

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-Baker^{1,*}

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

DOI 10.1016/j.stem.2007.11.007

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 hyper-

dynamic plasticity of chromatin proteins (Li and Xie, 2005; Meshorer 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 p21/27/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).

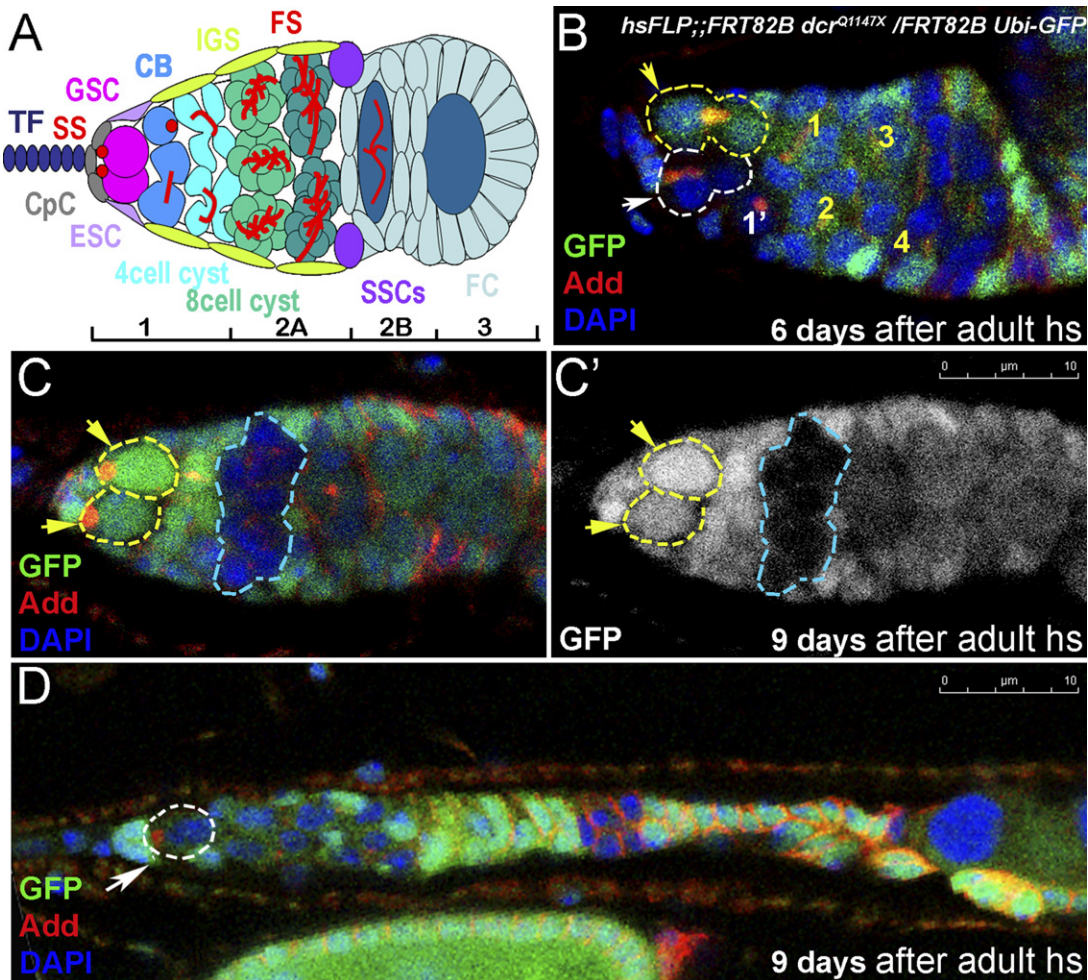


Figure 1. Adult-Induced *dicer-1* Mutant Germline Stem Cell Clones Are Lost from the Niche

(A) Diagram showing the germlarium. Germline stem cells (GSC, pink) indicated by anterior spectrosomes (SS, red) are located at the anterior end of the germlarium adjacent to the niche cap cells (CpC, gray). Terminal filament (TF; dark blue), escort stem cell (ESC, lavender), differentiated cystoblast (CB, blue), inner germarial sheath cell (IGS, lime), 4, 8, and 16 cell cysts (celadon, emerald, and green, respectively), marked by the presence of branched fusomes (FS, red), somatic stem cells (SSCs, violet), and follicle cells (FC, light blue) are as noted.

(B) Adult-induced *dicer-1*^{Q1147X} mutant GSCs divide slower than control GSCs (the mutant produced one progeny, whereas the control produced four progeny) and leave the niche producing cysts that move posteriorly (C).

(D) GSC loss coupled with a reduction in germline stem cell division results in smaller germlaria (a germlarium shown with a single *dicer-1* mutant germline stem cell). Red, Adducin; blue, DAPI; and green, GFP. Mutant clones are outlined with white dashed lines, control clones with yellow.

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-1*^{Q1147X}) 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^{Δ1}* 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 ($1.6 \pm 0.5\%$, $1.3 \pm 0.6\%$ of *miR-8^{Δ1}* 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, $14.1\% \pm 2.8\%$ of *bantam* mutant GSCs (*hsFLP;;ban^{Δ1} 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 ($6.2\% \pm 0.4\%$ 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^{L1170}/ban^{EP3622}*, *ban^{L1170}/ban^{Δ1}*, and *ban^{EP3622}/ban^{Δ1}*) exhibit similar mutant phenotypes as the *ban^{Δ1}* 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; $n = 36$ *Mad¹²* GSCs and $n = 42$ *WT* GSCs). These data suggest that larval/pupal-induced *Mad¹²*-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 *Mad¹²* mutations in GSCs of 3rd instar larva/early pupae, late pupae, and 1- to 4-day-old adult flies (Figure 4E). Interestingly, *Mad¹²* 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.

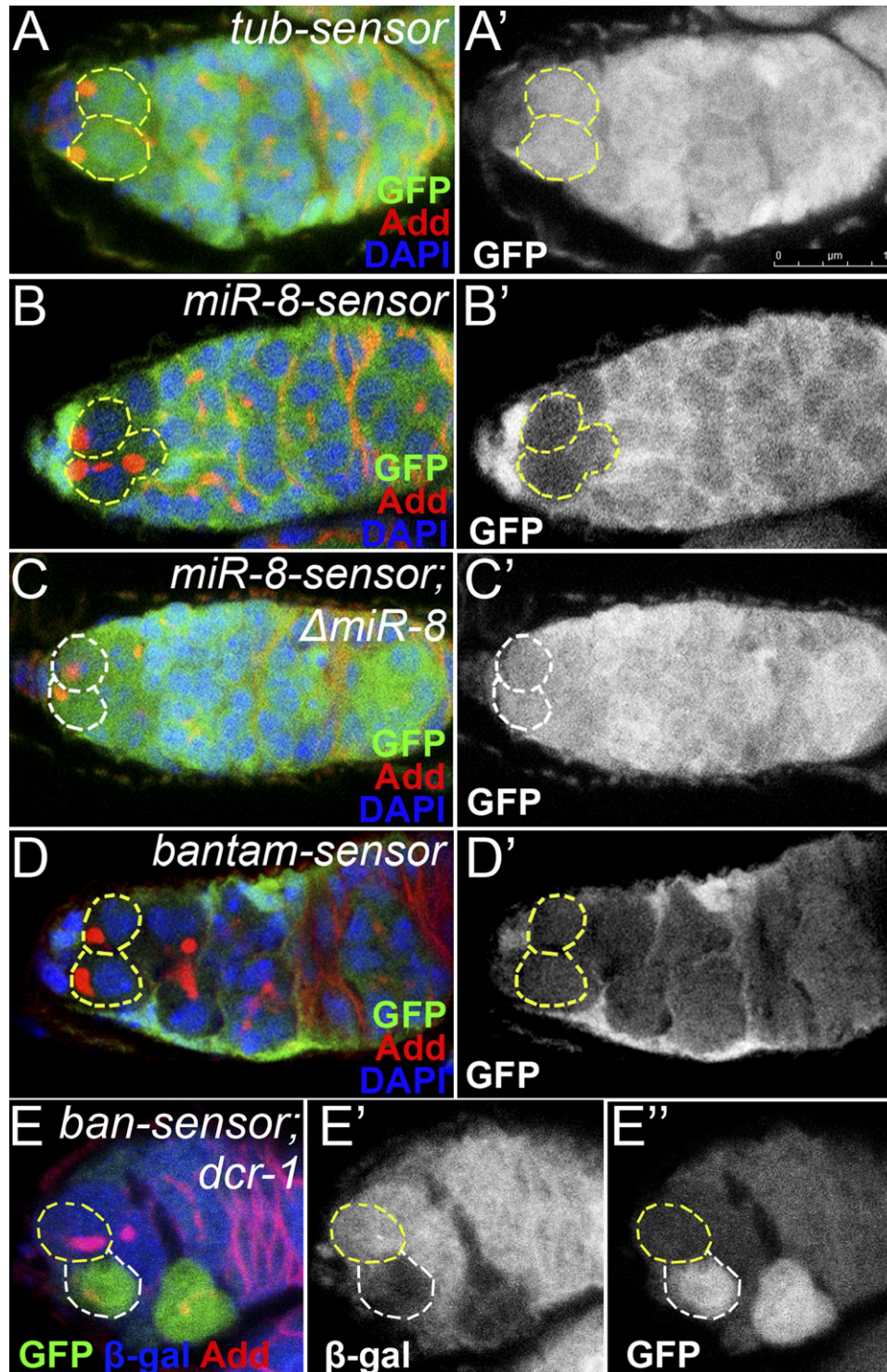


Figure 2. miRNA-GFP Sensors Are Expressed in Distinct Subsets of Cells in the Germarium

miRNA sensor expression patterns in (A, B, and D) wild-type, (C) *miR-8⁻¹*, 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*- (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-8⁻¹* 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; ban^{atub84BT:Avic/GFP-EGFP}/+; FRT82B dcr-1^{Q1147X}/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).

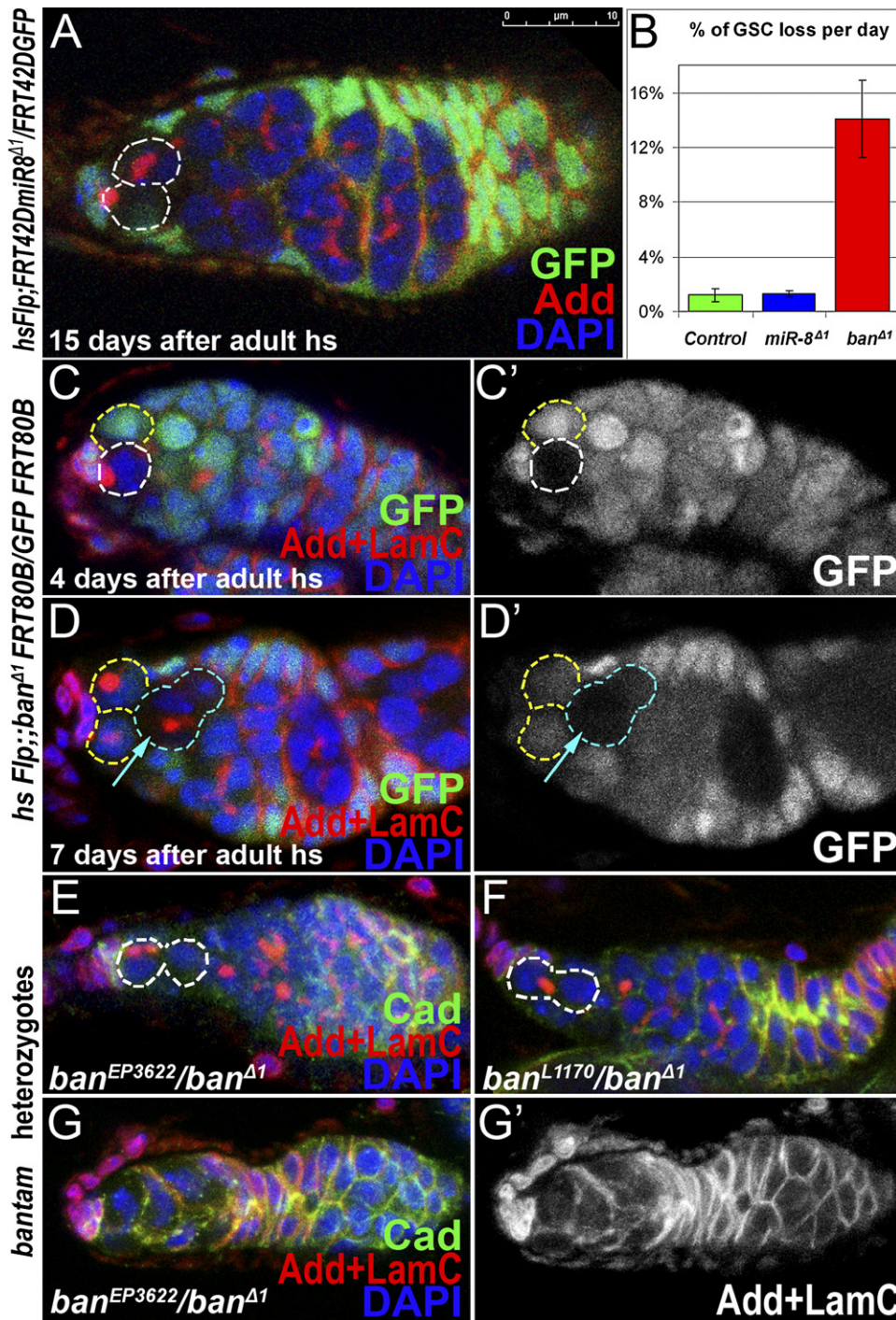


Figure 3. *bantam* miRNA Is Required for GSC Maintenance in the Niche

(A) *miR-8^{Δ1}*-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 *ban^{EP3622}/ban^{Δ1}* (E and G) and *ban^{L1170}/ban^{Δ1}* (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-1^{Q1147X}/FRT82B GFP* and *hsFLP; Mad¹² FRT40A/ GFP FRT40A; FRT82B dcr-1^{Q1147X}/+*). 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 germline 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).

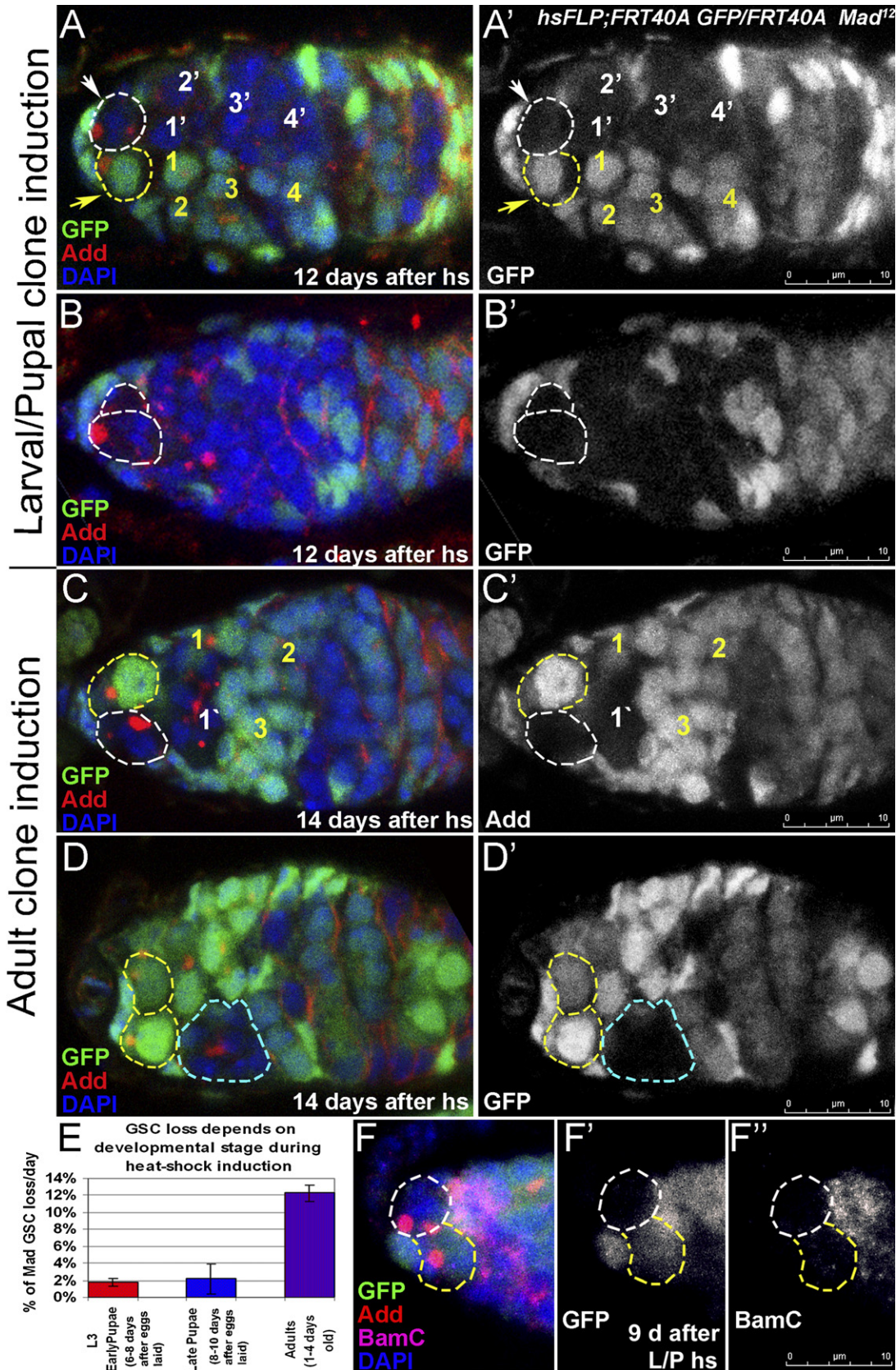
In order to determine whether Notch pathway function in GSC maintenance is Mad dependent during larval/pupal development, we analyzed whether the additional GSCs produced by increased Notch signaling during developmental stages require Mad signaling for their maintenance. We assayed whether ectopic GSCs 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 germlaria 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* suppress, 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)



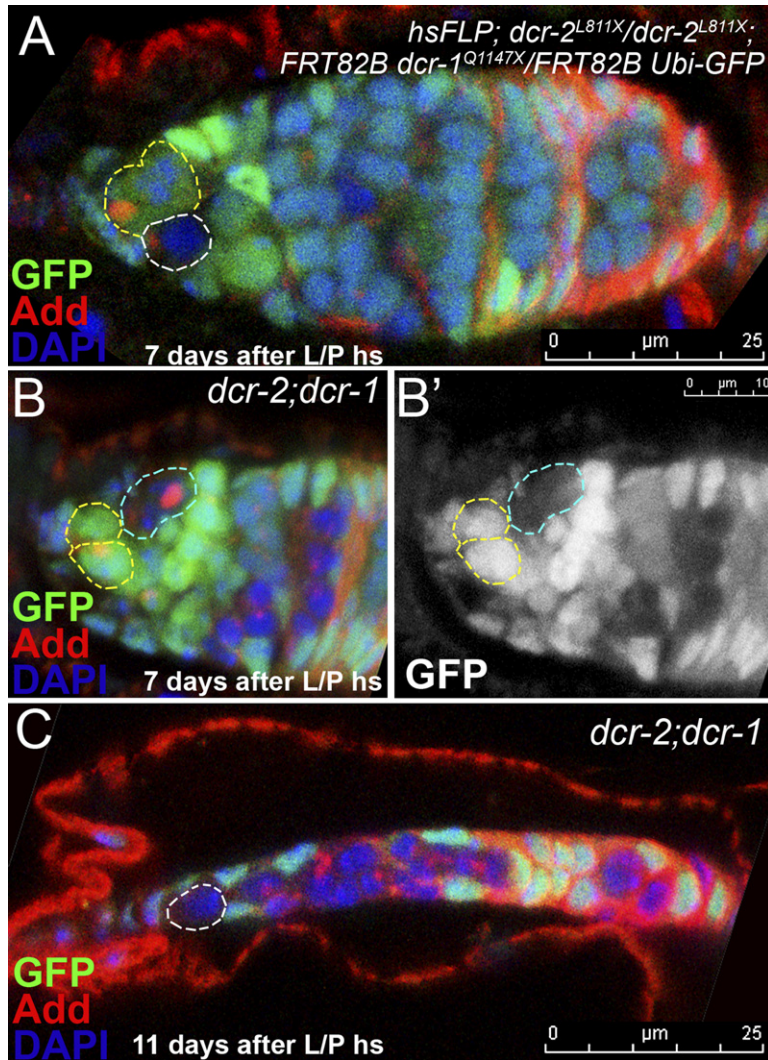


Figure 5. GSCs Lacking Dicer-1 Activity and Dicer-2 Activity from Pupal Stages onward Are Lost from the Niche

(A–C) Larval-induced *dicer-1* mutant GSCs in a *dicer-2*-mutant background (*hsFLP; dcr-2^{L811X}/dcr-2^{L811X}; FRT82B dcr-1^{Q1147X}/FRT82B Ubi-GFP*) do not divide normally and are readily lost from the niche (B). Recall, that when Dicer-2 activity is present, larval-induced *dicer-1* mutant GSCs are not lost from the niche. (C) Example of a severely reduced germarium with a single mutant GSC. GSCs are outlined with dashes: yellow, normal; white, mutant. GSCs departed from the niche are outlined with turquoise dashes. Red, Adducin; green, GFP; and blue, DAPI.

(Bilen et al., 2006). 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

Figure 4. Larval/Pupal-Induced *Mad¹²* Mutant GSCs Are Maintained in the Niche

Larval/pupal-induced *Mad¹²* mutant GSCs divide at the same frequency as control GSCs ([A]; Table S4) and are maintained in the niche 12 days after clonal induction ([B]; Table S1). However, as described before (Xie and Spradling, 1998), *Mad¹²*-mutant GSC clones induced during adulthood divide slower than nonclonal germline stem cells ([C]; Table S4) and are not maintained in the niche ([D]; 14 days after adult clonal induction, the *Mad*-mutant GSC has left the niche and become an 8 cell cyst). The bar graph shows that GSC maintenance has a development-dependent character: when *Mad¹²* mutation was induced during late larval/early pupal or even late pupal developmental stages, mutant GSCs were maintained; however, a clear GSC loss was observed for adulthood-induced *Mad¹²* GSCs (E). A differentiation factor, Bam, is not derepressed in mutant GSCs, showing that larval/pupal-induced *Mad¹²*-mutant GSCs maintain their SC identity. Red, Adducin; blue, DAPI; green, GFP; and purple, BamC; mutant GSCs are outlined with white dashed lines, control GSCs with yellow dashes.

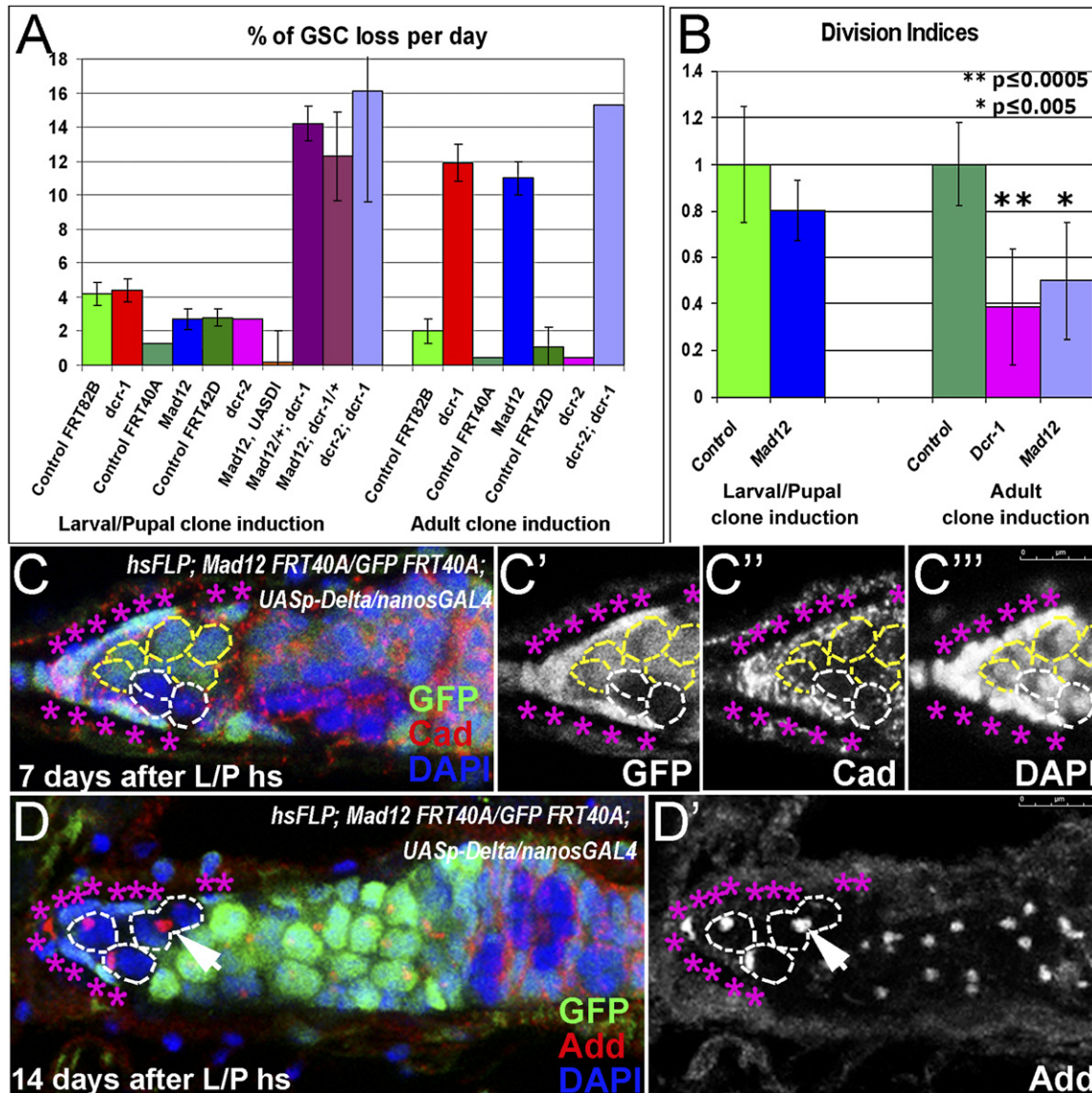


Figure 6. GSC Maintenance Is Governed by a Robust Redundant Mechanism during Development

(A) The percentage of GSC loss per day is similar to controls when either *dicer-1* or *Mad* is removed from GSCs during larval/pupal stages. In contrast, the GSCs are lost rapidly when either *dicer-1* or *Mad* is removed from the GSCs during adulthood. In addition, a synthetic maintenance defect is observed when both *Dicer-1* and *Mad* levels are reduced simultaneously during larval/pupal stages (*Mad*^{1/2}+/; *dcr-1* or *Mad*^{1/2}; *dcr-1*+/). Error bars represent standard error of the mean.

(B) GSCs lacking *Mad* activity from larval/pupal stages onward divide relatively normally compared to controls, whereas GSCs lacking either *Dicer-1* or *Mad* activity during adulthood divide significantly slower than controls. The bar graph shows the division indices at 14 days after larval/early pupal heat shock and 9 days after adult heat shock (see Table S4). Error bars represent average deviation.

(C and D) *Mad*^{1/2}-mutant GSCs in germline overexpressing *UASp-Delta* in the germline (*hsFLP*; *Mad*^{1/2} FRT40A/*Ubi-GFP FRT40A*; *UASp-Delta/nanosGAL4*) are maintained in the enlarged niche. Furthermore, the number of *Mad*-mutant GSCs in the niche is increased from the 7 to 14 day time point after larval/pupal clonal induction ([C] and [D], respectively); a dividing *Mad* mutant GSC is marked with an arrow. *Mad*-mutant GSCs are outlined with white dashed lines, cap cells identified with pink asterisks. Red, Cadherin (C) or Adducin (D); blue, DAPI; and green, GFP.

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*

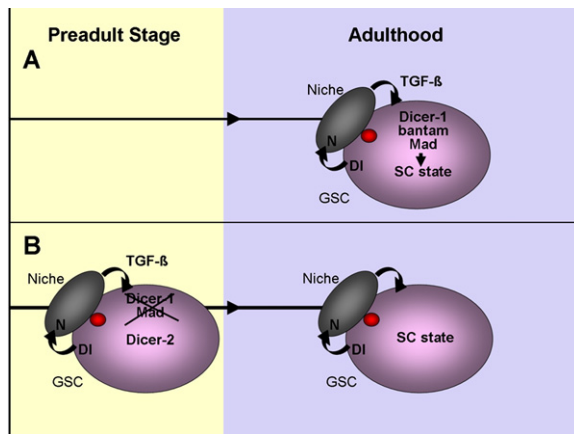


Figure 7. Model Showing Different Mechanisms for GSC Maintenance during Development and Adulthood

(A) TGF- β and Dicer-1 are required for GSC maintenance in adult *Drosophila* ovaries. We have identified *bantam* as the key miRNA in this process. *bantam* has previously been studied in cell cycle and cell death in *Drosophila* imaginal disc epithelial cells. However, in GSCs, the *bantam* target process that leads to GSC maintenance in the niche remains to be revealed.

(B) Surprisingly, we find that Mad and Dicer-1 are not required for GSC maintenance if these components are lost in GSCs during larval/pupal development. Preadult GSCs lacking Dicer-1 are no longer maintained if they also lack Dicer-2. We propose that the Dicer-2 pathway prevents GSC loss, possibly by chromatin remodeling during developmental stages, which as a consequence promotes stem cell fate during adulthood.

was recently reported (Karres et al., 2007). Further studies are required to test this hypothesis.

Robust Maintenance of Preadult GSCs

The presented work reveals the resilience of preadult stem cells to perturbations that cause GSC maintenance defects if introduced in adults. It seems logical that developing organisms would have a means of protecting their precious stem cells during the many intricate developmental processes that occur. We have shown that stem cells are still protected late in preadult development (during pupation), but not during adulthood. What protects the germline 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 morphological 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) pathway-dependent chromatin modifications have been previously observed in *Drosophila* (Grimaud 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 preadult GSCs. Further work will help to unravel the role of Dcr-2 in this process.

Overall, our study shows that in *Drosophila* young germline stem cells are better able to withstand perturbations that disrupt their maintenance than adult germline 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.

EXPERIMENTAL PROCEDURES

Fly Strains

We used the following mutant stocks: *eyFLP;;FRT82B dcr-1^{Q1147X}/TM3Sb*, *eyFLP;;FRT82B, eyFLP; FRT42D dcr-2^{L811X}/CyO* (Lee et al., 2004), *Mad¹² FRT40A/CyO* (Xie and Spradling, 1998), *FRT42D yki^{BS}* (Huang et al., 2005), *FRT42B isw²* (a gift from J. Tamkun), *pUASP Delta* (Jordan et al., 2006), *w⁻;Df(3L)ban^{d1} FRT80B/TM6, ban^{L1170}, ban^{EP3622}, w⁻;ban^{ScerUAS.T.Avic/GFP-EGFP} (pUAST-*bantam* [Brennecke et al., 2003]), *ban^{atub84BT.Avic/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;NGT40/SM6a;nanosGal4/TM3Sb* (Bloomington Stock Center). The *miR-8^{d1}* deletion line was generated by imprecise *P* element excision of *EP(2)2269*. *EP(2)2269* flies were isogenized with *w¹¹¹⁸* and balanced. Standard *P* element imprecise excision was carried out, and 300 individual excision stocks were screened by primers 5'-ATCACACGTTAACGTAACGTAACGGCAG-3' and 5'-AGATTCGAAAGCCCCACAGCAATC-3'. The *miR-8^{d1}* 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* sequence to 236 bp downstream of the mature sequence. The *miR-8^{d1}* deletion was recombined onto the *FRT42D* chromosome by using standard meiotic recombination protocols (Xu and Rubin, 1993). The recombinant *FRT42D miR-8^{d1}* lines were screened by PCR with primers 5'-AAATCTCACCGTCACCCAGTCGT-3' and 5'-AGAAACCAGCAGAAAGCAGCATCC-3'.*

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

pUASP-bantam: A partial *bantam* precursor sequence (584 nt) was amplified from *pUASP-EGFP-bantam* construct (Brennecke et al., 2003) by using the following primers: *bantam* forward, 5'-ATAGCGGCCGCGTAACTGGCAGCATATAATTC-3'; *bantam* reverse, 5'-ATTCTAGATTAAGCAGATTTAACATGTGG-3'. The amplified fragments were cloned into UASP plasmid using Not1 and Xba1.

pUASP-miR-8: A partial *miR-8* precursor sequence (729 nt) was amplified from adult fly genomic DNA with the following primers: *miR-8* forward, 5'-ATAGCGGCCGCGCGGTACACGCACATTTCAATA-3'; *miR-8* reverse, 5'-ATTCTAGAAATGGGAATTGGGAACGATCTCGC-3'. The amplified fragments were cloned into UASP plasmid with Not1 and Xba1.

miR-8-sensor: Two perfect complementary target sequences of *miR-8* separated by 16 nt were inserted downstream of *tub-GFP* plasmid into the 3' UTR of the *P* element in *CaSpeR4* with Not1 and Xba1. The following oligonucleotides containing the target sequences of *miR-8* were used: 5'-CGCCCTTGACATCTTTACCTGACAGTATTAA CGGAATATCCCTTTGACATCTTTACCTGACAGTATTATGAACCT-3'; 5'-TAGAGGTTCAATAACTGTCAAGTAAAGATGTCAAAGGGATATT CGCGTTAA TACTGTCAAGTAAAGATGTCAAAGGGC-3'.

Transgenic flies were generated by injection of purified plasmid DNA into *w¹¹¹⁸* *Drosophila* embryos (Rainbow Transgenic Flies Inc., CA). These flies were crossed with *w¹¹¹⁸*, and transformants with germline insertion of plasmid DNA were selected based on eye color. For *pUASP-bantam*, 27 independent transgenic lines were generated and three analyzed. For *pUASP-miR-8*, 34 independent transgenic lines were generated, and three were screened and showed no defects in GSC maintenance and kinetics. For *miR-8-sensor*, 27 independent transgenic lines were generated. Six out of seven examined lines show similar GFP expression patterns in the germlarium as shown in Figure 2.

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 (9 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 in a 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;;ban^{d1} FRT80B/Ubi-GFP FRT80B* flies at 37°C for 50 min twice daily 2 days in a row, with a 5 hour 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) × 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 *GFP^{+/+}* 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 (Shcherbata 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 SPE5) 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 Hybridoma 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. McKearin), 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 Cy5 (1:250, Jackson Immunoresearch).

Supplemental Data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at <http://www.cellstemcell.com/cgi/content/full/1/6/698/DC1/>.

ACKNOWLEDGMENTS

We thank Drs. A. Spradling, D. Pan, B. Calvi, S. Cohen, T.T. Su, T. Orr-Weaver, T. Xie, J. Tamkun, and R. Carthew for suggestions, flies, and reagents, Christian Nelson, Louise von Stechow, and Andriy Yatsenko for help with experiments, and Dr. Bradford Stadler for help with QPCR analysis. This work was supported by AHA fellowships for H.R.S. and S.H.R. and MOD and NIH grants and the Tietze Fellowship for H.R.B.

Received: April 24, 2007

Revised: September 11, 2007

Accepted: November 21, 2007

Published: December 12, 2007

REFERENCES

- Bilen, J., Liu, N., Burnett, B.G., Pittman, R.N., and Bonini, N.M. (2006). MicroRNA pathways modulate polyglutamine-induced neurodegeneration. *Mol. Cell* 24, 157–163.
- Brennecke, J., Hipfner, D.R., Stark, A., Russell, R.B., and Cohen, S.M. (2003). *bantam* encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene *hid* in *Drosophila*. *Cell* 113, 25–36.
- Chen, D., and McKearin, D. (2003). Dpp signaling silences *bam* transcription directly to establish asymmetric divisions of germline stem cells. *Curr. Biol.* 13, 1786–1791.
- Fuchs, E., Tumber, T., and Guasch, G. (2004). Socializing with the neighbors: stem cells and their niche. *Cell* 116, 769–778.
- Gilboa, L., and Lehmann, R. (2006). Soma-germline interactions coordinate homeostasis and growth in the *Drosophila* gonad. *Nature* 443, 97–100.
- Grimaud, C., Bantignies, F., Pal-Bhadra, M., Ghana, P., Bhadra, U., and Cavalli, G. (2006). RNAi components are required for nuclear clustering of Polycomb group response elements. *Cell* 124, 957–971.
- Hatfield, S.D., Shcherbata, H.R., Fischer, K.A., Nakahara, K., Carthew, R.W., and Ruohola-Baker, H. (2005). Stem cell division is regulated by the microRNA pathway. *Nature* 435, 974–978.
- Hatfield, S., and Ruohola-Baker, H. (2007). microRNA and stem cell function. *Cell Tissue Res.*, in press. Published online November 7, 2007. 10.1007/s00441-007-0530-3.
- Hatfield, S., Shcherbata, H.R., Ward, E.J., Reynolds, S., and Ruohola-Baker, H. (2007). microRNAs and their involvement in stem cell division. In *microRNAs; Biology, Function and Expression*, N. Clarke and P. Sanseau, eds. (Eagleview, PA: DNA Press), pp. 221–250.
- Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. (2005). The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the *Drosophila* Homolog of YAP. *Cell* 122, 421–434.
- Jin, Z., and Xie, T. (2007). Dcr-1 maintains *Drosophila* ovarian stem cells. *Curr. Biol.* 17, 539–544.
- Jordan, K.C., Schaeffer, V., Fischer, K.A., Gray, E.E., and Ruohola-Baker, H. (2006). Notch signaling through tramtrack bypasses the mitosis promoting activity of the JNK pathway in the mitotic-to-endocycle transition of *Drosophila* follicle cells. *BMC Dev. Biol.* 6, 16.
- Karres, J.S., Hilgers, V., Carrera, I., Treisman, J., and Cohen, S.M. (2007). The conserved microRNA miR-8 tunes atrophin levels to prevent neurodegeneration in *Drosophila*. *Cell* 131, 136–145.
- Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J., and Carthew, R.W. (2004). Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* 117, 69–81.
- Li, L., and Xie, T. (2005). Stem cell niche: structure and function. *Annu. Rev. Cell Dev. Biol.* 21, 605–631.
- Li, Z., and Li, L. (2006). Understanding hematopoietic stem-cell microenvironments. *Trends Biochem. Sci.* 31, 589–595.

Meshorer, E., Yellajoshula, D., George, E., Scambler, P.J., Brown, D.T., and Misteli, T. (2006). Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. *Dev. Cell* *10*, 105–116.

Nolo, R., Morrison, C.M., Tao, C., Zhang, X., and Halder, G. (2006). The bantam microRNA is a target of the hippo tumor-suppressor pathway. *Curr. Biol.* *16*, 1895–1904.

Pal-Bhadra, M., Bhadra, U., and Birchler, J.A. (2002). RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. *Mol. Cell* *9*, 315–327.

Pal-Bhadra, M., Leibovitch, B.A., Gandhi, S.G., Rao, M., Bhadra, U., Birchler, J.A., and Elgin, S.C. (2004). Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. *Science* *303*, 669–672.

Park, J.K., Liu, X., Strauss, T.J., McKearin, D.M., and Liu, Q. (2007). The miRNA pathway intrinsically controls self-renewal of *Drosophila* germline stem cells. *Curr. Biol.* *17*, 533–538.

Peng, J.C., and Karpen, G.H. (2007). H3K9 methylation and RNA interference regulate nucleolar organization and repeated DNA stability. *Nat. Cell Biol.* *9*, 25–35.

Pham, J.W., Pellino, J.L., Lee, Y.S., Carthew, R.W., and Sontheimer, E.J. (2004). A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in *Drosophila*. *Cell* *117*, 83–94.

Robins, H., Li, Y., and Padgett, R.W. (2005). Incorporating structure to predict microRNA targets. *Proc. Natl. Acad. Sci. USA* *102*, 4006–4009.

Shcherbata, H.R., Althausen, C., Findley, S.D., and Ruohola-Baker, H. (2004). The mitotic-to-endocycle switch in *Drosophila* follicle cells is

executed by Notch-dependent regulation of G1/S, G2/M and M/G1 cell-cycle transitions. *Development* *131*, 3169–3181.

Shcherbata, H.R., Hatfield, S., Ward, E.J., Reynolds, S., Fischer, K.A., and Ruohola-Baker, H. (2006). The MicroRNA pathway plays a regulatory role in stem cell division. *Cell Cycle* *5*, 172–175.

Song, X., Wong, M.D., Kawase, E., Xi, R., Ding, B.C., McCarthy, J.J., and Xie, T. (2004). Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the *Drosophila* ovary. *Development* *131*, 1353–1364.

Thompson, B.J., and Cohen, S.M. (2006). The Hippo pathway regulates the bantam microRNA to control cell proliferation and apoptosis in *Drosophila*. *Cell* *126*, 767–774.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I., and Moazed, D. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* *303*, 672–676.

Ward, E.J., Shcherbata, H.R., Reynolds, S.H., Fischer, K.A., Hatfield, S.D., and Ruohola-Baker, H. (2006). Stem cells signal to the niche through the Notch pathway in the *Drosophila* ovary. *Curr. Biol.* *16*, 2352–2358.

Xie, T., and Spradling, A.C. (1998). *decapentaplegic* is essential for the maintenance and division of germline stem cells in the *Drosophila* ovary. *Cell* *94*, 251–260.

Xu, T., and Rubin, G.M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* *117*, 1223–1237.

Zhu, C.H., and Xie, T. (2003). Clonal expansion of ovarian germline stem cells during niche formation in *Drosophila*. *Development* *130*, 2579–2588.