The Company of **Biologists**

STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Neuroblast niche position is controlled by Phosphoinositide 3-kinase-dependent DE-Cadherin adhesion

Susan E. Doyle, Matthew C. Pahl, Karsten H. Siller, Lindsay Ardiff and Sarah E. Siegrist*

ABSTRACT

Correct positioning of stem cells within their niche is essential for tissue morphogenesis and homeostasis. How stem cells acquire and maintain niche position remains largely unknown. Here, we show that a subset of brain neuroblasts (NBs) in *Drosophila* utilize Phosphoinositide 3-kinase (PI3-kinase) and DE-cadherin to build adhesive contact for NB niche positioning. NBs remain within their native microenvironment when levels of PI3-kinase activity and DE-cadherin are elevated in NBs. This occurs through PI3-kinase-dependent regulation of DE-Cadherin-mediated cell adhesion between NBs and neighboring cortex glia, and between NBs and their ganglion mother cell daughters. When levels of PI3-kinase activity and/or DE-Cadherin are reduced in NBs, NBs lose niche position and relocate to a non-native brain region that is rich in neurosecretory neurons, including those that secrete some of the *Drosophila* insulin-like peptides. Linking levels of PI3-kinase activity to the strength of adhesive attachment could provide cancer stem cells and hematopoietic stem cells with a means to cycle from trophic-poor to trophic-rich microenvironments.

KEY WORDS: Stem cell, PI3-kinase, DE-Cadherin, Shotgun, Niche, Neuroblast, *Drosophila*

INTRODUCTION

Most stem cells are surrounded by specialized groups of cells, collectively referred to as a niche, that provide trophic and structural support to stem cells as well as protection (Losick et al., 2011; Scadden, 2014). Stem cells receive information about physiological conditions and/or developmental states of the tissues in which they reside through their niche, and this ensures that the correct numbers and types of stem cell progeny are produced when needed. Niche to stem cell signaling is not unidirectional, however, and in many cases stem cells produce the cellular and molecular components of their own niche (Voog et al., 2008; Hsu et al., 2011; Sato et al., 2011). While investigating how neurogenesis becomes progressively restricted during development, we discovered that neural stem cells in the brain of *Drosophila* build and maintain their own adhesive stem cell niche through activation of Phosphoinositide 3-kinase (PI3-kinase) signaling, which mediates DE-Cadherin (Shotgun – FlyBase) adhesive cell contact between NBs and NB neighbors. These results are reported here.

All neurons within the *Drosophila* brain are generated either directly or indirectly from a population of asymmetrically dividing neural stem cells known as neuroblasts (NBs), which are self-renewing, multipotent progenitors (Doe, 2008; Homem and Knoblich, 2012). Following cell division, a large NB is regenerated and a smaller ganglion mother cell (GMC) daughter is produced that creates limited numbers of neurons and/or glia. About 100 NBs are located within the central region of each brain lobe and this number remains invariant throughout development because central brain NBs only undergo self-renewing asymmetric cell division. This feature, together with the fact that NBs can be identified based on cell size, expression of molecular markers and position, allows for the NB population to be easily tracked over time. Central brain NBs are specified during embryogenesis, proliferate throughout most of larval development, and terminate cell divisions during pupal stages before adult formation. All central brain NBs terminate cell divisions within 30 h after pupal formation, except for the eight mushroom body (MB) NBs (four per brain lobe, eight total) (Truman and Bate, 1988; Ito and Hotta, 1992; Siegrist et al., 2010). These NBs persist late and undergo apoptosis only shortly before animals emerge from their pupal case as young adults (Truman and Bate, 1988; Ito and Hotta, 1992; Siegrist et al., 2010). The difference in timing of termination of neurogenesis between MB and non-MB NBs is likely to be regulated by lineage-specific intrinsic differences among NBs themselves and by local differences within NB microenvironments.

Whether NBs reside within a molecularly and spatially defined stem cell niche has remained rather elusive; however, cortex glia is one cell type that might constitute the NB niche (Chell and Brand, 2010; Sousa-Nunes et al., 2011). Cortex glia ensheath NBs and their recently born progeny and have been shown to produce Insulin-like peptide 6 (Dilp6; Ilp6 – FlyBase) in response to feeding (Chell and Brand, 2010; Sousa-Nunes et al., 2011). Dilp6 in glia activates the highly conserved PI3-kinase growth signaling pathway in NBs and is sufficient to reactivate quiescent NBs (Chell and Brand, 2010; Sousa-Nunes et al., 2011). In *Drosophila*, PI3-kinase pathway activation is mediated largely through availability of any one of the seven Dilps to bind and activate the single Insulin-like tyrosine kinase receptor. In general, this leads to increased cell growth and proliferation, in part through increased protein synthesis (Engelman et al., 2006). Activation of PI3-kinase not only reactivates quiescent NBs, but also maintains NB proliferation rates throughout development, ensuring sufficient numbers of neurons and glia are produced for adult brain function. Levels of PI3-kinase activity remain high in NBs during development, but decrease in MB NBs prior to termination of neurogenesis (Siegrist et al., 2010). Decreasing levels of PI3-kinase activity in MB NBs ensures that MB NBs terminate cell divisions on time and prevents excess MB neurogenesis, which could be deleterious (Siegrist et al., 2010).

Beyond growth and proliferation control, we describe here a new role for PI3-kinase in regulating MB NB position. We find that MB NBs utilize PI3-kinase to build DE-Cadherin-containing adhesive contact sites that MB NBs use for niche attachment. MB NBs maintain position within their niche through contact with cortex glia

Department of Biology, University of Virginia, Charlottesville, VA 22903, USA.

*Author for correspondence (ses4gr@virginia.edu)

S.E.S., 0000-0003-0685-5387

DEVELOPMENT

and their GMC daughters. MB NB-GMC contact initiates during NB cell division, as PI3-kinase activity becomes localized along the developing cleavage furrow. This primes new NB-GMC contact sites for assembly of DE-Cadherin-containing adhesive complexes. To our knowledge, this is the first report demonstrating that PI3-kinase regulates stem cell niche position and that NB daughters provide NB niche attachment.

RESULTS

Ectopic NBs are located in the pars intercerebralis of the adult brain

During development, MB NBs, a subset of brain NBs that generate neurons important for memory and learning, are located on the dorsal brain surface, superficial to the MB calyces in each brain lobe. MB NBs remain located on the dorsal brain surface throughout development, until their elimination via apoptosis, which occurs shortly before animals emerge from their pupal case as young adults (Siegrist et al., 2010). One method previously used to ascertain

whether MB NBs undergo apoptosis during development employed the GAL4/UAS binary system to drive the baculovirus caspase inhibitor *UAS-p35* specifically in NBs using worGAL4 (Siegrist et al., 2010). In these animals (designated as p35 animals), in which NB apoptosis is blocked, we observed MB NBs located on the dorsal surface superficial to the MB calyces in brains of 1-day-old adults (Fig. 1A, top; quantified in 1D) (Siegrist et al., 2010). In addition, other cells expressing the NB transcription factor Deadpan (Dpn) were also observed in an anterior-medial brain region known as the pars intercerebralis (PI) (Fig. 1A,C). This was unexpected, as NBs are normally never found in the PI region and NBs are not thought to be migratory. Typically, NBs maintain position within the CNS throughout development (Truman and Bate, 1988; Broadus and Doe, 1995; Urbach et al., 2003).

To determine whether the ectopic Dpn-positive cells located in the PI region are NBs, we fed 1-day-old p35 adults fly food spiked with the thymidine analog EdU. This allows for all cells and their progeny to be positively marked after cell division. After 24 h of
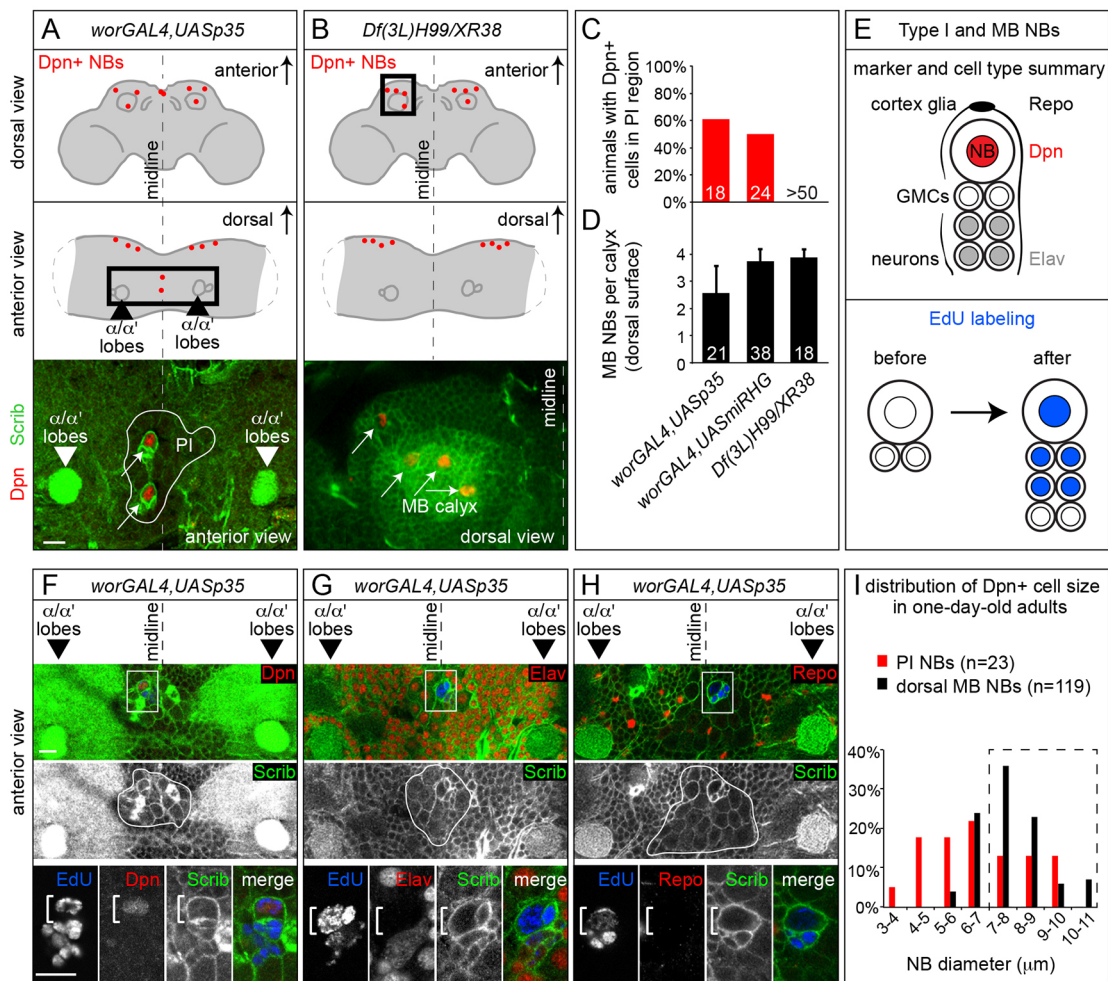
Fig. 1. Ectopic NBs are located in the PI region in brains of adult *Drosophila*. (A,B) Schematics of dorsal (top) and anterior (middle) views of 1-day-old adult brains of the indicated genotypes. Red spheres indicate the position of Dpn-expressing NBs. The boxed regions of PI in A and of calyx in B are shown in the confocal images (bottom). Arrows point to NBs labeled with Dpn (red) and the membrane marker Scrib (green); arrowheads indicate MB α/α′ lobes used as a landmark. PI region is outlined in white. The confocal image in B is a maximum intensity projection. (C) Percentage of 1-day-old adults with a Dpn-positive NB located in the PI region. Numbers on bars indicate the number of animals scored per genotype. (D) Average number of MB NBs per brain lobe located on the dorsal surface, superficial to MB calyx in 1-day-old adult brains. Numbers on bars indicate number of brain lobes scored per genotype. Error bars indicate s.d. (E) Schematic depicting markers used and pattern of EdU labeling after cell division. (F-H) Anterior view of p35 adult brains after 24 h of EdU labeling. Colored overlay (top) with single channel image (middle). NBs (white box, top) located in the PI region (white outline, middle) are shown under higher magnification (bottom). White brackets mark NBs. (I) Size distribution of Dpn-positive cells located in the PI region and of Dpn-positive MB NBs located on the dorsal surface. *n*, number of NBs scored at each location. Scale bars: 10 μm.

feeding, we observed EdU-positive MB NBs and their EdU-positive progeny located on the dorsal brain surface, as previously reported (data not shown) (Siegrist et al., 2010). In addition, we observed that the Dpn-positive cells located in the PI region also incorporated EdU and generated new EdU-positive progeny (Fig. 1E-H). New progeny generated at both locations, by either the MB NBs on the dorsal brain surface (data not shown) or by the ectopic Dpn-positive cells located in the PI region, expressed the early neuronal-specific transcription factor Elav (Fig. 1G), but not the glial marker Repo (Fig. 1H), nor Dpn, which marks NBs and intermediate progenitors from type II NB lineages (Fig. 1F). Together, this suggests that the ectopic Dpn-positive cells located in the PI region are NBs, similar to MB NBs and other type I NBs, but not type II NBs or their intermediate progenitors. Furthermore, we observed that some, but not all, of the ectopic PI NBs were the same size as MB NBs located on the dorsal brain surface (Fig. 1I, dashed outline), and all animals with one or more large PI NB (>7 µm average diameter, n=9) had a concomitant reduction in the number of MB NBs located on the dorsal surface. Together, this raised the possibility that some PI NBs are MB NBs that relocate from the dorsal brain surface to the more central PI region.

A second method used to block MB NB apoptosis combines two mutant chromosomes, $Df(3L)H99$ and $Df(3L)XR38$, in trans to reduce pro-apoptotic gene function in all cells (Peterson et al., 2002; Siegrist et al., 2010). Here, MB NBs were also observed on the dorsal brain surface, but no NBs were observed in the PI region in brains of 1-day-old $Df(3L)H99/XR38$ adults (Fig. 1B-D). Absence versus presence of PI NBs could be due to differences in activated

caspase activity in NBs. In $Df(3L)H99/XR38$ animals, apoptosis is blocked upstream of caspase activation, whereas in p35 animals apoptosis is blocked downstream of caspase activation. Caspase-inhibited epithelial cells derived from imaginal disks that initiate apoptosis but fail to die can invade neighboring tissue (Martin et al., 2009; Rudrapatna et al., 2013). To investigate this possibility, we used $worGAL4$ to drive expression of a second apoptotic inhibitor, UAS-$miRHG$, which blocks apoptosis upstream of caspase activation, similar to $Df(3L)H99/XR38$ animals, but only in NBs (designated as miRHG animals) (Siegrist et al., 2010). Both MB NBs and PI NBs were observed in brains of 1-day-old miRHG adults, as in p35 adults (Fig. 1C,D). We conclude that NB relocation to the PI region is independent of caspase activity and that differences in NB location could be due to differences in local or systemic NB environments.

MB NBs relocate from the dorsal brain surface to the central PI region during the pupal to adult transition

To determine whether PI NBs are MB NBs that migrate in from the dorsal brain surface, we assayed the number and location of Dpn-positive NBs in brains of miRHG and p35 animals over time. At 72 h after pupal formation (APF), all MB NBs were observed (eight total, four per brain hemisphere) on the dorsal brain surface superficial to the MB calyx in miRHG animals, as in wild-type controls (Fig. 2E; data not shown) (Siegrist et al., 2010). Occasionally, other Dpn-positive cells of unknown identity were also observed along the midline in miRHG animals (four total, one each in four of 23 brains) but not in wild-type controls. These
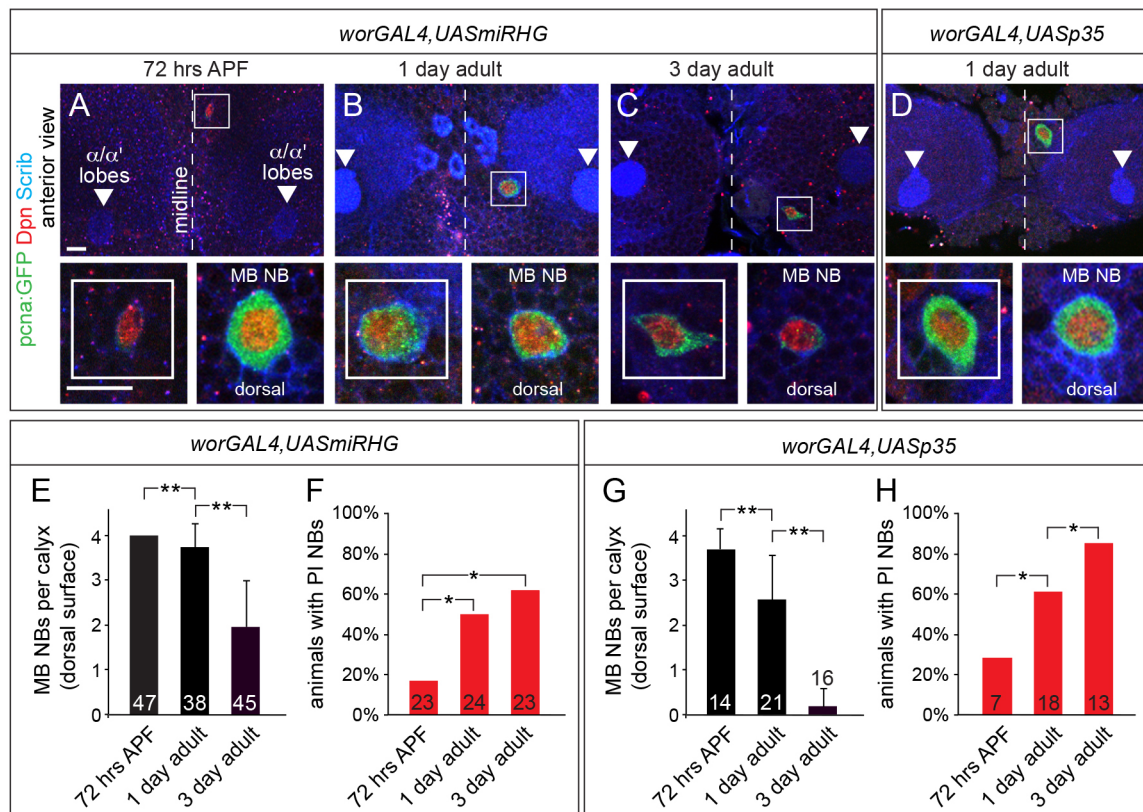
Fig. 2. Some ectopic PI NBs are MB NBs that migrate in from the dorsal surface. (A-D) Anterior brain views of the indicated genotypes. Colored overlay (top) with Dpn-positive NB (white box) shown at higher magnification (below left). For comparison, an MB NB located on the dorsal surface, superficial to the calyx in the same animal, is also shown (below right). (E,G) Average number of MB NBs per brain lobe located on the dorsal surface over time (APF, after pupal formation). Number of brain lobes scored is indicated. **P<0.001, one-tailed t-test. (F,H) Percentage of animals with Dpn-positive NBs located in the PI region. Number of animals scored at each time point is indicated. *P<0.05, one-tailed t-test. Error bars indicate s.d. Scale bars: 10 µm.

ectopic Dpn-positive cells were small and only weakly, if at all, expressed the S-phase activity reporter PCNA:GFP (Fig. 2A,F). Next, we assayed the number and location of Dpn-positive NBs in brains of 1-day-old miRHG adults. This is 24-36 h later, and in wild-type animals MB NBs are no longer present, having undergone apoptosis several hours earlier (Siegrist et al., 2010). On average, we observed fewer than four MB NBs per brain lobe located on the dorsal surface in miRHG animals (Fig. 2B,E) (Siegrist et al., 2010). Importantly, this reduction correlated with an increase in the number of animals with NBs located in the PI region (Fig. 2B,F). Many of these ectopic PI NBs were large and expressed PCNA:GFP strongly (Fig. 2B). Finally, we assayed the number and location of Dpn-positive NBs in brains of 3-day-old miRHG adults. We observed a further reduction in the number of MB NBs located on the dorsal brain surface and an increase in the number of adults with PI NBs (Fig. 2C,E,F). No more than eight Dpn-positive cells were ever observed in total in 1-day-old or in 3-day-old adult brains. Similar results were obtained for p35 animals (Fig. 2D,G,H). We conclude that some of the ectopic Dpn-positive NBs located in the PI region are MB NBs that migrate in from the dorsal surface. Furthermore, because MB to PI NB migration coincides with the time when MB NBs initiate reductions in cell growth and proliferation prior to apoptosis (Siegrist et al., 2010), we propose that both events might be linked and possibly triggered by changes in MB NB microenvironments.

PI3-kinase activation in MB NBs anchors them within their native niche

Changes in MB NB growth and proliferation prior to MB NB apoptosis are due in part to reductions in levels of PI3-kinase activity in MB NBs (Siegrist et al., 2010). Furthermore, levels of PI3-kinase activity modulate migratory cell behavior in many biological systems, including leukocytes, *Dictyostelium*, and hematopoietic stem cells (Kolsch et al., 2008; Buitenhuis, 2011). To determine whether MB to PI NB migration is due to changes in levels of PI3-kinase activity, we expressed *UAS-dp110* (*Pi3K21B* – FlyBase) or *UAS-dp60* (*Pi3K92E* – FlyBase) to increase or decrease the levels of PI3-kinase activity in MB NBs, respectively (Leevers et al., 1996; Weinkove et al., 1999). When MB NB levels of PI3-kinase activity were increased (*UAS-dp110*), there was a decrease in the number of 1-day-old miRHG adults with PI NBs (16% compared with 50%; Fig. 3A). Conversely, when MB NB PI3-kinase activity levels were decreased (*UAS-dp60*), the number of 1-day-old miRHG adults with PI NBs increased (92% compared with 50%; Fig. 3A). This indicates that MB NB location can be modulated by the levels of PI3-kinase activity and that high levels of PI3-kinase activity are required to keep MB NBs located within their native niche on the dorsal surface superficial to the MB calyx. Consistent with this notion, reducing levels of PI3-kinase activity caused MB NBs to relocate from the dorsal surface to the PI region in *Df(3L)H99/XR38* adults (47% compared with 0%; Fig. 3B,C). We conclude that PI3-kinase activation is required to anchor MB NBs within their native microenvironment on the dorsal brain surface. Furthermore, both MB to PI NB migration and decreased MB NB growth and proliferation are linked and triggered by reductions in the levels of PI3-kinase activity.

A PIP3 sensor localizes around the NB cortex and becomes enriched along the developing cleavage furrow during mitosis

PI3-kinase is a lipid kinase and when active converts phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-
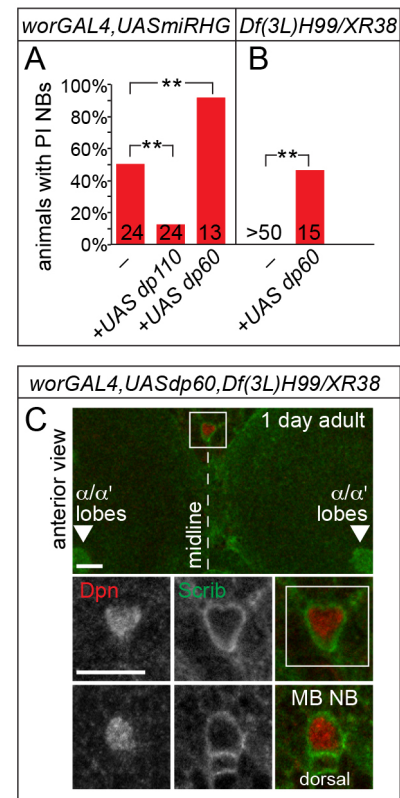
Fig. 3. PI3-kinase activation is required to anchor MB NBs in their niche. (A,B) Percentage of 1-day-old adults with Dpn-positive NBs located in the PI region. Genotypes are listed above with additional manipulations listed below. Column numbers indicate number of animals scored. **$P<0.001$, one-tailed *t*-test. (C) Anterior view of a 1-day-old adult brain. Colored overlay (top), with PI NB (white box) shown at higher magnification in single channel and colored overlay (middle). For comparison, an MB NB located on the dorsal surface, superficial to the MB calyx in the same animal, is also shown (bottom). Scale bars: 10 μm.

3,4,5-triphosphate (PIP3). To understand how PI3-kinase functions to regulate MB NB position, we assayed the localization of a PIP3 sensor in NBs over time. The PIP3 sensor used, UAS-splitVenus-GRP1-PH, contains the Venus fluorescent protein sequence split into two non-fluorescent halves, each fused to the PIP3-specific binding sequence of the GRP1-PH domain (Khuong et al., 2013). When co-expressed as UAS-transgenes under Gal4 control, the N-terminal and C-terminal Venus-GRP1-PH fragments are recruited to PIP3-rich plasma membrane domains where they complement to form a fluorescent Venus-GRP1-PH reporter molecule (Khuong et al., 2013). This bi-molecular sensor shows improved membrane:cytoplasmic signal to noise ratio compared with the conventional mono-molecular PH-GFP reporter (Britton et al., 2002; Khuong et al., 2013). We imaged isolated NBs dissociated from larval brains over time (Siegrist and Doe, 2006; Egger et al., 2013; Harzer et al., 2013). This method allows for unambiguous detection of the PIP3 sensor along the NB plasma membrane and for quantification of PIP3 sensor fluorescence intensity over time.

During interphase, we observed mostly uniform localization of the PIP3 sensor around the NB plasma membrane (Movie 1). During mitosis, however, localization of the PIP3 sensor became enriched along the developing cleavage furrow during early telophase (Fig. 4A-F,I, Movie 1). This resulted in accumulation of the PIP3 sensor along the newly generated NB-GMC contact after cytokinesis was complete (Fig. 4G,H, Movie 1). The same pattern
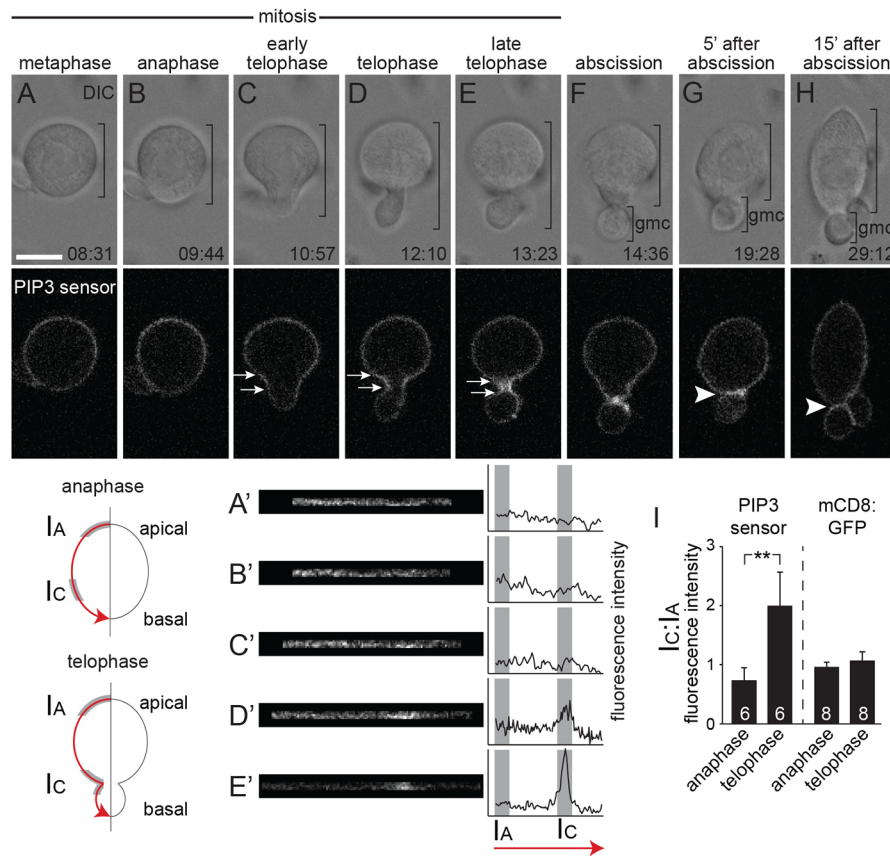
Fig. 4. PIP3 is found along the NB membrane and becomes enriched along the developing cleavage furrow during mitosis. (A-H) Series of still images of an isolated larval NB dividing in culture. PIP3, which was assayed using bi-molecular fluorescence complementation, becomes enriched along the developing NB cleavage furrow (arrows) and persists along the newly generated NB-GMC contact site (arrowheads). Time stamp corresponds to min:s after nuclear envelope breakdown. (A′-E′) Line scans of PIP3 sensor fluorescence along the NB cortex from apical to basal, with intensity profiles on right. (I) Ratio of PIP3 fluorescence along the cleavage furrow (IC, gray box) to fluorescence along the apical NB membrane (IA, gray box) increases from anaphase to telophase. This ratio remains unchanged in control NBs expressing membrane-bound mCD8:GFP. Number in columns indicates number of NBs scored. Only the first cell division was assayed. **$P<0.001$, two-tailed t-test. See also Movie 1. Error bars indicate s.d. Scale bar: 10 μm.

was observed in dividing NBs in brain explants (Movie 2) and in isolated cultured NBs that express the conventional mono-molecular PIP3 reporter (Movie 3) (Britton et al., 2002). No change in fluorescence intensity was observed along the cleavage furrow in NBs expressing the membrane-tethered GFP fusion protein mCD8:GFP (Fig. 4I). We conclude that PI3-kinase is active along the entire NB membrane, including where NBs make contact with cortex glia and where NBs make contact with their GMC daughters. This suggests that cell-cell interactions might regulate MB NB niche position.

PI3-kinase regulates DE-Cadherin-dependent MB NB positioning

DE-Cadherin (DE-Cad) is a well-known regulator of cell adhesion between many cell types, including *Drosophila* germline stem cells and their cap cell niche (Song et al., 2002). DE-Cad is also expressed throughout the brain, in glia and in NBs and their lineages (Akong et al., 2002; Dumstrei et al., 2003). We investigated whether DE-Cad regulates MB NB niche position. We overexpressed a wild-type version of DE-Cad in MB NBs and assayed the location of Dpn-positive NBs in brains of 1-day-old miRHG adults (Oda and Tsukita, 1999). We observed a decrease in the number of adults with PI NBs compared with miRHG adults (12% compared with 50%; Fig. 5A). Conversely, when a dominant-negative (DN) version of DE-Cad was expressed in MB NBs we observed an increase in the number of adults with PI NBs (67% compared with 50%; Fig. 5A) (Oda and Tsukita, 1999). Interestingly, the absence or presence of PI NBs correlated with the absence or presence of GFP-tagged transgene localization around the MB NB cell membrane (Fig. S1). We conclude that MB NBs in which apoptosis has been inhibited require DE-Cad to maintain niche position. Furthermore, because

MB NBs also utilize PI3-kinase to maintain niche position, this raised the possibility that PI3-kinase might regulate DE-Cad for MB NB niche attachment.

Next, we analyzed the fluorescence intensity of DE-Cad along the MB NB plasma membrane under conditions of high or low PI3-kinase activity (Fig. 5B-F). MB NBs were colabeled with Scrib to mark the MB NB membrane and we measured the average DE-Cad fluorescence per unit length of membrane to account for differences in NB cell size across genotypes (see Materials and Methods). PI3-kinase has known roles in growth regulation, and NB cell size can be modulated by PI3-kinase activity (Fig. S2B) (Chell and Brand, 2010; Siegrist et al., 2010). Assuming equal levels of DE-Cad molecules, we expect the average DE-Cad fluorescence intensity to be lower in larger cells and higher in smaller cells. However, we observed the opposite. Larger cells (*UAS-dp110*) have higher average DE-Cad fluorescence intensity than smaller cells (*UAS-dp60*) (Fig. S2A,B). This suggests that PI3-kinase activity regulates the levels of DE-Cad independently of NB cell size.

It is reported that cell adhesion strength correlates with total Cadherin protein levels along the plasma membrane (Steinberg and Takeichi, 1994). We multiplied the average DE-Cad fluorescence intensity along the MB NB membrane (Fig. S2A) by the length of the MB NB perimeter (Fig. S2B) for each genotype to calculate total fluorescence intensity of DE-Cad along the MB NB plasma membrane cross-section (Fig. 5G). We used this as a proxy for the total amount of DE-Cad protein along the MB NB plasma membrane and an indicator of adhesive strength. When levels of PI3-kinase activity are elevated (*UAS-dp110*) in miRHG MB NBs, there was an increase in total DE-Cad fluorescence along the NB membrane compared with miRHG MB NBs alone (Fig. 5G). Conversely, when levels of PI3-kinase activity were reduced (*UAS-*
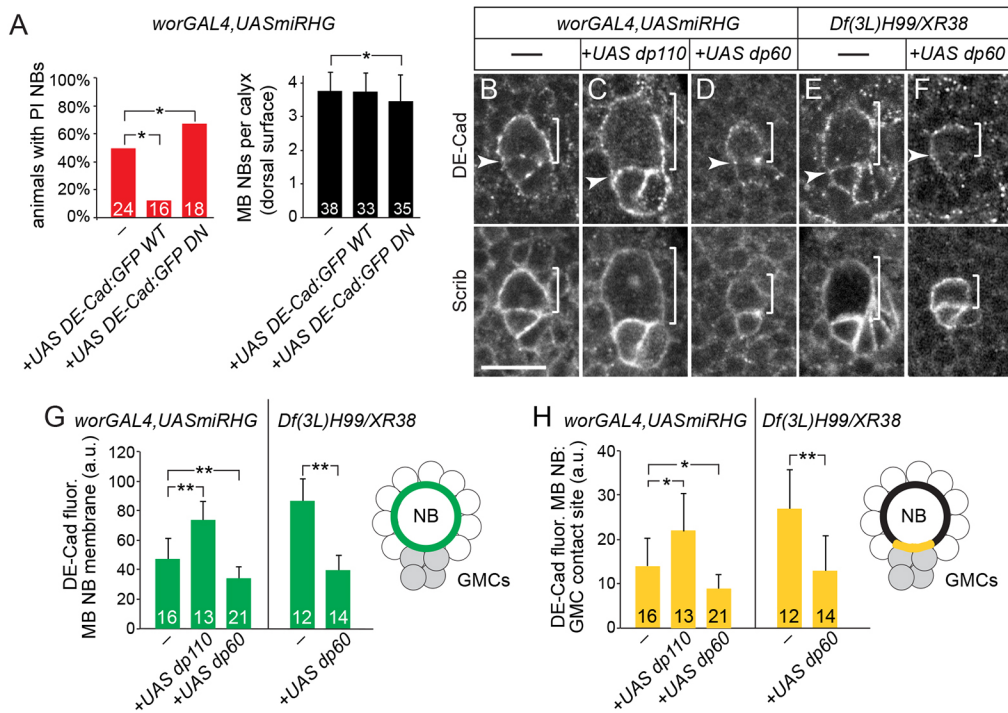
Fig. 5. PI3-kinase regulates the levels of DE-Cad along the MB NB cortex. (A) Percentage of 1-day-old adults with Dpn-positive PI NBs (left). Average number of MB NBs per brain lobe located on the dorsal surface, superficial to MB calyx in 1-day-old adult brains (right). Column numbers indicate number of animals scored. *$P<0.05$, one-tailed t-test. Genotype at top, with additional manipulation listed below. (B-F) Double-labeled MB NBs located on the dorsal surface in brains of 1-day-old adults. MB NBs in brackets; arrowhead marks MB NB-GMC contact sites. (G,H) Quantification of total DE-Cad fluorescence intensities along the entire MB NB membrane (G) or along the MB NB-GMC contact site only (H). Number of MB NBs scored is indicated. *$P<0.05$, **$P<0.001$, two-tailed t-test. Error bars indicate s.d. Scale bar: 10 µm.

$dp60$) in miRHG or $Df(3L)H99/XR38$ MB NBs, there was a reduction in total DE-Cad fluorescence along the MB NB membrane (Fig. 5G). Interestingly, total DE-Cad levels are elevated in H99/XR38 animals compared with miRHG animals, consistent with the observation that MB NBs in H99/XR38 animals maintain niche position, whereas MB NBs in miRHG animals do not. Levels of DE-Cad fluorescence specifically along MB NB-GMC contacts were also responsive to levels of PI3-kinase activity in MB NBs: fluorescence decreased when levels of PI3-kinase were reduced and increased when levels of PI3-kinase were elevated (Fig. 5H). We conclude that PI3-kinase regulates the levels of DE-Cad along the MB NB plasma membrane and that the strength of adhesive contact between MB NBs and cortex glia and/or between MB NBs and GMCs might determine whether MB NBs remain within or vacate their native microenvironment.

PI3-kinase mediates DE-Cad adhesion assembly along newly generated NB-GMC contact sites

We have shown that PI3-kinase activity localizes in a cell cycle-dependent manner, becoming enriched along the developing cleavage furrow during mitosis. We also found that levels of DE-Cad are reduced along MB NB-GMC contact sites when levels of PI3-kinase are reduced. Next, we asked whether PI3-kinase primes new NB-GMC contact sites for assembly of DE-Cad adhesive complexes, which could be important for MB NB niche positioning. To investigate this possibility, we imaged DE-Cad:GFP in cultured isolated NBs over time (Movie 4). Because cell-cell contact is required for Cadherin clustering (Yap et al., 1998), DE-Cad:GFP was only weakly detected along the NB membrane in isolated NBs (Fig. 6A, metaphase, Movie 4). This makes the *in vitro* culture method ideal for visualizing DE-Cad:GFP at NB-GMC contact sites in time-lapse. After the first cell division, DE-Cad:GFP became localized along the newly generated NB-GMC contact (Fig. 6A, arrow, 2′ after abscission, Movie 4). Localization of DE-Cad:GFP along the new NB-GMC contact site remained throughout interphase and, on average, increased twofold in relative

florescence intensity (Fig. 6A,C,D,E, Movie 4). In isolated NBs with reduced levels of PI3-kinase activity, we observed a delay in DE-Cad:GFP localization along the newly generated NB-GMC contact site (Fig. 6B,D, Movie 5). There was also a significant reduction in DE-Cad:GFP enrichment levels along the new NB-GMC contact site compared with control NBs, as seen *in vivo* (Fig. 6E). We conclude that PI3-kinase promotes the accumulation of DE-Cad along newly generated NB-GMC contact sites, consistent with the possibility that NBs use their newly generated daughter cells for niche attachment.

MB NBs physically separate from their GMC daughters *in vivo*

To gain insight into how MB NBs relocate from the dorsal surface to the more central PI region, we imaged MB NBs in brain explants from 1-day-old miRHG adults over time. MB NBs located on the dorsal surface were observed undergoing asymmetric cell division, as expected (data not shown) (Siegrist et al., 2010). In addition, in two of 14 brain explants we observed an MB NB physically separate from its GMC daughter (Fig. 7A, Movie 6). This was unexpected, as during development NBs normally always remain in contact with their newborn GMCs (Truman and Bate, 1988; Broadus and Doe, 1995; Urbach et al., 2003). Over time, the distance between the MB NB and its GMC daughter increased and, eventually, the MB NB left the imaging field of view (Fig. 7A, Movie 6). We conclude that MB NBs maintain position on the dorsal surface within their native microenvironment through contact with their GMC daughters and through contact with their cortex glia neighbors. Furthermore, loss of MB NB-GMC contact might be an early step in NB relocation from the dorsal surface to the PI region.

DISCUSSION

Proper positioning of neural stem cells within their niche ensures that correct numbers and types of stem cell progeny are produced where and when needed. Neural stem cells that lose niche position also lose niche support and protection. This could lead to premature stem cell loss, which is associated with certain types of human birth
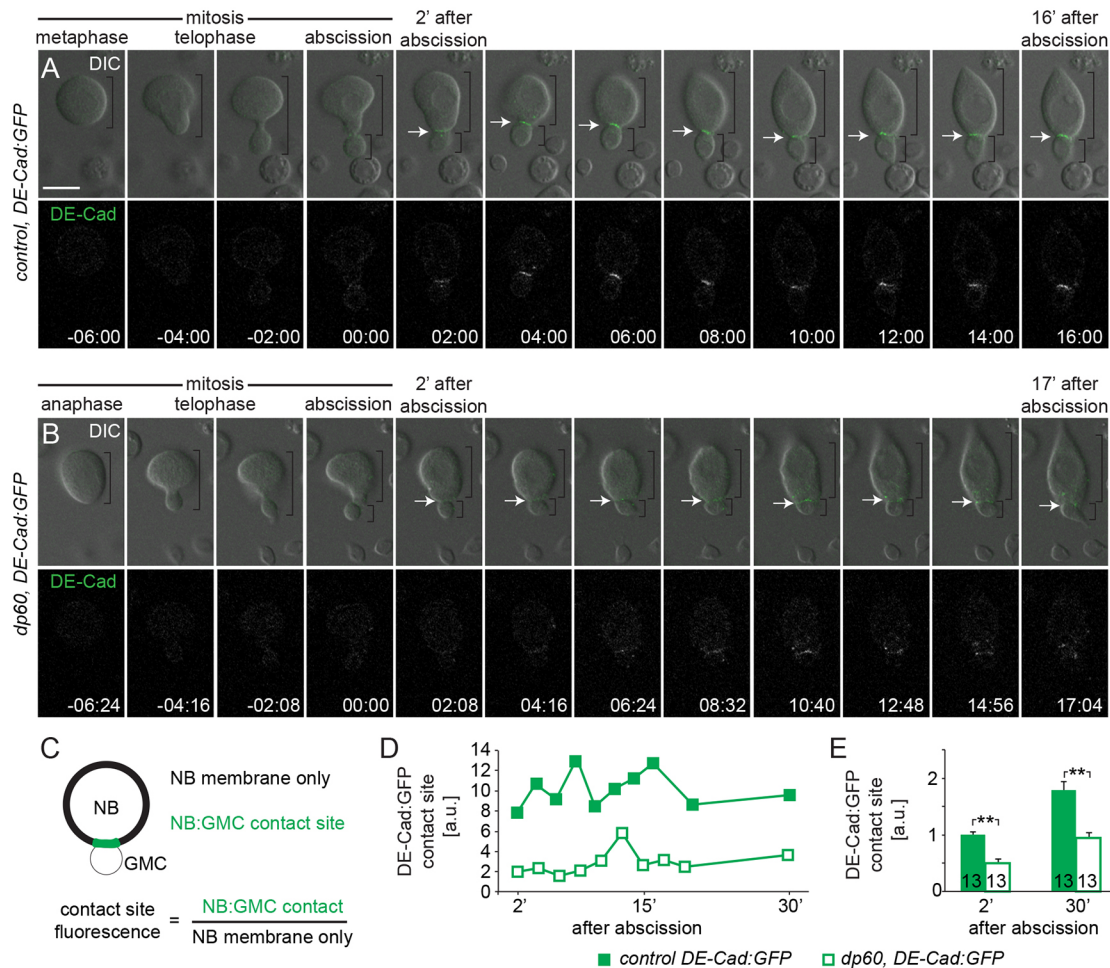
Fig. 6. PI3-kinase regulates DE-Cad along newly generated NB-GMC contacts. (A) Still images of an isolated larval NB dividing in culture. (Top) DIC image with DE-Cad:GFP fluorescence overlay in green. (Bottom) DE-Cad:GFP fluorescence in grayscale. Genotype is *worGAL4,UAS-DE-Cad:GFP*. See also Movie 4. Arrows indicate enrichment of DE-Cad:GFP along the newly generated NB-GMC contact site; large bracket indicates the dividing NB, and after cell division (00:00), the regenerated NB; small bracket indicates the newly generated GMC daughter. (B) Same as A, except genotype is *worGAL4,UAS-dp60,UAS-DE-Cad:GFP*. See also Movie 5. Time stamp is min:s starting at abscission. (C) Schematic of NB areas used to quantify DE-Cad:GFP fluorescence. (D) Quantification of DE-Cad:GFP enrichment at the NB-GMC contact site over time in control NBs (as in A) and when PI3-kinase is reduced (as in B). (E) Quantification of average relative enrichment of DE-Cad:GFP at the NB-GMC contact site at 2 min and at 30 min after abscission. **$P<0.001$, two-tailed *t*-test. Error bars indicate s.e.m. Scale bar: 10 μm.

defects as well as with cognition deficits in the elderly. In other cases, loss of stem cell niche positioning may lead to tumor formation, as stem cells outside of their native niche receive, and could respond to, unwanted growth factors and cytokines (Losick et al., 2011; Scadden, 2014). Understanding how neural stem cells maintain niche position will provide better insight into human disease and possibly also into how neurogenesis is terminated once development is complete. Based on the results described here, we propose the following model to explain how *Drosophila* neural stem cells can lose niche position (Fig. 7B).

During development, when nutrient conditions are favorable, cortex glia, which ensheath NBs and their newborn progeny, secrete Dilp6 (Chell and Brand, 2010; Sousa-Nunes et al., 2011). Local production of Dilp6 might ensure that levels of PI3-kinase activity in NBs remain high to sustain neurogenesis. Once development is complete, however, the glia niche could break down (Fig. 7B, orange). This could be a physical breakdown, such as the rearrangement or death of cortex glia, a breakdown in cell-cell signaling (i.e. no more local Dilp6), or possibly both. This could explain why levels of PI3-kinase activity in late-stage pupal MB

NBs are low compared with MB NBs during earlier larval stages (Siegrist et al., 2010). Small, growth-impaired MB NBs are then primed for elimination via apoptosis (Siegrist et al., 2010). If apoptosis fails, however, then MB NBs remain in adult brains, some on the dorsal surface in their native microenvironments and some migrating to the non-native PI region.

Whether MB NBs remain on the dorsal surface or relocate to the PI region is dependent on levels of PI3-kinase activity in MB NBs. High levels of PI3-kinase activity in MB NBs keep MB NBs positioned on the dorsal surface, whereas low levels promote MB to PI NB relocation. This is because high levels of PI3-kinase activity in MB NBs maintain high levels of DE-Cad around the NB membrane, which serves to anchor MB NBs to both cortex glia and their GMC daughters. The observation that PI3-kinase becomes enriched along the developing cleavage furrow during mitosis was unexpected. This allows for PI3-kinase to accumulate along the newly generated NB-GMC contact site after cytokinesis. This, together with the observation that MB NBs separate from their GMC daughters *in vivo* and that localization of DE-Cad along new NB-GMC contact sites depends on PI3-kinase activity, supports the
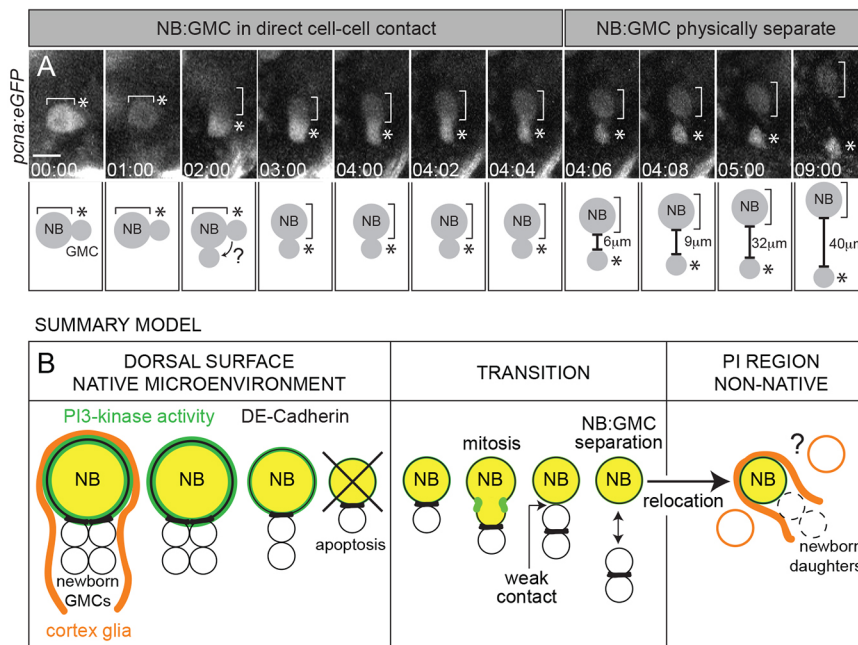
Fig. 7. MB NBs break contact with their GMC daughters and separate over time. (A) Maximum intensity projections (top) with schematic interpretation (bottom) of *pcna:GFP*-expressing MB NB from 1-day-old miRHG adult brain explant. Asterisks indicate the position of the most recently generated GMC daughter. Distance between MB NB and GMC daughter increases over time. See also Movie 6. (B) Model to explain how *Drosophila* neural stem cells can lose niche position. During development, MB NBs maintain niche position on the dorsal brain surface through contact with cortex glia and their GMC daughters. Towards the end of development, MB NBs undergo apoptosis. If, however, apoptosis fails, then some MB NBs can relocate to the non-native PI region. This is because levels of DE-Cad and PI3-kinase are reduced in MB NBs. For further details, see main text.

idea that MB NB-GMC contact contributes to niche attachment. Whether MB NBs also use cortex glia for niche attachment is certainly possible. In the future, it will be interesting to better understand the dynamic mechanical properties of NB adhesive contacts. For example, is adhesive strength between NBs and cortex glia and between NBs and GMCs constant throughout development or does adhesive strength change as NBs change their developmental programs? How do quiescent NBs, which have low levels of PI3-kinase activity and no recently generated GMC progeny, maintain niche position during the embryonic to larval transition? Are quiescent NBs insensitive to signals required to initiate and/or carry out PI migration? Alternatively, could physical forces generated during cell division itself be what triggers 'loosely' adherent NBs to detach and leave their native microenvironments?

How MB NBs with low levels of PI3-kinase activity physically relocate to the PI region remains unclear, as they must use a method other than conventional actin-based cell motility, which requires PI3-kinase activation at the leading edge (Kolsch et al., 2008). MB NBs could migrate via 'blebbing', where membrane protrusion is driven by intracellular pressure, when the plasma membrane disassociates from the underlying actin cytoskeleton (Charras and Paluch, 2008; Fackler and Grosse, 2008). Blebbs are frequently observed in cultured cells during cell division, in cells undergoing apoptosis, and in cells that migrate in 3D culture (Charras and Paluch, 2008; Fackler and Grosse, 2008). For reasons that remain unclear, once outside of their native microenvironment ectopic NBs are only ever observed in the PI region. This suggests that the PI region could provide a permissive environment for NB survival. In the future, it will be interesting to investigate whether the PI region, which is rich in neurosecretory neurons, provides trophic support for ectopic NBs and, if so, whether the PI is molecularly similar to the native MB NB microenvironment (Nässel and Winther, 2010).

Somewhat surprising is the fact that MB NBs do not seem to require PI3-kinase or DE-Cad for niche positioning during larval stages. This could be because cortex glia and GMCs function in a redundant manner to provide MB NB niche attachment. MB NBs, as well as other NBs, might in fact utilize both cell types for niche positioning: attachment to cortex glia could keep NBs

positioned globally, along the outside of the developing brain, while contact with GMCs might keep NBs positioned locally within the glial pockets. In fact, this is consistent with previous work showing NBs located at 'variable depths' when DE-Cad is disrupted in NB lineages but not in glia (Dumstrei et al., 2003). Towards the end of development, however, MB NB niche positioning does become dependent on levels of PI3-kinase activity and DE-Cad. At this time, MB NB proliferation rates decrease as PI3-kinase activity levels decline (Siegrist et al., 2010). This results in the production of fewer GMCs and, therefore, fewer GMCs for MB NB niche attachment. The combined loss of cell-cell interactions between MB NBs and cortex glia and between MB NBs and their GMC daughters could enable MB NBs to lose niche position and relocate to the PI. In the future, it will be important to determine whether MB NBs and other NBs utilize both cortex glia and GMCs for niche attachment during normal development.

Unlike *Drosophila*, neurogenesis continues throughout adulthood in the ventricular-subventricular zone (V-SVZ) in the lateral ventricle and in the subgranular zone of the dentate gyrus (SGZ) in the hippocampus in rodents. V-SVZ neural stem cells (NSCs), which generate olfactory bulb interneurons and oligodendrocytes, are highly polarized, contacting both blood vasculature and the cerebral spinal fluid located in the ventricles. This positions V-SVZ NSCs ideally to receive and respond to a variety of systemic and local signals (Lim and Alvarez-Buylla, 2014; Bjornsson et al., 2015). In adults, V-SVZ NSCs reside within pinwheel-like structures, formed by ependymal cells that line the ventricles. Location within this microarchitecture impacts V-SVZ NSC fate decisions, as neurogenesis continues only where ependymal cells form pinwheels (Mirzadeh et al., 2008; Conover and Shook, 2011). Like *Drosophila* NBs, V-SVZ NSCs in rodents also express the cell adhesion molecule E-Cadherin and, in rodents, loss of E-Cadherin in V-SVZ NSCs leads to premature depletion of the NSC pool (Karpowicz et al., 2009). In the future, it will be interesting to investigate whether NSC niche position within pinwheels in mammals is also controlled by PI3-kinase-dependent regulation of E-Cadherin adhesion.

MATERIALS AND METHODS

Fly rearing, stocks and genetics

The following fly stocks were used: *pcna:GFP* (Thacker et al., 2003), *UAS-N-Venus-PH-GRP$_1$* (Khuong et al., 2013), *UAS-C-Venus-PH-GRP$_1$* (Khuong et al., 2013), *tub:PHGFP* (Britton et al., 2002), *Df(3L)H99* (Bloomington *Drosophila* Stock Center, BL1576), *Df(3L)XR38* (Peterson et al., 2002), *worGAL4* (Albertson and Doe, 2003), *UAS-miRHG* (Siegrist et al., 2010), *UAS-p35* (BL5072), *UAS-dp60* (Weinkove et al., 1999), *UAS-dp110* (Leevers et al., 1996), *UAS-DECad:GFP* (Oda and Tsukita, 1999), *UAS-dNc9-1, DE-Cad DN* (Oda and Tsukita, 1999), *UAS-mCD8GFP* (BL5137). All animals were raised in uncrowded conditions at 25°C on Bloomington fly food.

Brain dissections, tissue preparation and antibody labeling

Adult and pupal brains were fixed, dissected, and stained using standard methods (Siegrist et al., 2010). White pupae were picked and aged accordingly at 25°C and adults staged from time of eclosion. We used GP-Scrib (kind gift from D. Bilder, University of California, Berkeley, CA, USA), Rat-DCAD2 (DHSB), Ms-Arm (N27A1, DSHB) and other antibodies as previously described (Siegrist et al., 2010). For EdU labeling, adults were fed 0.1 mg/ml EdU mixed in with heat-inactivated yeast paste with food dye for 24 h. Only brains from animals with colored guts were processed as described (Daul et al., 2010). Pupal and adult brains were mounted and imaged dorsal side up on a Leica scanning confocal microscope equipped with a 63×/1.4 NA oil-immersion objective and then remounted and imaged anterior side up. Only brains with an intact outer glial membrane were scored and included in the analysis.

Live imaging of whole-brain explants

One-day-old adult brains were dissected and transferred to a glass-bottom culture dish (MatTek, P35G-1.0-14-C) in D22 insect medium (pH 6.95; US Biological) supplemented with 10% FBS and 0.2 mg/ml insulin. Brains were imaged on an inverted Zeiss LSM 700 equipped with a 63×/1.4 NA oil-immersion objective. *z*-stacks (30×1.5 µm) were acquired every 2 min for 12 h. Larval brains were prepared as previously described and imaged every 15 s on a Leica SP8 (Siller and Doe, 2008). Confocal data sets were reconstructed using ImageJ (NIH).

Live imaging of disassociated NBs

Primary cultures were generated from brains of 30 third instar larva (96 h after larval hatching) using standard methods (Egger et al., 2013; Harzer et al., 2013). Disassociated cells were plated on poly-L-lysine-coated coverslips and allowed to settle for 30 min before imaging. NBs were identified based on their large size and pattern of division.

Fluorescence intensity measurements

Fluorescent pixels were measured using ImageJ. Only the first cell division of isolated NBs was included in the quantification. For PIP3 Venus and mCD8:GFP line scans, the NB membrane was manually segmented from the apical to basal pole. A five-pixel-wide line scan was performed with a rolling average of three along this line. The apical to basal line segment was then divided into five equal subsegments; subsegment IA is the most apical region, and segment IC is the fourth and comprises the cleavage furrow (or presumptive cleavage furrow for anaphase). For each NB, the integrated fluorescence intensity in segment IA and IC was calculated during anaphase and during telophase and expressed as a ratio (IC:IA).

Quantification of endogenous DE-Cad immunofluorescence in fixed specimens was performed as follows. Using Scrib staining as a membrane marker, the MB NB outline was manually traced and dilated into a three-pixel-wide ring-shaped region of interest (ROI); this is the total MB NB membrane region (Fig. 5G, green). The total MB NB membrane region was subdivided into two subregions: (1) the MB NB-GMC contact subregion where MB NBs contact their GMCs (Fig. 5H, yellow); and (2) the MB NB membrane-only subregion, which is where MB NBs are in contact with cortex glia but not their GMCs (Fig. 5H, black). For each MB NB, average DE-Cad fluorescence intensity was measured in the total MB NB membrane region, as well as in each of the two subregions. In addition, we measured

DE-Cad fluorescence in a randomly selected area neighboring, but not containing, MB NBs and their GMC progeny, in each analyzed image. This is the 'baseline region'. Baseline regions were outside of the Gal4 expression domain and therefore cells in these regions were not affected by Gal4-driven gene expression. In Fig. S2, we report normalized average fluorescence intensity across genotypes. This was calculated by dividing the average DE-Cad fluorescence intensity of the MB NB membrane region by the corresponding average DE-Cad fluorescence intensity of the baseline region. This ratio reflects the expression level of DE-Cad in MB NBs relative to its genetically unperturbed neighboring cells. In Fig. 5, to obtain a proxy of total surface DE-Cad along the MB NB membrane region or subregion, the normalized average DE-Cad fluorescence intensity (the ratio calculated above) of the MB NB membrane region (or subregion) was multiplied by the length of the MB NB perimeter (or subregion length). This approach allows for comparison of NB-specific changes in DE-Cad expression across samples and genotypes.

Quantification of GFP enrichment at NB-GMC contact sites in isolated NBs expressing DE-Cad:GFP in live culture employed a similar approach as above. DIC images were used to manually trace the NB outline. For each NB, average DE-Cad fluorescence intensity was measured in the two NB membrane subregions: (1) the NB-GMC contact subregion; and (2) the NB membrane-only subregion (described above). Background fluorescence, due to microscope acquisition noise, was measured in a region where no cells were located and was subtracted from the average GFP fluorescence intensity measured in each of the two NB membrane subregions. The relative enrichment of DE-Cad at the NB-GMC contact site was calculated by dividing the background-corrected DE-Cad fluorescence intensity of NB subregion 1 by the background-corrected DE-Cad fluorescence intensity of NB subregion 2.

Author contributions

S.E.S. designed and carried out all experiments, except primary cultures, which were performed by S.E.D.; and M.C.P., K.H.S. and L.A. established genetic lines for cadherin experiments. S.E.S. wrote the paper and K.H.S. provided comments.

References

Akong, K., McCartney, B. M. and Peifer, M. (2002). Drosophila APC2 and APC1 have overlapping roles in the larval brain despite their distinct intracellular localizations. *Dev. Biol.* **250**, 71-90.

Albertson, R. and Doe, C. Q. (2003). Dlg, Scrib and Lgl regulate neuroblast cell size and mitotic spindle asymmetry. *Nat. Cell Biol.* **5**, 166-170.

Bjornsson, C. S., Apostolopoulou, M., Tian, Y. and Temple, S. (2015). It takes a village: constructing the neurogenic niche. *Dev. Cell* **32**, 435-446.

Britton, J. S., Lockwood, W. K., Li, L., Cohen, S. M. and Edgar, B. A. (2002). Drosophila's insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. *Dev. Cell* **2**, 239-249.

Broadus, J. and Doe, C. Q. (1995). Evolution of neuroblast identity: seven-up and prospero expression reveal homologous and divergent neuroblast fates in Drosophila and Schistocerca. *Development* **121**, 3989-3996.

DEVELOPMENT

Buitenhuis, M. (2011). The role of PI3K/protein kinase B (PKB/c-akt) in migration and homing of hematopoietic stem and progenitor cells. *Curr. Opin. Hematol.* **18**, 226-230.

Charras, G. and Paluch, E. (2008). Blebs lead the way: how to migrate without lamellipodia. *Nat. Rev. Mol. Cell Biol.* **9**, 730-736.

Chell, J. M. and Brand, A. H. (2010). Nutrition-responsive glia control exit of neural stem cells from quiescence. *Cell* **143**, 1161-1173.

Conover, J. C. and Shook, B. A. (2011). Aging of the subventricular zone neural stem cell niche. *Aging Dis.* **2**, 149-163.

Daul, A. L., Komori, H. and Lee, C. Y. (2010). EdU (5-ethynyl-2'-deoxyuridine) labeling of Drosophila mitotic neuroblasts. *Cold Spring Harb. Protoc.* **2010**, pdb prot5461.

Doe, C. Q. (2008). Neural stem cells: balancing self-renewal with differentiation. *Development* **135**, 1575-1587.

Dumstrei, K., Wang, F. and Hartenstein, V. (2003). Role of DE-cadherin in neuroblast proliferation, neural morphogenesis, and axon tract formation in Drosophila larval brain development. *J. Neurosci.* **23**, 3325-3335.

Egger, B., van Giesen, L., Moraru, M. and Sprecher, S. G. (2013). In vitro imaging of primary neural cell culture from Drosophila. *Nat. Protoc.* **8**, 958-965.

Engelman, J. A., Luo, J. and Cantley, L. C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat. Rev. Genet.* **7**, 606-619.

Fackler, O. T. and Grosse, R. (2008). Cell motility through plasma membrane blebbing. *J. Cell Biol.* **181**, 879-884.

Harzer, H., Berger, C., Conder, R., Schmauss, G. and Knoblich, J. A. (2013). FACS purification of Drosophila larval neuroblasts for next-generation sequencing. *Nat. Protoc.* **8**, 1088-1099.

Homem, C. C. F. and Knoblich, J. A. (2012). Drosophila neuroblasts: a model for stem cell biology. *Development* **139**, 4297-4310.

Hsu, Y.-C., Pasolli, H. A. and Fuchs, E. (2011). Dynamics between stem cells, niche, and progeny in the hair follicle. *Cell* **144**, 92-105.

Ito, K. and Hotta, Y. (1992). Proliferation pattern of postembryonic neuroblasts in the brain of Drosophila melanogaster. *Dev. Biol.* **149**, 134-148.

Karpowicz, P., Willaime-Morawek, S., Balenci, L., DeVeale, B., Inoue, T. and van der Kooy, D. (2009). E-Cadherin regulates neural stem cell self-renewal. *J. Neurosci.* **29**, 3885-3896.

Khuong, T. M., Habets, R. L. P., Kuenen, S., Witkowska, A., Kasprowicz, J., Swerts, J., Jahn, R., van den Bogaart, G. and Verstreken, P. (2013). Synaptic PI(3,4,5)P3 is required for Syntaxin1A clustering and neurotransmitter release. *Neuron* **77**, 1097-1108.

Kolsch, V., Charest, P. G. and Firtel, R. A. (2008). The regulation of cell motility and chemotaxis by phospholipid signaling. *J. Cell Sci.* **121**, 551-559.

Leevers, S. J., Weinkove, D., MacDougall, L. K., Hafen, E. and Waterfield, M. D. (1996). The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth. *EMBO J.* **15**, 6584-6594.

Lim, D. A. and Alvarez-Buylla, A. (2014). Adult neural stem cells stake their ground. *Trends Neurosci.* **37**, 563-571.

Losick, V. P., Morris, L. X., Fox, D. T. and Spradling, A. (2011). Drosophila stem cell niches: a decade of discovery suggests a unified view of stem cell regulation. *Dev. Cell* **21**, 159-171.

Martin, F. A., Perez-Garijo, A. and Morata, G. (2009). Apoptosis in Drosophila: compensatory proliferation and undead cells. *Int. J. Dev. Biol.* **53**, 1341-1347.

Mirzadeh, Z., Merkle, F. T., Soriano-Navarro, M., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (2008). Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain. *Cell Stem Cell* **3**, 265-278.

Nässel, D. R. and Winther, A. M. (2010). Drosophila neuropeptides in regulation of physiology and behavior. *Prog. Neurobiol.* **92**, 42-104.

Oda, H. and Tsukita, S. (1999). Nonchordate classic cadherins have a structurally and functionally unique domain that is absent from chordate classic cadherins. *Dev. Biol.* **216**, 406-422.

Peterson, C., Carney, G. E., Taylor, B. J. and White, K. (2002). reaper is required for neuroblast apoptosis during Drosophila development. *Development* **129**, 1467-1476.

Rudrapatna, V. A., Bangi, E. and Cagan, R. L. (2013). Caspase signalling in the absence of apoptosis drives Jnk-dependent invasion. *EMBO Rep.* **14**, 172-177.

Sato, T., van Es, J. H., Snippert, H. J., Stange, D. E., Vries, R. G., van den Born, M., Barker, N., Shroyer, N. F., van de Wetering, M. and Clevers, H. (2011). Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature* **469**, 415-418.

Scadden, D. T. (2014). Nice neighborhood: emerging concepts of the stem cell niche. *Cell* **157**, 41-50.

Siegrist, S. E. and Doe, C. Q. (2006). Extrinsic cues orient the cell division axis in Drosophila embryonic neuroblasts. *Development* **133**, 529-536.

Siegrist, S. E., Haque, N. S., Chen, C.-H., Hay, B. A. and Hariharan, I. K. (2010). Inactivation of both Foxo and reaper promotes long-term adult neurogenesis in Drosophila. *Curr. Biol.* **20**, 643-648.

Siller, K. H. and Doe, C. Q. (2008). Lis1/dynactin regulates metaphase spindle orientation in Drosophila neuroblasts. *Dev. Biol.* **319**, 1-9.

Song, X., Zhu, C. H., Doan, C. and Xie, T. (2002). Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. *Science* **296**, 1855-1857.

Sousa-Nunes, R., Yee, L. L. and Gould, A. P. (2011). Fat cells reactivate quiescent neuroblasts via TOR and glial insulin relays in Drosophila. *Nature* **471**, 508-512.

Steinberg, M. S. and Takeichi, M. (1994). Experimental specification of cell sorting, tissue spreading, and specific spatial patterning by quantitative differences in cadherin expression. *Proc. Natl. Acad. Sci. USA* **91**, 206-209.

Thacker, S. A., Bonnette, P. C. and Duronio, R. J. (2003). The contribution of E2F-regulated transcription to Drosophila PCNA gene function. *Curr. Biol.* **13**, 53-58.

Truman, J. W. and Bate, M. (1988). Spatial and temporal patterns of neurogenesis in the central nervous system of Drosophila melanogaster. *Dev. Biol.* **125**, 145-157.

Urbach, R., Schnabel, R. and Technau, G. M. (2003). The pattern of neuroblast formation, mitotic domains and proneural gene expression during early brain development in Drosophila. *Development* **130**, 3589-3606.

Voog, J., D'Alterio, C. and Jones, D. L. (2008). Multipotent somatic stem cells contribute to the stem cell niche in the Drosophila testis. *Nature* **454**, 1132-1136.

Weinkove, D., Neufeld, T. P., Twardzik, T., Waterfield, M. D. and Leevers, S. J. (1999). Regulation of imaginal disc cell size, cell number and organ size by Drosophila class I(A) phosphoinositide 3-kinase and its adaptor. *Curr. Biol.* **9**, 1019-1029.

Yap, A. S., Niessen, C. M. and Gumbiner, B. M. (1998). The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. *J. Cell Biol.* **141**, 779-789.