and time-lapse imaging in *aurora-A* mutants show single neuroblasts generate multiple neuroblasts (ectopic self-renewal). This phenotype is due to two independent neuroblast defects: abnormal atypical protein kinase C (aPKC)/Numb cortical polarity and failure to align the mitotic spindle with the cortical polarity axis. *numb* mutant clones have ectopic neuroblasts, and Numb overexpression partially suppresses *aurora-A* neuroblast overgrowth (but not spindle

misalignment). We conclude that Aurora-A and Numb are novel inhibitors of neuroblast self-renewal and that spindle orientation regulates neuroblast self-renewal.

We next identified an *sgt1* (*suppressor-of-G2-allele-of-skp1*) mutant that had fewer neuroblasts. We found that *sgt1* neuroblasts have two polarity phenotypes: failure to establish apical cortical polarity at prophase and lack of cortical Scribble localization throughout the cell cycle. Apical cortical polarity was partially restored at metaphase by a microtubule-induced cortical polarity pathway. Double mutants lacking Sgt1 and Pins (a microtubule-induced polarity pathway component) resulted in neuroblasts without detectable cortical polarity and formation of "neuroblast tumors." Mutants in *hsp83* (encoding the predicted Sgt1-binding protein Hsp90), *LKB1*, or *AMPK α* all show a similar apical cortical phenotype (but no Scribble phenotype), and activated AMPK α rescued the *sgt1* mutant phenotype. We propose that an Sgt1/Hsp90-LKB1-AMPK pathway acts redundantly with a microtubule-induced polarity pathway to generate neuroblast cortical polarity, and the absence of neuroblast cortical polarity can produce neuroblast tumors.

This dissertation includes published and unpublished co-authored material.

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CHAPTER I

INTRODUCTION

This dissertation contains published and unpublished co-authored material. Chapter II was published in the journal *Genes and Development* with co-authors Cheng Yu Lee, Clemens Cabernard, Laurina Manning, Khoa D. Tran, Marcus Lanskey, Arash Bashirullah, and Chris Q. Doe. Chapter III is unpublished material with co-authors Doug Turnbull, Eric Johnson, and Chris Q. Doe.

How multicellular organisms are derived from a single cell has fascinated scientists for over a century. The unequal distribution of pigments in early amphibian embryos fostered the notion that the early oocyte and embryo contained intrinsic polarity (Spemann, 1927). Throughout the 20th century research has focused on the molecular mechanisms that underlie polarity, creating sophisticated tools to interrogate polarity across species of many Phyla.

Asymmetric cell division is critical for generating cellular diversity during development. This mode of division occurs when the mother cell divides giving rise to two cells with different fates. The molecular mechanisms of asymmetric cell division are just now coming into view, and many of the advancements have come from studies in invertebrate model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*. From these pioneering studies, a sequence of events has been proposed that model most intrinsic asymmetric cell divisions. First, the mother cell receives a signal that breaks cellular symmetry. Second, cell fate determinants are asymmetrically localized within the mother cell. Third, a mitotic spindle aligns along this polarity. Fourth, division occurs segregating the cell fate determinants to two distinct daughter cells.

Through this mode of division, a mother cell can create two daughter cells born at the same time with distinct cellular fates.

Asymmetric cell division is a fundamental property of stem cells. Stem cells divide asymmetrically to produce daughter cells that go on to differentiate into specific cells and tissues, while the stem cell maintains itself after many rounds of divisions, a process known as self-renewal. Stem cells have been identified in diverse tissues in mammals, maintain tissue homeostasis, and may contribute to disease states.

Asymmetric stem cell division has been described in skin (Fuchs*, 2006), muscle (Shinin et al., 2006), mammary glands (Cicalese et al., 2009), the hematopoietic system (Marciniak-Czochra et al., 2009) and the developing brain (Bultje et al., 2009). Recently, it has been proposed that mutations within stem cell populations can lead to tumor formation. In support of this hypothesis, mutations of tumor suppressors in stem cells cause tumor formation, but not in non-stem cell types (Barker et al., 2008). Further, many tumors display stem cells that are able to recapitulate the entire tumor when transplanted, unlike other cell types within the tumor. These “cancer stem cells” express many of the same molecular markers as the normal stem cell population from which the tumor arose (Lobo et al., 2007). Despite the importance of stem cell self-renewal and differentiation in development, tissue homeostasis, and tumor formation the molecular mechanisms governing these decisions are largely unknown.

Drosophila brain neuroblasts have many stem cell attributes—such as the ability to remain proliferative, undifferentiated, and nontumorous for a hundred or more cell divisions while generating post-mitotic neurons and glia—and thus they have recently served as a paradigm for studies of self-renewal versus differentiation. Neuroblasts

delaminate from the ventral neuroectoderm during embryogenesis dividing asymmetrically to create the neurons of the larval nervous system. The neuroblasts enter a stage of quiescence at the end of embryogenesis, only to reenter the cell cycle during larval stages to produce neurons of the adult fly (Urbach, 2003). The most prevalent neuroblast populations are termed Type I neuroblasts that divide into a large cell that remains a neuroblast and smaller cell termed the ganglion mother cell (GMC) that divides once again to produce two differentiated neurons (Doe, 1992). Type II neuroblasts are located in a specific region within the central brain and give rise to a different lineage than Type I neuroblasts. The small daughter cell of a Type II neuroblast forms an intermediate neural precursor (INP), which continues to divide in self-renewing asymmetric divisions giving rise to one INP and one GMC (Boone and Doe, 2008). Also within the brain specialized Type I neuroblasts are present in the optic lobes, the mushroom bodies, and the ventral nerve chord.

Neuroblast self-renewal requires proper establishment and maintenance of apical basal polarity within all populations of neuroblasts. During late G2 of the cell cycle the polarity proteins in the Par complex, which comprises of Bazooka (Baz; Par 3 in mammals), Par6, and atypical Protein Kinase C (aPKC) begin to form apical crescents within the neuroblast. As the neuroblast continues in mitosis another complex begins to form apically known as the Pins Complex, consisting of Partner of Incuteable (Pins), G α i, and Mushroom Body Defective (Mud). The adaptor protein Inscuteable (Insc) links the Par and Pins complexes and has been shown to physically interact with Baz and Pins (Knoblich et al., 1999; Schaefer et al., 2000). The Par complex is involved in segregating cell fate determinants to the basal side of the neuroblast through localization of the

adaptor proteins Miranda (Mira) and Partner of Numb (PON) (Schaefer et al., 2000). Miranda binds the cell fate determinants Prospero (Pros) (Ikeshima-Kataoka et al., 1997) and Brain Tumor (Brat) (Lee et al., 2006c). PON binds the Notch inhibitor Numb (Lu et al., 1998). Mislocalization of aPKC to the basal (GMC) cortex triggers ectopic neuroblast self-renewal, resulting in a dramatic expansion of the neuroblast population (Lee et al. 2006a), whereas depletion of Pros or Brat from the GMC also leads to GMC-neuroblast transformation and overproduction of neuroblasts (Bello et al. 2006; Betschinger et al. 2006; Lee et al. 2006b). Thus, establishing proper cortical polarity is essential for neuroblast self-renewal and GMC differentiation.

A second, more speculative pathway for regulating neuroblast self-renewal involves spindle orientation, mediated by the Pins complex. In theory, if the neuroblast mitotic spindle aligns orthogonal to the apical/basal polarity axis, both neuroblast progeny would inherit apical and basal proteins, and both might assume a neuroblast identity (similar to an epithelial cell division, which partitions apical and basolateral membrane domains equally into both siblings). The linkage between spindle and apical cortex is provided, in part, by the Mud protein (Mushroom body defective; the *Drosophila* NuMA ortholog), which directly binds both microtubules and cortical Pins protein; *mud* mutants show misalignment of the mitotic spindle without altering cortical polarity (Bowman et al. 2006; Izumi et al. 2006; Siller et al. 2006). *mud* mutant brains have an increase in neuroblast number (Prokop and Technau 1994; Bowman et al. 2006), and it is tempting to speculate that the symmetric divisions lead to a pair of sibling neuroblasts and thus to the observed increase in neuroblast number, but this has not been confirmed by clonal analysis.

To find additional regulators of neuroblast asymmetric self-renewal we performed a genetic mutagenesis screen. Each lobe of the *Drosophila* brain contains approximately one hundred central brain Type I neuroblasts. As each of these neuroblasts divides in a self-renewing manner, we reasoned that mutations affecting neuroblast self-renewal would affect neuroblast number. Mutations involved in promoting neuroblast self-renewal would cause brain lobes to contain less than one hundred neuroblasts. Conversely, mutations in genes that inhibit self-renewal would cause brain lobes to have more than one hundred neuroblasts. Using this methodology, we screened fly lines exposed to the mutagen ethyl methanesulfate (EMS) as well as molecularly defined p-element insertions.

We first found three EMS mutants showing massive increases in larval neuroblast numbers, and all are alleles of the evolutionary conserved mitotic kinase *auroraA* (*aurora-A*). Mammalian kinase is localized to centrosomes and is considered an oncogene (for review, see Giet et al. 2005). In *Drosophila*, Aurora-A is required for centrosome maturation, cell cycle progression, Numb protein localization during sense organ precursor asymmetric cell division, and astral microtubule length in S2 cells and larval neuroblasts (Glover et al. 1995; Berdnik and Knoblich 2002; Giet et al. 2002). Here we show that loss of Aurora-A leads to neuroblast brain tumors, which arise primarily due to defects in Numb localization and secondarily due to defects in spindle-to-cortical polarity alignment. Our data define a role for Numb in the GMC as an inhibitor of neuroblast self-renewal, and provide direct evidence that spindle orientation can regulate neuroblast self-renewal.

Despite the importance of the apical polarity proteins in regulating neuroblast

self-renewal, it remains unknown exactly how the Par and Pins complexes are initially localized to the apical cortex. The conserved polarity protein Cdc42 binds Par-6 and is required for apical localization of Par-6 and aPKC, but *cdc42* mutants still localize Baz to the apical cortex, albeit weakly (Atwood et al., 2007). The only mutant reported to abolish Baz apical localization is *lkb1* (Bonaccorsi et al., 2007), which encodes a serine/threonine kinase in the Par-4 kinase family; however, the authors only observed weak Baz localization in wild type neuroblasts, so the significance of the mutant phenotype is difficult to interpret.

As we continued to look for molecules involved in neuroblast self-renewal we identified a mutant with a reduced number of brain neuroblasts and defects in the earliest steps of generating neuroblast apical cortical polarity. We mapped the mutant to a region of the third chromosome and used a novel method to identify the mutant lesion, revealing a small deletion in the *suppressor of G2 allele of Skp1* (*sgt1*). Sgt1 is an evolutionary conserved co-chaperone of the molecular chaperone Hsp90. We demonstrate that Sgt1 promotes apical polarity by a Hsp90-Lkb1-AMPK signaling pathway.

CHAPTER II

***DROSOPHILA* AURORA-A KINASE INHIBITS NEUROBLAST SELF-RENEWAL BY REGULATING aPKC/NUMB CORTICAL POLARITY AND SPINDLE ALIGNMENT**

This work was published in volume 20 of the journal *Genes and Development* in December, 2006. Markus Lanskey and Khoa D. Tran provided preliminary mapping analysis, Laurina Manning contributed SOP analysis, Clemens Cabernard contributed time-lapse analysis, and Cheng-Yu Lee performed initial neuroblast number quantification, mapping data, and editorial support. Arash Bashirullah and Chris Q. Doe were principle investigators involved in the study. I performed and quantified expression analysis, quantified neuroblast numbers, constructed fly stocks and performed experiments for neuroblast clones, performed spindle orientation analysis in neuroblasts, constructed illustrative figures, and wrote the manuscript.

Materials and Methods

Fly stocks and genetics

All mutant chromosomes were balanced over *CyO*, *actin::GFP* or *TM3 actin::GFP*, *Ser*, *e*. We used Oregon R as wild type, and the following mutant chromosomes:

aPKC^{k06403} (Rolls et al. 2003) *aurora-A*⁸⁸³⁹ (this work) *aurora-A*¹⁴⁶⁴¹ (this work) *aurora-A*¹⁷⁹⁶¹ (this work) *aurora-A*^{87Ac-3} (Bloomington stock 6188) *FRT82B aurora-A*¹⁴⁶⁴¹ (this work) *UAS-aurora-A:GFP* (this work) *UAS-numb* (Knoblich et al. 1997) *worniu-Gal4* (Albertson and Doe 2003; Lee et al. 2006a) *cnn*^{HK21} (Megraw et al. 1999) *Df(3R)T-61* (Bloomington stock 3003) *elav-Gal4(C155)*, *UAS-mCD8:GFP*, *hsp70-FLP*; *tubulin-*

Gal80, FRT40A (Lee and Luo 1999) *elav-Gal4(C155), UAS-mCD8:GFP, hsp70-FLP;*
FRT82B tubulin-Gal80 (Lee and Luo 1999) *w; FRT82B, mira^{zz178}* (Caussinus and
Gonzalez 2005) *w; brat¹⁵⁰, FRT40A* (Betschinger et al. 2006) *w; numb², FRT40A* (this
work) *w; FRT82B pros¹⁷* (Reddy and Rodrigues 1999) *His2AvD:GFP* (Clarkson and
Saint 1999) *hs-flp(F38)* (Bloomington stock 5258) *actin-FRT-Draf+-FRT-nuc.lacZ*
(Struhl and Basler 1993) *actin-FRT-stop-FRT-Gal4 UAS-Notch-intra* (Struhl and
Greenwald 2001) *Notch-ts1* (Bloomington stock 2533; chromosome cleaned of
background mutations and provided by Ross Cagan, Washington University, St. Louis,
MO) *Notch-ts2* (Bloomington stock 3075). All clone experiments (MARCM, *actin-Gal4*,
or *actinnuc.lacZ*) were done using standard methods (Lee and Luo 2001; Lee et al.
2006b) by giving the larval progeny a 1-h 37°C heat shock at 24 h ALH, development at
25°C, and clone analysis at 96 h ALH.

Antibodies and imaging

Larvae brains were dissected in Schneider's medium (Sigma), 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 and blocked for 1 h in 1• PBS containing 1% BSA and 0.3% Triton X-100 (PBS-BT) supplemented with 0.01 M glycine and 0.1% goat normal serum. After blocking, specimens were washed in PBS-BT for 1 h and incubated with primary antibodies in PBS-BT overnight at 4°C. Brains were stained as described previously (Albertson and Doe 2003; Lee et al. 2006a). Pupal nota were dissected and stained as described previously (Manning and Doe 1999). We used guinea pig anti-Mira (1:400), rat anti-Mira (1:500), guinea pig anti-Numb (1:1000; J. Skeath), rat anti-Brat (1:100), mouse anti-Pros MR1A bioreactor supernatant (1:100), rabbit anti-aPKC ζ (1:1000; Santa Cruz

Biotechnology), rabbit anti-G α i (1:1000), mouse anti-Insc (1:1000; Bill Chia), rat anti-Pins (1:500), rat anti-Par6 (1:50), guinea pig anti-Baz (1:400), rabbit anti-Scribble (1:2500), mouse anti-Discs large (1:100; Developmental Study Hybridoma Bank [DSHB]), rabbit anti-Mud (1:1000), guinea pig anti-Dpn (1:1), mouse anti-Elav (1:50; DSHB), rat anti-Elav (1:10; DSHB), mouse anti-BrdU (1:50, Sigma), rabbit anti- β gal (1:1000), mouse anti- α -tubulin (1:2000; Sigma), Rat anti- α -tubulin (1:100; Serotec), mouse anti- γ -tubulin (1:2000; Sigma), rabbit anti-Aurora-A (1:200; J. Knoblich), rabbit anti-phosphohistoneH3 (1:1000; Upstate Biotechnology), rabbit anti-Cnn (1:1000; T. Kaufman), rabbit anti-GFP (1:1000; Torrey Pine), mouse anti-CD8 (1:100; Caltag), mouse anti-Hindsight “concentrated” (1:100; DSHB), and secondary antibodies from Molecular Probes. Antibodies without named sources were made in the laboratory; details are available on request.

BrdU pulse/chase experiments

Larvae were fed with BrdU (1 mg/mL; Roche) in media for 4 h, and then one pool was processed for BrdU staining (pulse experiments) and a second pool was grown without BrdU for 24 h before fixation and BrdU staining (pulse/chase experiments). Larval brains were dissected, processed, and antibody-stained as described above with the exception that larval brains were treated in 2N HCl for 30 min prior to primary anti-BrdU antibody staining.

Neuroblast counting and brain orientation

A larval brain lobe consists of the medially localized central brain and the laterally localized optic lobe. Neuroblasts can be unambiguously identified by expression of Wor, Dpn, and Mira and the absence of the neuronal/glial differentiation markers Elav

and Repo (Albertson and Doe 2003; Lee et al. 2006a). Central brain neuroblasts (the focus of this study) can be distinguished from optic lobe neuroblasts due to their medial–superficial location in the brain, larger size, and dispersed pattern (optic lobe neuroblasts laterally positioned in the brain and spaced very closely to each other, forming a ribbon that flanks and encircles the highly stereotypical epithelial-shaped optic lobe cells) (Lee et al. 2006a). All images of neuroblasts shown were collected from central brain; all brains were mounted with dorsal surface up and ventral surface down, and the midline is to the left in all panels.

Time-lapse imaging of larval neuroblasts

*aurora-A*¹⁴⁶⁴¹ recombined with *His2AvD:GFP* (Clarkson and Saint 1999) and crossed to *aurora-A*¹⁷⁹⁶¹. Late second and/or early third instar *aurora-A* mutant larvae were picked based on the lack of the dominant marker *Tubby* and dissected as previously described (Siller et al. 2005, 2006). Larvae were dissected and mounted in D-22 media (US Biological) with 1% bovine growth serum (BGS; HyClone) and imaged on a Bio-Rad Radiance 2000 confocal or Leica SP2 microscope using a 60• 1.4NA oil-immersion objective. Time-lapse sequences were processed using ImageJ and Imaris 5.0.1, 64-bit (Bitplane).

***aurora-A* Mutants Have Too Many Larval Neuroblasts**

To identify genes that regulate neuroblast self-renewal, we screened a collection of pupal lethal mutants (L. Wang, J. Evans, H. Andrews, R. Beckstead, C.S. Thummel, and A. Bashirullah, in prep.) for changes in central brain neuroblast number. Three mutants [*l(3)LL-8839*, *l(3)LL-14641*, *l(3)LL-17961*] had a massive increase in larval neuroblasts, developing >1000 central brain neuroblasts at stages where wild-type larvae

have only 95– 100 neuroblasts; in addition, the mutant neuroblasts were maintained into pupal stages, whereas wild-type pupae have few neuroblasts (Fig. 1A–C). The mutants had normal optic lobe and imaginal disc epithelial morphology (Fig. 1B[']; data not shown), unlike previously identified tumor suppressor mutants (for review, see Hariharan and Bilder 2006). The mutations were deficiency and complementation mapped to the *aurora-A* locus, and the *aurora-A*^{87ac-3} mutant (Glover et al. 1995) showed a similar supernumerary brain neuroblast phenotype (data not shown). Sequencing of the mutant alleles revealed that *l(3)LL-14641* has a single base change resulting in a V → E change at position 302, within the kinase activation loop (Cheetham et al. 2002); *l(3)LL-17961* has a single base change resulting in a D → N change at position 334 that is predicted to destabilize α -helical packing; and *l(3)LL-8839* has a single base change resulting in a K → stop change at position 377 that deletes the C terminus of the protein (Fig. 1D); thus these are new *aurora-A* alleles. The Aurora-A kinase is detected in the cytoplasm and on centrosomes in neuroblasts and sense organ precursors (Berdnik and Knoblich 2002), as is an Aurora-A:GFP fusion protein expressed in neuroblasts (data not shown), consistent with a role in neuroblast asymmetric cell division.

We next asked if the ectopic neuroblasts in *aurora-A* mutants had all the properties of wild-type neuroblasts. In wild-type brains, neuroblasts express the markers Deadpan (Dpn), Mira, Worniu (Wor); fail to express the neuronal differentiation markers nuclear Pros and Elav; and are proliferative based on their ability to incorporate BrdU (Fig. 1E). In *aurora-A* mutants, all ectopic neuroblasts have the same properties (Fig. 1F; data not shown). In addition, we used BrdU pulse/chase experiments to determine if *aurora-A* neuroblasts could generate neurons. Immediately after a BrdU pulse, both wild-

type and *aurora-A* mutant neuroblasts—but no neurons—incorporated BrdU (Fig. 1G,I); following a 24-h BrdU-free chase, all BrdU labeling was detected exclusively in the $Elav^+$ neurons (Fig. 1H,J). Thus, the ectopic neuroblasts in *aurora-A* mutant larvae show normal marker expression and proliferation, and are able to generate post-mitotic neurons.

Single *aurora-A* Mutant Neuroblasts Generate Clones Containing Multiple Neuroblasts

To determine the origin of the extra neuroblasts in the mutant brains, we generated positively marked clones in single neuroblasts within *aurora-A* mutant brains, at a low frequency of one to two per brain lobe to keep each clone well separated. In wild type, genetically marked βgal^+ clones induced in a single neuroblast always mark one large neuroblast and a population of smaller GMC/neuronal progeny (Fig. 1K). In contrast, single neuroblast clones induced in *aurora-A* mutants typically contained multiple neuroblasts as well as a pool of smaller GMC/ neuronal progeny (Fig. 1L). Thus, *aurora-A* mutant neuroblasts undergo occasional divisions in which both siblings assume the neuroblast fate, leading to an expansion of the neuroblast population (Fig. 1M). Importantly, neuroblast-specific expression of Aurora-A:GFP (*wor-gal4 UAS-aurora-A:GFP*) is able to significantly rescue the *aurora-A* supernumerary neuroblast phenotype, showing that the phenotype is due to loss of Aurora-A within neuroblast lineages (data not shown). We conclude that the evolutionarily conserved Aurora-A kinase is required to inhibit neuroblast self-renewal and promote GMC/neuronal differentiation.

Aurora-A Regulates aPKC and Numb Cortical Polarity

In wild-type neuroblasts, Baz/Par6/aPKC and Insc/Pins/Gai proteins are localized

to the apical cortex of metaphase neuroblasts (Fig. 2A–C; data not shown). In *aurora-A* mutants, we find that aPKC can be uniform cortical or show ectopic cortical patches, all other apical proteins assayed show normal localization (Fig. 2D–G, data not shown). Ectopic aPKC is observed in a small fraction of neuroblasts at early larval stages (48–72 h after larval hatching [ALH]) and becomes much more common at later stages when the increase in neuroblast number is the most dramatic (96–120 h ALH). *aPKC; aurora-A* double mutants completely suppress ectopic neuroblast formation (data not shown), showing that aPKC is required for the *aurora-A* supernumerary neuroblast phenotype. This rescue is specific (e.g., not simply due to *aPKC* mutants arresting the neuroblast cell cycle or inducing neuroblast cell death) because *aPKC* mutants can only weakly suppress the *brat* mutant supernumerary neuroblast phenotype (Lee et al. 2006b). We conclude that *aurora-A* mutants disrupt aPKC but not other tested apical polarity proteins; that aPKC delocalization coincides with the increase in neuroblast numbers; and that aPKC is required for the ectopic neuroblasts observed in *aurora-A* mutants.

Loss of the basal proteins Brat or Pros can generate supernumerary neuroblast phenotypes (Bello et al. 2006; Betschinger et al. 2006; Lee et al. 2006b), so we tested whether *aurora-A* mutants have defects in basal protein localization. Wild-type neuroblasts localize Numb, Miranda, Pros, and Brat to the basal cortex at metaphase (Fig. 2H–K). In *aurora-A* mutants, we observed normal Mira/ Pros/Brat protein basal crescents at metaphase (Fig. 2I–K), but Numb was either relatively normal (Fig. 2L) or delocalized around the cortex (Fig. 2M). This is similar to the Numb localization defects in *aurora-A* mutant mitotic sense organ precursors (Berdnik and Knoblich 2002; see below).

Numb Inhibits Neuroblast Self-Renewal

The role of Numb during larval neuroblast asymmetric division has never been investigated, so we assayed the phenotype of *numb* mutant clones generated in single larval neuroblasts. Wild-type clones always show one neuroblast and its family of neuronal progeny (Fig. 3A), whereas *numb* mutant clones always contain multiple neuroblasts plus neuronal progeny (Fig. 3B). Thus, the *numb* clones are similar to the *aurora-A* mutant brains in their ability to expand the neuroblast population while still producing differentiated Pros⁺ Elav⁺ neurons. Interestingly, the *numb* and *aurora-A* mutant phenotypes are distinct from *mira*, *pros*, or *brat* mutant single neuroblast clones, which contain few or no differentiated neurons (data not shown). We conclude that Aurora-A is required to restrict Numb to the basal cortex and that Numb inhibits neuroblast self-renewal.

We next tested whether Numb acts downstream from Aurora-A by using the neuroblast-specific *wor-Gal4* line to overexpress Numb in *aurora-A* mutant neuroblasts and GMCs. While *aurora-A*¹⁴⁶⁴¹ mutants have >450 neuroblasts per brain lobe, overexpression of Numb is sufficient to partially rescue the supernumerary neuroblast phenotype in *aurora-A*¹⁴⁶⁴¹ mutants (~175 neuroblasts per lobe) at the same larval stage (Fig. 3C–E); this is similar to the number of neuroblasts seen in mutants that affect spindle orientation but not cortical polarity such as *cnn* (Fig. 3C) or *mud* (Prokop and Technau 1994; Bowman et al. 2006). Thus, Numb overexpression appears to suppress the cortical polarity component of the *aurora-A* supernumerary neuroblast phenotype. We conclude that Numb acts downstream from Aurora-A to inhibit neuroblast self-renewal.

Aurora-A Regulates Alignment of the Mitotic Spindle With the Cortical Polarity Axis

We next tested whether spindle orientation defects could contribute to the production of ectopic neuroblasts in *aurora-A* mutants. In wild-type metaphase neuroblasts, the mitotic spindle is aligned within 15° of the center of the apical/basal cortical polarity axis (Siller et al. 2005, 2006; Bowman et al. 2006). In *aurora-A* metaphase neuroblasts, the mitotic spindle is essentially randomized relative to the apical/basal cortical polarity axis (Fig. 4B,C). Because some *aurora-A* neuroblasts have multiple centrosomes (wild type: 2.0, $n = 25$; *aurora-A*: 3.8, $n = 45$) and multipolar spindles, we only scored neuroblasts where a clear bipolar spindle could be observed. In addition, *aurora-A* mutant neuroblasts have defects in telophase spindle orientation, with ~15% of the neuroblasts dividing symmetrically (Fig. 4D,F; see below)—a phenotype that is never observed in wild-type neuroblasts (Fig. 4E). Spindle orientation defects are not due to loss of cortical Mud protein (which is essential for proper spindle orientation) (Bowman et al. 2006; Izumi et al. 2006; Siller et al. 2006), because Mud remains cortical in *aurora-A* mutant neuroblasts (Fig. 4G,H). Interestingly, overexpression of Numb does not rescue the *aurora-A* spindle orientation defect (Fig. 4D), consistent with Aurora-A regulating cell polarity and spindle orientation phenotypes via two distinct pathways (see Discussion). We conclude that Aurora-A is essential for proper alignment of the mitotic spindle to the neuroblast cortical polarity axis.

To determine if the *aurora-A* cell polarity and spindle orientation phenotypes are specific to neuroblasts, we also assayed asymmetric cell division of the pupal external sensory organ precursor (SOP). In wild-type pupae, the SOP localizes Numb and Pins to

the anterior cortex and Baz/Par6/aPKC to the posterior cortex, and the mitotic spindle is tightly aligned with the anterior/posterior cortical polarity axis (Fig. 4I,J; for review, see Betschinger and Knoblich 2004). In addition, we find that the spindle anchoring protein Mud is centrosomal and enriched at the anterior and posterior cortex (Fig. 4K). In *aurora-A* mutant SOPs, cortical polarity is established normally based on proper anterior Pins crescent formation, but Numb is delocalized (confirming previous findings; Berdnik and Knoblich 2002). Importantly, we observe nearly random spindle orientation relative to the SOP cortical polarity axis (Fig. 4L–N), similar to the neuroblast spindle orientation phenotype. The most frequent spindle orientation is along the proximodistal axis, perhaps reflecting a secondary spindle orientation cue from the planar cell polarity system. We conclude that Aurora-A is required in both neuroblasts and SOPs to align the mitotic spindle with the cortical polarity axis.

***aurora-A* Neuroblasts Divide Symmetrically to Expand the Neuroblast Population**

It has been proposed that physically symmetric cell divisions can lead to expansion of the larval neuroblast population during mutant or wild-type insect development (Nordlander and Edwards 1970; Farris et al. 1999; Bowman et al. 2006; Siller et al. 2006), but this attractive hypothesis has never been directly tested. Here we use time-lapse imaging to assay the frequency and cell fate consequences of neuroblast symmetric divisions in *aurora-A*¹⁴⁶⁴¹ mutants. We imaged histone:GFP (HisAvD:GFP) in explanted larval brains; these whole-brain cultures are healthy enough to allow us to follow >10 wild-type neuroblast cell divisions (see Materials and Methods). We assayed division symmetry by measuring both sibling nuclear size (with histone:GFP) and sibling cell size

(using background fluorescence signal). We found that wild-type neuroblasts always divide asymmetrically with regard to sibling cell and nuclear size (100%, $n = 11$) (Fig. 5A,D). In contrast, *aurora-A* mutant neuroblasts show both asymmetric cell divisions (Fig. 5B,D) and symmetric cell divisions (17%, $n = 42$) (Fig. 5C,D). Thus, *aurora-A*¹⁴⁶⁴¹ neuroblasts show a significant number of symmetric divisions; this could contribute to the supernumerary neuroblast phenotype if both progeny retained a neuroblast identity. In support of this model, we observed a neuroblast lineage tree containing two symmetric divisions followed by an asymmetric cell division (Fig. 5E). This shows that a symmetric neuroblast division can produce additional neuroblasts with the potential for asymmetric cell division—a hallmark of neuroblast identity—and suggests that symmetric divisions can expand the neuroblast population.

Reduction in mammalian Aurora-A kinase levels results in mitotic delay (Dutertre et al. 2002; Du and Hannon 2004). Time-lapse analysis of *aurora-A* mutant neuroblasts revealed delays in prometaphase and metaphase (Fig. 5F) as well as increased overall cell cycle length (data not shown). In addition, we observe an increased mitotic index (wild type: 7.6% prometaphase–metaphase, $n = 690$; *aurora-A*: 14.2% prometaphase–metaphase; $n = 2753$) in fixed preparations. It is formally possible that the cell cycle delays are the cause of the neuroblast cell polarity and supernumerary neuroblast phenotype (Fichelson and Gho 2004). However, we have previously shown that metaphase-arrested neuroblasts have normal cortical polarity, and that *Lis1* and *Glued* mutants have cell cycle delays without a matching neuroblast cortical polarity or supernumerary neuroblast phenotype (Spana and Doe 1995; Broadus and Doe 1997; Siller et al. 2005). We conclude that *aurora-A* mutant neuroblasts show cell cycle delays,

but that these delays do not prevent or cause the observed increase in brain neuroblasts.

Discussion

We have shown that mutations in *aurora-A* lead to a massive increase in larval brain neuroblasts. The major cause of this phenotype appears to be misregulation of neuroblast cortical polarity. One cortical polarity defect is increased basal localization of aPKC, which we previously showed was sufficient to induce ectopic neuroblasts (Lee et al. 2006a). Consistent with this hypothesis, *aPKC aurora-A* double mutants show strong suppression of the *aurora-A* supernumerary neuroblast phenotype, consistent with aPKC functioning downstream from Aurora-A. While it is possible that loss of aPKC suppresses the phenotype in a nonspecific way (e.g., by arresting neuroblast cell proliferation or inducing neuroblast apoptosis), we fail to observe similarly strong suppression of the *brat* supernumerary neuroblast phenotype in *aPKC brat* double mutants (Lee et al. 2006b). This shows that aPKC functions more specifically in the Aurora-A pathway than in the Brat pathway.

The only other detectable cortical polarity defect seen in *aurora-A* mutant neuroblasts is a delocalization of Numb from the basal cortex. A similar Numb defect is seen during asymmetric cell division of pupal SOPs in *aurora-A* mutants (Fig. 4K; Berdnik and Knoblich 2002), perhaps reflecting a specific and direct regulation of Numb by Aurora-A, although Numb is not phosphorylated by Aurora-A in vitro (Berdnik and Knoblich 2002). The importance of the Numb delocalization phenotype is revealed by the ability of Numb overexpression in neuroblasts to rescue most of the *aurora-A* mutant phenotype (all except the component due to spindle orientation defects; see below). Thus, Numb acts downstream from Aurora-A to inhibit neuroblast self-renewal. Numb joins

Mira/Pros/Brat as proteins that are partitioned into the GMC during neuroblast asymmetric cell division, where they function to inhibit neuroblast self-renewal (Fig. 6; Bello et al. 2006; Betschinger et al. 2006; Lee et al. 2006b).

Where does Aurora-A function to inhibit neuroblast self-renewal? Aurora-A appears to be required in the neuroblast lineage, and not in surrounding glial cells or nonneuronal tissues of the larva, because neuroblast-specific expression of either Aurora-A or the downstream component Numb can rescue most of the *aurora-A* supernumerary neuroblast phenotype. This shows that Aurora-A is not required outside the neuroblast lineage to inhibit neuroblast self-renewal. Within the neuroblast, Aurora-A appears to function in the cytoplasm and not at the centrosome, because *cnn* mutants lack all detectable Aurora-A centrosomal localization yet do not match the *aurora-A* supernumerary neuroblast phenotype. We conclude that Aurora-A acts in the neuroblast cytoplasm to promote aPKC/Numb cortical polarity and spindle-to-cortex alignment.

How does Numb inhibit neuroblast self-renewal in the GMC? Numb is a well-characterized inhibitor of Notch signaling that is segregated into the GMC (Frise et al. 1996; Guo et al. 1996; Zhong et al. 1996; O'Connor-Giles and Skeath 2003; Hutterer and Knoblich 2005), and Notch signaling is active in larval neuroblasts but not in GMCs (Almeida and Bray 2005). Thus the most obvious model is that Numb blocks Notch receptor signaling in the GMC. However, *Notch* mutant clones generated in larval neuroblasts do not affect neuroblast survival or clone size (Almeida and Bray 2005). Similarly, we have seen no change in neuroblast number in two different *Notch-ts* mutants (although we observed the expected small wing imaginal disc phenotype) (C.-Y. Lee, unpubl.). In addition, we did not observe supernumerary neuroblasts in larval

neuroblast clones overexpressing the constitutively active Notch intracellular domain (C.-Y. Lee, unpubl.), although the same Notch intracellular domain generates the expected sibling neuron phenotype when expressed in the embryonic CNS (J. Boone and K. Robinson, unpubl.). Thus, Notch is an excellent candidate for promoting neuroblast self-renewal, but additional experiments will be needed to test this model more rigorously. In this context, it is interesting to note that Notch promotes stem cell self-renewal in mammals (Androutsellis-Theotokis et al. 2006; Fan et al. 2006).

We show that *aurora-A* mutant neuroblasts have essentially random orientation of the mitotic spindle relative to the apical/basal cortical polarity axis, resulting in some neuroblasts dividing symmetrically (in size and cortical polarity markers). This phenotype may arise due to lack of astral microtubule interactions with the neuroblast cortex, as *aurora-A* mutant neuroblasts have reduced astral microtubule length (Giet et al. 2002). Alternatively, Aurora-A may affect spindle orientation by phosphorylating proteins required for spindle orientation, such as Cnn, Pins, or Mud. For example, Mud has a consensus Aurora-A/Ipl1 phosphorylation site within its microtubule-binding domain, and it will be interesting to determine if this site needs to be phosphorylated for Mud to bind microtubules. Spindle orientation defects only generate part of the supernumerary neuroblast phenotype in *aurora-A* mutant brains, however, because overexpression of Numb can rescue most of the phenotype without rescuing spindle alignment, and *cnn* or *mud* mutants have nearly random spindle alignment but only a modest increase in neuroblast number (Fig. 3C; Bowman et al. 2006). Thus, we propose that spindle orientation defects and cortical polarity defects combine to generate the dramatic supernumerary neuroblast phenotype seen in *aurora-A* mutants (Fig. 6).

Mammalian *aurora-A* has been termed an oncogene due to its overexpression in several cancers, its ability to promote proliferation in certain cell lines, and the fact that reduced levels lead to multiple centrosomes, mitotic delay, and apoptosis (for review, see Giet et al. 2005). However, an *in vivo* *aurora-A* mutant phenotype has not yet been reported. In contrast, we find that *aurora-A* loss-of-function mutations result in a neuroblast “brain tumor” phenotype, including prolonged neuroblast proliferation during pupal stages when wild-type neuroblasts have stopped proliferating. *aurora-A* mutants do not, however, have the imaginal disc epithelial overgrowth seen in other *Drosophila* tumor suppressor mutants (for review, see Hariharan and Bilder 2006), and *aurora-A* mutant neuroblasts have a delay in cell cycle progression. We propose that the *aurora-A* supernumerary neuroblast phenotype is not due to loss of growth control or a faster cell cycle time, but rather due to a cell fate transformation from a differentiating cell type (GMC) to a proliferating cell type (neuroblast).

We conclude that Aurora-A restrains neuroblast numbers using two pathways: first by promoting Numb localization into the GMC, and second by promoting alignment of the mitotic spindle with the cortical polarity axis. Absence of the first pathway leads to increased neuroblasts at the expense of GMCs, whereas absence of the second pathway leads to increased neuroblasts due to symmetric cell division. It will be interesting to determine whether mammalian Aurora-A uses one or both pathways to regulate stem cell asymmetric division and self-renewal.

Although we demonstrate that Aurora-A is required for proper aPKC/Numb polarity and spindle orientation, many aspects of neuroblast self-renewal are still unknown. How is Baz localized to the apical cortex? What are the molecular

mechanisms underlying spindle orientation? As our initial screen was largely successful, we continued screening other mutant lines to find additional regulators of neuroblast self-renewal.

CHAPTER III

SGT1 ACTS VIA AN LKB1/AMPK PATHWAY TO ESTABLISH CORTICAL POLARITY IN LARVAL NEUROBLASTS

This chapter contains unpublished co-authored material. Doug Turnbull developed the mapping/sequencing scheme used to identify the *sgt1* mutant. Chris Q. Doe and Eric Johnson were principle investigators for this work. I was the primary contributor for the rest of the work, prepared the illustrative figures, and wrote the manuscript.

Materials and Methods

Identification and sequencing of the $sgt1^{s2383}$ mutant

The *sgt1^{s2383}* mutant allele was originally found in a P-element screen of the 3rd chromosome. The P-element mapped to 66E1-2, but *sgt1^{s2383}* over a deficiency removing this region was viable without a CNS phenotype; additional deficiency mapping revealed that the lethality and CNS phenotype mapped to *Df(3R)6147* at 84F6-F13. To further define the region, rescue constructs were made using gap-repair and Φ C31 mediated transgenesis (Venken et al., 2009). Using the tilling BAC 33N15, we made two overlapping 60kb and 28kb rescue constructs; only the 60kb construct rescued the *l(3)s2383* phenotype, narrowing the relevant region to approximately 13 genes. To identify the lesion in this interval, we used a biotin mediated “sequence capture” technique followed by deep sequencing. Genomic DNA from both wild type and mutant larva were prepared for deep sequencing. Using BAC33N15 as a template, biotin incorporated “probes” were created. The biotin labeled probes were individually hybridized with wild type or mutant DNA and purified over streptavidin beads. The

genomic DNA was then eluted from the beads, and sequenced using Illumina deep sequencing, revealing a 15 nucleotide in frame deletion within the first exon of *sgt1*^{s2383}; this allele will be called *sgt1* for simplicity. In addition, a P element allele of *sgt1* (C01428; from the Harvard Exelixis collection) failed to complement and showed the same phenotype as *sgt1*^{s2383}.

Fly stocks

Df(3R)6147 was obtained from the Bloomington stock center. The *lkb1*^{4A4-2} and *lkb1*^{4A4-11} mutant stocks (Mirouse et al., 2007) were kindly provided by Daniel St. Johnston; the strong or null *ampkα* mutants *ampk*¹, *ampk*² and the *UAS-GFP-lkb1* and *UAS-ampkα (T-D)* stocks were gifts from Jay Brenman; *UAS-apkcRNAi* was obtained from the Vienna Stock Center. *pins*^{p62} was obtained from Bill Chia (Singapore); *hsp83*^{13F3} and *hsp83*⁵⁸² were obtained from Howard Lipshitz (Toronto). The *sgt1*^{s2383} *pins*^{p62} double mutants were generated by recombination. *insc-gal4* (aka *1407-gal4*), *worniu-gal4*, and *tubulin-gal4* were all used for rescue experiments. Mutant larva were identified by lack of balancer chromosome markers *Tubby* or GFP.

Antibody staining, drug treatment, and imaging

Third instar larval brains were dissected and fixed as previously described (Lee et al., 2006a). Neuroblast counting and BrdU labeling were done as previously described (Lee et al., 2006a). Dissected brains were washed several times in PBS-T (PBS with 0.1% TritonX-100) and blocked for 1hr in PBS-BT (PBS with 0.1% Triton and 1% BSA). Brain preparations were incubated overnight at 4C with primary antibodies diluted in PBS-BT. All analyses were performed with the mitotic markers rat anti- α -tubulin (1:3000; Serotec, Kidlington, Oxford, UK) or rabbit anti-phospho-histone H3 (1:1000,

Upstate, West Grove, PA) and one or more of the following antibodies: guinea pig anti-Bazooka (1:1000, Doe lab), rabbit anti-aPKC (1:1000; Sigma, St. Louis, MO), rabbit anti-PAR6 (1:100; Doe lab), rat anti-Pins (1:500; Doe lab), rabbit anti-Inscuteable (1:500; Bill Chia), rabbit anti-G α i (1:500; Doe lab), mouse anti-Discs Large (1:500; Developmental Hybridoma Studies Bank [DHSB], Iowa), anti-rabbit Scribble (1:5000; Doe lab), guinea pig anti-Miranda (1:400; Doe lab), rabbit anti-Miranda (1:1000; Doe lab), rabbit anti-phosphoT385AMPK α (1:100; Cell Signaling, Beverly, MA), and guinea pig anti-Numb (1:100; Jim Skeath). After 3 washes with PBS-BT the samples were incubated for 2 hours at room temperature with the appropriate secondary antibody (FITC-conjugated IgG, Rhodamine Red-X-conjugated IgG, and Cy5-conjugated IgG; Invitrogen, Eugene, OR). All secondary antibodies were diluted from a 0.5 mg/ml stock solution and used at 1:400. Brains washed in PBT, mounted in VectaShield medium, and examined with a Bio-Rad Radiance 2000 confocal microscope using a 63X 1.4NA oil immersion objective and a Zeiss LSM 700 confocal microscope using a 40X 1.3NA oil immersion objective. Image processing was performed using ImageJ and Photoshop (Adobe), and figures were assembled in Illustrator (Adobe).

To inhibit microtubule formation, dissected wild type and *sgt1* mutant brains were placed in 10 μ g/ml Colcemid (Sigma) diluted in Schneider's medium (Sigma) for 1hr. Brains were fixed and antibody stained as described above.

A Genetic Screen for Neuroblast Self-Renewal Mutants Identifies *sgt1*

To find potential regulators of neuroblast self-renewal, we screened homozygous P-element induced mutations for altered larval neuroblast numbers, using Miranda (Mira)

and Deadpan (Dpn) to identify neuroblasts and Scribble (Scrib) to outline the neuroblast cell cortex and highlight general neuroanatomical landmarks (Lee et al., 2006c) (Figure 1A). One mutant that showed a modest decrease in neuroblast number, and larval/pupal lethality, was *l(3)s2383* which contains a P element at 66E1-66E2 on the left arm of the third chromosome (Figure 7A,B). To confirm that the P element insertion caused the phenotype, we assayed the *l(3)s2383* chromosome *in trans* to a deficiency that removed chromosomal region 66E1-66E2. Surprisingly, these larvae were completely normal for neuroblast number and viability, showing that the mutation mapped to elsewhere on the third chromosome. We used deficiency mapping to localize the mutation to the 84F6-84F13 region of the right arm of the third chromosome (Figure 7B). We found that *l(3)s2383 / Df(3R)6147* larvae showed the same neuroblast number and larval/pupal lethality phenotypes as *l(3)s2383* homozygotes. Phenotypic analysis of overlapping deficiencies in the region allowed us to map the mutation to a 90kb interval, and we confirmed the mutation was in this interval by genomic DNA rescue of the phenotype (data not shown; and see Materials and Methods).

To identify the DNA lesion responsible for the phenotype, we used a novel “sequence capture” strategy combined with Illumina deep sequencing (Figure 8A) to identify an in frame 15 nucleotide deletion in the first exon of the *sgt1* gene, also known as *CG9617* (Fig 8B; and see Materials and Methods). We henceforth call this allele *sgt1^{s2383}*. To confirm that the *sgt1^{s2383}* lesion is responsible for the observed neuroblast and viability phenotype, we crossed *sgt1^{s2383}* with the known *sgt1^{c01428}* mutation (Martins et al., 2009). We found that *sgt1^{s2383}* fails to complement *sgt1^{c01428}*, and that *sgt1^{s2383} / sgt1^{c01428}* larvae have the same phenotype as *sgt1^{s2383}* homozygotes. Furthermore, the fact

that the phenotype of *sgt1*^{s2383} / *sgt1*^{c01428} is identical to that of *sgt1*^{s2383} / *Df(3R)6147*, indicated that *sgt1*^{s2383} is a strong or null allele for CNS function. The lesion in *sgt1*^{s2383} removed 15 nucleotides in frame, leading to a five amino acid deletion in the Chord and Sgt1 (CS) domain that mediates Sgt1-Hsp90 protein interactions in other organisms (Zhang et al., 2010). We conclude that *Drosophila* Sgt1 is required for maintaining normal numbers of larval neuroblasts and for viability.

***sgt1* Is Required for Apical Protein Localization in Prophase Neuroblast**

To investigate the cellular origin of the neuroblast depletion phenotype in *sgt1* mutants, we assayed neuroblast cell cycle progression and neuroblast cell polarity. We confirmed a recent report (Martins et al., 2009) that *sgt1* mutants have slower cell cycle progression, cytokinesis failure, polyploidy, multiple centrosomes, and malformed mitotic spindles (Figure 9 and data not shown). In this paper, we focus on the previously unreported cell polarity aspects of the phenotype. First, we assayed neuroblast apical cortical polarity ("apical cortex" is defined as the cortical domain that is partitioned into the sibling neuroblast, whereas "basal cortex" is defined as the domain segregated into the GMC). The first sign of neuroblast asymmetry occurs during late G2 and early prophase when Par complex proteins (e.g. Baz, aPKC), Pins complex proteins (e.g. Pins), and the Par/Pins linker protein Insc are localized to an apical cortical crescent (Figure 10A; quantified in A'). In contrast, *sgt1* mutant prophase neuroblasts typically showed cytoplasmic or undetectable localization of these proteins (Figure 10B; quantified in B'). Interestingly, during metaphase we found a substantial rescue of apical cortical polarity (Fig 10C,D; quantified in C',D'); the basal proteins Miranda and Numb fairly normal at metaphase (Figure 11). We conclude that Sgt1 is required for establishing apical cortical

polarity during prophase, and during metaphase an Sgt1-independent pathway can generate apical/basal cortical protein localization.

We next analyzed Dlg and Scrib cortical localization in wild type and *sgt1* mutant neuroblasts, for two reasons. First, we had observed a complete lack of Scrib cortical localization in our original screen (Figure 1A), and second, Dlg is part of the Pins complex (Siegrist and Doe, 2005) so we wanted to know if it was delocalized in prophase neuroblasts similar to Pins. In wild type interphase and mitotic neuroblasts, Scrib was always uniform cortical (Figure 12A; 100% uniform cortical, n=50), while Dlg was invariably uniform cortical throughout the cell cycle plus enriched with Pins at the apical cortex during mitosis (Figure 12B; 100% uniform cortical n=53, 94% apical enriched, n=34). In contrast, *sgt1* mutant neuroblasts showed a complete loss of cortical Scrib localization during metaphase (Figure 12C; 3% cortical, n=75) and interphase (Figure 7A, and data not shown); they also showed a loss of the uniform cortical pool of Dlg protein at interphase and mitosis, with retention of the pool of Dlg asymmetrically co-localized with Pins at the apical cortex (Figure 12D; 2% uniform cortical n=45, 83% apical enriched, n=29). We conclude that Sgt1 is essential for targeting Dlg/Scrib to the neuroblast cortex, except where Dlg is co-localized with Pins.

Microtubules Induce Apical Cortical Polarity in *sgt1* Metaphase Neuroblasts

What is the Sgt1-independent pathway that generates Pins and Par complex apical cortical localization in metaphase neuroblasts? We previously defined a microtubule-induced cortical polarity pathway that could induce Pins/G α i/Dlg cortical polarity (Siegrist and Doe, 2005), so here we test whether this pathway induces Pins and Par

complex apical cortical polarity at metaphase in *sgt1* mutant neuroblasts. We used the microtubule inhibitor colcemid to abolish spindle microtubules in *sgt1* mutant and control neuroblasts. Wild type neuroblasts treated with the colcemid retain apical cortical crescents of Dlg, Pins and Insc; the first two proteins are Pins complex members and the latter is a linker protein that can associate with either the Pins complex via direct interaction with Pins or the Par complex via direct interaction with Baz (Figure 13B, top row; quantified in figure legend). Strikingly, *sgt1* mutant neuroblasts treated with colcemid lack all detectable apical cortical polarity (Figure 13B, bottom row; quantified in figure legend). We conclude that microtubules are required to establish apical cortical polarity in the absence of Sgt1.

***sgt1, pins* Double Mutant Neuroblasts Lack All Detectable Cortical Polarity and Form Neuroblast Brain “Tumors”**

The loss of both Sgt1-dependent and microtubule-dependent cortical polarity pathways is predicted to generate a completely apolar neuroblast (see above). The developmental consequence of a complete loss of neuroblast polarity has never been assayed, so we tested (a) whether *sgt1, pins* double mutants abolish both Par and Pins complex polarity, and if so (b) what is the fate of such apolar neuroblasts. We found that *sgt1, pins* double mutant neuroblasts establish little or no apical or basal cortical polarity (Figure 14F-J and data not shown). The developmental consequences of this loss of polarity are the formation of ectopic neuroblasts throughout the brain (Figure 15N). This is in striking contrast to the *sgt1* and *pins* single mutant brains, which are each smaller with reduced neuroblast numbers (Figure 15L,M). We propose that loss of all cortical polarity in the

double mutants results in symmetric neuroblast division to form two neuroblasts and expand the pool of brain neuroblasts (see Discussion).

Reducing Hsp90 Levels Mimics the *sgt1* Mutant Phenotype

To determine the mechanism by which Sgt1 promotes apical cortical polarity and Dlg/Scrib cortical localization, we started by determining if Sgt1 worked together with its evolutionarily-conserved binding partner, Hsp90. Sgt1 directly interacts with Hsp90 via its CS domain in all organisms tested (Nony et al., 2003; Lee et al., 2004; Lingelbach and Kaplan, 2004; Catlett and Kaplan, 2006), and our *sgt1*^{s2383} allele generates an Sgt1 protein lacking 5 amino acids within CS domain (Figure 2B). We obtained mutants in *hsp83* (which encodes the Hsp90 protein) and examined the larval brain neuroblast phenotype in *hsp83*^{13F3}/*hsp83*⁵⁸² (van der Straten et al., 1997; Lange et al., 2000). We find that *hsp83* mutants have neuroblast phenotypes that are similar to that of *sgt1* mutants: loss of apical cortical polarity at prophase (Figure 16B,C; quantified in Figure 16G). However, there is no loss of cortical Scrib in *hsp83* mutants (Figure 16C), suggesting that Sgt1 promotes Dlg/Scrib localization by an Hsp90-independent mechanism (see Discussion). The similarity of the *sgt1* and *hsp83* apical cortical polarity phenotypes, and the fact that the Sgt1^{s2383} protein has a small deletion within the conserved Hsp90-binding CS domain, leads us to conclude that Sgt1 and Hsp90 act together to promote apical protein localization in neuroblasts.

Sgt1 Acts Via an LKB1/AMPK Pathway to Generate Apical Cortical Polarity, but Not for Scrib Cortical Localization

Sgt1 has been shown to act through Polo kinase to regulate neuroblast cell cycle progression and cytokinesis (Martins et al., 2009). We confirmed that misexpression of Polo rescues these neuroblast cell cycle phenotypes, but found no rescue of the neuroblast cortical polarity phenotypes (data not shown). Thus, we investigated other downstream effectors of Sgt1 for a role in establishing apical protein localization.

lkb1 mutants are reported to have defects in neuroblast apical polarity that are similar to the *sgt1* mutant phenotype (Bonaccorsi et al., 2007). We confirm that *lkb1* mutants had a nearly identical neuroblast polarity phenotype as *sgt1* mutants, with the striking exception of showing normal Scrib cortical localization (Figure 16D; quantified in Figure 16G). We conclude that Sgt1 and LKB1 are both required to establish apical cortical polarity in neuroblasts.

LKB1 kinase can activate multiple members of the AMP-activated kinase (AMPK) family, such as Par-1, AMPK, Sik, NUAC, and others (Lizcano et al., 2004). Both Par-1 and AMPK activity are required for generating cell polarity in multiple cell types and organisms (Goldstein and Macara, 2007; Williams and Brenman, 2008), so we tested whether Par-1 or AMPK functions downstream of Sgt1/LKB1 in generating apical localization of Par complex proteins in neuroblasts. First we examined the localization and function of Par-1; we observed high levels of the protein in cortex glia that ensheath the neuroblast, but no sign of polarized localization within the neuroblast and no change in neuroblast polarity in *par-1* mutant MARCM clones (data not shown). Next we assayed *AMPK α* mutants, and found a similar defect in apical protein localization as seen

in *sgt1* and *lkb1* single mutants (Figure 16E; quantified in Figure 16G). As with *lkb1* mutants, we saw normal cortical localization of Scrib, confirming that Sgt1 acts in an LKB1/AMPK-independent pathway to promote Scrib cortical localization. Importantly, we found that neuroblast-specific expression of constitutively activated AMPK α (Lee et al., 2007) (*tub-gal4 UAS-ampk α^{TD}*) substantially rescued the *sgt1* mutant phenotype (Figure 16F; quantified in Figure 16G). We conclude that an Sgt1 – Hsp90 – LKB1 – AMPK pathway is used to establish apical cortical polarity in prophase neuroblasts, whereas an Sgt-specific pathway is used to establish corticalDlg/Scrib localization (Figure 16H).

Discussion

Here we show that the evolutionary-conserved protein Sgt1 acts with Hsp90, LKB1 and AMPK to promote apical localization of the Par and Pins complexes in prophase neuroblasts. The first step in Sgt1-induced Par polarity is likely to be Sgt1/Hsp90 activating LKB1: Sgt1 and Hsp90 are known to interact in all organisms tested (Nony et al., 2003; Lee et al., 2004; Lingelbach and Kaplan, 2004; Catlett and Kaplan, 2006). We propose that Sgt1/Hsp90 proteins function together based on multiple lines of evidence: (1) they show conserved binding from plants to humans; (2) our *sgt1*^{s2383} mutant results in a five amino acid deletion within the CS domain, which is the Hsp90 binding domain (Catlett and Kaplan, 2006; Zhang et al., 2008); (3) *sgt1* and *hsp83* have similar cell cycle phenotypes (Figure 9)(Lange et al., 2000); and (4) *sgt1* and *hsp83* have similar neuroblast polarity phenotypes (Figure 16B,C). The Sgt1/Hsp90 complex either stabilizes or activates client proteins (Zuehlke and Johnson, 2010); we suggest that

Sgt1 activates LKB1 because we were unable to rescue the *sgt1* mutant phenotype simply by overexpressing wild type LKB1 protein (data not shown).

LKB1 is a "master kinase" that activates at least 13 kinases in the AMPK family (Lizcano et al., 2004). We suggest that LKB1 activates AMPK because overexpression of phosphomimetic, activated AMPK α can rescue the *lkb1* and *sgt1* mutant phenotype (Figure 16F and data not shown). It remains unclear how AMPK activity promotes apical protein localization. An antibody to activated AMPK α (anti-phosphoT385-AMPK α , Mirouse et al., 2007) shows spindle and cytoplasmic staining that is absent in *ampka* mutants, and centrosomal staining that persists in *ampka* null mutants (data not shown). AMPK activity is thought to directly (Lee et al., 2007) or indirectly (Bultot et al., 2009) activate myosin regulatory light chain to promote epithelial polarity (Lee et al., 2007; Miranda et al., 2010), and could use a similar pathway to generate apical protein localization in neuroblasts. AMPK is activated by a rise in AMP/ATP levels that occur under energy stress or high metabolism; AMP binds to the γ regulatory subunit of the heterotrimeric complex and results in allosteric activation of the α subunit (Hardie, 2007). *ampka* mutants grown under energy stress have defects in apical/basal epithelial cell polarity in follicle cells within the ovary (Mirouse et al., 2007). In contrast, *ampka* mutants grown on nutrient rich food still show defects in embryonic epithelial polarity (Lee et al., 2007), neuroblast apical polarity (this work) and visceral muscle contraction (Bland et al., 2010). Larval neuroblasts, embryonic ectoderm, and visceral muscle may have a high metabolic rate, require a low basal level of AMPK activity, or use a different mechanism to activate AMPK than epithelial cells.

Sgt1/Hsp90/LKB1/AMPK are all required for apical Par/Pins complex localization, but Sgt1 must act via a different pathway to promote Dlg/Scrib cortical localization, because only the *sgt1* mutant affects Dlg/Scrib localization, and overexpression of activated AMPK α is unable to restore cortical Scrib in *sgt1* mutants (Figure 16). The mechanism by which Sgt1 promotes Dlg/Scrib cortical localization is unknown.

We have shown that *sgt1* mutants lack Par/Pins apical polarity in prophase neuroblasts, but these proteins are fairly well polarized in metaphase neuroblasts. The rescue of cortical polarity is microtubule dependent, probably occurring via the previously described microtubule-dependent cortical polarity pathway containing Pins, Dlg and Khc-73 (Siegrist and Doe, 2005). The polarity defects still observed in metaphase neuroblasts may be due to the poor spindle morphology in *sgt1* mutant neuroblasts (Figure 9). The lack of microtubule-induced polarity at prophase, despite a robust microtubule array in prophase neuroblasts, suggests that the microtubule-induced cortical polarity pathway is activated at metaphase. Activation of the pathway could be via expression of the microtubule-binding protein Khc-73; via phosphorylation of Pins, Dlg or Khc-73 by a mitotic kinase like AuroraA (Johnston et al., 2009); or via a yet unknown pathway.

It was somewhat surprising that the *sgt1, pins* double mutants had increased numbers of brain neuroblasts, because each single mutant had reduced neuroblast numbers (this work) (Lee et al., 2006b). This paradox can be resolved by the observation that in each single mutant, neuroblasts retain some cortical polarity, but the *sgt1, pins* double mutants lack all known neuroblast cortical polarity. We propose that the apolar

double mutant neuroblasts partition cell fate determinants equally to both siblings, and that both siblings frequently or invariably assume a neuroblast identity. This is supported by our recent finding that when the neuroblast spindle is aligned orthogonal to a normal apical/basal polarity axis, such that both siblings inherit equal amounts of apical and basal cortical proteins, the siblings always acquire a neuroblast identity (Cabernard and Doe, 2009). Thus, equal partitioning of apical/basal cell fate determinants (in spindle orientation mutants) or failure to establish any cortical polarity (*sgt1*, *pins* mutants) appears to result in neuroblast/neuroblast siblings and an expansion of the neuroblast population.

CHAPTER IV

CONCLUSION

Stem cells are present from the early embryo, through development, and in to adult. The balance between production of stem cells and differentiating cells is critical to the development and homeostasis of an organism. An imbalance between these decisions has been conceptually and experimentally linked with tumorigenesis (an increase in stem cell production) and tissue degeneration (loss of stem cell population). Work in *Drosophila* neuroblasts has revealed that establishment and maintenance of stem cell polarity is critical for balancing self-renewal versus differentiation. Importantly, almost all of the molecules involved in neuroblast asymmetry are conserved in vertebrates. Further, vertebrate stem cells asymmetrically localize these proteins including Par3 (neural stem cells) (Bultje et al., 2009), Numb (mammary stem cells) (Cicalese et al., 2009), and Inscutable/Pins/Mud (skin stem cells) (Poulson and Lechler, 2010). Clearly, *Drosophila* neuroblasts have provided insight into how stem cells regulate self-renewal versus differentiation and provide a powerful model to look for additional regulators of stem cell biology.

Here we describe a genetic screen that found additional regulators of neuroblast self-renewal. Both molecules that we discovered are involved in proper regulation of mitotic kinases. Given the essential roles of kinases during cell division, it is not surprising that cell kinases would have an important role in stem cell divisions. Most work in vertebrates has focused exclusively on the role of cell kinases in cell cycle progression. We find, however, that not only are mitotic kinases involved in the progression of the cell cycle they are key regulators of asymmetric stem cell division.

Specifically, both AuroraA kinase and, Sgt1 through activation of AMPK signaling, are required for proper cell fate localization. Further, AuroraA is also involved in proper mitotic spindle alignment. These two phenomena, asymmetric protein localization and spindle alignment, are key regulatory steps in vertebrate stem cell biology (Bultje et al., 2009). Whether AuroraA and Sgt1 play similar roles in vertebrate stem cells remains to be determined.

Although AuroraA has not been looked at in vertebrate stem cell populations, it has emerged as a regulator of tumorigenesis. AuroraA has been classified as an oncogene, being overexpressed in several types of tumors and linked with poor prognosis. Amplification or overexpression of AuroraA has been associated with lung tumors (Lo Iacono et al., 2011), glioblastoma (Barton et al., 2010) and oral carcinomas (Tanaka et al., 2005). Consistent with the role for AuroraA in *Drosophila* neuroblasts as a tumor suppressor, however, AuroraA has also been identified as a tumor susceptibility gene in mouse and human tumors (Ewart-Toland et al., 2003). Several polymorphisms have been linked to specific tumor types including esophageal (Kimura et al., 2005), gastric (Ju et al., 2006), breast (Cox et al., 2006), lung (Davies et al., 2005), pancreas, (Chen et al., 2007), and prostate (Matarasso et al., 2007). Further work needs to be done to look at the specific mechanisms that underlie AuroraA in mammalian tumor suppression, specifically the function of AuroraA in stem cell biology including polarity regulation and spindle orientation.

We demonstrate that the chaperone complex containing Sgt1 and Hsp90 promotes apical neuroblast polarity. Although the role of Sgt1 in vertebrate stem cell self-renewal has not been tested, emerging evidence suggests that Hsp90 may be an important factor

in stem cell biology. Hsp90 induction has been shown to induce neural differentiation of embryonal carcinoma stem cells (Afzal et al., 2011). Also, specific inhibition of Hsp90 protects neural stem cells (Wang et al., 2011) and oligodendrocyte precursors (Cid and Alcazar, 2010) from apoptosis and differentiation. These studies suggest that Hsp90 may be a key factor in inhibiting stem cell self-renewal, however, work in rat mesenchymal stem cells (MSC) suggests that Hsp90 is involved in promoting MSC survival (Gao et al., 2010). Hsp90 inhibition has long been a target for the treatment of tumors (Giménez Ortiz and Montalar Salcedo, 2010). Given the specific roles of Hsp90 in different stem cell populations, and the roles of stem cells in promoting tumorigenesis, it will be critical to understand the molecular mechanisms of Hsp90 in stem cell self-renewal and differentiation.

In neuroblasts, we have shown that the Sgt1-Hsp90 complex regulates neuroblast self-renewal by activation of Lkb1 and AMPK signaling. Lkb1 has long been known to be an Hsp90 client protein (Boudeau et al., 2003). Interestingly, Lkb1 has recently been shown to be required for maintaining hematopoietic stem cell (HSC) homeostasis (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010). Conditional knock down of Lkb1 in hematopoietic tissues led to a progressive loss of all blood cell types (Nakada et al., 2010), suggesting that Lkb1 has a critical role in promoting HSC self-renewal. The loss of HSC was due to apoptosis (Gurumurthy et al., 2010). Unlike in *Drosophila* neuroblasts, AMPK was not downstream of Lkb1 in HSC, as activated AMPK could not rescue Lkb1 depleted HSCs, and AMPK deficient HSC had no discernable phenotype (Nakada et al., 2010). Lkb1 activates a large family of AMPK-related kinases, and further study needs to be done to determine if they are involved in Lkb1 function in HSC.

Also, determining if Hsp90 functions with Lkb1 in HSC and other stem cell populations, and whether AMPK is downstream will be intriguing.

The causal relationship between stem cell function and tumor formation is still controversial. Given the roles of cell cycle kinases in stem cell homeostasis and tumor progression, however, may provide a conceptual link between the two. There is a growing body of evidence that mutations within stem cells may be the initial steps in tumor formation. Recent work has demonstrated that intestinal tumors can be generated when the polarity regulator adenomatous polyposis complex (APC) is mutated specifically in stem cells, but not in non-stem cell types (Barker et al., 2008). Targeted mutation of the *p53* locus in mouse mammary stem cells is sufficient to generate mammary tumors (Cicalese et al., 2009). Also, glioblastoma models have demonstrated that tumors arise with the appearance of abnormal stem cell populations (Tabatabai, 2011), suggesting the stem cells have defects in asymmetric cell division. Addressing the roles of stem cells and cancer stem cells in model organisms, including *Drosophila*, may be able to provide information on how stem cells become malignant.

The link between cell polarity and stem cell function in vertebrates is coming in to view. From *Drosophila* to mice polarity seems to be essential for many stem cell functions. These characteristics suggest that proper establishment of polarity is a conserved mechanism required for proper stem cell function. As the molecular mechanisms of polarity establishment become elucidated, including the roles of kinases, drugs or techniques that target stem cell polarity may be useful to revert aspects of stem cell disease.

APPENDIX

FIGURES

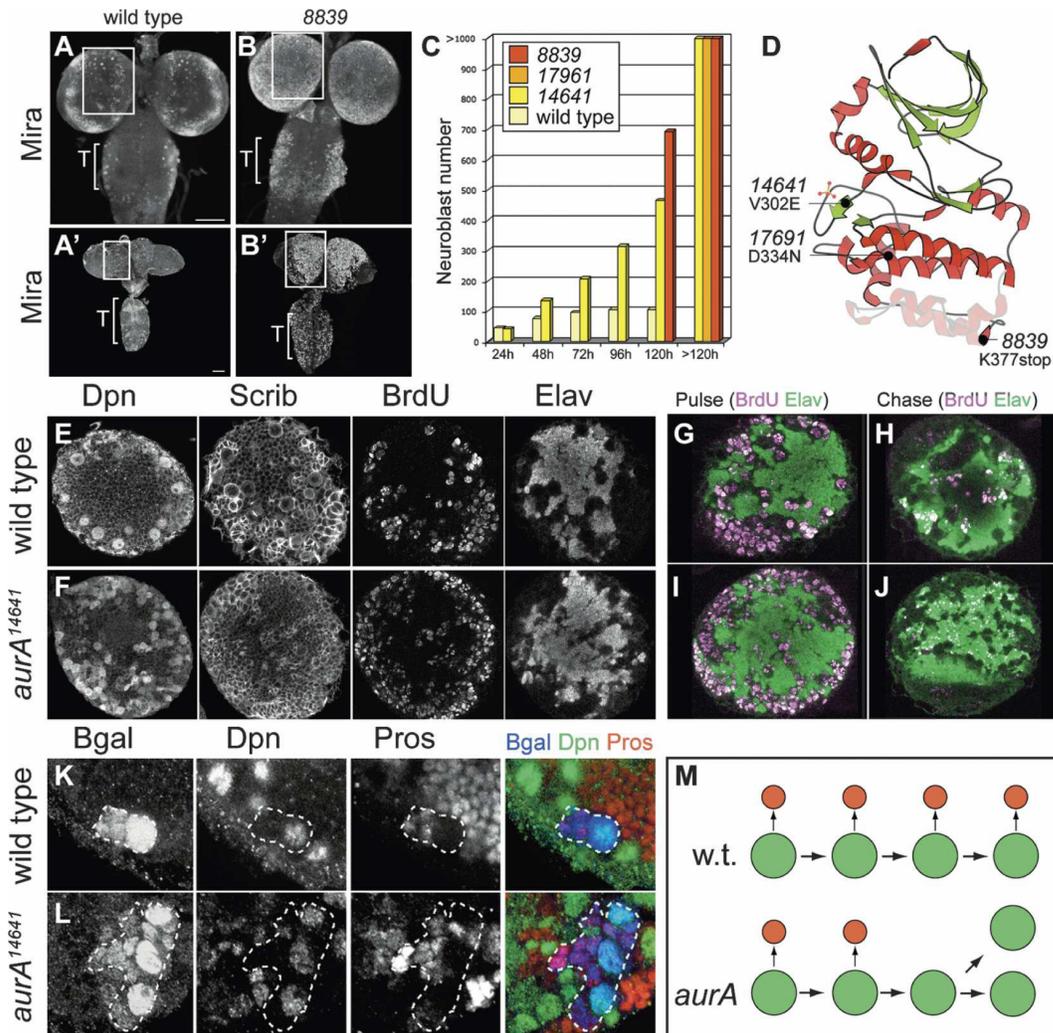


Fig. 1. *aurora-A* mutants have too many larval neuroblasts. (A,B) Larval brains at 96 h ALH showing increased Mira+ neuroblasts in the mutant central brain (boxed) and thorax (T). (A',B') Pupal brains at 12–18 h after pupariation showing increased numbers of proliferative Dpn+ neuroblasts and expanded CNS size. (C) Quantification of brain neuroblast numbers (using Dpn or Mira markers). (D) Molecular lesions in *aurora-A* alleles superimposed on the crystal structure of human Aurora-A (Cheetham et al. 2002; Nowakowski et al. 2002). Lightly shaded region is absent in *aurora-A*⁸⁸³⁹. (E,F) *aurora-A* mutant neuroblasts are correctly specified. Wild-type (E) and *aurora-A*¹⁴⁶⁴¹ (F) neuroblasts are Dpn+ Elav– and incorporate BrdU, showing that they are specified correctly and are proliferative. (G–J) *aurora-A* mutant neuroblasts can proliferate and generate neuronal progeny. Wild-type (G,H) and *aurora-A*¹⁴⁶⁴¹ (I, J) 96-h-ALH larvae incorporate BrdU into neuroblasts following a 4-h BrdU pulse (G,I), and “chase” the BrdU into post-mitotic neurons following a 24-h BrdU-free chase (H,J). (K–M) *aurora-A* mutant neuroblasts generate multiple neuroblasts. Single-neuroblast clones identified by β gal expression (circled with the dotted line). (K) In wild type, clones contain one Dpn+ neuroblast and many nuclear Pros+ progeny. (L) In *aurora-A*¹⁴⁶⁴¹, clones contain multiple Dpn+ neuroblasts and nuclear Pros+ progeny. (M) Summary. (Green) Neuroblasts; (red) GMCs.

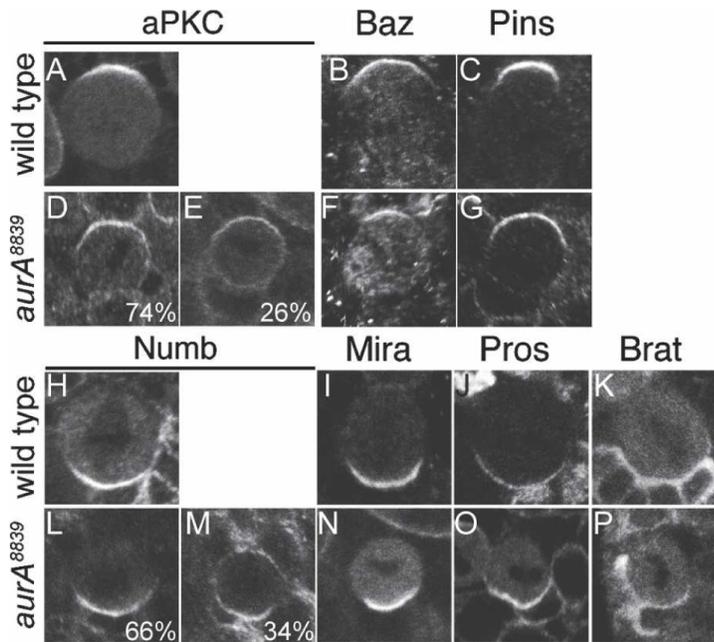


Fig. 2. *aurora-A* neuroblasts have ectopic aPKC and delocalized Numb. (A–C,H–K) Wild-type mitotic neuroblasts at 120 h ALH show normal apical protein localization (A–C) and basal protein localization (H–K). (D–G,L–P) *aurora-A*⁸⁸³⁹ mutant mitotic neuroblasts at 120 h ALH show delocalization of aPKC (26%, n = 47) and Numb (34%, n = 69). Baz, Pins, Mira, Pros, Brat (B,C,I–K) and Gαi, Insc, and Pon (data not shown) have essentially normal localization.

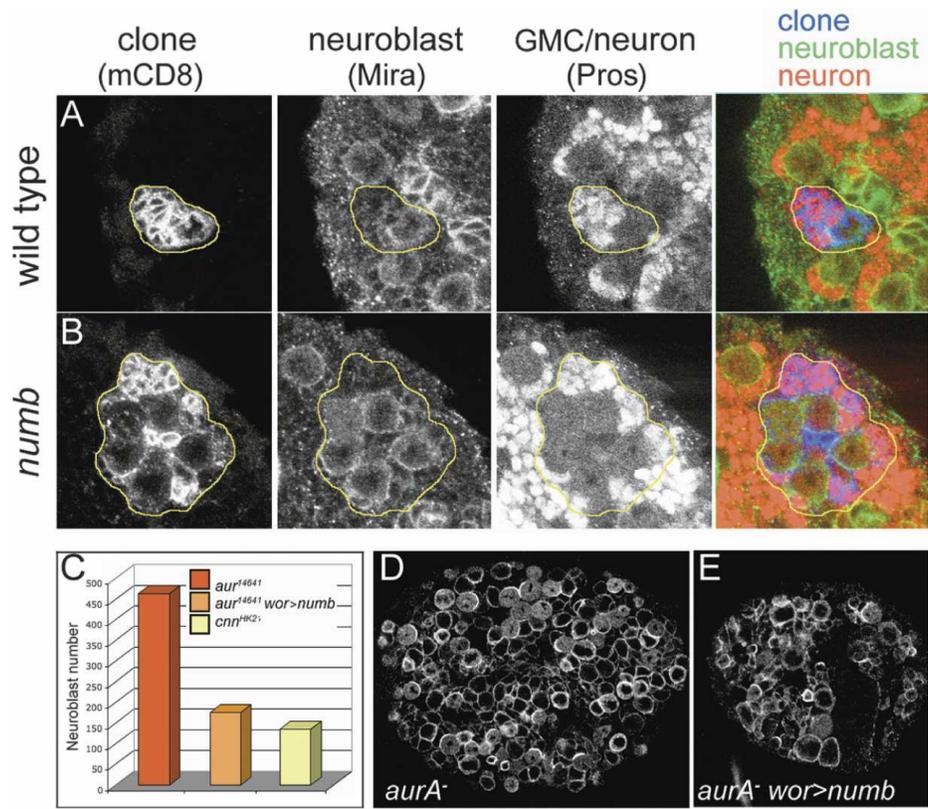


Fig. 3. Numb acts downstream from Aurora-A to inhibit neuroblast self-renewal. (A,B) Numb inhibits neuroblast self-renewal. (A) Wild-type neuroblast clones always contain a single neuroblast and multiple differentiated progeny. (B) *numb*² mutant clones contain multiple neuroblasts. Clones were induced by standard methods at 0–24 h ALH and processed at 72 h ALH for mCD8 (clone marker), Mira (neuroblast marker), and Pros (GMC/ neuron marker). (C–E) Numb overexpression can suppress supernumerary neuroblast formation in *aurora-A* mutants. (C) Quantification of Mira+ neuroblast numbers for a 120-h-ALH brain lobe of the indicated genotypes. (D,E) Single optical section 8 μ m below the dorsal surface of a 120-ALH brain lobe stained for the Mira neuroblast marker in *aurora-A*¹⁴⁶⁴¹ (D) or *aurora-A*¹⁴⁶⁴¹ *wor-gal4 UAS-numb* (E) larvae.

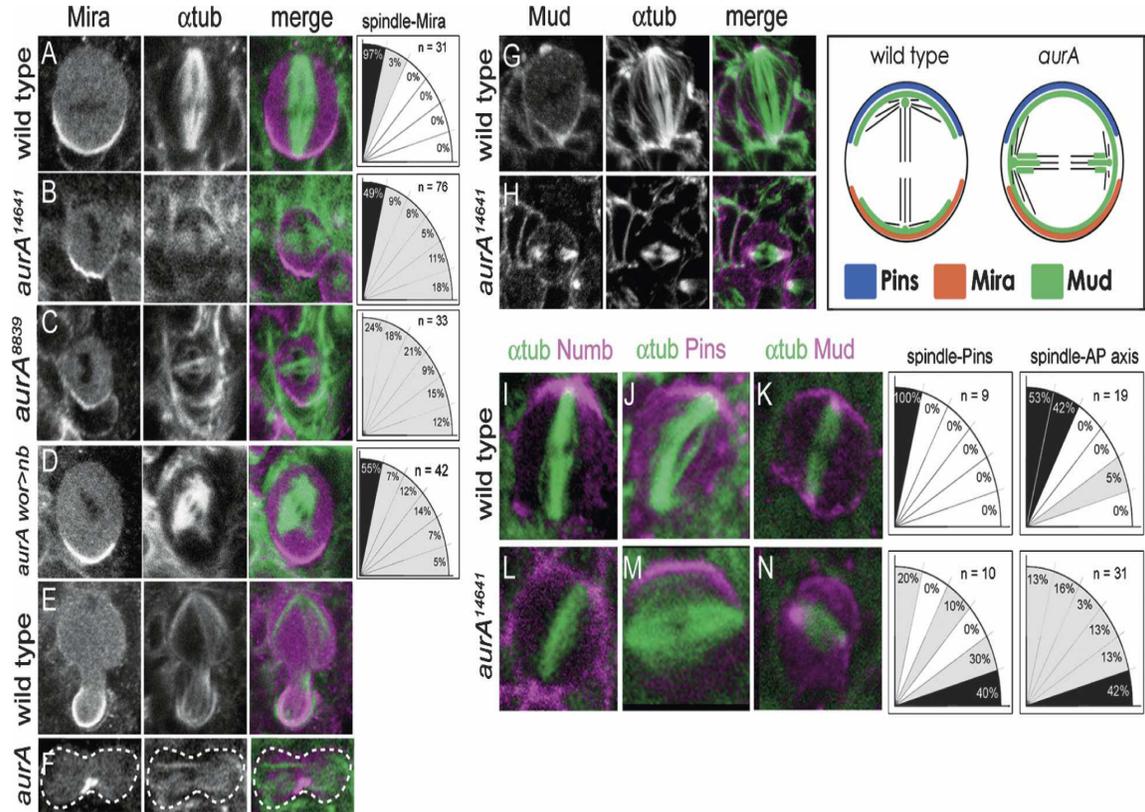


Fig. 4. *aurora-A* neuroblasts and SOPs fail to align the mitotic spindle with the cortical polarity axis. (A–F) *aurora-A* mutant neuroblasts fail to align the mitotic spindle with the cortical polarity axis. (A) Wild-type metaphase (A) or telophase (E) 120-h-ALH neuroblasts showing tight alignment of the mitotic spindle (α -tub, α -tubulin) with the apical/basal cortical polarity axis (Mira, Miranda basal marker). (B,C,F) *aurora-A*⁸⁸³⁹ mutant larval metaphase (B,C) or telophase (F) 120-h-ALH neuroblasts showing misalignment of the mitotic spindle relative to the apical/basal cortical polarity axis. Quantification of metaphase spindle orientation is shown at right (black, >40% within the 15° sector; gray, 1%–39%; white, 0%). (G,H) The Mud spindle anchoring protein is cortical and centrosomal in both wild type (G) and *aurora-A*¹⁴⁶⁴¹ (H) metaphase neuroblasts, although *aurora-A*¹⁴⁶⁴¹ mutants have increased Mud staining on the spindle. (Right panel) Summary. (I,J) Wild type and *aurora-A*¹⁴⁶⁴¹ mutant mitotic SOPs at 16–20 h APF; anterior up, labeled for the indicated cortical polarity marker (magenta) and the α -tubulin spindle marker (green). The spindle is often orthogonal to the cortical polarity axis. Quantification of metaphase spindle orientation is shown at right.

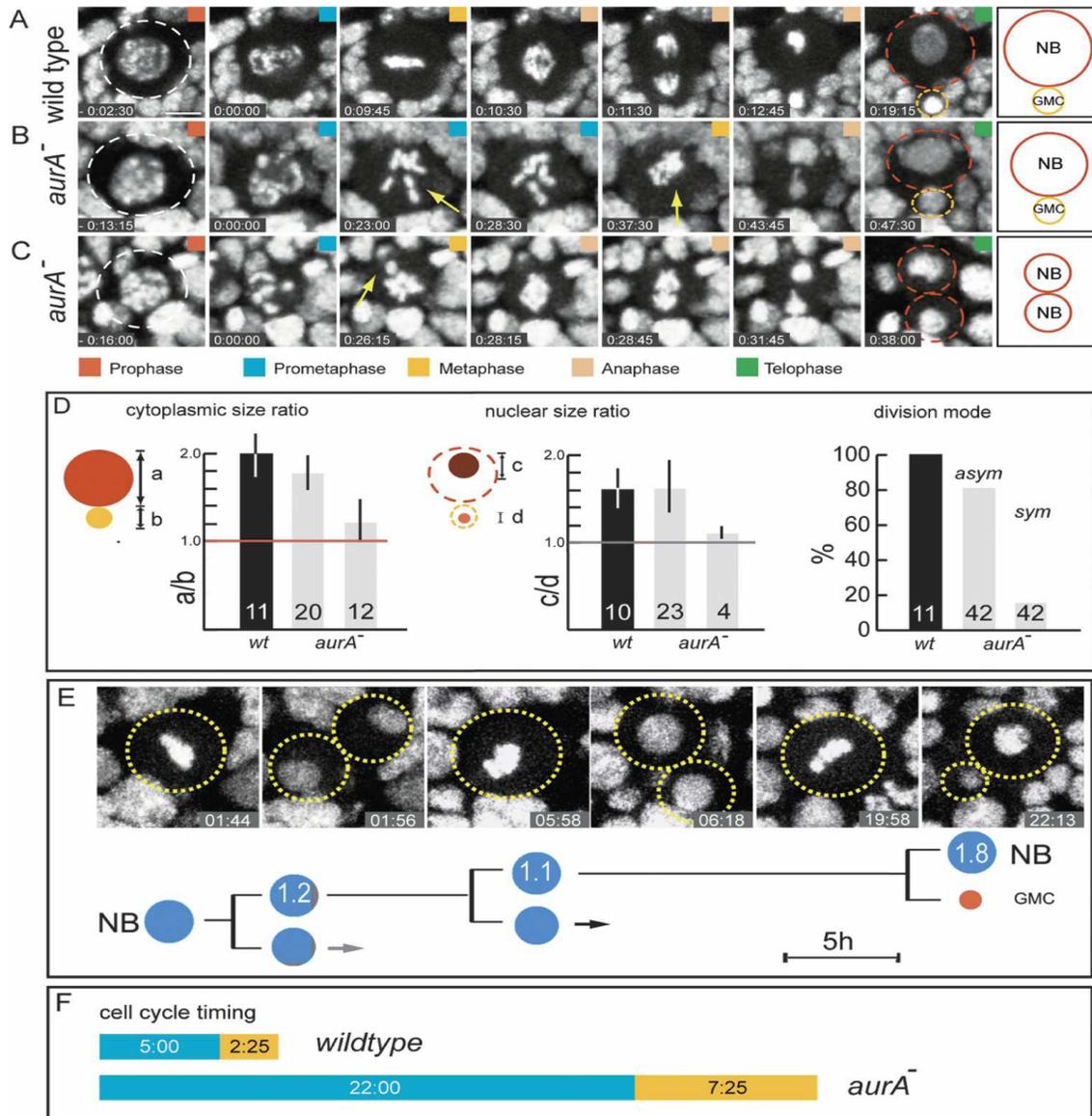


Fig. 5. *aurora-A* neuroblasts undergo symmetric cell division to expand the neuroblast population. (A–D) *aurora-A* mutant neuroblasts (NB) can divide symmetrically. (A) Wild-type neuroblasts divide asymmetrically. (B) The majority of *aurora-A* mutant neuroblasts divide asymmetrically. (C) A minority of *aurora-A* mutant neuroblasts undergo symmetric cell division. Bar, 5 μ m. (D) Quantification of neuroblast sibling cell size and nuclear size in wild type and *aurora-A* mutants. Data are derived from movies of wild-type and *aurora-A*¹⁴⁶⁴¹/*aurora-A*¹⁷⁹⁶¹ neuroblasts expressing histone:GFP (*His2AvD:GFP*) under its native promoter (Clarkson and Saint 1999). Neuroblasts and GMCs are outlined in the first and last panel. 0:00:00 (hours:minutes:seconds) indicates nuclear envelope breakdown; colored boxes indicate cell cycle stages. (E) Example of an *aurora-A* neuroblast lineage tree. The neuroblast divides twice symmetrically and then undergoes a fully asymmetric cell division. White numbers are the ratio of sibling cell diameters; 1.0–1.25, symmetric; >1.5, asymmetric. (Gray arrow) Neuroblast initiated division in the last frame of the movie; (black arrow) neuroblast remained in interphase. (F) *aurora-A* mutants have delays in cell cycle progression. *aurora-A* neuroblasts dividing symmetrically or asymmetrically have similar delays and are pooled for analysis. (Blue) Prometaphase; (yellow) metaphase. Numbers are in minutes:seconds (mean values); n = 11 for each genotype.

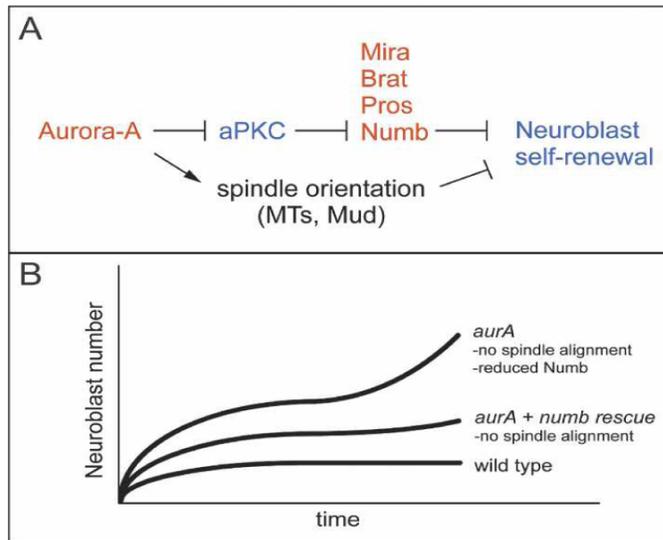


Fig. 6. Aurora-A inhibits neuroblast self-renewal. (A) Aurora-A inhibits aPKC basal localization and promotes Numb basal localization; Numb inhibits neuroblast self-renewal. Aurora-A independently promotes spindle–cortex alignment. Although aPKC mutants affect all known basal proteins (Numb, Mira, Brat, and Pros), *aurora-A* mutants only show detectable changes in Numb localization. (B) Schematic illustrating the consequences of losing one or both Aurora-A pathways for inhibiting neuroblast self-renewal. (Top line) *aurora-A* mutants have defects in both basal Numb localization and spindle alignment; loss of both pathways leads to massive increases in neuroblast numbers. (Middle line) *aurora-A* mutants overexpressing Numb have defects only in the spindle alignment pathway and have a smaller increase in neuroblast numbers (comparable to *mud* or *cnn* mutants) (Fig. 3C; Siller et al. 2006; Bowman et al. 2006). (Bottom line) Wild-type neuroblast numbers. Time axis depicts 0–120 h ALH.

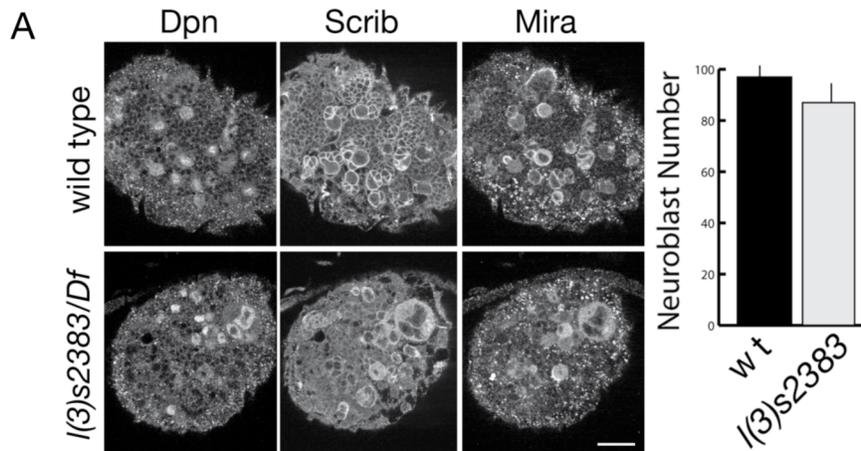


Fig. 7. *l(3)s2383* is required for larval brain development. (A) Wild type and *l(3)s2383 / Df(3R)Exel6147 (l(3)s2383 / Df)* third instar larval brains stained for the neuroblast markers Miranda (Mira) and Deadpan (Dpn) plus Scribble (Scrib) which decorates the cortex of all cells in the brain. Right: histogram of the average neuroblast number per brain lobe (bar, standard deviation in each genotype). Note that the mutant brains have fewer neuroblasts and cytoplasmic Scribble.

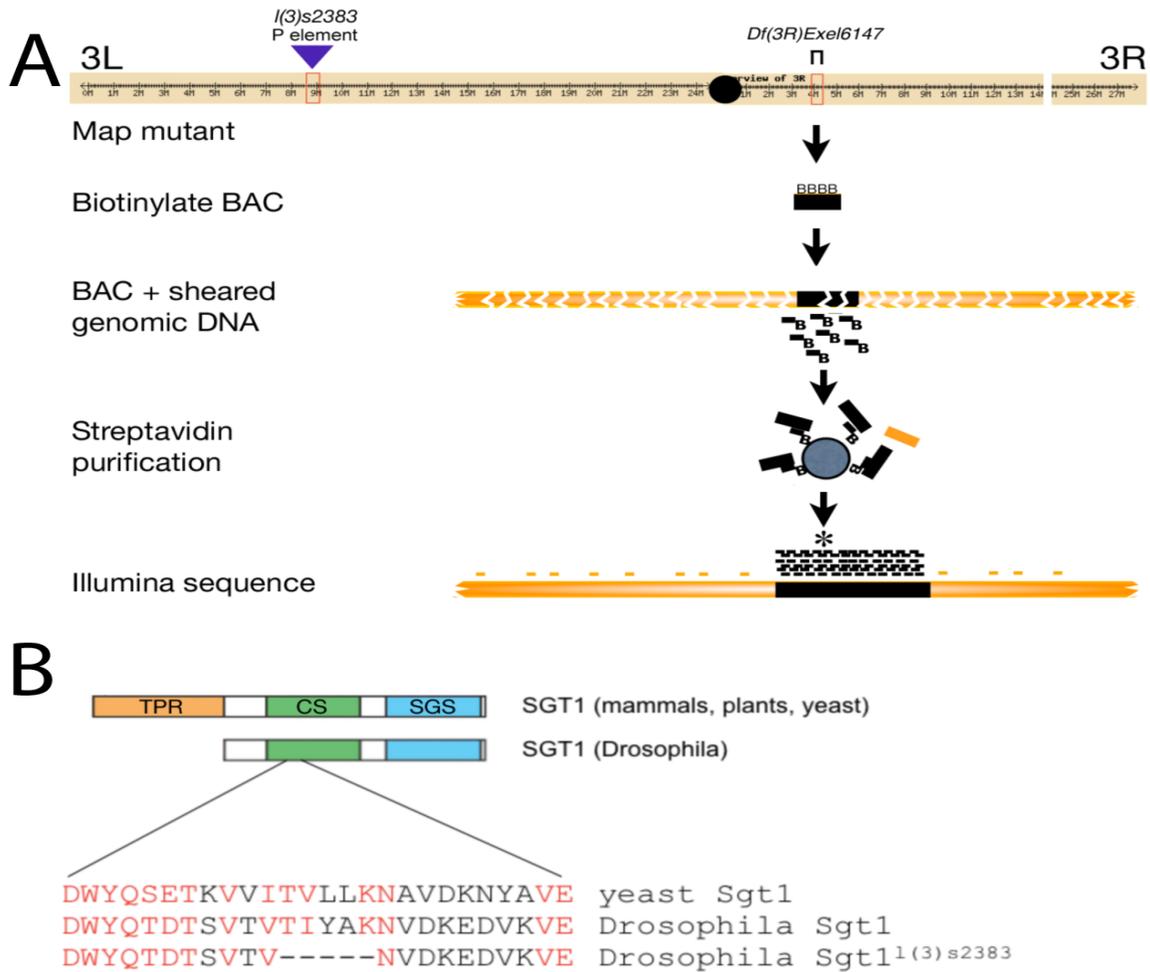


Fig. 8. Schematic of the “sequence capture” and deep sequencing strategy. (A) Although the chromosome was generated in a P element mutagenesis, we found a 15 nucleotide deletion unrelated to the P element insertion that showed the mutant phenotype when transheterozygous to *Df(3R)Exel6147*. **(B)** The *l(3)s2383* mutation resulted in an in-frame five amino acid deletion in the Drosophila Sg1 protein, within the N-terminal CS domain (green); the C-terminal Sg1-specific SGS domain is shown (white).

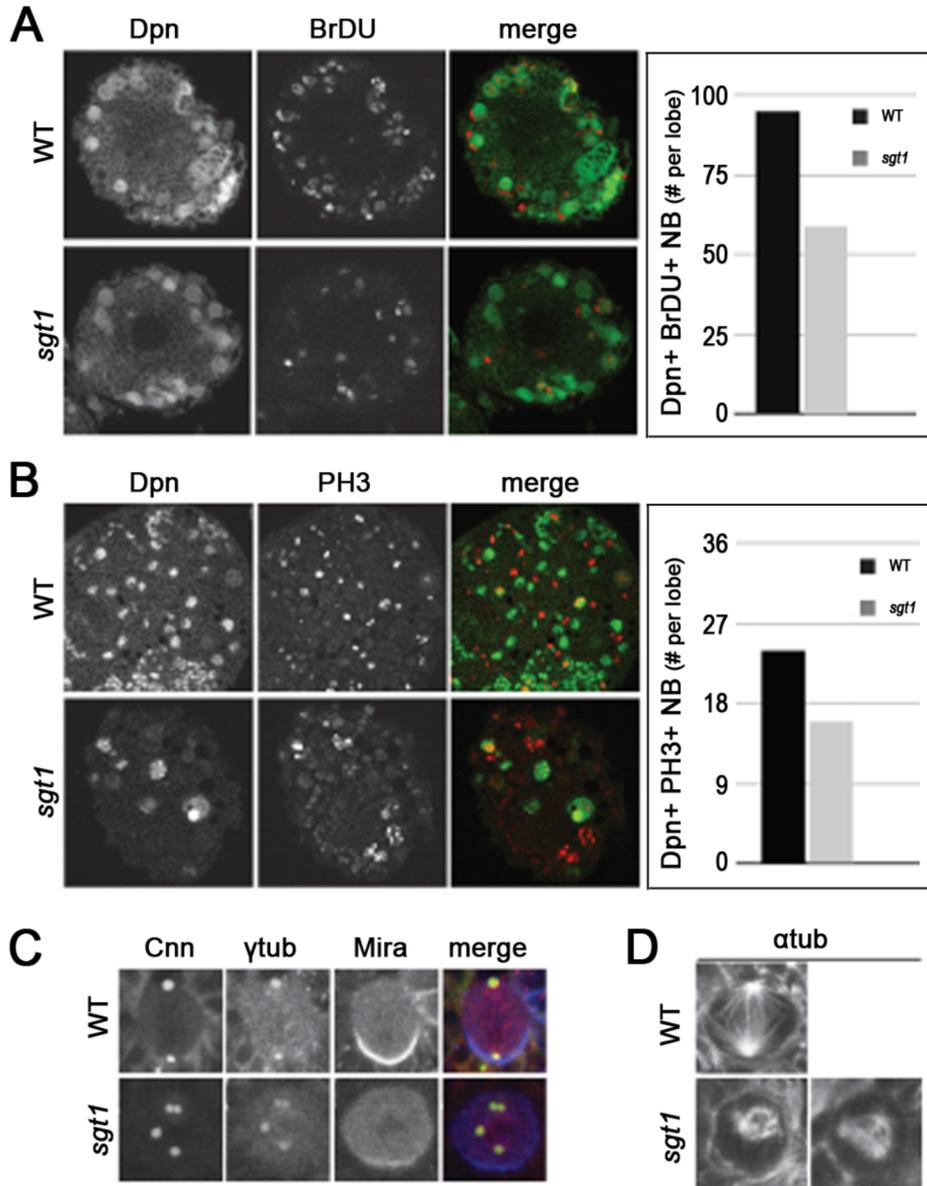


Fig. 9. *sgt1* mutant neuroblasts have a slow cell cycle, supernumerary centrosomes, and spindle morphology defects. (A) Wild type or *sgt1* mutant brains from larvae exposed to BrdU for two hours (quantified to right). In *sgt1* mutants, fewer neuroblasts incorporate BrdU, indicating a slower cell cycle (B) Wild type or *sgt1* larval brains stained for the mitotic marker phospho-histoneH3 (PH3). In wild type, about 25 of the 100 Deadpan-positive neuroblasts (Dpn) are PH3+ (quantified to right), whereas *sgt1* mutants show fewer PH3+ neuroblasts. (C) Larval brain neuroblasts stained for the centrosomal markers Centrosomin (Cnn) and gamma-tubulin (γ tub) plus the neuroblast marker Miranda (Mira). In wild type, virtually all mitotic neuroblasts contain two centrosomes. In *sgt1* mutants, many mitotic neuroblasts contain more than two centrosomes. (D) Larval brain neuroblasts stained for the spindle marker alpha-tubulin (α tub) and the neuroblast marker Miranda (not shown).

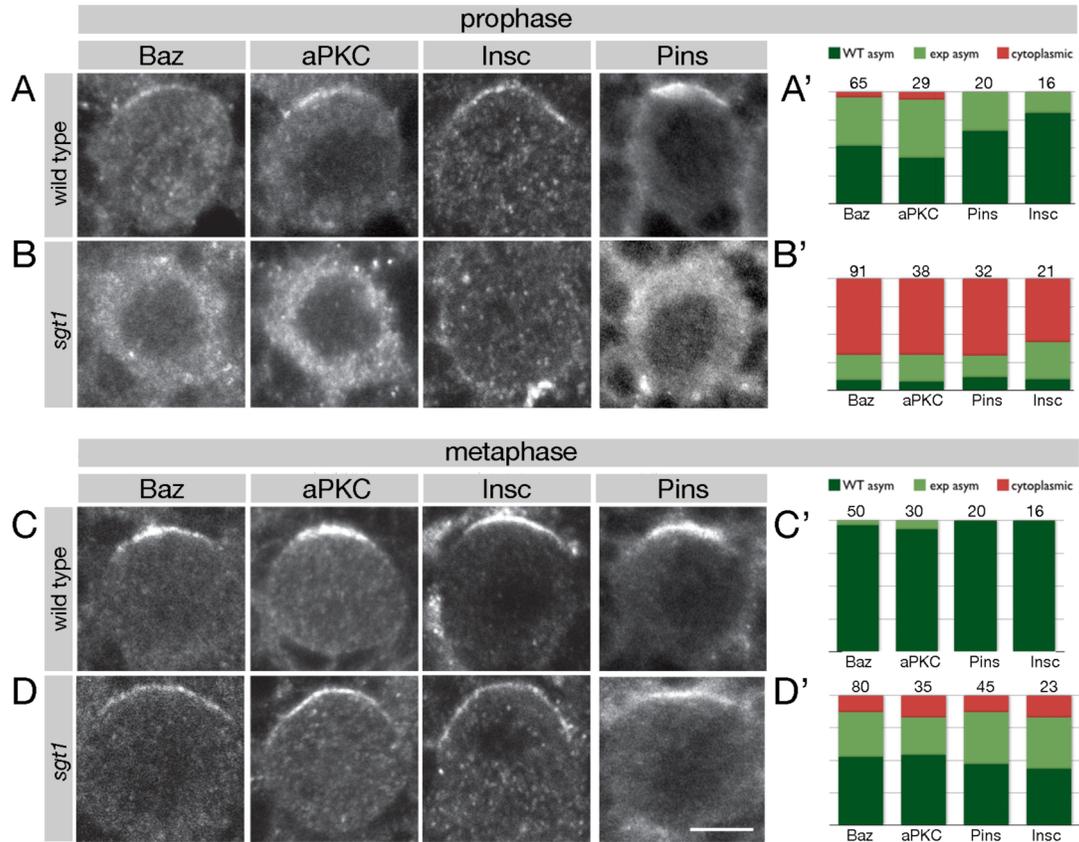


Fig. 10. *sgt1* mutant neuroblasts have defects in establishing apical cortical polarity. Wild type and *sgt1*^{s2383} / *sgt1*^{s2383} (*sgt1*) mutant larval brain neuroblasts stained for the indicated cortical proteins plus α -tubulin (not shown) and phospho-histone H3 (PH3; not shown). Prophase neuroblasts were identified as PH3+ without a bipolar spindle; metaphase neuroblasts were identified as PH3+ with a bipolar spindle. Scale bar, 5 μ m. **(A)** Wild type prophase neuroblasts show apical enrichment of the Par complex (Baz, aPKC) and Pins complex (Pins) and Insc; quantification in (E). **(B)** *sgt1* prophase neuroblasts show a nearly complete loss of apical localized Par complex (Baz, aPKC), Pins complex (Pins) and Insc; quantification in (F). **(C)** Wild type metaphase neuroblasts show apical enrichment of the Par complex (Baz, aPKC) and Pins complex (Pins) and Insc; quantification in (G). **(D)** *sgt1* metaphase neuroblasts have recovered substantial apical protein localization compared to their prophase phenotype, although it is not fully back to wild type levels; quantification in (H). **(E-G)** Quantification of the phenotypes shown in A-D. Dark green, normal asymmetric; light green, expanded asymmetric; red, cytoplasmic. Number of neuroblasts scored shown in each bar.

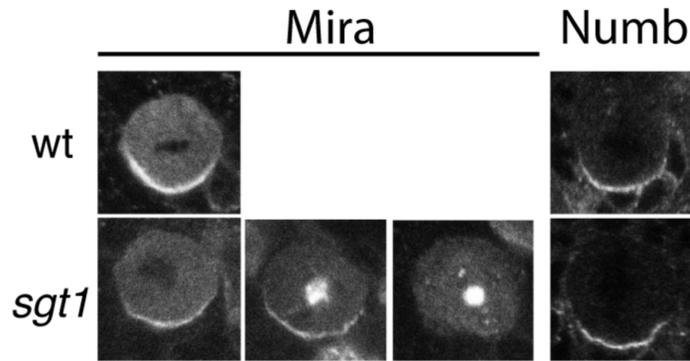


Fig. 11. Localization of basal proteins in *sgt1* mutant metaphase neuroblasts . In *sgt1* mutants, Miranda shows normal basal asymmetric localization 53%, basal with ectopic 27%, and cytoplasmic 20% of neuroblasts. Numb is relatively normal (86%).

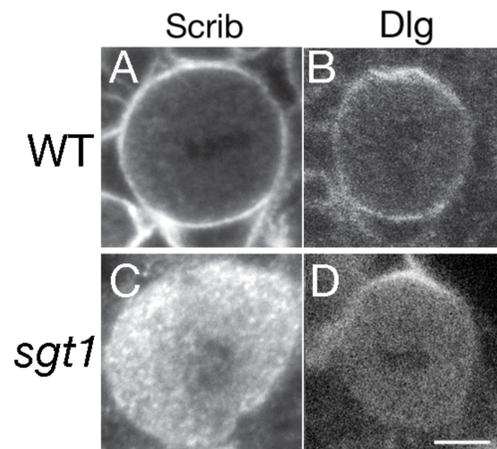


Fig. 12. *sgt1* mutant neuroblasts have defects in Dlg/Scrib cortical localization. (A, B) Wild type prophase or metaphase larval neuroblasts; Scrib is uniform cortical (100%, n=50), and Dlg is uniform cortical with apical enrichment at metaphase (100%, n=53). (C, D) *sgt1^{s2383} / sgt1^{s2383}* (*sgt1*) metaphase larval neuroblasts; Scrib is cytoplasmic (4%, n=75), and Dlg is mostly cytoplasmic except for persistent apical enrichment at metaphase (83%, n=29). Scale bar, 5 μ m.

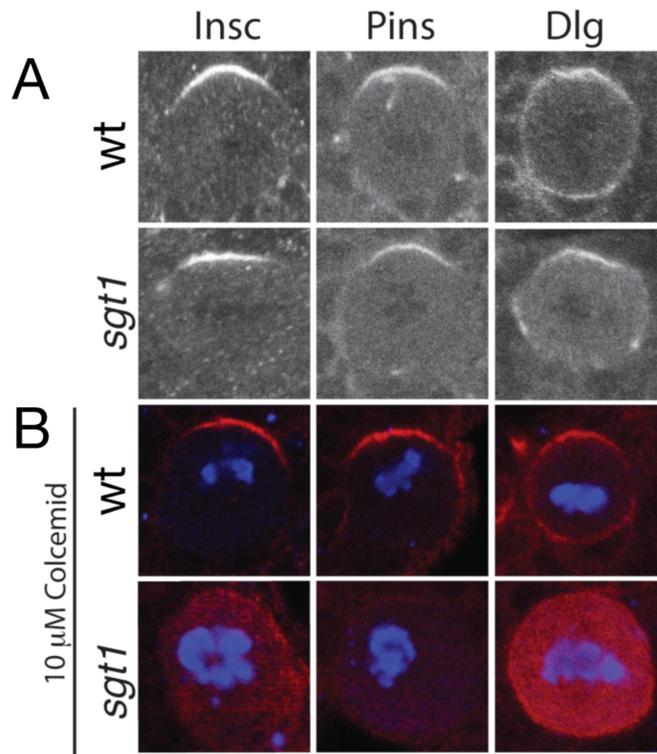


Fig. 13. Sgt1 and microtubules act in redundant pathways to generate apical protein localization in metaphase neuroblasts. Wild type and *sgt1*^{s2383} / *sgt1*^{s2383} (*sgt1*) metaphase larval neuroblasts stained for the indicated Pins complex proteins and the mitotic marker phospho-histone H3 (not shown in A; blue in B). Scale bar, 5 μm. (A) Wild type and *sgt1* mutant neuroblasts show apical localization of Insc and Pins proteins, and apical enrichment of Dlg protein. Quantification of apical asymmetry: WT (Pins, 100%, n=50; Insc, 100%, n=40; Dlg, 94%, n=34) *sgt1* (Pins, 83%, n=35; Insc, 82%, n=23; Dlg, 83%, n=29)(B) Wild type and *sgt1* mutants treated with 10 μM Colcemid to depolymerize microtubules. Wild type neuroblasts show normal apical localization of the Pins complex (Pins, 100%, n=20; Insc, 100%, n=26; Dlg, 100%, n=20), whereas *sgt1* mutants lack detectable cortical localization of Pins complex proteins (Pins, 6%, n=31; Insc, 14%, n=22; Dlg, 7%, n=15).

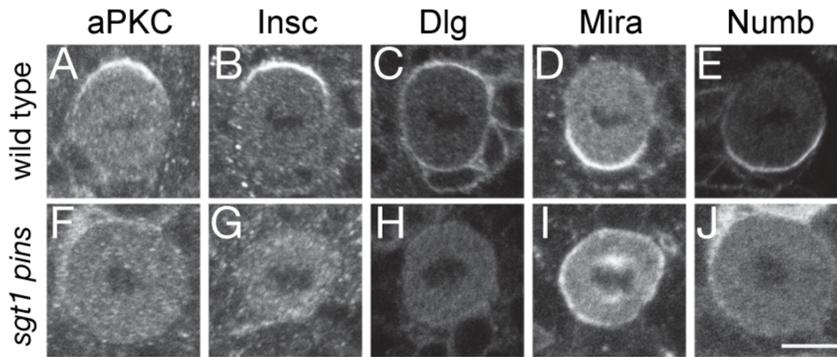


Fig. 14. *sgt1*, *pins* double mutants larval brains have apolar neuroblasts. Wild type, *sgt1*^{s2383} / *sgt1*^{s2383} (*sgt1*) single mutants, *pins*^{p62} / *pins*^{p62} (*pins*) single mutants, or *sgt1*, *pins* double mutant larval brains stained for the indicated cortical polarity proteins. Scale bar, 5 μ m. (A-E) Wild type metaphase neuroblasts showing normal cortical polarity; apical up. Quantification of asymmetric localization for each protein: aPKC, 100%, n=30; Insc, 100%, n=16; Dlg, 83%, n=29; Mira, 100%, n=20; Numb, 100%, n=20. (F-J) *sgt1*, *pins* double mutant metaphase neuroblasts lack all cortical polarity. Quantification of asymmetric localization for each protein: aPKC, 6%, n=64; Insc, 5%, n=39; Dlg, 0%, n=45; Mira, 10%, n=79; Numb, 7%, n=41.

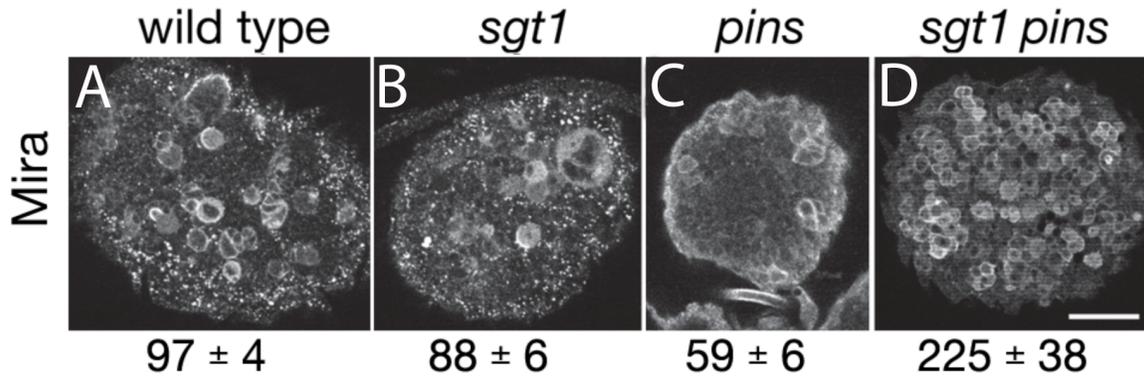


Fig. 15. (A-D) *sgt1*, *pins* double mutants larval brains have ectopic neuroblasts. Third instar larval brains stained for the neuroblast marker Miranda (Mira); the number of central brain neuroblasts in each genotype is given below each panel (average \pm standard deviation). (A) Wild type showing normal neuroblast numbers. (B,C) *sgt1* and *pins* single mutant brains showing reduced neuroblast numbers. (D) *sgt1*, *pins* double mutant brain showing a large increase in neuroblast numbers. Scale Bar 25 μ M.

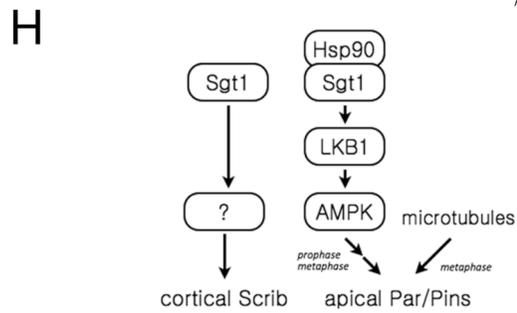
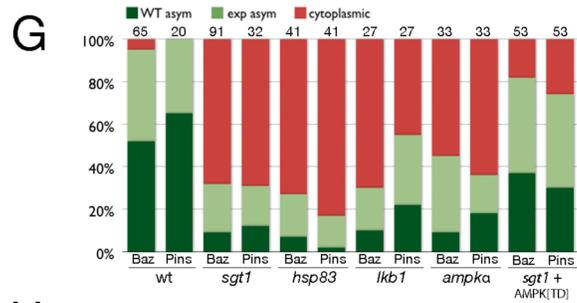
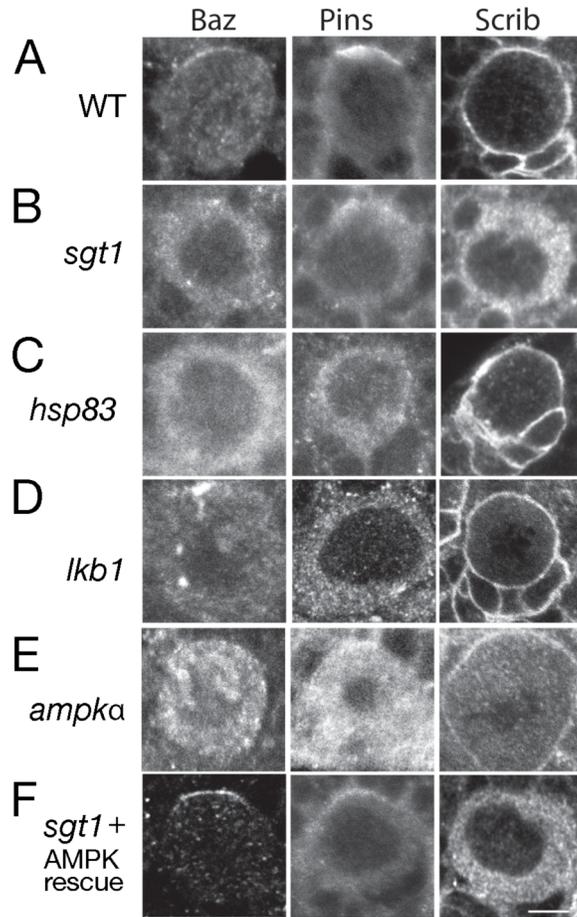


Fig. 16. *sgt1*, *hsp83*, *LKB1*, and *AMPK α* have similar neuroblast polarity phenotypes.

Prophase larval brain neuroblasts stained for the Par complex protein Bazooka (Baz), the Pins complex protein Pins, and the cortical protein Scribble (Scrib). Scale bar, 5 μ m. **(A)** Wild type. Baz and Pins form apical cortical crescents; Scrib is uniform cortical. **(B)** *sgt1^{s2383} / Df(3R)6147* (*sgt1*). Baz and Pins are delocalized; Scrib is cytoplasmic. **(C)** *hsp83^{13F3} / hsp83⁵⁸²* (*hsp83*). Baz and Pins are delocalized; Scrib is uniform cortical. **(D)** *lkb1^{4A4-2} / lkb1^{4A4-2}* (*lkb1*). Baz and Pins are delocalized; Scrib is uniform cortical. **(E)** *ampk^l / ampk^l* (*ampk α*). Baz and Pins are delocalized; Scrib is uniform cortical. **(F)** *sgt1^{s2383} / Df(3R)6147; tub-gal4 UAS-ampk^{TD}* (*sgt1* + AMPK rescue). Baz and Pins form apical cortical crescents; Scrib is uniform cortical. **(G)** Quantification of the Baz and Pins phenotypes for the genotypes listed in A-F. Dark green, normal asymmetric; light green, expanded asymmetric; red, cytoplasmic. Number of neuroblasts scored shown in each bar. **(H)** Model for Sg1 regulation of neuroblast cortical polarity. Sg1 activates LKB1 4 which activates AMPK to promote apical Par/Pins complex localization by an unknown mechanism; spindle microtubules provide a redundant pathway generating Par/Pins apical localization at metaphase. Sg1 acts by an Hsp90/LKB1/AMPK-independent mechanism to promote Scribble (Scrib) uniform cortical localization.

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