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/glial 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 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 (Passegue 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. ApPKC, 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 ApPKC 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, ApPKC is not required for proliferation in brat mutant and the downstream target(s) of ApPKC 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 ApPKC to regulate NB self-renewal. AurA is required for the asymmetric localization of ApPKC and preventing it from localizing to the basal cortex. We also suggest that Numb is a major target of AurA and ApPKC 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, ApPKC, 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 Insuteable [Insc] [Fig. 1A [wild type], A’ [l(3)LL-8839 [aurA8839]], data not shown] were produced. Genetic analyses and sequencing of these mutations, aurA8839 [Lys 377 to a stop codon], aurA14041 [Val 302 to Glutamate] and aurA17961 [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, aurA<sup>R7Ac−</sup>, also shows a similar tumorous larval brain phenotype [data not shown]. These alleles of aurA represent an allelic series, with aurA8839 showing the strongest phenotype. The aurA8839 tumor phenotype and lethality are fully rescued by an auragfp transgene expressed with a NB-specific driver, worniu-Gal4 [worniu-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 aurA8839 larval NBs [Fig. 1C’]. These data indicate that loss of AurA function is responsible for the overproliferation phenotype, and that aurA8839 is either a strong hypomorphic or null allele.

**Loss of AurA leads to larval NB overgrowth**

We quantified central brain NB numbers in aurA8839 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 aurA8839 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<sup>8839</sup> 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’].
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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 aurA mutant larval brains, as the numbers of cells labeled by Caspase-3 [Supplementary Fig. 1A'] or DIAP-1 (data not shown) in aurA 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/Cip1/PUMA family of CDK inhibitors (Lane et al. 1996), by wor-Gal4 in the aurA mutant. The tumor phenotype of aurA 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 aurA mutant larval NBs [C], [D] Quantification of wild-type and aurA central brain NB numbers from 24- to 96-h-ALH. n = 20 per time point per genotype. [E,E'] aurA larval brain (E') can grow to a massive size compared with wild-type brains (E). Arrows point to the brain lobes.

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 aurA 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
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 aurA mutant 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; aur8839, 187.9 ± 16.4; aPKC;aur8839 double mutant, 109 ± 17.1). Thus, AurA may suppress self-renewal by preventing aPKC from localizing to the basal cortex.

We also found that in aurA mutant 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 aurA mutant, 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 aurA 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
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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 overexpressed, 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/H11033). The centrosome number in aurA mutants is also abnormal, and NBs with one (data not shown) to three (Fig. 4A/H11032/H11033) 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 aurA8839 larval NBs Mud cortical localization was no longer asymmetric, and was uniformly cortical [Fig. 4C’,

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 aurA8839 [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 aurA8839 [E’] compared with wild-type [E] NBs. [F,F’] aPKC is delocalized to the entire cortex in aurA8839 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 aurA8839 larval NBs [I]. Pon [J’] is also often delocalized to the entire cortex in aurA8839 larval NBs, while they are seen as crescents in wild-type NBs [J].
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 numb15 NB MARCM clones in larval brains. numb15 clones contained a greater number of cells compared with wild-type clones (Fig. 5E, numb15, 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). numb15 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 aurA8839 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-
volved 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 (Nact, the intracellular domain of Notch) in NB clones also leads to the overproliferation of larval NBs (Fig. 6B,B′). These mutant clones (O/E Nact, 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 numb15 (93%, n = 15; Fig. 6D′) and aurA8839 (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 CycE and dMyc are drastically increased when Nact is overexpressed (Supplementary Fig. 4E–G′ and legend).

A Notch temperature-sensitive (ts) mutant (Nts−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 Notchts−1 mutant.
(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 Nts-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...
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; Carmena 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 Spdo, 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; Carmena 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 NB daughter 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 Notchts-1;aurA, we cannot ex-
clude the possibility that additional mechanisms may be involved.

Materials and methods

Fly strains

Fly strains used were aurA^{AB360} [l(3)LL8839], aurA^{14641} [l(3)LL14641], aurA^{17961} [l(3)LL17961], warnut-Gal4, and brat^{14} [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^{+1}, UAS-Numb-GFP [Y.N. Jan], UAS-actNotch [S. Artavanis-Tsakonas], yw, hs-Flp, act-FRT-γ’-FRT-Gal4, UAS-LacZ[als] [J. Knoblich], and TubGal80^{+/−} 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, J. Skeath), mouse anti-BrdU (1:20, Roche), mouse anti-Pros (1:10, DSHB), rabbit anti-dMyc (1:5, B. Edgar), 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-Spd (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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