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Brat Is a Miranda Cargo Protein that Promotes Neuronal Differentiation and Inhibits Neuroblast Self-Renewal

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Summary

An important question in stem cell biology is how a cell decides to self-renew or differentiate. *Drosophila* neuroblasts divide asymmetrically to self-renew and generate differentiating progeny called GMCs. Here, we report that the Brain tumor (Brat) translation repressor is partitioned into GMCs via direct interaction with the Miranda scaffolding protein. In *brat* mutants, another Miranda cargo protein (Prospero) is not partitioned into GMCs, GMCs fail to downregulate neuroblast gene expression, and there is a massive increase in neuroblast numbers. Single neuroblast clones lacking Prospero have a similar phenotype. We conclude that Brat suppresses neuroblast stem cell self-renewal and promotes neuronal differentiation.

Introduction

Asymmetric cell division is important for generating cell diversity during development and for maintaining stem cell identity (Betschinger and Knoblich, 2004; Lechler and Fuchs, 2005; Yamashita et al., 2003). *Drosophila* neuroblasts are an excellent model system for investigating both asymmetric cell division and stem cell self-renewal (Betschinger and Knoblich, 2004; Lee et al., 2005). Mitotic neuroblasts have distinct apical and basal cortical domains, and the mitotic spindle aligns with the axis of cortical polarity, resulting in asymmetric cell division. The large apical sibling is a self-renewing neuroblast, and the small basal sibling is a ganglion mother cell (GMC) that typically generates two postmitotic neurons. Apical cortical proteins include atypical protein kinase C (aPKC), Bazooka (Baz/Par3), Par6, Inscuteable (Insc), Partner of Inscuteable (Pins), and Gαi. These apical proteins regulate neuroblast spindle orientation, spindle asymmetry, sibling cell size asymmetry, and targeting of proteins to the basal cortex (Betschinger and Knoblich, 2004). Basal cortical proteins include Numb, Miranda (coiled-coil scaffold), and two Miranda cargo proteins—Prospero (homeodomain transcriptional repressor) and Staufen (RNA binding protein that localizes *prospero* mRNA) (Broadus et al., 1998; Ikeshima-Kataoka et al., 1997; Knoblich et al., 1995; Rhyu et al., 1994; Schuldt et al., 1998; Spana and Doe, 1995). Miranda basal localization requires the amino-terminal portion of the protein (1–405 aa), and the adjacent coiled-coil central domain of Miranda is required for basal localization of Prospero and Staufen cargo proteins (Fuerstenberg et al., 1998; Ikeshima-Kataoka et al., 1997; Matsuzaki et al., 1998; Schuldt et al., 1998). To date, the only cell fate determinants known to be partitioned into the GMC are Numb and Prospero; Numb has no known function in GMCs (Skeath and Doe, 1998), whereas Prospero is thought to promote GMC exit from the cell cycle (Li and Vaessin, 2000). It is unknown whether there are other cell fate determinants segregated into the GMC, and whether the Miranda scaffolding protein has cargo proteins other than Prospero and Staufen.

Drosophila neuroblasts have also emerged as a model system for investigating stem cell self-renewal. Larval neuroblasts can produce hundreds of GMCs without losing cell volume, losing proliferation potential, or differentiating (Lee et al., 2005; Rolls et al., 2003); in contrast, a GMC typically undergoes a reductive division to generate only two postmitotic neurons (Lee and Luo, 2001). Apical proteins, which are partitioned into the neuroblast at cytokinesis, would be excellent candidates for promoting neuroblast self-renewal. Indeed, we have recently shown that *aPKC* mutants have fewer neuroblasts, whereas aPKC overexpression can induce ectopic neuroblast renewal and massively increase the population of larval neuroblasts (Lee et al., 2005). Conversely, basal proteins would be excellent candidates for promoting neuronal differentiation and inhibiting neuroblast self-renewal; consistent with this model, it has recently been shown that *prospero*, *miranda*, and *numb* mutant brain tissue can form metastatic tumors when transplanted into wild-type adult hosts (Caussinus and Gonzalez, 2005). However, the cellular phenotype of *prospero*, *miranda*, and *numb* mutants has not been determined, so it remains unclear whether they induce tumor formation as a consequence of genomic instability (Caussinus and Gonzalez, 2005) or by allowing GMC-to-neuroblast cell fate transformations.

We have been screening for mutants that affect larval neuroblast self-renewal and asymmetric cell division (C.-Y.L. and C.Q.D.), and we identified a mutation in *brain tumor* (*brat*) that resulted in ectopic larval brain neuroblasts. Brat is a translational repressor containing an evolutionarily conserved NHL (NCL-1, HT2A, and LIN-41) domain that recruits Pumilio and Nanos to block *hunchback* mRNA translation during early embryogenesis (Sonoda and Wharton, 2001; Arama et al., 2000), and *brat* mutants are pupal lethal with a brain overgrowth phenotype (Arama et al., 2000). Here, we report that *brat* mutants have ectopic neuroblasts and fail to differentiate GMCs. We show that Brat protein is asymmetrically partitioned into GMCs via direct interaction with the Miranda basal scaffolding protein, and that, in *brat* mutants, the Miranda cargo protein Prospero fails to be partitioned into GMCs. Thus, Brat is a Miranda binding protein that is partitioned into the GMC, where it

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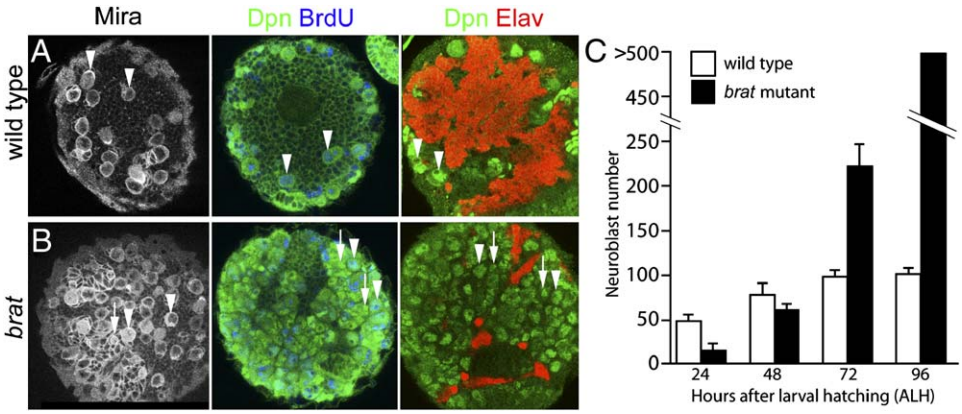

Figure 1. *brat* Mutants Have Ectopic Neuroblasts

(A and B) Images of single brain lobes of wild-type or $brat^{11}/brat^{11}$ mutants 96 hr after larval hatching (ALH) stained for the neuroblast markers Miranda (Mira) and Deadpan (Dpn) and the neuronal marker Elav and assayed for proliferation by performing a 4 hr BrdU pulse prior to fixation. Each panel shows a single optical section. Representative neuroblasts, arrowheads; GMCs, arrows.

(C) Quantification of neuroblast numbers per brain lobe was based on Dpn staining in wild-type (wt; white bars) and $brat^{11}/brat^{11}$ mutants (*brat*; black bars). Average neuroblast number and standard deviation (in parentheses): 24 hr ALH wild-type, 47.1 (5.0) and *brat*, 16.4 (5.1); 48 hr ALH wild-type, 79 (14.2) and *brat*, 61.2 (8.1); 72 hr ALH wild-type, 100 (5.7) and *brat*, 222.4 (27.2); 96 hr ALH wild-type, 103 (4.1) and *brat*, >500 (n.a.). The lower numbers of neuroblasts in 24–48 hr ALH brat mutant larvae are likely due to organismal developmental delay, as *brat* mutants are slower to hatch into larvae and slower to undergo each larval molt (data not shown).

inhibits neuroblast self-renewal and promotes neuronal differentiation.

Results

brat Mutants Have Supernumerary Brain Neuroblasts

In order to identify genes that regulate larval neuroblast self-renewal and asymmetric cell division, we screened a collection of transposable P element-induced lethal mutants for an altered number of brain neuroblasts or mislocalized cell polarity markers (C.-Y.L. and C.Q.D.). We identified a mutation in *l(2)k06028*, a weak loss-of-function allele of *brat*, that resulted in increased brain neuroblast numbers (Figure S1; see the Supplemental Data available with this article online).

To determine the *brat* null mutant phenotype, we quantified neuroblast numbers in $brat^{11}$ homozygous mutant larvae from 24 to 96 hr after larval hatching (ALH). We identified neuroblasts by using molecular and proliferation markers. Neuroblasts express the markers Deadpan (Dpn), Worniu, and Miranda (each is also very transiently present in newborn GMCs); lack the neuronal marker Elav and the glial marker Repo; and are proliferative based on their ability to incorporate BrdU. In wild-type larvae, the number of proliferating neuroblasts gradually increased from 45 neuroblasts at 24 hr ALH to 100 neuroblasts at 72 hr ALH and remained at ~100 neuroblasts until the onset of metamorphosis (Figures 1A and 1C) (Datta, 1995; Lee et al., 2005). These 100 neuroblasts generate many thousands of $Elav^+$ postmitotic neurons comprising the larval and adult brain (Figure 1A). In *brat* mutant larvae, we observed three phenotypes. First, we detected a dramatic increase in neuroblast number—over 500 by 96 hr ALH and an estimated several thousand by 120 hr ALH (Figures 1B and 1C; data not shown). Second, we observed a corresponding decrease in $Elav^+$ neurons (Figure 1B), suggesting that the ectopic neuroblasts may be generated at the expense of neurons. Finally, we noticed that the GMCs adjacent to each neuroblast failed to downregulate neuroblast marker expression (e.g., maintaining Dpn, Miranda, and Worniu staining; Figure 1B and data not shown). The fate of these cells will be addressed in the next section. We conclude that *brat* mutants have a late-onset increase in proliferative neuroblasts and a corresponding decrease in differentiated neurons. The fact that the neuroblast population expands simultaneously with loss of neurons strongly suggests that at least some GMCs are developing as neuroblasts rather than neurons (see Discussion).

brat Mutants Have a Delay in GMC Differentiation

To further characterize the fate of the small "GMCs" that maintain neuroblast marker expression in *brat* mutant brains, we performed BrdU pulse-chase experiments. Our reasoning was that BrdU incorporation can be used to identify cells that continue to proliferate (they initially incorporate BrdU but rapidly dilute it out during the chase period) and cells that differentiate (they incorporate BrdU during their terminal division and maintain BrdU during the chase period). In wild-type larvae, a 4 hr pulse of BrdU followed by immediate fixation and BrdU staining revealed BrdU incorporation in all neuroblasts and a few adjacent newborn, smaller GMCs (Figures 1A and 2A). After a 24 hr BrdU-free chase, however, all BrdU labeling was restricted to the pool of $Elav^+$ neurons born during the BrdU pulse window (Figure 2B). Thus, the proliferative neuroblasts dilute out BrdU incorporation, whereas postmitotic $Elav^+$ neurons retain BrdU incorporation (Figures 2A and 2B, schematic).

We next assayed *brat* mutant larvae in these pulse-chase experiments. Immediately after the 4 hr BrdU pulse, nearly all neuroblasts were labeled with BrdU as well as adjacent smaller, presumptive GMCs (Figures 1B and 2C). After a 24 hr chase, each Dpn^+ large neuroblast was surrounded by "GMCs" that maintained Dpn,

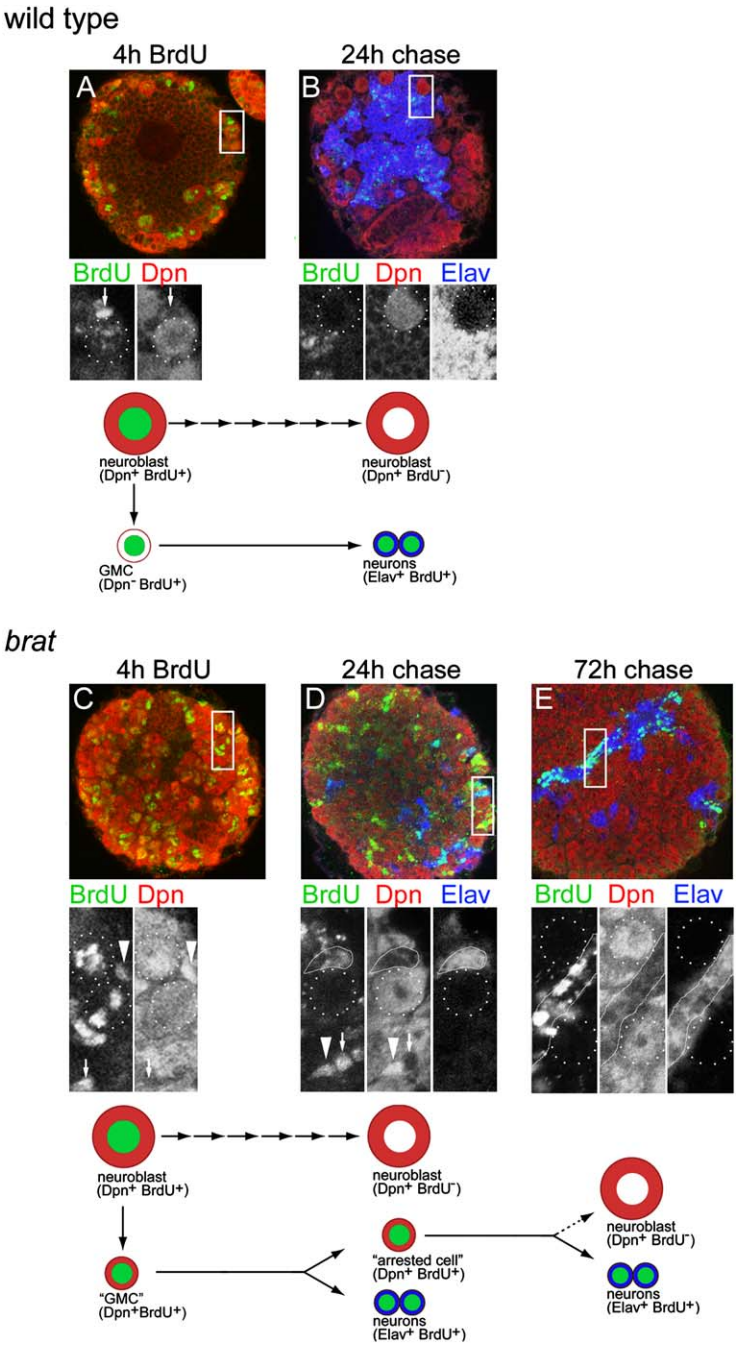

Figure 2. *brat* Mutants Have a Failure in GMC Differentiation

(A–E) Wild-type or $brat^{11}/brat^{11}$ mutant larvae 72 hr ALH were given a 4 hr BrdU pulse and either fixed immediately or chased in BrdU-free media for 24 or 72 hr. One brain lobe stained for the indicated markers is shown in each panel, and high-magnification single-label images of the boxed region are shown below each panel. Dpn^+ large neuroblasts, dotted circles; Dpn^- $BrdU^+$ GMCs, arrows; Dpn^+ $BrdU^+$ "GMCs," arrowheads; $Elav^+$ neurons, solid outline (in [D] and [E]). Schematic summaries: Dpn, red; BrdU, green; Elav, blue. (A and B) Wild-type. (A) A 4 hr BrdU pulse labels all neuroblasts and newborn GMCs. (B) After a 24 hr chase, BrdU can only be detected in $Elav^+$ neurons (the large Dpn^+ neuroblasts have diluted out BrdU). (C–E) *brat* mutants. (C) A 4 hr BrdU pulse labels all neuroblasts and many small newborn "GMCs" that fail to downregulate Dpn. (D) After a 24 hr chase, the large neuroblasts have diluted out BrdU, but BrdU is observed in small Dpn^+ "GMCs" (arrowhead) and $Elav^+$ neurons (solid outline). (E) After a 72 hr chase, virtually all BrdU is restricted to the $Elav^+$ neurons (solid outline).

did not express Elav, and did not dilute out BrdU (Dpn^+ $BrdU^+$ $Elav^-$) (Figure 2D); these are likely to be "arrested GMCs" that have neither differentiated nor resumed proliferation. We also detected a small number of $BrdU^+$ $Elav^+$ neurons, indicating that some GMCs are able to differentiate normally (Figure 2D). After a 72 hr chase, there were many fewer $BrdU^+$ cells overall, and the remaining $BrdU^+$ cells were virtually all $Elav^+$ (Figure 2E), showing that some small Dpn^+ cells seen at 24 hr post-BrdU eventually differentiate into neurons. Based on the steep decline in the number of Dpn^+ $BrdU^+$ small cells during the 24–72 hr chase interval, and the corresponding increase in neuroblast number during this interval (Figure 1C), we propose that some Dpn^+ "GMCs" enlarge into neuroblasts and reenter the cell cycle (Figures 2C–2E, schematic; see Discussion). We conclude that *brat* neuroblasts generate GMC-sized progeny that are cell cycle delayed and continue to express the neuroblast markers Dpn, Miranda, and Worniu (Figure 2 and data not shown); some of these "GMCs" differentiate into neurons, but many appear to develop into proliferative neuroblasts.

Brat Is Asymmetrically Partitioned into the GMC during Neuroblast Division

brat mutants show GMC gene regulation defects, so we next assayed for Brat protein localization in neuroblasts and GMCs of the embryonic and larval CNS. In wild-type

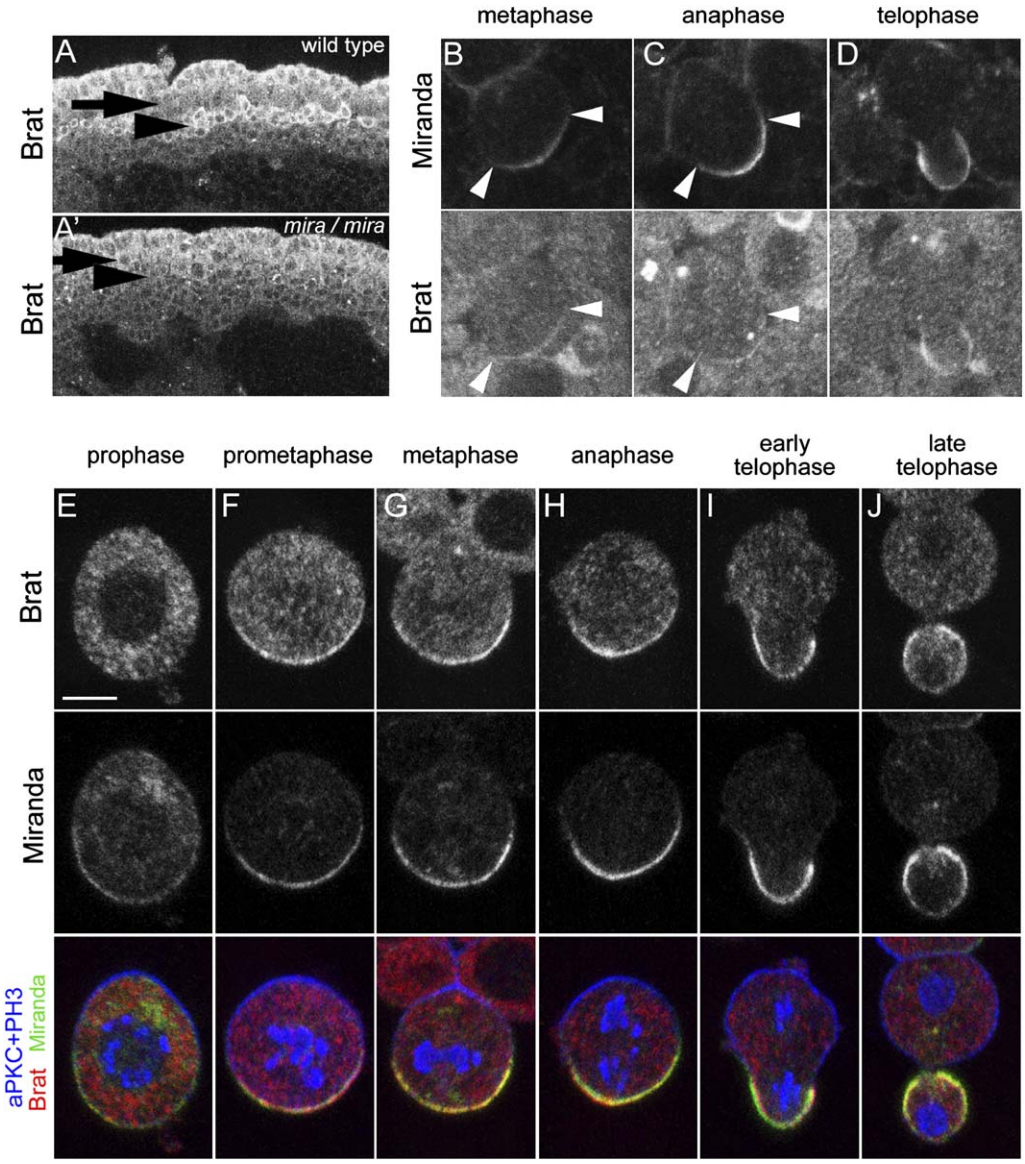

Figure 3. Brat and Miranda Colocalize and Are Partitioned into the GMC during Embryonic Neuroblast Asymmetric Division

(A and A′) Wild-type and *miranda*^{AB78}/*miranda*^{AB78} mutant stage-11 embryos. Wild-type embryos have high Brat protein levels in the GMC layer (arrowhead) compared to the neuroblast layer (arrow), but this difference is abolished in *miranda* mutants. Apical is up.

(B–D) Wild-type embryonic neuroblasts in vivo. Brat and Miranda colocalize in a basal crescent (between arrowheads) and are partitioned into the GMC. Apical is up.

(E–J) Wild-type embryonic neuroblasts isolated in vitro. Isolated neuroblasts undergo normal asymmetric cell division (Broadus and Doe, 1997; Grosskortenhaus et al., 2005; Siegrist and Doe, 2006) and reveal the basal cortex better due to the absence of associated GMCs. Brat and Miranda colocalize throughout the cell cycle and are partitioned specifically into the GMC. Apical is up (defined by the position of the aPKC crescent). The scale bar is 5 μm.

embryos, Brat was detected at a low level in neuroblasts, and it was highly enriched in the GMC layer (Figure 3A). Examination of mitotic embryonic neuroblasts revealed that Brat was colocalized with the basal scaffolding protein Miranda from prometaphase through telophase, and both proteins were partitioned into the GMC after cytokinesis (95% basal, n = 25; Figures 3B–3D; Table 1). To obtain a clearer view of the Brat protein basal crescent, we cultured embryonic neuroblasts in vitro by using standard methods; these neuroblasts undergo normal asymmetric cell divisions (Broadus and Doe, 1997; Grosskortenhaus et al., 2005; Siegrist and Doe, 2006). Again, we find that Brat and Miranda are precisely colocalized in a basal protein crescent from prometaphase through telophase, with both proteins being specifically partitioned into the newborn GMC (Figures 3E–3J). In addition, larval neuroblasts showed the identical colocalization of Brat and Miranda in basal cortical crescents throughout the cell cycle, resulting in the partitioning of both proteins into the newborn GMC (Figures S2A–S2C). In fact, Brat/Miranda colocalization was maintained even in mutant genotypes where Miranda was mislocalized: in *aPKC* mutants, both Miranda and Brat were uniform cortical in metaphase neuroblasts, and in

Table 1. Quantification of Neuroblast Cortical Polarity in Wild-Type and Mutant Genotypes

	Wild-Type[a]	*brat* Mutant[a]
aPKC[b]		
Apical (metaphase)	100% (n = 43)	60% (n = 136)
Prospero[b]		
Basal (metaphase)	91.4% (n = 35)	5.1% (n = 59)
Basal (telophase)	88% (n = 25)	7.9% (n = 63)
		miranda Mutant[a]
Brat[b]		
Basal (metaphase)	96% (n = 25)	0% (n = 20)

[a] Genotypes: wild-type, heterozygous siblings (*brat*/+ or *miranda*/+); *brat* mutant, $brat^{11}$ homozygotes; *miranda* mutant, $miranda^{AB78}$ or $miranda^{L44}$ homozygotes.
[b] aPKC and Prospero protein localization scored in larval central brain neuroblasts at 120 hr ALH; Brat protein localization scored in embryonic neuroblasts. n, number of neuroblasts scored; apical localization, complete restriction to apical quadrant; basal localization, detectable protein at the basal quadrant.

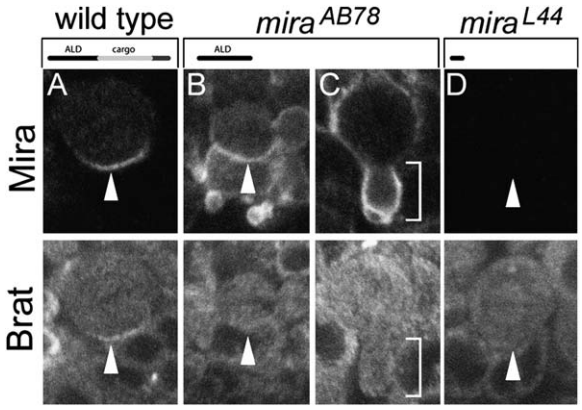

Figure 4. Miranda Is Required for Brat Basal Asymmetric Localization

(A–D) (A) Wild-type and (B–D) *miranda* mutant embryonic neuroblasts double labeled for Miranda (Mira) and Brat; panels were acquired by using identical confocal settings. The $Miranda^{AB78}$ protein forms a basal crescent but lacks the central cargo binding domain, whereas the $Miranda^{L44}$ protein is undetectable. All neuroblasts are in metaphase, except for the neuroblast in (C), which is in telophase; all neuroblasts are at stage 10, except for the neuroblast in (B), which is at stage 13 and is thus smaller. Miranda protein domains present in each genotype are diagrammed at the top (ALD, asymmetric localization domain; cargo, Prospero/Staufen cargo binding domain). Apical is up. The arrow or bracket indicates basal Miranda protein.

lgl mutant neuroblasts, both Miranda and Brat were predominantly cytoplasmic at metaphase (Figures S2D and S2E). We conclude that Brat and Miranda are invariably colocalized in mitotic neuroblasts, and that Brat is partitioned into the GMC during neuroblast asymmetric cell division.

Brat Is a Miranda Cargo Protein

To test whether asymmetric localization of Brat is mediated by Miranda, we examined Brat protein localization in two *miranda* mutant genotypes: $miranda^{L44}$, a genetic null that makes no detectable protein, and $miranda^{AB78}$, which generates a protein of 405 amino acids that lacks the central Prospero/Staufen binding domain and C-terminal domain (Fuerstenberg et al., 1998). The $miranda^{AB78}$ homozygous embryos lacked Brat enrichment in the GMC layer, instead showing equalized Brat protein levels between the neuroblast and GMC layers (Figure 3A′). Examination of mitotic neuroblasts in both *miranda* mutant genotypes revealed that Brat protein was completely delocalized into the cytoplasm (100% delocalized, n = 20; Table 1; Figure 4). We conclude that Brat is a Miranda cargo protein that requires the Miranda coiled-coil cargo binding domain (405–830 aa) for proper basal targeting into the GMC.

Independently of our genetic screen, we identified Miranda in a yeast two-hybrid screen for proteins that bind the Brat NHL domain (B.D.W and R.P.W.). All *brat* alleles exhibiting brain overgrowth, including the genetic null $brat^{11}$, that have been sequenced possess molecular lesions in the NHL domain (Sonoda and Wharton, 2001; Arama et al., 2000). We identified six different clones encoding Miranda; each clone contains a portion of the Miranda central cargo binding domain (data not shown). We next tested Miranda binding to a panel of Brat proteins containing single alanine substitutions, which collectively decorate both surfaces of the NHL domain (Edwards et al., 2003). We observed that mutations mapping to the "top surface" of the NHL domain had no effect on Miranda/Brat association, but that several mutations mapping to the "lower surface" of the NHL domain prevented Miranda/Brat binding (Figure 5). In particular, the substitution of glycine 774 or tyrosine 829 completely abolished Miranda binding to the NHL domain (Figure 5); both mutants also abolish Brat/Pumilio association (Edwards et al., 2003; Sonoda and Wharton, 2001), suggesting that Miranda and Pumilio have overlapping binding surfaces on the Brat NHL domain. We conclude that the Brat NHL domain interacts with the Miranda cargo domain, confirming our genetic evidence that Brat is a Miranda cargo protein.

brat Mutants Have Defects in Prospero and aPKC Asymmetric Localization

Brat protein is partitioned into the newborn GMC, and *brat* mutants have defects in GMC development. However, it is possible that the *brat* phenotype arises in the neuroblast and not the GMC, based on fact that mislocalization of aPKC beyond its normal apical cortical domain during neuroblast cell division is sufficient to generate ectopic larval brain neuroblasts (Lee et al., 2005). Here, we test whether the Brat protein, which forms a basal cortical crescent in mitotic neuroblasts, has a role in establishing or maintaining neuroblast cortical polarity.

In wild-type neuroblasts, aPKC, Baz, and Pins were all localized to the apical cortex at metaphase (Figure 6A; Table 1; data not shown). In *brat* mutants, Baz and Pins showed normal localization, but aPKC was occasionally delocalized to the basal cortex (40%, n = 136; Figure 6B; Table 1). Ectopic aPKC was not sufficient to cause the *brat* mutant phenotype, however, because *brat aPKC* double mutants still developed ectopic neuroblasts, and misexpression of Lgl3A (which is a potent suppressor of aPKC-induced ectopic neuroblasts [Lee et al., 2005]) was unable to suppress the ectopic neuroblasts in *brat* mutants (Figure S3).

Examination of the basal cortical markers Miranda and Prospero in *brat* mutants revealed a more striking

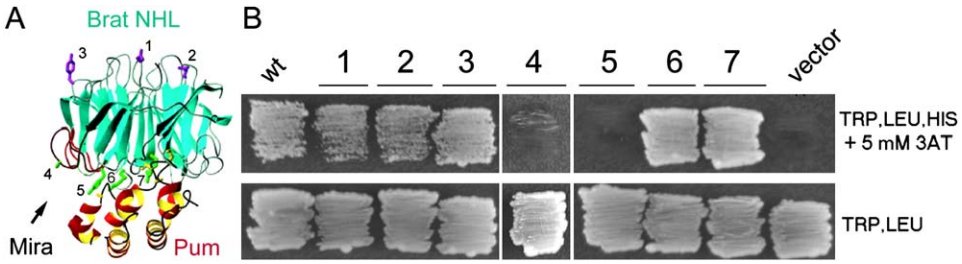

Figure 5. Miranda Directly Interacts with the Brat NHL Domain

(A) Diagram of the Brat NHL domain (turquoise). The Brat NHL domain interacts with both Pumilio (Pum; red/yellow; Edwards et al., 2003) and Miranda (Mira) on the "lower" surface of the NHL domain. The numbers 1–7 represent the following Brat mutations used in the yeast two-hybrid experiments: (1) Y859A, (2) D1012A, (3) E970A, (4) G774A, (5) Y829A, (6) R847A, (7) R875A (Edwards et al., 2003).

(B) Yeast two-hybrid experiments were performed as described previously (Edwards et al., 2003); interaction between Brat and Mira is required for growth in the absence of histidine (top row). Substitutions on the "lower" surface of the NHL domain block interaction both with Pum and with Mira (mutants 4 and 5) or just Pum (mutants 6 and 7); substitutions on the "top surface" (mutants 1–3) have no effect on Mira or Pum binding. The smallest portion of Mira common to the 6 different clones isolated in the screen consists of amino acids 481–579; exclusion of a single clone that encodes a weakly interacting Mira fragment changes the minimal interaction domain to residues 344–579.

phenotype: although Miranda localization was normal, Prospero was almost always absent from the basal cortex (metaphase 95% absent, n = 59; telophase 92% absent, n = 63; Figure 6B; Table 1). In addition, we observed a significant decrease in overall Prospero levels in *brat* mutant brains (Figure 6, right-most panels; Figure S1). We conclude that Brat promotes Prospero localization into the GMC and normal Prospero levels in the brain.

prospero Mutant Neuroblast Clones Maintain Neuroblast Gene Expression and Fail to Differentiate GMCs

To test whether the absence of Prospero was sufficient to mimic the *brat* mutant phenotype, we removed Prospero function from single neuroblast lineages by using the MARCM technique (Lee and Luo, 2001). We generated a low frequency of *prospero*^{17} null mutant clones in the thoracic ganglia, central brain, and optic lobes of the larval CNS. In every case, the *prospero* mutant clone was nearly exclusively composed of Dpn^{+} Elav^{-} cells, and it was almost completely devoid of Elav^{+} neurons (Figures 7A–7C). We conclude that Prospero is required to promote GMC differentiation, and thus loss of Prospero is sufficient to account for this aspect of the *brat* mutant phenotype.

Discussion

Brat Is a Miranda Cargo Protein

Here, we show that the Brat translational repressor directly interacts with the Miranda central domain and is a Miranda cargo protein specifically partitioned into the GMC daughter cell during neuroblast asymmetric cell division. To our knowledge, Brat is the first Miranda cargo protein identified since the original finding that Prospero and Staufen were shown to be Miranda cargo proteins over 8 years ago (Fuerstenberg et al., 1998; Ikeshima-Kataoka et al., 1997; Shen et al., 1998). Prospero is a homeodomain transcriptional repressor, and Staufen is an RNA binding protein that interacts with *prospero* mRNA. It is unknown whether Miranda has other cargo proteins in addition to Brat, Prospero, and Staufen, and it is unclear whether all three known cargo proteins can associate with a single Miranda protein.

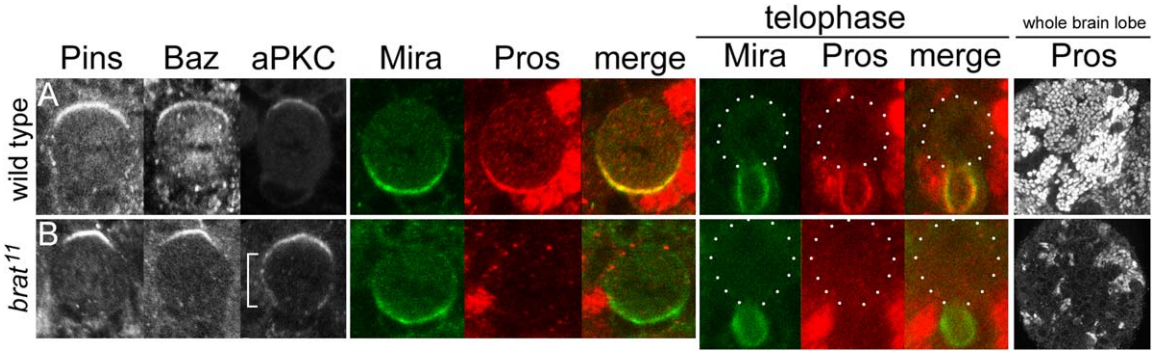

Figure 6. *brat* Mutants Fail to Localize Prospero Protein into GMCs and Have Reduced Prospero Levels

Third instar larval neuroblasts in wild-type and *brat*^{11}/*brat*^{11} mutants stained for the indicated proteins.

(A) In wild-type, Bazooka (Baz), Partner of Inscuteable (Pins), and aPKC are localized to the apical cortex, and Miranda (Mira) and Prospero (Pros) are localized to the basal cortex. Right panel: Prospero protein staining in a single brain lobe.

(B) In *brat* mutants, Baz, Pins, and Mira show normal localization, while aPKC shows partial extension into the basal cortical domain (bracket); Pros is undetectable at the basal cortex. Right panel: Prospero protein levels are reduced in *brat* brain lobes. Dotted lines mark the apical cortex in telophase neuroblasts. Apical is up.

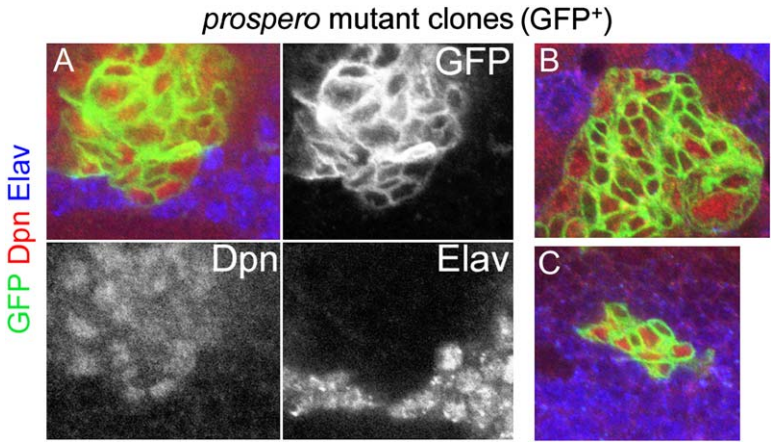

Figure 7. Single Neuroblast *prospero* Mutant Clones Have Ectopic Neuroblast Self-Renewal at the Expense of GMC Differentiation

(A–C) A *prospero*^{17} null mutant clone induced in the (A) thoracic ganglia, (B) central brain, and (C) optic lobe. The mutant clone is marked with GFP (green), the Dpn neuroblast marker (red), and the Elav GMC/neuron marker (blue). One optical section is shown; anterior is up.

brat Mutant Neuroblasts Have Defects in Prospero and aPKC Localization

It is unknown how Brat promotes Prospero basal localization. We favor the model that Brat protein stabilizes Prospero/Miranda interactions, so that Prospero protein is cytoplasmic in the absence of Brat. We don't see an obviously elevated level of cytoplasmic Prospero in *brat* mutant neuroblasts, but delocalization of Prospero protein from the basal crescent might not be visible over background. Alternatively, *brat* mutant neuroblasts may fail to transcribe or translate *prospero* in neuroblasts. This would most likely be an indirect effect, as Brat has only been shown to have translational repressor function (Sonoda and Wharton, 2001). We have been unable to detect *prospero* mRNA in wild-type larval neuroblasts, despite robust levels in GMCs (unpublished data), so we have been unable to test this possibility.

We also observed that some *brat* mutant neuroblasts show expanded aPKC cortical crescents, in some cases reaching the basal cortex. This phenotype appears specific for aPKC, because other apical cortical proteins (e.g., Baz, Pins) are unaffected. Brat might repress aPKC translation, leading to increased aPKC protein levels in *brat* mutants. Alternatively, the absence of Prospero or other basal cortical proteins may indirectly affect aPKC localization.

brat Mutants Show Ectopic Neuroblast Self-Renewal and Fail to Differentiate GMCs

brat mutant brains show a dramatic increase in the number of large, proliferating Dpn$^+$ neuroblasts between 48 and 96 hr ALH. Where do these hundreds of extra neuroblasts come from? They are unlikely to come from outside the brain, or from dedifferentiation of neurons or glia, although we can't formally rule out these models. They are likely to derive from the pool of Dpn$^+$ neuroblasts in the brain, because these are the primary pool of proliferating cells in the larval central brain, and thus the best candidates to generate the thousands of extra cells found in the hypertrophied *brat* mutant brains.

We favor a model in which a subset of *brat* mutant "GMCs" enlarge into proliferative neuroblasts (Figure 8).

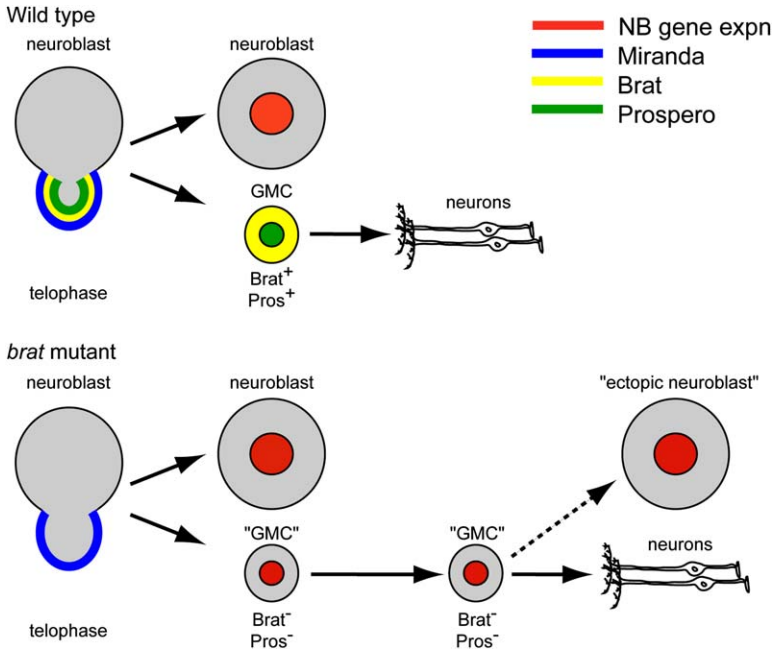

Figure 8. Brat Is a Miranda Cargo Protein Required for Inhibiting Neuroblast Self-Renewal and Promoting GMC Differentiation

Top, wild-type. Neuroblasts partition Miranda (Mira), Brat, and Prospero (Pros) into GMCs. In GMCs, Mira is degraded, Brat is cytoplasmic, Pros is nuclear, and neuroblast genes are downregulated. GMCs differentiate into two postmitotic neurons. Bottom, *brat* mutant. Neuroblasts partition Miranda (Mira), but not Brat or Pros, into GMCs. GMCs maintain neuroblast gene expression and show delayed differentiation; some ultimately form neurons, while others appear to enlarge into proliferative neuroblasts. NB gene expression indicates Dpn, Mira, and Worniu.

This model is supported by several lines of evidence. (1) *brat* mutant GMCs maintain neuroblast-specific gene expression (Dpn, Miranda, Worniu); (2) *brat* mutants show an inverse relationship between increasing neuroblast number and decreasing neuronal number over time, consistent with GMCs forming neuroblasts instead of neurons; (3) *brat* mutant GMCs can be labeled by a BrdU pulse at their birth, yet most lose BrdU incorporation during the chase interval, showing that they either reenter the cell cycle or undergo cell death, and that cell death is not consistent with the brain overgrowth phenotype; (4) *brat* mutant telophase profiles show that all GMCs are born as small Miranda$^+$ cells, ruling out physically or molecularly symmetric neuroblast divisions as a mechanism for increasing the neuroblast population; and (5) *brat* mutants show cell enlargement in other tissues, and a similar cell growth phenotype has been observed in mutants in the *C. elegans brat* ortholog (Frank et al., 2002).

What is the cellular origin of the *brat* mutant phenotype? *brat* mutant GMCs are compromised in three ways: they lack Brat translational repression activity, lack Prospero, and some may have ectopic aPKC. Loss of Brat translational repression activity could well play a role in the ectopic neuroblast self-renewal phenotype, because all *brat* mutants disrupting the NHL translational repression domain exhibit a brain tumor phenotype, and Brat has been previously shown to negatively regulate cell growth (Frank et al., 2002). Loss of Prospero also plays a role in the *brat* phenotype: *prospero* mutant GMCs have a failure to downregulate neuroblast gene expression and a failure in neuronal differentiation, similar to *brat* mutants. *prospero* null mutant embryos also show a slight delay in neuronal differentiation (Broadie and Bate, 1993), although they appear to undergo normal neuroblast self-renewal (Doe et al., 1991; Vaessin et al., 1991). Finally, ectopic aPKC can also mimic aspects of the *brat* phenotype, including formation of supernumerary large Dpn$^+$ neuroblasts (Lee et al., 2005). Interestingly, the mammalian paralogs of *Drosophila* aPKC (aPKCλ/ζ) are expressed in neural progenitors of the ventricular zone (Manabe et al., 2002), and the mammalian Prospero ortholog Prox1 is expressed in differentiating neurons of the subventricular zone (Oliver et al., 1993). Thus, identifying Prospero transcriptional targets and aPKC phosphorylation targets may provide further insight into the molecular mechanism of neural stem cell self-renewal in both *Drosophila* and mammals.

Experimental Procedures

Fly Stocks and Genetics

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

mira^{L44} and *mira^{AB78}* (Fuerstenberg et al., 1998; Ikeshima-Kataoka et al., 1997)
brat^{11} and *brat$^{l(2)k06028}$* (Arama et al., 2000)
lgl^{334} (Lee et al., 2005)
aPKC^{k06403} (Rolls et al., 2003)
worniu-gal4 (Lee et al., 2005)
UAS-lgl3A (Betschinger et al., 2003; Lee et al., 2005)

MARCM experiments were performed by using published methods (Lee and Luo, 2001) by crossing *elav-Gal4(C155), UAS-mCD8:GFP, hsp70-FLP; FRT82B tubulin-Gal80* to *FRT82B prospero^{17}* and giving the larval progeny a 1 hr 37ºC heat shock at 24 hr ALH followed by clone analysis at 96 hr ALH.

Antibody Staining and Neuroblast Culture

Larvae were dissected in Schneider's medium (Sigma), fixed in 100 mM PIPES (pH 6.9), 1 mM EGTA, 0.3% Triton X-100, 1 mM $MgSO_4$ containing 4% formaldehyde for 25 min, and blocked for 1 hr in 1× PBS containing 1% BSA and 0.3% Triton X-100 (PBS-BT). After blocking, specimens were washed in PBS-BT for 1 hr and incubated with primary antibodies in PBS-BT overnight at 4ºC. Embryos were stained as described previously (Spana and Doe, 1995). We used rat anti-Brat (1:100), guinea pig anti-Dpn (1:1000; J. Skeath), guinea pig anti-Miranda (1:400), rat anti-Miranda (1:500), rat anti-Pins (1:1000), mouse anti-Elav (1:50), mouse anti-BrdU (1:50; Sigma), mouse anti-Prospero bioreactor supernatant (1:100), rabbit anti-βgal (1:1000), rat anti-Elav (1:10; Developmental Study Hybridoma Bank), mouse anti-α-tubulin (1:2000; Sigma), rabbit anti-aPKCζ (1:1000; Santa Cruz Biotech.), rabbit anti-phosphohistoneH3 (1:1000; Upstate), rat anti-Par6 (1:50), guinea pig anti-Baz (1:400), rabbit anti-Scribble (1:2500), and secondary antibodies from Molecular Probes (details are available upon request). Embryonic neuroblasts (4–5 hr) were isolated in vitro, cultured for 30 min, and antibody stained by using published methods (Grosskortenhaus et al., 2005).

Yeast Two-Hybrid Experiments

We used the Brat NHL domain as a bait and screened a library of plasmids encoding activation domain fusions to cDNAs from 0–4 hr embryos (Dahanukar et al., 1999). Experiments were done as described previously (Edwards et al., 2003).

BrdU Pulse-Chase Experiments

Larvae were fed BrdU (1 mg/ml; Roche, Indianapolis, IN) in media for 4 hr, and then one pool was processed for BrdU staining (pulse experiments; Figure 1) and a second pool was grown without BrdU for 24 or 72 hr before fixation and BrdU staining (pulse-chase experiments; Figure 2). 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 antibody staining; BrdU detection was performed by following the manufacturer's protocol (details are available upon request).

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 Worniu, Dpn, and Miranda and the absence of the neuronal/glial differentiation markers Elav and Repo (Albertson and Doe, 2003; Lee et al., 2005). Central brain neuroblasts (the focus of this paper) 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 close to each other, forming a ribbon that flanks and encircles the highly stereotypical epithelial shape optic lobe cells [Lee et al., 2005]). All images of neuroblasts shown were collected from central brain, and all brains were mounted with dorsal surface up and ventral surface down; the midline is to the left in all panels.

Supplemental Data

Supplemental Data including three figures are available at http://www.developmentalcell.com/cgi/content/full/10/4/441/DC1/.

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