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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-

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Numb. These results reveal a novel mechanism by which Numb activity is tightly controlled, and highlight the novel role of Dronc 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-Deadpan (1:1000), mouse anti-Elav (1:50), chicken anti-GFP (1:3000), mouse anti-p-Histone3 (1:1000), rabbit anti-Miranda (1:1000), mouse anti-Myc4A6 (1:500), guinea pig anti-Numb (1:1000), rabbit anti-aPKC (1:500), rabbit anti-Pon (1:1000) and guinea pig anti-Spdo (1:1000). Quantification of neuroblast numbers was carried out at 96 hours ALH at 25°C. Larvae of each genotype were dissected and stained with neuroblast marker Miranda or Deadpan. Central brain neuroblasts can be distinguished from optic lobe neuroblasts based on their medial-superficial location, larger size and dispersed pattern.

^{32}P labeling, Co-IP assays in HEK-293T cells and western blotting

Numb-WT and -TS4A plasmids were transfected into HEK-293T cells using Fugene6 (Roche). Approximately 28 hours post-transfection, the cells were incubated with 100 nM okadaic acid and 1 μM aPKC inhibitor for 2 hours before adding 0.2 μCi μl^{-1} ^{32}P-labeled orthophosphate and culturing for additional 4 hours. Cell lysates were prepared in 50 mM Tris (pH 7.5), 100 mM NaCl, 1% Trinton X-100 containing protease inhibitor cocktails (Roche) plus phosphatase inhibitors NaF and β-glycerophosphate. Numb proteins were immunoprecipitated with agarose-conjugated rabbit anti-Myc (Sigma). For co-IP, corresponding tagged constructs were transfected into HEK-293T cells as described above. The proteins were immunoprecipitated using either mouse anti-Flag or rabbit anti-Myc agarose (Sigma), and bound proteins were blotted with either rabbit anti-Myc antibody (Millipore) or mouse anti-Flag antibody (Sigma).

Two or three brains from third instar larvae were homogenized in a buffer containing 50 mM Tris (pH 7.2), 150 mM NaCl, 5 mM EDTA and 1 mM DTT with the addition of protease inhibitor (Roche) and phosphatase inhibitor (Sigma) cocktails. The extracts were run on SDS-PAGE and transferred to PVDF membrane. Endogenous Numb was detected with guinea pig anti-Numb antibody (1:20,000).

In vitro phosphorylation and dephosphorylation assays

The cDNA inserts encoding Numb-WT or -TS4A (117-556 amino acids) were tagged with His tag at the C terminus and cloned into the *pGEX6P-1* vectors. Recombinant proteins were produced in bacteria according to standard protocols. Approximately 0.5~1 μg Numb-WT and -TS4A proteins were incubated with 2 μl of human PLK1 (ProQinase) in a buffer containing 50 mM Tris (pH 7.2), 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 50 μM ATP and 5 mM β-glycerophosphate. The phosphorylation reaction was extended for 1 hour at 30°C with the addition of [γ-^{32}P] ATP (PerkinElmer, 3 μCi per sample).

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 $MnCl_2$]. The reactions were terminated with SDS sample buffer and subjected to SDS-PAGE followed by autoradiography.

TUNEL assay

ApopTag direct fluorescein in situ apoptosis detection kits (Millipore) were used to detect apoptotic cells in larval brain in accordance with the manufacturer's instructions.

Statistical analysis

The *t*-test program in Microsoft Excel was used for comparing different genotypes. *P* values less than 0.05 were considered to be statistically significant.

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). The sequences flanking these residues resemble phosphorylation sites for Polo kinase, with at least one negatively charged D/E residues at the –3 to +3 positions (Kelm et al., 2002; Nakajima et al., 2003; Barr et al., 2004; Yamaguchi et al., 2005; Mbefo et al., 2010) (see Fig. S1 in the supplementary material). To test whether Polo regulates the in vivo phosphorylation of Numb, we analyzed Numb protein in *polo* mutant flies. Wild-type Numb is present as multiple bands on SDS-PAGE gel. 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,Cb,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

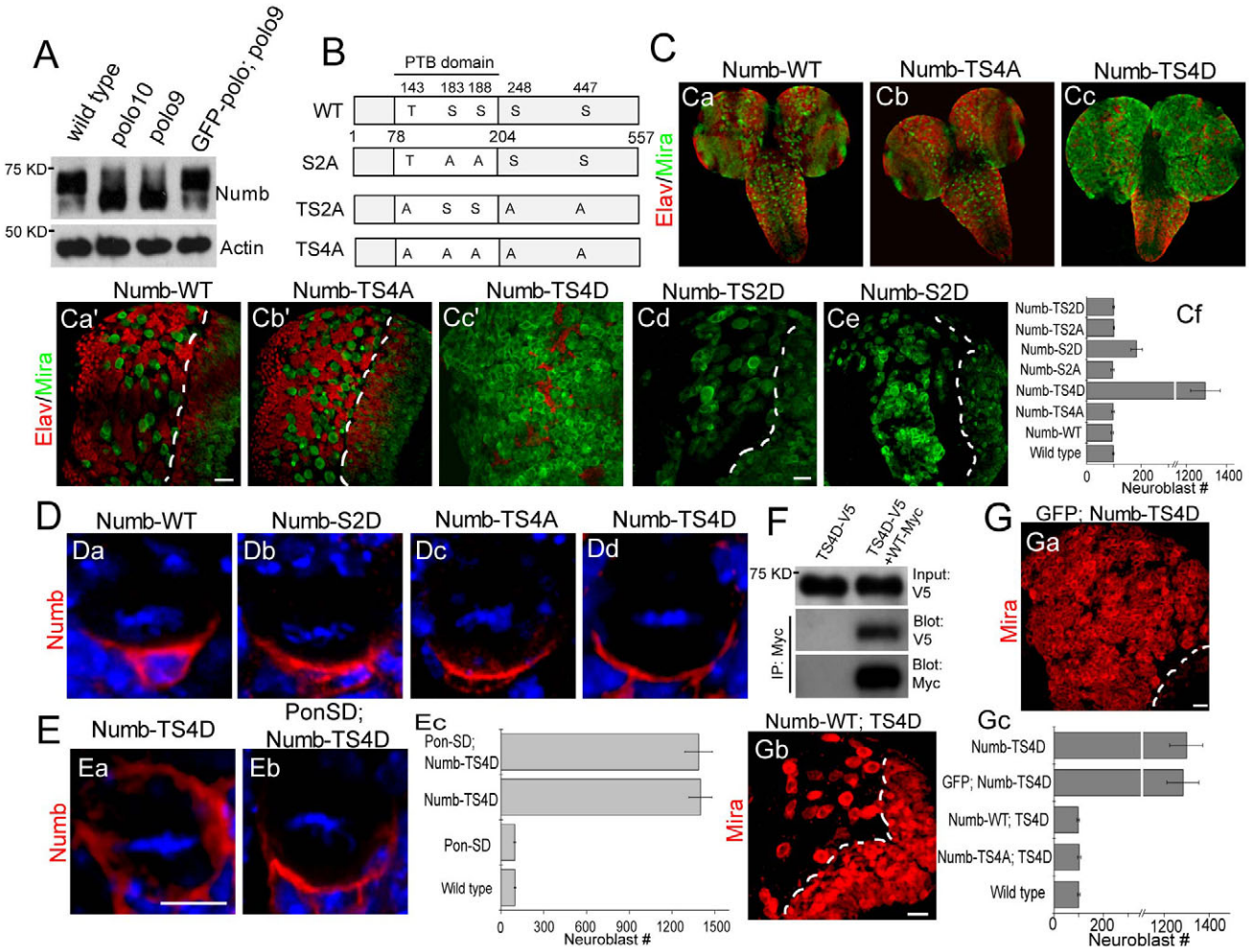

Fig. 1. A phospho-mimetic form of Numb induces ectopic neuroblasts. (**A**) The mobility of endogenous Numb is changed in *polo* mutants and is restored by Polo-GFP. (**B**) Schematic diagram of sites mutated in different Numb transgenes. The S/T residues mutated in Numb-S2A, -TS2A and -TS4A are shown in this diagram. The corresponding residues are changed to D in Numb-S2D, -TS2D and -TS4D. (**C**) Effects of expression of various forms of Numb on neuroblast number. Numb-WT (Ca), -TS4A (Cb), -TS4D (Cc), -TS2D (Cd) and -S2D (Ce) were expressed in neuroblasts using *insc-Gal4*. Neuroblasts are marked by Miranda (green, Ca-Ce) and differentiated neurons by Elav (red, Ca′-Cc′). Dashed lines separate central brain (left) from optic lobe (right) neuroblasts. Quantification of neuroblast number is shown in Cf. (**D**) Localization of Numb-WT (Da), -S2D (Db), -TS4A (Dc) and -TS4D (Dd) proteins (red). (**E**) Pon-SD rescues the mislocalization (Ea,Eb) but not the ENF effect (Ec) of Numb-TS4D. (**F**) Co-IP between Numb-TS4D and Numb-WT in HEK-293T cells. Lysates containing Numb-TS4D-V5 alone or both Numb-TS4D-V5 and Numb-WT-Myc were immunoprecipitated with anti-Myc and the presence of Numb-TS4D-V5 in the IP complex was detected with anti-V5. (**G**) Suppression of Numb-TS4D induced neuroblast overproliferation by Numb-WT (Gb), but not by GFP (Ga). Miranda is shown in red. Quantification of neuroblast number is shown in Gc. Scale bars: 20 μm in C,G; 5 μm in D,E. For additional data, see Figs S1, S2 in the supplementary material. Data are mean±s.e.m.

type, as indicated by the proliferation markers phospho-H3 (p-H3) and cyclin E (CycE) (see Fig. S2B′,C′ 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′). 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). In the case of Numb-TS4D, most of the neuroblasts showed normal basal crescents (Fig. 1Dd), but some neuroblasts showed compromised Numb localization at metaphase (20%, n=20) and telophase (12%, n=25) (Fig. 1Ea; see Fig. S2F,G,I-K in the supplementary material). As Numb-S2D is localized normally yet it can induce ENF, its effect on neuroblast homeostasis is unlikely to be due to aberrant localization. However, the mislocalization of Numb-TS4D in some neuroblasts and its stronger ENF phenotype suggest that aberrant localization might contribute to the stronger effects of Numb-TS4D. To test this possibility, we tried rescuing Numb-TS4D localization defect using Pon-SD, a mutant form of Pon mimicking the phosphorylated form that is required for Numb asymmetric

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 *Dll-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).

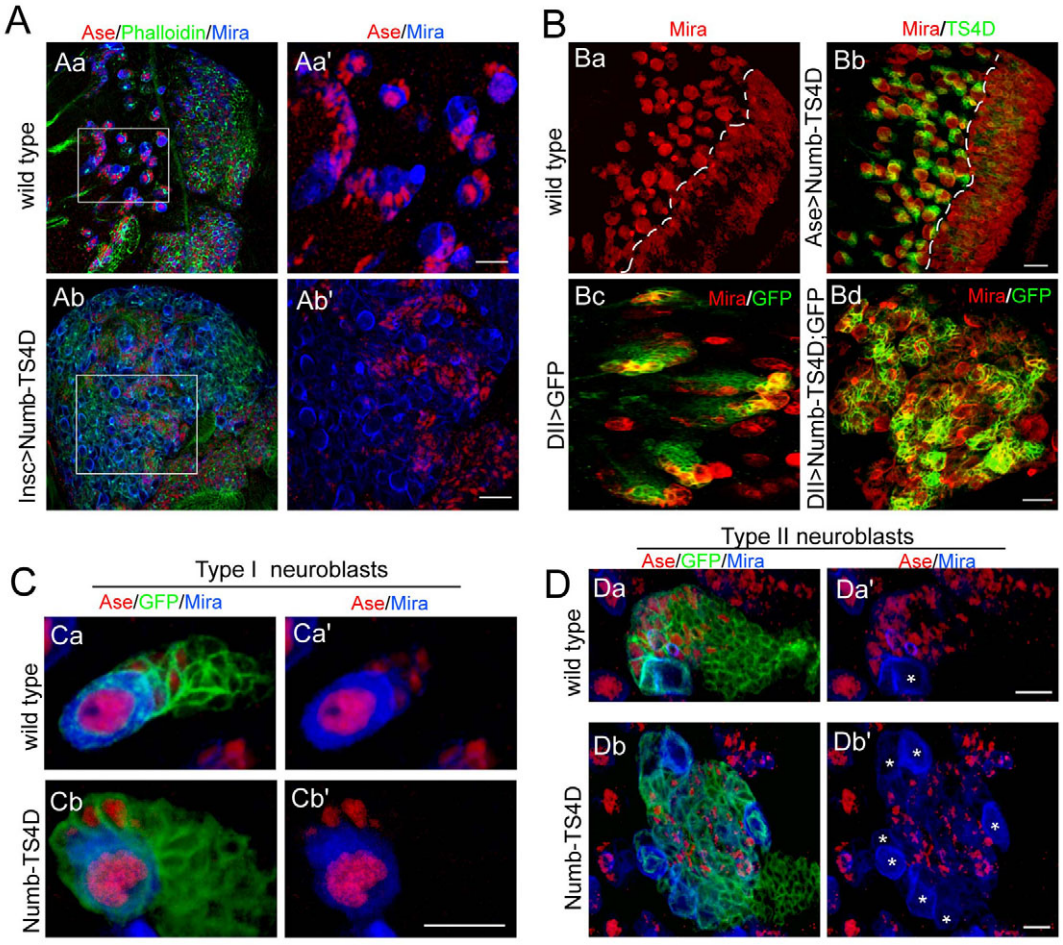

Fig. 2. Numb-TS4D specifically affects type II neuroblasts. (**A**) Ectopic neuroblasts induced by Numb-TS4D are Asense negative (Ase^{-}, red) and Miranda positive (Mira^{+}, blue). Magnified regions are boxed and shown in the right panels (Aa′,Ab′). (**B**) Specific expression of Numb-TS4D in type II neuroblasts using *Dll-Gal4* leads to ENF (Bd), whereas neuroblast number is not affected when Numb-TS4D is expressed in type I neuroblasts using *asense-Gal4* (Bb). (**C**,**D**) Clonal analysis of Numb-TS4D effect on type II neuroblast homeostasis. Wild-type or Numb-TS4D-expressing MARCM clones are marked with GFP (green). Numb-TS4D has no effects on Ase^{+} Mira^{+} type I neuroblast (Cb), but induced ectopic Ase^{-} Mira^{+} type II neuroblasts (Db, labeled as asterisks). Scale bars: 20 μm in A,B; 10 μm in C,D.

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 was significantly reduced compared with Numb-WT. The remaining ^{32}P label in Numb-TS4A probably represents phosphorylation at other sites (Smith et al., 2007). This result indicated that at least some, if not all, of the five sites mutated in Numb-TS4A are phosphorylated in HEK-293T cells. Consistent with this notion, 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 with PP2A resulted in removal of the phosphate groups (Fig. 3C), suggesting that PP2A and Polo exert opposite effects on Numb phosphorylation at these sites.

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 focused our analysis on the type II lineage, considering that Numb-TS4D affects only this lineage. We performed three lines of genetic interaction studies to test the possible regulation of Numb by Polo and PP2A. First, we analyzed the effects of ectopic Numb-WT or Numb-TS4A on *mts* mutant phenotypes. *mts* mutant type II neuroblast clones contain multiple neuroblasts (3.1±0.5 neuroblasts per clone, 15/17 clones observed; Fig. 3Db). Numb-TS4A completely rescued this phenotype, resulting in a single neuroblast per clone as in the control (Fig. 3Dd). By contrast, Numb-WT was unable to do so (Fig. 3Dc). As loss of asymmetric aPKC localization was observed in *PP2A* mutants (Chabu and Doe, 2009; Wang et al., 2009), it is possible that the inability of Numb-WT to rescue *mts* mutant phenotype is due to its phosphorylation by mislocalized aPKC, whereas Numb-TS4A somehow resisted aPKC phosphorylation. To rule out this possibility, we compared the extent of Numb-WT and Numb-TS4A phosphorylation by aPKC in vitro. Both Numb-WT and Numb-TS4A were efficiently phosphorylated by aPKC (see Fig. S4 in the supplementary material). Thus, Numb activity responds to PP2A in vivo and the phospho-sites mutated in Numb-TS4A are important for PP2A to antagonize phospho-Numb-induced ENF. Second, we tested for genetic interactions between Numb and Polo. As *Dll-Gal4*-directed overexpression of a constitutively active form of Polo (Polo-CA) in the whole brain caused embryonic lethality, we resorted to clonal analysis of Polo-CA in type II neuroblasts. As shown in Fig. 3Ea, ectopic neuroblasts in type II neuroblast clones were induced by Polo-CA, presumably through boosting endogenous phospho-Numb level (Fig. 3Ea). Interestingly, co-expression of Numb-TS4A effectively suppressed this phenotype (Fig. 3Eb). For unknown reasons, we were unable to generate type II neuroblast clones co-expressing Polo-CA and Numb-WT, despite multiple attempts. Nevertheless, the Numb-TS4A result supports the notion that the effect of Polo-CA on neuroblast homeostasis is mediated at least in part through phosphorylation of endogenous Numb at the sites mutated in Numb-TS4A. Third, to further test whether Polo and PP2A exert antagonistic effects on endogenous Numb, we co-expressed *mts*-RNAi knockdown and *Polo* overexpression transgenes specifically in type II neuroblasts. We used a GFP-tagged wild-type Polo transgene (*UAS-GFP-Polo-WT*) for this experiment. Knockdown of *mts* by RNAi using *Dll-Gal4* (Fig. 3Fa), or overexpression of *GFP-Polo-WT* by itself (see Fig. S5 in the supplementary material), had no effect on type II neuroblasts; however, co-expression of *mts*-RNAi together with *GFP-Polo-WT* resulted in an ~ 25% increase of ectopic neuroblasts (7.6±0.5 neuroblasts, 9/11 clones observed; Fig. 3Fb). We conclude that Polo and PP2A regulate Numb phosphorylation and activity in an antagonistic manner.

Numb-TS4D impairs Sanpodo (Spdo) endocytosis and fails to antagonize Notch activity

We next investigated the possible mechanisms by which Numb-TS4D affected neuroblast homeostasis. Numb is an endocytic protein and it antagonizes Notch activity (Guo et al., 1996; Santolini et al., 2000). We first asked whether the TS4D mutations affected the ability of Numb to inhibit Notch, as measured with the mγGFP 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,

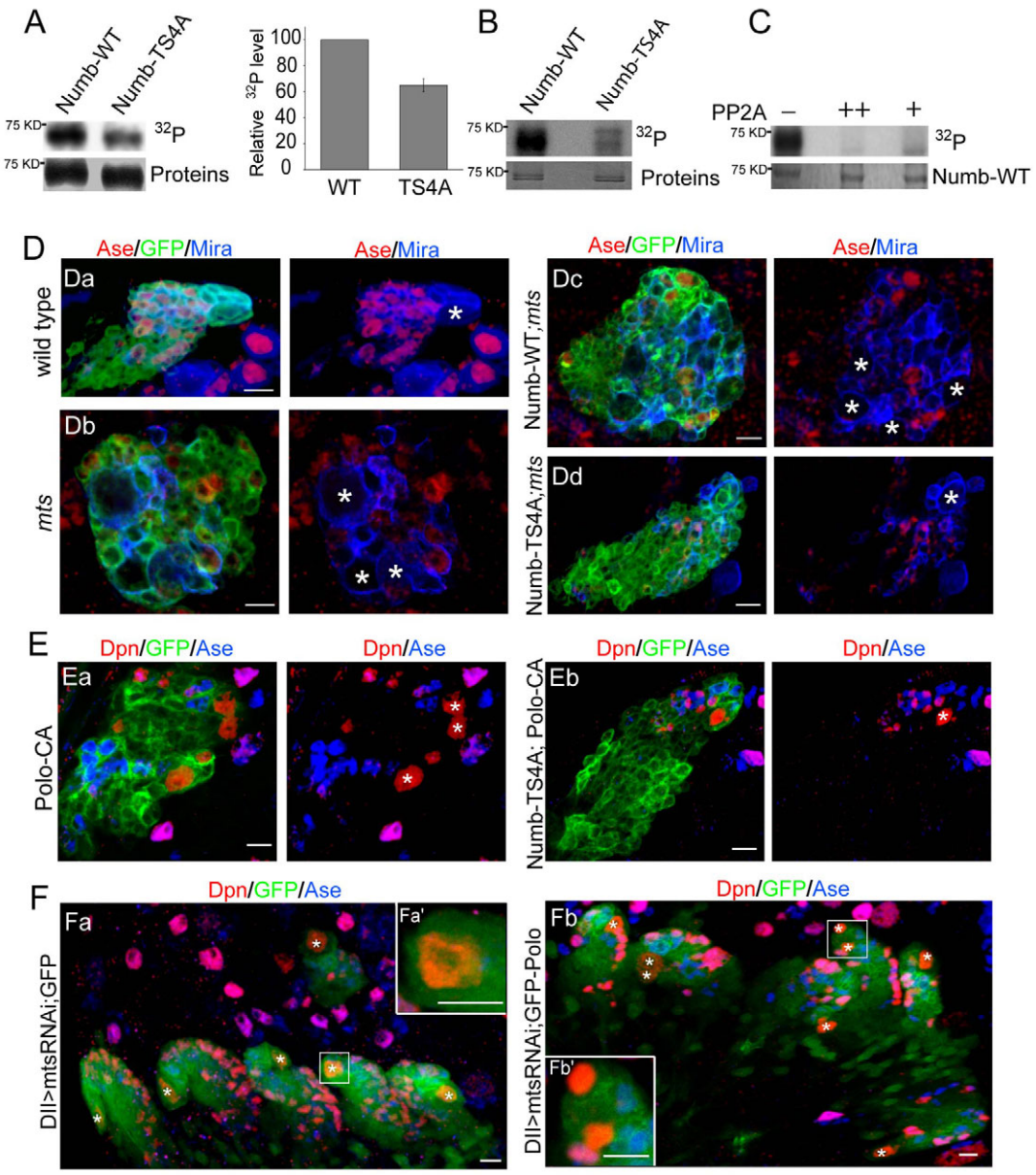

Fig. 3. Numb phosphorylation is regulated by Polo and PP2A. (**A**) In vivo ^{32}P labeling of Numb proteins in HEK-293T cells. ^{32}P-positive signal was normalized with the total amount of Numb protein on the same blot. Upper panel, autoradiography; lower panel, western blotting. Data on the right are mean±s.e.m. (**B**) In vitro phosphorylation of purified Numb-WT and Numb-TS4A by human PLK1. Upper panel, autoradiography; lower panel, Coomassie Blue staining of the same gel. (**C**) In vitro dephosphorylation of human PLK1-phosphorylated Numb-WT by PP2A. High (++, 15 ng/μl) and low (+, 5 ng/μl) concentrations of PP2A were used. Upper panel: autoradiography; lower panel, Coomassie Blue staining of the same gel. (**D**) Genetic interactions of Numb-WT or -TS4A with *mts* mutant. *mts*^{299} MARCM clones are marked with GFP (green). Type II neuroblasts are marked with asterisks. Numb-TS4A (Dd), but not Numb-WT (Dc), rescues ectopic neuroblasts in *mts* mutant (Db). (**E**) Co-expressing Numb-TS4A (Eb) inhibits ENF in type II lineage caused by Polo-CA (Ea). MARCM clones are marked with GFP (green). Type II lineage neuroblasts (Dpn^{+}Ase^{-}) are marked with asterisks. (**F**) Overexpression of GFP-Polo increases type II neuroblast number in Mts RNAi background. Type II lineage is labeled by a GFP transgene driven by *Dll-Gal4* and neuroblasts are marked with asterisks. Regions of interest in *Dll-Gal4>mts*RNAi (Fa) and *Dll-Gal4>mts*RNAi + *GFP-Polo* (Fb) animals are boxed and shown at higher magnification in Fa′,Fb′. Scale bars: 10 μm. For additional data see Figs S3-S5 in the supplementary material.

inhibition of Notch activity using either *Notch*^{ts} 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 *numb*^{15} 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 *numb*^{15} phenotype by cytoplasmically localized

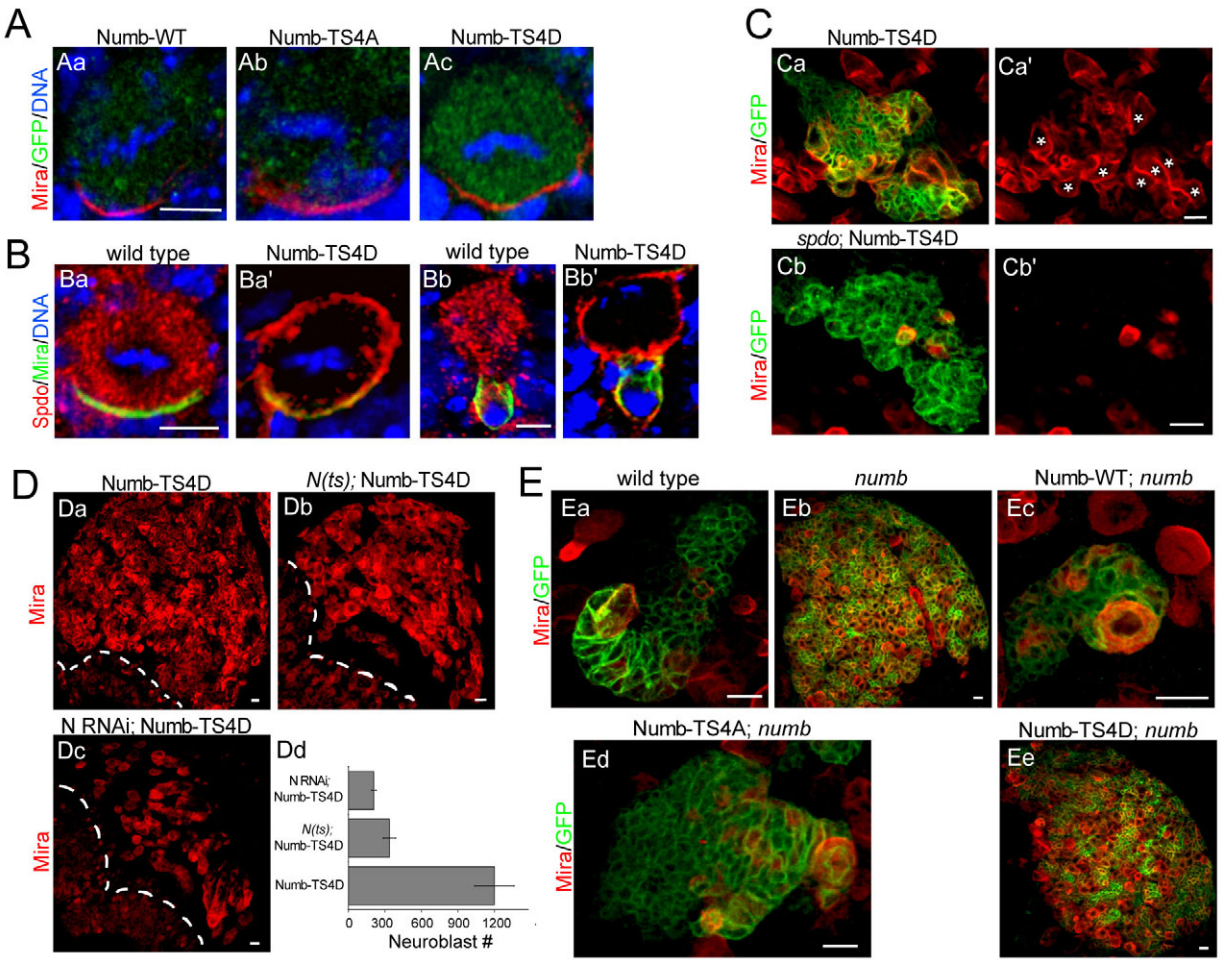

Fig. 4. Numb-TS4D loses its tumor suppressor activity. (**A**) Notch activity is elevated by Numb-TS4D (Ac), but not Numb-WT (Aa) or -TS4A (Ab). Notch reporter mγGFP was co-expressed with Numb-WT, -TS4A or -TS4D in neuroblasts. Metaphase neuroblasts were stained for GFP (green), Miranda (red) and DNA (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. *spdo*^{G104} 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 *numb*^{15} 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.

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 interactions between Numb and Dronc. Dronc-ΔN, 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 is 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). To examine whether the interaction between Dronc and Numb was compromised by TS4A or TS4D mutations in Numb, we carried out co-IP assay between Dronc-ΔN and Numb-WT, -TS4A or -S4D. Strikingly, compared with Numb-WT or -TS4A, Numb-TS4D exhibited a dramatic reduction of binding to Dronc (Fig. 5D). By contrast, the interaction between Numb and Pon, another Numb PTB domain-interacting protein, was not affected by the TS4D mutations (see Fig. S8B in the supplementary material). This result suggests that Numb phosphorylation mimicked by the TS4D mutations specifically impairs Numb interaction with Dronc.

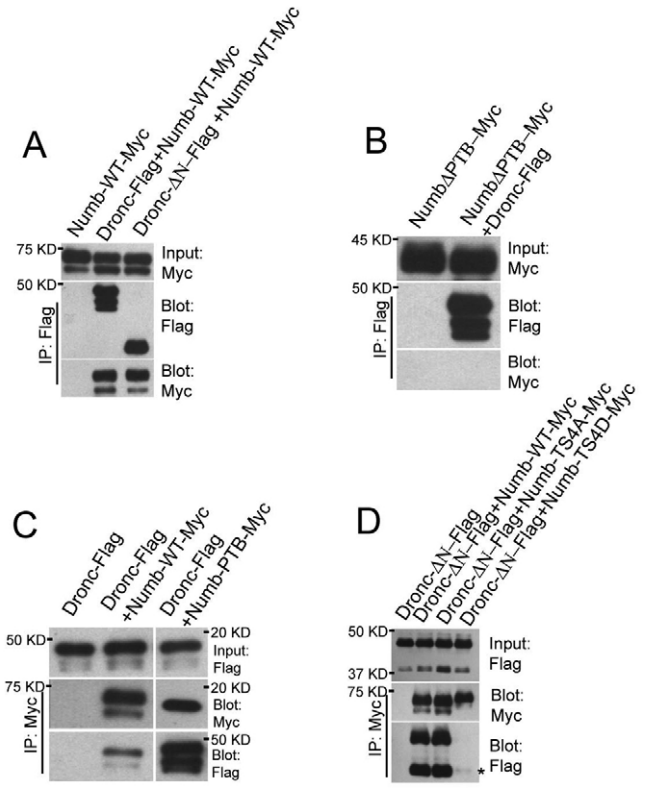

Fig. 5. Dronc is a novel binding partner of Numb. (**A**) Dronc physically interacts with Numb and the N-terminal CARD domain is dispensable for Dronc binding to Numb. Cells co-transfected with the Numb-Myc and Dronc-Flag constructs were subjected to co-IP assays in HEK-293T cells and the bound proteins in the IP complex were detected with Flag or Myc antibody. (**B**,**C**) The PTB domain of Numb is required and sufficient for Numb to bind to Dronc. Numb constructs with either the PTB domain deleted (B) or having only the PTB domain (C) were co-transfected with Flag-tagged Dronc and the interaction was examined by co-IP. (**D**) Numb-TS4D exhibits impaired binding to Dronc. Numb proteins were immunoprecipitated from cell extracts and bound Dronc-ΔN detected by western blot. Asterisk indicates residual binding of Dronc-ΔN to Numb-TS4D compared with the negative control. For additional data see Fig. S8 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 not 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 Notch^{intra}, a constitutively active form of Notch, was unable to suppress Notch^{intra}-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^{CAAX} 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* or *brat* 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

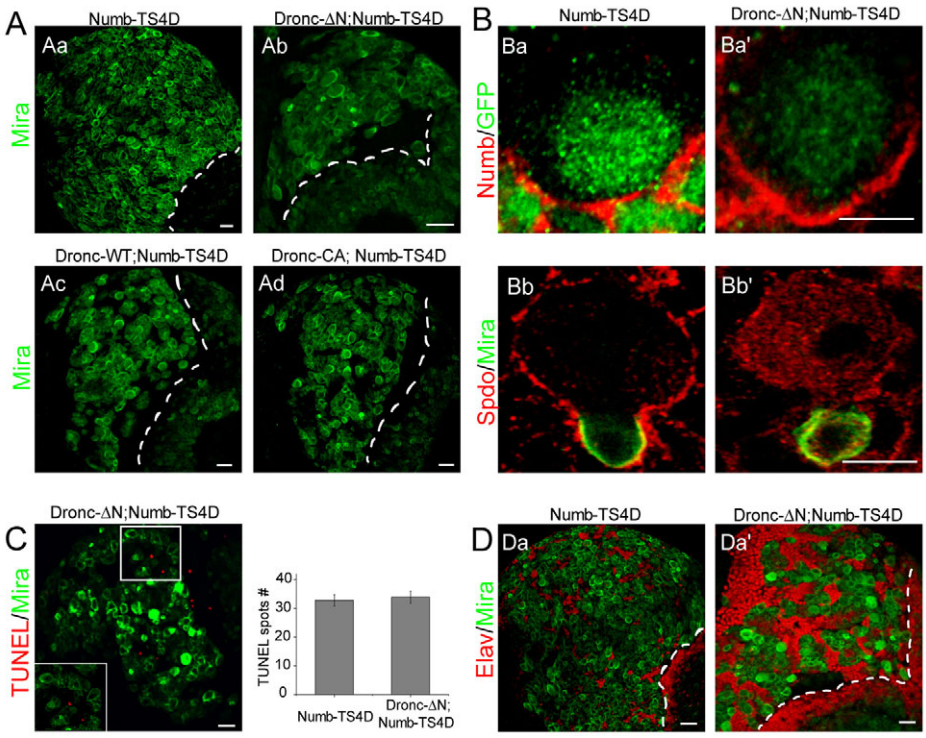

Fig. 6. The rescue of Numb-TS4D effect by Dronc is apoptosis independent. (**A**) Dronc suppresses Numb-TS4D-induced ENF. Dronc-ΔN (Ab), Dronc-WT (Ac) and Dronc-CA (Ad) were co-expressed with Numb-TS4D, and the effect on neuroblast number is quantified in Fig. S9A in the supplementary material. (**B**) Elevated Notch activity and ectopic Spdo cortical localization caused by Numb-TS4D (Ba,Bb) are rescued by Dronc-ΔN (Ba′,Bb′). (**C**) Larval brains co-expressing Numb-TS4D and Dronc-ΔN were stained for TUNEL (red) and Miranda (green). Note that TUNEL^{+} signals, as shown in the magnified inset, were not found in the neuroblasts. Graph shows quantification of TUNEL^{+} spots (mean±s.e.m.). (**D**) Dronc promotes neuroblast differentiation. Larval brains expressing Numb-TS4D (Da) or co-expressing Dronc-ΔN and Numb-TS4D (Da′) were stained for Miranda (green) and neuronal marker Elav (red). Scale bars: 20 μm in A,C,D; 5 μm in B. Central brain neuroblasts are located to the left of the broken line. For additional data, see Fig. S9 in the supplementary material.

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 *numb*^{S52F} mutant, which exhibits a weak loss of Numb activity (Bhalerao et al., 2005). MARCM clones of *numb*^{S52F} 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 *numb*^{S52F} (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

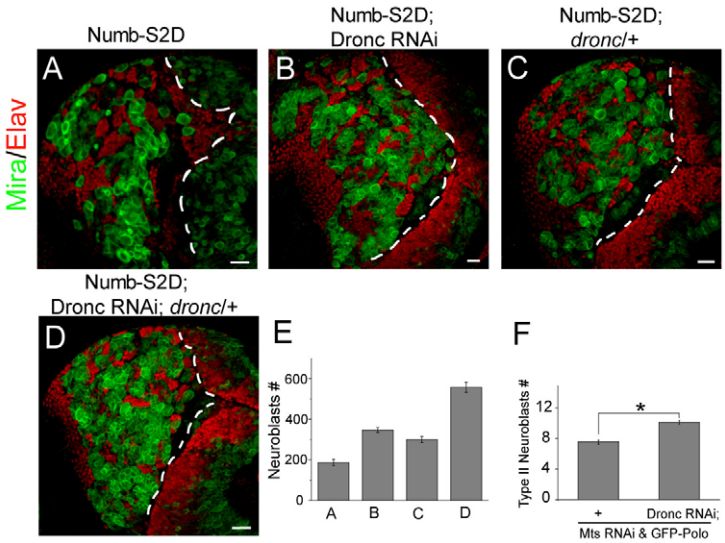

Fig. 7. Reduction of Dronc activity augments ENF caused by Numb-S2D. (**A-E**) Decreasing Dronc levels in Numb-S2D background (A) by Dronc RNAi (B), removal of one copy of *dronc* (C) or through combination of Dronc RNAi with *dronc* heterozygosity (D) leads to increased neuroblasts (Mira, green) at the expense of differentiated neurons (Elav, red). Quantification of neuroblast number is shown in E. Scale bars: 20 μm. Data are mean±s.e.m. (**F**) The number of type II neuroblasts is increased by ~25% when *Dronc RNAi* is co-expressed with *mts* RNAi and *GFP-Polo*, compared with co-expression of *mts* RNAi and *GFP-Polo* only. Data are mean±s.e.m. **P*<0.01. For additional data, see Figs S10, S11 in the supplementary material.

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., 1998; Lee 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; Mbefo 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 phospho-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 phospho-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 $numb^{S52F}$ mutant, 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 developmental process 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 Beyaert, 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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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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