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## Stage-specific differences in the requirements for germline stem cell maintenance in the *Drosophila* ovary

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### Abstract

In this study, we uncover a role for microRNAs 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* microRNA 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- $\beta$  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 Dcr-2 dependent redundancy of GSC maintenance mechanisms 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 (Boiani et al., 2005). Adult stem cells are undifferentiated cells that 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; 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.

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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). *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, the niche furthermore controls the GSC maintenance via TGF-beta pathway (Chen and McKearin, 2003; Ward et al., 2006; Xie and Spradling, 1998).

Previous work has demonstrated that microRNAs, small (21-23nt) RNA molecules that can regulate gene expression, are required for normal stem cell function in mouse, *Drosophila* and plants [for reviews see (Hatfield, 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 G<sub>1</sub>/S transition concomitant with increased expression of CDK-inhibitor p21/27/Dacapo, suggesting that microRNAs are required for stem cells to bypass the normal G<sub>1</sub>/S checkpoint. Hence loss of the microRNA pathway might inactivate a mechanism that makes stem cells sensitive to environmental signals that normally control the cell cycle at the G<sub>1</sub>/S transition (Hatfield et al., 2005; (Shcherbata et al., 2006)..

Here, we show that in addition to stem cell division, microRNAs are also required for stem cell maintenance. Furthermore, we identify *bantam* as a key microRNA 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

### microRNAs are required for adult GSC maintenance

To assess the requirement for microRNAs in stem cells during different stages of development, we generated germline stem cells (GSC) 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<sup>Q1147X</sup>*) generated during adult life showed a defect in germline stem cell division kinetics (Figure 1B; S Table 4; S 1A-1C), 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, S 3A). 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 1D, S 3B). On average 12% of *dicer-1* mutant GSCs were lost per day, while only 2% were lost in the control group (Figure 6A, S 1D). 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

Since Dicer-1 and, therefore, microRNA function is required for adult GSC maintenance, we analyzed which microRNA(s) is/are responsible for this phenotype. By using sensor constructs for *miR-8* and *bantam* we found that these microRNAs are expressed in GSCs (Figure 2). While the control sensor lacking microRNA binding sites shows uniform GFP expression, including the GSCs (Figure 2A), the GFP expression of *miR-8*- and *bantam*-sensors are highly reduced in the wild type GSCs (Figure 2B,D), but are up-regulated in *miR-8<sup>Δ1</sup>* and *dcr-1* mutant GSCs (Figure 2C,E). These data indicate that *miR-8* and *bantam* are expressed in adult GSCs.

Since *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<sup>Δ1</sup>* mutant GSCs lost/day; Figure 3A, 3B; S Table 4). However adult-generated GSCs mutant for *bantam* showed maintenance and cell division defects (Figure 3C,D; S Table 1, 4). On average  $14.1\% \pm 2.8\%$  of *bantam* mutant GSCs (*hsFLP*; *ban<sup>Δ1</sup> FRT80B/Ubi-GFP FRT80B*) were lost per day, while no loss was observed in the control group (*hsFLP*; *FRT80B/arm lacZ FRT80B*; S Table 1). When *bantam* clones were generated in preadult stages the loss was not as dramatic ( $6.2 \pm 0.4\%$  per day; S Table 1). 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<sup>L1170</sup>/ban<sup>EP3622</sup>*, *ban<sup>L1170</sup>/ban<sup>Δ1</sup>* and *ban<sup>EP3622</sup>/ban<sup>Δ1</sup>*) exhibit similar mutant phenotypes as the *ban<sup>Δ1</sup>* clones (but in a lower frequency), suggesting that the defects are due to the loss of *bantam* function and not due to second site mutations (Figure 3E-G; S 4B-E). These data show that *bantam* and *dicer-1* mutant defects in GSC maintenance are similar and therefore suggest that *bantam* is a key microRNA 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 (Figure 4A-4B, 6A), but was essential if the *Mad* mutation was introduced in GSCs during adulthood [Figure 4D, 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 (Figure 4C, 6B; S 4). 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- $\beta$  signaling within the GSC niche blocks germline stem cell differentiation by silencing *Bam*. In the absence of Mad, *Bam* is de-repressed and the GSC differentiates (Chen and McKearin, 2003; Song et al., 2004; Xie and Spradling, 1998). Since GSCs lacking the transcription factor Mad, a key component of the TGF- $\beta$  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<sup>12</sup>* GSCs and n=42 *WT* GSCs). These data suggest that larval/pupal-induced *Mad<sup>12</sup>* 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<sup>12</sup>* mutations in GSC of 3rd instar larva/early pupae, late pupae and 1-4 days old adult flies (Figure 4E). Interestingly, *Mad<sup>12</sup>* 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.

## Preadult Mad and Dicer-1 interact in GSC maintenance

Since both the 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; Mad<sup>12</sup> FRT40A/+; FRT82B dcr-1<sup>Q1147X</sup>/FRT82B GFP* and *hsFLP; Mad<sup>12</sup> FRT40A/GFP FRT40A; FRT82B dcr-1<sup>Q1147X</sup>/+*). 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 further investigate the role of small interfering RNAs 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 (Figure 5, 6A). However the Dicer-2 pathway alone is not required for larval/pupal or adult germline stem cell maintenance (Figure 6A, S Table 3). 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 microRNA processing, as previous biochemical studies showed that Dicer-2 does not appear to process microRNAs (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; Pal-Bhadra et al., 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 germ line 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 following larval/pupal clonal induction (*hsFLP; Mad<sup>l2</sup> FRT40A/Ubi-GFP FRT40A; pUASP-Delta/nanosGAL4*, Figure 6A,  $0.2 \pm 1.8\%$  loss/day); the number of germlaria containing mutant GSCs remains the same in the two timepoints analyzed. However, unlike the *Mad* mutant GSCs described above, in the Delta overexpression background the number of *Mad* GSCs increases ( $\sim 2$  mutant GSCs to  $\sim 3$  mutant GSCs/7 days; Figure 6C-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* microRNA are required for adult stem cell maintenance (Figure 7A). Second, preadult stem cells have a youthful resilience that is lost at adulthood. Thus, in developing animals if certain key components required for adult germline stem cell maintenance are lost, the animal can overcome this loss and maintain the stem cells throughout life (Figure 7B).

### Bantam function in GSCs

The microRNA *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 polyglytamine protein Ataxin-3, which is mutated in the human polyglutamine disease spinocerebellar ataxia type 3 (SCA3) (Bilen et al., 2006). These studies suggest that *bantam* microRNA 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* microRNA is essential for the stem cell maintenance in the niche (Figures 2-3, S 4) and appears to be acting independently of Hippo-pathway as *yorkie* mutant GSCs are maintained in the niche; S Table 1. Many questions remain about this new function of *bantam*, such as: 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 in the

niche [(Hatfield et al., 2005), Figure 6A]. Interestingly, the 3' UTR of *Mad* is a validated target of *bantam* microRNA in S2 cells (Robins et al., 2005). It is therefore possible that *bantam* microRNA 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 microRNAs in germline stem cells to ensure their maintenance in the niche. Similar fine-tuned regulation of Atrophin by *miR-8* was recently reported (Karres et al., 2007). Further studies are required to test this hypothesis.

### Robust Maintenance of Preadult Germline Stem Cell

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 siRNA pathway. As RNAi pathway-dependent chromatin modifications have been previously observed in *Drosophila* (Grimaud et al., 2006; Lee et al., 2004; Pal-Bhadra et al., 2002; Pal-Bhadra et al., 2004; Peng and Karpen, 2007; Verdel et al., 2004), one possibility is that Dcr-2 acts through stem cell chromatin 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.

## Materials and Methods

### Fly Strains

We used the following mutant stocks: *eyFLP*; ;*FRT82B dcr-1<sup>Q1147X</sup>/TM3Sb*, *eyFLP*; ;*FRT82B*, *eyFLP*; *FRT 42D dcr-2<sup>L811X</sup>/CyO* (Lee et al., 2004), *Mad<sup>12</sup> FRT40A/CyO* (Xie and Spradling, 1998), *FRT42D yki<sup>B5</sup>* (Huang et al., 2005), *FRT42B iswi<sup>2</sup>* (a gift from J.Tamkun), *pUASP Delta* (Jordan et al., 2006), *w<sup>-</sup>; ;Df(3L)ban<sup>Δ1</sup> FRT80B/TM6*, *ban<sup>L1170</sup>*, *ban<sup>EP3622</sup>*, *w<sup>-</sup>; ban<sup>Scer</sup>UAS:T:Avic/GFP-EGFP [pUAST-bantam* (Brennecke et al., 2003)], *ban<sup>Δtub84BT</sup>: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*, *w*; ;*NGT40/SM6a;nanosGal4/TM3Sb* (Bloomington Stock Center). The *miR-8<sup>Δ1</sup>* deletion line was generated by imprecise P-element excision of *EP(2) 2269*. *EP(2)2269* flies were isogenized with *w<sup>1118</sup>* and balanced. Standard P element imprecise excision was carried out and 300 individual excision stocks were screened by primers 5' - ATCACACGTTAACGTAACGTAACGGCAG and 5' - AGATTCGAAAGCCCCACACGCACAATC. The *miR-8<sup>Δ1</sup>* deletion removes 1316 bp of genomic DNA, including the 23bp mature *miR-8* microRNA. The deletion spans from 1057bp upstream of the mature *miR-8* sequence to 236bp downstream of the mature sequence. The *miR-8<sup>Δ1</sup>* deletion was recombined onto the *FRT42D* chromosome using standard meiotic recombination protocols (Xu and Rubin, 1993). The recombined *FRT42D miR-8<sup>Δ1</sup>* lines were screened by PCR with primers 5' - AAATCTTCACCGTCACCCAGTCGT and 5' - AGAAACCAGCAGAAAGCAGCATCC.

### 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) using the following primers:

*bantam* forward: 5'-ATAGCGGCCGCGTAACTGGCAGCATATAATTC;

*bantam* reverse: 5'-ATTCTAGATTATAGGCAGATTAAACATGTGG.

The amplified fragments were cloned into UASP plasmid using *NotI* and *XbaI*.

**pUASP-miR-8**—A partial *miR-8* precursor sequences (729 nt) were amplified from adult fly genomic DNA with the following primers:

*miR-8* forward: 5'-ATAGCGGCCGCGCGGTACACGCACATTTCAATA;

*miR-8* reverse: 5'-ATTCTAGAAATGGGAATTGGGAACGATCTCGC.

The amplified fragments were cloned into UASP plasmid with *NotI* and *XbaI*.

**miR-8-sensor**—Two perfect complementary target sequences of *miR-8* separated by 16 nucleotides were inserted downstream of *tub-GFP* plasmid into the 3'UTR of the P-element in CaSpeR4 with *NotI* and *XbaI*. The following oligonucleotides containing the target sequences of *miR-8* were used:

5'-  
CCGCCCTTGACATCTTTACCTGACAGTATTAACGCGAATATCCCTTTGACAT  
C TTTACCTGACAGTATTATGAACCT;

5'-  
TAGAGGTTTCATAATACTGTCAGGTAAGATGTCAAAGGGATATTCGCGTTA  
A TACTGTCAGGTAAGATGTCAAGGGC.

Transgenic flies were generated by injection of purified plasmid DNA into *w<sup>1118</sup>* *Drosophila* embryos (Rainbow Transgenic Flies Inc., CA). These flies were crossed with *w<sup>1118</sup>* and transformants with germline insertion of plasmid DNA were selected based on eye color. For *pUASP-bantam* 27 independent transgenic lines were generated and 3 analyzed. For *pUASP-miR-8* 34 independent transgenic lines were generated, 3 screened and showed no defects in GSC maintenance and kinetics. For *miR-8-sensor* 27 independent transgenic lines were generated. 6 out of 7 examined lines show similar GFP expression patterns in the germarium as shown in Figure 2.

### Generation of clones, maintenance, kinetic- and statistical-analysis

*Drosophila melanogaster* stocks were raised on standard cornmeal-yeast-agar-medium at 25°C. Clones were induced 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 & 7 days after crosses were set up) for 1hr at 37°C two 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 & 10 days after crosses were set up) for 1hr at 37°C two 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 2-4 days old F<sub>1</sub> adult females in empty vials for 50 minutes two days in a row in a 37°C water bath.

Adult-induced *bantam* clones were generated by heat-shocking 2-4 day old *hsFLP;;ban<sup>Δ1</sup>FRT80B/Ubi-GFP FRT80B* flies at 37°C for 50 minutes twice daily 2 days in a row, with a five 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 (S Table 1-3) was determined by comparison of the percentage of germaria with clonal GSCs between two different time points after clonal induction:

$$\text{GSC loss per day} = (\% \text{ of clonal GSC at timepoint1} - \% \text{ of clonal GSC at timepoint2}) \times 100\% / \% \text{ of clonal GSC at timepoint 1} / \text{elapsed time}$$

The relative division index (S Table 4) 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 using 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 measurement 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*<sup>+/+</sup> cyst. This cyst developed from the first daughter cell of the clonal GSC (*GFP*<sup>-</sup>) after heat-shock induced mitotic recombination. Cyst production from homozygous clonal GSCs was divided by the cyst production from heterozygous non-clonal GSCs in the same germarium to obtain the division index. The student 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, 4-6) or by using anti-GFP-directly conjugated with Alexa 488 (Figures 2-3). 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) and 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), 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 and anti-guinea pig (1:500, Molecular Probes), goat-anti-rat Cy5 (1:250, Jackson Immunoresearch).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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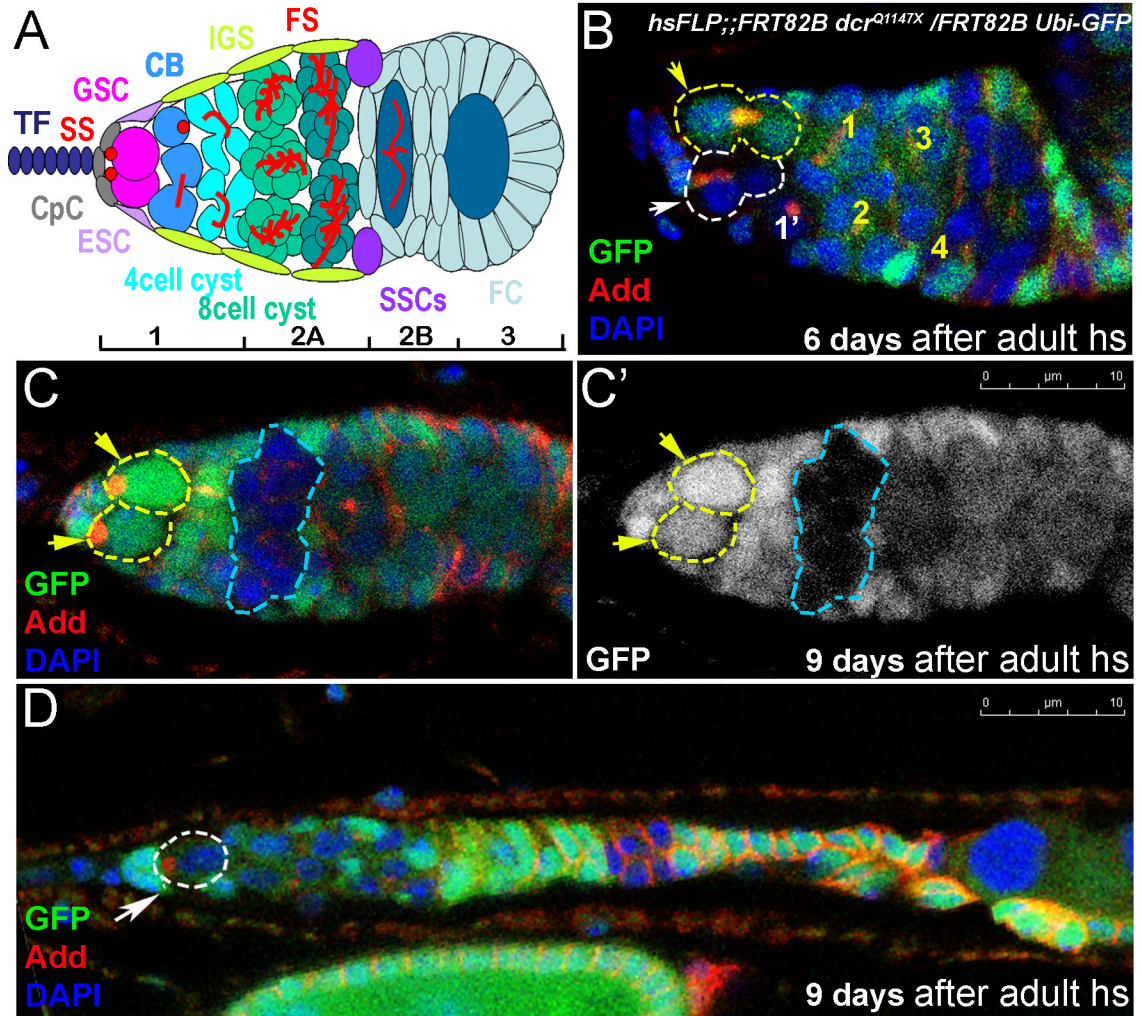
### References

- Bilen J, Liu N, Burnett BG, Pittman RN, Bonini NM. MicroRNA pathways modulate polyglutamine-induced neurodegeneration. *Mol Cell* 2006;24:157–163. [PubMed: 17018300]
- Boiani M, Gentile L, Gambles VV, Cavaleri F, Redi CA, Scholer HR. Variable reprogramming of the pluripotent stem cell marker Oct4 in mouse clones: distinct developmental potentials in different culture environments. *Stem Cells* 2005;23:1089–1104. [PubMed: 15955835]

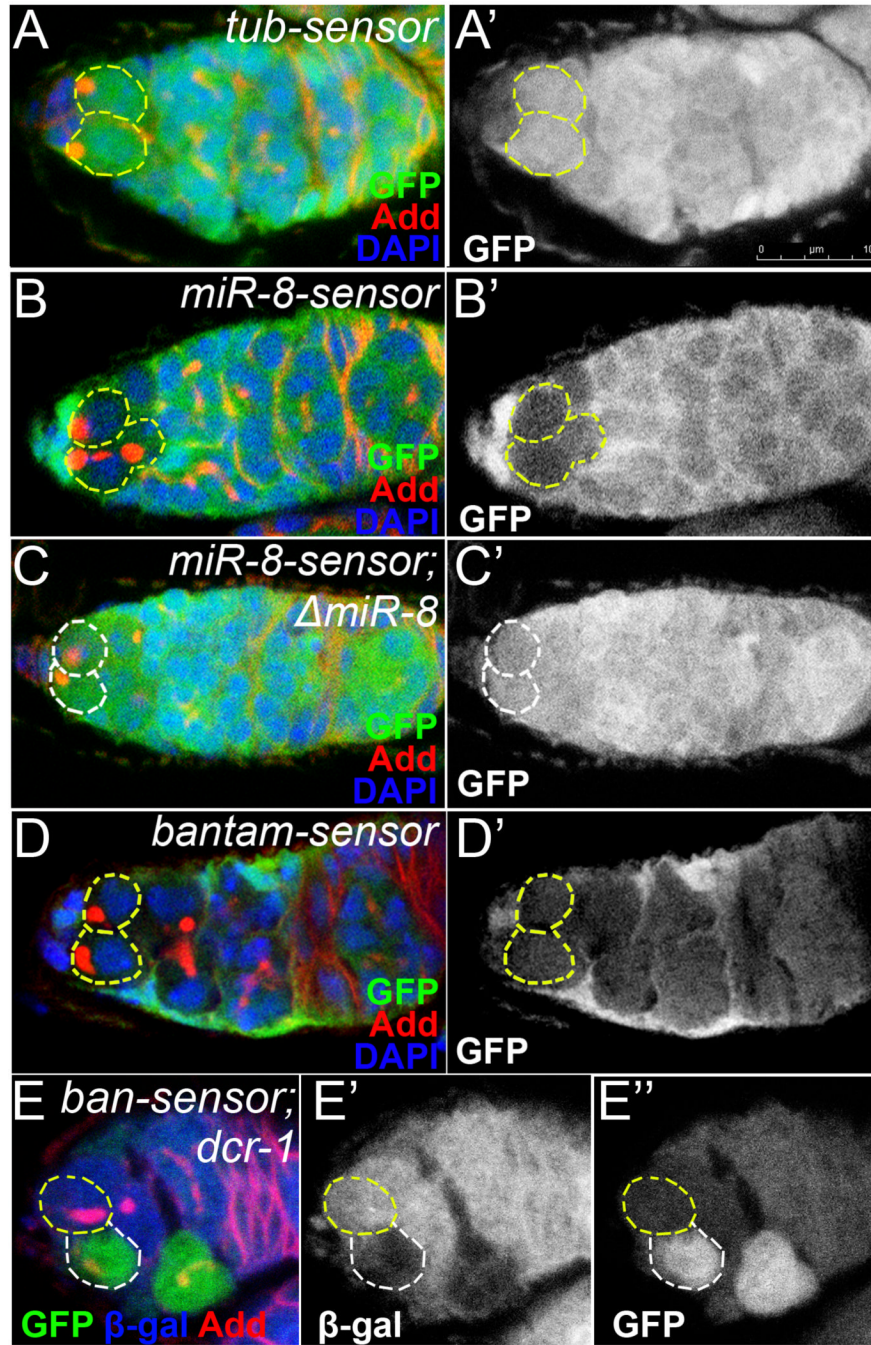


- Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM. bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in *Drosophila*. *Cell* 2003;113:25–36. [PubMed: 12679032]
- Chen D, McKearin D. Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. *Curr Biol* 2003;13:1786–1791. [PubMed: 14561403]
- Fuchs E, Tumber T, Guasch G. Socializing with the neighbors: stem cells and their niche. *Cell* 2004;116:769–778. [PubMed: 15035980]
- Gilboa L, Lehmann R. Soma-germline interactions coordinate homeostasis and growth in the *Drosophila* gonad. *Nature* 2006;443:97–100. [PubMed: 16936717]
- Grimaud C, Bantignies F, Pal-Bhadra M, Ghana P, Bhadra U, Cavalli G. RNAi components are required for nuclear clustering of Polycomb group response elements. *Cell* 2006;124:957–971. [PubMed: 16530043]
- Hatfield, S.; Shcherbata, HR.; Ward, EJ.; Reynolds, S.; Ruohola-Baker, H. microRNAs and their involvement in Stem Cell division. In: Clarke, N.; Sanseau, P., editors. *microRNAs; Biology, Function and Expression*. DNA Press; 2007.
- Hatfield SD, Shcherbata HR, Fischer KA, Nakahara K, Carthew RW, Ruohola-Baker H. Stem cell division is regulated by the microRNA pathway. *Nature* 2005;435:974–978. [PubMed: 15944714]
- Huang J, Wu S, Barrera J, Matthews K, Pan D. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the *Drosophila* Homolog of YAP. *Cell* 2005;122:421–434. [PubMed: 16096061]
- Jin Z, Xie T. Dcr-1 maintains *Drosophila* ovarian stem cells. *Curr Biol* 2007;17:539–544. [PubMed: 17306537]
- Jordan KC, Clegg NJ, Blasi JA, Morimoto AM, Sen J, Stein D, McNeill H, Deng WM, Tworoger M, Ruohola-Baker H. The homeobox gene mirror links EGF signalling to embryonic dorso-ventral axis formation through notch activation. *Nat Genet* 2000;24:429–433. [PubMed: 10742112]
- Jordan KC, Schaeffer V, Fischer KA, Gray EE, Ruohola-Baker H. 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* 2006;6:16. [PubMed: 16542414]
- Karres JS, Hilgers V, Carrera I, Treisman J, Cohen SM. The Conserved microRNA MiR-8 Tunes Atrophin Levels to Prevent Neurodegeneration in *Drosophila*. *Cell* 2007;131:136–145. [PubMed: 17923093]
- Lee YS, Nakahara K, Pham JW, Kim K, He Z, Sontheimer EJ, Carthew RW. Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* 2004;117:69–81. [PubMed: 15066283]
- Li L, Xie T. Stem cell niche: structure and function. *Annu Rev Cell Dev Biol* 2005;21:605–631. [PubMed: 16212509]
- Li Z, Li L. Understanding hematopoietic stem-cell microenvironments. *Trends Biochem Sci* 2006;31:589–595. [PubMed: 16911868]
- Meshorer E, Yellajoshula D, George E, Scambler PJ, Brown DT, Misteli T. Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. *Dev Cell* 2006;10:105–116. [PubMed: 16399082]
- Nolo R, Morrison CM, Tao C, Zhang X, Halder G. The bantam microRNA is a target of the hippo tumor-suppressor pathway. *Curr Biol* 2006;16:1895–1904. [PubMed: 16949821]
- Pal-Bhadra M, Bhadra U, Birchler JA. RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. *Mol Cell* 2002;9:315–327. [PubMed: 11864605]
- Pal-Bhadra M, Leibovitch BA, Gandhi SG, Rao M, Bhadra U, Birchler JA, Elgin SC. Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. *Science* 2004;303:669–672. [PubMed: 14752161]
- Park JK, Liu X, Strauss TJ, McKearin DM, Liu Q. The miRNA pathway intrinsically controls self-renewal of *Drosophila* germline stem cells. *Curr Biol* 2007;17:533–538. [PubMed: 17320391]
- Peng JC, Karpen GH. H3K9 methylation and RNA interference regulate nucleolar organization and repeated DNA stability. *Nat Cell Biol* 2007;9:25–35. [PubMed: 17159999]
- Pham JW, Pellino JL, Lee YS, Carthew RW, Sontheimer EJ. A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in *Drosophila*. *Cell* 2004;117:83–94. [PubMed: 15066284]

- Robins H, Li Y, Padgett RW. Incorporating structure to predict microRNA targets. *Proc Natl Acad Sci U S A* 2005;102:4006–4009. [PubMed: 15738385]
- Shcherbata HR, Althausen C, Findley SD, Ruohola-Baker H. 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* 2004;131:3169–3181. [PubMed: 15175253]
- Shcherbata HR, Hatfield S, Ward EJ, Reynolds S, Fischer KA, Ruohola-Baker H. The MicroRNA pathway plays a regulatory role in stem cell division. *Cell Cycle* 2006;5:172–175. [PubMed: 16357538]
- Song X, Wong MD, Kawase E, Xi R, Ding BC, McCarthy JJ, Xie T. 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* 2004;131:1353–1364. [PubMed: 14973291]
- Thompson BJ, Cohen SM. The Hippo pathway regulates the bantam microRNA to control cell proliferation and apoptosis in *Drosophila*. *Cell* 2006;126:767–774. [PubMed: 16923395]
- Verdel A, Jia S, Gerber S, Sugiyama T, Gygi S, Grewal SI, Moazed D. RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* 2004;303:672–676. [PubMed: 14704433]
- Ward EJ, Shcherbata HR, Reynolds SH, Fischer KA, Hatfield SD, Ruohola-Baker H. Stem cells signal to the niche through the Notch pathway in the *Drosophila* ovary. *Curr Biol* 2006;16:2352–2358. [PubMed: 17070683]
- Xie T, Spradling AC. decapentaplegic is essential for the maintenance and division of germline stem cells in the *Drosophila* ovary. *Cell* 1998;94:251–260. [PubMed: 9695953]
- Xu T, Rubin GM. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* 1993;117:1223–1237. [PubMed: 8404527]
- Zhu CH, Xie T. Clonal expansion of ovarian germline stem cells during niche formation in *Drosophila*. *Development* 2003;130:2579–2588. [PubMed: 12736203]

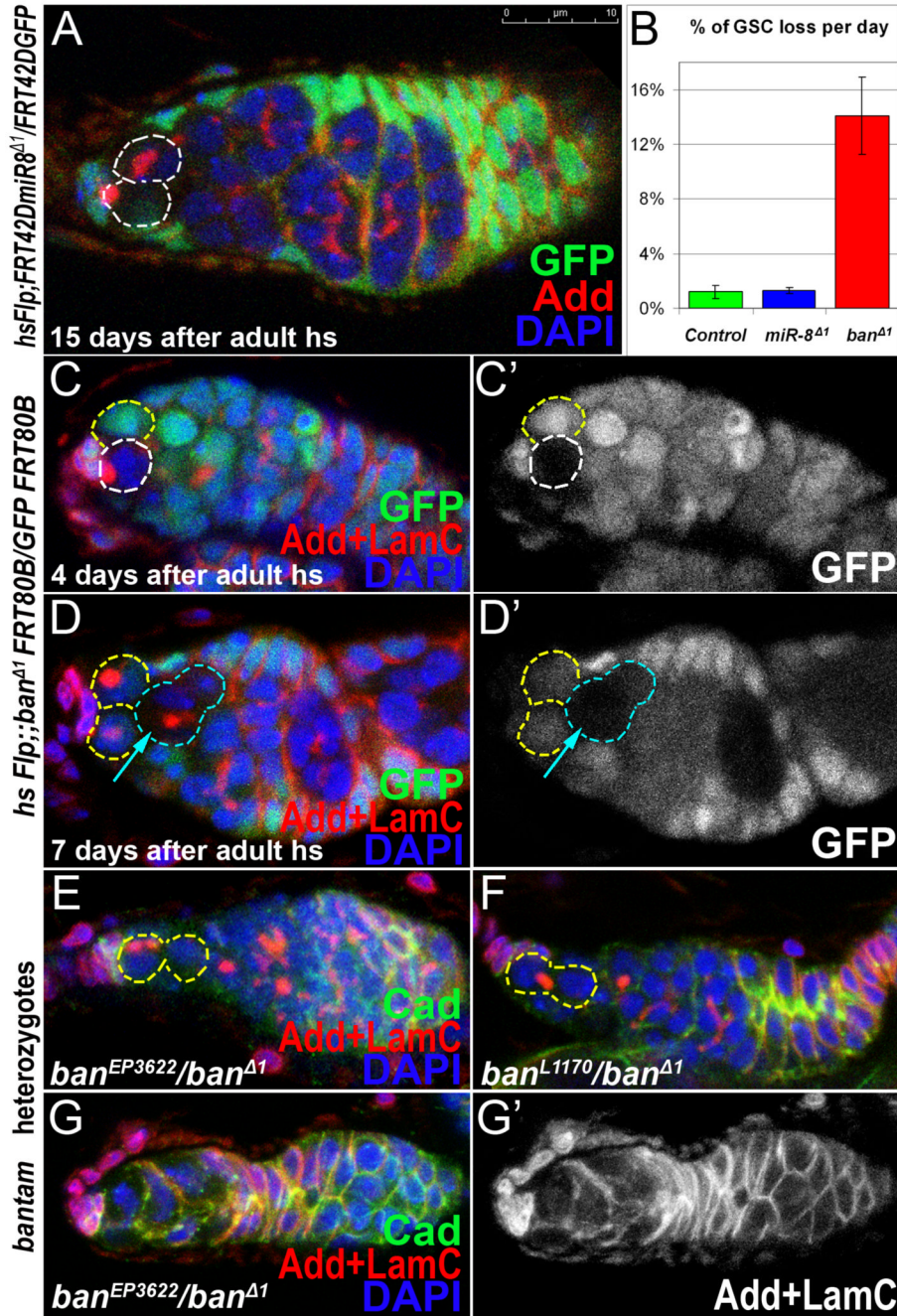


**Figure 1. Adult-induced *dicer-1* mutant germline stem cell clones are lost from the niche**  
 (A) Diagram showing the germline stem cell niche. Germline stem cells (GSC, pink) indicated by anterior spectrosomes (SS, red) are located at the anterior end of the germium adjacent to the niche cap cells (CpC, grey). Terminal filament (TF; dark blue), escort stem cell (ESC, lavender), differentiated cystoblast (CB, blue), inner germarial sheath cell (IGS, lime), germ cell cyst (cyst, celadon, emerald and green), marked by the presence of a branched fusomes (FS, red), somatic stem cells (SSCs, violet), follicle cells (FC, light blue).  
 (B) Adult induced *dicer-1*<sup>Q1147</sup> mutant GSCs divide slower than control GSCs (the mutant produced one progeny, while the control produced 4 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 germaria (a germline stem cell shown with a single *dicer-1* mutant germline stem cell). Red=Adducin, Blue=DAPI, Green=GFP, mutant clones outlined with white dashed lines, control clones with yellow.



**Figure 2. microRNA-GFP sensors are expressed in distinct subsets of cells in the germlarium** MicroRNA sensor expression patterns in (A, B and D) wild-type and (C) *miR-8<sup>Δ1</sup>* mutant germaria. Sensors 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<sup>Δ1</sup>* mutant germaria. (E) *bantam-sensor* is responsive to the dicer; in *dcr-1* clones, marked by the absence of β-gal (E''), the level of GFP fluorescence is higher than that in a non-clonal neighbor (E'). In E native GFP expression by one copy of *bantam-sensor* is analyzed (*hs Flp*;

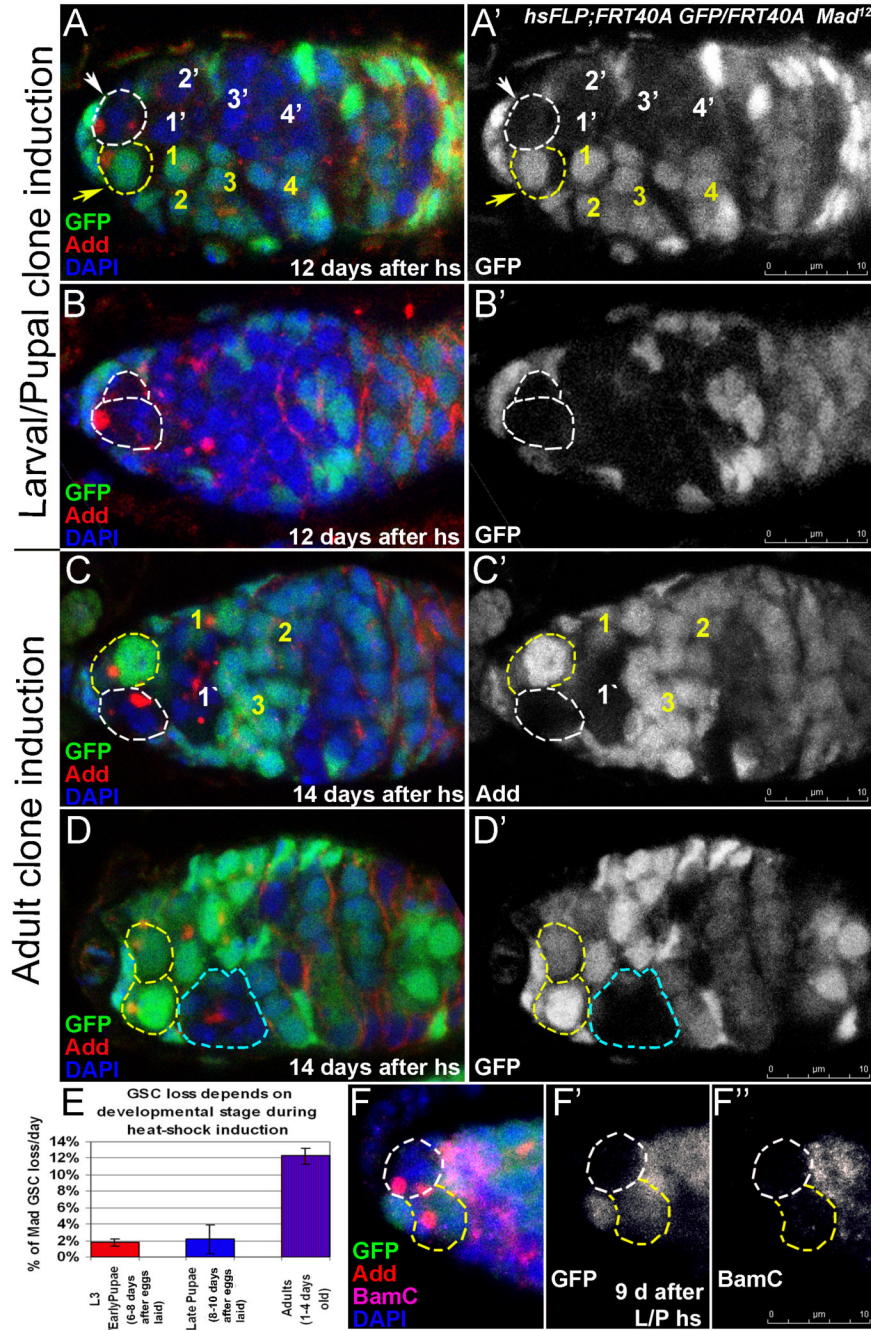
*ban<sup>Δtub84B</sup>:Avic/GFP-EGFP/+; FRT82B dcr-1<sup>Q1147X</sup>/FRT82B arm-lacZ*). Red=Adducin, Blue=DAPI (A-D) or β-gal (E), Green=GFP, GSCs marked with white dashed lines.



**Figure 3. *bantam* microRNA is required for GSC maintenance in the niche**

(A) *miR-8<sup>Δ1</sup>* mutant germline stem cells are maintained in the niche and divide properly 14 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. *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<sup>EP3622</sup>/ban<sup>Δ1</sup>* (E-G) and *ban<sup>L1170</sup>/ban<sup>Δ1</sup>* (F) exhibit mutant phenotypes similar to *bantam* clones: germaria are reduced in size and have a single GSC (E,F) or no GSC (G).

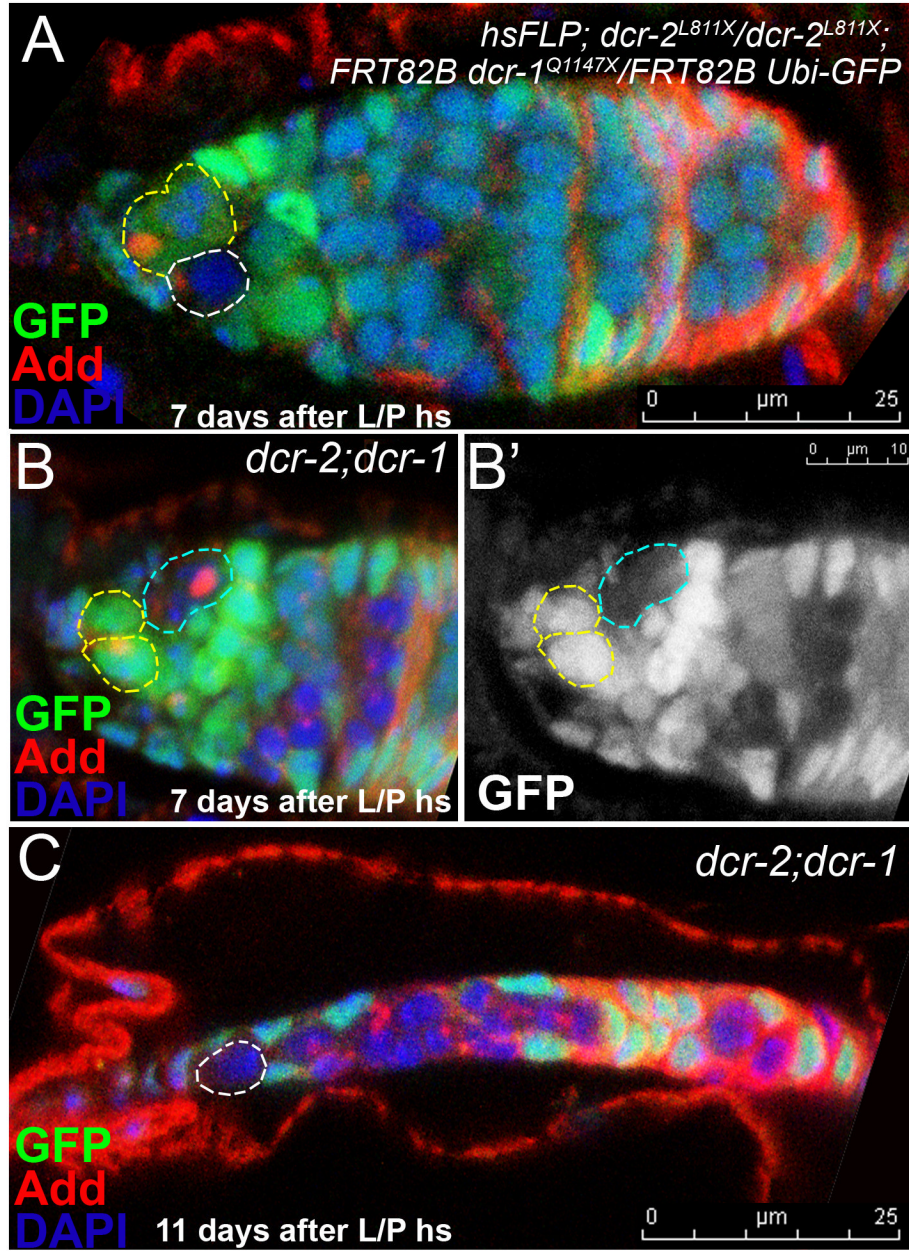
Red=Adducin (A) or Adducin+LaminC (C-G), Blue=DAPI, Green=GFP (A-D) or Cadherin (E-G); mutant GSCs or cysts outlined with white dashed lines, departed or differentiated stem cells with turquoise dashes.



**Figure 4. Larval/pupal-induced *Mad*<sup>12</sup> mutant GSCs are maintained in the niche**  
 Larval/pupal-induced *Mad*<sup>12</sup> mutant GSCs divide at the same frequency as control GSCs (A; S Table 4) and are maintained in the niche 12 days after clonal induction (B; S Table 1). However, as described before (Xie and Spradling, 1998), *Mad*<sup>12</sup> mutant GSC clones induced during adulthood divide slower than non-clonal germline stem cells (C; S Table 4) and are not maintained in the niche (D; 14 days after adult clonal induction, the *Mad* mutant GSC has left the niche and produced a 8-cell cyst). A bar graph showing that GSC maintenance has a development-dependent character: when *Mad*<sup>12</sup> 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 *Mad*<sup>12</sup> GSCs (E). A differentiation factor Bam

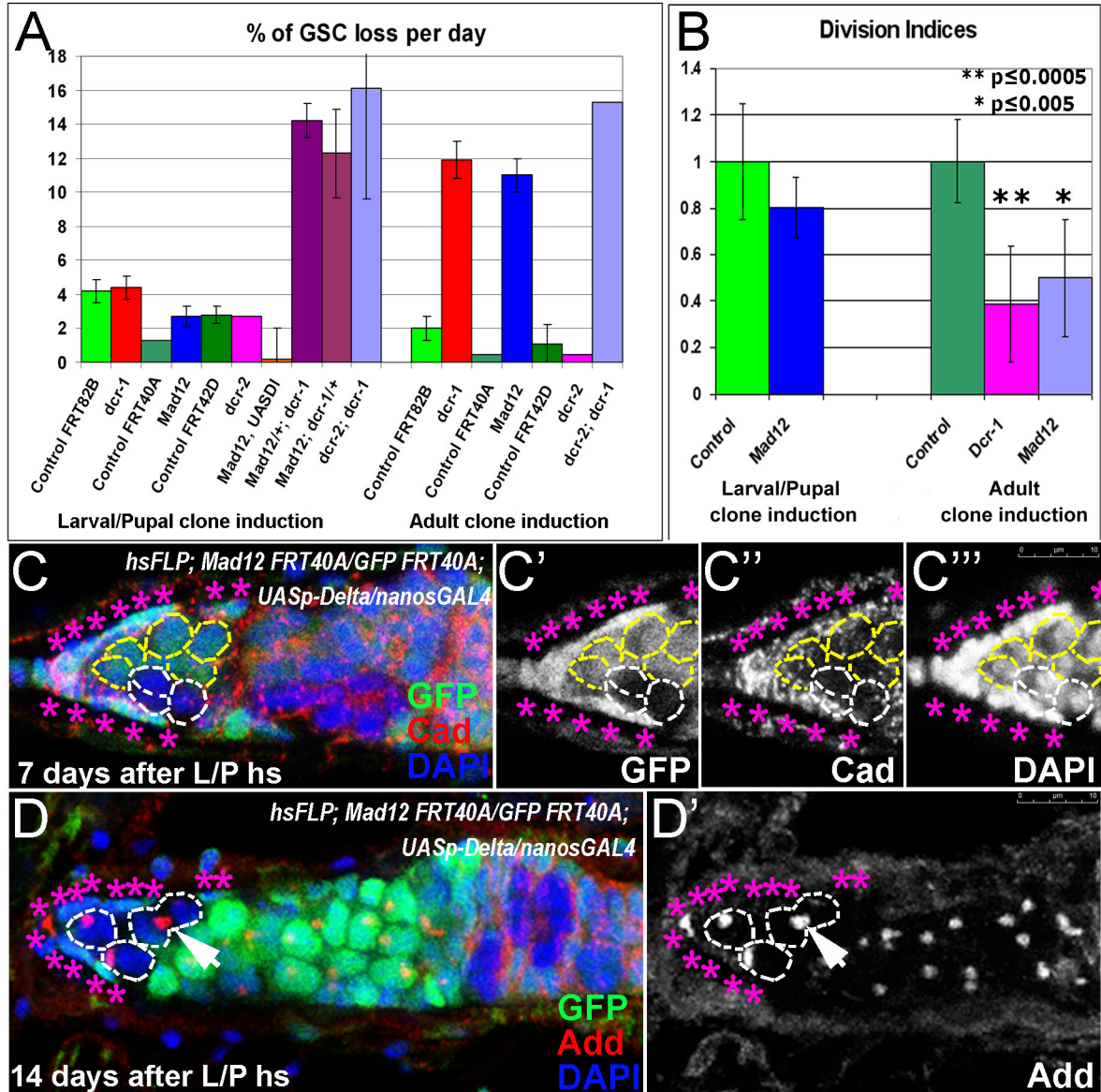


is not derepressed in mutant GSCs, showing that larval/pupal-induced *Mad12* mutant GSCs maintain their SC identity. Red=Adducin, Blue=DAPI, Green=GFP, Purple=BamC, mutant GSCs outlined with white dashed lines, control GSCs with yellow dashes.



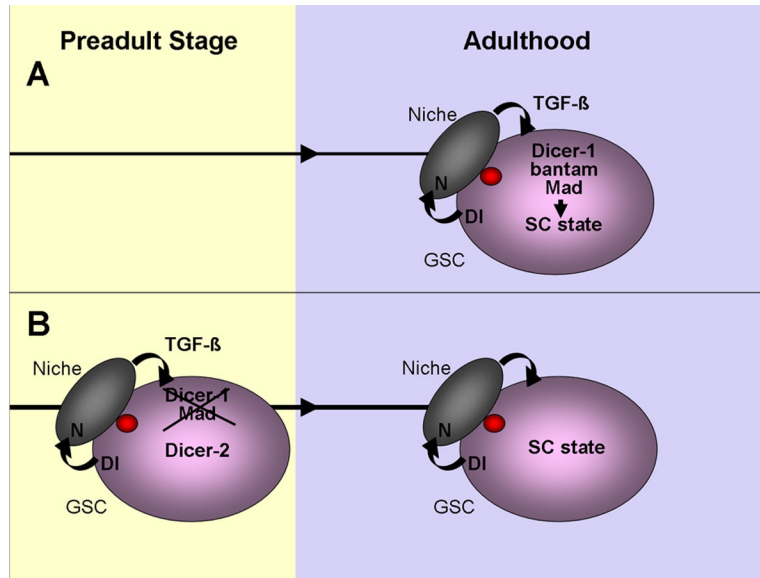
**Figure 5. GSCs lacking Dicer-1 activity and Dicer-2 activity from pupal stages onwards are lost from the niche**

(A-C) Larval-induced *dicer-1* mutant GSCs in a *dicer-2* mutant background (*hsFLP; dcr-2<sup>L811X</sup>/dcr-2<sup>L811X</sup>; FRT82B dcr-1<sup>Q1147X</sup>/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 outlined with dashes: yellow=normal, white=mutant. GSCs departed from the niche are outlined with turquoise dashes. Red=Adducin, Green=native GFP, Blue=DAPI.



**Figure 6. The 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 synergetic maintenance defect is observed when both *dicer-1* and *Mad* levels are reduced simultaneously during larval/pupal stages (*Mad<sup>12/+</sup>; dcr-1* or *Mad<sup>12</sup>; dcr-1/+*). (B) Germline stem cells lacking *Mad* activity from larval/pupal stages onwards divide relatively normally compared to controls. Whereas, germline stem cell 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 heatshock and 9 days after adult heatshock (see S Table 4). (C, D) *Mad<sup>12</sup>* mutant GSCs in germlaria over-expressing *UASp-Delta* in the germline (*hsFLP; Mad<sup>12</sup> 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 7 to 14

day timepoint after larval/pupal clonal induction (C and D respectively, a dividing *Mad* mutant GSC marked with an arrow. *Mad* mutant GSCs outlined with white dashed lines, cap cells identified with pink asterisks. Red=Cadherin (C) or Adducin (D), Blue=DAPI, Green=GFP.



**Figure 7. Model showing different mechanisms for GSC maintenance during development and adulthood**

(A) TGF- $\beta$  and Dicer-1 are required for GSC maintenance in adult *Drosophila* ovary. We have identified bantam as the key microRNA in this process. Bantam has previously been studied in cell cycle and cell death in *Drosophila* imaginal disc epithelial cells. However, in GSC 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.