Dronc caspase exerts a non-apoptotic function to restrain phospho-Numb-induced ectopic neuroblast formation in Drosophila

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SUMMARY

Drosophila neuroblasts have served as a model to understand how the balance of stem cell self-renewal versus differentiation is achieved. Drosophila Numb protein regulates this process through its preferential segregation into the differentiating daughter cell. How Numb restricts the proliferation and self-renewal potentials of the recipient cell remains enigmatic. Here, we show that phosphorylation at conserved sites regulates the tumor suppressor activity of Numb. Enforced expression of a phospho-mimetic form of Numb (Numb-TS4D) or genetic manipulation that boosts phospho-Numb levels, attenuates endogenous Numb activity and causes ectopic neuroblast formation (ENF). This effect on neuroblast homeostasis occurs only in the type II neuroblast lineage. We identify Dronc caspase as a novel binding partner of Numb, and demonstrate that overexpression of Dronc suppresses the effects of Numb-TS4D in a non-apoptotic and possibly non-catalytic manner. Reduction of Dronc activity facilitates ENF induced by phospho-Numb. Our findings uncover a molecular mechanism that regulates Numb activity and suggest a novel role for Dronc caspase in regulating neural stem cell homeostasis.

KEY WORDS: Numb, Dronc (Nc), Neural stem cell, Drosophila, Tumor suppression

INTRODUCTION

Stem cells possess the unique ability to self-renew and differentiate, which is crucial for development and tissue homeostasis. Disruption of this balance leads to aberrant proliferation and, in turn, contributes to tumorigenesis (Caussinus and Gonzalez, 2005; Morrison and Kimble, 2006). Although the underlying mechanisms appear diverse among different species and stem cell types, asymmetric cell division (ACD) has emerged as one common theme (Guo et al., 1996; Petersen et al., 2002; Noctor et al., 2004; Neumuller and Knoblich, 2009). Drosophila neural stem cells (NSCs) known as neuroblasts have been instrumental for studying ACD (Knoblich et al., 1995; Doe, 2008; Wu et al., 2008). During each division, factors controlling self-renewal and differentiation are unequally segregated along the apical/basal axis, leading to renewal of an apical NSC and concurrent budding off of a basal ganglion mother cell (GMC) that has limited proliferation potential. Numb is one key factor that segregates predominantly into the differentiating daughter cell (Uemura et al., 1989; Rhyu et al., 1994; Spana et al., 1995). Several components have been implicated in controlling Numb asymmetric localization (Lu et al., 1998; Lee et al., 2006a; Smith et al., 2007; Wang et al., 2007; Wirtz-Peitz et al., 2008). Among these factors, aPKC has been found to control Numb localization directly through phosphorylation (Smith et al., 2007). Partner of Numb (Pon) acts as an adaptor protein to recruit Numb (Lu et al., 1998). Recently, Polo kinase was shown to phosphorylate Pon and indirectly regulate the asymmetric localization of Numb (Wang et al., 2007). Although Numb is a crucial regulator of neural stem cell homeostasis (Lee et al., 2006a; Wang et al., 2006), how its activity is regulated is not understood.

Two neuroblast lineages with distinct spatial position and intrinsic properties are characterized in Drosophila (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). Type II neuroblast lineage differs from type I in that the type II lineage generates intermediate neural progenitors (INPs). INPs undergo a maturation process and multiple rounds of asymmetric division to produce GMCs and differentiated progenies. Therefore, expansion of neuronal populations can be accomplished through the INPs. However, unrestrained proliferation of INPs carries the risk of impaired NSC homeostasis and ultimately tumorigenesis. Loss of numb function in NSCs has been suggested to result in INPs regaining similar properties as the parental NSC (Bowman et al., 2008). The molecular mechanism underlying Numb function in this process is not well understood.

To identify genes that impinge on Numb to control neuroblast homeostasis, we have focused on Numb phosphorylation events that may regulate its activity, considering that Numb is a phosphoprotein in vivo (Tokumitsu et al., 2005; Tokumitsu et al., 2006; Smith et al., 2007). Here, we show that phosphorylation at conserved sites modulates the tumor suppressor activity of Numb, and that Polo kinase and protein phosphatase 2A (PP2A) appear to act antagonistically in this process. Expression of a phospho-mimetic Numb-TS4D abolished Numb activity and led to ectopic neuroblast formation (ENF). We identify Dronc caspase (Nedd2-like caspase, Nc – FlyBase) as a novel binding partner of Numb that can attenuate Numb-TS4D-induced ENF. Interestingly, Dronc exerts its function in an apoptosis-independent, and possibly non-catalytic, manner. In addition, we show that reducing Dronc activity enhances the brain tumor-initiating potential of phospho-
Numb. These results reveal a novel mechanism by which Numb activity is tightly controlled, and highlight the novel role of Drone in NSC homeostasis.

MATERIALS AND METHODS

Molecular cloning

Point mutations in Numb were introduced into a pSK-Numb construct using the QuickChange II XL site-directed mutagenesis kit (Stratagene). The mutations in pSK-Numb were screened and confirmed by DNA sequencing before subcloning into the pUASt vector.

Fly genetics

To generate Numb-WT, -S2A, -TS2A, -TS4A and Numb-S2D, -TS2D, -TS4D, GFP-Polo-WT transgenic flies, the corresponding cDNA constructs generated in pUASt vector were sequenced before being injected into w embryos to obtain transgenic lines, either by ourselves or using BestGene (Chino Hills, CA, USA). All other fly stocks and GAL4 lines were obtained from the Bloomington Drosophila Stock Center or have been previously described (Meier et al., 2000; Kanuka et al., 2005; Xu et al., 2005; Wang et al., 2007; Wang et al., 2009).

To generate single neuroblast clones in MARCM analysis, larvae were heat-shocked at 37°C for 90 minutes 24 hours ALH (after larval hatching), and further aged for 3 days at 25°C before analysis.

Immunohistochemistry

Larval brain tissues were fixed in 4% formaldehyde according to standard procedures (Wang et al., 2007). The primary antibodies used were: rabbit anti-Asense (1:1000), guinea pig anti-Baz (1:500), rat anti-CycE (1:100), guinea pig anti-Numb antibody (1:20,000). Transgenes with comparable expression levels were chosen. Upon phosphatase treatment, the slow-migrating bands collapsed into fast-migrating bands (data not shown), suggesting that the former represents phospho-Numb. In polo mutant larval brain extracts, there is a reduction in the level of phospho-Numb, which was restored by a Polo-GFP transgene (Fig. 1A). Thus, Polo controls the phosphorylation status of Numb.

To test whether these two putative phospho-sites (residues S183, S188) are functionally important, we generated transgenic flies overexpressing phospho-mimetic (S2D) and non-phosphorylatable (S2A) forms of Numb. In addition, we generated transgenic flies expressing TS2D and TS2A forms of Numb carrying mutations at three other potential Polo phosphorylation sites that match the consensus sequence Q/D/E-X-S/T-Φ (residues T143, S248, S447), and TS4D and TS4A forms carrying mutations in all five candidate sites (Fig. 1B; see Fig. S1 in the supplementary material). Transgenes with comparable expression levels were chosen. Upon overexpression in larval brain neuroblasts using the binary UAS-Gal4 system (Brand and Perrimon, 1993), the non-phosphorylatable forms of Numb (S2A, TS2A, TS4A) did not affect neuroblast number compared with Numb-WT and the control (Fig. 1Ca,Ch,Cf). By contrast, Numb-S2D led to a significant increase of neuroblasts (Fig. 1Ce,Cf), suggesting that phospho-Numb affects neuroblast homeostasis. Although Numb-TS2D transgenics had comparable number of neuroblasts as wild-type control (Fig. 1Cd,Cf), combining TS2D and S2D mutations as in Numb-TS4D caused a more dramatic increase of neuroblasts than Numb-S2D (Fig. 1Cc,Cf), suggesting possible synergy among these five sites. In subsequent studies, we focused on Numb-TS4D. Neuroblasts in Numb-TS4D are more proliferative than the wild.

RESULTS

Overexpression of Numb-TS4D causes excess neuroblast production

Given that mammalian Numb can functionally substitute for Drosophila Numb, even though they are localized differently (Zhong et al., 1996), we hypothesized that potential Numb activity-regulating sites are conserved. Two conserved residues, S183 and S188, are located within the phospho-tyrosine binding (PTB) domain that is essential for Numb activity (Frise et al., 1996).

In vitro phosphorylated Numb-WT protein was passed through a Zebra desalt spin column (Thermo Scientific) to remove unincorporated cold and hot ATP. The elution was divided into equal aliquots and incubated with different amounts of PP2A (Millipore) at 30°C for 2 hours in a phosphatase buffer [20 mM MOPS (pH 7.0), 2 mM DTT, 100 mM NaCl, 2 mM MnCl2]. The reactions were terminated with SDS sample buffer and subjected to SDS-PAGE followed by autoradiography.

In vitro phosphorylated Numb-WT protein was passed through a Zebra desalt spin column (Thermo Scientific) to remove unincorporated cold and hot ATP. The elution was divided into equal aliquots and incubated with different amounts of PP2A (Millipore) at 30°C for 2 hours in a phosphatase buffer [20 mM MOPS (pH 7.0), 2 mM DTT, 100 mM NaCl, 2 mM MnCl2]. The reactions were terminated with SDS sample buffer and subjected to SDS-PAGE followed by autoradiography.
type, as indicated by the proliferation markers phospho-H3 (p-H3) and cyclin E (CycE) (see Fig. S2B/C/H11032/C/H11032 in the supplementary material). On the other hand, the number of embryonic lethal abnormal visual system (Elav)-positive postmitotic neurons was significantly reduced (Fig. 1Cc/C/H11032/C/H11032). Taken together, we conclude that Numb-TS4D leads to excess production of neural stem cells at the expense of neuronal differentiation.

Numb-TS4D acts in a dominant-negative fashion to increase neuroblast number

Numb-S2D and Numb-TS4D-induced ENF might be caused by either mislocalization of Numb or impairment of its activity. To distinguish between these possibilities, we examined the localization pattern of Numb-S2D and Numb-TS4D. In larval brain neuroblasts, Numb-S2D and Numb-TS4A were asymmetrically localized in the same way as Numb-WT (Fig. 1Da-Dc). The corresponding residues are changed to D in Numb-S2D, -TS2D and -TS4D. The localization of Numb-WT, Numb-S2D, -TS2D and -TS4D was examined in larval brain neuroblasts. The localization of Numb-WT, Numb-S2D, -TS2D and -TS4D was examined in larval brain neuroblasts. The localization of Numb-WT, Numb-S2D, -TS2D and -TS4D was examined in larval brain neuroblasts. The localization of Numb-WT, Numb-S2D, -TS2D and -TS4D was examined in larval brain neuroblasts. The localization of Numb-WT, Numb-S2D, -TS2D and -TS4D was examined in larval brain neuroblasts. The localization of Numb-WT, Numb-S2D, -TS2D and -TS4D was examined in larval brain neuroblasts.
localization (Wang et al., 2007). Although Pon-SD fully restored Numb-TS4D localization at metaphase and telophase (Fig. 1Eb and data not shown, 100%, n=15), it was unable to rescue ENF (Fig. 1Ec), suggesting that the effect of Numb-TS4D on NSC homeostasis is probably due to impairment of Numb activity rather than to localization. In addition, other apical and basal polarity markers, including the key neuroblast self-renewal factor aPKC, were localized normally in Numb-TS4D-expressing neuroblasts (see Fig. S2E-K in the supplementary material).

The effect of Numb-TS4D on NSC homeostasis is reminiscent of that seen in numb-null mutants, suggesting that Numb-TS4D might act in a dominant-negative fashion to interfere with endogenous Numb function. To test this possibility, we examined whether Numb-TS4D binds to Numb-WT. In co-immunoprecipitation (co-IP) experiments, Numb-WT and Numb-TS4D could form a complex (Fig. 1F). Consistent with Numb-TS4D acting through a dominant-negative mechanism, co-expression of Numb-WT or Numb-TS4A efficiently suppressed Numb-TS4D-induced ENF (Fig. 1Gb,Gc). By contrast, a GFP control failed to do so (Fig. 1Ga,Gc).

**Numb-TS4D specifically affects Type II neuroblasts**

Our data described so far suggest that Numb-TS4D impairs neuroblast self-renewal/proliferation control. To identify the neuroblast lineages affected by Numb-TS4D, we used specific markers Asense (Ase) and Miranda (Mira) to distinguish type I from type II lineages. Type II neuroblasts are Mira⁺ Ase⁻, whereas type I neuroblasts are Mira⁻ Ase⁺ (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). When Numb-TS4D was expressed using insc-Gal4 in both type I and type II lineages, the ectopic neuroblasts were mostly Mira⁺ Ase⁻, indicating that the type II neuroblasts are affected (Fig. 2Ab). Consistently, when Numb-TS4D was exclusively expressed in type II lineage with Dil-Gal4 (Izergina et al., 2009), ENF was also observed (Fig. 2Bd). By contrast, expression of Numb-TS4D in type I lineage using asense-Gal4 had no obvious effect (Fig. 2Bb).

To further examine at which stage Numb-TS4D affects type II lineage specification, we specifically expressed Numb-TS4D with dFezf/Erm-Gal4, which is expressed in mature INPs, GMCs and differentiated neurons (Weng et al., 2010). Numb-TS4D expression in these cell types was unable to induce ENF (data not shown).
Although it is possible that dFezf/Erm-Gal4 may be too weak a driver to induce Numb-TS4D overexpression phenotype, it is also possible that Numb-TS4D acts in a cell type not covered by dFezf/Erm-Gal4 to induce ENF. To further assess the lineage specificity of the Numb-TS4D effect, we performed clonal analysis using MARCM (Lee and Luo, 1999). Type II neuroblast clones expressing Numb-TS4D contained ectopic neuroblasts (Fig. 2Db), whereas Numb-TS4D-expressing type I neuroblast clones always contained a single neuroblast as in the control (Fig. 2Cb). Thus, Numb-TS4D specifically affects type II neuroblast homeostasis.

**Polo and PP2A act antagonistically to regulate Numb phosphorylation and activity**

To test whether the sites mutated in Numb-TS4A/TS4D are phosphorylated in vivo, we conducted metabolic labeling in HEK-293T cells. Numb-WT or Numb-TS4A labeled with $^{32}$P-orthophosphate were immunoprecipitated from cell extracts and subjected to autoradiography and western blot analysis. $^{32}$P-positive signal was normalized with the total amount of Numb protein. As shown in Fig. 3A, $^{32}$P incorporation into Numb-TS4A positive signal was normalized with the total amount of Numb protein. Moreover, GST fusion proteins of Numb-WT showed robust phosphorylation by human polo-like kinase (PLK1) in vitro, whereas Numb-TS4A displayed only background level of phosphorylation (Fig. 3B). By contrast, the control GST-Fis1 protein was not phosphorylated by human PLK1 under the same conditions (see Fig. S3 in the supplementary material). Moreover, treatment of human PLK1-phosphorylated Numb-WT showed robust phosphorylation by human polo-like kinase (PLK1) in vitro, whereas Numb-TS4A displayed only background level of phosphorylation (Fig. 3B). By contrast, the control GST-Fis1 protein was not phosphorylated by human PLK1 under the same conditions (see Fig. S3 in the supplementary material). Moreover, treatment of human PLK1-phosphorylated Numb-WT showed robust phosphorylation by human polo-like kinase (PLK1) in vitro, whereas Numb-TS4A displayed only background level of phosphorylation (Fig. 3B). By contrast, the control GST-Fis1 protein was not phosphorylated by human PLK1 under the same conditions (see Fig. S3 in the supplementary material).

We next examined whether Polo and PP2A exhibit genetic interactions with Numb in vivo that are consistent with their antagonistic effects on Numb phosphorylation. PP2A acts as a tumor suppressor by modulating multiple pathways, including cell cycle progression (Westermarck and Hahn, 2008), and mutations in Drosophila PP2A catalytic subunit (mts) affect neuroblast homeostasis (Chabu and Doe, 2009; Wang et al., 2009). We therefore asked whether the TS4D mutations affected the ability of Numb to inhibit Notch, as measured with the $\gamma$GFP reporter (Almeida and Bray, 2005). Neuroblasts expressing Numb-TS4D exhibited significantly increased Notch activity compared with Numb-TS4A or Numb-WT (Fig. 4A), suggesting that aberrant Notch activity in Numb-TS4D might contribute to uncontrolled neuroblast proliferation. Spdo acts as a key component of Notch pathway, and its association with the plasma membrane is tightly regulated by Numb in other cell types (O’Connor-Giles and Skeath, 2003). We therefore asked whether elevated Notch activity observed in Numb-TS4D is correlated with aberrant Spdo membrane association. In wild-type neuroblasts, Spdo was apparently cytoplasmic at both metaphase and telophase stages (Fig. 4Ba,Bb). By contrast, neuroblasts expressing Numb-TS4D exhibited significantly enriched Spdo membrane localization at similar stages (Fig. 4Ba’Bb’), indicating that Numb-TS4D lost its ability to promote Spdo endocytosis. Enhanced Spdo membrane association was also observed in Numb-TS4D MARCM clones (see Fig. S6 in the supplementary material). Although membrane association of Spdo was affected by Numb-TS4D, the physical interaction between Numb and Spdo, as reported previously (O’Connor-Giles and Skeath, 2003), was not affected by the TS4D mutations (see Fig. S7A in the supplementary material), suggesting that the effect of Numb-TS4D on neuroblast homeostasis might not be due to alteration of its binding to Spdo.

We further tested whether abnormal Spdo endocytosis and elevated Notch signaling were responsible for the Numb-TS4D effects on neuroblast homeostasis. In $spdo$-null mutant clones, Numb-TS4D-induced ENF was abolished (Fig. 4Cb). Moreover,
inhibition of Notch activity using either NotchT mutant or Notch RNAi suppressed Numb-TS4D-induced ENF (Fig. 4Db-Dd). These results support the observation that aberrant Notch signaling contributes to Numb-TS4D effect on NSC homeostasis.

The effect of Numb-TS4D on neuroblast homeostasis prompted us to examine whether Numb-TS4D impaired the tumor suppressor activity of Numb. In numb mutant clones, an ENF phenotype was observed (Lee et al., 2006a; Wang et al., 2006; Bowman et al., 2008). Numb-WT and Numb-TS4A were able to readily suppress this phenotype in numbT mutant clones (100% penetrance, n=10; Fig. 4Ec,Ed). By contrast, Numb-TS4D was unable to do so (100% penetrance, n=12; Fig. 4Ee). As the rescue in this setting largely depends on Numb activity rather than localization, as indicated by the rescue of numbT phenotype by cytoplasmically localized...
human Numb (see Fig. S7B-D in the supplementary material), these data support the observation that Numb activity is disrupted in Numb-TS4D.

**Identification of Dronc as a novel binding partner of Numb**

To better understand the mechanism by which Numb-TS4D affects NSC homeostasis, we focused on identifying new Numb-interacting proteins whose interaction with Numb might be affected by the TS4D mutations. In a yeast two-hybrid screen using the PTB domain of Numb as bait, we identified Dronc, an initiator caspase involved in apoptosis (Dorstyn et al., 1999), as a novel binding partner of Numb. Using co-IP assays, we mapped the domains mediating the interaction between Numb and Dronc. Dronc-D, with the N-terminal caspase activation and recruitment domain (CARD) deleted, showed similar affinity to Numb as did full-length Dronc, indicating that CARD is dispensable for Dronc binding to Numb (Fig. 5A). Further mapping experiments showed that the last ~100 amino acids of Dronc are required for its binding to Numb (see Fig. S8A in the supplementary material). In addition, we found that the PTB domain of Numb is necessary and sufficient for association with Dronc (Fig. 5B,C).

**Fig. 4. Numb-TS4D loses its tumor suppressor activity.** (A) Notch activity is elevated by Numb-TS4D (Ac), but not Numb-WT (Aa) or -TS4A (Ab). Notch reporter mGFP was co-expressed with Numb-WT, -TS4A or -TS4D in neuroblasts. Metaphase neuroblasts were stained for GFP (green), Miranda (red) and DNA (blue). (B) Effects of Numb-TS4D on Spdo endocytosis. Spdo shows enhanced cortical localization in Numb-TS4D-expressing neuroblasts (Ba’,Bb’), compared with its cytoplasmic localization in the control (Ba,Bb). (C) Effects of spdo mutation on Numb-TS4D-induced ENF. spdoG104 mutant MARCM clones are marked with GFP (green). Canonical neuroblasts are identified as Miranda-positive cells with a diameter of around 10 μm (red). The smaller Miranda-positive cells in Cb’ are INPs. (D) Inhibition of Notch activity with either a temperature-sensitive allele (Db) or RNAi (Dc) suppresses the effect of Numb-TS4D. Neuroblast number is quantified in Dd. Data are mean±s.e.m. (E) Effects of Numb-WT (Ec), -TS4A (Ed) and -TS4D (Ee) on the ENF phenotype in numb mutant (Eb). The corresponding Numb transgenes were introduced into numb15 MARCM clones marked with GFP (green), and neuroblasts were stained with Miranda (red). The clones in Eb and Ee are bigger, as indicated by the scale bars. Scale bars: 5 μm in A,B; 10 μm in C-E. For additional data, see Figs S6, S7 in the supplementary material.
Overexpression of Dronc suppresses Numb-TS4D-induced ENF

To test the possibility that the compromised interaction of Numb-TS4D with Dronc might mediate its effect on neuroblast homeostasis, we overexpressed in Numb-TS4D background Dronc-WT and Dronc-ΔN (Meier et al., 2000). Both forms of Dronc were able to attenuate Numb-TS4D-induced ENF (Fig. 6Ab,Ac; see Fig. S9A in the supplementary material). Dronc-ΔN displayed a stronger effect than Dronc-WT, suggesting that CARD might exert an inhibitory effect on Dronc function in this context. Concomitant with the inhibition of ENF, Dronc-ΔN also suppressed ectopic Spdo membrane association and aberrant Notch activity caused by Numb-TS4D (Fig. 6B).

There are two possibilities that could account for the rescue of Numb-TS4D effects by Dronc. First, cleavage of Numb-TS4D by Dronc through its caspase activity (Dorstyn et al., 1999; Hawkins et al., 2000) might occur in vivo, leading to reduced Numb-TS4D protein level. However, we did not observe alteration of Numb-TS4D protein levels after co-expressing Dronc (data not shown). Second, ectopic Dronc might compete with Numb-TS4D to bind to endogenous Numb and therefore partially restore Numb function. To test whether endogenous Numb was involved, we analyzed the effect of Dronc-ΔN in numb-null mutant clones with or without Numb-TS4D co-expression. In the absence of endogenous Numb, Dronc-ΔN failed to suppress Numb-TS4D-induced ENF (see Fig. S9B in the supplementary material). This data suggests that the rescuing effect of Dronc on Numb-TS4D requires endogenous Numb.

The suppressive effect of Dronc on Numb-TS4D is apoptosis independent

Dronc is known as an initiator caspase with pro-apoptotic function (Quinn et al., 2000; Chew et al., 2004; Daish et al., 2004; Waldhuber et al., 2005; Xu et al., 2005). To test whether Dronc acts through an apoptotic mechanism to suppress Numb-TS4D-induced ENF, we performed a TUNEL assay. The specificity of this assay was supported by the detection of increased TUNEL+ cells in p53-overexpressing larval brains (see Fig. S9C in the supplementary material). No significant difference in the number of TUNEL+ cells was observed in Numb-TS4D background with or without Dronc-ΔN co-expression (Fig. 6C), indicating that overexpression of Dronc-ΔN in this context did not promote apoptosis. Of note, the few apoptotic cells detected by TUNEL+ staining in the central brain were apparently neuroblasts (Fig. 6C). Moreover, overexpression of Dronc-WT or Dronc-ΔN alone did not affect neuroblast number (data not shown), suggesting that Dronc does not induce neuroblast apoptosis in the central brain under the conditions used. These data indicated that Dronc probably exerts its effect on Numb-TS4D in a non-apoptotic mechanism.

Caspases are involved in the regulation of diverse developmental processes, including differentiation (Fernando et al., 2002; Arama et al., 2003; Fujita et al., 2008). To test whether Dronc exerts its effect by promoting differentiation, we co-stained larval brain with the neuroblast marker Miranda and the pan-neuronal marker Elav. Accompanying the dramatic reduction of neuroblast number in animals co-expressing Dronc-ΔN and Numb-TS4D, the number of Elav-positive neurons was significantly increased (Fig. 6D). This result indicated that Dronc has the capacity to limit neuroblast proliferation and initiate neuronal differentiation in the Numb-TS4D background. Next, we examined whether overexpression of Dronc was also able to suppress ENF in other genetic backgrounds. First, we found that co-expression of Dronc with NotchΔnin, a constitutively active form of Notch, was unable to suppress NotchΔnin-induced ENF (data not shown). This result suggests that Dronc may act upstream of Notch to mediate Numb function. Second, we tested the effects of Dronc overexpression in an aPKC<sup>Wts</sup> overexpression background, which leads to ENF in both type I and type II lineages (Lee et al., 2006b; Bowman et al., 2008), and in lgl<sup>or</sup> mutant backgrounds, which produce ENF largely in the type II lineage (Betschinger et al., 2006; Bowman et al., 2008). However, none of these ENF phenotypes could be inhibited by the co-expression of Dronc (data not shown), suggesting that Dronc might specifically regulate the effect of phospho-Numb on neuroblast homeostasis.

A non-catalytic form of Dronc also suppresses Numb-TS4D effects

To further test whether the rescuing effect of Dronc on Numb-TS4D relies on activation of downstream caspases, we co-expressed p35 [a baculovirus-derived protein that inhibits the activities of downstream caspases (Hay et al., 1994)] and Dronc in...
the Numb-TS4D background. Enforced expression of p35 was unable to prevent Dronc from suppressing ENF in Numb-TS4D background (data not shown), suggesting that the effect of Dronc is unlikely to be due to activation of downstream caspases. To test whether the rescuing effect of Dronc on Numb-TS4D requires its caspase activity, we co-expressed with Numb-TS4D a catalytically inactive form of Dronc (Dronc-CA), which has the Cys in the caspase active site replaced by Ala. Surprisingly, Dronc-CA was able to attenuate Numb-TS4D-induced ENF almost as effectively as Dronc-WT, suggesting that Dronc may act in this process in a catalytic activity-independent manner (Fig. 6Ad; see Fig. S9A in the supplementary material).

Reduction of Dronc activity facilitates ENF induced by partial loss of Numb

Knowing that enforced expression of Dronc is sufficient to attenuate ENF caused by Numb-TS4D, we next investigated the normal requirement for Dronc in neuroblast homeostasis. In well-characterized dronc-null mutants with cell death defects (Xu et al., 2005), the number of neuroblasts appeared normal (data not shown), indicating that loss of dronc on its own has no significant effect on neuroblast homeostasis under normal conditions. It is possible that the pleiotropic effects of complete loss of Dronc might mask its effect on neuroblast self-renewal. To test whether partial loss of dronc might predispose animals to ENF upon impairment of Numb activity, we made use of Numb-S2D, which displays a milder ENF phenotype than Numb-TS4D. Knocking down Dronc using RNAi significantly augmented Numb-S2D-induced ENF (Fig. 7B,E). This enhancement was not due to prevention of neuroblast apoptosis, as the total number of TUNEL+ cells in the central brain did not change (data not shown). Consistent with the RNAi result, removal of one copy of dronc also led to enhanced ENF in Numb-S2D background (Fig. 7C,E). Moreover, Dronc RNAi in dronc heterozygous background showed further enhancement of Numb-S2D phenotypes (Fig. 7D,E). These data suggest that reduction of dronc function renders the animal more susceptible to ENF caused by impairment of Numb function. We further tested the idea that the effect of Dronc RNAi on neuroblast number is due to disinhibition of the stimulating effect of phospho-Numb on ENF. For this purpose, we introduced Dronc RNAi into mts-RNAi and GFP-Polo-WT co-expression background. As shown earlier, co-expression of mts-RNAi and GFP-Polo-WT led to ENF in type II lineage (Fig. 3F), presumably because of increased phosphorylation of endogenous Numb. Reducing Dronc activity by RNAi further increased this ENF by ~25% (Fig. 7F), supporting that the effect of Dronc is due to modulation of phospho-Numb.

We further explored the physiological relevance of the biochemical interaction between Numb and Dronc. Co-expression of Numb had no effect on the eye degeneration phenotype caused by Dronc overexpression (see Fig. S10 in the supplementary material), suggesting that Numb may not regulate the pro-apoptotic activity of Dronc in this setting. To examine the potential role of Dronc in regulating Numb function, we introduced Dronc RNAi into numb512F mutant, which exhibits a weak loss of Numb activity (Bhalerao et al., 2005). MARCM clones of numb512F mutant alone showed a mild ENF phenotype in type II neuroblast lineage. Interestingly, although Dronc RNAi alone had no effect on type II neuroblast number, it was able to further increase the number of ectopic neuroblasts induced by numb512F (see Fig. S11 in the supplementary material), indicating that Dronc-Numb interaction is normally involved in neuroblast homeostasis.

DISCUSSION

Proper balance of the self-renewal versus differentiation of stem cells is crucial for tissue homeostasis. Disruption of this process could contribute to tumorigenesis (Neumuller and Knoblich, 2009). Numb has been identified as a key player that limits the proliferation potential of neuroblasts and INPs (Bowman et al., 2008). Here, we elucidate the mechanisms of Numb action in this
process and uncover a novel mechanism by which Numb activity is regulated at the post-translational level. Our results suggest a model in which phosphorylation of Numb at conserved sites within its functionally important PTB domain impairs its association with the caspase Dronc and attenuates its tumor suppressor activity in type II neuroblasts.

As a defining feature of Numb protein is its asymmetric localization in stem cells and progenitors (Rhyu et al., 1994), previous studies of Numb have been focused on the control of its asymmetric localization. A number of factors have been identified to regulate Numb localization, including its binding partner Pon and kinases such as aPKC, Aurora A and Polo (Lu et al., 2006a; Smith et al., 2007; Wang et al., 2007; Wirtz-Peitz et al., 2008). In this study, we present evidence that phosphorylation of Numb at the putative Polo sites primarily affect Numb activity in negatively regulating Notch signaling through promoting the endocytosis of Spdo. Although not all the identified Polo phosphorylation sites in Numb perfectly match the optimal consensus sequence initially defined for Polo (Nakajima et al., 2003), the Polo consensus sequence being defined is evolving (Barr et al., 2004), and specific characterized phosphorylation sites in other Polo substrates actually do not conform to the above consensus sequences (Toyoshima-Morimoto et al., 2001; Casenghi et al., 2003; Jackman et al., 2003; Yamaguchi et al., 2005; Mbofo et al., 2010; Yim and Erikson, 2010; Jang et al., 2011; Rizkallah et al., 2011). A common feature appears to be negatively charged residues surrounding the S/T residues; all five sites identified in Numb have this feature. Moreover, we provide evidence that the sites we identified are responsive to phosphorylation controlled by Polo and PP2A. More importantly, phosphorylation of Numb at these sites has a significant effect on NSC homeostasis.

Polo kinase was shown to also control Numb asymmetric localization by phosphorylating Pon, an adaptor protein for Numb. Loss of Numb asymmetry in polo mutants contributes to ENF (Wang et al., 2007). The increased neuroblasts in polo mutants largely occur in the type I lineage (Y.O. and B.L., unpublished). Here, we demonstrate that overexpression of Polo impairs Numb activity and leads to ENF in type II lineage. In this situation, Pon is presumably also phosphorylated by Polo. However, its positive effect on Numb asymmetric localization is likely to be overridden by impairment of Numb activity by Polo. This underlines the importance of Numb activity regulation in vivo and further indicates that Polo kinase acts on diverse targets to control neuroblast homeostasis. We show that phosphorylation of Numb by Polo is probably antagonized by PP2A action in type II lineage, which presumably serves to fine-tune Numb activity through dephosphorylation. Interestingly, the relationships between Polo and PP2A in type I lineage is different from that in type II lineage. In type I neuroblasts, overexpression of Polo can rescue PP2A loss-of-function phenotype (Y.O. and B.L., unpublished), consistent with Polo being positively regulated by PP2A at the transcription level (Wang et al., 2009). Elucidation of the mechanisms mediating these differential effects will help us to understand the distinct behaviors of neuroblasts in these two lineages.

Deregulation of Numb phosphorylation contributes to loss of Numb activity and eventually leads to unrestrained ENF. Given the conservation of the phosho-sites identified in this study and the potential role of Numb in tumor suppression in mammals (Pece et al., 2004), mutations in Numb itself or in some kinases/phosphatases that affect Numb phosphorylation under pathophysiological conditions could contribute to cancer in humans. In lung and breast cancer tissues, Polo expression is upregulated (Holtrich et al., 1994; Yuan et al., 1997; Ando et al., 2004). It is possible that under such pathophysiological conditions, Numb becomes hyperphosphorylated and consequently loses its antagonistic effect on Notch signaling, which could have detrimental consequences on tissue homeostasis. The phosphorylation sites of Numb identified in this study are conserved in mammals. It would be interesting to test in the future whether this phosho-epitope could be detected in human tumor samples.

We demonstrate that ENF induced by phospho-Numb occurs specifically in type II lineages, consistent with Numb primarily acting in type II lineage to restrict the proliferation of INPs (Bowman et al., 2008). It is conceivable that Numb is also phosphorylated by Polo kinase in type I lineage. However, certain unidentified factors might block the effect of phospho-Numb on type I neuroblasts. It is also possible that type I and type II lineages might employ different molecular mechanisms to control their stem cell self-renewal and differentiation, considering their different origin and modes of neurogenesis. Consistent with this notion, the Numb/Notch pathway has been suggested to be dispensable in the type I lineage (Bowman et al., 2008).
The prominent brain tumor phenotype induced by Numb-TS4D provides an excellent system with which to identify novel molecules involved in controlling NSC homeostasis. We show here that Dronc, a newly identified binding partner of Numb, is involved in regulating neuroblast homeostasis. Overexpression of Dronc is sufficient to attenuate Numb-TS4D-induced ENF without promoting neuroblast apoptosis. At the mechanistic level, we show that Dronc appears to act upstream of Notch to regulate Numb function, apparently in a process that does not strictly depend on its catalytic activity. Importantly, reduction of dronc function results in neuroblasts being more susceptible to the effect of phospho-Numb on neuroblast homeostasis. In addition, Dronc RNAi is able to further increase ectopic neuroblasts in numbSS2F mutants, indicating that Dronc-Numb interaction is normally involved in regulating neuroblast homeostasis. Accumulating evidence suggests that caspases, in addition to their pro-apoptotic functions, also participate in other developmenal processes without inducing cell death (Geisbrecht and Montell, 2004; Huh et al., 2004a; Huh et al., 2004b; Kuranaga et al., 2006; Kuranaga and Miura, 2007; Maelfait and Beyeart, 2008; Murray et al., 2008). For example, Dronc has been implicated in a non-autonomous role in compensatory proliferation (Huh et al., 2004a; Wells et al., 2006). It would be interesting to examine in the future whether Dronc transduces a signal from the neighboring niche cells via cell-cell interaction to establish neuroblast homeostatic control. It is also worth noting that mice deficient for caspase 2 (Casp2), which is closely related to Dronc in Drosophila, develop normally as their wild-type siblings; however, the fibroblasts from Casp2 null animals are easily transformed when challenged with oncogenic insults (Ho et al., 2009). The downstream effectors mediating this effect are not known. It would therefore be interesting to test whether the Numb/Dronc pathway identified here is generally involved in stem cell and cancer biology.

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