

# Adult Neurogenesis in *Drosophila*

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## SUMMARY

Adult neurogenesis has been linked to several cognitive functions and neurological disorders. Description of adult neurogenesis in a model organism like *Drosophila* could facilitate the genetic study of normal and abnormal neurogenesis in the adult brain. So far, formation of new neurons has not been detected in adult fly brains and hence has been thought to be absent in *Drosophila*. Here, we used an improved lineage-labeling method to show that, surprisingly, adult neurogenesis occurs in the medulla cortex of the *Drosophila* optic lobes. We also find that acute brain damage to this region stimulates adult neurogenesis. Finally, we identify a factor induced by acute damage, which is sufficient to specifically activate the proliferation of a cell type with adult neuroblast characteristics. Our results reveal unexpected plasticity in the adult *Drosophila* brain and describe a unique model for the genetic analysis of adult neurogenesis, plasticity, and brain regeneration.

## INTRODUCTION

Many animal tissues, including the brain, contain slow cycling cells whose proliferation has important functions during normal tissue homeostasis and disease (Gould, 2007; Kempermann, 2012; Lledo et al., 2006). In the adult brain, neurogenesis contributes to neural plasticity, damage repair, regeneration (Ohira, 2011; Wang et al., 2011), and is an important aspect of cortical function necessary, for example, for the integration of new memories (Deng et al., 2010; Zhao et al., 2008).

Previous work in *Drosophila* has detected no neurogenesis in the adult brain (Kato et al., 2009; Siegrist et al., 2010), suggesting that tissue stability has been favored over plasticity. Here, we used an improved method to show that adult proliferation and neurogenesis occur in the *Drosophila* optic lobes (OLs).

## RESULTS

### A Method to Detect Proliferation: “Perma-Twin”

In order to identify dividing cells in the *Drosophila* adult, we developed a mitotic recombination-dependent lineage-labeling method based on the twin-spot MARCM system (Yu et al.,

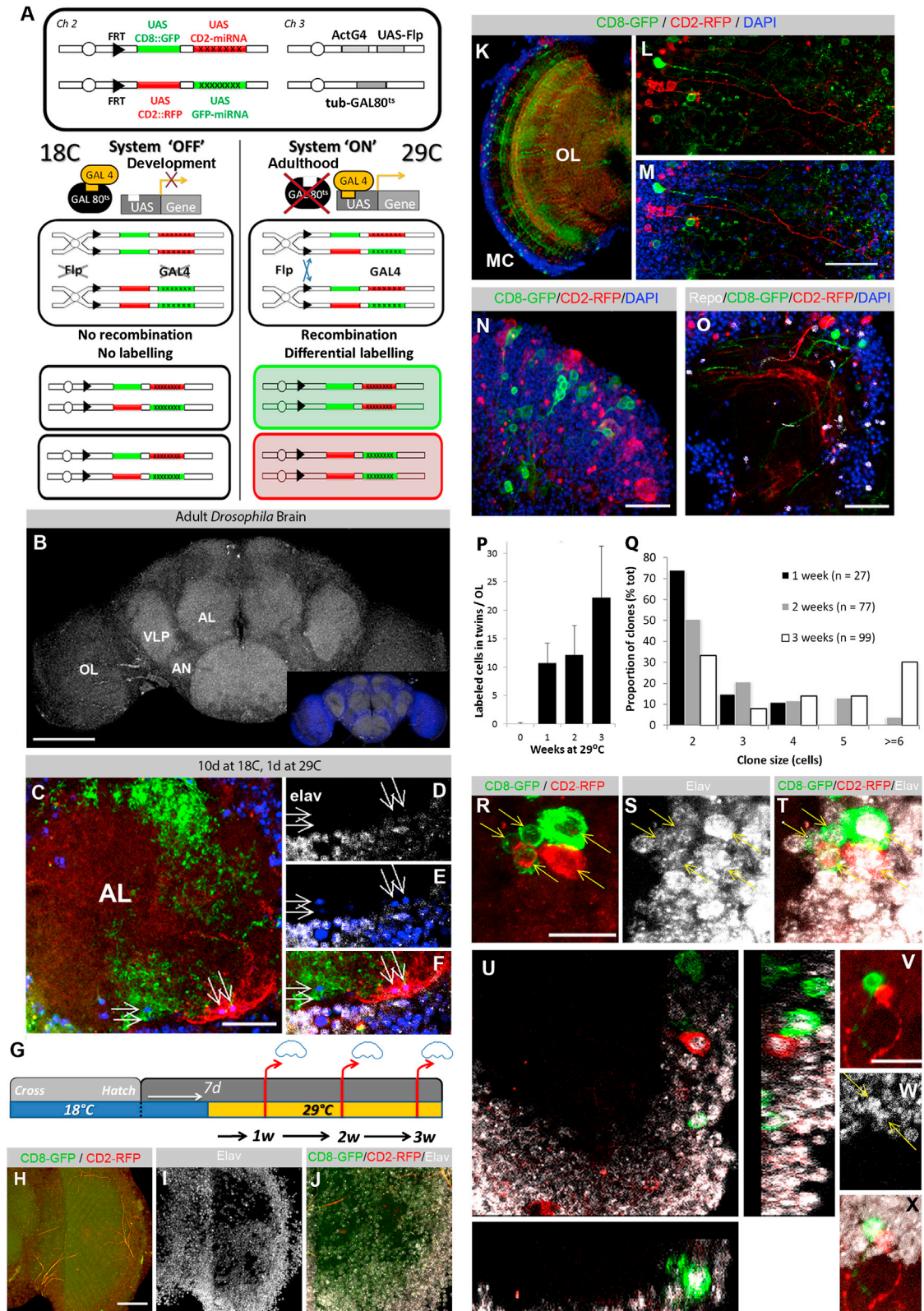
2009) with sustained functional capacity by adding a continuous and ubiquitous source of Flipase that could be switched “on” or “off” with a simple temperature shift from 18°C to 29°C (Figure 1A). For simplicity, we will refer to this twin-spot-based method as the perma-twin genotype because it permanently marks proliferating cells by producing twin clones upon division: one labeled with membrane GFP and the other with membrane RFP (Figure 1A). Because the perma-twin system is constantly active once adult flies are shifted to 29°C, it encompasses the advantage to label even cells proliferating at very low rates (Figures 1A, S1A, and S1B).

We first tested the perma-twin method in the adult intestine (Figures S1C–S1N), where proliferation has been described (Fox and Spradling, 2009; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Flies of perma-twin genotype kept always at 18°C and dissected 9 days after hatching showed no labeled cells (Figures S1C–S1E), confirming that cell markage can be efficiently inactivated (“off state”) during development and adult life. When perma-twin flies were kept at 18°C during development and adults were shifted after eclosion for 9 days at 29°C (on), we observed clones appearing in the posterior midgut (Figures S1F–S1H), but not in the hindgut, which normally shows a sporadic-to-nonproliferative activity in the adult if undamaged (Fox and Spradling, 2009). In a third experiment, we kept the flies throughout embryonic and larval stages always at 29°C (on) to confirm that the whole adult gut was labeled, and therefore, all divisions were traced (Figures S1I–S1K). Finally, we tested perma-twin flies lacking the Flipase required for recombination and could not observe any labeling in the adult intestine even when flies were kept always at 29°C (Figures S1L–S1N), showing that there are no spontaneous recombination events leading to false-positive-labeled cells.

Previous reports showed that glial cells continue to proliferate during adulthood in the *Drosophila* brain (Kato et al., 2009; von Trotha et al., 2009). To confirm this, we raised perma-twin flies at 18°C (off), including embryonic and larval development plus the first 10 days after hatching. Ten-day-old adult perma-twin flies were then shifted for 1 day to 29°C and dissected. As reported by Kato et al. (2009) and von Trotha et al. (2009), labeled cells that were negative for the neuronal marker Elav (Robinow and White, 1991) appeared around the antennal lobe (AL) (Figures 1B–1F).

### Proliferation in the Adult *Drosophila* Brain

Because adult neurogenesis is normally based on slow proliferating progenitors (Abrous et al., 2005; Mizrahi et al., 2006), we decided to look for adult-born neurons in *Drosophila*. To this



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end, perma-twin flies were kept at 18°C (off) during embryonic and larval development, as well as during the first week post-hatching (Figure 1G). Those flies contained no labeled cells (18 out of 25) or one single-labeled cell (7 out of 25) (Figures 1H–1J). Flies were then transferred to 29°C and brains dissected 1, 2, and 3 weeks after the temperature shift (Figure 1G). Using this method, we observed green- and red-labeled cells next to each other in the medulla cortex of the OLs (Figures 1K–1N). The occurrence of labeled green and red cells next to each other, at a high frequency and that increase over time in the on state, contrasts with the very seldom appearance of single-labeled cells in the off state (Figure 1P). The tagged clones did not appear to be normal glial cells because they were negative for the glial marker Repo (Xiong et al., 1994) (Figure 1O). Production of marked clones after mitotic recombination depends upon subsequent cell division and is, therefore, a direct means to assay proliferation. We consequently measured the size of the clones (cells per clone), which indeed increased over time (Figures 1P and 1Q). In fact, the total number of labeled cells in twins per OL increased from 10.7 in the first week to 22.3 in the third week (Figure 1P). Although 74% of clones at 1 week consisted of just two cells, and the maximal clone size detectable was four cells (black bars,  $n = 27$  clones counted), already 16.9% of the clones comprised five to six cells by week 2 (gray bars,  $n = 77$ ). Finally, after 3 weeks, 30% of the clones were larger than six cells (white bars,  $n = 99$ ) (Figure 1Q). Notably, the trend toward bigger clones correlated with a decreased representation of small clones, indicating that a subset of the initially detected small clones had undergone consecutive rounds of divisions over time.

Clones of two cells detected at 3 weeks (Figure 1Q, white bars) might correspond to terminal divisions (Figures 1V–1X), but bigger clones also grew symmetrically (Figures S2A and S2B). Altogether, these results show proliferation in the adult *Drosophila* brain after the first week of adulthood. We have focused here on compact clones, where green cells grow next to red cells, to better understand their growth rates. However,

migration is likely to occur and will need to be analyzed in the future. Based on our data, we estimate an average of at least four to six division events per OL per week in compact clones.

### Adult Neurogenesis in *Drosophila*

Because the labeled cells identified in the medulla cortex of the OLs were negative for the glial marker Repo (Figure 1O) and showed a neuron-like morphology (Figures 1L–1N), we decided to stain for the neuronal marker Elav. All newly generated twins in the medulla cortex contained at least one Elav-positive (Elav+) cell when left to mature for 2 and 3 weeks after perma-twin activation (40 out of 40 clones) (Figures 1R–1X). Adult-born neurons seem to have normal projections, connecting the medulla with either the lobula or with both lobula and lobula plate (Tm and TmY neurons, respectively) (Morante and Desplan, 2008) (Figures 1K–1M). This reveals that adult neurogenesis occurs in the medulla cortex of the OLs (Figures 1B and 1K).

### Brain Regeneration in the Medulla Cortex

Subsequently, we asked whether acute damage could further stimulate neurogenesis. To test this, the right eye of adult flies was punctured with a thin sterile filament, which was introduced through the eye into the OL to mechanically injure the medulla (Figure 2A). This procedure created acute damage to the right OL, detectable by localized Caspase activation (Figure 2B) and neuronal death, assessed by TUNEL labeling (Figures 2C–2I). One-week-old adult perma-twin flies kept always at 18°C (off) were subjected to the same mechanical injury protocol. The punctured perma-twin flies were allowed to recover for 2 hr at 25°C before being shifted to 29°C and dissected 5 or 9 days later (Figure 2J). Labeled cells that were negative for glial marker Repo appeared in the damaged region (Figures 2K and 2L). Therefore, the brains were stained for the neuron marker Elav, and the number of labeled neurons was quantified (Figure 2M). Five days after injury, foci of labeled cells, which stained positive for Elav, appeared around damaged areas in the right OL, which were absent in control left OLs (Figures 2N–2Q). At 9 days after

### Figure 1. Adult Neurogenesis in *Drosophila*

(A) Scheme depicting the perma-twin system. The labeling of the cells depends on two fusion reporters (*UAS-CD8::GFP* and *UAS-CD2::RFP*) and two corresponding inhibitors (*UAS-driven miRNA transgenes*, *UAS-GFP-Mir* and *UAS-CD2-Mir* that can silence the expression of the reporters) (Yu et al., 2009). The use of a thermosensitive Gal 80 (*Gal80<sup>ts</sup>*), a repressor of Gal4, allows the conditional expression of Gal4 protein driven by the ubiquitous actin promoter (*act-G4*) and needed to activate the Flipase, which mediates recombination of chromosome arms during cell divisions. When flies are kept at 18°C during development, the system is off, and no cells are labeled. When shifted to 29°C during adulthood, the system is on in all tissues, and dividing cells are labeled.

(B) Adult *Drosophila* brain is shown. Inset shows overlay with DAPI; red dotted line marks OL border to central brain. AN, antennal nerve; VLP, ventrolateral protocerebrum. Scale bar represents 100  $\mu\text{m}$ .

(C–F) Dividing cells appear around AL (C; arrows indicate GFP/RFP cells), which are Elav negative (D–F, Elav is shown in gray). Scale bar represents 20  $\mu\text{m}$ .

(G) Experimental conditions for long-term analysis of perma-twin clones in adult brains. Flies were kept at 18°C during development and 7 days after hatching, then shifted to 29°C to activate perma-twin labeling, and brains were dissected every week.

(H–J) Control brains of 7-day-old adult flies (always at 18°C, system off) do not show GFP/RFP clones in the OL. (H) Merge of GFP and RFP channels. (I) Staining for the neuronal marker Elav. (J) Merge of Elav staining (neurons) and RFP/GFP channels. Scale bar represents 50  $\mu\text{m}$ .

(K–N) After activation of the system, clones appear in the medulla cortex (MC) of the adult OL (K and N), with neuronal morphology projecting processes through the lobula (L and M). Clones are 3 weeks old. Scale bars represent 20  $\mu\text{m}$ .

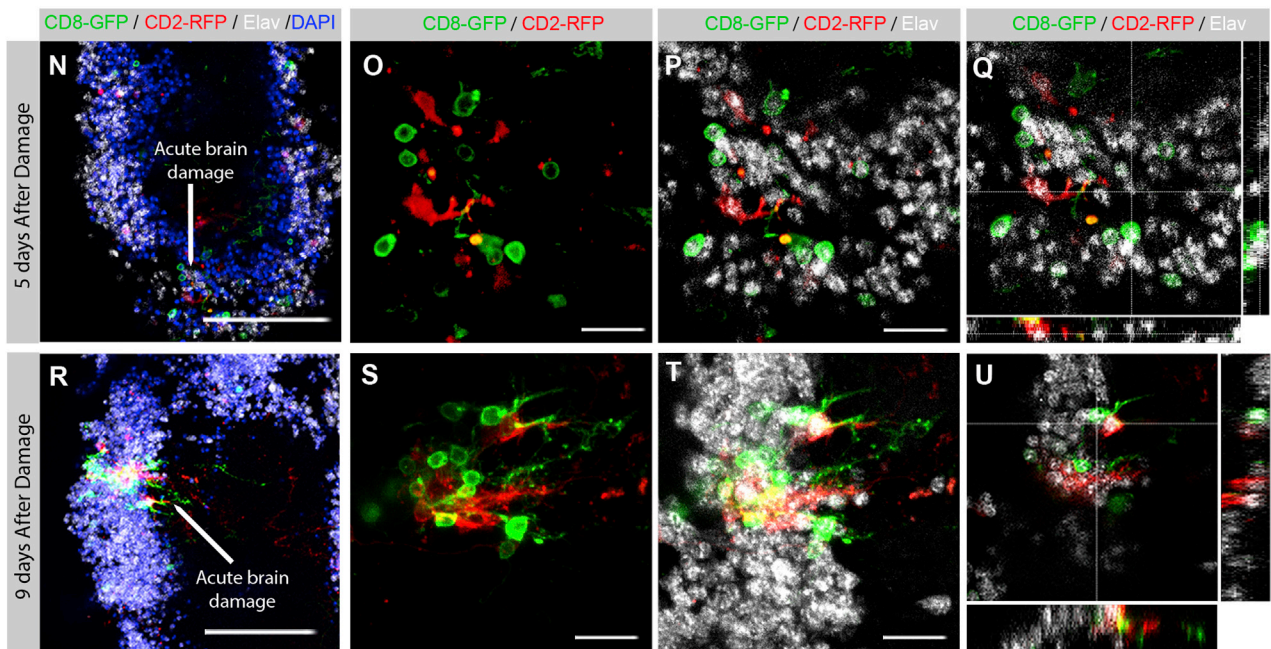
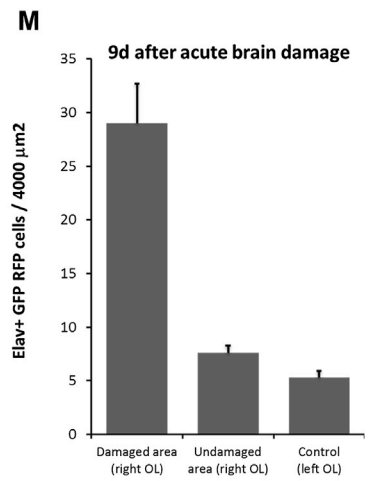
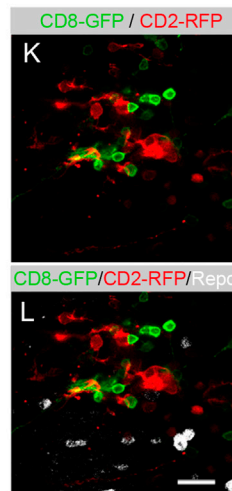
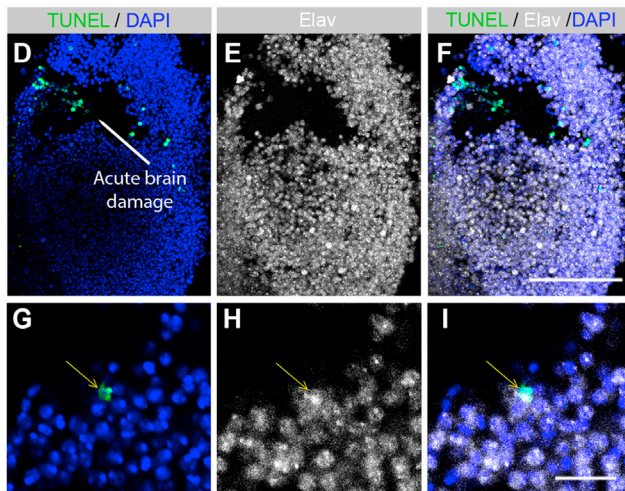
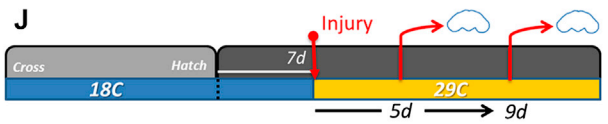
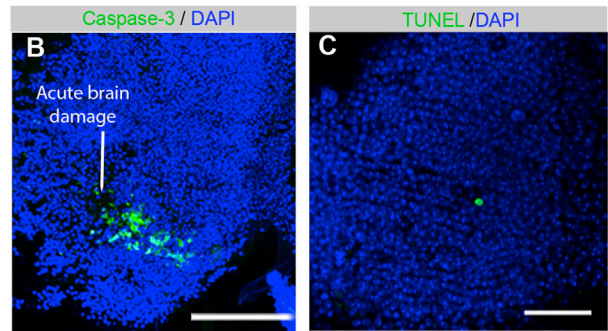
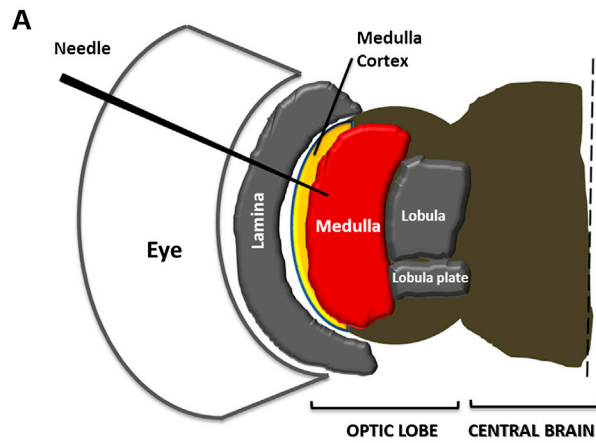
(O) Generated clones are not glial cells (Repo-negative) Scale bar represents 20  $\mu\text{m}$ .

(P) Total number of cells in labeled twin clones per OL at different time points is shown. Error bars indicate 0.5 SD.

(Q) Size distribution of clones detected 1, 2, or 3 weeks after activation of the perma-twin system during adulthood (in percentage [%] of total clones analyzed,  $n$ ).

(R–X) Neurons are generated during adulthood, assessed by Elav staining and perma-twin labeling. Symmetric divisions occur by either expanding the clone size (R–U, 2-week-old clone) or generating two differentiated neurons (V–X) (U, orthogonal view of clone shown in R–T, showing Elav coexpression). Scale bars represent 10  $\mu\text{m}$ .

See also Figures S1 and S2.



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injury, the needle insertion site was lined by numerous marked cells. Individual clones could not be distinguished anymore, but all labeled areas contained Elav+ cells (Figures 2R–2U), indicating that neurogenesis was locally increased in the damaged areas ( $29 \pm 3.7$  Elav+ cells/4,000  $\mu\text{m}^2$ , based on 40  $\mu\text{m}$  z stacks), compared to injury-distant sites in the right OL ( $7.6 \pm 0.7$  Elav+ cells/4,000  $\mu\text{m}^2$ ) or the undamaged left OL ( $5.3 \pm 0.6$  cells/4,000  $\mu\text{m}^2$ ) (Figure 2M). Taken together, our results clearly reveal that neurogenesis normally occurs in the adult fly brain and is enhanced in response to acute brain damage to the OLs.

### Adult Neuroblasts

Next, we tried to identify some of the potential precursor cells of the adult-born neurons using specific lineage markers. *Drosophila* neurogenesis has been well studied in larval brains (Maurange, 2012; Sousa-Nunes et al., 2010; Ulvklo et al., 2012), where two classes of neuroblasts were described (Boone and Doe, 2008; Egger et al., 2008; Knoblich, 2010; Weng and Lee, 2011). Type I neuroblasts express Deadpan (Dpn) and Asense (Ase); type II neuroblasts only Dpn, but not Ase (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). We therefore looked for several-described progenitor/neuroblast markers, including Ase, Dpn, Earmuff (Weng et al., 2010), and Miranda (Ikeshima-Kataoka et al., 1997) (Figures S3A–S3F; data not shown). In adult OLs, only Dpn-positive (Dpn+) cells were detectable (Figure S3F). Dpn+ cells were scattered throughout the medulla cortex (Figures 3A, 3B, and 3C–3E') and were neither positive for Elav (Figures 3F–3H) nor Repo (Figures 3I–3K), defining a cell type in the adult fly brain. Intriguingly, Dpn+ cells were also found in the central brain (ventrolateral protocerebrum) (Figures S3G–S3I), but we focus here on the population of the OLs.

Several lines of evidence strongly supported the view that Dpn+ cells are quiescent progenitors stimulated upon damage. First, most Dpn+ cells in nondamaged brains showed rather cytoplasmic expression of the transcription factor Dpn (Figures 3C–3E'), and we found that Dpn localization could change upon damage and become nuclear in cells surrounding the injured brain areas (Figures 3L–3N). Second, perma-twin clones contain Dpn+ cells, both during physiologic neurogenesis (Figures 3O–3Q) and upon brain damage induction (Figures 3R–3T). Third, as soon as 24 hr after damage, Dpn+ cells proliferated, showing mitotic markers (Dpn+/PH3+, Figure S3J), but

not expressing asymmetric division markers (i.e., Miranda) (Figures 3U–3W and S3M). Finally, Dpn+ cells appear in clusters of three to eight cells after damage, and those clusters did not come from differentiated glia, via dedifferentiation, because a *flip-out* construct to permanently mark glial lineages (*UASflip, ubi > stop > GFP; repoGal4/Gal80ts*) (Figures S3N and S3O) did not generate Dpn+ cells marked with GFP (Figures 3X–3Z).

### dMyc Is Induced upon Injury

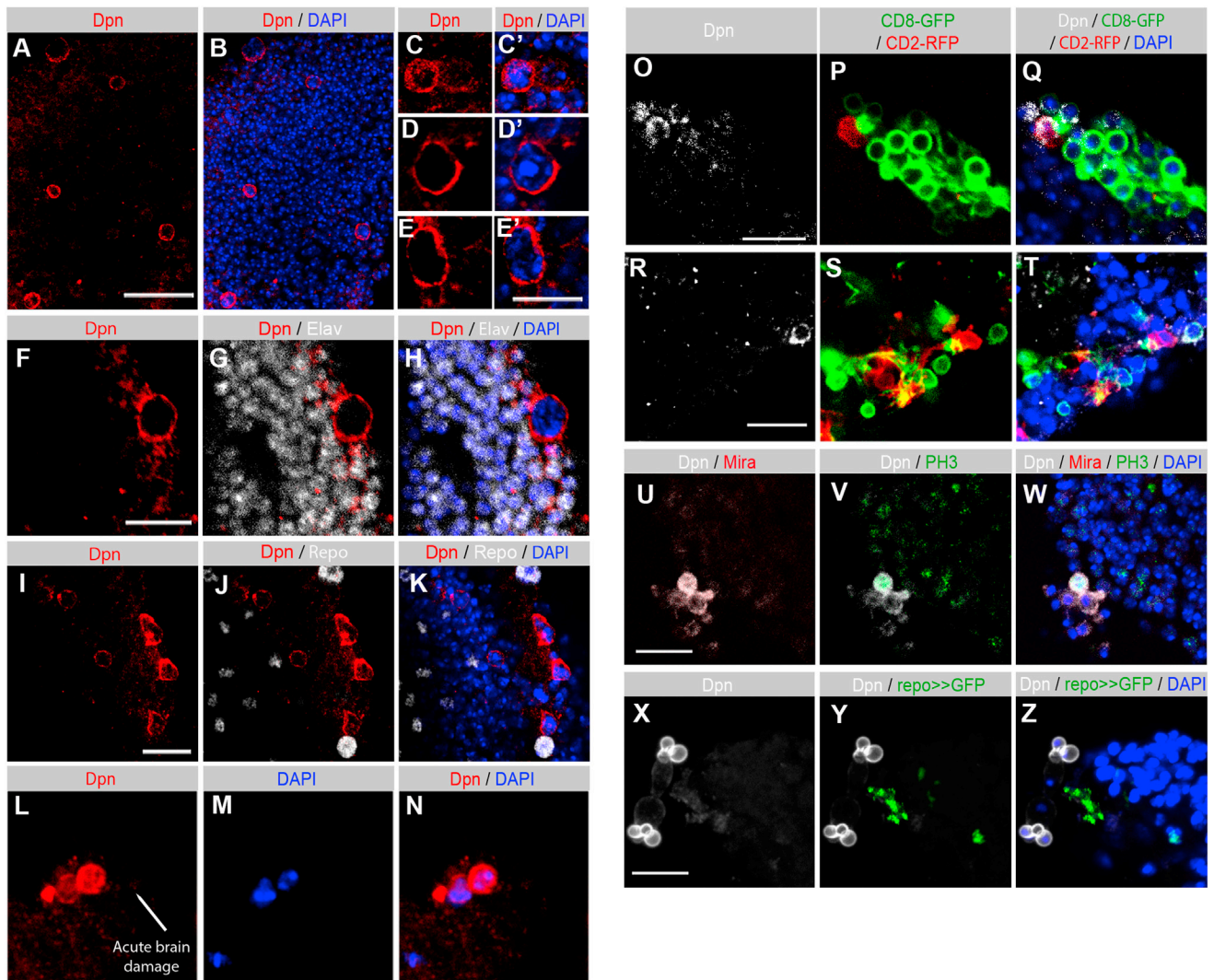
These results suggested that most adult Dpn+ cells are quiescent during homeostatic conditions and activated upon injury. But what are the factors that activate the progenitors? Because dMyc is a marker of proliferating larval neuroblasts (Betschinger et al., 2006; Siegrist et al., 2010), we stained for dMyc 48 hr after acute damage to the OL injury and saw upregulation of dMyc expression around the wound (Figure 4A) and in Dpn+ cells, even in cells when Dpn had not yet relocated to the nucleus (48 hr after brain damage) (Figures 4B–4D).

### dMyc Is Sufficient to Activate Quiescent Neuroblasts

To mimic this pulse of dMyc expression, we performed an experiment in undamaged brains with flies containing *UAS-dmyc* and *hsp70-Gal4*, where overexpression of Gal4 and dMyc was activated with a heat shock at 37°C. Flies were dissected 24 hr later and stained with anti-Dpn and PH3 antibodies. The mitotic marker PH3 revealed a wave of division with many Dpn+ cells coordinated in mitosis (Figures 4E–4H) followed by an increase of duplets of cells with nuclear localization of Dpn (Figures 4I–4K). Five days after heat shock induction of dMyc, we could observe that the number of Dpn+ cells had significantly increased compared to control flies lacking *UASdmyc*, from 39 to 53 Dpn+ cells/OL ( $p < 0.01$ ) (Figure 4L), but Dpn was again cytoplasmic (Figures 4M–4P), and Dpn+ cells were no longer dividing, as revealed by the PH3 mitotic marker (Figure 4O). To test if neurogenesis occurred upon dMyc induction, we used EdU incorporation, a thymidine analog used to witness DNA replication (Salic and Mitchison, 2008). We found EdU/Elav double-positive cells in the OL in flies fed with EdU during 19 days after dMyc induction (Figures S4A–S4C). We applied the same technique to assess neurogenesis after damage using *elav-Gal4, UAS-CD8-GFP* flies, and we also found EdU/CD8-GFP double-positive cells 2 weeks after damage, revealing de novo neurons (Figures S4D–S4F). Most glial cells (Repo+) were not

### Figure 2. Acute Brain Damage Induces Neurogenesis in the Adult Brain

(A) Scheme depicting the acute brain damage protocol. The right OL was damaged by introducing a thin needle through the eye into the medulla (red area). (B and D–I) Needle insertion caused activation of Caspase-3 (B; green) and neuronal cell death (D–I), verified by TUNEL (green) and Elav staining (white). Scale bars represent 50  $\mu\text{m}$  (B and D–F) and 10  $\mu\text{m}$  (insets in G–I). (C) Uninjured control brain. (J) Experimental conditions to analyze neurogenesis in response to brain damage. Perma-twin flies were kept at 18°C up to 7 days after hatching (system off) when damage to the right OL was performed, then flies were shifted to 29°C to activate perma-twin labeling, and brains were dissected 5 and 9 days after brain damage. (K and L) Labeled clones that appeared 9 days after injury in the damaged region were negative for the glial marker Repo. (M) Quantification of newly generated neurons 9 days after brain damage. The graph depicts the number of Elav+ and GFP or RFP cells in damaged and undamaged areas of the right OL and control areas in the left OL. A square area of 4,000  $\mu\text{m}^2$  (z sections 40  $\mu\text{m}$  deep) was analyzed for the three different regions. Error bars are shown as  $\pm$  SD ( $n \geq 5$  brains). (N–U) Representative images of right OLs 5 days (N) and 9 days (R) after brain damage showing the extent of proliferation (GFP and RFP cells) around the needle insertion site (white bar), DAPI (blue), Elav (white). Scale bar represents 50  $\mu\text{m}$ . Insets of the damaged areas in (N) and (R) reveal proliferation (GFP and RFP labeling) of neurons (Elav+, white) 5 days (O–Q) and 9 days after brain damage (S–U). Scale bar represents 10  $\mu\text{m}$ . z Sectioning confirms cells that are double positive for Elav and GFP or RFP (panels Q and U).



**Figure 3. Identification of Dpn+ Cells in the Adult Brain**

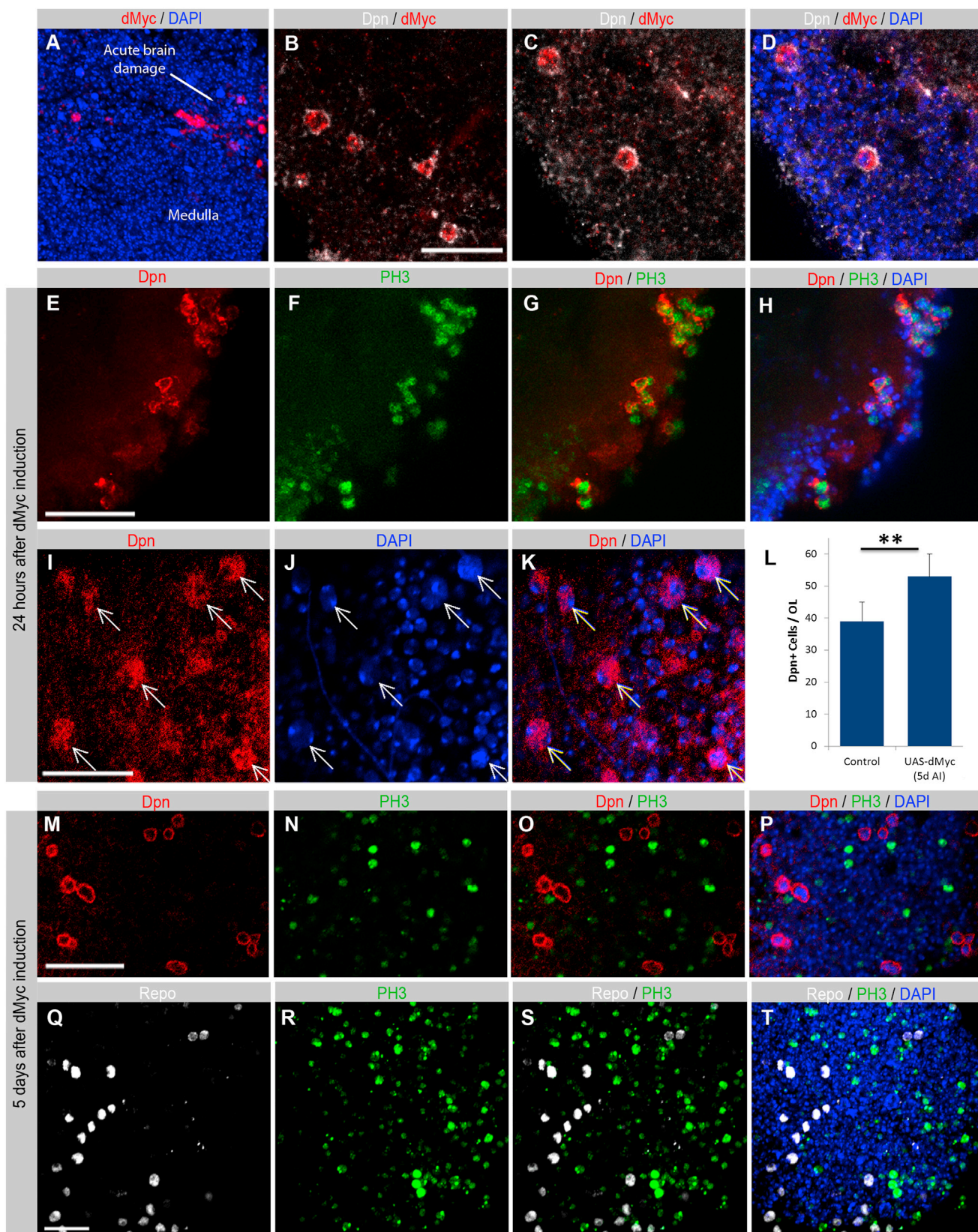
(A and B) Images showing a scattered population of Dpn+ cells (red) in the medulla cortex of the adult OLs. Scale bar represents 50  $\mu\text{m}$ . (C–E) In few cases, Dpn signal (red) appears slightly nuclear (DAPI, blue C and C'), but in most of the cases, it is rather cytoplasmic (D–E'). Scale bar represents 10  $\mu\text{m}$ . (F–K) Dpn+ cells (red) are neither neurons (F–H; Elav-negative, white) nor glia (I–K; Repo-negative, white). (L–N) Nuclear Dpn (red) can be found in proximity to the injury zone (white line) 72 hr after brain damage. (O–T) Dpn+ cells (white) show proliferative capacity because they are found in perma-twin clones (GFP, RFP) in the adult brain during physiologic neurogenesis (O–Q) or upon acute brain damage (R–T) (2 weeks and 9 days after perma-twin labeling, respectively). Scale bars represent 10  $\mu\text{m}$ . (U–W) Dpn+ cells proliferate 24 hr after damage but are negative for the asymmetric division marker Miranda (Dpn is indicated in white, PH3 in green, and Mira in red). Scale bar represents 10  $\mu\text{m}$ . (X–Z) The proliferative Dpn+ cells 48 hr after damage did not come from differentiated glia, assessed by repoG4-lineage tracing. Scale bar represents 10  $\mu\text{m}$ . See also Figure S3.

induced to proliferate by the dMyc pulse, but we cannot exclude some dMyc-induced gliogenesis (Figures 4Q–4T). Therefore, dMyc is activated upon damage in Dpn+ cells and is sufficient to activate their proliferation and Dpn nuclear translocation even in the absence of brain injury.

## DISCUSSION

Here, we find that adult neurogenesis occurs in the medulla cortex of the *Drosophila* OLs. Previous approaches to discover cell

turnover in the adult brain had detection limits. First, traditional mitotic-recombination labeling used pulses of Flipase. Second, the scarce appearance of neurons incorporating EdU or BrdU could be due to the reagents not reaching the neurons efficiently and/or the well-known negative effects of BrdU and EdU during cell-cycle progression, evoking a senescence and DNA damage response, inducing cell death, inhibiting expansion of neural progenitor cells, and repressing neuronal and oligodendroglial differentiation (Diermeier-Daucher et al., 2009; Lehner et al., 2011; Ross et al., 2011; Taupin, 2007). Only when massive



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proliferation is induced, EdU detects neurogenesis (Figure S4A). For those reasons, proliferating cells with slower cell-turnover kinetics may have been missed (Figures S1A and S1B).

The perma-twin method overcomes those complications and identifies a neurogenetic region in the OLs. We also find that acute brain damage further stimulates adult neurogenesis. The regenerative potential of the medulla cortex may have evolved to cope with injury caused by accidents or predators and could be used to follow up how increased adult neurogenesis helps overcome the symptoms of acute brain damage. Understanding this regenerative process in the adult brain may shed light on strategies to promote functional regeneration after injury or degenerative neurological diseases.

Finally, we identify the *Drosophila* homolog of the proto-oncogene Myc, d-Myc, as a critical factor induced by acute damage, which is sufficient to specifically activate the proliferation of a cell type with characteristics of adult neuroblasts. The lineage in the adult appears to be less dependent on asymmetric divisions than during development, which may allow the brain to cope with different degrees of damage.

Our results reveal unexpected plasticity in the adult *Drosophila* brain and describe a model for the genetic analysis of adult neurogenesis, brain regeneration, and adult neural plasticity. In the future, it would be interesting to determine what extent adult neurogenesis in *Drosophila* represents exclusively a replacement mechanism for lost neurons, or is a process that offers an expanded capacity in response to experience and, if so, how experience regulates the cellular composition of the adult brain. Finally, the *Drosophila* adult-born neurons represent a model for understanding the fundamental question of how newly created neurons integrate into the existing neuronal circuitry.

## EXPERIMENTAL PROCEDURES

### Perma-Twin Flies

Flies with the final genotype *w; FRT40A, UAS-CD8-GFP, UAS-CD2-Mir/FRT40A, UAS-CD2-RFP, UAS-GFP-Mir; act-Gal4 UAS-flp/Gal80<sup>ts</sup>* were kept at 18°C up to 1 week after hatching and then shifted to 29°C to activate the system and perform experiments. Additional fly stocks were *erm-Gal4* (B. Egger) and *elav-Gal4* (S. Sprecher).

### Myc Overexpression Experiments

Flies of the genotype *y w hsp70-flp; CyO/lf; UAS-dMyc/hsp70-Gal4* were heat shocked 1 hr at 37°C to induce Gal4 and dMyc expression. Flies were kept at 29°C after heat shock and dissected 24 hr and up to 5 days later.

### Stainings

TUNEL (Roche) and EdU (Invitrogen) staining was performed according to the supplier's protocol. Other immunostaining was performed as referred to in Extended Experimental Procedures.

### Image Acquisition and Clonal Analysis

Images were acquired in a Leica TCS SP2 or SP5 confocal microscope. Only clones containing at least one green and one red cell in contact were counted.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2013.05.034>.

## LICENSING INFORMATION

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### Figure 4. Dpn+ Cells Upregulate dMyc upon Damage, and dMyc Overexpression Is Sufficient to Trigger Proliferation of Dpn+ Cells

(A–D) Forty-eight hours after OL puncture, high dMyc levels (red) are detected around damaged regions (white line) (A). Nuclei are shown in blue (DAPI). (B–D) Dpn+ cells (white) show strong dMyc expression (red) 48 hr after brain damage. Nuclei are stained with DAPI in (D). Scale bar represents 10  $\mu$ m.

(E–K) To mimic damage-induced dMyc expression, a pulse of dMyc was provided by activating *UAS-dmyc* with a heat shock-inducible Gal4. Twenty-four hours after dMyc induction, specific proliferation of Dpn+ cells (red), assessed by phospho-histone H3 staining (PH3 is shown in green), was observed (E–H), along with increased nuclear translocation of Dpn (I–K). Arrows in (I)–(K) indicate cells with nuclear Dpn localization. Scale bars represent 10  $\mu$ m.

(L) dMyc overexpression results in a significant increase of Dpn+ cells (\*\* $p \leq 0.001$ , Student's t test) 5 days after activation of the dMyc pulse. The graph shows the total number of Dpn+ cells per OL in *UASdMyc* and control flies ( $n = 9$  OL). Error bars indicate SD.

(M–P) Five days after the dMyc pulse, twins and clusters of Dpn+ cells (red) are detected in the OLs, suggesting clonal divisions. Note that proliferation of Dpn+ neuroblast-like cells (red) has ceased at this time point (negative PH3 staining is indicated in green), but another cell population (positive for PH3) keeps proliferating. Scale bar represents 20  $\mu$ m.

(Q–T) The proliferating cells (PH3 is shown in green) observed 5 days after the dMyc pulse are not glial cells (Repo-negative is indicated in white), suggesting the existence of another progenitor triggered to proliferate by the initial rounds of divisions of Dpn+ cells. Scale bar represents 20  $\mu$ m.

See also Figure S4.



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## EXTENDED EXPERIMENTAL PROCEDURES

### Design and Construction of Perma-Twin Flies

First, *act-Gal4* was recombined on the third chromosome with *UAS-flp*.; Based on the previously described twin-spot system (Yu et al., 2009) the following stocks were generated and crossed for perma-twin experiments: *w*; *FRT40A*, *UAS-CD8-GFP*, *UAS-CD2-Mir*; *act-Gal4 UAS-flp/TM6B* and *w*; *FRT40A*, *UAS-CD2-RFP*, *UAS-GFP-Mir*; *tub-Gal80<sup>ts</sup>/TM6B* flies yielding progeny of genotype *w*; *FRT40A*, *UAS-CD8-GFP*, *UAS-CD2-Mir/ FRT40A*, *UAS-CD2-RFP*, *UAS-GFP-Mir*; *act-Gal4 UAS-flp/tub-Gal80<sup>ts</sup>*.

### Other Fly Genotypes

For glia-lineage experiments flies of the genotype *UAS-Flp*, *ubi > stop > GFP*; *repo-Gal4/tub-Gal80ts* were used. For EdU incorporation experiments after damage, flies of the genotype *elav-Gal4/UAS-CD8-GFP* were used (*elav-Gal4* from S. Sprecher).

### Brain Dissection and Immunostaining

Brains were dissected in chilled S2 media, fixed in 4% PFA 20 min at RT, washed twice in PBS-Triton-X 1% at least 20 min, blocked with PBT 1%/1% BSA 20 min and incubated with primary (over night at 4°C) and secondary antibodies (1-2h at RT). Brains were mounted in Vectashield with DAPI (Vector Labs) containing a spacer to avoid compression of optic lobes. The following antibodies were used: rat monoclonal anti-Elav (1:50), mouse monoclonal anti-Repo (1:50) (Developmental Studies Hybridoma Bank); rabbit polyclonal anti cleaved Caspase3 (1:50, Cell Signaling); guinea pig polyclonal anti-Dpn (1:400 and 1:200, a gift from S. Tohr and J. Knoblich, respectively); rat anti-Dpn (1:10) (in combination with Biotin-Streptavidin amplification) and rat anti-Mira (1:2000) (gift of C. Cabernard), guinea pig polyclonal anti-dMyc (Rhiner et al., 2010) (1:50, F.A. Martín); polyclonal rabbit anti-PH3 (1:100, Cell Signaling).

### dMyc Staining

Flies were kept at 29°C after damage, dissected 48h later and stained for dMyc, using TSA system, as for wing discs (Portela et al., 2010) (only Figure 4A). Immunostaining for other antibodies was performed as referred in Extended Experimental Procedures.

### TUNEL Staining

Brains were dissected 24h after damage and stained for Elav and TUNEL (Roche) as for wing discs (Lolo et al., 2012).

### EdU Experiments

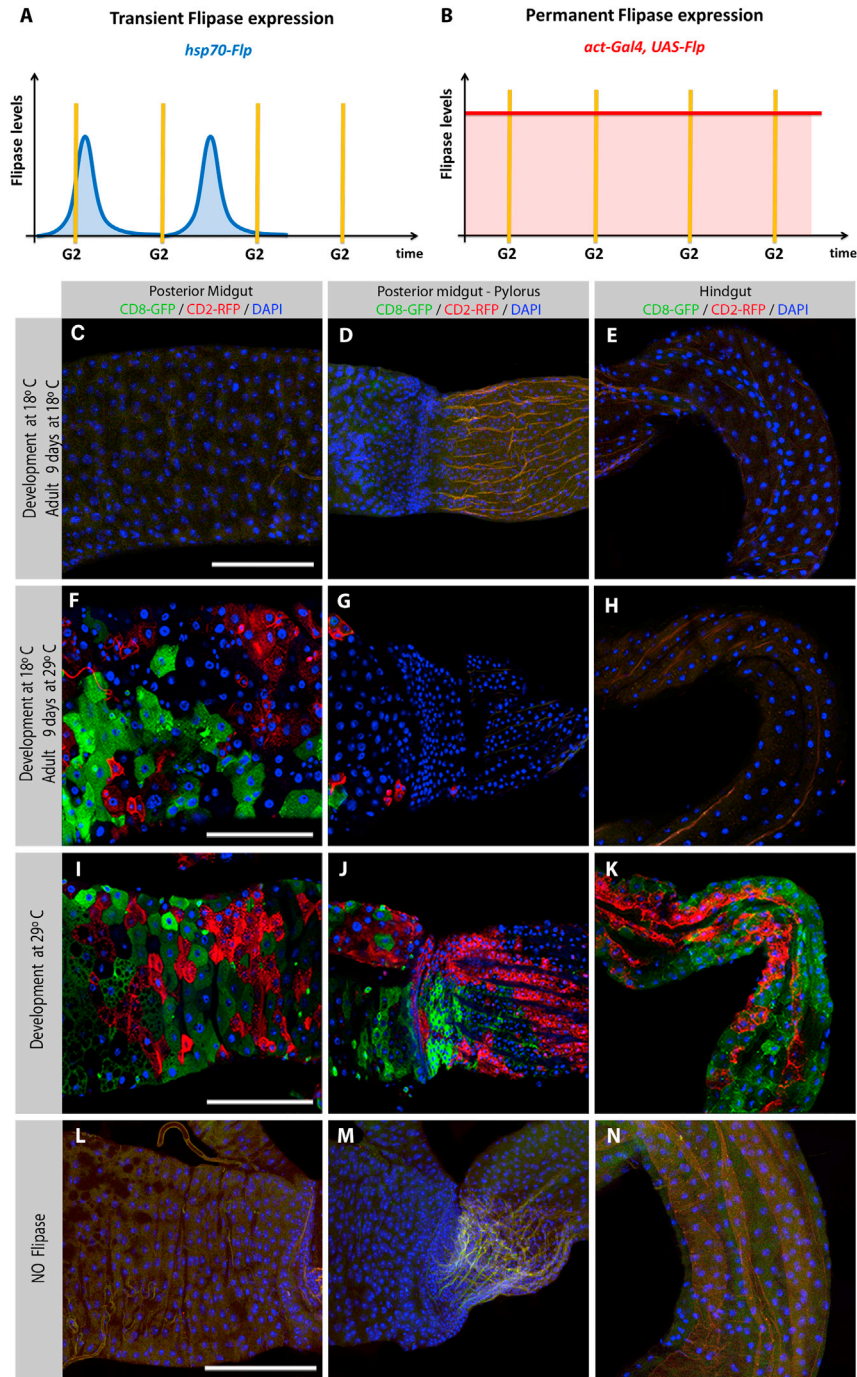
Flies were fed continuously with fresh yeast supplemented with EdU at 100 μM final concentration since dMyc induction until dissection 19 days later. Detection reaction was performed according to supplier instructions (Invitrogen).

### Optic Lobe Puncture

A thin sterile filament (Ø 0.1mm, Fine Science Tools) was introduced through the right eye of adult flies to the level of the optic lobes.

### Image Acquisition and Clonal Analysis

Optic lobes were scanned from anterior to posterior covering most of the medulla cortex surface. Only clones containing at least one green and one red cell in contact were counted. For large clones (>4 cells), we only considered those containing clusters of green and red cells extending in opposite directions. We excluded clones with intermingled green and red cells as they could be generated by the merging of small independent clones. Images were acquired in a Leica TCS SP2 or SP5 confocal microscope.



**Figure S1. Perma-Twin System Validation, Related to Figure 1**

(A) Transient availability of Flipase (*hsp70-flp*) can fail to induce recombination events (and labeling) of slow dividing cells.

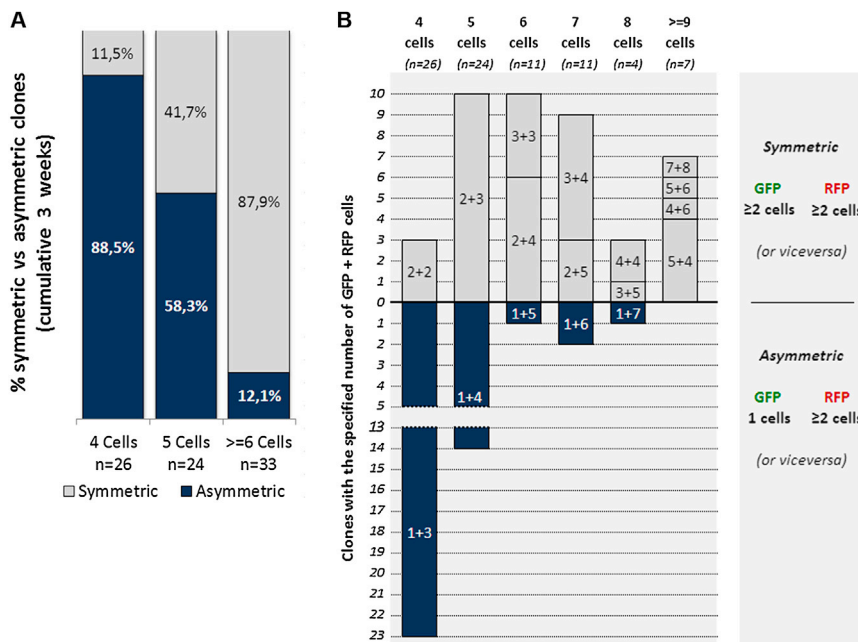
(B) The Perma-twin system provides constant Flipase levels during adulthood under the control of the ubiquitous *actin* promoter (*act-GAL4, UAS-Flp*) ensuring that all mitotic recombination events are traced.

(C–N) Validation of the conditional activation of the perma-twin system in the fly adult intestine: (C–E) no labeled cells are present in intestine of flies kept always at 18°C (perma-twin markage inactivated by Gal80<sup>ts</sup>, 'OFF' state).

(F–H) Flies kept at 18°C during development ('OFF') and shifted to 29°C after hatching during 9 days (system 'ON') only show labeled cells in the posterior midgut, but not in the hindgut, as reported previously (Fox and Spradling, 2009; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006).

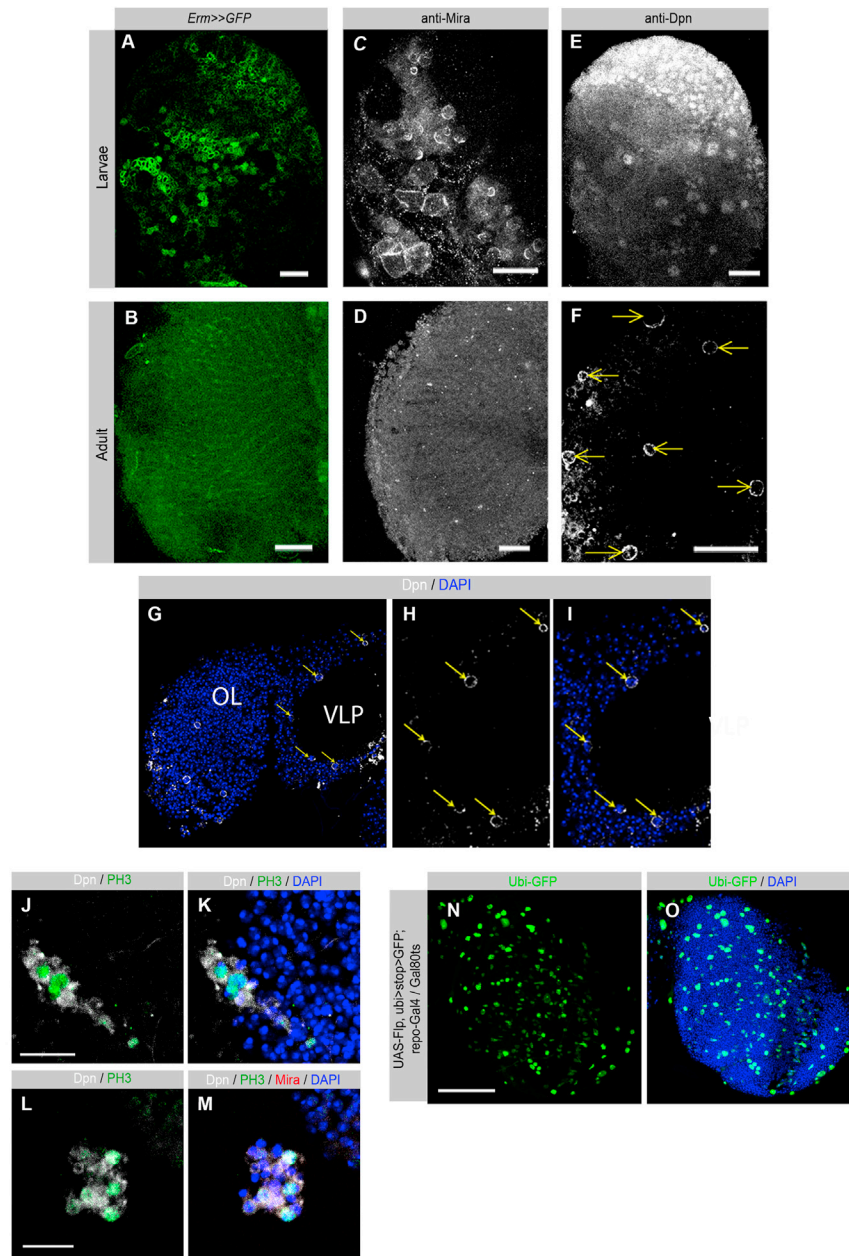
(I–K) When flies were allowed to develop at 29°C (always 'ON'), the entire intestine is labeled, as expected.

(L–N) In the absence of UAS Flipase no cell markage was observed, excluding the possibility of non-Flipase-dependent spontaneous recombinations and thereby appearance of false positives (scale bars, 100 μm).



**Figure S2. Symmetric Distribution of Perma-Twin Clones, Related to Figure 1**

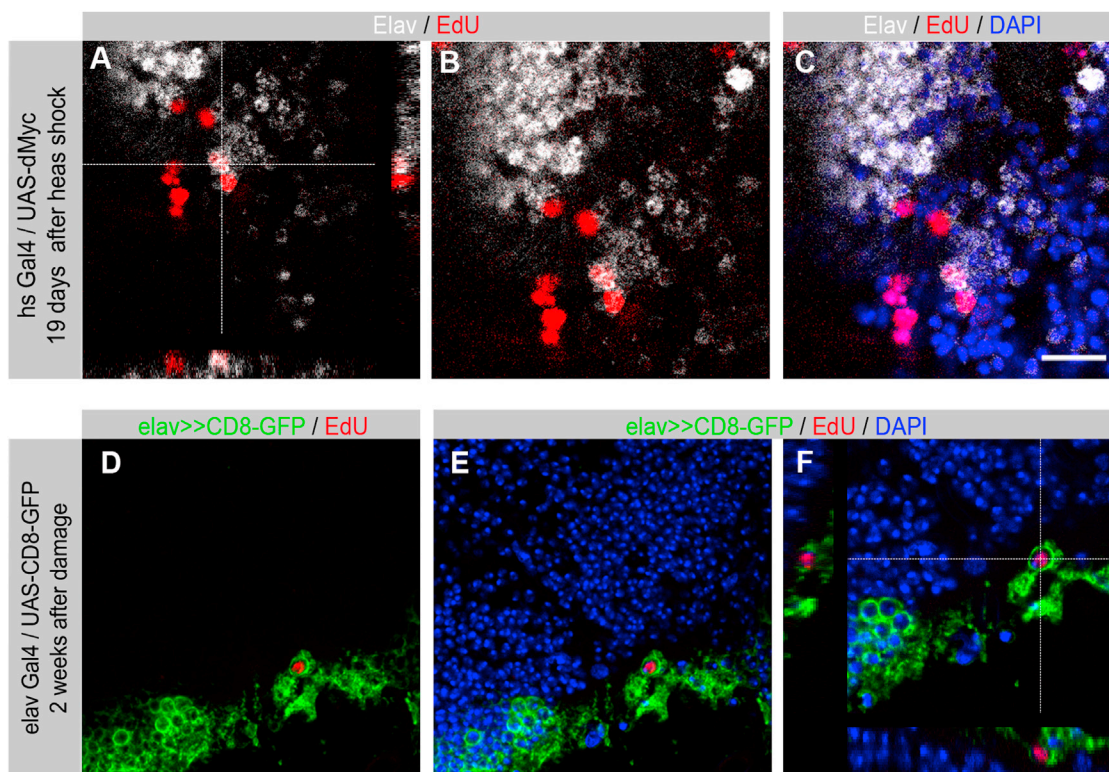
(A) Clones grow in a symmetric way over time (gray bars), assessed by the presence of more than one red and one green cells per compact clone.  
 (B) Specific distribution of green and red cells per clone shows symmetric expansion.



**Figure S3. Dpn+ Cells and Their Proliferation, Related to Figure 3**

(A–M) Expression of several known larval neuroblast (NB) markers was tested in larval and adult brains. Earmuff-GAL4 line (A and B) is active in larval but not adult brains. Also Miranda (Mira, C and D), a carrier protein involved in distribution of cell polarity markers during larval NB asymmetric divisions, is just expressed in larval brain, but not adult optic lobes. Only Deadpan (Dpn, E and F), a marker for Type I and Type II larval NBs, is expressed in both larval and adult fly brains, either in optic lobes as well as in central brain (ventrolateral protocerebrum, G–I, Dpn in white) Scale bars, 20  $\mu$ m. Adult Dpn-positive cells proliferate 24 hr after acute brain damage forming clusters (J and K), that are negative for Miranda (L and M), suggesting symmetric divisions (Dpn, white; PH3, green; Mira, red; scale bar, 10  $\mu$ m).

(N and O) A Flip-out cassette was used to assess dedifferentiation of glial cells to a Dpn+ state after damage (*w; UAS-Flip, ubi > stop > GFP; repo-Gal4/tub-Gal80<sup>ts</sup>*). Gal4 was kept inactivated by Gal80<sup>ts</sup> during development at 18°C and just activated during adulthood at 29°C.



**Figure S4. dMyc- and Damage-Induced Neurogenesis, Related to Figure 4**

(A–C) Elav/EdU cells appear 19 days after dMyc induction. A pulse of dMyc was provided by activating *UAS-dmyc* with a heat-shock inducible Gal4. Flies were fed on EdU for 19 days after heat shock (AHS) and dissected (EdU, red; Elav, white; DAPI, blue; scale bar 10 $\mu$ m).

(D–F) Flies also show incorporation of EdU in neurons 2 weeks after damage, witnessing de novo neurons (EdU, red; elav-Gal4, UAS-CD8-GFP). For EdU staining, anti-GFP antibody was used to overcome GFP bleaching by EdU. GFP signal is restricted to the surface of the optic lobes due to low penetration of the anti-GFP antibody.