Aurora-A acts as a tumor suppressor and regulates self-renewal of *Drosophila* neuroblasts

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The choice of self-renewal versus differentiation is a fundamental issue in stem cell and cancer biology. Neural progenitors of the *Drosophila* post-embryonic brain, larval neuroblasts (NBs), divide asymmetrically in a stem cell-like fashion to generate a self-renewing NB and a Ganglion Mother Cell (GMC), which divides terminally to produce two differentiating neuronal/glia daughters. Here we show that Aurora-A (AurA) acts as a tumor suppressor by suppressing NB self-renewal and promoting neuronal differentiation. In *aurA* loss-of-function mutants, supernumerary NBs are produced at the expense of neurons. AurA suppresses tumor formation by asymmetrically localizing atypical protein kinase C (aPKC), an NB proliferation factor. Numb, which also acts as a tumor suppressor in larval brains, is a major downstream target of AurA and aPKC. Notch activity is up-regulated in *aurA* and *numb* larval brains, and Notch signaling is necessary and sufficient to promote NB self-renewal and suppress differentiation in larval brains. Our data suggest that AurA, aPKC, Numb, and Notch function in a pathway that involved a series of negative genetic interactions. We have identified a novel mechanism for controlling the balance between self-renewal and neuronal differentiation during the asymmetric division of *Drosophila* larval NBs.

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Drosophila larval NBs divide asymmetrically to produce a larger NB daughter that is capable of self-renewal, and a smaller Ganglion Mother Cell (GMC) daughter that divides once to produce progeny, which undergo terminal differentiation into two post-mitotic neurons/glia. Studies primarily in the embryonic ventral cord have revealed the mechanism of NB asymmetric divisions, which appears to be conserved between embryonic and larval NBs (for reviews, see Jan and Jan 2001; Betschinger and Knoblich 2004; Wodarz 2005; Yu et al. 2006). The asymmetric division of NBs involves the asymmetric localization and segregation of cell fate determinants Numb, Prospero (Pros), Brat, and their adaptor proteins Partner of Numb (Pon) and Miranda (Mira) into the basal GMC (Rhyu et al. 1994; Knoblich et al. 1995; Ikeshima-Kataoka et al. 1997; Shen et al. 1997; Lu et al. 1998). During NB asymmetric divisions, the mitotic spindle is oriented along an axis perpendicular to the epithelial

layer and an asymmetric spindle is generated to give rise to two unequal-sized daughter cells with distinct cell fates. NB asymmetric divisions are controlled by an apically localized complex of proteins that include the *Drosophila* homologs of the conserved Par3 (Bazooka, Baz)/Par6 (DmPar6)/atypical protein kinase C (aPKC) proteins (Kuchinke et al. 1998; Wodarz et al. 2000; Petronczki and Knoblich 2001), Inscuteable (Insc) (Kraut et al. 1996), and heterotrimeric G proteins G α i (Schaefer et al. 2001; Yu et al. 2003) and their regulators Partner of Insc (Pins) (Yu et al. 2000), Locomotion defects (Loco) (Yu et al. 2005), and a Pins-interacting protein mushroom body defective (Mud) (Bowman et al. 2006; Izumi et al. 2006; Siller et al. 2006). The asymmetric localization of G α i requires G β (Schaefer et al. 2001; Yu et al. 2003) and G γ (Fuse et al. 2003) and its membrane localization requires Ric-8 (Hampoezel et al. 2005; Wang et al. 2005). Basal protein localization and segregation are mediated by apical proteins through cortically localized tumor suppressors, Discs large (Dlg) and Lethal (2) giant larvae (Lgl) (Ohshiro et al. 2000; Peng et al. 2000). Phosphorylation of Lgl by apically localized aPKC leads to Lgl inactivation, while nonphosphorylated Lgl at the basal cor-

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tex is active and allows the localization of basal proteins (Betschinger et al. 2003).

Drosophila larval NBs have emerged as a model system for studying stem cell self-renewal as larval NBs, unlike embryonic NBs, share many features of stem cells as they can undergo growth and self-renewal for extended periods and produce a large number of progeny (see reviews Jan and Jan 2001; Betschinger and Knoblich 2004; Wodarz 2005; Yu et al. 2006). There is increasing support for the idea that tumors can arise from tumor stem cells in which the normal control of self-renewal versus differentiation is disturbed (Pasque 2006). The regulation of self-renewal versus differentiation, associated with asymmetric cell divisions, is tightly controlled during *Drosophila* larval brain development. Perturbation of the asymmetric division process can lead to uncontrolled proliferation and aberrant terminal differentiation. When transplanted into wild-type adults, mutant larval brain tissue from *pins*, *mira*, *numb*, or *pros* can form malignant tumors that rapidly kill the host (Caussinus and Gonzalez 2005). Recent studies have also suggested that NBs utilize the asymmetric localization/segregation machinery to distribute “proliferation factors” to the NB daughter and “differentiation factors” to the GMC daughter during asymmetric cell division. aPKC, which is inherited by NBs, is necessary to promote NB self-renewal, and when a membrane targeted version is overexpressed and localized to the entire cortex, leads to NB overgrowth (Lee et al. 2006a). In contrast, “differentiation factors” such as tumor suppressors Brain tumor (Brat) and Prospero (Pros), which are asymmetrically segregated into the GMC daughter act to suppress self-renewal and promote differentiation in GMC. Loss of Brat or Pros results in massive production of NBs at the expense of neurons (Bello et al. 2006; Betschinger et al. 2006; Lee et al. 2006b). Delocalization of aPKC and Brat/Pros in *lethal giant larvae* (*lgl*) mutants can lead to tumor formation in larval brains (Betschinger et al. 2006; Lee et al. 2006a,b). However, the mechanism of self-renewal versus differentiation is still largely unknown. For example, aPKC is not required for proliferation in *brat* mutant and the downstream target(s) of aPKC for the regulation of NB proliferation remains unclear. It appears that additional novel mechanisms might act to suppress excessive NB self-renewal.

Here we show that Aurora-A (AurA) acts as a tumor suppressor by suppressing NB self-renewal and promoting neuronal differentiation during larval brain development. We show that AurA acts upstream of aPKC to regulate NB self-renewal. AurA is required for the asymmetric localization of aPKC and preventing it from localizing to the basal cortex. We also suggest that Numb is one major target of AurA and aPKC in regulating NB self-renewal. The overproliferation phenotype of *aurA* or *numb* mutants is due to the up-regulation of Notch activity, but appears to be largely independent of Brat and Pros. Thus, our data provides evidence for a novel pathway that involves AurA, aPKC, Numb, and Notch for the regulation of neuroblast (NB) self-renewal versus neuronal differentiation.

Results

AurA acts as a novel tumor suppressor in Drosophila larval brains

We screened a collection of pupal lethal mutants (L. Wang, J. Evans, H. Andrews, R. Beckstead, C.S. Thummel, and A. Bashirullah, in prep.) to isolate mutants that are defective in asymmetric cell division or self-renewal of larval NBs. Three recessive EMS-induced pupal lethal mutants (Fig. 1A; data not shown) belonging to a single complementation group were identified. In these mutants, supernumerary NBs that express both Mira and Inscuteable (Insc) [Fig. 1A [wild type], A' [l(3)LL-8839 (*aurA*⁸⁸³⁹)]]; data not shown] were produced. Genetic analyses and sequencing of these mutations, *aurA*⁸⁸³⁹ (Lys 377 to a stop codon), *aurA*¹⁴⁶⁴¹ (Val 302 to Glutamate) and *aurA*¹⁷⁹⁶¹ (Asp 344 to Asparagine), revealed that they each harbor either a single missense or a nonsense mutation in the conserved C-terminal kinase domain of *aurora-A* (*aurA*) (Fig. 1B). An available strong hypomorphic allele, *aur*^{87Ac-5}, also shows a similar tumorous larval brain phenotype (data not shown). These alleles of *aurA* represent an allelic series, with *aurA*⁸⁸³⁹ showing the strongest phenotype. The *aurA*⁸⁸³⁹ tumor phenotype and lethality are fully rescued by an *aurA*-GFP transgene expressed with a NB-specific driver, *worniu-Gal4* (*wor-Gal4*) (data not shown). In addition, AurA protein, which normally localizes on both centrosomes in wild-type metaphase NBs (Fig. 1C), is undetectable on the centrosomes of *aurA*⁸⁸³⁹ larval NBs (Fig. 1C'). These data indicate that loss of AurA function is responsible for the overproliferation phenotype, and that *aurA*⁸⁸³⁹ is either a strong hypomorphic or null allele.

Loss of AurA leads to larval NB overgrowth

We quantified central brain NB numbers in *aurA*⁸⁸³⁹ mutant larvae from 24 h to 96 h after larval hatching (ALH). Larval NBs can be identified unambiguously by the expression of the markers Worniu, Deadpan (Dpn) and Mira (which is also transiently present in newborn GMCs) and the absence of the neuronal marker, Elav (Betschinger et al. 2006; Lee et al. 2006a,b). Wild-type larval central brains have 31 ± 6 NBs at 24 h ALH and the NB number increases to 89 ± 16 by 96 h ALH just prior to metamorphosis (Figs. 1D, 2A,B; Lee et al. 2006a). In *aurA*⁸⁸³⁹ mutant larvae, NBs start with a similar number at 24 h ALH. However, NB number increases dramatically to 371 ± 85 at 96 h ALH (Figs. 1D, 2A',B') and to more than a thousand at 120 h ALH during an extended larval life (data not shown). Before puparium formation, the *aurA*⁸⁸³⁹ larval brains (Fig. 1E', arrow) can grow up to 10 times the wild-type size (Fig. 1E, arrow). BrdU incorporation experiments indicate a large increase in the number of S-phase cells in *aurA* mutant (Fig. 2C') compared with wild-type brains (Fig. 2C). A significant increase of mitotic cells labeled by phospho-Histone H3 was also observed in *aurA* brains (Fig. 2D [wild type], D').

Figure 1. *AurA* is a novel tumor suppressor gene. (A,A') Wild-type (A) and *aurA⁸⁸³⁹* (A') larval central brain regions at 96 h ALH were stained with the NB markers Mira (red) and Insc (green); DNA staining is in blue in all panels. (B) Schematic representation of AurA domains and mutated sites for *aurA⁸⁸³⁹*, *aurA¹⁴⁶⁴¹*, and *aurA¹⁷⁹⁶¹*. (C,C') *AurA* is undetectable in *aurA⁸⁸³⁹* mutant larval NBs. *AurA* in wild-type metaphase NBs (C) is localized to both centrosomes, but is undetectable in *aurA⁸⁸³⁹* metaphase NBs (C'). (D) Quantification of wild-type and *aurA⁸⁸³⁹* central brain NB numbers from 24- to 96-h-ALH. $n = 20$ per time point per genotype. (E,E') *aurA⁸⁸³⁹* larval brain (E') can grow to a massive size compared with wild-type brains (E). Arrows point to the brain lobes.

There is also a significant decrease in cells expressing the neuronal markers, *Elav* (Fig. 2E') and nuclear *Pros* (Fig. 2F'), in *aurA* compared with wild-type brains (Fig. 2E,F), suggesting that the increase in NB-like cells occurs at the expense of differentiated neurons.

Mammalian *AurA* is thought to be oncogenic, as overexpression of mammalian *AurA* causes cell transformation (Zhou et al. 1998; Meraldi et al. 2002). Human *AurA* phosphorylates P53, a tumor suppressor that regulates apoptosis, leading to its degradation and thus facilitating the oncogenic transformation of cells (Katayama et al. 2004). However, overexpression of *AurA* with *wor-Gal4* did not affect NB number in the larval brains (data not shown). Apoptosis also appears not to be affected in *aurA⁸⁸³⁹* mutant larval brains, as the numbers of cells labeled by Caspase-3 (Supplementary Fig. 1A') or DIAP-1 (data not shown) in *aurA⁸⁸³⁹* are similar to those of wild-type brains (Supplementary Fig. 1A; data not shown). These data suggest that *AurA* acts via a novel mechanism in regulating *Drosophila* larval NB proliferation.

CycE/CDK2 is an important cell cycle regulator and CycE levels were shown to be up-regulated in imaginal discs of tumor suppressor mutants including *warts*, *salvador*, and *hippo* (Vidal and Cagan 2006). We also observed a dramatic increase in the number of CycE-positive cells in *aurA* larval brains (Fig. 2G') compared with wild type (Fig. 2G). To investigate whether this is a causal effect, we overexpressed *Dacapo*, a member of the P21/P27 family of CDK inhibitors (Lane et al. 1996), by *wor-Gal4* in the *aurA⁸⁸³⁹* mutant. The tumor phenotype

of *aurA⁸⁸³⁹* is largely suppressed (Fig. 2I–L) by overexpressing *Dacapo*. However, overexpression of CycE alone does not cause overgrowth of larval NBs (Betschinger et al. 2006; data not shown), suggesting that elevated levels of CycE are necessary but not sufficient to induce tumor formation. In addition to CycE, *dMyc*, another important growth factor, is also up-regulated in *aurA⁸⁸³⁹* mutant (Fig. 2H') compared with wild-type larval brains (Fig. 2H). *aurA* mutant also generate weak NB overproliferation phenotype in mitotic clones by the Mosaic Analysis with a Repressible Cell Marker (MARCM) system (Supplementary Fig. 2; Lee et al. 2000), presumably due to protein perdurance in the clone.

Asymmetric localization of *aPKC* and *Numb* requires *AurA* function

Tumor suppressors *Brat* (96%, $n = 27$, Fig. 3A'; wild type, 100%, $n = 19$, Fig. 3A) and *Pros* (96%, $n = 25$, Fig. 3B'; wild type, 100%, $n = 16$, Fig. 3B) remain asymmetrically localized in *aurA⁸⁸³⁹* mutants. Consistently, asymmetric localization (94%, $n = 36$, Fig. 3C'; wild type, 100%, $n = 20$, Fig. 3C) and segregation (100%, $n = 18$, Fig. 3D'; wild type, 100%, $n = 20$, Fig. 3D) of *Mira*, an adaptor protein of *Brat* and *Pros*, is also unaffected in *aurA* mutants. Localization of *Inscuteable* (*Insc*) (97%, $n = 32$, Fig. 1A'; wild type, 100%, $n = 21$, Fig. 1A) and *Bazooka* (97%, $n = 34$, Fig. 3E'; wild type, 100%, $n = 15$, Fig. 3E), remain asymmetric in *aurA* mutants.

Apically localized *aPKC* is a NB proliferation factor

Figure 2. AurA acts to suppress self-renewal and promote neuronal differentiation. (A–H'), Confocal single-scanning images of wild-type (A–G) and *aurA* mutant (A'–G') larval brains at 96 h ALH stained with NB markers Mira (A,A', in red), Dpn (B,B', in green), BrdU incorporation and labeling (C,C', in black), phospho-Histone H3 (D,D', in red), neuronal marker Elav (E,E', in red), nuclear Pros (F,F', in green), and CycE (G,G', in red). (H,H'), The number of dMyc-expressing cells is increased in *aurA* mutant (H', in green) compared with wild type (H, in green). (I–L) NB overgrowth is dependent on CDK2/CycE activity. *UAS-dap; aurA⁸⁸³⁹* (data not shown) and *wor-Gal4, UAS-dap* (*wor > dap*); *aurA⁸⁸³⁹* brains at 96 h ALH were stained with Mira (I, in green), Dpn (J, in green), and Elav (K, in red). (L) Quantification of NB numbers in *UAS-dap; aurA⁸⁸³⁹* (control) and *wor > dap; aurA⁸⁸³⁹* brains at 96 h ALH. *n* = 20 for each genotype. In panels that contain both central brain and optic lobe, the approximate margin between the two regions is marked by a dashed line with the central brain region to the left and optic lobe to the right (optic lobe NBs are smaller than central brain NBs). Note that in *aurA* mutants, both regions are enlarged compared with wild type. Posterior brain regions are imaged in all panels.

that seems to act independently of Brat (Lee et al. 2006a,b). We tested whether AurA regulates NB self-renewal by modulating aPKC function. Indeed, in *aurA⁸⁸³⁹* larval metaphase NBs, asymmetric localization of aPKC is strongly affected and it is delocalized to the entire cortex with punctuate staining (58%, *n* = 33 at 96 h ALH; Fig. 3F'). This phenotype is first observed at 48 h ALH (31.8%, *n* = 22) and is more severe in NBs from 120-h-ALH larvae (88.4% of delocalization, *n* = 69) in which most of maternal AurA had been presumably depleted. In this context, Mira remains largely asymmetrically localized (76.1% normal crescent, 15.2% reduced crescent, and 8.6% missing or cytoplasmic; *n* = 46), presumably because Baz and Insc remain asymmetrically localized. Overexpressed aPKC-CAAX (a membrane-targeted version of aPKC) causes ectopic localization of aPKC to both the apical and basal cortex and results in tumor formation (Lee et al. 2006a). Delocalization of aPKC in *aurA* mutants raises the interesting possibility that overproliferation of *aurA* may result from ectopic localization of aPKC on the cortex. To examine whether this is the case, we generated an *aPKC;aurA* double mutant. We found that *aPKC* significantly suppresses the *aurA* overproliferation phenotype at 68 h ALH (number of NB per brain lobe for each genotype: wild type,

67.2 ± 10.6; *aPKC*, 53.2 ± 13.5; *aurA⁸⁸³⁹*, 187.9 ± 16.4; *aPKC;aurA⁸⁸³⁹* double mutant, 109 ± 17.1) (Fig. 7C–G, below). Thus, AurA may suppress self-renewal by preventing aPKC from localizing to the basal cortex.

We also found that in *aurA⁸⁸³⁹* larval metaphase NBs (at 96 h ALH), Numb levels are strongly reduced, and its asymmetric localization is also compromised (24.7% weakly cortical localization; 28.8% strongly reduced crescent, *n* = 73) (data not shown) compared with wild-type NBs (100% crescents, *n* = 16, Fig. 3G,H). Delocalization of Numb has been observed at 48 h ALH (32% weakly cortical or strongly reduced crescent, *n* = 25) in *aurA⁸⁸³⁹*, and these defects were more prominent (66.7% weakly cortical with punctate staining, Fig. 3G'; 29.8% strongly reduced crescent, Fig. 3G"; *n* = 57) in *aurA⁸⁸³⁹* metaphase NBs from 120-h-ALH larvae. During anaphase/telophase, Numb is often missegregated to both daughter cells (57.9%, *n* = 19, Fig. 3I') in *aurA* larval NBs, whereas segregation is exclusively to one daughter in wild type (100%, *n* = 12, Fig. 3I). These are consistent with the previous finding that AurA is required for the asymmetric localization of Numb in sensory organ precursors (SOPs) of the *Drosophila* peripheral nervous system (Berdnik and Knoblich 2002). Pon, an adaptor protein required for the asymmetric localization of Numb

Figure 3. AurA is required for asymmetric localization of aPKC and Numb/Pon and regulates spindle alignment. Brat (*A,A'*), Pros (*B,B'*), and Mira (*C,C'*), remain asymmetrically localized in both wild-type (*A–C*) and *aurA*⁸⁸³⁹ (*A'–C'*) mutant metaphase NBs; DNA staining is in blue in all panels. Mira is exclusively segregated to the GMC at telophase in both wild-type (*D*) and *aurA* mutant (*D'*) NBs. (*E,E'*) Baz has a slight expansion to the cortex but remains asymmetrically localized in *aurA*⁸⁸³⁹ (*E'*) compared with wild-type (*E*) NBs. (*F,F'*) aPKC is delocalized to the entire cortex in *aurA*⁸⁸³⁹ NBs (*F'*) in contrast to a crescent seen in a wild-type NB (*F*). (*G–G'*) During metaphase, Numb is either seen as weakly scattered to the entire cortex (*G'*) or a strongly reduced crescent (*G''*) in NBs from 120-h-ALH larvae. Numb is delocalized to the entire cortex in 24.7% of NBs and Numb crescent is reduced in 28.8% NBs from 96-h-ALH larvae (*n* = 73). (*H*) Ectopic cortical localization of aPKC leads to delocalization of Numb. When aPKC-CAAX is overexpressed, Numb is delocalized in metaphase NBs. At telophase, Numb asymmetric segregation is defective in *aurA*⁸⁸³⁹ larval NBs (*I'*) compared with that in wild-type larval NBs (*I*). Pon (*J'*) is also often delocalized to the entire cortex in *aurA*⁸⁸³⁹ larval NBs, while they are seen as crescents in wild-type NBs (*J*).

(Lu et al. 1998), was also often delocalized to the entire cortex of *aurA* metaphase NBs (34.1%, $n = 41$, 96 h ALH, Fig. 3J'; 58.6%, $n = 29$, 120 h ALH) in contrast to wild-type metaphase NBs (100% crescents, $n = 11$, Fig. 3J). We then tested to see whether delocalization of aPKC to the entire NB cortex might affect asymmetric localization of Numb. Under conditions in which aPKC-CAAX is over-expressed, where it localizes uniformly to the NB cortex and causes overproliferation (Lee et al. 2006a), Numb also delocalized to the entire cortex (86.2%, $n = 29$, Fig. 3H). These data further suggest that AurA acts upstream of aPKC in controlling NB self-renewal, probably by regulating Numb function.

AurA is required for proper spindle orientation

aurA has a function in centrosome separation and chromosome segregation (Glover et al. 1995; Giet et al. 2002). We investigated whether AurA might be required for proper orientation of the mitotic spindle. In wild-type

metaphase NBs, the mitotic spindle, as judged by the positions of centrosomes, orients perpendicular to the Mira crescent (Fig. 4A,B). In 60% of *aurA* metaphase NBs, the spindle appears to be misaligned relative to the Mira crescent (Fig. 4A',B'). Centrosomin (CNN) is absent in the majority of metaphase NBs and appears cytoplasmic (Fig. 4A''). The centrosome number in *aurA* mutants is also abnormal, and NBs with one (data not shown) to three (Fig. 4A''') centrosomes can be observed. *mud* NBs also exhibit spindle orientation and centrosome organization defects (Bowman et al. 2006; Izumi et al. 2006; Siller et al. 2006) similar to those seen for *aurA*. Mud is required for proper spindle orientation in NBs and it is normally localized to both centrosomes and forms an apical crescent in wild-type NBs (Fig. 4C). Given that both AurA and Mud localize to the centrosomes and exhibit similar defects in spindle orientation, they may act together to regulate spindle orientation. Indeed, in *aurA*⁸⁸³⁹ larval NBs Mud cortical localization was no longer asymmetric, and was uniformly cortical (Fig. 4C',

Figure 4. AurA controls mitotic spindle orientation through regulating asymmetric localization of Mud. (A,A') The mitotic spindle is often misoriented relative to the Mira crescent in *aurA*¹⁷⁹⁶¹ mutants (A'), in contrast to wild type (A) in which the spindle orientation inferred by two CNN-labeled centrosomes is always aligned perpendicular to the Mira crescent. CNN staining is often lost (A'') or strongly reduced (A') in *aurA*⁸⁸³⁹ mutant NBs. In *aurA* mutants, one (data not shown) or three (A''') centrosomes can also be observed. The quantification of the distribution of spindle orientation in wild-type (B) and *aurA*¹⁷⁹⁶¹ mutant (B') NBs based on NBs that have two CNN⁺ centrosomes. (C–D') AurA is required for Mud asymmetric cortical localization but not its centrosomal localization. (C) In wild-type metaphase NBs, Mud is asymmetrically localized as a cortical crescent at the apical cortex as well as to both centrosomes. (C') In contrast, in *aurA* metaphase NBs, Mud is delocalized to almost the entire cortex but remains localized to both centrosomes (arrow). (D,D') Mira is asymmetrically localized in both wild-type NBs (D) and *aurA* mutant (D').

arrow), while its centrosomal localization was unaffected (Fig. 4C'). These observations suggest that AurA may act through Mud to regulate spindle orientation in larval NBs.

Numb acts as a tumor suppressor that functions downstream from AurA

To examine whether Numb might be a downstream target of AurA and aPKC in regulating NB self-renewal, we generated *numb*¹⁵ NB MARCM clones in larval brains. *numb*¹⁵ clones contained a greater number of cells compared with wild-type clones (Fig. 5E, *numb*¹⁵, 342 ± 82; wild type, 38 ± 18) and most of them express Dpn (Fig. 5B) and Mira (Supplementary Fig. 3B) in contrast to wild-type NB clones in which only a single cell expresses Dpn (Fig. 5A) and Mira (Supplementary Fig. 3A). *numb*¹⁵ clones contain very few neurons expressing Elav (Fig. 5C [wild type], D) and nuclear Pros (Supplementary Fig. 3C,D [wild type]). Thus, similar to AurA, Numb acts as a tumor suppressor to suppress self-renewal and promote

differentiation during larval brain development. Ectopic expression of Numb significantly suppresses the *aurA* overproliferation phenotype (178 ± 40 NBs per brain lobe at 96 h ALH and 302 ± 63 NBs per brain lobe at 120 h ALH, Fig. 5F',G) compared with control *aurA*⁸⁸³⁹ mutant (352 ± 94 NBs per brain lobe at 96 h ALH, and >1000 at 120 h ALH, Fig. 5F,G), suggesting that Numb acts downstream from AurA to regulate NB self-renewal. In this case, Numb expressed at high levels remains mislocalized on the cortex in a significant proportion of *aurA* mutant NBs (25%, *n* = 24 at 96 h ALH) (data not shown), indicating that the level of Numb has probably exceeded the threshold necessary to suppress proliferation in a proportion of these cells.

The role of Notch in NB proliferation

There is increasing evidence that Notch—a transmembrane receptor that is cleaved to release its intracellular domain, which directly participates in transcriptional regulation (Louvi and Artavanis-Tsakonas 2006)—is in-

Figure 5. AurA acts upstream of Numb to suppress tumor formation. (A–E) Numb acts as a tumor suppressor during larval brain development. (A', B') NB MARCM clones marked by CD8-GFP in third instar larval brains. Most cells in *numb*¹⁵ clones express Dpn (B) but very few cells express Elav (D), whereas in wild-type clones usually only a single cell (the NB) expresses Dpn (A) and most cells express Elav (C). (E) Quantification of the number of cells per clone in wild-type and *numb*¹⁵ clones. (F–G) Overexpression of Numb (*nb*)-GFP suppresses the NB overproliferation phenotype in *aurA*⁸⁸³⁹ brains. Overexpression of Numb-GFP using *wor*-Gal4 in *aurA*⁸⁸³⁹ mutant background (*wor>nb-GFP; aurA*⁸⁸³⁹) reduces brain size (F') compared with the control *wor*-Gal4, *aurA*⁸⁸³⁹ (F) at 120 h ALH, and the NB number is also significantly reduced (G; *n* = 20 per genotype per time point).

involved in the control of proliferation versus differentiation (Go et al. 1998; Androutsellis-Theotokis et al. 2006; Ferres-Marco et al. 2006; Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). Numb can antagonize Notch signaling to specify distinct sibling cell fates during asymmetric divisions of various progenitor cells (Rhyu et al. 1994; Spana and Doe 1996; Skeath and Doe 1998). Overexpression of a Notch dominant-active form (*N^{act}*, the intracellular domain of Notch) in NB clones also leads to the overproliferation of larval NBs (Fig. 6B, B'). These mutant clones (O/E *N^{act}*, 306 ± 99 cells per clone) (Fig. 6C) were of much larger size than control clones (53 ± 11 cells per clone) (Fig. 6C) and contained mostly NBs that expressed Mira (Fig. 6A [control], B) and Dpn (Supplementary Fig. 4A [control], B), and were essentially devoid of cells expressing Elav (Supplementary Fig. 4C [control], D). A previous study (Almeida and Bray 2005) suggested that Notch does not play a role in controlling NB proliferation in larval ventral nerve cord (VNC). The discrepancies in our findings may be due to differences in which proliferation of VNC and central brain NBs are regulated. To test whether Notch might be hyperactivated in *aurA* mutants, we examined the membrane localization of Sanpodo (Spdo), which is required

to activate Notch but is inhibited by Numb during GMC and SOP asymmetric divisions (O'Connor-Giles and Skeath 2003; Le Borgne et al. 2005). In wild-type interphase larval NBs, Spdo displayed a weak cortical staining and in punctate structures throughout the cytoplasm (100%, *n* = 38; Fig. 6D). In both *numb*¹⁵ (93%, *n* = 15; Fig. 6D') and *aurA*⁸⁸³⁹ (57%, *n* = 21; Fig. 6D'') larval NBs, Spdo was localized strongly to the cortex. In addition, *aurA* larval NBs expressed elevated levels of Notch (Fig. 6E') compared with wild type (Fig. 6E) using antibodies against Notch intracellular domain or extracellular domain (data not shown). The number of cells expressing CycE and dMyc are drastically increased when *N^{act}* is overexpressed (Supplementary Fig. 4E–G' and legend).

A *Notch* temperature-sensitive (*ts*) mutant (*N^{ts-1}*), when shifted to the restrictive temperature from the first instar larval stage onward, produced far fewer NBs in third instar larval brains (49 ± 9.8, *n* = 27; Fig. 6F', G) compared with wild type (89 ± 16.3, *n* = 10; Fig. 6F, G). Moreover, only 1.1% of the NBs are dividing in the mutant brains (*n* = 276; Fig. 6F'), as evidenced by cytoplasmic Dpn, compared with 18% in wild type (*n* = 293; Fig. 6F, arrowhead). The number of CycE-expressing NBs was also drastically decreased in the *Notch*^{*ts-1*} mutant

Figure 6. Notch activity is up-regulated in *aurA* mutants and Notch is necessary and sufficient for NB self-renewal. (A–B') Overexpression of activated Notch (the intracellular domain of Notch, N^{act}) induced NB overgrowth and tumor formation. (A',B') Mitotic clones were marked by β -galactosidase (β -Gal). In N^{act} clones, most cells express Mira (B), whereas only one cell (NB) expresses Mira in control clone (A). (C) Quantification of the number of cells per clone in control and activated Notch clones. (D,D'') Spdo localization in wild-type (D), *numb*¹⁵ (D'), and *aurA*⁸⁸³⁹ (D'') mutant larval NBs. (D) In wild-type NBs, Spdo is mostly observed as weak punctate structures on the cortex as well as throughout the cytoplasm. In *numb*¹⁵ (D') and *aurA*⁸⁸³⁹ (D'') mutants, Spdo is almost exclusively localized at the cortex. (E,E') Notch levels indicated by an antibody against its intracellular domain are up-regulated in *aurA* mutant brains (E') compared with wild type (E). (F,F') Notch is a critical proliferation factor for larval NBs. *Notch*^{ts-1} third instar larval brains (68 h ALH, 29°C from larval hatching) contain fewer NBs, marked by Dpn in green (F') compared with wild type (F). Note that wild-type larval NBs exhibit either nuclear (arrow) or cytosolic (arrowhead) Dpn signals, whereas most of the *Notch*^{ts-1} larval NBs exhibit nuclear (arrow) but not cytosolic Dpn. (G) Quantification of NB numbers per brain lobe for wild type and *Notch*^{ts-1}. Genotypes are *hs-FLP; actin-FRT-y⁺-FRT-Gal4, UAS-nlsLacZ(A,A')*, and *hs-FLP; actin-FRT-y⁺-FRT-Gal4, UAS-nlsLacZ/UAS-N^{act}* (B,B').

(Supplementary Fig. 4H') compared with wild-type brains (Supplementary Fig. 4H). Thus, Notch is necessary and sufficient to promote NB self-renewal during larval brain development. To further investigate whether AurA acts through regulation of Notch, we analyzed whether loss-of-Notch could suppress the *aurA* overgrowth phenotype. In double-mutant *N^{ts-1}; aurA⁸⁸³⁹* larval brains, the NB overgrowth phenotype is largely suppressed (Fig. 7A',B, 99 ± 22.9 NBs per brain lobe) compared with *aurA⁸⁸³⁹* (Fig. 5A,B, 400 ± 30.5 NBs per brain lobe), suggesting that AurA acts upstream of Notch to control NB self-renewal.

Discussion

Here we described a novel function of AurA as a tumor suppressor involved in the regulation of larval NB self-renewal. When *aurA* function is compromised, mutant NBs acquire some features of cancer stem cells. They divide to generate a large number of daughter cells capable of self-renewal. This excessive self-renewal occurs at the expense of neuronal differentiation, suggesting

that the normally asymmetric NB divisions have been altered such that the mutant NBs can divide symmetrically to generate two NB-like daughters. Cell cycle regulator CycE and cell growth factor dMyc are expressed in most of these tumor-like cells. We show that up-regulation of CycE is required for *aurA* overgrowth phenotype. AurA also regulates proper orientation of the mitotic spindle probably by controlling asymmetric localization of Mud. Both proteins are localized to centrosomes and are required for centrosome function. Centrosome abnormality and chromosome segregation defects in *aurA* could lead to aneuploidy, and many cancer cells exhibit centrosome defects and chromosome instability (Badano et al. 2005). Mammalian AurA when overexpressed can be oncogenic. However, future studies on its possible role as a tumor suppressor will be particularly interesting.

Our data suggest that *aurA* negatively regulates *aPKC* function to regulate NB self-renewal. *aPKC* appears to act as a NB proliferation factor since overexpression of a modified membrane-targeted version, *aPKC-CAAX*, which exhibits ectopic cortical localization throughout

Figure 7. Genetic interactions of *AurA* with *Notch* and *aPKC*. (A,A') *Notch^{ts-1}* suppresses the *aurA* overproliferation phenotype. Brains double mutant for *Notch^{ts-1}; aurA⁸⁸³⁹* do not display overgrowth of NBs (A') as indicated by the Dpn staining compared with *aurA⁸⁸³⁹* (A) at 68 h ALH at 29°C. (B) Quantification of NB numbers per brain lobe for *Notch^{ts-1}; aurA⁸⁸³⁹* and *aurA⁸⁸³⁹* alone. (C–G) *aPKC* significantly suppresses NB overgrowth phenotype in *aurA*. Larval brains of wild-type (C), *aPKC* (D), *aurA⁸⁸³⁹* (E), and *aPKC; aurA⁸⁸³⁹* double mutant (F) were stained with Dpn and number of NBs per brain lobe were quantified at 68 h ALH (G). *n* = 20. (H) A model showing a pathway composed of *AurA*, *aPKC*, *Numb*, and *Notch* in the regulation of larval NB self-renewal. *AurA* is also involved in spindle orientation through regulating *Mud* asymmetric localization. However, in our model the proposed role for *aPKC* is formal and our genetic analysis does not imply a direct molecular role of *AurA* on *aPKC*.

the NB cortex, leads to overproliferation and tumor formation (Lee et al. 2006a), similar to loss of *aurA*. *AurA* is required for the asymmetric localization of *aPKC* and restrict *aPKC* to the cortical region associated with the future NB daughter and loss of *aurA* results in delocalization of *aPKC* to the entire cortex. Consistent with and supporting this notion, we have shown that loss of *aPKC* can suppress, albeit partially, the *aurA* mutant overgrowth phenotype.

In contrast to the well-studied role of *Numb* as a cell fate determinant during asymmetric divisions of embryonic GMCs, SOPs, or muscle progenitors (Rhyu et al. 1994; Spana and Doe 1996; Carmona et al. 1998), a role for *Numb* during NB asymmetric divisions has not been described. We show here that *Numb* also acts as a tumor suppressor in *Drosophila* larval brains, and that *Numb* is a key downstream target of *AurA* and *aPKC* in the regulation of NB self-renewal. In both *aurA* mutant NBs or NBs overexpressing *aPKC-CAAX*, the asymmetric localization of *Numb* is compromised and the resultant overgrowth phenotype is consistent with that of *numb* loss-of-function. *numb* and *aurA* mutant NBs also share several common features including excessive self-renewal at the expense of neuronal differentiation as well as the membrane enrichment of *Spd*, a positive regulator of

Notch signaling. Our data suggest that *AurA* positively regulates *Numb* function. Genetic analysis is consistent with the notion that this is achieved through the negative regulation of *aPKC* that in turn negatively regulates *Numb*.

Numb is known to be a negative regulator of Notch signaling (Rhyu et al. 1994; Spana and Doe 1996; Carmona et al. 1998). Our findings indicate that *Notch* is necessary and sufficient for promoting larval NB proliferation and suppressing neuronal differentiation. Our genetic epistasis studies suggest that an *AurA*–*aPKC*–*Numb*–*Notch* genetic hierarchy acts to regulate self-renewal of *Drosophila* neural progenitor cells (Fig. 7H). During a wild-type larval NB asymmetric division, *aurA* acts to negatively regulate *aPKC* and restrict its localization to the cortical region associated with the future NB daughter; *aPKC* negatively regulates *Numb* and ensures that its localization/activity is restricted to the future GMC where *Numb* acts to antagonize *Notch*. The net effect is that *Notch* is asymmetrically activated in the NB daughter where it acts to promote self-renewal and suppress differentiation. Although our data suggest that *aurA* acts through the *aPKC*/*Numb*/*Notch* pathway, given the partial suppression seen in the double mutants *aPKC; aurA* and *Notch^{ts-1}; aurA*, we cannot ex-

clude the possibility that additional mechanisms may be involved.

Materials and methods

Fly strains

Fly strains used were *aurA*⁸⁸³⁹ [l(3)LL8839], *aurA*¹⁴⁶⁴¹ [l(3)LL14641], *aurA*¹⁷⁹⁶¹ [l(3)LL17961], *worniu-Gal4*, and *brat*¹¹ (C.Q. Doe); *aPKC* (A. Wodarz and D. Bilder); *UAS-aurA-GFP* (J. Knoblich); *UAS-CycE* (P. O'Farrells); *UAS-Dacapo* (P. O'Farrells); *UAS-aPKC-CAAX* (C.Q. Doe); *numb*¹⁵ (J. Knoblich); *Notch*^{ts-1}, *UAS-Numb-GFP* (Y.N. Jan); *UAS-actNotch* (S. Artavanis-Tsakonas); *yw*, *hs-Flp*; *act-FRT-y⁺-FRT-Gal4*, *UAS-LacZ(nls)* (J. Knoblich); and *Tub-Gal80^{ts}* and *UAS-Sanpodo* (J. Skeath).

Clonal analysis

To generate NB clones, 24-h-ALH larvae were heat-shocked at 37°C for 90 min and further aged for 3 d at 25°C.

Immunohistochemistry

For larval brain immunofluorescence stainings, larvae were dissected in PBS and fixed for 20 min in 4% formaldehyde, and processed similar to embryo stainings as described (Yu et al. 2000). Antibodies used were rabbit anti-Insc (1:1000), mouse anti-Mira (1:50, F. Matsuzaki), guinea pig anti-Dpn (1:1000, J. Skeath), mouse anti-Worniu (1:500), rabbit anti-phospho-Histone H3 (1:1000, Sigma), rat anti-Elav (1:10, DSHB), mouse anti-CycE (1:10, H. Richardson), mouse anti-dMyc (1:5, B. Edgar), rabbit anti-AurA (1:100, D. Glover), mouse anti-Pros (1:10, DSHB), rabbit anti-Brat (1:100, J. Knoblich), rat anti-Brat (1:100, R.P. Wharton), guinea pig anti-Numb (1:1000, J. Skeath), rabbit anti-Pon (1:500, Y.N. Jan), rabbit anti-Mud (1:200, F. Matsuzaki), rabbit anti-GFP (1:500, Molecular Probes), guinea pig anti-Spdo (1:1000, J. Skeath), rabbit anti-cleaved-Caspase-3 (1:75, Cell Signaling Technology), mouse anti-BrdU (1:20, Roche), mouse anti-Diap-1 (1:200, B. Hay), rabbit anti-CNN (1:5000, E. Schejter), and rabbit and mouse anti-β-Gal (1:1000, Promega).

BrdU labeling

Proliferating cells within whole brains were detected as previously described (Ceron et al. 2001). Dissected larval tissue was given a 40-min pulse of 37.5 μg/mL BrdU in Shields and Sang 3M insect medium. Tissue was then fixed for 15 min in 3.7% formaldehyde, and DNA denatured with 2N HCl for 40 min, before washing in PBS and incubating with anti-BrdU.

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