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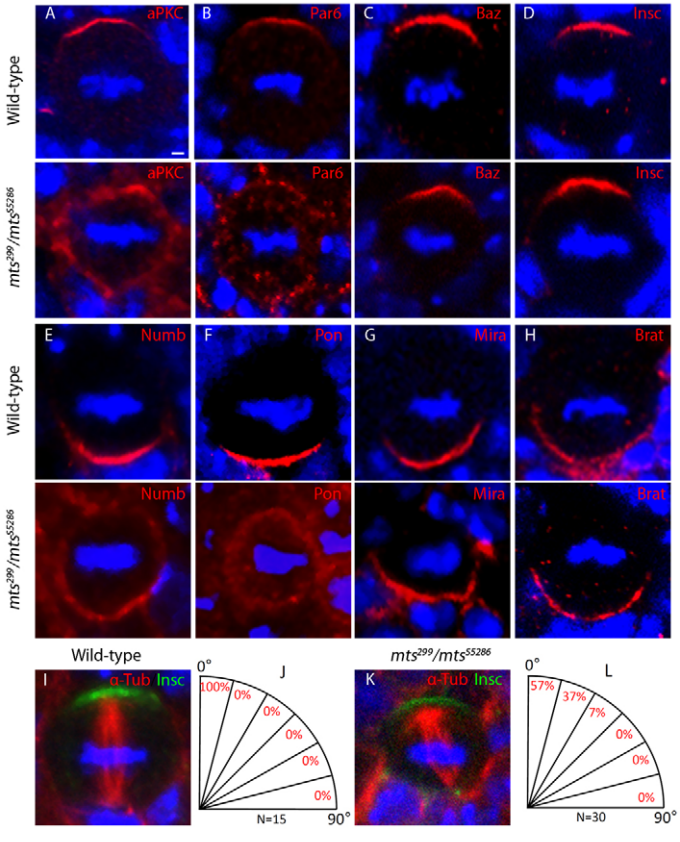
An incorrect version of Fig. 4, in which the same panel appears twice (upper panel of Fig. 4B and lower panel of Fig. 4C), was provided post-acceptance. The correct Fig. 4 appears below.

The authors apologise to readers for this mistake.



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Protein phosphatase 2A regulates self-renewal of *Drosophila* neural stem cells

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Drosophila larval brain neural stem cells, also known as neuroblasts, divide asymmetrically to generate a self-renewing neuroblast and a ganglion mother cell (GMC) that divides terminally to produce two differentiated neurons or glia. Failure of asymmetric cell division can result in hyperproliferation of neuroblasts, a phenotype resembling brain tumors. Here we have identified *Drosophila* Protein phosphatase 2A (PP2A) as a brain tumor-suppressor that can inhibit self-renewal of neuroblasts. Supernumerary larval brain neuroblasts are generated at the expense of differentiated neurons in *PP2A* mutants. Neuroblast overgrowth was observed in both dorsomedial (DM)/posterior Asense-negative (PAN) neuroblast lineages and non-DM neuroblast lineages. The PP2A heterotrimeric complex, composed of the catalytic subunit (Mts), scaffold subunit (PP2A-29B) and a B-regulatory subunit (Tws), is required for the asymmetric cell division of neuroblasts. The PP2A complex regulates asymmetric localization of Numb, Pon and Atypical protein kinase C, as well as proper mitotic spindle orientation. Interestingly, PP2A and Polo kinase enhance Numb and Pon phosphorylation. PP2A, like Polo, acts to prevent excess neuroblast self-renewal primarily by regulating asymmetric localization and activation of Numb. Reduction of PP2A function in larval brains or S2 cells causes a marked decrease in Polo transcript and protein abundance. Overexpression of Polo or Numb significantly suppresses neuroblast overgrowth in *PP2A* mutants, suggesting that PP2A inhibits excess neuroblast self-renewal in the Polo/Numb pathway.

KEY WORDS: PP2A, Self-renewal and differentiation, *Drosophila*, Neural stem cell, Neuroblast, Asymmetric division

INTRODUCTION

Drosophila larval brain neuroblasts, also known as neural stem cells (NSCs), divide asymmetrically to give rise to a self-renewing daughter, that continues to divide asymmetrically, and a ganglion mother cell (GMC), that is committed to differentiation. The mechanisms of neuroblast asymmetric division have been studied primarily in embryonic neuroblasts; these mechanisms appear to be conserved between embryonic and larval brain neuroblasts (Doe, 2008; Gonczy, 2008; Gonzalez, 2007; Knoblich, 2008; Wu et al., 2008). During asymmetric division of embryonic neuroblasts, cell fate determinants Numb, Prospero (Pros), Brain tumor (Brat), and their adaptor proteins Partner of Numb (Pon) and Miranda (Mira) are basally localized and segregated into the GMC (Ikeshima-Kataoka et al., 1997; Knoblich et al., 1995; Lu et al., 1998; Rhyu et al., 1994; Shen et al., 1997). The mitotic spindle is oriented along an axis perpendicular to the epithelial layer and an asymmetric spindle is generated to give rise to two unequally sized daughter cells with a distinct cell fate. The localization of basal proteins and mitotic spindle orientation are controlled by apically localized proteins that include Bazooka (a *Drosophila* homolog of Par3), Par6 and Atypical protein kinase C (aPKC) (Kuchinke et al., 1998; Petronczki and Knoblich, 2001; Wodarz et al., 2000), Inscuteable (Insc) (Kraut et al., 1996), and heterotrimeric G protein Gαi (Schaefer et al., 2001; Yu et al., 2003) and its regulators Partner of Insc (Pins, also known as Raps) (Yu et al., 2000), Locomotion defects (Loco) (Yu et al., 2005) and a Pins-interacting protein known as Mushroom body defect (Mud) (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). Cortically localized Gβ and Gγ regulate asymmetric localization of Gαi (Schaefer et al., 2001; Yu et al., 2003), and Ric8a (DmRic8) is required for Gαi membrane localization (Hampoelz et al., 2005; Wang et al., 2005). Basal protein localization and segregation are mediated by apical proteins through cortically localized tumor suppressors Discs large, Scribbled and Lethal (2) giant larvae (Albertson et al., 2004; Albertson and Doe, 2003; Ohshiro et al., 2000; Peng et al., 2000).

Drosophila larval brain neuroblasts have recently emerged as a model for studying stem cell self-renewal and tumorigenesis. Failure of asymmetric cell division can result in their hyperproliferation, a phenotype resembling brain tumors. Mutant larval brain tissue from *pins*, *mira*, *numb* or *pros*, when transplanted into wild-type adults, can form malignant tumors that rapidly kill the host (Caussinus and Gonzalez, 2005). Recent studies suggest that neuroblasts utilize the asymmetric localization/segregation machinery to distribute 'proliferation factor' to the neuroblast daughter and 'differentiation factor' to the GMC daughter during asymmetric division. aPKC, a neuroblast proliferation factor, which acts at least partially through regulating Numb asymmetry, leads to neuroblast overgrowth when overexpressed in the entire cortex (Lee et al., 2006a; Lee et al., 2006b; Wang et al., 2006). Proteins that are asymmetrically segregated into the GMC, including Numb, Pon, Pros, Brat and Mira, are 'differentiation factors' and have brain tumor-suppressor function to prevent neuroblast overgrowth (Bello et al., 2006; Betschinger et al., 2006; Caussinus and Gonzalez, 2005; Choksi et al., 2006; Lee et al., 2006a; Lee et al., 2006b; Lee et al., 2006c; Wang et al., 2006; Wang et al., 2007). Numb antagonizes Notch signaling to inhibit neuroblast overproliferation, whereas Notch promotes

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neuroblast self-renewal at larval central brains (Wang et al., 2006). Cell cycle regulators Aurora A and Polo also have brain tumor-suppressor function and both inhibit neuroblast overproliferation primarily by regulating Numb asymmetry (Lee et al., 2006a; Wang et al., 2006; Wang et al., 2007). Polo can directly phosphorylate Pon on S611, which localizes Pon and subsequently Numb asymmetrically (Wang et al., 2007). Aurora A has been shown recently to phosphorylate Par6 to allow aPKC to phosphorylate and asymmetrically localize Numb (Wirtz-Peitz et al., 2008).

Despite studies of protein kinases during asymmetric division of neuroblasts, very little is known about the role of protein phosphatases in this process. Mammalian protein phosphatase 2A (PP2A, also known as Ppp2ca) is a tumor suppressor that regulates cell cycle regulation, various signaling pathways and tumorigenesis (Li et al., 2002; Westermarck and Hahn, 2008). Somatic mutations of PP2A have been reported in human lung, breast, and colon cancers as well as melanomas (Westermarck and Hahn, 2008). PP2A was shown to bind to viral oncoprotein simian virus (SV40) small t (ST) splicing form that is able to transform mammalian cells (Chen et al., 2007b; Cho et al., 2007). In addition, RalA and cMyc have been shown to be tumorigenic substrates of PP2A (Junttila et al., 2007; Sablina et al., 2007). However, it is unclear whether PP2A regulates neural stem cell homeostasis. Here we show that *Drosophila* PP2A behaves as a brain tumor-suppressor that regulates the balance of neural stem cell self-renewal and differentiation.

MATERIALS AND METHODS

Fly stocks

Fly culture and crosses were performed according to standard procedures and flies were raised at 25°C unless otherwise indicated. *Drosophila* stocks used in this study are: mts^{299} (this work); tws^{60} (T. Uemura, Kyoto University, Yoshida Konoe-cho, Sakyo-ku, Kyoto, Japan); Ase-Gal4 (Y. Lee, University of Massachusetts Medical School, Worcester, MA, USA); UAS-Mts, wdb^{dw} and wdb^{IP} (S. Eaton, Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstr, Dresden, Germany); *par1Δ16* and UASPar1 (D. St Johnston, Wellcome/CRC Institute and Department of Genetics, University of Cambridge, Cambridge, UK); $aPKC^{06403}$ (C. Doe, University of Oregon, USA, Eugene, OR, USA); GFP-Polo (C. Sunkel, Universidade do Porto, Porto, Portugal); UAS-venus-Polo (this study); $wrd^{KG01108}$ $wdb^{12\text{-}1es}$ (T. Megraw, University of Texas Southwestern Medical Center, Dallas TX, USA); UAS-Numb-GFP (Y. N. Jan, UCSF, San Francisco, CA, USA). PP2A RNAi stocks are from the Vienna *Drosophila* RNAi Center and all other fly stocks are from Bloomington.

Antibodies and immunohistochemistry

Immunohistochemistry on larval brain tissues was performed as described (Wang et al., 2006). The primary antibodies used were: guinea pig anti-Dpn (1:1000, J. Skeath, Washington University School of Medicine, St. Louis, MO, USA), mouse anti-Mira (1:50, F. Matsuzaki, Riken Center for Developmental Biology, Chuo-ku, Kobe, Japan), mouse anti-Worniu (1:500), rat anti-Elav (1:10, DSHB), mouse anti-Pros (1:10, DSHB), mouse anti-CycE (1:10, H. Richardson, Peter MacCallum Cancer Centre, Melbourne, Australia), rabbit anti-phospho-Histone H3 (1:1000, Sigma), mouse anti-dMyc [1:5, B. Edgar, Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA, USA], mouse anti-BrdU (1:20, Roche), anti-Baz (1:500, A. Wodarz, Universitätsmedizin Göttingen, Göttingen, Germany), rabbit anti-PKCζ C20 (1:500, Santa Cruz Biotechnology), rabbit anti-Par6 (1:500, J. Knoblich, Institute of Molecular Biotechnology GmbH, Vienna, Austria), rabbit anti-Insc (1:1000), guinea pig anti-Numb (1:1000, J. Skeath), rabbit anti-Pon (1:500, Y. N. Jan), rabbit anti-GFP (1:500, Molecular Probes), rabbit anti-S7Numb (1:500, K. Kaibuchi, Nagoya University, Showa, Nagoya, Aichi, Japan). Antibodies for western blotting were: guinea pig anti-Numb (1:1000, J. Skeath), mouse anti-Polo (1:100, C. Sunkel), rabbit anti-pS611Pon (1:500, B. Lu, Stanford University, Palo Alto, CA, USA), rabbit anti-PKCζ C20 (1:1000; Santa Cruz Biotechnology), mouse anti-α-tubulin (1:1000, Millipore), rabbit anti-p-aPKC (1:1000, Abcam).

Production of dsRNA

Individual DNA fragments ranging from approximately 400 to 800 bp in length were amplified either from cDNA clones from the *Drosophila* Genomics Resource Center (for *mts*, *PP2A-29B* and *wdb*) or an S2 cell cDNA library (for *tws*, *B56-1* and *PR-72/CG4733*). PCR primers contain a T7 RNA polymerase binding site at the 5′ end (Table 1). Double stranded RNA (dsRNA) was synthesized from 1 μg of the purified PCR product by incubating a 20 μl reaction at 37°C for 24 hours using the MEGAscript T7 Transcription Kit (Ambion). The RNA products were ethanol precipitated at –20°C and resuspended in diethyl pyrocarbonate-treated water. To anneal the single stranded RNA, samples were incubated for 30 minutes at 65°C and cooled slowly (4 hours to overnight) to room temperature.

Cell culture, dsRNA and drug treatment

Drosophila S2 cells were maintained in Shields and Sang M3 insect medium (Sigma-Aldrich), and supplemented with 10% fetal bovine serum (FBS; Hyclone). For dsRNA treatment, a final concentration of 20 μg/ml of the specified dsRNA was used. Calyculin A (Calbiochem) and the proteasome inhibitor MG132 were used at 30 nM and 100 μM, respectively. Production of dsRNA was performed as described previously (Sathyanarayanan et al., 2004).

Reverse transcriptase (RT)-PCR

Total RNA was isolated from S2 cells with TRI reagent (Sigma-Aldrich) followed by DNase I treatment (Sigma-Aldrich) as described by the manufacturer. Target messages were reverse transcribed using the ProtoScript First Strand cDNA Synthesis Kit (NEB). PCR was performed using the Expand High Fidelity PCR System (Roche). Targets to be amplified were approximately 250-750 bp in length, with PCR primers designed to traverse at least 1 intron.

Table 1. Primers used for making dsRNA

Gene	CG number		Primers* (5′ to 3′)
PP2A-A (*PP2A-29B*)	CG17291	F	GACTTCTGCGCCAATCTGGAC
		R	ATCAATGACGCTGGCCTCCAG
PP2A-C (*mts*)	CG7109	F	ATGCATCGCTAATCGATACAC
		R	GTACACCTGTGTGATCTGGC
PR55 (*tws*)	CG6235	F	GTTAATTCGGATCAGGAGACC
		R	TTGCGATCGAAGACGCGGAAG
B56-1	CG7913	F	CCTGAAGACTGTTTTACATCG
		R	CGTTCAAAAACATAACCTCC
B56-2 (*wdb*)	CG5643	F	GAGGACGATCCGACACTGGAG
		R	CATGATCGGCATGATGACCGC
PR72	CG4733	F	GATCCAGATACGTGCACACAG
		R	GAA-ACCACACAAAGTCCGTG

*T7 promoter sequence 5′-TTAATACGACTCACTATAGGGAGA-3′ precedes both forward (F) and reverse (R) primers.

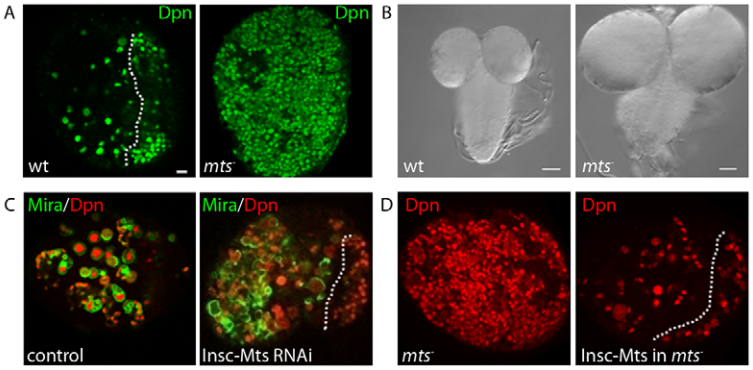

Fig. 1. Microtubule star (Mts) is a novel brain tumor-suppressor in *Drosophila* larval brains.
(**A**,**B**) Supernumerary larval brain neuroblasts (A) and highly enlarged larval brains (B) form in mts^{299} mutants (right panels). (**C**) *mts RNAi* expressed using the Insc-Gal4 driver results in supernumerary neuroblasts in the larval brains. (**D**) Overexpression of wild-type *mts* can completely rescue the brain tumor phenotype of mts^{299}. Neuroblasts are marked by Dpn or Mira, white dotted lines mark the margin between the central brain and the optic lobe; central brain to the left in A,C,D. Scale bars: 10 μm in A for A,C,D; 1 μm in B.

Western blotting

Larval brains or S2 cells were homogenized in RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) and western blotting was carried out according to standard procedures.

Neuroblast quantification and brain orientation

Quantifications of larval central brain neuroblast numbers were done on samples 24 hours to 96 hours after larval hatching (ALH) at 25°C. For each genotype, 4- to 6-hour embryo collections were obtained from a bottle with 100-200 flies. Embryos were allowed to develop for 20 hours at 25°C before hatching. Thereafter, time-course experiments were performed 24 hours, 48 hours, 72 hours and 96 hours ALH according to the midpoint of the 4- to 6-hour time window. Around 15-20 larvae from each time point were dissected, and stained with anti-Dpn antibody (1:1000). Larval brains were mounted with their dorsal side up. Central brain neuroblasts can be distinguished from optic lobe neuroblasts on the basis of their medial-superficial location in the brain, larger size and dispersed pattern.

BrdU labeling

Dissected larval tissue was given a 40-minute pulse of 37.5 mg/ml BrdU in Shields and Sang 3M insect medium, and fixed for 15 minutes in 3.7% formaldehyde. DNA was denatured with 2 M HCl for 40 minutes, and tissue was washed with PBS and incubated with anti-BrdU.

Spindle orientation quantification

Larval neuroblasts were stained with rat anti-α-Tubulin (1:250, Abcam) and rabbit anti-Insc. Confocal images were captured and were used for quantification. One line was drawn parallel to the metaphase spindle (indicated by Tubulin) and another line perpendicular to the apical crescent (marked by Insc). The angle at which the two lines met was then measured.

RESULTS

Microtubule star (Mts) is a novel brain tumor-suppressor in *Drosophila*

We identified Microtubule star (Mts), which encodes the catalytic subunit of Protein phosphatase 2A (PP2A), as a brain tumor-suppressor from a genetic screen in *Drosophila*. We isolated mts^{299}, a hypomorphic allele of *mts*, that produced supernumerary neuroblasts [marked by Deadpan (Dpn), Fig. 1A] and highly enlarged larval brain lobes (Fig. 1B) and has a mis-sense mutation (aspartic acid 197 to asparagine) in *Mts*. mts^{299} flies survive to the pupal stage, whereas *mts*-null ($mts^{XE\text{-}2258}$) flies die during embryogenesis (Snaith et al., 1996). In $mts^{299}/mts^{XE\text{-}2258}$ trans-heterozygotes, neuroblast overproliferation is also observed, although they die as second instars (see Fig. S1A in the supplementary material). RNAi-mediated knockdown of *mts* using the neuroblast-specific driver Inscuteable-Gal4 (Insc-Gal4) also caused neuroblast overproliferation in the larval brains (Fig. 1D). A transgene expressing wild-type *mts* fully rescued the neuroblast overproliferation phenotype of *mts* mutants (Fig. 1E). These data indicate that reduced Mts function can cause neuroblast overgrowth in the larval brain.

PP2A is a conserved serine/threonine phosphatase that functions as a trimeric protein complex composed of a catalytic subunit (C, Mts in *Drosophila*), a scaffold subunit (A, PP2A-29B), and one of the variable regulatory B (Twins), B′ (B56-1 and Widerborst) or B″ (PR-72) subunits (Li et al., 2002; Westermarck and Hahn, 2008). Mammalian PP2A has been implicated in various processes including cell cycle progression, cell death regulation and tumorigenesis (Janssens et al., 2005; Westermarck and Hahn, 2008). *Drosophila* PP2A functions in mitosis and *PP2A* mutants display mitotic abnormalities in the dividing neuroblasts (Chen et al., 2007a; Deak et al., 2003). We speculated that these distinct phenotypes seen with different *PP2A* mutants were due to in part to varying degrees of catalytic impairment. To test this, we examined the effects on BrdU incorporation in larval brains in which *mts* function was attenuated to various levels by driving *mts* RNAi in vivo using the Insc-Gal4 driver at various temperatures. *mts* RNAi is expected to reduce Mts function most at 30°C, and least at 13°C, as Gal4 activity is known to be temperature-dependent, with higher activity at a higher temperature. Interestingly, at both 13°C and 18°C, *mts* RNAi results in more BrdU incorporation compared with wild type, whereas at 25°C or 30°C, the *mts* RNAi brain had much less BrdU incorporation than wild type (see Fig. S2 in the supplementary material). This suggests that partial loss-of-function of Mts results in overproliferation of neuroblasts, whereas more severe perturbation of Mts function prevents cells from dividing. This is consistent with the inability to generate *mts*-null mutant clones in bristles (Hannus et al., 2002), which is likely to be due to the essential cell division function of Mts. Thus, PP2A has dual functions as a brain tumor-suppressor and a cell cycle regulator.

PP2A can inhibit excess self-renewal of neuroblasts

To examine the role of Mts in neuroblast self-renewal and differentiation, we first quantified central brain neuroblast numbers in mts^{299}/mts^{S5286} trans-heterozygotes. Both wild type and *mts* mutants have similar numbers of central brain neuroblasts at 24 hours after larval hatching (ALH). However, *mts* mutants generated around 1000 neuroblasts that are marked by Mira or Worniu at 96 hours ALH, whereas the wild type had only around 100 neuroblasts (Fig. 2A-C). Conversely, neuronal differentiation in *mts* mutants is impaired, as neurons marked by Embryonic lethal abnormal vision (Elav) or nuclear Prospero (Pros) are drastically reduced (Fig. 2D,E). Consistent with *mts* mutants producing a neuroblast overgrowth phenotype, the number of cells expressing markers associated with proliferation, including CycE (Fig. 2F), phospho-

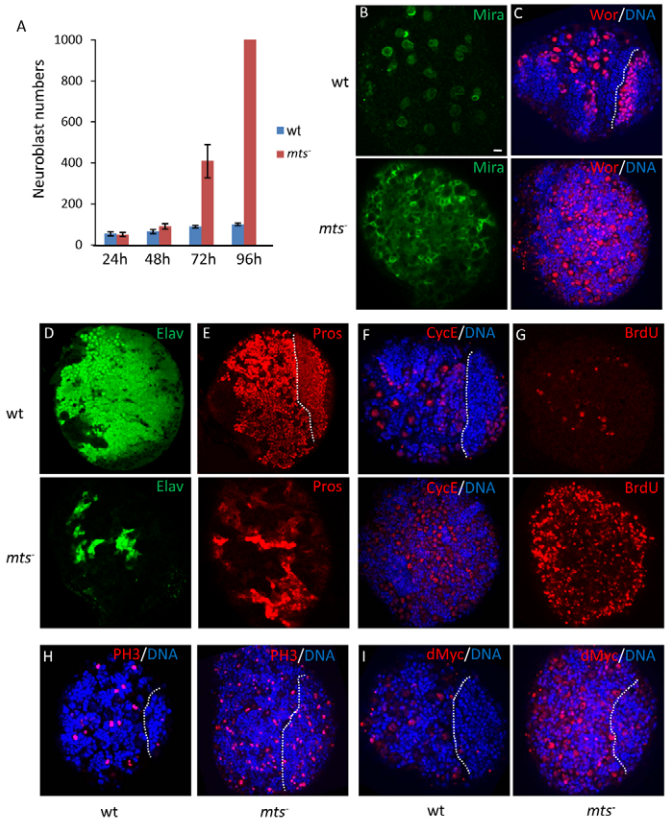

Fig. 2. PP2A can suppress neuroblast overproliferation and promote neuronal differentiation. (**A**) Quantification of neuroblast numbers in wild type (wt) and mts^{299}/mts^{55286} mutants from 24 hours to 96 hours ALH; n=20 per time point per genotype. (**B-I**) Confocal single scanning images of wild-type (upper panels in B-G) and mts^{299}/mts^{55286} mutant (lower panels in B-G) larval brains at 96 hours ALH that were examined for neuroblast markers Mira (B) and Wor (C), neuronal markers Elav (D) and Pros (E), G1/S cyclin CycE (F), cell proliferation markers BrdU (G) and phospho-Histone H3 (PH3, H), or cell growth factor Myc (I). DNA is blue. Dotted lines indicate the margins between central brains (to the left) and optic lobes. Scale bar: 10 μm.

Histone H3 (Fig. 2H) and the cell growth factor dMyc/c-Myc (Fig. 2I) are increased. An increase of BrdU-incorporation is also observed in *mts* mutants (Fig. 2G). Notch was previously shown to be a neuroblast proliferation factor in larval central brains (Wang et al., 2006). In *mts* mutants, the number of Notch-positive cells is increased (data not shown). Taken together, these findings show that neuroblast overproliferation occurs at the expense of neuronal differentiation in *mts* mutants.

Recently, it was reported that transit-amplifying intermediate progenitor cells exist in Asense (Ase)-negative dorsomedial (DM)/posterior Asense-negative (PAN) neuroblast lineages, but not Ase-positive non-DM neuroblast lineages in the larval brain (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). We show that Mts can inhibit excess neuroblast self-renewal in both Ase-negative DM and Ase-positive non-DM neuroblast lineages, as mts^{299} larval brains contain an increased number of both Ase^{+} and Ase^{-} neuroblasts compared with wild type (Fig. 3A). This is in contrast to the overgrowth of *brat* mutants that formed almost entirely Ase^{-} neuroblasts in larval brains, as Brat appears to act only in DM/PAN lineages (Bowman et al., 2008). *mts* RNAi using an Ase-Gal4 driver expressed only in Ase-positive non-DM neuroblast lineages caused neuroblast overproliferation in larval brains (Fig. 3B), although this was weaker than the phenotype derived from *mts* RNAi by Insc-Gal4, which drives expression in both neuroblast lineages (Fig. 1D). In addition, mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 1999) clone analysis in mts^{299} also suggests neuroblast overgrowth in both neuroblast lineages (Fig. 3C,D). The *mts*-null mutant is unable to generate MARCM clones (not shown), consistent with the cell lethal phenotype described previously (Hannus et al., 2002). When mts^{299} MARCM clones that were induced during the early larval stage were kept to adulthood, some of the clones in the adult brains had multiple phospho-Histone $H3^{+}$ and/or Dpn^{+} cells; this was never seen in wild-type MARCM clones in adult brains (Fig. 3E), suggesting that some of the mutant cells continue to proliferate until adulthood. These results indicate that Mts can inhibit excess self-renewal of *Drosophila* neural stem cells.

The protein product of Mts, the catalytic subunit of PP2A, normally functions in a heterotrimeric protein complex (Janssens et al., 2005). To ascertain the function of the A and B regulatory subunits of PP2A during larval brain neuroblast self-renewal, we examined the single A subunit (*PP2A-29B*) as well as the multiple B subunits [*twins* (*tws*), *widerborst* (*wdb*), *B56-1* and *PR-72*] using in vivo RNAi or by mutations. Subunit A of PP2A can inhibit neuroblast overgrowth, as *PP2A-29B* RNAi, as well as trans-heterozygotes between two lethal P elements $PP2A\text{-}29B^{RS}$ and $PP2A\text{-}29B^{EP}$, which survive to the third instar larval stage, displayed neuroblast overproliferation in larval brains (Fig. 3F and see Fig. S1C,E in the supplementary material). In tws^{60} mutant brains, the number of neuroblasts is also slightly increased compared with wild type (see Fig. S1D,E in the supplementary material). This weak phenotype observed in tws^{60} is likely to be due to other defects including apoptosis and/or cell division in tws^{60}. Consistently, a significant number of cells in tws^{60} larval brains are labeled by activated Caspase 3 (also known as Decay), in contrast with wild-type brains in which very few cells are labeled (see Fig. S1F in the supplementary material). Furthermore, a weak adult-lethal *tws* mutant was shown to cause bristle duplication (Shiomi et al., 1994), suggesting that a mild perturbation of Tws function can lead to cell fate transformation. Other B regulatory subunits including Wdb, B56-1 and PR-72 do not obviously influence neuroblast self-renewal. wdb^{IP} or wdb^{dw} MARCM clones, as well as *B56-1* and *PR-72* RNAi knockdown, do not generate any defects in the self-renewal of larval brain neuroblasts (data not shown). It is possible that some B subunits are redundant for the regulation of neuroblast self-renewal, as both B56 subunits were shown to have redundant functions in regulating cell death (Li et al., 2002). However, in a double mutant of *B56 wdb* ($wrd^{KG01108}$ $wdb^{12\text{-}1es}$), we did not observe any defect in asymmetric division or self-renewal of neuroblasts (data not shown). Therefore Tws appears to be the B regulatory subunit of PP2A that controls the self-renewal of larval brain neuroblasts.

PP2A regulates asymmetric protein localizations as well as mitotic spindle orientation

To explore whether PP2A regulates asymmetric cell division of neuroblasts, the distribution of asymmetrically localized proteins were examined in *PP2A* mutants. The asymmetric localization of most polarity proteins, including aPKC (Fig. 4A; 70.0%, n=30), Par6 (Fig. 4B; 33.3%, n=30), Baz (Fig. 4C; 36.4%, n=48) and Insc (Fig. 4D; 35.4%, n=48) are affected to various degrees in mts^{299} and

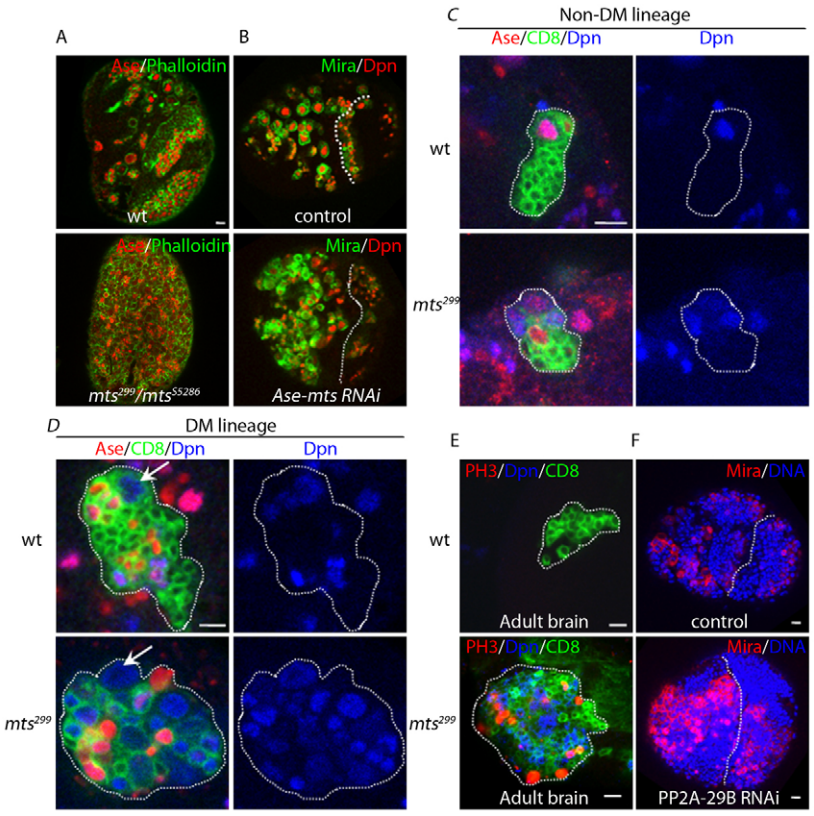

Fig. 3. Mts inhibits neuroblast overgrowth in both Ase-positive non-DM and Ase-negative DM neuroblast lineages. (**A**) Large numbers of both Ase^{-} and Ase^{+} (red) neuroblasts are seen in *mts*^{299}/*mts*^{55268} mutants. Phalloidin-labeled actin is green. (**B**) *mts* RNAi in Ase^{+} neuroblast lineages is sufficient to cause larval brain neuroblast overproliferation. Neuroblasts are marked with Dpn (red) and Mira (green). (**C**,**D**) Excess neuroblasts are observed in both non-DM (C) and DM (D) neuroblast lineages compared with wild type. MARCM clones are marked by CD8 (green), neuroblasts are marked by Dpn (blue, right), Asense is red. DM lineage clones are located at the dorsal-medial region, whereas non-DM clones are located at the dorsal-anterior region of larval brains and the neuroblast is Asense-positive. Note that in the wild-type DM lineage (upper panel), the neuroblast is Asense-negative (arrow), whereas in *mts*^{299} mutants (lower panel), multiple neuroblasts are generated and it is difficult to trace the Asense-negative neuroblast (the putative neuroblast is indicated by the arrow). (**E**) *mts*^{299} MARCM clones kept to adulthood have multiple cells expressing phospho-Histone H3 (red) and/or Dpn (blue). This was never seen in wild-type MARCM clones in adult brains. (**F**) *PP2A-29B* RNAi generates supernumerary larval brain neuroblasts. *PP2A-29B* RNAi is controlled under the Insc-Gal4 driver. Mira (red) marks neuroblasts, DNA is blue. Dotted lines mark the outline of clones in C,D and E, and mark the margin between central brain and optic lobe, with central brain to the left, in B and F. Scale bars: 10 μm.

mts$^{XE-2258}$ trans-heterozygotes (*mts*-null). Among the basal proteins, localizations of Numb (Fig. 4E; 71.7%, *n*=46) and Pon (Fig. 4F; 68%, *n*=25) are most severely disrupted, whereas Mira (Fig. 4G; 14.6%, *n*=48) and Brat (Fig. 4H; 33.3%, *n*=30), are mildly perturbed in *mts* mutants. Phenotypic penetrance follows the order of *mts*^{299}/*mts*$^{XE-2258}$>*mts*^{299}/*mts*^{S5286}>*mts*^{299}. Mitotic spindle orientation was also compromised in *mts* mutants (Fig. 4K,L). The mitotic spindle was misaligned with the cortical crescent in 44% of cells in the *mts*^{299}/*mts*$^{XE-2258}$ mutant (Fig. 4K,L; *n*=30). These asymmetric division defects of *mts* mutants were fully rescued by a wild-type *mts* transgene (see Fig. S1B in the supplementary material).

We then ascertained that PP2A subunits A and B also regulate asymmetric division of neuroblasts. In larval brains of *PP2A-29B*^{RS}/*PP2A-29B*^{EP} mutants, asymmetric distributions of aPKC (Fig. 5A; 84.2%, *n*=19), Numb (Fig. 5B; 56.3%, *n*=16) and Pon (Fig. 5C; 80%, *n*=15) were lost. Mitotic spindle misorientation can be observed in *PP2A-29B*^{RS}/*PP2A-29B*^{EP} (Fig. 5E). In *tws*^{60} (pupal lethal) and *tws*^{02424} (embryonic lethal) trans-heterozygotes, asymmetric localization of aPKC, Numb and Pon, as well as mitotic spindle orientation, was also affected (Fig. 5A-C). In a *tws*^{60}/tws^{02424} mutant, asymmetric localizations of aPKC (Fig. 5A; 75%, *n*=20), Numb (Fig. 5B; 74.1%, *n*=27) and Pon (Fig. 5C; 71.4%, *n*=21) were disrupted. It was shown recently in S2 cells that *PP2A* RNAi leads to defects in cell cycle progression (Chen et al., 2007a), so our current data do not rule out cell cycle delays as the cause of neuroblast polarity defects. It was shown in *C. elegans* embryos that DNA replication defects delay cell division and disrupt cell polarity (Encalada et al., 2000). However, metaphase-arrested neuroblasts induced either by a microtubule depolymerizing drug or by mutations in *cdc20* (*fzy*) or *cdh1* (*rap*) display normal cortical polarity (Broadus and Doe, 1997; Slack et al., 2007). Our data suggest that the PP2A heterotrimeric complex is required for neuroblast polarity and appears to act upstream of polarity proteins including aPKC to control neuroblast asymmetry.

PP2A and Polo enhance Numb phosphorylation and asymmetric localization

The PP2A-dependent defects in the asymmetric division of neuroblasts resemble those seen in *polo* loss-of-function mutants. Polo kinase was previously identified as a brain tumor-suppressor that mediates the asymmetric localization and segregation of Numb by phosphorylating Pon, an adaptor protein for Numb (Wang et al., 2007). This prompted us to ascertain whether PP2A might act to prevent excess self-renewal by regulating Numb asymmetry, similar to Polo (Wang et al., 2007). The neuroblast overgrowth phenotype was significantly, although not completely, suppressed by overexpression of Numb-GFP in *mts* mutants (Fig. 6A-C). These data indicate that PP2A suppresses neuroblast overgrowth, at least in part, by regulating Numb asymmetry/function. We therefore tested if Numb might be a substrate for PP2A dephosphorylation. Numb proteins were detected as two bands, with the higher band representing the modified form of Numb (Rhyu et al., 1994). If Numb is a substrate of PP2A, the modified form of Numb, which is the putative phosphorylated form, will increase in *PP2A* mutants. However, surprisingly, hyperphosphorylated Numb is reduced in larval brains of *mts*^{299}/*mts*$^{XE-2258}$ trans-heterozygotes or by *mts* in vivo RNAi (Fig. 6D and data not shown), indicating that Numb is unlikely to be a direct substrate of PP2A. A similar reduction in the levels of phosphorylated Numb was also observed in *PP2A-29B*$^{-}$ and *tws*^{60}/*tws*^{02424} larval brains (data not shown).

We speculate that PP2A may activate a kinase that phosphorylates Numb. Three protein kinases, aPKC, Polo and Aurora A, were previously shown to regulate asymmetric division of *Drosophila*

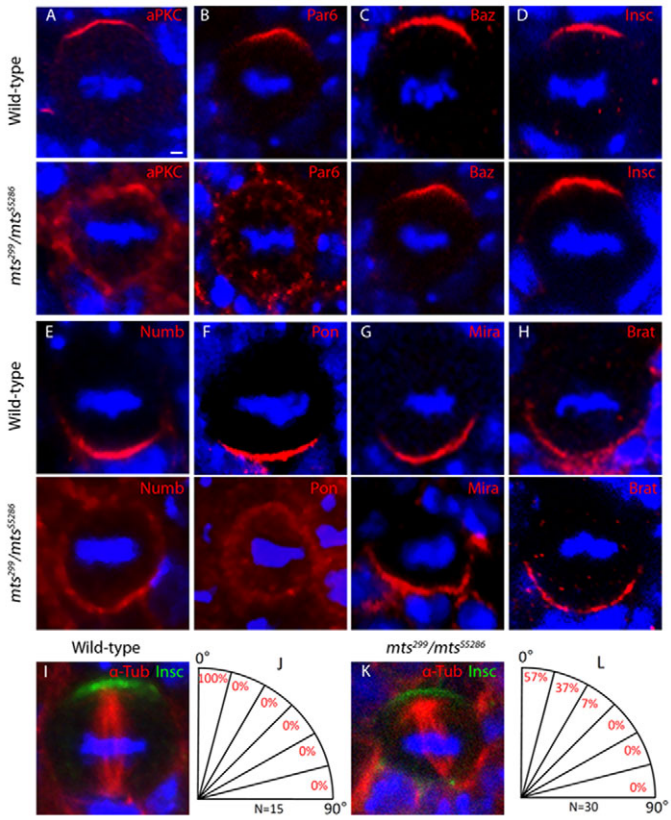

Fig. 4. Mts is required for aPKC, Numb and Pon cortical polarity and proper mitotic spindle orientation. Larval brain neuroblasts from wild type (upper panels) or mts^{299}/mts^{S5286} mutants (lower panels) were examined for localization of aPKC (**A**), Par6 (**B**), Baz (**C**), Insc (**D**), Numb (**E**), Pon (**F**), Mira (**G**) and Brat (**H**). (**I-L**) PP2A is required for proper mitotic spindle orientation. Wild-type (I) and mts^{299}/mts^{S5286} mutant (K) neuroblasts were triple-labeled for α-Tubulin (red), Insc (green) and DNA (blue). Mitotic spindle orientation in wild type (J) and mts^{299}/mts^{S5286} mutants (L) was quantified. Scale bar: 1 μm.

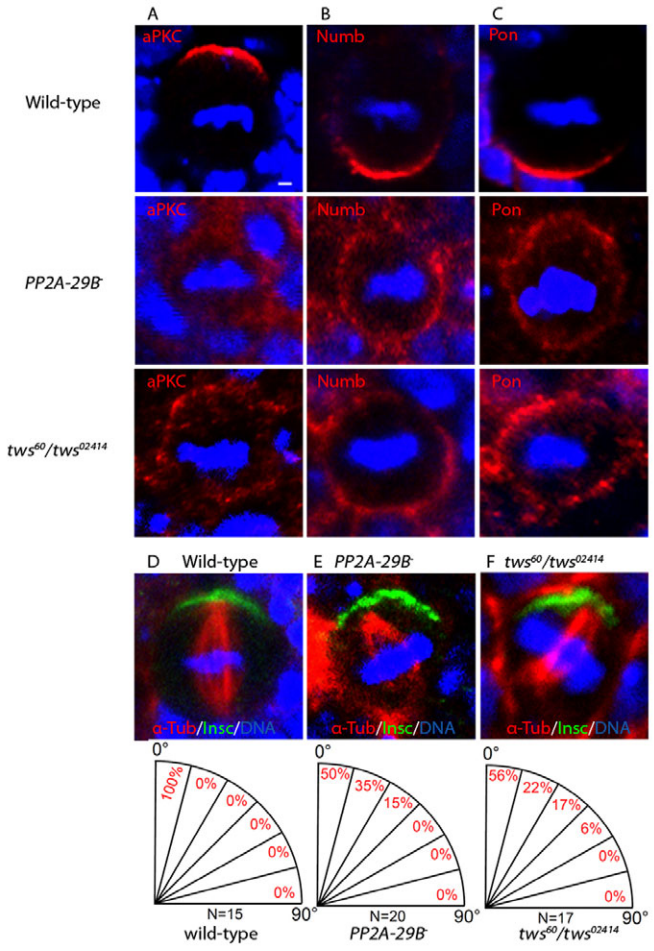

Fig. 5. PP2A A and B subunits regulate the asymmetric division of neuroblasts. Asymmetric localization of aPKC (**A**), Numb (**B**) and Pon (**C**) is disrupted in the brains of $PP2A\text{-}29B^{RS}/PP2A\text{-}29B^{EP}$ (middle) and tws^{60}/tws^{02424} (bottom) trans-heterozygote larvae. In *tws* mutants, the asymmetric localizations of aPKC (A; 75%, *n*=20), Numb (B; 74.1%, *n*=27) and Pon (C; 71.4%, *n*=21) were disrupted. In *PP2A-29B* mutants, asymmetric distributions of aPKC (A; 84.2%, *n*=19), Numb (B; 56.3%, *n*=16) and Pon (C; 80%, *n*=15) were lost. In contrast with wild type (**D**), mitotic spindle misorientation can be observed in $PP2A\text{-}29B^{RS}/PP2A\text{-}29B^{EP}$ (**E**) and tws^{60}/tws^{02424} (**F**) mutants. α-Tubulin (red) marks the mitotic spindle and Insc (green) represents asymmetrically localized proteins. DNA is blue. Quantifications of mitotic spindle orientation are shown in D-F (lower panels). % in A-C, the percentage of neuroblasts in which mislocalization of the proteins was observed; in D-F, the percentage of neuroblasts with the measured angles between the mitotic spindle and the midline of the crescent. Scale bar: 1 μm.

neural stem cells (Betschinger et al., 2003; Lee et al., 2006a; Rolls et al., 2003; Wang et al., 2006; Wang et al., 2007). aPKC phosphorylates Numb and leads to its asymmetric localization (Lee et al., 2006b; Smith et al., 2007). Five aPKC phosphorylation sites, including Serine (Ser) 52 of Numb (corresponding to Ser7 in murine Numb), are evolutionarily conserved. Aurora A phosphorylates Par6 and allows aPKC to phosphorylate Numb and release it from one side of the cell cortex (Wirtz-Peitz et al., 2008). Polo can phosphorylate Pon directly, which leads to the asymmetric localization of Pon and subsequently Numb (Wang et al., 2007). Loss of Numb phosphorylation in *PP2A* mutants is unlikely to be due to the compromised function of aPKC, as aPKC protein levels are upregulated in both *PP2A* and *polo* mutants (Fig. 7B). Furthermore, phosphorylation of Numb by aPKC, which is recognized by anti-pSer7Numb antibody, is enhanced despite the overall reduction of Numb phosphorylation in *PP2A* mutants (Fig. 7C). Therefore, loss of Numb phosphorylation in *PP2A* mutants appears to be aPKC-independent. However, aPKC activity that is probed by an antibody against the autophosphorylated aPKC remains unchanged after *mts* knockdown (Fig. 7C), indicating that increased Numb phosphorylation is not due to increased aPKC activity in *PP2A* mutants. The neuroblast overproliferation phenotype of *mts* brains was partially suppressed by the loss-of-function mutation $aPKC^{06403}$ (see Fig. S4 in the supplementary material). This suppression is consistent with the role for aPKC in neuroblast proliferation. In this double mutant the expected loss of Numb phosphorylation at the Ser52 aPKC site may contribute to this suppression.

We thus tested whether bulk Numb phosphorylation requires Polo or Aurora A kinases, two brain tumor-suppressors that regulate Numb asymmetry. Interestingly, Numb phosphorylation is similarly lost in *polo* mutant larval brains, whereas it is not obviously affected in *aurA* mutant trans-heterozygous [$aurA^{8839}$ (strong hypomorphic allele)/$aurA^{87Ac\text{-}3}$ (amorph allele)] brains (Fig. 6D). Therefore Polo,

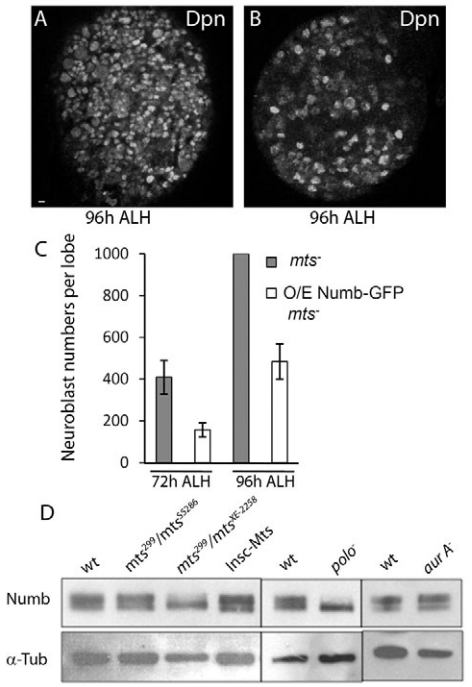

Fig. 6. PP2A primarily acts upstream of Numb to regulate neuroblast self-renewal. (**A-C**) Ectopic expression of Numb-GFP using Insc-Gal4 significantly suppresses the neuroblast overgrowth phenotype in mts^{299}/mts^{S5286} mutants (A) and Insc-Gal4 mts^{299}/mts^{S5286} (B) was used as control. Dpn marks neuroblasts. Scale bar: 10 μm. (C) Quantification of neuroblast numbers at 72 hours ALH and 96 hours ALH. O/E, overexpressed. (**D**) Numb phosphorylation is abolished in $mts^{299}/mts^{XE-2258}$ and $polo^{9}/polo^{11}$ larval brains. Numb phosphorylation appears to be largely present in a weaker mts^{299}/mts^{S5286} trans-heterozygote. Ectopic expression of Mts (Insc-Mts) results in increased Numb phosphorylation. Numb phosphorylation is present in $aurA^{8839}/aurA^{87Ac-3}$ ($aurA^{-}$).

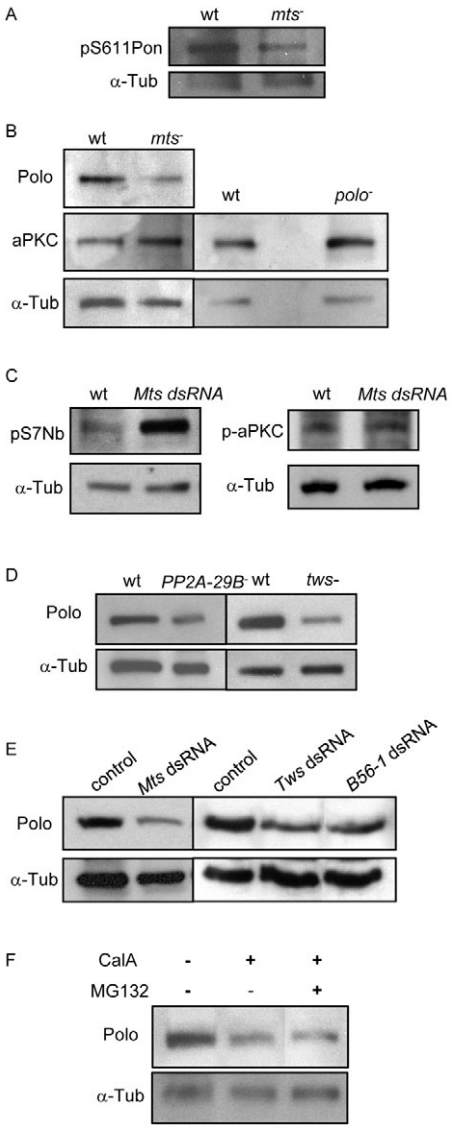

Fig. 7. Polo protein abundance depends on PP2A. (**A**) Phosphorylation of Pon on Ser611 is strongly reduced in $mts^{299}/mts^{XE-2258}$ mutants. (**B**) In $mts^{299}/mts^{XE-2258}$ larval brains, Polo protein levels are strongly decreased, whereas aPKC levels are increased. aPKC protein levels are increased in $polo^{9}/polo^{11}$ larval brains. (**C**) Numb phosphorylation by aPKC (pS7Nb) is increased but aPKC autophosphorylation remains unchanged after *mts* knockdown in S2 cells. (**D**) Polo protein levels are reduced in $PP2A\text{-}29B^{RS}/PP2A\text{-}29B^{EP}$ and tws^{60}/tws^{02414} larval brains. (**E**) Polo protein levels are significantly reduced in S2 cells upon *mts* RNAi, *tws* RNAi or *B56-1* RNAi (E) but not after *wdb* or *PR-72* RNAi (data not shown). (**F**) Polo protein levels are reduced in S2 cells upon treatment with Calyculin A (CalA), or cotreatment with CalA and MG132. α-Tubulin served as a loading control. The quantifications of changes of protein levels are normalized by the loading controls in the following ratios: (A) wt:mts^{-}, 1:0.38; (B) wt:mts^{-}, 1:0.34 (Polo); wt:mts^{-}, 1:1.39 (aPKC); wt:$polo^{-}$, 1:1.43 (aPKC); (C) wt:mts^{-}, 1:4.58 (pS7Nb); wt:mts^{-}, 1:0.93 (p-aPKC); (D) wt:$PP2A\text{-}29B^{-}$, 1:0.4; wt:tws^{-}, 1:0.26; (E) control:*mts* dsRNA, 1:0.27; control:tws^{-}:$B56^{-}$1, 1:0.31:0.36; (F) –:+–:++, 1:0.35:0.35.

but not Aurora A, is crucial for the bulk phosphorylation of Numb (Wang et al., 2007). Although Aurora A is required for phosphorylation of Numb by aPKC (Wirtz-Peitz et al., 2008), other kinase(s) can also phosphorylate Numb on phosphorylation sites that might be different from aPKC phosphorylation sites, and could therefore account for Numb phosphorylation in *aurora A* mutants. Phosphorylation of Ser611Pon is also strongly reduced in *mts* mutants (Fig. 7A), similar to *polo* mutants. Thus, both PP2A and Polo are crucial for Numb and Pon phosphorylation. We also explored the possible involvement of several other kinases, such as Par1 kinase, in neuroblast self-renewal. However, neither overexpression of Par1 nor *par1*-null MARCM clones showed any neuroblast proliferation phenotype (see Fig. S3 in the supplementary material). Therefore, similar to Polo, PP2A regulates asymmetric localization of aPKC, Pon and Numb, as well as mitotic spindle orientation. In addition, both PP2A and Polo decrease aPKC protein levels and enhance Numb and Pon phosphorylation. Thus, they may act in the same pathway during neuroblast asymmetric divisions.

PP2A acts in Polo/Numb pathway to inhibit neuroblast overgrowth

We next examined how PP2A might regulate Polo function to facilitate asymmetric division. In $mts^{299}/mts^{XE-2258}$ mutant or *mts* RNAi larval brains Polo protein levels were dramatically reduced (Fig. 7B and data not shown), suggesting that PP2A is required for Polo expression. Polo protein levels were also strongly reduced in $PP2A\text{-}29B^{RS}/PP2A\text{-}29B^{EP}$ and tws^{60}/tws^{02414} mutant larval brains

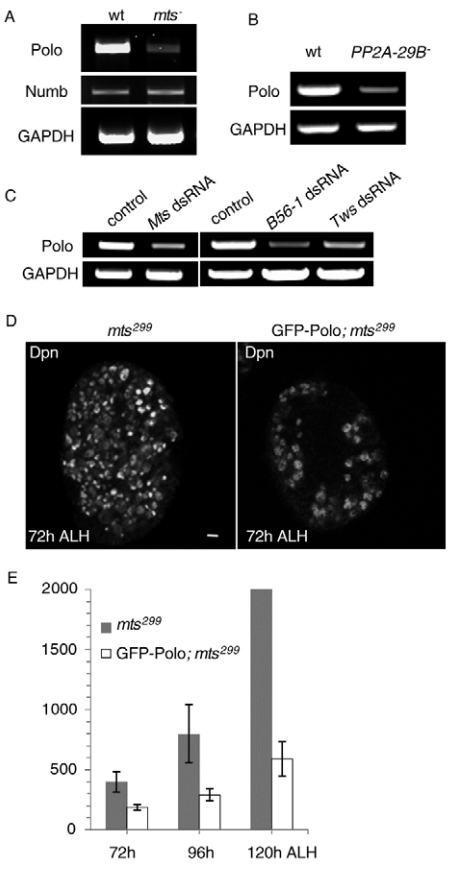

Fig. 8. PP2A promotes Polo expression to inhibit neuroblast overgrowth. (**A-C**) *polo* transcript levels are significantly reduced in $mts^{299}/mts^{XE\text{-}2258}$ (A) and $PP2A\text{-}29B^{RS}/PP2A\text{-}29B^{EP}$ (B) larval brains, and in S2 cells upon *mts*, *tws* or *B56-1* (C) knockdown. (**D**,**E**) Overexpression of GFP-Polo significantly suppresses neuroblast overgrowth in mts^{299}. mts^{299} mutants with overexpression of GFP-Polo have a significantly reduced number of neuroblasts compared with mts^{299} at 72 hours, 96 hours and 120 hours ALH. Neuroblasts are marked by Dpn. Scale bar: 10 μm. (E) Quantification of central brain neuroblast numbers in control mts^{299} and GFP-Polo; mts^{299} brains. The quantifications of changes of transcript levels are normalized by the loading controls in the following ratios: (A) wt:mts^{-}, 1:0.125 (*polo*); wt:mts^{-}, 1:1.22 (*numb*); (B) wt:$PP2A\text{-}29B^{-}$, 1:0.24; (C) control:*mts* dsRNA, 1:0.08; control:*B56-1* dsRNA:*tws* dsRNA, 1:0.17:0.38.

(Fig. 7D). Polo abundance was similarly reduced in S2 cells after RNAi knockdown of *mts* or the two B regulatory subunits *twins* or *B56-1* (Fig. 7E), but not significantly changed by RNAi knockdown of *wdb* or *PR-72* (data not shown). Similar reduction of Polo protein levels were also observed in S2 cells treated with Calyculin A, an inhibitor of PP2A and PP1 (Fig. 7F), indicating that PP2A might be required for Polo expression.

To investigate how PP2A regulates Polo protein expression, S2 cells were cotreated with both Calyculin A and MG132, a proteasome inhibitor. In these cells, Polo protein levels remain reduced similar to cells treated with Calyculin A alone (Fig. 7F). We therefore tested whether PP2A sustains *polo* transcript abundance in the larval brains. *polo* transcript levels were significantly decreased in $mts^{299}/mts^{XE\text{-}2258}$ larval brains (Fig. 8A). This effect on *polo* transcript levels appears specific for *polo*, as *mts* mutations did not significantly affect transcript levels of *numb*, *baz* or *lgl* (Fig. 8A, and data not shown). *polo* transcript levels were also dramatically reduced in $PP2A\text{-}29B^{RS}/PP2A\text{-}29B^{EP}$ larval brains (Fig. 8B) or in S2 cells upon *mts*, *tws* or *B56-1* knockdown (Fig. 8C). These data suggest that PP2A sustains *polo* transcript levels in both larval brains and S2 cells. Although both Tws and B56-1 are important for *polo* expression in S2 cells, B56-1 does not appear to be required for the asymmetric division of neuroblasts. The introduction of GFP-Polo (genomic construct) or Insc-venus-Polo is able to significantly suppress the neuroblast overproliferation phenotype in *mts* mutants (Fig. 8D,E and data not shown), further supporting the model that PP2A functions upstream of Polo to inhibit excess neuroblast self-renewal.

DISCUSSION

Mammalian PP2A is a tumor suppressor that participates in malignant transformation by regulating multiple pathways (Westermarck and Hahn, 2008). However, it is unknown whether PP2A controls neural stem cell self-renewal. Our data explicitly show that the *Drosophila* PP2A trimeric complex confers brain tumor-suppressor activity and controls the balance of self-renewal and differentiation of neural stem cells. We show that *PP2A* mutation leads to neural stem cell overproliferation in *Drosophila* larval brains, which is associated with dramatically reduced neuronal differentiation. Cell cycle genes including CycE, and phospho-Histone H3 and growth factor Myc are upregulated in *PP2A* mutants, consistent with the neuroblast overgrowth phenotype. Neuroblasts overproliferate in *PP2A* mutant MARCM clones. When these mutant clones that were generated at larval stages are kept until adulthood, neural stem cells continue to proliferate in adult brains, which is never observed for wild-type clones. Therefore, PP2A can inhibit excess self-renewal and promote neuronal differentiation of neural stem cells.

This overgrowth of neural stem cells in *PP2A* mutants is a consequence of defects in the asymmetric division of neural stem cells. PP2A regulates asymmetric protein localization as well as mitotic spindle orientation. In our previous study, we showed that Polo kinase is a brain tumor-suppressor that regulates Numb/Pon and aPKC asymmetric localization, as well as mitotic spindle orientation (Wang et al., 2007). Although *polo* mutants displayed pleiotropic phenotypes during asymmetric divisions, Polo primarily regulates asymmetric division of neural stem cells by regulating Numb asymmetry (Wang et al., 2007). Polo directly phosphorylates Pon on Ser611, which leads to the asymmetric localization of Pon and subsequently Numb (Wang et al., 2007). Strikingly similar to Polo, PP2A also regulates the asymmetric localization of aPKC, Pon and Numb, and is required for Pon phosphorylation on Ser611. Interestingly, both PP2A and Polo are required for Numb phosphorylation, which may be important for Numb asymmetric localization or activity on the cortex. Thus, Numb is a major downstream factor for both PP2A and Polo in regulating neural stem cell self-renewal. Consistent with this, overexpression of Numb, but not PonS611D, a phospho-mimetic form of Pon, in *polo* mutants significantly rescues the neuroblast overgrowth phenotype (Wang et al., 2007).

We further discovered that PP2A functions upstream of Polo/Numb in the same pathway to control self-renewal of neuroblasts. Polo transcript and protein abundance is dependent on PP2A function. The expression of several other genes, including *numb*, *baz* and *lgl*, are not affected by PP2A knockdown, suggesting that the downregulation of *polo* in *PP2A* mutants appears to be specific. Moreover, overexpression of GFP-Polo or Numb can largely suppress neuroblast overgrowth in *PP2A* mutants, suggesting that PP2A primarily acts in the Polo/Numb pathway to inhibit neuroblast overgrowth. Our discovery suggests that PP2A and Polo, both of which are crucial brain

tumor-suppressors and cell cycle regulators, can function in the same pathway to regulate stem cell self-renewal and tumorigenesis. Currently, it is not clear how PP2A, which is a protein phosphatase, promotes *polo* expression. It is conceivable that PP2A dephosphorylates a transcription factor and consequently activates it to allow *polo* transcription. Alternatively, PP2A may dephosphorylate a protein that is required for *polo* mRNA stabilization.

PP2A is involved in a broad range of cellular processes including signal transduction, transcriptional regulation and cell cycle control (Westermarck and Hahn, 2008). PP2A regulates the Wnt/Wingless signaling pathway and affects the degradation of β-catenin, a transcription factor and the central molecule of this pathway (Eichhorn et al., 2009). Two of the components of Wnt/Wingless signaling pathway, Adenomatous polyposis coli (APC) and Shaggy (also known as GSK3), do not regulate neuroblast polarity [(Rusan et al., 2008) and data not shown]. So it remains to be determined whether Wnt/Wingless signaling plays a role in neuroblast polarity. Mammalian PP2A directly dephosphorylates oncogene cMyc and tumor suppressor p53, both of which are transcription factors (Eichhorn et al., 2009; Junttila et al., 2007). Future studies should identify potential substrate(s) of PP2A that can promote *polo* expression and control neural stem cell self-renewal.

Interestingly, we also observed that cMyc protein levels were increased in *PP2A* mutants (data not shown), suggesting that PP2A may have a conserved role in modulating cMyc protein and suppressing its function. However, ectopic expression of cMyc alone does not induce brain tumor formation in *Drosophila* (Betschinger et al., 2006), suggesting that PP2A can regulate multiple pathways to affect neural stem cell self-renewal. However, the PP2A/Numb pathway appears to be one of the major pathways by which PP2A controls the balance of self-renewal and differentiation in *Drosophila*, as overexpression of Polo or Numb can largely suppress neural stem cell overgrowth in *PP2A* mutants. Furthermore, PP2A may regulate Numb function and activity by both promoting *polo* expression and antagonizing aPKC phosphorylation of Numb. Whether mammalian PP2A also regulates stem cell polarity will be of great interest for future study.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/13/2287/DC1

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