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Short Article

Apical/Basal Spindle Orientation Is Required for Neuroblast Homeostasis and Neuronal Differentiation in *Drosophila*

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SUMMARY

Precise regulation of stem cell self-renewal/differentiation is essential for embryogenesis and tumor suppression. *Drosophila* neural progenitors (neuroblasts) align their spindle along an apical/basal polarity axis to generate a self-renewed apical neuroblast and a differentiating basal cell. Here, we genetically disrupt spindle orientation without altering cell polarity to test the role of spindle orientation in self-renewal/differentiation. We perform correlative live imaging of polarity markers and spindle orientation over multiple divisions within intact brains, followed by molecular marker analysis of cell fate. We find that spindle alignment orthogonal to apical/basal polarity always segregates apical determinants into both siblings, which invariably assume a neuroblast identity. Basal determinants can all be localized into one sibling without inducing neuronal differentiation, but overexpression of the basal determinant Prospero can deplete neuroblasts. We conclude that the ratio of apical/basal determinants specifies neuroblast/GMC identity, and that apical/basal spindle orientation is required for neuroblast homeostasis and neuronal differentiation.

INTRODUCTION

Drosophila and mammalian neural progenitors have apical/basal polarity: mammalian neuroepithelial cells have a miniscule apical membrane domain flanked by adherens junctions and a large basolateral domain, whereas *Drosophila* neuroblasts have an apical cortical domain and a basal cortical domain without mature adherens junctions (reviewed in Knoblich, 2008). Thus, both *Drosophila* and mammalian progenitors have the potential to align their mitotic spindle along the apical/basal axis to divide asymmetrically ("apical/basal division") or to align their spindle orthogonal to the polarity axis to divide symmetrically ("orthogonal division"). Studies on mammalian progenitors have generated conflicting results on the relationship of spindle orientation, cell polarity, and sibling cell fate. Early studies concluded that orthogonal divisions result in a symmetric division to generate two progenitors or two neurons, whereas oblique or apical/basal divisions generated progenitor and neuron siblings (Chenn and McConnell, 1995; Kosodo et al., 2004; Zigman et al., 2005). However, more recent studies report that spindle orientation does not affect progenitor/neuron fates, but only cell position relative to the ventricular zone (Konno et al., 2008; Morin et al., 2007). Thus, the role of spindle orientation in regulating mammalian progenitor/neuron cell fates is controversial.

Drosophila neuroblasts are an ideal system to investigate the role of spindle orientation in regulating progenitor self-renewal versus differentiation. There is a steady-state of ~100 proliferating neuroblasts in each larval brain lobe, and thus neuroblasts balance self-renewal with neuronal production throughout larval life (reviewed in Doe, 2008). Mitotic neuroblasts have apical/basal cortical polarity, including many evolutionarily conserved proteins with similar localization in mammalian neural progenitors. The apical cortical domain is defined by the presence of Bazooka (Baz; Par-3 in mammals), Par-6, atypical protein kinase C (aPKC), Partner of Inscuteable (Pins; LGN/AGS3 in mammals); the basal cortical domain is defined by the presence of Miranda (Mira), Prospero (Pros), Brain tumor (Brat), and Numb proteins (reviewed in Knoblich, 2008). In wild-type neuroblasts, the mitotic spindle invariably aligns with the apical/basal polarity axis, resulting in an asymmetric cell division producing an apical neuroblast and a basal ganglion mother cell (GMC) that is committed to neuronal differentiation. Mutations that disrupt neuroblast spindle orientation have been identified, including *mushroom body defective* (*mud*; related to mammalian NuMA) (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006), *centrosomin* (*cnn*) (Lucas and Raff, 2007; Megraw et al., 2001), and *lissencephaly1* (*lis1*) (Siller and Doe, 2008). These proteins have no detectable effect on apical/basal cortical polarity, and in each mutant, 5%–15% of the metaphase neuroblasts show spindle orientation orthogonal to the apical/basal cortical polarity axis (Bowman et al., 2006; Izumi et al., 2006; Megraw et al., 2001; Siller et al., 2006; Siller and Doe, 2008). *mud* and *cnn* mutants have a slight increase in brain neuroblast numbers (Bowman et al., 2006; Lee et al., 2006a), but the origin of these neuroblasts is unknown. They could arise by many possible mechanisms: spindle orientation defects generating neuroblast/neuroblast siblings, earlier brain patterning defects, altered cell cycle length, subtle or unknown alterations in cortical polarity, or changes outside the brain. In addition, it is unknown whether *mud* mutants generate both neuroblast/neuroblast and GMC/GMC divisions, whether segregation of the apical or basal cortical domain correlates with specific sibling cell fates, or whether other spindle orientation mutants show a similar increase in brain neuroblast numbers.

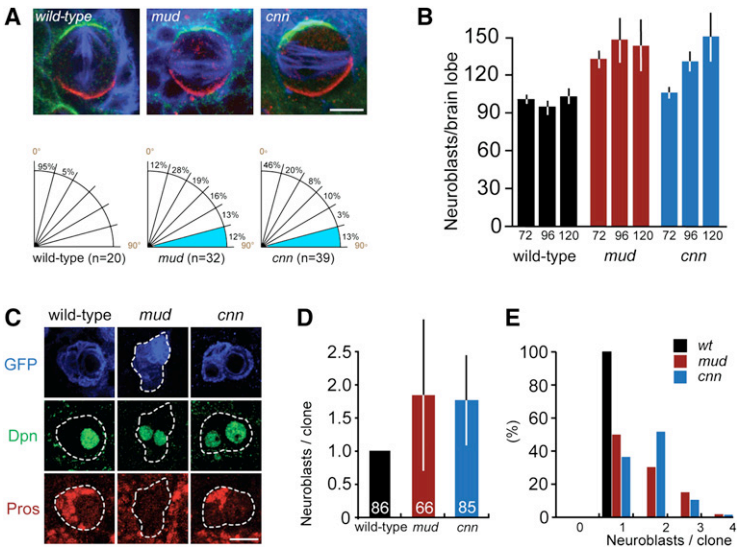

Figure 1. Spindle Orientation Mutants Have Normal Cortical Polarity and an Increase in Neuroblast Numbers

(A) Cortical polarity and spindle orientation. Top: wild-type, mud^4/mud^3, and cnn^{HK21}/cnn^{HK21} mutant larval neuroblasts have normal cortical polarity at metaphase (aPKC, green; Mira, red; spindle, blue). Bottom: spindle orientation in the same genotypes; percentage of spindles aligned in each 15° bin is indicated. Note that 12%–13% of the mutant neuroblasts show "orthogonal" spindle alignment (blue shading). Scale bar, 5 μm.

(B) Brain neuroblast numbers are increased in mud^4/mud^3 and cnn^{HK21}/cnn^{HK21} mutant brains. Each time point is hours after larval hatching; error bars represent standard deviation.

(C–E) Single neuroblast clones show that *mud* and *cnn* clones increase but never decrease neuroblast numbers. See Experimental Procedures for alleles. (C) Single optical section through a wild-type or homozygous mutant clone (blue), Deadpan neuroblast marker (Dpn; green), nuclear Prospero GMC/neuron marker (Pros; red). Clone border, dashed lines. Scale bar, 10 μm. (D) Average neuroblast number per clone. Error bars, standard deviation. Number of cells scored in bars. (E) Percentage of clones containing 0, 1, 2, 3, and 4 neuroblasts; clones containing zero neuroblasts were never observed.

RESULTS AND DISCUSSION

Spindle Orientation Defects in Neuroblasts Result in Neuroblast/Neuroblast Siblings but not GMC/GMC Siblings

It is critical for our studies to use mutants that affect spindle orientation but not cortical polarity, so we first confirmed that the spindle orientation mutants *mud* and *cnn* have no detectable effect on apical/basal cortical polarity (see Figure S1 available online). We found that 12%–13% of the metaphase neuroblasts in these mutants showed aberrant spindle orientation orthogonal to the apical/basal cortical polarity axis (Figure 1A), and that both had an increased number of brain neuroblasts (Figure 1B). Thus, these two mutants are appropriate tools for studying the role of spindle orientation in regulating neuroblast self-renewal versus differentiation.

To determine if *mud* and *cnn* mutants act autonomously within neuroblast lineages to increase neuroblast number, as predicted if neuroblast spindle orientation defects lead to increased brain neuroblast numbers, we generated GFP-marked mutant clones within single neuroblasts using the MARCM technique (Lee and Luo, 1999). Clones were induced in first instar larvae and analyzed in third instar larvae. Mutant clones were identified by GFP expression and scored for the neuroblast markers Deadpan (Dpn) or Mira and the GMC/neuron marker nuclear Pros (nPros) (Lee et al., 2006b). Wild-type single neuroblast clones always contained a single large Dpn^+ $nPros^-$ neuroblast and several smaller $nPros^+$ GMC/neurons (Figures 1C–1E). In contrast, *mud* and *cnn* mutant single neuroblast clones often contained two or more Dpn^+ $nPros^-$ neuroblasts; the multiple neuroblasts in a clone were of similar size and were always tightly adjacent (Figures 1C–1E). Importantly, we never observed mutant clones containing zero neuroblasts, which would be expected if defects in spindle orientation resulted in some divisions producing GMC/GMC siblings (Figure 1E), and live imaging confirms that GMC/GMC siblings are never generated (see below). These data are consistent with a model in which *mud* and *cnn* mutant neuroblasts generate neuroblast/neuroblast sibling cells, but not GMC/GMC sibling cells.

Spindle Orientation Orthogonal to the Apical/Basal Cortical Polarity Axis Invariably Generates Two Sibling Neuroblasts

To determine whether ectopic neuroblasts in *mud* and *cnn* mutants arise occasionally or invariably from neuroblast orthogonal divisions, we performed live imaging of neuroblast cell lineages within intact larval brains (Siller et al., 2005). This method allowed us to track individual neuroblasts from mitotic spindle orientation through to subsequent sibling cell fates. Spindle orientation was monitored with a microtubule-associated Cherry::Jupiter fusion protein, cortical polarity was monitored using the basal marker GFP::Mira, and neuroblast/GMC cell fates were determined by multiple cell biological criteria (subsequent cell division profile, cell lineage, cell cycle length, and cell size; see Experimental Procedures). Wild-type neuroblasts always showed apical/basal spindle orientation, production of unequally sized daughter cells, and partitioning of the basal cortical marker GFP::Mira into the smaller daughter cell (n = 18; Figure 2A; Movie S1). As expected, *cnn* and *mud* mutant neuroblasts also frequently showed normal apical/basal spindle orientation, divided asymmetrically, and generated neuroblast/GMC siblings (Figure 2B, divisions 2–5; Figure 2C, divisions 2–6).

Importantly, a subset of *cnn* and *mud* mutant neuroblast divisions showed spindle orientation orthogonal to the apical/basal polarity axis, allowing us to determine the role of spindle orientation in neuroblast self-renewal versus differentiation. Live imaging showed that neuroblasts undergoing orthogonal divisions always generated equally sized siblings that both invariably assumed a neuroblast identity based on their ability to maintain a neuroblast-like short cell cycle and ability to subsequently undergo asymmetric cell division (*cnn* mutants: 8%, n = 212, Figure 2B, division 1; *mud* mutants: 16%, n = 256, Figure 2C, division 1; Movie 2; quantified in Figures 2D and 2E). To provide an independent molecular assay of sibling cell identity, we performed correlative microscopy (see Experimental Procedures) in which we used live imaging to identify orthogonal neuroblast cell divisions and then subsequently fixed and stained the identical neuroblast lineage for molecular marker expression. We found that neuroblast orthogonal divisions always generated

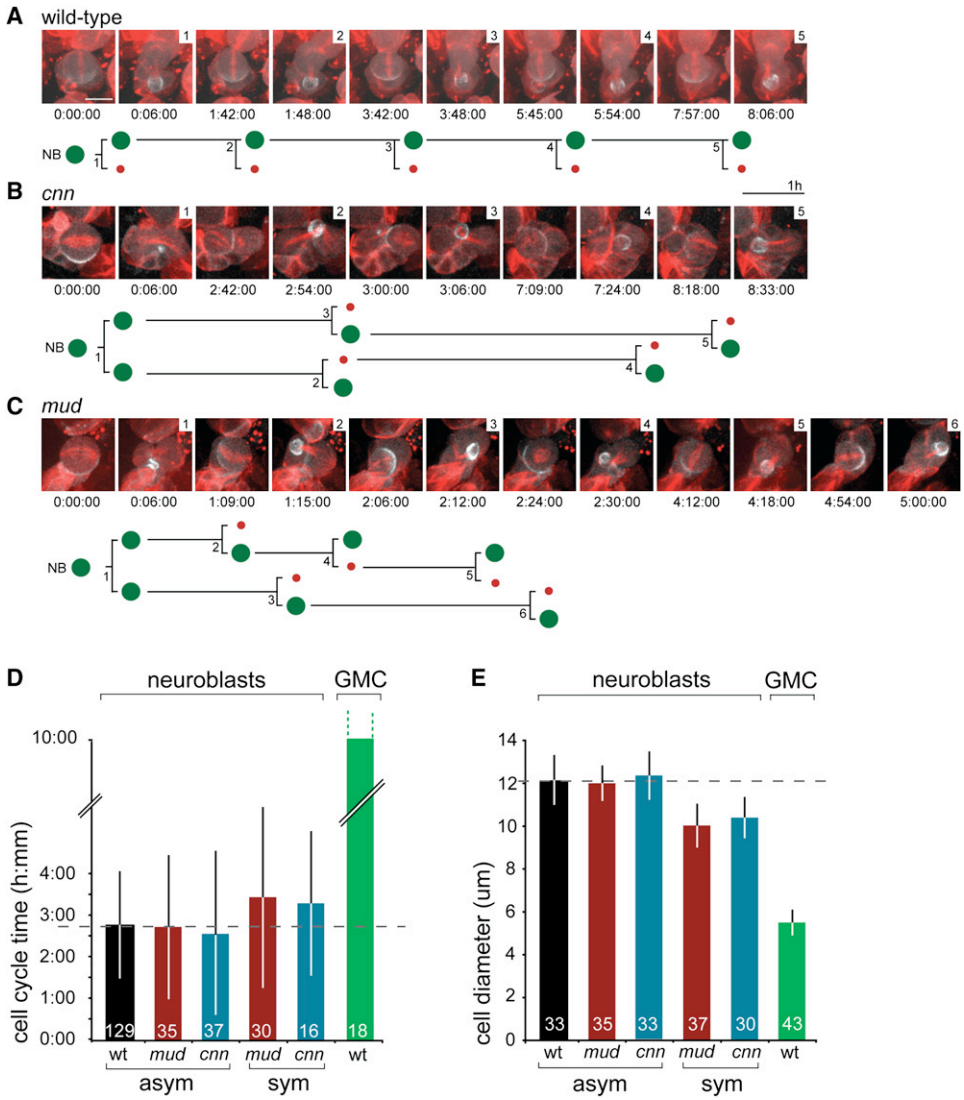

Figure 2. Time-Lapse Imaging Shows that Neuroblasts with Orthogonal Spindle Orientation Generate Two Neuroblast Sibling Cells Based on Cell Division and Cell Cycle Profiles

(A) Wild-type neuroblasts always have apical/basal spindle alignment, divide unequally, and generate one neuroblast (green in lineage tree) and one GMC (red in lineage tree). Each pair of images shows metaphase/telophase of a cell division, which are numbered in the top right of each pair of panels and at the corresponding point in the lineage tree below. Spindle labeled with Cherry::Jupiter (red); basal cortical domain labeled with GFP::Mira (white); images acquired every 3 min. Neuroblast/GMC identities shown in lineage tree; for neuroblast/GMC identification criteria, see Experimental Procedures. Time scale is hours:minutes: seconds; scale bar is 10 μm.

(B and C) *cnn* or *mud* mutant brain neuroblasts imaged as described for (A). Mutant neuroblasts can divide with spindle orthogonal to the polarity axis (division 1 in each sequence) to generate equally sized sibling cells, which both take a neuroblast identity based on their ability to divide physically asymmetrically (divisions 2–5), large size, and short cell cycle time (see Experimental Procedures).

(D) Neuroblasts have a shorter cell cycle than GMCs. Asym, asymmetric apical/basal divisions; symm, symmetric orthogonal divisions. See Experimental Procedures for neuroblast/GMC identification criteria. Error bars, standard deviation. Number of cells scored, in bars. Wild-type average (wt), dashed line.

(E) Cell diameter is similar in neuroblasts dividing asymmetrically to form neuroblast/GMC siblings (asym) or neuroblasts dividing symmetrically to form neuroblast/neuroblast siblings (sym). GMCs are distinctly smaller. Error bars, standard deviation. Number of cells scored, in bars. Wild-type average, dashed line.

two siblings that expressed the neuroblast marker Deadpan (Dpn) and lacked the differentiation marker nPros (n = 10; Figures 3B–3D; Figure S2; Movies S3 and S4). We conclude that neuroblast orthogonal divisions always generate two equally sized cells that assume a neuroblast identity: they have a short cell cycle, can divide asymmetrically, express the neuroblast marker Dpn, and lack the GMC/neuronal marker nPros. Thus, altering neuroblast spindle orientation from apical/basal to orthogonal results in the invariant production of two sibling neuroblasts, based on both cell biological and molecular criteria.

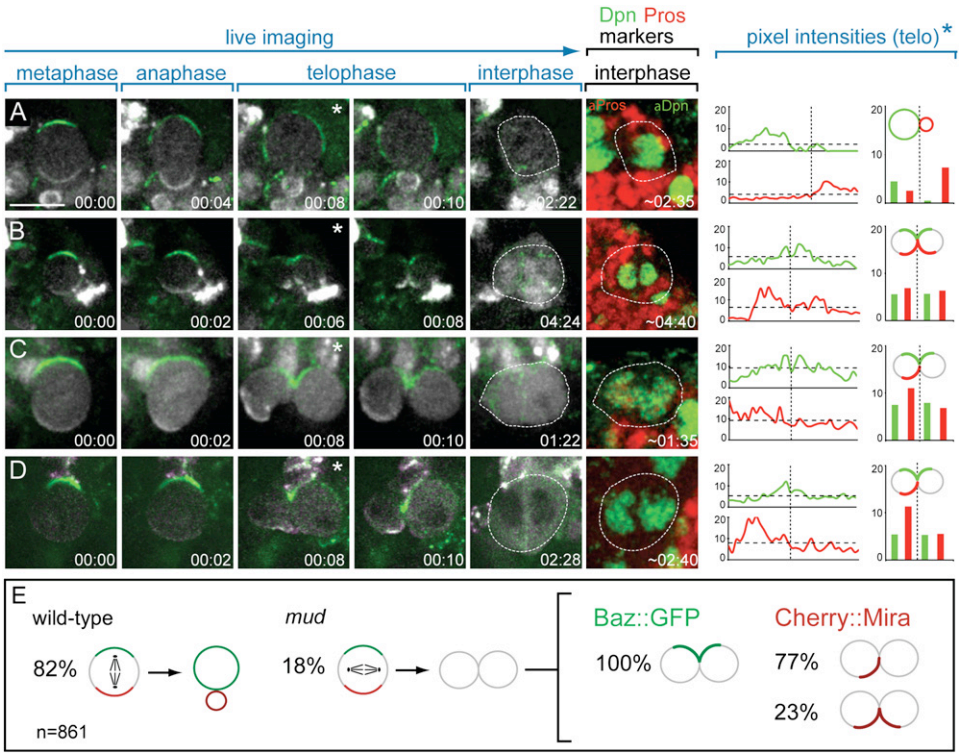

Figure 3. Inheritance of the Apical Cortical Domain, but Not the Basal Cortical Domain, Predicts Neuroblast/GMC Identity

Correlative imaging showing time-lapse of apical (Baz::GFP, green) and basal (Cherry::Mira, white) protein localization during *mud* mutant larval neuroblast cell lineages, followed by fixation and staining for cell fate molecular markers (neuroblast, Dpn; GMC, nuclear Pros).

(A–D) Inheritance of the apical cortical marker Baz::GFP correlates with neuroblast cell fate, but the basal cortical marker Cherry::Mira can partition into neuroblast or GMC, during both apical/basal (A) or orthogonal (B–D) neuroblast divisions. All imaged *mud* neuroblast divisions (n > 300) showed complete cleavage to form a pair of mononucleated siblings. Right columns: the level of Baz::GFP (green) and Cherry::Mira (red) partitioned to each sibling cell at telophase is quantified as pixel intensity plots for marked cells (*), and the average is shown in the histograms. Furrow position, vertical dashed line. Time scale is hours:minutes. Scale bar, 10 μm.

(E) Quantification of data shown in (A–D).

Localization of Apical and Basal Cortical Domains during Orthogonal Spindle Orientation: Only the Apical Domain Correlates with Cell Fate Specification

Neuroblasts dividing orthogonally to the apical/basal polarity axis invariably generate two sibling neuroblasts (see above). To determine how apical/basal cortical determinants correlate with cell fate specification—if they correlate at all—we quantified the partitioning of apical or basal cortical domains in *cnn* or *mud* mutant orthogonal neuroblast divisions. As expected, wild-type or mutant neuroblasts with apical/basal spindle orientation always segregated the majority of the apical marker Baz::GFP into the neuroblast, and the majority of the basal marker Cherry::Mira into the GMC (100%, n = 699; Figure 3A; Movie S3; data not shown). In contrast, *mud* mutant neuroblasts with orthogonal spindle orientation always segregated the apical marker Baz::GFP equally into both sibling cells (100%, n = 162; Figures 3B–3E; Movies S4 and S5). The apical protein aPKC is also symmetrically partitioned during orthogonal divisions (Figure S3). The basal marker Cherry::Mira could also be segregated equally to both siblings (23%, n = 162; Figure 3B; Movies S4 and S5), but surprisingly was more frequently partitioned unequally to only one sibling (77%, n = 162; Figures 3C–3E; Movie S6). Similar results were obtained with *cnn* mutant neuroblasts (Figure S3). Clearly the segregation of all basal determinants into just one sibling was insufficient to induce neuronal differentiation, as all orthogonal divisions generated two sibling neuroblasts. We conclude that the apical cortical domain is perfectly correlated with acquisition of neuroblast identity, whereas the basal cortical domain is insufficient to specify GMC identity.

The Apical:Basal Cortical Polarity Ratio Determines Sibling Cell Fate

Orthogonal neuroblast divisions always partition apical proteins into both siblings and always generate two neuroblasts; basal proteins can all be localized into one sibling without inducing differentiation. It is thus tempting to conclude that only apical proteins are used to specify cell fate. However, an alternative model is that cell fate is determined by the ratio of apical:basal proteins and that a sibling containing half the apical proteins and all of the basal proteins (e.g., Figures 3C and 3D, left sibling) still has an apical:basal ratio high enough to promote neuroblast identity.

We can distinguish between these two models by increasing the amount of the basal cell fate determinant Prospero: the "apical dominant" model predicts no effect on neuroblast identity, whereas the "apical:basal ratio" model predicts at least

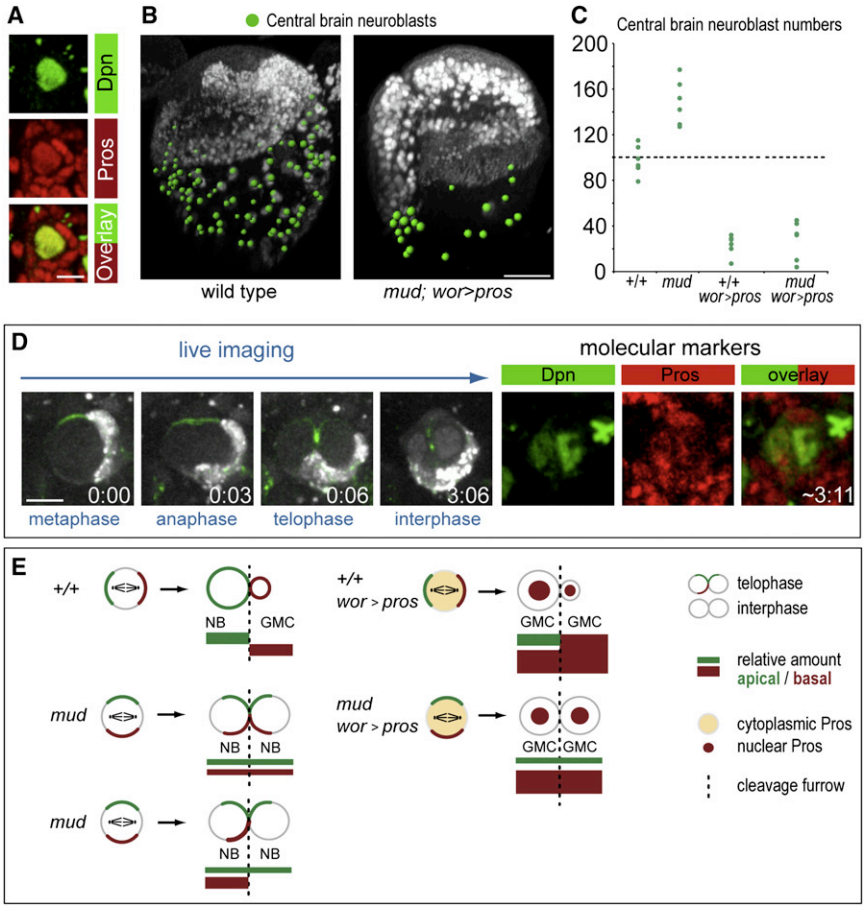

Figure 4. Overexpression of the Prospero Basal Determinant Results in Neuroblast Depletion in Wild-Type and *mud* Mutant Larval Brains

(A) Wild-type late third instar brain expressing *pros* using the neuroblast driver *wor-Gal4* results in ectopic nuclear Pros (red) in the neuroblast (Dpn, green). Scale bar, 10 μm.

(B) Wild-type and *mud* late third instar brain expressing *pros* using the neuroblast driver *wor-Gal4* results in depletion of neuroblast number (central brain neuroblasts identified as large Dpn^+ cells and marked with green dots). *wor-Gal4* is not expressed in the optic lobe, and there is no phenotype in these neuroblasts (white). Scale bar, 50 μm.

(C) Wild-type late third instar brains expressing *pros* using the neuroblast driver *wor-Gal4* quantified for neuroblast numbers (identified as large Dpn^+ cells). Green dots represent independent brain hemispheres assayed (n = 6 for all genotypes). Wild-type average, dashed line.

(D) Correlative imaging showing timelapse of apical (Baz::GFP, green) and basal (Cherry::Mira, white) protein localization during *mud* mutant larval neuroblast cell lineages overexpressing Prospero (*mud Baz::GFP/Y*; *wor-Gal4 UAS-cherry::mira/UAS-pros*) shown to the left, followed by fixation and staining for cell fate molecular markers (neuroblast, Dpn; GMC, nuclear Pros) shown to the right. Time-lapse imaging shows that both apical and basal markers are normally localized, which is most easily visualized at metaphase (time 0:00). The neuroblast siblings are subsequently marked by Deadpan (Dpn, green) and contain ectopic nuclear Prospero (Pros, red). Time scale is hours:minutes. Scale bar, 10 μm

(E) Model showing that the ratio of apical:basal cortical polarity markers determines neuroblast and GMC identity. See text for details. Green equals apical determinants of neuroblast identity. Red equals basal determinants of GMC identity. Light red equals cytoplasmic Prospero in mitotic neuroblasts. NB, neuroblast; GMC, ganglion mother cell; dashed line, cleavage furrow.

some loss of neuroblast identity. We found that overexpressing Prospero in neuroblasts results in coexpression of nuclear Prospero and the neuroblast marker Deadpan (Figure 4A) and a striking depletion of larval neuroblasts (Figures 4B and 4C). Importantly, we observed no change in the localization or function of apical cortical proteins: Baz::GFP formed an apical crescent, and aPKC was able to exclude Miranda from the apical cortex (Figure 4D). Thus, increasing the amount of the cell fate determinant Prospero, without altering apical cortical proteins, is sufficient to block neuroblast specification or maintenance, resulting in a decrease in neuroblast numbers. We conclude that the ratio of apical:basal cortical polarity markers is important for determining neuroblast/GMC identity and that apical/basal spindle orientation maintains neuroblast homeostasis and promotes neuronal differentiation by allowing the production of a basal cell with a high basal:apical ratio of cell fate determinants (Figure 4E).

In this study, we have used a combination of genetic mutants that specifically disrupt spindle orientation without affecting cell polarity, live imaging of apical/basal spindle orientation for multiple neuroblast divisions within intact larval brains, and correlative microscopy to determine the molecular profile of terminal progeny of the imaged lineages. Our results show that apical/basal spindle orientation is essential for maintaining neuroblast pool size and promoting neuronal differentiation: direct observation shows that all mutant neuroblasts with orthogonal spindle orientation generate two neuroblast siblings, whereas all mutant and wild-type neuroblasts with apical/basal spindle orientation generate neuroblast/GMC siblings. This provides strong evidence that spindle orientation defects in these mutants lead to the observed increase in neuroblast numbers, rather than other possible defects including brain patterning, nonautonomous effects in glia or GMCs, or altered cell polarity.

Analysis of orthogonal divisions reveals that only apical proteins are correlated with cell fate (being 100% correlated with neuroblast identity), whereas inheritance of all the basal proteins by one sibling is insufficient to induce neuronal differentiation. This is strikingly similar to mammalian embryonic neural stem cells, where only the apical cortical domain is correlated with self-renewal, while the basolateral and adherens junctional domains distribute independently of cell fate (Marthiens and ffrench-Constant, 2009). Nevertheless, we show that the apical cortical domain is not the sole determinant of cell fate, but rather it is the ratio of apical:basal proteins that specifies

neuroblast/GMC identity (Figure 4E). This model is supported by the observation that increasing levels of the apical determinant aPKC can switch GMCs into neuroblasts (Lee et al., 2006b), and that decreasing the levels of basal determinants can turn GMCs into neuroblasts (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006b; Lee et al., 2006c; Wang et al., 2006). A high apical:basal ratio may promote neuroblast identity by inactivating basal proteins (Betschinger et al., 2003; Smith et al., 2007), increasing cell size (Kawamura and Carlson, 1962), promoting cell proliferation (Chabu and Doe, 2008; Rolls et al., 2003), or altering centrosome composition/function (Rebollo et al., 2007; Rusan and Peifer, 2007). Conversely, a high basal:apical ratio may promote differentiation via Prospero repression of genes promoting cell proliferation (Dyer, 2003; Li and Vaessin, 2000) or neuroblast identity (Choksi et al., 2006), by Brain tumor suppression of Myc-dependent cell growth (Betschinger et al., 2006), and/or by Numb inhibition of Notch-dependent neuroblast self-renewal (Lee et al., 2006a; Lee et al., 2006c; Wang et al., 2007; Wang et al., 2006).

In wild-type *Drosophila* neuroblasts, the mitotic spindle is always aligned with the apical/basal axis, which maintains neuroblast pool size and allows neuronal differentiation (this study). In other insects and mammals, regulated spindle orientation may allow switching between neural progenitor expansion and homeostasis. In the honeybee *Apis*, mushroom body neuroblasts expand via symmetric divisions prior to switching to an asymmetric division mode to generate neurons (Farris et al., 1999). Neuroblast expansion may be due to an increased apical:basal determinant ratio or a phase of orthogonal spindle orientation. Similarly, mammalian neural progenitors switch between phases of progenitor expansion, homeostasis, and depletion (Chenn and McConnell, 1995; Fish et al., 2006; Kosodo et al., 2004; Zigman et al., 2005). Clues that spindle orientation plays an important role come from the analysis of mammalian mutants *CDK5RAP2* and *lis1*, which cause microcephaly in mammals (Bond et al., 2005; Fish et al., 2006); the orthologous *Drosophila* mutants *cnn* and *lis1* both disrupt spindle orientation, but not cortical polarity, and lead to an increase in neuroblast numbers (this study). However, the respective contribution of apical/basal determinant ratio and spindle orientation remains to be determined in mammals, primarily due to the lack of candidate cell fate determinants and the difficulty of performing correlative microscopy within intact brain tissue. Our results suggest that concurrent live imaging of cell polarity, spindle orientation, and sibling cell fate will be necessary to determine the role of spindle orientation in regulating mammalian neural stem cell self-renewal versus differentiation.

EXPERIMENTAL PROCEDURES

Fly Stocks and Genetics

Mutant chromosomes were balanced over *CyO actin::GFP*, *TM3 actin::GFP, Ser e*, or *TM6B Tb*. The following alleles/allelic combinations and strains were used: *yw* (Bloomington stock center), *Baz::GFP* (Buszczak et al., 2007), mud^4/Y, mud^4/mud^3, cnn^{HK2}. For MARCM clones, we recombined mud^4 and cnn^{HK21} onto *FRT19A* and *FRTG13* (Bloomington stock center), respectively, and crossed to the following lines: *yw, hsFLP, tubP-Gal80, FRT19A; UAS-CD8::GFP/(CyO); TubGal4/TM6B Tb* (gift from B. Bello). *yw, hsFLP70; tubP-Gal80, FRTG13/(CyO); TubGal4, UAS-CD8::GFP/TM6C Sb*. cnn^{HK21} was recombined onto the *wor-Gal4* chromosome and *UAS-GFP::Mira* (K. Siller, personal communication). mud^4 was recombined onto the *Baz::GFP* chromosome. Lines used for live imaging consisted of the following genotype: *yw; wor-Gal4, UAS-cherry::jupiter, UAS-GFP::Mira*. *yw; wor-Gal4, UAS-GFP::mira/(CyO); UAS-His2B::mRFP1*. *yw; wor-Gal4, UAS-GFP::mira,* cnn^{HK21}*/CyO; UAS-cherry::jupiter/TM6B*. mud^4*, Baz::GFP; wor-Gal4, UAS-cherry::mira*. For Prospero overexpression experiments, *UAS-prosL*^{17U2} (Manning and Doe, 1999) was crossed into a mud^4 mutant background and expressed with *wor-Gal4*. *mud* mutant larvae expressing *pros* were raised at 25°C for 3 days and another 24 hr at 30°C prior to dissection. Transgenic lines were generated by Eric Spana, Model System Genomics, Duke University.

Antibody Staining

The following antibodies were used: rabbit anti-Insc (1:1000), rat anti-Dpn (1:1), guinea-pig anti-Dpn (1:2000, Jim Skeath), guinea-pig anti-Bazooka (1:1000), mouse anti-Pros (1:1000), guinea-pig anti-Mira (1:1000), rabbit or mouse anti-GFP (Roche, 1:500), rabbit anti-aPKC (1:1000, Santa Cruz Biotechnology), rat anti-Pins (1:400), mouse or rat anti-Tubulin (1:1000, Serotec). Secondary antibodies were used from Molecular Probes. Immunostaining experiments including those after live imaging recordings: larval brains were fixed in 4% paraformaldehyde for 20 min, with washes and stainings performed as described previously (Siller et al., 2006).

Constructs

The *pUAST-cherry::jupiter* construct was generated by using the *jupiter* cDNA clone LD21358. Since this clone contained a point mutation close to the C terminus, causing a premature stop codon, the frameshift was corrected by PCR. The corrected cDNA was cloned in frame with cherry cDNA AY678264 introducing a BglII restriction site and a 6 nucleotide linker sequence. The resulting fusion construct was subcloned into pUAST with EcoR1 (5′) and XbaI (3′). The *pUAST-cherry::mira* construct was generated by cloning the *cherry* cDNA upstream and in frame with *mira* cDNA containing a BglII restriction site in between and a 6 nucleotide linker sequence. The resulting fusion construct was cloned into pUAST, and standard methods were used to generate transgenic flies.

Imaging and Measurements

Wild-type, mud^4, or cnn^{HK21} third instar larvae (72–96 hr after egg laying) were picked based on the lack of the *actin::GFP* balancer and/or the expression of the corresponding fluorescent fusion proteins and dissected and mounted in Schneider's insect media (Sigma) supplemented with 1% bovine growth serum (BGS; HyClone), 0.5 mM ascorbic acid, and the fat bodies of 10 wild-type larvae. Movies were acquired on a Bio-Rad Radiance 2000 point scanning confocal or McBain spinning disc confocal microscope equipped with a Hamamatsu EM-CCD camera, using a 60× 1.4NA oil-immersion lens. Images were acquired every 3-5 min with a spacing of 1 μm between Z-sections. Images with voxel sizes between 0.21 μm (spinning disc) and 0.43 μm (Bio-Rad) were acquired. Brains were oriented with the dorsal side facing the coverslip. The top 20–25 μm of the dorsal brain was imaged. Time-lapse sequences were processed, and diameter and intensity measurements acquired, using ImageJ and Imaris 5.7.2 and 6.2, 64 bit (Bitplane). Fixed preps were imaged on a Leica SP2 confocal microscope and analyzed with Imaris 5.7.2 and 6.2, 64 bit (Bitplane). Statistical analysis was performed in Microsoft Excel.

Correlative Microscopy

Molecular marker staining following live imaging was done by removing brains from the imaging chamber and fixing in 4% paraformaldehyde for 20 min. Washes and stainings, using anti-Dpn (rat or guinea-pig) and anti-Prospero (mouse), and confocal microscopy were performed as described above. Imaged neuroblasts were identified within the stained brains based on the unique pattern of Cherry::Mira accumulation in certain lineages, which provided landmarks to map and correlate imaged and fixed neuroblasts using Imaris software.

Neuroblast and GMC Identification

We did not analyze the eight "type II" neuroblast lineages in the dorsoposterior brain that have more complex lineages (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008) or optic lobe neuroblasts. In time-lapse imaging experiments shown in Figure 2, neuroblasts were defined by the following

cell biological criteria: cell cycle time of less than 4 hr, diameter >8 μm, and ability to perform physically asymmetric apical/basal cell divisions. GMCs were defined as cells that had a cell cycle length >10 hr and a cell size <6 μm. In fixed brains, neuroblasts were defined as Dpn^+ nuclear $Pros^-$ cells >8 μm in diameter.

Apical/Basal and Orthogonal Divisions

In metaphase neuroblasts, apical/basal divisions are defined as spindle orientation ±15° of the apical/basal polarity axis, and orthogonal divisions are defined as spindle orientation ±15° of the axis orthogonal to the apical/basal polarity axis; all other divisions are termed oblique. Time-lapse analysis shows that all neuroblast divisions with oblique spindle orientation resolve into physically asymmetric cell divisions with spindle and cortical polarity aligned; all neuroblast divisions with orthogonal spindle orientation are resolved into physically symmetric divisions with cleavage bisecting the apical domain. In fixed preparations, it is difficult to recognize orthogonal divisions at telophase due to the smaller size of the sibling cells, short length of telophase, and the lack of a Mira "cortical ring" in the budding GMC (Siller and Doe, 2008); we do observe them in fixed samples (Figure S4), but they are more easily detected by time-lapse analysis where all mitotic cells can be followed into cytokinesis.

SUPPLEMENTAL DATA

Supplemental data include four figures and six movies and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00250-0/.

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