Stage-Specific Differences in the Requirements for Germline Stem Cell Maintenance in the *Drosophila* Ovary

Halyna R. Shcherbata,1 Ellen J. Ward,1 Karin A. Fischer,1 Jenn-Yah Yu,1 Steven H. Reynolds,1 Chun-Hong Chen,2 Peizhang Xu,2,3,4 Bruce A. Hay,2 and Hannele Ruohola-Baker1,*

1Department of Biochemistry, Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA 98195, USA
2Division of Biology, MC 156-29, California Institute of Technology, Pasadena, CA 91125, USA
3Howard Hughes Medical Institute
4Department of Physiology
University of California, San Francisco, San Francisco, CA 94158-0725, USA
*Correspondence: hannele@u.washington.edu
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SUMMARY

In this study, we uncover a role for microRNAs (miRNAs) in *Drosophila* germline stem cell (GSC) maintenance. Disruption of Dicer-1 function in GSCs during adult life results in GSC loss. Surprisingly, however, loss of Dicer-1 during development does not result in a GSC maintenance defect, although a defect is seen if both Dicer-1 and Dicer-2 function are disrupted. Loss of the *bantam* miRNA mimics the Dicer-1 maintenance defect when induced in adult GSCs, suggesting that *bantam* plays a key role in GSC self-renewal. Mad, a component of the TGF-β pathway, behaves similarly to Dicer-1: adult GSC maintenance requires Mad if it is lost during adult life, but not if it is lost during pupal development. Overall, these results show stage-specific differential sensitivity of GSC maintenance to certain perturbations and suggest that there may be a Dicer-2-dependent GSC maintenance mechanism during development that is lost in later life.

INTRODUCTION

The formation of embryonic tissues and the regeneration of adult tissues in the animal kingdom depend on stem cell populations. Embryonic stem cells are considered pluripotent due to their ability to differentiate into almost any cell type if placed in an appropriate context. Adult stem cells are undifferentiated cells that mainly reside in microenvironments known as niches, and they possess the ability to produce an undifferentiated stem cell and a daughter cell that can differentiate (Fuchs et al., 2004).

Stem cell function has shown recently to be controlled by concerted actions of extrinsic signals from its respective regulatory niche and intrinsic factors, including hyperdynamic plasticity of chromatin proteins (Li and Xie, 2005; Mesheror et al., 2006). However, not all stem cells remain in their niches continuously. For example, hematopoietic stem cells can relocate from their niche in adult animals (Li and Li, 2006). Yet, it is thought that many adult stem cells can only be fully functional in an appropriate niche. It is therefore important to understand how stem cell maintenance in the niche is regulated.

One of the most fundamental processes a developing animal needs to accomplish is to set aside and protect its precious stem cell population to replenish injured or lost tissues during adult life. At the moment, little is known about the processes involved in establishing stem cells during development, though communication between stem cells and their environment is suggested to be a key regulator of the homeostasis of the process (Gilboa and Lehmann, 2006; Ward et al., 2006). The *Drosophila* GSC niche has been extensively studied and has been an instructive model for understanding niche-stem cell communications. The GSC-niche interaction has shown to be reciprocal; stem cells communicate to niche through the Delta ligand, and the niche furthermore controls GSC maintenance via the TGF-β pathway (Chen and McKearin, 2003; Ward et al., 2006; Xie and Spradling, 1998).

Previous work has demonstrated that miRNAs, small (21–23 nt) RNA molecules that can regulate gene expression, are required for normal stem cell function in mouse, *Drosophila*, and plants (for reviews see Hatfield et al., 2007; Hatfield and Ruohola-Baker, 2007; Shcherbata et al., 2006). Detailed analysis in *Drosophila* GSCs using cell-cycle stage markers revealed that *dcr-1*-deficient GSCs were delayed in the p21/p27/Dacapo-dependent G1/S transition concomitant with increased expression of CDK-inhibitor p21/p27/Dacapo, suggesting that miRNAs are required for stem cells to bypass the normal G1/S checkpoint. Hence, loss of the miRNA pathway might inactivate a mechanism that makes stem cells sensitive to environmental signals that normally control the cell cycle at the G1/S transition (Hatfield et al., 2005; Shcherbata et al., 2006).
Here, we show that, in addition to stem cell division, miRNAs are also required for stem cell maintenance. Furthermore, we identify bantam as a key miRNA required for germline stem cell maintenance in adults. Importantly, Dicer-1 activity is required for germline stem cell maintenance in adults, but surprisingly, its activity is dispensable for maintenance if lost during development. Interestingly, we find that Dicer-2 is required for this developmental resistance of GSCs to loss of Dicer-1 function; if both dcr-1 and dcr-2 are absent in preadult GSCs, the GSCs are not maintained. Similarly, we find that Mad activity is required for GSC maintenance if lost in the adult, but not if it is lost at a younger stage. Our data therefore suggest that Drosophila ovarian GSCs have differential and stage-specific requirements for maintenance during development and in adults and that at earlier stages Dcr-2-dependent adaptive mechanisms may exist that allow GSCs to withstand perturbations that are not tolerated in the adult.

RESULTS

miRNAs Are Required for Adult GSC Maintenance

To assess the requirement for miRNAs in stem cells during different stages of development, we generated germline stem cells (GSCs) that developed in normal conditions throughout larval and pupal stages but lacked Dicer-1 during adult stages. These dicer-1 mutant germline stem cells (hsFLP::FRT82Bdcr-1Q1147X) generated during adult life showed a defect in germline stem cell division kinetics (Figure 1B and Table S4 and Figures S1A–S1C, available...
online), similar to that shown previously for dicer-1 GSCs generated during late larval/early pupal stages (Hatfield et al., 2005). To our surprise, these mutant GSCs showed an additional phenotype: a maintenance defect (Figure 1C and Figure S3A). Similar findings were described recently (Jin and Xie, 2007; Park et al., 2007). Adult-induced dicer-1 mutant GSCs divide slowly and leave the niche. In many cases, a wild-type GSC replaces the departed mutant GSC (Figure 1C). In other cases when there are two mutant GSCs, both GSCs may leave the niche, resulting in an empty germarium (Figure S3B). On average, 12% of dicer-1 mutant GSCs were lost per day, whereas only 2% were lost in the control group (Figure 6A and Figure S1D). This is in sharp contrast to dicer-1 mutant preadult germline stem cells, which are not lost (Figure 6A; Hatfield et al., 2005).

**bantam Is Required for Adult GSC Maintenance**

Because Dicer-1 and, therefore, miRNA function are required for adult GSC maintenance, we analyzed which miRNA(s) is responsible for this phenotype. By using sensor constructs for mir-8 and bantam, we found that these miRNAs are expressed in GSCs (Figure 2). Although the control sensor lacking miRNA binding sites shows uniform GFP expression, including the GSCs (Figure 2A), the GFP expression of mir-8- and bantam-sensor is highly reduced in the wild-type GSCs (Figures 2B and 2D) but is upregulated in mir-8 R- and dcr-1 mutant GSCs (Figures 2C and 2E). These data indicate that mir-8 and bantam are expressed in adult GSCs.

Because mir-8 and bantam are expressed in GSCs, we tested whether they are required for GSC maintenance. GSCs, mutant for mir-8, showed no obvious maintenance or cell division defects during preadult or adult stages (1.6 ± 0.5%, 1.3 ± 0.6% of mir-8 R-mutant GSCs lost/day; Figures 3A and 3B and Table S1). However, adult-generated GSCs mutant for bantam showed maintenance and cell division defects (Figures 3C and 3D and Tables S1 and S4). On average, 14.1% ± 2.8% of bantam mutant GSCs (hsFLP; ban R FRT80B/Ubi-GFP FRT80B) were lost per day, whereas no loss was observed in the control group (hsFLP; FRT80B/arm lacZ FRT80B; Table S1). When bantam clones were generated in preadult stages, the loss was not as dramatic (6.2% ± 0.4% per day; Table S1). However, given the existing evidence that all miRNA production in flies is strictly dependent on Dcr-1 function, it is surprising to note that the bantam larval/pupal clones appear to have a stronger phenotype than the dcr-1 clones. This apparent difference in phenotypic severity may be attributable to differences in gene product perdurance and/or to the inherent variability in the GSC loss assay. Heteroallelic combinations of bantam mutants (ban R1770 R ban EP3622, ban R1770 R ban R, ban EP3622 R ban R, and ban EP3622 R ban EP3622) exhibit similar mutant phenotypes as the ban R clones (but at a lower frequency), suggesting that the defects are due to the loss of bantam function and not due to second site mutations (Figures 3E–3G and Figures S4B–S4E). These data show that bantam and dicer-1 mutant defects in GSC maintenance are similar and therefore suggest that bantam is a key miRNA in assuring the maintenance of adult GSCs.

**Mad-Mutant GSCs Are Maintained if the Mutation Is Induced during Development**

The results described above present an unexpected scenario in which a mutation causes a maintenance defect when the deficiency is introduced during adult stages, but not if it is introduced during late larval/pupal stages. To address the generality of the phenomenon, we tested whether a well-studied component of the GSC maintenance pathway, the transcription factor Mad, fits this paradigm. Similar to dicer-1, Mad was not essential for GSC maintenance if the defect was induced during late larval/early pupal stages (Figures 4A, 4B, and 6A) but was essential if the Mad mutation was introduced in GSCs during adulthood (Figures 4D and 6A; Xie and Spradling, 1998). In addition, as shown before (Xie and Spradling, 1998), these adult-induced Mad-mutant GSCs were defective not only in maintenance but also in normal cell-cycle kinetics (Figures 4C and 6B and Table S4). These data show a similarity between Mad and dicer-1 mutants: both maintain the adult GSCs if the mutation is introduced during pupal development. However, if the mutations are introduced during adult life, Mad and Dicer-1 are essential for normal GSC maintenance.

TGF-β signaling within the GSC niche blocks germline stem cell differentiation by silencing Bam. In the absence of Mad, Bam is derepressed and the GSC differentiates (Chen and McKearin, 2003; Song et al., 2004; Xie and Spradling, 1998). Because GSCs lacking the transcription factor Mad, a key component of the TGF-β pathway, from late larval/pupal developmental stages onward were maintained in the niche, we decided to test whether the differentiation factor Bam was still repressed. Interestingly, we found Bam being repressed in this case (Figure 4F; n = 36 Mad R GSCs and n = 42 WT GSCs). These data suggest that larval/pupal-induced Mad R-mutant GSCs silence Bam by a mechanism other than transcriptional repression by Mad.

**Period of Competence of Preadult Stem Cells Extends through Pupal Development and Ends at Adulthood**

Our data suggest that the miRNA pathway and Mad activity are dispensable when they are lost in young GSCs, but they are essential when they are lost in older GSCs. To identify the latest stage of development at which GSCs are able to overcome the loss of Mad activity for GSC maintenance, we introduced Mad R mutations in GSCs of 3rd instar larva/early pupae, late pupae, and 1- to 4-day-old adult flies (Figure 4E). Interestingly, Mad R GSCs were lost only after adult clonal induction, suggesting that the period of competence of preadult GSC maintenance extends through late pupal stages, but not into adulthood. Previous studies have shown that GSCs already reside in a niche at the late pupal stage (Zhu and Xie, 2003), suggesting that this resilience is not a result of major differences in the morphological environment of GSCs during development and adulthood.
Figure 2. miRNA-GFP Sensors Are Expressed in Distinct Subsets of Cells in the Germarium

miRNA sensor expression patterns in (A, B, and D) wild-type, (C) miR-8<sup>−</sup><sup>−</sup>, and (E) dcr-1 mutant germaria. Sensor expression patterns determined by staining homozygous lines with anti-GFP antibodies (A–D). High GFP levels are observed in control (A), but not in miR-8<sup>−</sup><sup>−</sup> (B) or bantam-sensor GSCs (D), suggesting that miR-8 and bantam are expressed in GSCs. (C) Consistent with this, miR-8-sensor GFP levels increase substantially in homozygous miR-8<sup>−</sup><sup>−</sup> mutant germaria. (E) bantam-sensor is responsive to Dicer; in dcr-1 clones, marked by the absence of β-gal (E‘), the level of GFP fluorescence is higher than that in a nonclonal neighbor (E‘‘). In (E), native GFP expression by one copy of bantam-sensor is analyzed (hs Flp; bay<sup>b3</sup> w<sup>1118</sup>; tub84BT:Avic/GFP-EGFP/+; FRT82B dcr-1<sup>Q1147X</sup>/FRT82B arm-lacZ). Red, Adducin; blue, DAPI (A–D) or β-gal (E); green, GFP. GSCs are marked with dashed lines (white indicates mutant, yellow wild-type or control).
Figure 3. bantam miRNA Is Required for GSC Maintenance in the Niche

(A) miR-8Δ1-mutant germline stem cells are maintained in the niche and divide properly 15 days after adult heat shock.

(B) Graph showing that the bantam mutant GSCs are lost 11 times faster from the niche compared to miR-8 or control GSCs.

(C and D) bantam-mutant GSC clones (C), 4 days after clonal induction) are not maintained in the niche (D), 7 days after clonal induction.

(E–G) Germaria from bantam heteroallelic mutants banEP3622/banΔ1 (E and G) and banL1170/banΔ1 (F) exhibit mutant phenotypes similar to bantam clones; germaria are reduced in size and have a single GSC (E and F) or no GSC (G).

Red, Adducin (A) or Adducin+LaminC (C–G); blue, DAPI; and green, GFP (A–D) or Cadherin (E–G); mutant GSCs or cysts are outlined with dashed lines, departed or differentiated stem cells with turquoise dashes (white indicates mutants, yellow controls).
Preadult Mad and Dicer-1 Interact in GSC Maintenance

Because both Mad and Dicer-1 are required during adult stages but are not required if the components are lost during preadult stages, we tested whether they interact during earlier development to maintain germline stem cells in the niche. Specifically, we reduced the level of Mad in a dicer-1 clonal background or reduced the level of Dicer-1 in a Mad clonal background (hsFLP; Mad12 FRT40A/+; FRT82B dcr-1Q1475/FRT82B GFP and hsFLP; Mad12 FRT40A/ GFP FRT40A; FRT82B dcr-1Q1475/+). In both cases, the clones were induced during late larval/early pupal stages. Interestingly, when both Mad and Dicer-1 activities were reduced at the same time, a clear maintenance defect was observed after preadult clone induction (Figure 6A). These data show that Dicer-1 and Mad interact genetically during developmental stages (a synthetic GSC maintenance defect).

Preadult Germline Stem Cells Lacking Both Dicer-1 and Dicer-2 Activities Are Lost from the Niche

To investigate whether another short RNA producing enzyme contributes to GSC maintenance, we tested the role of small interfering RNAs (siRNAs) in preadult germline stem cells, we tested dcr-2; dcr-1 double mutants and observed a strong maintenance defect when the double mutants were induced during larval/pupal development (Figures 5 and 6A). However, the Dicer-2 pathway alone is not required for larval/pupal or adult germline stem cell maintenance (Figure 6A and Table S3). These data indicate that dicer-1 and dicer-2 interact genetically in some manner to maintain preadult germline stem cells. The Dicer-2 contribution to the dicer-2; dicer-1 germline stem cell maintenance phenotype is not likely to be due to defective miRNA processing, as previous biochemical studies showed that Dicer-2 does not appear to process miRNAs (Lee et al., 2004; Pham et al., 2004). Furthermore, we did not observe any reduction of mature bantam levels by QPCR analysis in dcr-2 homozygous animals compared to the control animals, suggesting that Dicer-2 does not have a major role in bantam processing (data not shown). Interestingly Dicer-2 is known to act through the RNAi pathway to modify chromatin (Grimaud et al., 2006; Lee et al., 2004; Pal-Bhadra et al., 2002, 2004; Peng and Karpen, 2007; Verdel et al., 2004), raising the possibility that chromatin modification contributes to the robust maintenance behavior of preadult germ line stem cells.

Notch Pathway Does Not Require Mad Activity during Development

In contrast to our observations with Mad and dicer-1 clones, GSC maintenance requires Notch signaling from the GSCs to the niche throughout development. In the absence of Neuralized (required for proper processing of Delta or Serrate ligands), GSCs are not maintained in the niche. This Notch signaling requirement is observed in both late larval/early pupal and adult clones (Ward et al., 2006). Furthermore, an increase in Notch ligand production in the germline results in an enlarged niche, which in turn supports additional GSCs. This niche expansion can be induced after pupal development (Ward et al., 2006).

In order to determine whether Notch pathway function in GSC maintenance is Mad dependent during larval/pupal development, we analyzed whether the additional GSCs produced by increased Notch signaling during developmental stages require Mad signaling for their maintenance. We assayed whether ectopic GSCs induced by overexpression of Delta were maintained in the niche if they were also mutant for Mad. Our clonal analysis shows that the ectopic GSCs produced during development do not require Mad for their maintenance in the niche. Similar to the Mad-mutant GSCs described above, we find that the Mad, pUASP-Delta-mutant GSCs are not lost from the niche after larval/pupal clonal induction (hsFLP; Mad12 FRT40A/Ubi-GFP FRT40A; pUASP-Delta/nanosGAL4, Figure 6A, 0.2% ± 1.8% loss/day); the number of germaria containing mutant GSCs remains the same in the two time points analyzed. However, unlike the Mad-mutant GSCs described above, in the Delta overexpression background, the number of Mad GSCs increases (approximately two mutant GSCs to approximately three mutant GSCs/7 days; Figures 6C and 6D), indicating that the Mad-mutant GSCs can divide and are recruited to and maintained in the enlarged niche. Thus, the extra GSCs produced by increased Notch signaling behave similarly to normal GSCs: they do not require Mad activity for maintenance in the niche if the Mad mutation is introduced during preadult stages. Therefore, ectopic GSCs as well as wild-type stem cells have a period of competence during preadult stages that ensures their maintenance within the niche even in the absence of Mad.

DISCUSSION

We draw two important conclusions from this work. First, Dicer-1 and, more specifically, bantam miRNA are required for adult stem cell maintenance (Figure 7A). Second, preadult stem cells have a youthful resilience that is lost at adulthood. Thus, if certain key components required for adult germline stem cell maintenance in developing animals are lost, the animal can overcome this loss and maintain the stem cells throughout life (Figure 7B).

bantam Function in GSCs

The miRNA bantam has been previously found to promote tissue growth in Drosophila imaginal discs (Brennecke et al., 2003). In addition, removing one copy of the endogenous bantam gene has shown to enhance, and overexpression of bantam suppresses, the severity of Hid overexpression-induced apoptosis in the eye (Brennecke et al., 2003). Based on these results, a hypothesis was put forward that bantam simultaneously stimulates cell proliferation and inhibits apoptosis. Furthermore, recent studies have revealed that bantam overexpression mitigates degeneration induced by the pathogenic polyglutamine protein Ataxin-3, which is mutated in the human polyglutamine disease spinocerebellar ataxia type 3 (SCA3).
GSC Maintenance in the Drosophila Ovary

**A**

GFP Add DAPI

12 days after hs

**A’**

hsFLP;FRT40A GFP/FRT40A Mad

GFP

**B**

GFP Add DAPI

12 days after hs

**B’**

GFP

**C**

GFP Add DAPI

14 days after hs

**C’**

Add

**D**

GFP Add DAPI

14 days after hs

**D’**

GFP

**E**

GSC loss depends on developmental stage during heat-shock induction

<table>
<thead>
<tr>
<th>Stage</th>
<th>% of Mad GSC lost/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3</td>
<td>14%</td>
</tr>
<tr>
<td>Early Pupal stage</td>
<td>12%</td>
</tr>
<tr>
<td>Late Pupal stage</td>
<td>10%</td>
</tr>
<tr>
<td>Early Adult stage</td>
<td>8%</td>
</tr>
<tr>
<td>Adult (14 days old)</td>
<td>4%</td>
</tr>
</tbody>
</table>

**F**

GFP Add BamC DAPI

9 d after L/P hs

**F’**

GFP

BamC

**F’’**

GFP

BamC
These studies suggest that bantam miRNA can also suppress neuronal degeneration. The Hippo-tumor-suppressor pathway has emerged as a key regulator for bantam expression in Drosophila imaginal discs in regulating cell division (Nolo et al., 2006; Thompson and Cohen, 2006).

The present work supports a different view of bantam action in Drosophila GSCs, adding new possibilities to the repertoire of bantam’s functions. In the adult stem cell population, bantam miRNA is essential for the stem cell maintenance in the niche (Figures 2 and 3 and Figure S4) and appears to be acting independently of the Hippo pathway as yorkie mutant GSCs are maintained in the niche (Table S1). Many questions remain about this new function of bantam. What biological process is defective in bantam-mutant GSCs that results in their loss from the niche? What are the targets of bantam, and what are the pathways that regulate bantam expression in GSCs? In theory, the biological process and the targets of bantam in GSCs might be the same as those involved with imaginal disc cell-cycle control. However, cell-cycle defects alone cannot account for the GSC loss as dicer-1-mutant GSCs that are generated during preadult stages show adult GSC division defects but are maintained normally.

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in the niche (Hatfield et al., 2005; Figure 6A). Interestingly, the 3' UTR of Mad is a validated target of bantam miRNA in S2 cells (Robins et al., 2005). It is therefore possible that bantam miRNA may directly regulate Mad in GSCs. However, in this scenario, loss of bantam should result in Mad overexpression, yet the bantam mutant phenocopies Mad loss-of-function phenotypes. One potential explanation is that high levels of Mad are just as deleterious to germline stem cells as the lack of Mad activity. If this is the case, then Mad levels would need to be finely tuned by miRNAs in germline stem cells to ensure their maintenance in the niche. Similar fine-tuned regulation of Atrophin by miR-8...
Cells. Further analysis of these findings might ultimately lead to insights into cancer stem cell resilience and even help to reveal ways to rejuvenate failing and/or aging stem cells.

**EXPERIMENTAL PROCEDURES**

**Fly Strains**

We used the following mutant stocks: eyFLP::FRT82B dcr-1
t(T1427) TM3Sb, eyFLP::FRT82B, eyFLP::FRT42 dcr-2(G171)CyO (Lee et al., 2004), Mad
t FRT40A/Cyo (Xie and Spradling, 1998), FRT42D y(w)
(Huang et al., 2005), FRT42B is(wt) (a gift from J. Tamkun), pUASP-De
t (Jordan et al., 2006), w¹; Df3L, ban/GAL4, FRT80B/TM6, ban¹; T1110
banP35D, w¹; banGAL4, UAS-T;Anc/GFP-EGFP (pUAST-bantam [Brennecke et al., 2003]), banP35D;Anc/GFP-EGFP (bantam-sensor [Brennecke et al., 2003]), hsFLP::FRT82B Ubi-GFP/TM3Sb, hsFLP::Ubi-GFP FRT40A/Cyo, yw hsFLP::FRT42D Ubi-GFP/Cyo, hsFLP::Ubi-GFP FRT80B/TM3, and w;PGT40/SMa; nanosGal4/TM3Sb (Bloomington
Stock Center). The miR-8¹ deletion line was generated by imprecise
P element excision of EP(2)2266. EP(2)2269 flies were isogenized with
FRT42D and balanced. Standard P element imprecise excision was car
ried out, and 300 individual excision stocks were screened by primers
5’-ATCCACGTAACTGTAATGGTGACAGGGAATCTGC-3’ and 5’-AGATCG
AAAGCCCCACACCCACATC-3’. The miR-8¹ deletion removes
1316 bp of genomic DNA, including the 23 bp mature miR-8 miRNA.
The deletion spans from 1057 bp upstream of the mature miR-8 se
quence to 236 bp downstream of the mature sequence. The miR-8¹ dete
letion was recombined onto the FRT42D chromosome by using standard meiotic recombination protocols (Xu and Rubin, 1993). The recombined FRT42D miR-8¹ lines were screened by PCR with primers
5’-AAATCTTCACCAGCCACCCAGTG-3’ and 5’-AAGACAGAAGCCCAGCACATCC-3’.

**Generation of pUASP-bantam, pUASP-miR-8, and miR-8-sensor**

**pUASP-bantam**: A partial bantam precursor sequence (584 nt) was
amplified from pUAST-EGFP-bantam construct (Brennecke et al.,
2003) by using the following primers: bantam forward, 5’-ATACGG
GCCCGGTTAACCTGGCACTATAATTTCC-3’; bantam reverse, 5’-ATT
CTAGATTGAGCGACTTAAACATGGG-3’. The amplified fragments
were cloned into UASP plasmid using NotI and XbaI.

**pUASP-miR-8**: A partial miR-8 precursor sequence (729 nt) was am
plified from adult fly genomic DNA with the following primers: miR-8 forward,
5’-ATACGGCGCCGCACGCTACACGACATTTCAATA-3’; miR-8 reverse,
5’-ATCTGAGAAATGGAGATTGGAGAAGATCCTGG-3’. The amplified fragments
were cloned into UASP plasmid using NotI and XbaI.

**miR-8-sensor**: Two perfect complementary target sequences of
miR-8 separated by 16 nt were inserted downstream of
pUASP-bantam and balanced. Standard
P element excision was carried out, and 300 individual excision stocks were screened by primers
5’-ATCCACGTAACTGTAATGGTGACAGGGAATCTGC-3’ and 5’-AGATCG
AAAGCCCCACACCCACATC-3’. The miR-8¹ deletion removes
1316 bp of genomic DNA, including the 23 bp mature miR-8 miRNA.
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5’-AAATCTTCACCAGCCACCCAGTG-3’ and 5’-AAGACAGAAGCCCAGCACATCC-3’.

Robust Maintenance of Preadult GSCs

The presented work reveals the resilience of preadult stem
cells to perturbations that cause GSC maintenance de
fects if introduced in adults. It seems logical that developing
organisms would have a means of protecting their
precious stem cells during the many intricate develop
mental processes that occur. We have shown that stem
cells are still protected late in preadult development (dur
ning pupation), but not during adulthood. What protects the
germine stem cells prior to adulthood in
*Drosophila*? As the niche has already formed by pupal stage, we suggest that the period of competency does not reflect a morpho
logical difference in the niche at different time points. Instead, however, we found that the preadult competence requires Dcr-2. Dcr-2 activity is shown to be required for
the siRNA pathway. As RNA interference (RNAi) path
way-dependent chromatin modifications have been previ
ously observed in *Drosophila* (Grimsdau et al., 2006; Lee et al., 2004; Pal-Bhadra et al., 2002, 2004; Peng and Karpen, 2007; Verdel et al., 2004), one possibility is that
Dcr-2 acts through stem cell chromatin remodeling in pre
adult GSCs. Further work will help to unravel the role of
Dcr-2 in this process.

Overall, our study shows that in *Drosophila* young germ
line stem cells are better able to withstand perturbations that disrupt their maintenance than adult germine stem
cells. Further analysis of these findings might ultimately lead to insights into cancer stem cell resilience and even help to reveal ways to rejuvenate failing and/or aging stem cells.
Generation of Clones, Maintenance, and Kinetic and Statistical Analysis

Drosophila melanogaster stocks were raised on standard cornmeal-yeast-agar medium at 25°C. Clones were induced by using the hsFLP-FRT system for mitotic recombination.

Larval/early pupal germline clones were produced by heat shocking third instar larvae/early pupae (usually 6 and 7 days after crosses were set up) for 1 hr at 37°C 2 days in a row and dissected at different time points after the last heat shock. Late pupal germline clones were produced by heat shocking late pupae (8 and 10 days after crosses were set up) for 1 hr at 37°C 2 days in a row. The flies eclosed 1–2 days after the last heat shock. Adult heat-shock germline clones were induced by heat shocking 2- to 4-day-old F1, adult females in empty vials for 50 min 2 days in a row at 37°C water bath. The time points were calculated from the last heat shock.

Adult-induced bantam clones were generated by heat shocking 2- to 4-day-old hsFLP;banFRT80B/U6i-GFP FRT80B flies at 37°C for 50 min twice daily 2 days in a row, with a 5 h recovery period between daily heat shocks. Flies for this were collected for 2 days after they began eclosing and then kept on wet yeast at 25°C until dissected. They were turned over into fresh vials with wet yeast every other day.

The germline stem cell loss per day (Tables S1–S3) was determined by comparison of the percentage of germaria with clonal GSCs between two different time points after clonal induction. GSC loss per day = (percentage of clonal GSC at time point 1 – percentage of clonal GSC at time point 2) x 100% / percentage of clonal GSC at time point 1 / elapsed time.

The relative division index (Table S4) for a marked GSC is determined by the number of cysts generated by a marked GSC divided by the number of unmarked cysts generated by an unmarked GSC in the same germaria (Hatfield et al., 2005). Division frequencies were measured with germaria containing one GFP-positive GSC and one clonal (GFP-negative) GSC. The total number of cysts from a GSC that are produced in a given time window provides a measure of GSC division frequency. In our case, the time window spanned from the first heat-shock treatment to the time of harvesting the adults. Therefore, we limited our counts to the region of the germarium that was anterior to the easily identifiable G4+/- cyst. This cyst developed from the first daughter cell of the clonal GSC (GFP+) after heat-shock-induced mitotic recombination. Cyst production from homozygous clonal GSCs was divided by the cyst production from heterozygous nonclonal GSCs in the same germarium to obtain the division index.

A Student’s t test was used to determine the statistical significance.

Staining Procedures

Antibody stainings and confocal microscopy were performed as described previously (Sherchbera et al., 2004). GFP was detected either by analyzing the native GFP (Figures 1 and 4–6) or by using anti-GFP directly conjugated with Alexa 488 (Figures 2 and 3). A confocal laser-scanning microscope (Leica SPES) was used in this study. We used the following mouse monoclonal antibodies: Engrailed (1:20), Armadillo (1:40), Adducin (1:20), anti-DE-Cadherin (1:50), and Lamin C (1:20) from the Developmental Studies Hybirdoma Bank and anti-p-Mad (1:500, P. ten Dijke), guinea pig anti-CycE (1:500, T. Orr-Weaver), rat anti-Bam-C (1:1000, D. Mckeanin), and rabbit anti-GFP directly conjugated with Alexa 488 (1:3000, Invitrogen). Secondary antibodies were Alexa 488, 568, 633, or 647 goat anti-mouse, anti-rabbit, anti-guinea pig (1:500, Molecular Probes), and goat-anti-rat Cy3 (1:250, Jackson Immunoresearch).

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