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Argonaute 1 regulates the fate of germline stem cells in *Drosophila*

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The Argonaute-family proteins play crucial roles in small-RNA-mediated gene regulation. In *Drosophila*, previous studies have demonstrated that Piwi, one member of the PIWI subfamily of Argonaute proteins, plays an essential role in regulating the fate of germline stem cells (GSCs). However, whether other Argonaute proteins also play similar roles remains elusive. Here, we show that overexpression of Argonaute 1 (AGO1) protein, another subfamily (AGO) of the Argonaute proteins, leads to GSC overproliferation, whereas loss of *Ago1* results in the loss of GSCs. Combined with germline clonal analyses of *Ago1*, these findings strongly support the argument that *Ago1* plays an essential and intrinsic role in the maintenance of GSCs. In contrast to previous observations of Piwi function in the maintenance of GSCs, we show that AGO1 is not required for *bag of marbles* (*bam*) silencing and probably acts downstream or parallel of *bam* in the regulation of GSC fate. Given that AGO1 serves as a key component of the miRNA pathway, we propose that an AGO1-dependent miRNA pathway probably plays an instructive role in repressing GSC/cystoblast differentiation.

KEY WORDS: Argonaute protein, *Ago1*, miRNA, GSC self-renewal, *Drosophila*

INTRODUCTION

In *Drosophila* ovary, germline stem cells (GSC) provide an attractive system for investigating the regulatory mechanisms that determine stem cell fate (Lin, 2002; Spradling et al., 2001). Studies from several laboratories have identified the genes that are essential for GSC fate determination. Both the *bag of marbles* (*bam*) and *benign gonial cell neoplasm* (*bgcn*) genes are known to act autonomously in the germline and are required for cystoblast (CB) differentiation (Lavoie et al., 1999; McKearin and Ohlstein, 1995). By contrast, the BMP ligands *glass bottom boat* (*gbb*) and *decapentaplegic* (*dpp*) are expressed in the niche cap cells and play an essential role in GSC maintenance by silencing *bam* transcription to repress GSC differentiation (Chen and McKearin, 2003a; Song et al., 2004; Xie and Spradling, 1998).

Independent of the *bam* transcriptional silencing pathway, the self-renewal of GSCs is also controlled by two translational repressors, Pumilio (Pum) and Nanos (Nos) (Forbes and Lehmann, 1998; Lin and Spradling, 1997). Pumilio is not required for *bam* silencing, but acts either downstream of *bam* or parallel to *bam* action (Chen and McKearin, 2005; Szakmary et al., 2005). Recent data also showed that Pelota, another putative translational repressor, plays a similar role in controlling GSC fate in a *bam*-independent manner (Xi et al., 2005). These findings suggest that cell-autonomous translational control could contribute a great deal to GSC regulation.

The Argonaute family proteins play the central roles in small-RNA-mediated gene regulation (Parker and Barford, 2006). In *Drosophila*, two subfamilies (AGO and PIWI subfamilies) of Argonaute proteins have been characterized. Piwi, a member of the PIWI subfamily protein, has been shown to associate with repeat associated small interfering RNA (rasiRNA or piRNA) (Vagin et al., 2006) and exhibit target cleavage RNA activity (Grivna et al., 2006; Saito et al., 2006). By contrast, as members of the AGO subfamily of Argonaute proteins, Argonaute 1 (AGO1) and Argonaute 2 (AGO2) have been shown to be involved in miRNA-mediated gene regulation and in siRNA-mediated mRNA degradation, respectively (Lee et al., 2004; Okamura et al., 2004). In *Drosophila* ovary, Piwi has been shown to play essential roles in the maintenance of GSCs via the *bam* silencing pathway and the regulation of GSC division (Chen and McKearin, 2005; Cox et al., 2000; Szakmary et al., 2005); however, whether AGO1 is also involved in this biological process remains unclear. The role of the miRNA pathway in GSC fate determination has been explored in *Drosophila* ovary. Previous studies showed that loss of the *loquacious* (*loqs*) gene, which encodes a partner protein of Dicer1 (Dcr1) in miRNA biogenesis, leads to GSC maintenance defects (Forstemann et al., 2005; Jiang et al., 2005; Park et al., 2007). However, the role of Dcr1 in the maintenance of GSC is contradictory (Hatfield et al., 2005; Jin and Xie, 2007), because an extensive functional analysis of *Dcr1* in GSCs suggested that the miRNA pathway plays an important role in the control of GSC division, rather than GSC self-renewal (Hatfield et al., 2005). Thus the role of miRNAs in GSC fate determination is still uncertain and remains to be elucidated. Here we show that overexpression of AGO1 leads to GSC overproliferation, whereas loss of *Ago1* results in the loss of GSCs. Combined with germline clonal analyses of *Ago1*, these findings strongly support the argument that *Ago1* plays an essential and intrinsic role in the maintenance of GSCs and that an AGO1-dependent miRNA pathway plays at least a partial instructive role in repressing GSC/CB differentiation. Furthermore, in contrast to *piwi*, we show that *Ago1* is not required for *bam* silencing and probably acts downstream or parallel of *bam* in the regulation of GSC maintenance.

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MATERIALS AND METHODS

Drosophila genetics

All flies were maintained under standard culture conditions. bam^{86} is a null allele for *bam* as described previously (McKearin and Ohlstein, 1995; Ohlstein et al., 2000). bam^{BG} is a strong allele for *bam* as described previously (Chen and McKearin, 2005). $piwi^{EP}$ and *P{hs-gal4}* have been described previously (Chen and McKearin, 2005; Cox et al., 2000) $Ago1^{k08121}$ has been described previously (Jin et al., 2004; Kataoka et al., 2001; Williams and Rubin, 2002), and $loqs^{f00791}$ was a strong allele for *loqs* from the Exelixis Collection and described previously (Forstemann et al., 2005; Jiang et al., 2005). *P{bamp-gfp}* was described by Chen and McKearin (Chen and McKearin, 2003b), and *P{hs-Ago1}* (a gift from Dr T. Uemura, Kyoto University, Japan) was used for rescuing *Ago1* mutants (Kataoka et al., 2001). $Ago1^{EMS}$ is generated through an EMS mutagenesis; sequencing results showed that the $Ago1^{EMS}$ allele contains a C-T switch, which changes amino acid 212 Q to a stop codon in the AGO1 B form and changes amino acid 246 Q to a stop codon in the putative AGO1 A and C forms. The resulting truncated proteins lack both the PAZ and PIWI domains, which are essential for Argonaute protein function (Miyoshi et al., 2005; Okamura et al., 2004; Shi et al., 2004), indicating that $Ago1^{EMS}$ is a null allele for the *Ago1* gene; to obtain more mutants for the further analysis of *Ago1* function in GSCs, we performed a mutagenesis through imprecise mobilization of the P-element in $Ago1^{k08121}$. Fifteen alleles, which we referred to as $Ago1^{1}$~$Ago1^{15}$, were recovered, and one of them, $Ago1^{14}$, was characterized as having a 16 kb deletion that covers the *Ago1* coding sequence, indicating that $Ago1^{14}$ is another null allele for the *Ago1* gene.

Immunohistochemistry and microscopy

Ovaries were prepared for reaction with antibodies as described previously (McKearin and Ohlstein, 1995). Monoclonal anti-Bam antibody (McKearin and Ohlstein, 1995) was used at a 1:500 dilution, polyclonal anti-Vasa antibody (Santa) was used at 1:200 dilution, polyclonal anti-GFP antibody (Invitrogen) was used at a 1:5000 dilution, monoclonal anti-Hts antibody was used at a 1:500 dilution, and mouse anti-AGO1 (a gift from Dr M. Siomi and Dr H. Siomi, Institute for Genome Research, University of Tokushima, Japan) (Miyoshi et al., 2005) was used at a 1:200 dilution. Secondary antibodies used were goat anti-mouse Alexa 568, goat anti-rabbit Alexa 488, and goat anti-rat Cy3 (Molecular Probes), all at 1:200. All samples were examined by Zeiss microscope and images were captured using the Zeiss Two Photon Confocal LSM510 META system supported by the State Key Laboratory of Biomembrane and Membrane Biotechnology and Institute of Zoology, CAS. Images were further processed with Adobe Photoshop 6.0.

Phenotypic assay for quantification of GSC maintenance in mutant adult ovaries

Ovaries isolated from wild-type and different mutant flies of different ages were incubated with anti-Hts antibody, anti-Vasa antibody and DNA dyes to identify terminal filament cells, fusomes and germ cells. We scored as GSCs any Vasa-positive germ cells at the anterior position that appeared close to cap cells or to the basal cells of terminal filaments and also carried spherical fusomes at the anterior position or extending fusomes when a GSC was dividing.

Germline clonal analysis

FLP/FRT-mediated recombination was used to generate *Ago1* mutant GSC and PGC clones. To generate GSC clones, *w; FRTG13, Ago1/CyO* (*w; FRTG13/CyO* as the control) males were crossed to virgin females of *w hsFlp; FRTG13, ubi-gfp*, and 3-day-old female progenies lacking the *CyO* chromosome underwent heat-shock treatment at 37°C for 60 min twice daily at 12 hourly intervals. Ovaries dissected from *{hs-flp; frtG13, /frtG13, ubi-gfp}* or *{hs-flp; frtG13, Ago1/ frtG13, ubi-gfp}* were stained with anti-GFP and anti-Hts antibodies for quantification of GSC clones. GSC clones were identified by the lack of GFP expression and carrying anterior-positioned spectrosome. To analyze GSC establishment, for adult GSC assay, the heat-shock treatment was started at the early third larval stage or early to induce PGC clones. GSC clones with negative GFP in *{hs-flp; frtG13, Ago1/ frtG13, ubi-gfp}* newly eclosed females were quantified to calculate the rate of GSC clones. In this experiment, *{hs-flp; frtG13, / frtG13, ubi-gfp}* was used as the FRT control. For pupa GSC clonal assay, the progenies from the cross of *w; FRTG13, Ago1/CyO* and *w hsFlp; FRTG13, ubi-gfp* began to be treated by constitutive heat-shock from the first instar larval stage; meanwhile, the progenies from the cross of *w; FRTG13 /CyO* and *w hsFlp; FRTG13, ubi-gfp* were used as FRT control. After staining with anti-GFP and anti-Hts antibodies, female gonads containing GFP-negative germ cells were examined and putative GSC clones were identified by their anterior position close to the terminal filament and their lack of GFP expression.

RESULTS

Ectopic AGO1 expression increases the number of GSC-like cells

To explore the potential role of *Ago1* in the regulation of GSC fate, we overexpressed *Ago1* in germaria by expressing an *Ago1* cDNA under the control of the heat-shock promoter *P{hs-Ago1}* and then applying daily heat-shock treatment (Kataoka et al., 2001). In this study, we distinguished GSCs from differentiated germ cells by using anti-Vasa and anti-Hts antibodies to visualize germ cells and fusomes, respectively. The fusome (also called a 'spectrosome' in GSCs/CBs) is a germ-cell-specific organelle that is morphologically spherical in GSCs/CBs or extends from anterior position to posterior position when a GSC is dividing, but is branched in differentiated cysts (Fig. 1A). To assess the potential role of ectopic AGO1, we scored the number of germ cells carrying spectrosomes per germarium that came from wild type, and *P{hs-Ago1}* flies at 0, 6 and 10 days heat-shock treatment. As a positive control we used $piwi^{EP}$; *P{hs-gal4}* to induce ectopic GSC-like cells by overexpression of *piwi* as described previously (Cox et al., 2000). As shown in Fig. 1B, before heat-shock treatment, we observed averages of 3.3 (n=128), 3.2 (n=102) and 3.2 (n=95) spectrosome-containing germ cells per germarium in wild-type, *P{hs-Ago1}*, and $piwi^{EP}$; *P{hs-gal4}*, respectively, suggesting that there was no difference in the number of spectrosome-containing germ cells among wild-type, *P{hs-Ago1}*, and $piwi^{EP}$; *P{hs-gal4}* flies without heat-shock treatment. However, after 6 days of heat-shock treatment (three times per day), we observed averages of 4.9 (n=105) and 5.6 (n=123) spectrosome-containing germ cells per germarium in *P{hs-Ago1}* and $piwi^{EP}$; *P{hs-gal4}* ovaries, respectively. We noted that 25.7% (n=105) of *P{hs-Ago1}*, and 43.7% (n=145) of $piwi^{EP}$; *P{hs-gal4}*, germaria contained more than six spectrosome-containing germ cells (Fig. 1B). As ovaries from wild-type control females undergoing the same treatment carried only 3.3 spectrosome-containing germ cells per germarium (n=131), we excluded the possibility that the increase in the number of spectrosome-containing germ cells could be due to heat-shock treatment. We also found that, by extending heat-shock induction to 10 days, the average number of spectrosome-containing germ cells in *P{hs-Ago1}* and $piwi^{EP}$; *P{hs-gal4}* ovaries was increased to 5.9 (n=176) and 6.5 (n=103) per morphological normal germarium (tumorous germaria found in *P{hs-gal4}* were excluded in this quantification). In this case, we found that 56.5% (n=176) of *P{hs-Ago1}* and 64.2% (n=112) $piwi^{EP}$; *P{hs-gal4}* germaria contained more than six spectrosome-containing germ cells. However, for wild-type control under the same conditions, the number of spectrosome-containing germ cells was still maintained at 3.2 (n=115) per germarium. Thus, similar to *piwi*, the ectopic expression of AGO1 could also increase the number of GSC-like cells. Interestingly, in contrast to overexpression of *piwi*, a certain percentage (variable at 5-10%, n>100) of *P{hs-Ago1}* germaria were morphologically tumorous-like, when they were treated with 10 day heat-shock. As shown in Fig. 1C, GSC-like cells (Fig. 1C left and right panels and see Fig. S3 in the supplementary material) and differentiated cysts (Fig. 1C

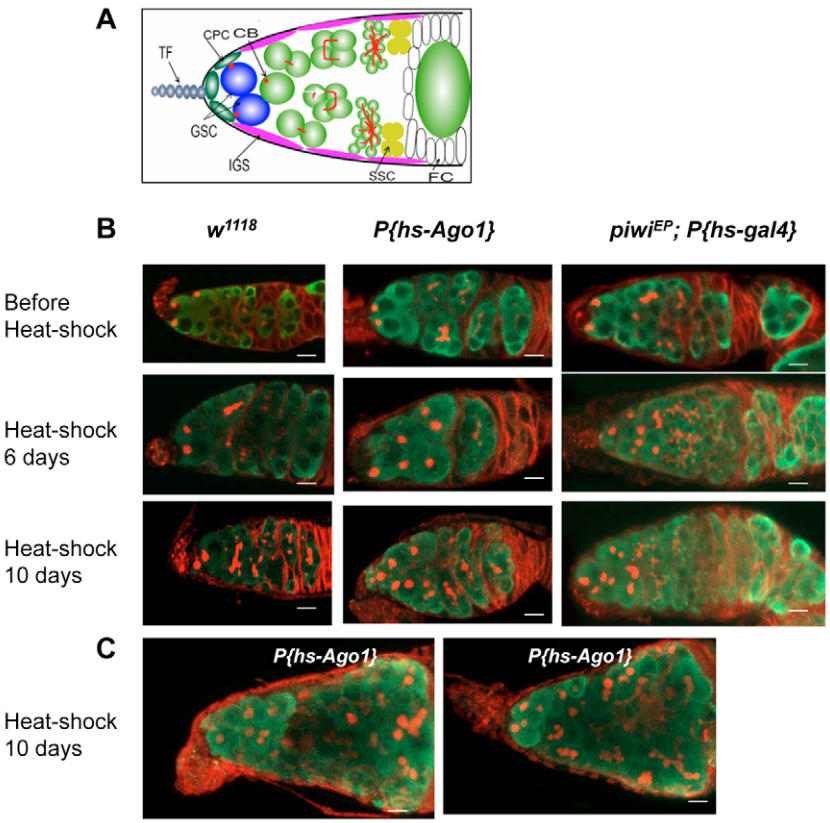

Fig. 1. Ectopic expression of Ago1 increases the number of GSCs. (**A**) A schematic diagram of a *Drosophila* germarium with different cell types labeled by different colors: GSCs blue, CBs and cysts green, terminal filaments (TFs) gray, cap cells (CPCs) deep green, inner germarium sheath cells (IGSs) pink, follicle cells (FCs) and fusomes red. (**B**) Ovaries collected from wild-type (w^{1118}), *P{hs-Ago1}* and *piwiEP; P{hs-gal4}* flies before heat-shock and after 6 days and 10 days heat-shock treatment, as indicated, were stained with anti-Vasa (green) and anti-Hts (red) antibodies. Vasa-positive germ cells carrying spectrosomes were undifferentiated germ cells (GSCs/Cb and GSC-like cells). (**C**) A germarium from 10-day heat shock was morphologically tumorous; many GSC-like cells carrying spectrosomes (both panels) and differentiated germ cells carrying branched fusomes (right panel) were observed. Scale bar: 10 μm.

right panel) were filled in these tumorous germaria. Thus, the increased AGO1 activity could also potentially induce the overproliferation of GSC-like cells, probably by delaying GSC/CB differentiation, and disrupt normal oogenesis.

The loss of *Ago1* leads to defects in GSC maintenance

The increase in GSC-like cells induced by *Ago1* overexpression suggests that *Ago1* could play a role in preventing GSC differentiation for their self-renewal. To explore this possibility, we analyzed the phenotype in *Ago1* loss-of-function mutants. One well-characterized *Ago1* mutant, $Ago1^{k08121}$ (Jin et al., 2004; Kataoka et al., 2001; Williams and Rubin, 2002), leads to a loss of function of *Ago1* that causes homozygous mutant lethality at the embryonic stage. To analyze the specificity of the $Ago1^{k08121}$ allele in germ cell development we generated revertants of the $Ago1^{k08121}$ allele. We found that the homozygous animals of precise excision lines were as viable and fertile as wild type, indicating that, apart from *Ago1*, no other genetic background mutations in the $Ago1^{k08121}$ flies could possibly be affecting lethality or germ cell development. This finding is also consistent with previous data showing that $Ago1^{k08121}$ specifically results in the loss of function of *Ago1* (Jin et al., 2004; Kataoka et al., 2001; Williams and Rubin, 2002). To obtain more mutant alleles with different genetic backgrounds for further analysis of *Ago1* function in GSCs, we performed EMS and imprecise excision mutagenesis to generate an EMS allele ($Ago1^{EMS}$) and an imprecise excision line ($Ago1^{14}$) that were null for *Ago1* (see Materials and methods for details).

All three mutants of *Ago1* ($Ago1^{k08121}$, $Ago1^{EMS}$ and $Ago1^{14}$) used in this work are lethal at embryonic stage, but homozygous and trans-heterozygous mutant animals could survive to adulthood when the transgene *P{hs-Ago1}* was introduced and the flies were treated with daily heat shock. Thus, by immediately withdrawing the heat-shock treatment after adult eclosion, the animals became progressively *Ago1*-deficient as the protein decayed (Fig. 2F). We found that ovaries from 3-day-old *Ago1* mutant flies (Fig. 2A) showed no phenotype differences compared to wild-type control flies. Strikingly, however, 15-day-old *Ago1* mutant ovaries exhibited strong defects in GSC maintenance. As illustrated in Fig. 2 and Table 1, for 15-day-old *Ago1*-deficient ovaries (from *hs-Ago1*; $Ago1^{k08121}$/ $Ago1^{k08121}$ following heat-shock withdrawal after eclosion), only a few germaria (6.3%, *n*=79) had a normal-looking structure containing two stem

Table 1. Phenotypic assay for *Ago1*-deficient flies

Genotype*	% germaria containing 2 or 3 GSCs	% germaria containing 1 GSC	% germaria containing only cysts	% empty germaria
Control wild-type	99.2 (*n*=245)	0.8 (*n*=245)	0 (*n*=245)	0 (*n*=245)
Hs-Ago1;$Ago1^{k08121}$/$Ago1^{k08121}$	6.3 (*n*=79)	19.0 (*n*=79)	45.6 (*n*=79)	29.1 (*n*=79)
Hs-Ago1;$Ago1^{k08121}$/$Ago1^{14}$	10.6 (*n*=113)	23.9 (*n*=113)	60.1 (*n*=113)	5.3 (*n*=113)
Hs-Ago1;$Ago1^{k08121}$/$Ago1^{EMS}$	10.3 (*n*=68)	26.5 (*n*=68)	60.3 (*n*=68)	2.9 (*n*=68)

n, Number of germaria examined.
*Progeny from wild-type and different crosses (*hs-Ago1;Ago1*/Cyo × *hs-Ago1;Ago1*/*Cyo*) were treated with daily heat shock until adult eclosion. 15-day-old mutant and wild-type control flies were analyzed for quantification of GSC number.

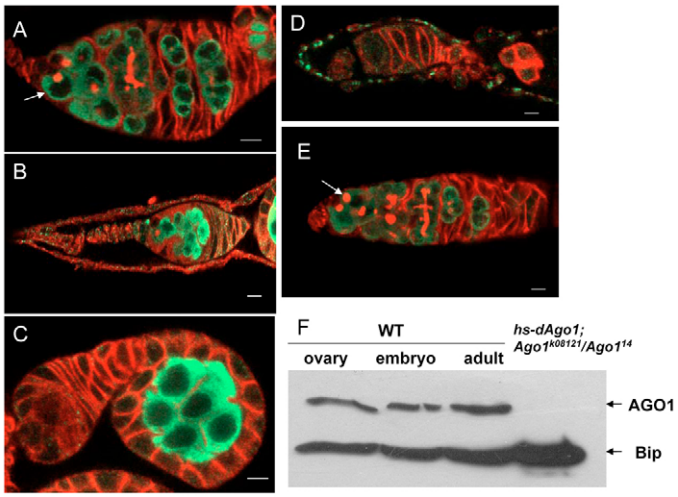

Fig. 2. Loss of *Ago1* disrupts GSC maintenance in *Drosophila*. Ovaries collected from *{hs-Ago1; Ago1/Ago1}* flies undergoing different treatments were stained with anti-Vasa (green) and anti-Hts (red) antibodies. (**A**) Example of a germarium from an *{hs-Ago1; Ago1^{k08121}/Ago1^{k0812}}* animal 3 days after withdrawal of heat shock, in which two GSCs were maintained well. (**B-D**) In *Ago1* mutants germ cell development was severely defective. There were no GSCs maintained at day 15 after withdrawal of heat shock. Allele combinations of (B) *Ago1^{k08121}/Ago1^{k08121}*, (C) *Ago1^{k08121}/Ago1^{14}* and (D) *Ago1^{k08121}/Ago1^{EMS}* are shown. (**E**) Example of the germarium of a fly undergoing constitutive heat shock, in which two GSCs were maintained well. (**F**) AGO1 is expressed almost equally in ovary, embryo and adult. In *Ago1^{k08121}/Ago1^{14}* mutant flies rescued by the transgene *P{hs-Ago1}*, the AGO1 protein was undetectable at day 15 after withdrawal of heat-shock treatment. Scale bar: 10 μm.

cells, and about 19.0% (n=79) of germaria contained a single stem cell; most of the germaria (74.6%, n=79) contained either differentiated germ cells with branched fusomes only (45.6%) or no germ cells at all (29.1%). In the latter case, germaria composed only of somatic cells were still visible by staining with Hoechst and anti-Hts antibody. We observed similar results with the other two combinations of different *Ago1* alleles (*hs-Ago1*; *Ago1^{k08121}/Ago1^{EMS}* and *hs-Ago1*; *Ago1^{k08121}/Ago1^{14}*) undergoing the same treatment (Fig. 2 and Table 1). It would therefore appear that, in contrast to controls, progressively reducing *Ago1* activity causes the loss of GSCs. To further exclude the possibility that the phenotype seen in germ cells from these alleles is due to genetic background differences, we treated mutant flies by constitutive daily heat-shock treatment once the fly eclosed. We found that the loss of GSCs and other developmental abnormalities of the germ cells caused by *Ago1* deficiency could be rescued by exogenous AGO1 protein (Fig. 2E). Taken together, these results suggest that AGO1 plays an important role in germline stem cell maintenance and possibly controls other aspects of germ cell development as well.

AGO1 is required intrinsically for the establishment and maintenance of GSCs

The loss of germline stem cells in *Ago1* mutant ovaries suggests that *Ago1* is required in either GSCs or somatic cells (or possibly both). To test whether *Ago1* is required intrinsically for GSC maintenance, we first examined the expression pattern of AGO1 in germarium. We found via immunostaining that the AGO1 protein is ubiquitously expressed in both germ cells and somatic cells, indicating that AGO1 could function in either cell type (see Fig. S1A in the supplementary material). To explore whether AGO1 functions as a cell-autonomous factor for maintaining GSC fate, we used an FLP-FRT-mediated mitotic recombination technique to generate marked mutant GSCs, then calculated the life span of the marked mutant GSCs by quantifying their loss rate (Xie and Spradling, 1998). The marked mutant GSCs were identified by the lack of GFP expression and by carrying an anterior-positioned spectrosome. The three alleles (*Ago1^{k08121}*, *Ago1^{EMS}* and *Ago1^{14}*) of *Ago1* were used to generate marked mutant GSC clones for analysis of *Ago1* function in GSCs. The percentages of Ubi-GFP-marked GSCs were measured at 2, 10 and 20 days after heat-shock treatment (AHST). As shown in Fig. 3A-C and Table 2, for wild-type control GSC clones, the percentage of marked clones was about 30.0% at day 2, which we considered the initial percentage, and this was reduced to 19.2% by day 20 AHST, suggesting that the marked clone loss rate was roughly 36% over the 20 day period; however, for *Ago1^{k08121}* mutant GSC clones under the same conditions, the initial percentage of marked clones was measured at 30.3%, but this fell to 3.9% at day 20 AHST, suggesting a loss rate for *Ago1^{k08121}* of about 90% during the testing period (Fig. 3D-F and Table 2). We obtained similar results with the two other alleles, *Ago1^{14}* and *Ago1^{EMS}*. We observed that all the marked GSC clones were lost (100% loss rate) in both these alleles over the 20 day testing period, as shown in Table 2. Taken together, these findings support the argument that AGO1 is intrinsically required for GSC maintenance.

The loss of GSCs in the *Ago1* mutants could be due to either differentiation or a reduction in cell viability. We noticed that the marked *Ago1* mutant stem cells had normal DNA staining and could also develop into normal cysts; thereby we excluded the possibility

Table 2. Clonal analyses of *Ago1* deficiency in GSCs

Genotype	Days after stopping heat shock	% marked GSCs	Relative % marked GSCs	Total number of germaria examined
Control *hs-flp;FRTG13,ubi-gfp/FRTG13*	2	30.0	100	160
	10	25.0	83.3	104
	20	19.2	64.0	73
Ago1^{k08121} hs-flp;FRTG13,ubi-gfp/FRTG13, Ago1^{k08121}	2	30.3	100	132
	10	12.5	41.3	104
	20	3.9	12.9	128
Ago1^{14} hs-flp;FRTG13,ubi-gfp/FRTG13, Ago1^{14}	2	30.0	100	101
	10	12.6	41.6	103
	20	0	0	137
Ago1^{EMS} hs-flp;FRTG13,ubi-gfp/FRT G13, Ago1^{EMS}	2	15	100	80
	10	0	0	102

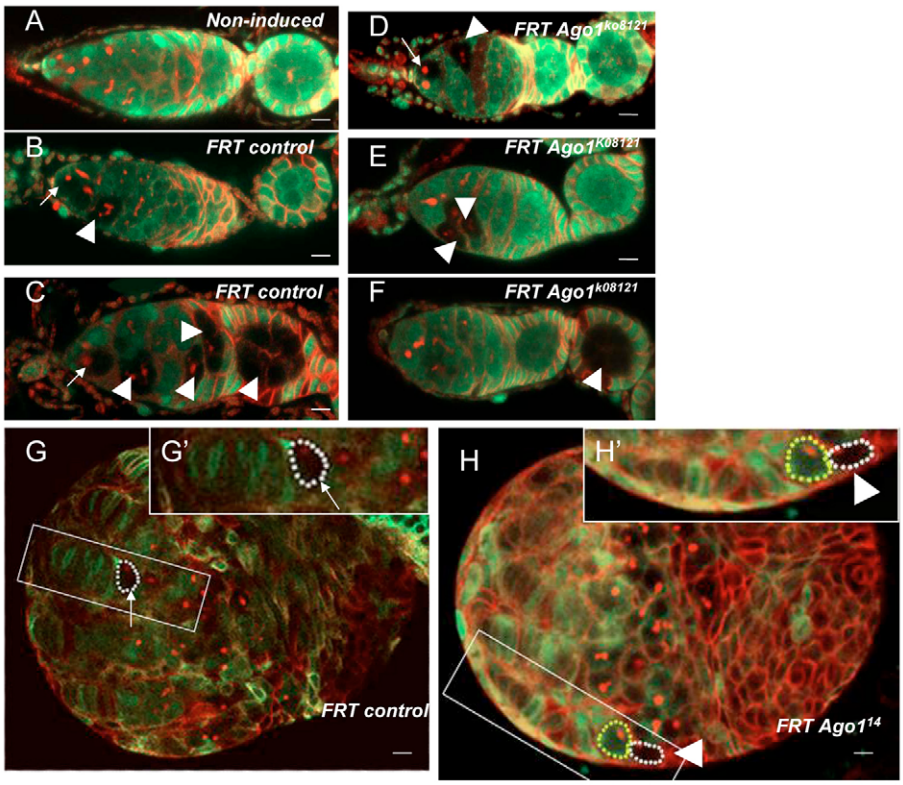

Fig. 3. AGO1 is intrinsically required for the establishment and maintenance of GSCs in *Drosophila*. Ovaries dissected from flies *{hs-flp; frtG13, / frtG13, ubi-gfp}* without heat shock (**A**), *{hs-flp; frtG13, / frtG13, ubi-gfp}* at day 2 AHST (**B**), *{hs-flp; frtG13, / frtG13, ubi-gfp}* at day 10 AHST (**C**), *{hs-flp; frtG13, Ago1*^{k08121}*/ frtG13, ubi-gfp}* at day 2 AHST (**D**), and *{hs-flp; frtG13, Ago1*^{k08121}*/ frtG13, ubi-gfp}* at day 10 AHST (**E**,**F**) were stained with anti-GFP (green) and anti-Hts (red) antibodies. GFP-negatively marked GSCs and cysts were indicated by arrows and arrowheads, respectively. Gonads were dissected from *{hs-flp; frtG13, / frtG13, ubi-gfp}* (**G**,**G′**) and *{hs-flp; frtG13, Ago1*^{14}*/ frtG13, ubi-gfp}* (**H**,**H′**) with constitutive heat-shock treatment at hour 3 after pupa formation were stained with anti-GFP (green) and anti-Hts (red) antibodies. G′ and H′ were the amplified images from G and H, as indicated, respectively. A putative GSC with negatively marked GFP (an anterior germ cell in a germarium) is indicated by an arrow. A posterior germ cell with negatively marked GFP is indicated by an arrowhead. Scale bar: 10 μm.

of stem cell loss due to cell death. To further confirm this conclusion, we examined the apoptosis of *Ago1* mutant GSC clones. In more than 200 GSC clones examined, there were no dying GSCs observed by TUNEL assay (see Fig. S2 in the supplementary material). We therefore concluded that *Ago1* is essential for regulating GSC self-renewal in an intrinsic manner and not essential for GSC survival.

We then investigated whether *Ago1* could be involved in the establishment of GSCs. We marked wild-type, mutant *Ago1*^{k08121} and *Ago1*^{EMS} primordial germ cells (PGCs) before the early third instar stage by the FLP-FRT-mediated recombination technique and examined the efficiency of these marked PGCs to differentiate into GSCs after adult eclosion. We observed that, for wild type, 17.9% (40/223) of GSCs were marked; however, only about 4.9% of *Ago1*^{k08121} (10/206) and 1.8% of *Ago1*^{EMS} (3/167) GSC clones were marked under the same experimental conditions. Interestingly, we also found that, when we started to induce *Ago1*^{14} PGC clones as early as the first instar larva stage and gave constitutive heat-shock induction until pupa formation, 23.2% (n=82) of adult germaria were empty, without any germ cells in the case of *Ago1*^{14} PGC clone induction. Under the same conditions, the rate of the marked GSC clones was observed at only 1.2% (n=82), in contrast to 13.7% (n=51) of that for wild-type FRT controls under exactly the same experimental conditions. We also examined the gonads in the early pupa stage. As shown in Fig. 3G,G′,H,H′, we observed that only 2.6% (n=79) of *Ago1*^{14} anterior germ cells (putative GSCs) (Asaoka and Lin, 2004; Zhu and Xie, 2003) were marked (GFP-), in contrast to 24.2% (n=99) of anterior germ cells (putative GSCs) in FRT controls.

Given that the half-life of *Ago1*^{14} mutant GSCs is no less than 5 days (based on our *Ago1* clonal assay), and that very low rate of *Ago1* GSC clones from PGC clones were marked compared with FRT controls, we excluded the possibility that *Ago1* contributed only to GSC maintenance and proposed that *Ago1* could be important for GSC establishment.

To explore whether *Ago1* is involved in controlling the rate of GSC division, we investigated the ability of *Ago1* mutant GSC-producing cysts at day 10 post-heat-shock inductions. For FRT controls, we found 52 germline cyst clones in the presence of 19 GSC clones, whereas for *Ago1*^{14} mutants we observed 30 cyst clones in the presence of 13 GSC clones. As the relative percentages of marked GSCs were 83.3 and 41.6% for FRT controls and *Ago1*^{14} at day 10 after heat-shock induction, respectively (shown in Table 2), this suggests that 83.3% of FRT control cysts and 41.6% of cysts came from the examined GSC clones. So we deduced that each FRT control GSC clone could produce an average of 2.3 cyst clones, whereas each *Ago1*^{14} GSC clone could produce an average of 0.96 cyst clones. Consistent with the previous findings of the clonal assay for *Dcr1* mutant GSCs (Hatfield et al., 2005), it appears that the loss of *Ago1* reduces the rate of GSC division.

The microRNA pathway is not required for bam silencing and probably acts downstream of or parallel to *bam* action

It has been shown previously that BMP/Dpp-dependent *bam* silencing represents the primary pathway for GSC self-renewal (Chen and McKearin, 2003a; Song et al., 2004). To test whether *Ago1* is involved in *bam* silencing in GSCs, we examined BamC expression in both wild-type and marked *Ago1* mutant GSC clones. As shown in Fig. 4A,B, both wild-type (n>100) and *Ago1* GSC clones (n>100) were BamC-negative, suggesting that *Ago1* is not required for *bam* silencing. As *Ago1* is a key component of the miRNA pathway, and given the potential role of miRNAs in stem cell biology, we decided to explore whether other components of the miRNA pathway might modulate GSC fate in a similar manner as well. One good candidate for such a modulator is Loquacious (Loqs), which functions together with Dcr1 and AGO1 to guide miRNA biogenesis (Forstemann et al., 2005; Jiang et al., 2005; Saito

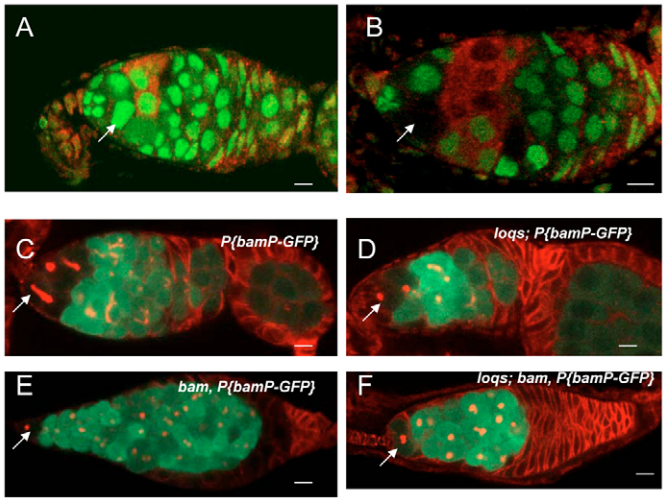

Fig. 4. Two components of the miRNA pathway, AGO1 and Loqs, are not required for bam silencing in *Drosophila*. Ovaries dissected from flies *{hs-flp; frtG13, Ago1^{k08121}/ frtG13, ubi-gfp}* with heat-shock treatment were stained with anti-BamC (red). Two wild-type GSCs that are GFP-positive are negative for BamC (indicated by an arrow) (**A**), and a negatively marked GFP GSC, indicated by an arrow, was also BamC-negative (**B**). Ovaries dissected from *P{bamP-GFP}* (**C**), loqs; *P{bamP-GFP}* (**D**), *bam, P{bamP-GFP}* (**E**), *loqs; bam, P{bamP-GFP}* (**F**) were stained with anti-GFP (green) and anti-Hts (red) antibodies. GSCs with negative GFP expression are indicated by arrows. Scale bar: 10 μm.

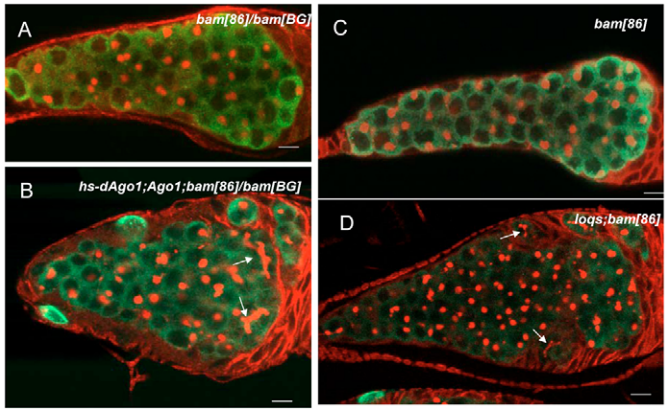

Fig. 5. In *Drosophila*, germ cells could differentiate in both *Ago1, bam* and *loqs, bam* double mutants. Ovaries dissected from *bam^{86}/bam^{BG}* mutants (15 days old) (**A**), *hs-dAgo1; Ago1^{k08121} bam^{86}/bam^{BG}* mutants (15 days old) (**B**), *bam^{86}* mutant (15 days old) (**C**) and *loqs^{f00971}; bam^{86}* double mutants (15 days old) (**D**) were stained with anti-Vasa (green) and anti-Hts (red) antibodies. Branched fusomes in *loqs* and *bam* mutant germaria are indicated by arrows. Scale bar: 10 μm.

et al., 2005). Consistent with two previous studies, we also found that the *loqs^{f00971}* mutant displays defects in GSC maintenance (see Table S1 in the supplementary material) (Forstemann et al., 2005; Jiang et al., 2005). To determine whether *loqs* is required for *bam* silencing, we then examined the expression of *bam* reporters in *loqs* mutant GSCs, as described previously (Chen and McKearin, 2005). As shown in Fig. 4D, 85.5% (n=101) of newly eclosed *loqs*-deficient flies carrying *P{bamP-GFP}* reporters showed a completely negative GFP pattern in putative GSCs (Fig. 4D). As *loqs* mutants cause complete GSC loss in some cases (about 36.3% at day 2 after eclosion), we further examined *P{bamP-GFP}* reporters (Chen and McKearin, 2003b) in *loqs* and *bam* double mutant flies that preserve GSCs in a majority of cases. In contrast to what was observed in *piwi* and *bgcn* double mutants (Chen and McKearin, 2005), our results revealed that, as with GFP patterns in the wild-type and *bam* single mutants (Fig. 4C,E), 87% of GSCs (n=150 germaria) exhibited a completely GFP-negative pattern in *loqs* and *bam* double mutants (Fig. 4F), indicating that the regulation of GSCs mediated by *loqs* does not require *bam*$^{+}$ activity.

To investigate the genetic relationship between *loqs* and *bam*, we began by examining the fusome behavior of germ cells in *bam* single mutants and *loqs*, *bam* double mutants. As described previously, in *bam* single mutants, we stained ovaries with anti-Hts and anti-Vasa antibodies from 7-day-old flies and found that all the germ cells were non-differentiated, GSC-like cells carrying either spherical fusomes or associated spherical fusomes between two germ cells (Fig. 5C). By contrast, the same-age ovaries lacking both *loqs* and *bam* produced a much more complex phenotype. We found that, even though most of the germaria were also tumorous, about 50% (n=100) of the germaria contained some germ cell clusters with highly branched fusomes (Fig. 5D). The appearance of germ cell clusters with branched fusomes suggested that these cells were undergoing differentiation. It appears that germ cells can still differentiate, provided that they lack *loqs*, even in a *bam* mutant background. This finding was different from the recent study showing that none of germ cells can differentiate in *loqs* and *bam* double mutant ovaries (Park et al., 2007). In order to further verify this, we next generated *Ago1* and *bam* double mutant flies by using the transgene *P{hs-Ago1}* with daily heat-shock treatment. Consistent with the analysis of *loqs* and *bam* double mutants in this study, we found that about 80% of tumorous germaria (n=102) (Fig. 5B) from 15-day-old flies with {*hs-dAgo1; Ago1^{k08121}; bam^{86}/bam^{BG}*} (immediately withdrawing the heat-shock treatment after adult eclosion) contained differentiated germ cells with highly branched fusomes (Fig. 5B); by contrast, in ovaries (n>100) from *bam* single mutants at the same age {*bam^{86}/bam^{BG}*}, no differentiated germ cell was observed (Fig. 5A).

Taking these results together, we conclude that *Ago1* and *loqs* are not involved in a *bam*-silencing pathway to regulate GSC fate. Given that *bam*$^{+}$ is dispensable for the loss of GSCs in *loqs* or *Ago1* mutants, we propose that the microRNA pathway probably represses GSC differentiation downstream of or parallel to *bam*.

DISCUSSION

In *Drosophila*, five members of Argonaute proteins have been characterized as constituting two distinct subfamilies (Gunawardane et al., 2007). As members of the PIWI subfamily, Aubergine (Aub) and Piwi play important roles for pole cell formation (Harris and Macdonald, 2001; Megosh et al., 2006). Piwi has been shown to be crucial for the maintenance of GSCs. A recent study showed that AGO3, another member of PIWI subfamily, has a similar function to Piwi and associates with rasiRNAs (Gunawardane et al., 2007). These findings suggest that PIWI subfamily Argonaut proteins play important roles in development. In this study, we analyzed the function of AGO1, a member of the AGO subfamily of Argonaute proteins in GSCs. We showed that overexpression of AGO1 leads to GSC overproliferation, whereas loss of *Ago1* results in the loss of GSCs. Combined with germline clonal analyses of *Ago1*, these findings strongly suggest that AGO1, as a member of the AGO subfamily also plays an essential role in the maintenance of GSCs. Given that an AGO1 serves as an important component in the

miRNA pathway, we propose that the AGO1-dependent miRNA pathway plays at least a partial instructive role in repressing GSC/CB differentiation. Furthermore, in contrast to previous observations of Piwi function in GSCs, we found that *Ago1* is not required for *bam* silencing and probably acts downstream of or parallel to *bam* action in the regulation of GSC maintenance.

Previous work has shown that Dcr1, another key component in the miRNA pathway, is important for controlling the GSC division rate but is dispensable for maintaining GSC self-renewal (Hatfield et al., 2005). Based on the data that Loqs functions selectively in the biogenesis of specific miRNAs (Park et al., 2007), and the recent results showing that *Ago1* and *Ago2* act in a partially redundant manner to control key steps in the midblastula transition and segmental patterning (Meyer et al., 2006), we speculate that Dcr1 may have more functions than either *loqs* or *Ago1* alone (or together). It is possible that *Dcr1*, *loqs* and *Ago1* are all required for GSC maintenance; however, in some cases, even in the absence of Loqs and AGO1, Dcr1 can collaborate with AGO2 to execute some specific miRNA functions. Recent data have shown that the Notch/Delta signal plays an important role in controlling both niche and GSC fates (Ward et al., 2006). Previous data also demonstrated that Notch signaling was negatively regulated by the miRNA pathway (Kwon et al., 2005). Therefore, it is possible that Dcr1 is not only required for GSC maintenance, but also required for some specific miRNA function to promote GSC differentiation. In *Dcr1*-null GSCs, the loss of certain classes of miRNAs causes GSCs to differentiate; however, the loss of different miRNAs might lead to the upregulation of *Delta* activity in GSCs, which in turn upregulates Notch activity in somatic cells. Conversely, as a feedback signal, overexpression of *Notch* in somatic cells represses or delays GSC differentiation; therefore the determination of *Dcr*1-null GSC fate is balanced back to normal. Hence it is likely that the miRNAs play key roles in GSC maintenance.

Importantly in this study, we showed, for the first time, that overexpression of *Ago1* can potentially repress GSC/CB differentiation and result in the over-proliferation of GSC-like cells, suggesting that AGO1-dependent miRNAs play at least a partial instructive role in regulating GSC fate. Given the multiple functions of AGO1 in the miRNA pathway, the increase in GSC-like cells could be interpreted to mean that the overexpression of *Ago1* probably enhances either the efficiency of specific miRNA(s) production and/or the stability of mature miRNAs to repress the transcriptional or translational activity of the target mRNAs required for the differentiation of pre-cystoblasts (pre-CBs)/CBs, thereby resulting in delayed differentiation of GSCs/CBs.

In the previous model, both BMP/Dpp-dependent *bam* transcriptional silencing and the *bam*-independent pathway are required for GSC maintenance (Chen and McKearin, 2005; Maines et al., 2007; Szakmary et al., 2005; Xi et al., 2005). Our genetic evidence suggests that the regulation of GSC self-renewal mediated by the miRNA pathway acts in a *bam*-silencing-independent manner. Given the role of miRNAs in translational regulation, we favor a model in which the translational control of GSC fate determination may be partially via the miRNA pathway, although the possibility remains that some selective miRNAs could directly modulate the stability of specific mRNAs required for GSC/CB differentiation. Similarly, two other groups reported that Dcr-1 and Loqs, both important components of the miRNA pathway, are also required for GSC maintenance (Jin and Xie, 2007; Park et al., 2007). The question becomes how the microRNA pathway regulates the fate of GSC. Previous and current studies showed that *Dcr1*, *loqs* and *Ago1* are all not involved in *bam* transcriptional silencing (Hatfield et al., 2005; Park et al., 2007), suggesting that regulation of GSC fate by microRNAs does not go through a *dpp*-dependent *bam* silencing pathway. A recent study (Park et al., 2007) showed that no germ cells can differentiate in *loqs* and *bam*; however, in our study, we observed that at least 10% of germ cells started to differentiate in *loqs; bam* double mutants (this study), as well as in *loqs; bgcn* double mutant ovaries (data not shown). Consistently, a similar phenotype was observed in the analysis of *Ago1*; *bam* double mutants, suggesting that Loqs and AGO1 probably act independently of Bam action.

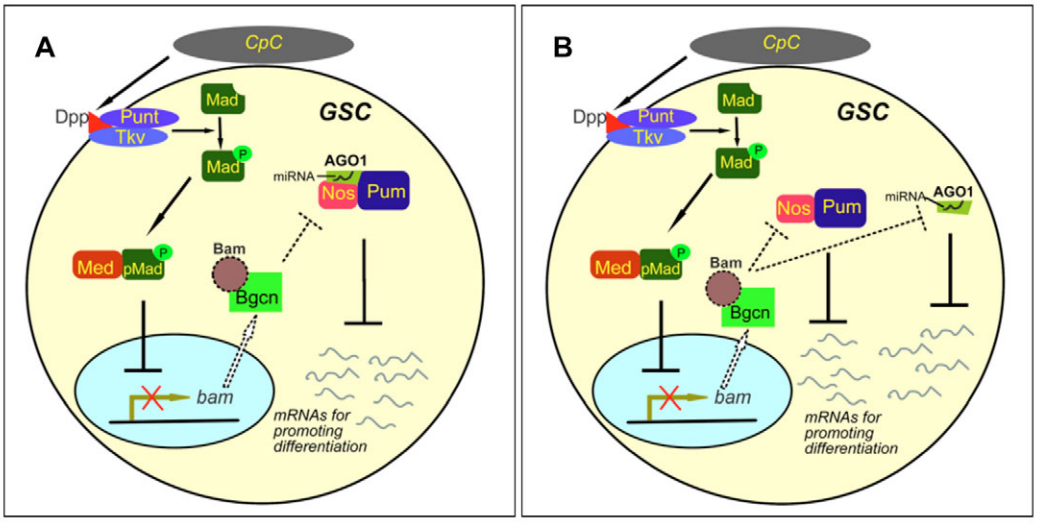

Fig. 6. Model of Ago1-dependent miRNAs in GSC fate determination. In the tip of the *Drosophila* germarium, BMP/Dpp, as short-range signals from niche cells perceived directly by GSCs, represses *bam* transcription and results in Bam/Bgcn complex activity loss, thereby depressing both the Pumilio/Nanos complex (Nos, Pum) and GSC-specific miRNA activities. The Pumilio/Nanos complex and GSC-specific miRNAs could function together to repress the translation of mRNAs for GSC/CB differentiation (**A**), or they might function separately to repress the translation of different groups of mRNA for GSC/CB differentiation (**B**).

Given that the *Ago1*-dependent microRNA pathway plays a major role in translational control, we propose that, aside from the *bam* silencing pathway, the *Ago1* contributes to GSC fate determination either in conjunction (Fig. 6A) or in parallel (Fig. 6B) with the pathway of translational control of Nos/Pum. Overall, our data suggest that miRNA, as an important global regulatory mechanism, plays vital roles in stem cell biology.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/23/4265/DC1

References

Asaoka, M. and Lin, H. (2004). Germline stem cells in the Drosophila ovary descend from pole cells in the anterior region of the embryonic gonad. *Development* **131**, 5079-5089.

Chen, D. and McKearin, D. (2003a). Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. *Curr. Biol.* **13**, 1786-1791.

Chen, D. and McKearin, D. M. (2003b). A discrete transcriptional silencer in the bam gene determines asymmetric division of the Drosophila germline stem cell. *Development* **130**, 1159-1170.

Chen, D. and McKearin, D. (2005). Gene circuitry controlling a stem cell niche. *Curr. Biol.* **15**, 179-184.

Cox, D. N., Chao, A. and Lin, H. (2000). piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* **127**, 503-514.

Forbes, A. and Lehmann, R. (1998). Nanos and Pumilio have critical roles in the development and function of Drosophila germline stem cells. *Development* **125**, 679-690.

Forstemann, K., Tomari, Y., Du, T., Vagin, V. V., Denli, A. M., Bratu, D. P., Klattenhoff, C., Theurkauf, W. E. and Zamore, P. D. (2005). Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. *PLoS Biol.* **3**, e236.

Grivna, S. T., Beyret, E., Wang, Z. and Lin, H. (2006). A novel class of small RNAs in mouse spermatogenic cells. *Genes Dev.* **20**, 1709-1714.

Gunawardane, L. S., Saito, K., Nishida, K. M., Miyoshi, K., Kawamura, Y., Nagami, T., Siomi, H. and Siomi, M. C. (2007). A slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in Drosophila. *Science* **315**, 1587-1590.

Harris, A. N. and Macdonald, P. M. (2001). Aubergine encodes a Drosophila polar granule component required for pole cell formation and related to eIF2C. *Development* **128**, 2823-2832.

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.

Jiang, F., Ye, X., Liu, X., Fincher, L., McKearin, D. and Liu, Q. (2005). Dicer-1 and R3D1-L catalyze microRNA maturation in Drosophila. *Genes Dev.* **19**, 1674-1679.

Jin, P., Zarnescu, D. C., Ceman, S., Nakamoto, M., Mowrey, J., Jongens, T. A., Nelson, D. L., Moses, K. and Warren, S. T. (2004). Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway. *Nat. Neurosci.* **7**, 113-117.

Jin, Z. and Xie, T. (2007). Dcr-1 maintains Drosophila ovarian stem cells. *Curr. Biol.* **17**, 539-544.

Kataoka, Y., Takeichi, M. and Uemura, T. (2001). Developmental roles and molecular characterization of a Drosophila homologue of Arabidopsis Argonaute1, the founder of a novel gene superfamily. *Genes Cells* **6**, 313-325.

Kwon, C., Han, Z., Olson, E. N. and Srivastava, D. (2005). MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling. *Proc. Natl. Acad. Sci. USA* **102**, 18986-18991.

Lavoie, C. A., Ohlstein, B. and McKearin, D. M. (1999). Localization and function of Bam protein require the benign gonial cell neoplasm gene product. *Dev. Biol.* **212**, 405-413.

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.

Lin, H. (2002). The stem-cell niche theory: lessons from flies. *Nat. Rev. Genet.* **3**, 931-940.

Lin, H. and Spradling, A. C. (1997). A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the Drosophila ovary. *Development* **124**, 2463-2476.

Maines, J. Z., Park, J. K., Williams, M. and McKearin, D. M. (2007). Stonewalling Drosophila stem cell differentiation by epigenetic controls. *Development* **134**, 1471-1479.

McKearin, D. and Ohlstein, B. (1995). A role for the Drosophila bag-of-marbles protein in the differentiation of cystoblasts from germline stem cells. *Development* **121**, 2937-2947.

Megosh, H. B., Cox, D. N., Campbell, C. and Lin, H. (2006). The role of PIWI and the miRNA machinery in Drosophila germline determination. *Curr. Biol.* **16**, 1884-1894.

Meyer, W. J., Schreiber, S., Guo, Y., Volkmann, T., Welte, M. A. and Muller, H. A. (2006). Overlapping functions of argonaute proteins in patterning and morphogenesis of Drosophila embryos. *PLoS Genet.* **2**, e134.

Miyoshi, K., Tsukumo, H., Nagami, T., Siomi, H. and Siomi, M. C. (2005). Slicer function of Drosophila Argonautes and its involvement in RISC formation. *Genes Dev.* **19**, 2837-2848.

Ohlstein, B., Lavoie, C. A., Vef, O., Gateff, E. and McKearin, D. M. (2000). The Drosophila cystoblast differentiation factor, benign gonial cell neoplasm, is related to DExH-box proteins and interacts genetically with bag-of-marbles. *Genetics* **155**, 1809-1819.

Okamura, K., Ishizuka, A., Siomi, H. and Siomi, M. C. (2004). Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev.* **18**, 1655-1666.

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.

Parker, J. S. and Barford, D. (2006). Argonaute: a scaffold for the function of short regulatory RNAs. *Trends Biochem. Sci.* **31**, 622-630.

Saito, K., Ishizuka, A., Siomi, H. and Siomi, M. C. (2005). Processing of pre-microRNAs by the Dicer-1-Loquacious complex in Drosophila cells. *PLoS Biol.* **3**, e235.

Saito, K., Nishida, K. M., Mori, T., Kawamura, Y., Miyoshi, K., Nagami, T., Siomi, H. and Siomi, M. C. (2006). Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome. *Genes Dev.* **20**, 2214-2222.

Shi, H., Ullu, E. and Tschudi, C. (2004). Function of the Trypanosome Argonaute 1 protein in RNA interference requires the N-terminal RGG domain and arginine 735 in the Piwi domain. *J. Biol. Chem.* **279**, 49889-49893.

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.

Spradling, A., Drummond-Barbosa, D. and Kai, T. (2001). Stem cells find their niche. *Nature* **414**, 98-104.

Szakmary, A., Cox, D. N., Wang, Z. and Lin, H. (2005). Regulatory relationship among piwi, pumilio, and bag-of-marbles in Drosophila germline stem cell self-renewal and differentiation. *Curr. Biol.* **15**, 171-178.

Vagin, V. V., Sigova, A., Li, C., Seitz, H., Gvozdev, V. and Zamore, P. D. (2006). A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* **313**, 320-324.

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.

Williams, R. W. and Rubin, G. M. (2002). ARGONAUTE1 is required for efficient RNA interference in Drosophila embryos. *Proc. Natl. Acad. Sci. USA* **99**, 6889-6894.

Xi, R., Doan, C., Liu, D. and Xie, T. (2005). Pelota controls self-renewal of germline stem cells by repressing a Bam-independent differentiation pathway. *Development* **132**, 5365-5374.

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.

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

Table S1. Phenotypic assay for *loqs* mutant flies

Genotype	% germaria containing 2 or 3 GSCs	% germaria containing 1 GSC	% germaria containing only cysts	% empty germaria
2-day-old wild-type	100	0	0	0
	n=78	*n*=78	*n*=78	*n*=78
7-day-old wild-type	100	0	0	0
	n=123	*n*=123	*n*=123	*n*=123
2-day-old *loqs*^{f00791}	30	33.6	34.5	1.8
	n=110	*n*=110	*n*=110	*n*=110
7-day-old *loqs*^{f00791}	4.9	33.0	33.0	25.1
	n=103	*n*=103	*n*=103	*n*=103

n, Number of germaria examined.