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Inactivation of both *foxo* and *reaper* promotes long-term adult neurogenesis in *Drosophila*

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Summary

Neurogenesis continues throughout adulthood in the brains of many animals, including some insects [1–3], and relies on the presence of mitotic neural stem cells. However, in mammals, unlike the liver or skin, the regenerative capacity of the adult nervous system is extremely limited. Using the *Drosophila* brain as a model, we investigate the mechanisms that restrict adult neurogenesis, with the eventual goal of devising ways to replace neurons lost by injury or disease. We find that in *Drosophila*, adult neurogenesis is absent due to the elimination of neural stem cells (neuroblasts) during development by cell death. Prior to their elimination, neuroblasts undergo a developmentally regulated reduction in their growth and proliferation rates due to downregulation of insulin/PI3 kinase signaling, resulting in increased levels of nuclear Foxo. These small, growth-impaired neuroblasts are often eliminated by caspase-dependent cell death and not exclusively by terminal differentiation as has been previously proposed[4]. Eliminating Foxo, together with inhibition of pro-apoptotic proteins promotes long-term survival of neuroblasts and sustained neurogenesis in the adult mushroom body (mb), the center for learning and memory in *Drosophila*. Foxo likely activates an autophagic cell death response, since simultaneous inhibition of ATG1 (autophagy-specific gene 1) and apoptosis also promotes long-term mb neuroblast survival. mb neurons generated in the adult incorporate into the existing mb neuropil suggesting that both neuroblast identity and neuronal pathfinding cues are intact. Thus neurogenesis can be induced in adults by antagonizing pathways that normally function to eliminate neural stem cells during development.

Results and Discussion

The *Drosophila* brain provides an excellent model system in which to investigate how neurogenesis becomes progressively restricted because neurons are generated only during development and not in adulthood[5–7]. All neurons within the central brain are generated from the asymmetric cell divisions of neural stem cells known as neuroblasts[8–10]. Neuroblasts are large in size and mitotically active, likely dividing every one to two hours during later larval development[6, 11]. Because central brain neuroblasts divide only asymmetrically and never symmetrically, their numbers remain constant. Therefore they can

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be followed unambiguously over time using neuroblast-specific molecular markers[12]. Since neurogenesis at all stages of development is characterized by the presence of neuroblasts, we first examined whether adult *Drosophila* brains have neuroblasts. We detected no cells displaying the molecular signature of neuroblasts in young (3–5 days post-eclosion) adult brains i.e. expression of Deadpan (Dpn), a neuroblast transcription factor, Miranda (Mira), a cargo carrier of cell fate determinants and nuclear exclusion of Prospero (Pros) (n>10 adult brains). Consistent with previous studies[5¯7], we also found no evidence for cell proliferation in these young adult brains using either MARCM[13], a genetic technique which positively marks cells only after cell division, or by administering BrdU, a thymidine analogue. Therefore adult neurogenesis is likely absent because no actively dividing neuroblasts are present in the adult *Drosophila* brain.

Reduction in neuroblast growth precedes neuroblast disappearance

To understand why adult neuroblasts are not present, we examined brains at earlier developmental stages when neuroblasts are still present. Using an antibody to Dpn and an E2F-responsive GFP reporter (*pcna:eGFP*)[14] (Figure1A,D), we identified 100 (mean, S.D.±2.73, n=8 brain lobes) large neuroblasts within the central brain of each brain lobe in mid L3 larvae (96 hours ALH, after larval hatching) during the height of neurogenesis (Figure1B,C). This is consistent with previous reports[6, 12]. Approximately 30 hours later, at five hours after pupal formation (APF), neuroblast number remained unchanged yet neuroblast cell size and mitotic activity were reduced (Figure1B,C), suggesting that neuroblast growth and division rates were changing. By 30 hours APF, only a few small neuroblasts remained, other than the mb neuroblasts (Figure1B and data not shown) (see below). This coincided with a complete shut down in cell proliferation as assessed by the lack of *pcna:eGFP* expressing cells (compare Figure1A to1D). Thus during early pupal stages, most central brain neuroblasts decrease their rates of growth and proliferation, raising the possibility that these changes trigger neuroblast disappearance and terminate neurogenesis.

Of all the neuroblasts in the central brain, only the mb neuroblasts remain midway through pupal development[6, 15], and are all located on the dorsal surface of the central brain, superficial to the mb calyx (Figure 1D,E). At 15 hours APF, mb neuroblasts can be positively identified among the remaining central brain neuroblasts (Figure1A), solely based on their size (average diameter=11.6 μm, S.D.±0.93, n=48). They remained large and mitotically active, similar to larval neuroblasts until 72 hours APF (Figure1B,C,E,F). After this, we observed a significant decrease in mb neuroblast cell size, followed 6 hours later by a strong reduction in their mitotic activity (Figure1E,F). By 96 hours APF, approximately ten hours before adult eclosion, essentially no mb neuroblasts were detected (Figure1E). Therefore all neuroblasts including the late persisting mb neuroblasts experience a reduction in growth prior to their disappearance, suggesting that a common lineage-independent mechanism links a decrease in growth with neuroblast disappearance.

Mushroom body neuroblasts are eliminated via Reaper-dependent cell death

A recent study[4] concluded that the most likely explanation for neuroblast disappearance within the central brain is terminal differentiation, which requires Pros to localize to the neuroblast nucleus after the final cell division. Therefore, we first examined mb neuroblasts for evidence of nuclear Pros at the time that best approximates when the final cell divisions of these neuroblasts occur (Figure1E). While nuclear localization of Pros could be transient and hence difficult to observe, we and others[15] found no evidence of Pros in the nuclei of mb neuroblasts at 84 or 90 hours APF (Figure S1C,D). This suggests that a mechanism other than terminal differentiation could account for mb neuroblast disappearance. Because neuroblasts located in the abdominal segments of the ventral nerve cord are eliminated by apoptosis[16, 17], we next tested whether central brain neuroblasts, including the mb neuroblasts also

undergo cell death. Indeed, at 25 and 90 hours APF, when either central brain or mb neuroblast number was dramatically declining, we observed neuroblasts displaying characteristics of dying cells, i.e. no nuclear envelope, activated caspase, and fragmented DNA (Figure2A–C, FigureS1A,B). Taken together, these results suggest that at least some central brain neuroblasts including the mb neuroblasts are eliminated by apoptosis and not differentiation.

If this were the case, then central brain neuroblasts would persist in animals with reduced levels of the Reaper-family of pro-apoptotic regulators. We examined brains from animals transheterozygous for two overlapping deletions *Df(3L)H99* and *XR38* [17]. These animals, hereafter referred to as *rpr* mutants, were homozygous-null for *reaper* (*rpr*) and heterozygous for *hid*, *grim*, and *sickle*, all four being pro-apoptotic genes[18]. At 30 hours APF, *rpr* mutant brains had more neuroblasts than wild type controls (Figure2G). This was not due to the initial specification of extra neuroblasts or symmetric neuroblast cell divisions, since *rpr* mutant larval brains have a normal number of large, Dpn-expressing neuroblasts at earlier stages (data not shown). Central brain neuroblasts aberrantly persisted even at 48 hours APF (Figure2D,E,G), yet their numbers were considerably reduced. We also observed transient persistence of mb neuroblasts in brains of young adult *rpr* mutants (Figure2H,I,K, MovieS1,S2). This delay in neuroblast disappearance could be due to residual pro-apoptotic activity in *rpr* mutants. Therefore, we used MARCM[13] to generate neuroblasts that are homozygous null for *reaper*, *hid* and *grim* (*H99*). We observed Dpn-expressing cells in *H99* mutant mb neuroblast clones only (FigureS1E), in brains of young (3–5 days post eclosion) adult animals (n>40 central brain neuroblast clones scored), suggesting that neuroblasts are still eliminated despite removal of additional pro-apoptotic genes. Unfortunately the low frequency of *H99* mutant mb neuroblast clones precluded the use of this method for a detailed study of the fate of persisting adult mb neuroblasts at all stages. Therefore we engineered a transgene, *UAS-RHG* miRNA, which generates microRNAs that simultaneously inhibit *reaper*, *hid* and *grim* and targeted expression of this transgene to neuroblasts using *worniuGAL4* (hereafter referred to as *RHG* miRNA) (FigureS2). In the brains of young *RHG* miRNA adults, the only neuroblasts observed were mb neuroblasts, whose persistence, again, was only transient (Figure2J,K). Similar results were observed following *worniuGAL4* induced expression of baculovirus p35, an inhibitor of active caspases (Figure2F,G,K). Thus, Reaper-family proteins and caspase activation are necessary for the proper timing of neuroblast removal. In their absence, neuroblast elimination is only delayed, suggesting that an alternative caspase-independent pathway compensates to ensure neuroblast removal. Interestingly, neuroblasts persisted longer in *rpr* mutant adults than in those expressing *RHG* miRNA, which could be due to downregulation of *worniuGAL4* (FigureS1F,G). This may indicate that neuroblasts rescued from an apoptotic fate have decreased transcription of neuroblast-specific genes, which may contribute to their disappearance via an alternative mechanism.

Cell death-inhibited mushroom body neuroblasts have impaired growth

Next, we examined the fate of the persisting mb neuroblasts in the brains of young (3–5 days post eclosion) *rpr* mutant adults. Persisting mb neuroblasts expressed both *pcna:eGFP* and Mira, showed an enrichment of membrane bound Scribble (Scrib), and excluded Pros from the nucleus, all indicators of proper neuroblast cell fate (FigureS3A,B,D). They also expressed the mb specific marker, Eyeless (Ey) and failed to express Elav or Repo, two markers characteristic of differentiated neuronal and glial cell fates respectively (FigureS3C,E). However, the persisting adult mb neuroblasts were only half the size of mb neuroblasts present during earlier stages of development (Figure4A) and they divided slowly (FigureS3F–H), generating very few new adult mb neurons (FigureS3I–L). Thus, growth of persisting mb neuroblasts may be impaired, which could contribute to neuroblast elimination via a caspase-independent pathway.

Consistent with this possibility, we observed that changes in the subcellular localization of the transcription factor Foxo correlate with changes in mb neuroblast cell size. Foxo was mostly cytoplasmic in wild type larval neuroblasts, when growth rates are high, consistent with elevated insulin/PI3 kinase signaling[19, 20] (Figure3A,E). However, more Foxo was observed in the nucleus in 72-hour-old pupal mb neuroblasts (Figure3C,E), when neuroblast size decreases (Figure1E,F), suggesting that insulin/PI3K signaling is reduced in these cells. This increase in nuclear Foxo was equivalent to that observed in larval neuroblasts that expressed a dominant negative Insulin receptor (InR DN) transgene (Figure3B,E). Levels of nuclear Foxo were even further elevated in the persisting mb neuroblasts observed in *rpr* mutant adults (Figure3D,E). Thus, the Reaper-independent pathway that eliminates neuroblasts may be activated by a reduction in insulin/PI3 kinase signaling.

Next we asked whether insulin/PI3 kinase activity regulates mb neuroblast cell death. We overexpressed Dp60 in mb neuroblasts to inhibit PI3 kinase and observed that mb neuroblasts undergo premature cell death (Figure3F,G). Conversely mb neuroblasts persist slightly longer in *foxo* mutant mb neuroblasts as well as in mb neuroblasts that express a constitutively active insulin receptor transgene, yet are still eliminated via cell death (Figure3F,H, and data not shown). Therefore levels of insulin/PI3 kinase signaling regulate timing of mb neuroblast caspase-induced cell death.

Foxo restricts growth and survival of apoptosis-inhibited mushroom body neuroblasts

To examine the consequences of maintaining insulin/PI3 kinase signaling in mb neuroblasts with decreased function of Reaper-family proteins, we either removed *foxo* function[19] or overexpressed *Dp110*, the catalytic subunit of PI3 kinase[21] ($Dp110^{o/e}$). We observed Dpn-expressing mb neuroblasts in 2-week-old and even in 1-month-old *RHG* miRNA, *foxo* mutant adults, as well as in $Dp110^{o/e}$, *rpr* mutant adults (Figure3I,K,4I, FigureS4A). In marked contrast, no neuroblasts were observed at these later time points in *RHG* miRNA expressing adults or those mutant only for either *rpr* or *foxo* (Fig3H, FigureS5A,C). Thus activation of the insulin/PI3 kinase pathway overcomes the caspase-independent mechanism that eliminates mb neuroblasts in *rpr* mutants and promotes long-term survival of mb neuroblasts in adult brains.

Does insulin/PI3 kinase activation suppress autophagy in persisting mb neuroblasts? Autophagic cell death due to downregulation of PI3 kinase signaling and apoptosis are both required for proper elimination of the *Drosophila* larval salivary gland[22]. To determine whether elimination of Foxo promotes long-term survival of *RHG* miRNA mb neuroblasts via inhibition of autophagy, we expressed a dominant-negative version of the autophagy-promoting protein ATG1[23] in *RHG* miRNA mb neuroblasts. Again, we observed Dpn-expressing mb neuroblasts in one month old *ATG1 DN*, *RHG* miRNA animals, but not in *ATG1 DN* neuroblast expressing animals alone. Similar to *foxo* mutant animals, *ATG1 DN* mb neuroblasts also have a slight delay in their disappearance. Therefore downregulation of insulin/PI3 kinase may serve as a potent back-up mechanism to ensure mb neuroblast elimination via activation of an autophagic cell death response.

Next we assayed the behavior of these long-term persisting adult mb neuroblasts. We observed that some, but not all, 2-week-old *RHG* miRNA, *foxo* mutant mb neuroblasts were large in size and generated many new progeny compared to young (3–5 days post eclosion) neuroblasts that either expressed *RHG* miRNA or were mutant for *rpr* (Figure4A–C,D,F,H, FigureS4F,H). Similarly, some young (3–5 day post eclosion) *RHG* miRNA, *foxo* mb neuroblasts were also larger in size and generated many more progeny (Figure4A,B,E), suggesting that increased levels of nuclear Foxo also restrict proliferation and growth in mb neuroblasts inhibited for Reaper family proteins. Interestingly, we observed a strong correlation between mb neuroblast cell size and progeny number in 2-week-old *RHG* miRNA, *foxo* mutant adults, but not in

younger mutant adults (Figure4B,C, FigureS6A). Thus, activation of the growth-promoting insulin/PI3 kinase pathway sustains not only long-term survival of adult mb neuroblasts but also increases their proliferation and growth rate. Furthermore, Foxo inactivation may indirectly allow long-term persisting adult mb neuroblasts to acquire growth properties common to neuroblasts during development.

Long-term surviving adult mb neuroblasts generate new mb neurons

Lastly, we analyzed the morphology of the newborn cells by visualizing mCD8:GFP, which perdures in daughter cells following neuroblast cell divisions (Figure4I,K, FigureS3B). Dendrites appeared to project normally into the mb calyx (Figure4J, arrow), while the trajectory of axons was more variable (Figure4K). We observed bundles of axons that projected normally through the mb pedunculus (Figure4K, right) and some that mistargeted, bifurcating prematurely and projecting anterior of the pedunculus (Figure4K,left). Axon pathfinding defects could arise from limited guidance cues in the fully-developed adult brain. Alternatively, some of these supernumerary neurons may have an inappropriately specified identity. We observed no defects in the mushroom body structure of *RHG* miRNA or *foxo* mutant adults. Thus, at least a subset of supernumerary mb neurons generated from long-term surviving mb neuroblasts appear to incorporate into the existing mature adult mushroom body structure.

Conclusions

Our findings demonstrate that two pathways act in concert to eliminate mb neuroblasts and terminate neurogenesis (Figure4L). Downregulation of insulin/PI3 kinase signaling occurs first and may activate both autophagy and a program of caspase-dependent cell death. In the absence of one of these cell death pathways, mb neuroblasts persist, but only transiently. Thus a failsafe mechanism likely exits to ensure mb neuroblast elimination, similar to salivary gland cells [22].

The reduction in growth that precedes neuroblast apoptosis appears to be developmentally regulated since it occurs at an earlier time in central brain neuroblasts than in mushroom body neuroblasts. This may be due to either local differences in microenvironments or differences in the ability of neuroblasts to respond to circulating insulin-like peptides[24]. Moreover, the extended survival of mb neuroblasts under these conditions, but not other central brain neuroblasts, suggests that additional mechanisms such as terminal differentiation still function to ensure elimination of most neuroblasts[4، 25]. Indeed, during mammalian development, neural progenitors are eliminated via cell death and by terminal differentiation. The relative importance of death and differentiation for neuroblast elimination may be lineage dependent. Finally because cricket adult mb neuroblasts proliferate in response to insulin in explant cultures[26], a common mechanism may regulate adult neurogenesis among insects and possibly in more distantly related metazoans. Our findings may represent an important first step towards devising ways to manipulate the regenerative capacity of adult brains in diverse species and provide insight into how aberrantly persisting neural stem cells behave *in vivo*.

Experimental Procedures

We used the following fly stocks: *w;FRT80B,Df(3L)H99, XR38, foxo*^{25}, *UAS-GFP,tub-GAL4;FRT80B,tub-GAL80, worniu-GAL4[12], UAS-p35, UAS-flp, hsp70flp; act5c-FRT-stop-FRT-tau:lacZ, tub-GAL80[ts], UAS-mCD8:GFP, UAS-Dp60, UAS-Dp110, UAS-InRDN, UAS-ATG1DN (UAS-ATG1*^{K38Q}*), pcnaeGFP*, and OregonR. We replaced eGFP with GAL4 in the pRD119 transformation vector[14] to generate *pcnaGAL4*. We used the miRNA mir6.1 method[27] to generate *UAS-RHG* miRNA. *rpr, hid, grim* sequences (FigureS2) were subcloned into one of three stem-loop units and ligated into pUAST or pGMR. Transgenic animals were generated by BestGene (Chino Hills, CA).

Neuroblast clones were induced by shifting freshly eclosed adults (*pcnaGAL4, UASflp, tubGAL80[ts],act5c-FRT-stop-FRT-tauLacZ*;Df(3L)H99/Df(3L)XR38) to 30°C or to 37°C for 15 mins (hsflp;act>stop>tauLacZ;H99/XR28). MARCM animals were shifted to 37°C for 1 hour at white pupal stages. Adults were fed BrdU (.5mg/ml) mixed with yeast paste and food dye for 24 or 48 hrs.

Adult and pupal brains were fixed for 25 mins. in 4% paraformaldeyde/PEM buffer (Pipes, EGTA, $MgCl_2$) and processed using standard methods[12]. White pupae were picked (t=0 hours) and aged accordingly. We used Lamin (DHSB, #ADL67.10) and other antibodies previously described[12, 20]. For BrdU labeling, brains were incubated in 2N HCl for 30 min. before processing. Cell death was assayed using a TUNEL kit (Roche). Labeling reaction proceeded for 1 hour at 37°C. All images collected on a confocal microscope and images processed with ImageJ, Photoshop, and Illustrator software.

Brains were imaged from the ventral to dorsal surface. When neuroblasts were too small to be distinguished by size, we counted only isolated Dpn-expressing cells. The small Dpn-expressing cells generated from intermediate progenitors that reside mostly in clusters on the dorsal brain surface were not counted. Average neuroblast diameter was determined by measuring the length of two perpendicular lines each passing through neuroblast center. Average pixel intensity was determined for three separate equal sized regions in the cytoplasm and in the nucleus, and then averaged. Student's two-tailed t-test was performed for statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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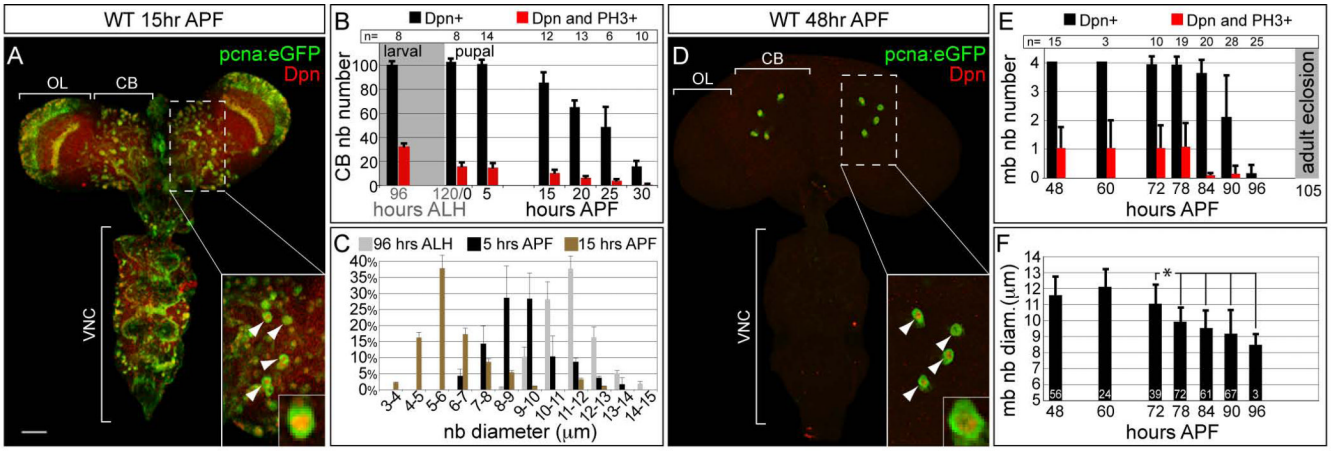

Figure 1. Neuroblast growth becomes restricted during pupal development

(A,D) Z projection of the brain (optic lobe, OL, and central brain, CB) and ventral nerve cord (VNC) from wild type (WT) animals (pcna:eGFP transgenic) at 15 (A) or 48 (D) hours after pupal formation (APF). Neuroblasts express Deadpan (Dpn, red) and the E2F reporter pcna:eGFP (GFP, green). (D) Only the four mushroom body (mb) neuroblasts (nbs) located on the dorsal CB surface persist late (arrowheads in A,D). Dotted box denotes area of CB magnified in larger inset with one of the four mb neuroblasts in small inset. Scale bar (A) equals 50μm. (B,E) Quantitation of CB, (including the mb neuroblasts) (B) and mb (E) neuroblast number and number of mitotic neuroblasts based on PH3 (phospho-histone H3) per brain lobe over time. n=number of brain lobes scored per time point. (C) Distribution of CB neuroblast cell size over time. The average diameter of all CB neuroblasts from three brain lobes (3 animals) was measured for each of 3 time points. (F) Quantitation of the average diameter (diam.) of mb neuroblasts over time. Number of mb neuroblasts measured for each time point shown as white number in black columns. *P-value <0.001. Columns represent mean, ±std.dev in this and all subsequent figures. ALH, after larval hatching.

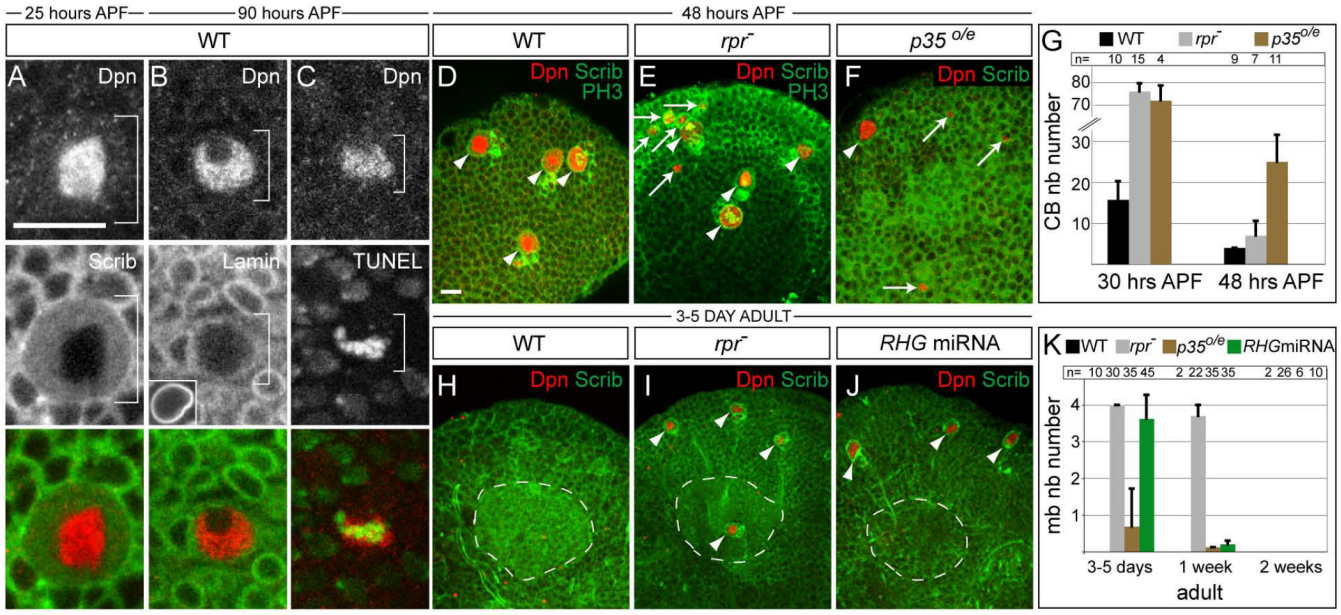

Figure 2. Reaper-family pro-apoptotic regulators and caspase activation are required for the proper timing of neuroblast cell death

(A–C) Wild type (WT) mb neuroblasts undergo cell death (B,C). Developmental time and genotype are listed above panels and markers are listed within panels in this and all subsequent figures. (A–C) Top two rows, single channel images with colored overlay in bottom row, showing co-localization of cell death and neuroblast markers (B,C). Mb neuroblast (brackets) at early pupal stages (A), days prior to mb neuroblast cell death (B,C). DNA fragmentation (C, TUNEL) and nuclear envelope breakdown, as indicated by an absence of Lamin staining (B), are hallmarks of cell death (compare with Lamin staining of a mb neuroblast with an intact nuclear envelope, B, inset). (D–F, H–J) Z-projections of the dorsal, anterior CB surface. Aberrantly persisting neuroblasts are marked with white arrows and mb neuroblasts are marked with arrowheads. In *rpr*$^{-}$ and *RHG* miRNA adults, the aberrantly persisting neuroblasts are the mb neuroblasts. Only one of the 4 mb neuroblasts is shown in panel F. (H–J) The mb calyx is outlined by the white dash. (G,K) Quantitation of Dpn-expressing CB or mb neuroblast number per brain lobe over time. n=number of brain lobes scored at each time point. *worniuGAL4* is used to induce UAS transgene expression (F,G,J,K). Scale bar (A,D) equals 10μm. see also FigureS1,S2.

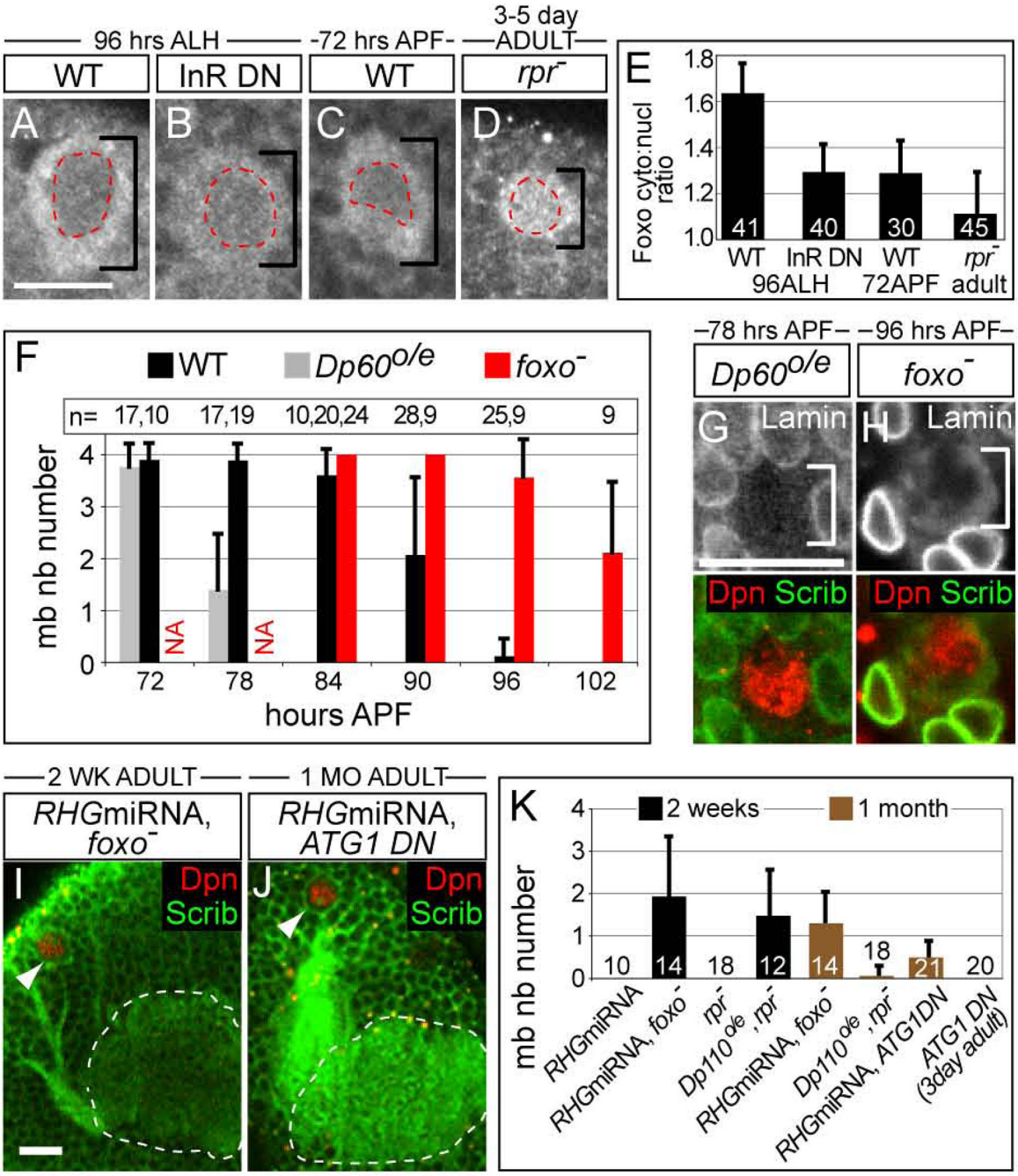

Figure 3. Increased levels of nuclear Foxo restrict long-term survival of mb neuroblasts

(A–D) Subcellular localization of Foxo in neuroblasts. All neuroblasts imaged under same settings for direct comparison. Neuroblasts are marked with black brackets and their nuclei are outlined in red. (E) Quantitation of the ratio of cytoplasmic to nuclear Foxo protein, based on fluorescence pixel intensities. White number in black columns equals number of neuroblasts scored for each genotype/time. (F) Quantitation of mb neuroblast number over time. (mean, std deviation). Dp60 overexpression (o/e) inhibits PI3K signaling leading to premature mb neuroblast cell death (F,G), while *foxo* mutant mb neuroblasts delay death (F,H). n equals number of brain lobes scored for each genotype at each time point (F). (G,H) Dying mb neuroblasts lack a nuclear envelope. Compare Lamin staining with inset in Figure2B. Top

panels are single image and bottom row is overlay. (I,J) Z-projections of the dorsal brain surface showing persisting adult mb neuroblasts (arrowheads) near the mb calyx (white outline). (K) Quantitation of mb neuroblast number over time. Numbers in columns equals number of brain lobes scored for each genotype/time. *worniuGAL4* is used to induce UAS transgene expression (B,E,F,G,I–K). Scale bar (A,G,I) equals 10μm. see also FigureS3.

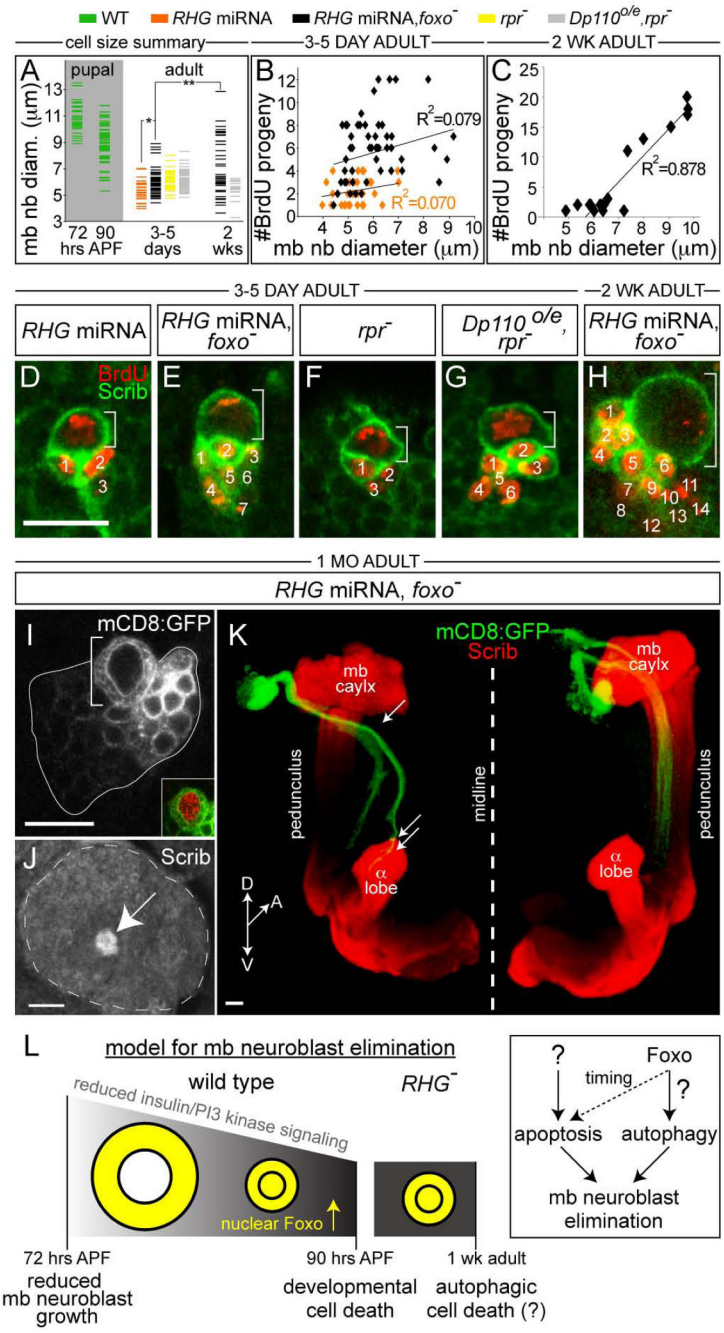

Figure 4. Long-term surviving mb neuroblasts proliferate and generate new adult mb neurons

(A) Summary of mb neuroblast cell size over time. Genotypes color-coded at top for panels a–c. Each dash (A) or diamond (B,C) represents one neuroblast. (A) *P-value <0.003, **P-value <0.0003. (B,C) Number of BrdU progeny generated in 24 hours relative to mb neuroblast cell size. Foxo restricts mb neuroblast proliferation rate and cell size. Mb neuroblast cell size and proliferation rate correlate in 2-week-old *RHG* miRNA, *foxo*$^{-}$ adults (C) but not in 3–5 day adults (B). R^2=coefficient of determination for a linear correlation. (D–H) Mb neuroblasts (white brackets) and progeny (numbered) following a 24 hour BrdU treatment. (H) Only 14 of 18 progeny are shown. (I,K) Month-old adult mb neuroblasts (I, bracket) generate new mb neurons, cell bodies (I, outline) and axons (K). Inset is double-labeled neuroblast, mCD8:GFP

(green) and Dpn (red). genotype: *worniuGAL4,UASmCD8:GFP,UAS-RHG*miRNA,*foxo*$^{-}$/foxo^{-} (K) Reconstruction of the mushroom body (red) with overlay of newborn adult neurons (mCD8:GFP, green). (J) Dendrites from new neurons project normally to the mb calyx (arrow, 2-week adult). (K,right) Axons project either normally through the mb pedunculus or mistarget (K,left). Single arrow marks premature axon bifurcation (K,left) and double arrow marks termination at the top of the α lobe. (L) model (see discussion). *worniuGAL4* is used to induce UAS transgene expression (A–E,G–K). Scale bar (D,I–K) equals 10μm. see also FigureS4.