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MicroRNAs downregulate Bag of marbles to ensure proper terminal differentiation in the *Drosophila* male germline

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SUMMARY

In many adult stem cell lineages, the continuous production of functional differentiated cells depends on the maintenance of progenitor cells in an undifferentiated and proliferative state, as well as the subsequent commitment to proper terminal differentiation. In the *Drosophila* male germline stem cell (GSC) lineage, a key differentiation factor, Bag of marbles (Bam), is required for the transition from proliferative spermatogonia to differentiating spermatocytes. We show that *bam* mRNA, but not Bam, is present in spermatocytes, suggesting that *bam* is regulated post-transcriptionally. Consistent with this, repression of Bam accumulation is achieved by microRNAs via the *bam* 3′UTR. When the *bam* 3′UTR was substituted with the 3′UTR of a constitutively expressed *α-Tubulin*, Bam became stabilized in spermatocytes. Moreover, such a persistent expression of Bam in spermatocytes was recapitulated by specifically mutating the putative *miR-275/miR-306* recognition site at the *bam* 3′UTR. In addition, overexpression of *miR-275* or *miR-306* in spermatogonial cells resulted in a delay of the proliferation-to-differentiation transition and resembled the *bam* loss-of-function phenotype, suggesting that these microRNAs are sufficient to downregulate Bam. Finally, the failure of Bam downregulation in spermatocytes affected spermatid terminal differentiation and resulted in increased male sterility. Our results demonstrate that microRNAs control the stem cell differentiation pathway through regulating Bam, the downregulation of which is crucial for proper spermatid terminal differentiation.

KEY WORDS: Post-transcriptional regulation, microRNA, Bag of marbles, Spermatocyte, Differentiation, *Drosophila*

INTRODUCTION

Adult stem cells sustain tissue homeostasis by consistently providing differentiated cells lost by injury or turnover (Morrison and Spradling, 2008). To retain an adequate stem cell population while generating differentiated cells, many adult stem cells asymmetrically divide into two daughter cells. One daughter cell self-renews as a new stem cell and the other daughter cell differentiates (Knoblich, 2008). In order to generate a sufficient number of differentiated cells in many adult stem cell lineages, the daughter cells from a stem cell asymmetric division undergo a proliferative stage called transit-amplification before activating a gene expression program required for terminal differentiation (Clarke and Fuller, 2006).

The *Drosophila melanogaster* germline stem cell (GSC) lineages have been used to study molecular mechanisms that determine the transition from the transit-amplification stage to the differentiation stage. In *Drosophila* testes, a GSC asymmetrically divides to produce a self-renewed GSC and a gonialblast, which undergoes exactly four rounds of mitosis as transit-amplifying spermatogonial cells. The resulting 16 spermatogonial cells then enter the spermatocyte stage, in which a robust transcription program is activated to express genes for meiotic divisions and terminal differentiation (Fuller, 1993; White-Cooper, 2010). Throughout spermatogenesis, germ cells divide and differentiate in a syncytium. It is only during the late stage of spermiogenesis that the cytoplasm of spermatids is separated by a process called individualization, in order to produce individual and functional haploid sperm (Fuller, 1993; Lindsley and Tokuyasa, 1980).

The transition from the transit-amplifying stage to the terminal differentiation stage should be tightly controlled. Failure of such control may lead to tumor-containing, ever-dividing transit-amplifying cells or to a lack of terminally differentiated cells, which leads to tissue dystrophy or to infertility. In the *Drosophila* male germline lineage, *bag of marbles* (*bam*) is essential for the proper transition from the proliferative to differentiating spermatocyte stages (Gönczy et al., 1997; McKearin and Spradling, 1990). Bam is detected in four- to 16-cell spermatogonia, with levels peaking at eight-cell spermatogonia (Gönczy et al., 1997). It has been shown that accumulation of Bam to a threshold level is required for the transition from spermatogonia to spermatocytes (Insco et al., 2009). However, Bam is not detected in GSCs (Insco et al., 2009; Monk et al., 2010; Schulz et al., 2004). If Bam is ectopically expressed in GSCs, it causes GSC premature differentiation or cell death, leading to GSC loss (Schulz et al., 2004; Sheng et al., 2009). Therefore, expression of Bam needs to be tightly controlled during spermatogenesis.

We report that in the *Drosophila* male GSC lineage, *bam* mRNA is present but Bam is undetectable in spermatocytes. Repression of Bam protein accumulation is achieved by microRNAs via the *bam* 3′UTR. Failure to repress Bam protein accumulation in spermatocytes leads to spermiogenesis defects and male sterility. Our results demonstrate endogenous functions of microRNAs in controlling stem cell differentiation pathway through regulating a key differentiation factor. Although Bam is important for initiating GSC differentiation, our data show that its downregulation is crucial for proper spermatid terminal differentiation.

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MATERIALS AND METHODS

Fly husbandry and fertility test

Flies were raised on standard molasses medium at 25°C. Wild-type flies were *y,w*. The *sa-GFP* (Chen et al., 2005), *nos-Gal4* (Kiger et al., 2001) and *bam-GAL4* (Chen and McKearin, 2003b) flies have been described previously. The $bam^{\Delta 86}$ (BL5427), *Df(3R)FDD-0247627* (Deficiency line for *bam*, BL27401), *UAS-GFP* (BL4775), *UAS-Gal4* (BL5939) and $dcr\text{-}1^{Q1147X}$ (BL32066) flies were obtained from Bloomington Stock Center. The *hs-FLP; Act5c-FRT-stop-FRT-Gal4, UAS-GFP* (Secombe et al., 2007) were obtained from J. Secombe (Albert Einstein College of Medicine, Bronx, NY, USA). The following stocks were generated in this study: *bam-HA bam3′*, *bam-HA tub3′*, *bam-HA* with the second half of *bam* 3′UTR substituted with *α-tubulin84E* 3′UTR, *bam-HA* with the first half of *bam* 3′UTR substituted with *α-tubulin84E* 3′UTR, *bam-HA bam3′* with the putative *miR-275/miR-306*-binding site mutated, *bam-HA bam3′* with the putative *miR-7*-binding site mutated and *bam-HA bam3′* with both putative *miR-317*-binding sites mutated (supplementary material Table S1). All seven constructs described above were incorporated at the attP40 site as transgenes using the ϕC31 integrase (Groth et al., 2004). *UAS-miR-7*, *UAS-miR-275*, *UAS-miR-306*, *UAS-miR-317* (supplementary material Table S2), *UAS-GFP-miR-275SP* and *UAS-GFP-miR-306SP* (supplementary material Tables S3 and S4) flies were generated in this study and the transgenes were introduced into flies by P-element-mediated transformation via embryo injection.

For efficient SP expression, *bam-Gal4, UAS-Gal4*/*UAS-GFP-microRNA-SP*; $dcr\text{-}1^{Q1147X}$ /*UAS-GFP-microRNA-SP* flies were raised at 29°C. For the fertility test, newly eclosed single males were mated with 2 *y,w* virgin females. Sterility was determined if there was no crawling larvae 5-7 days after the crosses. Testes from sterile flies were dissected and the testis morphology was examined using phase-contrast microscopy.

In situ hybridization

In situ hybridization was performed as previously described (Morris et al., 2009). DIG-labeled antisense *bam* probes were synthesized from PCR products amplified using a set of primers (forward 5′-ACCAGCAGTTGGACCACAAT-3′ and reverse 5′-GGATCCTAATACGACTCACTATAGGGAGAATGCGCAGCCTTGATCCAAT-3′). The probes *miR-275* (33020-01) and *miR-306* (33047-01) were purchased from EXIQON. Images were taken with a Zeiss Axioplan microscope.

Overexpression clones

Clones co-expressing *UAS-miR275* or *UAS-miR306* with the GFP marker were generated using flies with the following genotypes: *hs-FLP*; *Act5c-FRT-stop-FRT-Gal4, UAS-GFP/UAS-miR275*, *UAS-miR275/+* or *hs-FLP*; *Act5c-FRT-stop-FRT-Gal4, UAS-GFP/UAS-miR306*; *UAS-miR306/+*. Clones were induced by heatshock treatment of pupae at day 8 and day 9 for 2 hours at 37°C each time. After the second heat shock, flies were placed in 25°C incubator for 3 days, followed by dissection for immunostaining.

Immunostaining and phalloidin staining

Immunostaining was performed as previously described (Insco et al., 2009). Primary antibodies were rat anti-HA (Roche, 3F10) at 1:50, rabbit anti-Vasa (Santa Cruz, sc-30210) at 1:200, chicken anti-GFP (Abcam, ab13970) at 1:1000, mouse anti-Fas3 (DSHB, 7G10) at 1:100, mouse anti-Bam (DSHB) at 1:20 and mouse anti-Lamin C (DSHB, LC28.26) at 1:20. Secondary antibodies were the Alexa Fluor-conjugated series (1:200; Molecular Probes). For phalloidin staining, testes were dissected in PBS and fixed in 1 ml 4% formaldehyde in PBS for 30 minutes at room temperature. After fixation, testes were rinsed twice with 0.1% Triton X-100 in PBS (PBST) for 10 minutes at room temperature and then incubated with Alexa Fluor 568 phalloidin (Invitrogen, A12380) in the dark for 2 hours at room temperature. Testes were rinsed again twice with PBST, for 10 minutes each time, and mounted with Vectashield medium (Vector H-1200). Images were taken using the Zeiss LSM 510 Confocor 3 NLO system and processed using Adobe Photoshop.

Quantitative RT-PCR

Oligonucleotide primers were designed for *bam* (forward 5′-ACTCAGCGCATGGAGAGATTGCTA-3′ and reverse 5′-AGTAGCGGTGCTCCAGATCCATTT-3′) and a housekeeping gene, *RpL32* (forward 5′-CATGCTGCCCACCGGATTCAAGAAG-3′ and reverse 5′-CTCGTTCTCTTGAGAACGCAGGCGA-3′). Testes cDNA preparation and RT-PCR were performed as described previously (Chen et al., 2011). Each PCR reaction was performed in duplicates and Ct numbers were averaged, at least three independent biological replicates were used. The relative expression level was calculated by $2^{Ct(Rpl32)-Ct(bam)}$, where *Rpl32* was used as an internal control.

Counting number of germ cells per spermatocyte cyst

Testes were dissected in PBS and cut at about half way from the testis tip using forceps. Then, the testes tips were transferred onto a microscope slide and covered with a coverslip. PBS on the slide was carefully drained using a Kimwipe until cysts were flattened to single-cell-layer. The number of cells in spermatocyte cyst was counted using phase contrast microscopy. Images were taken on a Zeiss Axioplan microscope using the Openlab software.

Bioinformatics search of microRNA-binding sites

Potential microRNA binding sites at *bam* 3′UTR were identified using mirBase (http://www.mirbase.org/).

RESULTS

Post-transcriptional downregulation of Bam protein in spermatocytes through *bam* 3′UTR

Bam protein is detected in 4-16 spermatogonial cells with a peak expression at the eight-cell spermatogonial stage (Fig. 1A) (Gönczy et al., 1997; Insco et al., 2009). To identify the *bam* mRNA pattern, *in situ* hybridization was performed using antisense *bam* probe in wild-type testes. Two waves of *bam* transcript were detected. The first wave was in the spermatogonial cells (Fig. 1B, single asterisk) and the second wave was in early spermatocytes (Fig. 1B, double asterisk). As controls, such specific signals were not detected using sense *bam* probe in wild-type testes (supplementary material Fig. S1A) or using antisense *bam* probe in transcript null $bam^{\Delta 86}/Df$ testes (Gönczy et al., 1997; McKearin and Ohlstein, 1995) (supplementary material Fig. S1B). The presence of *bam* mRNA, but absence of Bam protein, indicated that accumulation of Bam protein in spermatocytes is repressed post-transcriptionally.

The 3′UTR is a common recognition site of post-transcriptional regulators (Lai, 2002; Stark et al., 2005). To investigate whether *bam* is regulated post-transcriptionally via its 3′UTR, the *bam-HA* genomic construct (including the endogenous *bam* promoter, 5′UTR, coding sequence tagged with HA at the 3′ end and 3′UTR), which rescues the *bam*-null phenotype in both male and female gonads (Li et al., 2009), was used to generate transgenic flies. In addition, the *bam* 3′UTR sequence was replaced with the 3′UTR of a constitutively expressed *α-tubulin84E* gene (Gan et al., 2010). Then, both *bam-HA* with its endogenous 3′UTR (*bam-HA bam3′*) and the one with *a-tubulin84E* 3′UTR (*bam-HA tub3′*) transgenes were integrated into fly genome using the φC31 integrase-mediated site-directed method in order to ensure the expression of both transgenes in the same genomic context (Groth et al., 2004). In order to distinguish spermatogonia and spermatocytes precisely, we used a spermatocyte-specific cellular marker, Sa-GFP (Chen et al., 2005) (Fig. 1C,D). Immunostaining experiments showed the presence of Bam in spermatogonial cells in testes from *bam-HA bam3′* males (Fig. 1C), which is consistent with previous reports (Gönczy et al., 1997; Insco et al., 2009). By contrast, Bam was present in both spermatogonia and spermatocytes in testes from *bam-HA tub3′* males (Fig. 1D). These results demonstrate that the 3′UTR of *bam* is required for the repression of Bam protein accumulation in spermatocytes.

Conversely, Bam needs to be silenced in both male (Fig. 2A) and female (Fig. 2C,C′) GSCs to prevent precocious differentiation (Ohlstein and McKearin, 1997; Schulz et al., 2004; Sheng et al.,

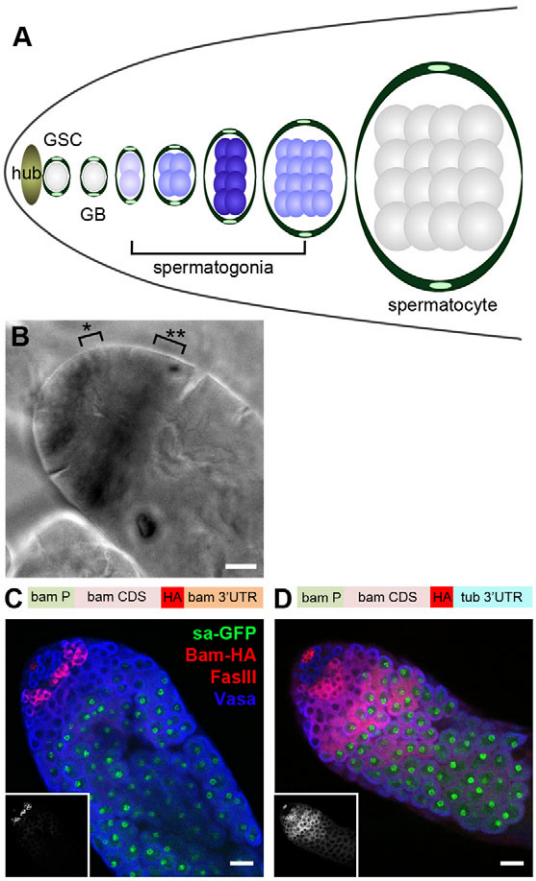

Fig. 1. Downregulation of Bam in spermatocytes requires the *bam* 3′UTR. (**A**) *Drosophila* male germline stem cell lineage. Different shades of blue in germ cells represent Bam expression patterns and levels in wild-type testes (GSC, germline stem cell; GB, gonialblast). (**B**) Two waves of *bam* transcript expression revealed by *in situ* hybridization using *bam* riboprobe: one in spermatogonia (single asterisk); another in early spermatocytes (double asterisk). (**C**,**D**) Top: structure of *bam-HA bam3′* (C) and *bam-HA tub3′* (D) constructs. Bottom: immunofluorescence images of testes from males containing the *sa-GFP* (green) transgene, stained with anti-Fas3 (red, labels hub cells only), anti-HA (red) and anti-Vasa (blue, labels germ cells). Insets show only red channel representing HA and Fas3. Scale bars: 20 μm.

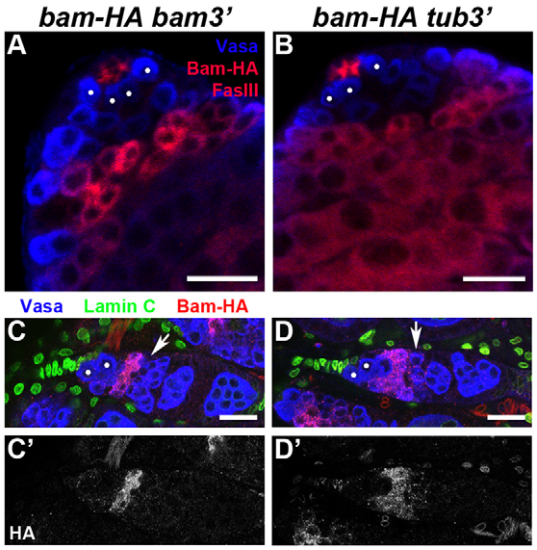

Fig. 2. Substitution of the *bam* 3′UTR with the *α-tubulin84E* 3′UTR does not lead to ectopic Bam expression in GSCs or in ovary. (**A-D′**) Immunofluorescence images of testis apical tip stained with anti-Fas3 (red), anti-HA (red) and anti-Vasa (blue) from *bam-HA bam3′* (A) and *bam-HA tub3′* (B) males. Ovarioles stained using anti-Lamin C, a marker for terminal filament and cap cells (green), anti-HA (red) and anti-Vasa (blue) in *bam-HA bam3′* (C) and *bam-HA* tub3′ (D) germarium. Bam-HA was detected in cystoblasts and early developing cysts, but not from germarium region 2A (arrows) and beyond. (C′,D′) HA staining only. GSCs are labeled with white dots. Scale bars: 20 μm.

2009). However, substitution of the *bam* 3′UTR with the *α-tubulin84E* 3′UTR did not lead to ectopic Bam expression in either male (Fig. 2B) or female (Fig. 2D,D′) GSCs, suggesting that the absence of Bam in GSCs is most likely to be regulated transcriptionally, consistent with previous reports (Chen and McKearin, 2003a; Chen and McKearin, 2003b).

In *Drosophila* ovaries, Bam is detected in cystoblasts, the immediate daughter cells of female GSCs, which will undergo differentiation, as well as in young developing cysts. However, it is not detected from germarium region 2a and beyond (Fig. 2C,C′) (Ohlstein and McKearin, 1997). In contrast to the results in testes (Fig. 1D), persistent *bam* expression was not detected in later stage germ cells in *bam-HA tub3′* ovaries (Fig. 2D,D′). Together, these data reveal that the post-transcriptional regulation of Bam via the *bam* 3′UTR to prevent accumulation of Bam in later-stage germ cells is male specific.

Although post-transcriptional regulation can be achieved by mRNA degradation or by translational repression, these two mechanisms are not mutually exclusive (He and Hannon, 2004; Hesketh, 2004). To study whether the endogenous *bam* 3′UTR is required for downregulating *bam* mRNA, quantitative RT-PCR was performed to compare the *bam* transcript level in testes from *bam-HA bam3′* and *bam-HA tub3′* transgenic males, including both transgenic and endogenous *bam* transcript. We found that levels of *bam* mRNA were 2.4-fold in *bam-HA bam3′* testes and 3.7-fold in *bam-HA tub3′* testes, respectively, compared with wild-type testes (Fig. 3A). The higher level of *bam* transcript in *bam-HA tub3′* testes than in *bam-HA bam3′* testes suggest that the *bam* 3′UTR could be used to destabilize *bam* mRNA.

When Bam is stabilized in spermatogonia, previous studies have demonstrated that the transition from spermatogonia to spermatocyte occurs prematurely, leading to spermatocyte cysts with fewer than 16 cells (Insco et al., 2009). Therefore, we next studied whether the *bam* 3′UTR-dependent post-transcriptional regulation of Bam affects the spermatogonia-to-spermatocyte transition. We counted the number of spermatocytes in intact cysts from either *bam-HA bam3′* or *bam-HA tub3′* testes, using both phase-contrast microscopy (Fig. 3B,C) and the spermatocyte-specific Sa-GFP marker (Fig. 3B′,C′). We found that the percentage of testes containing at least one eight-cell spermatocyte cyst had no significant difference between these two strains (Fig. 3D, P=0.93), indicating that both transgenes behaved similarly in regulating the spermatogonia-to-spermatocyte transition.

Post-transcriptional downregulation of Bam in spermatocytes depends on putative microRNA-binding sites at *bam* 3′UTR

MicroRNAs commonly act through 3′UTRs of target transcripts to destabilize mRNAs or repress translational initiation (He and

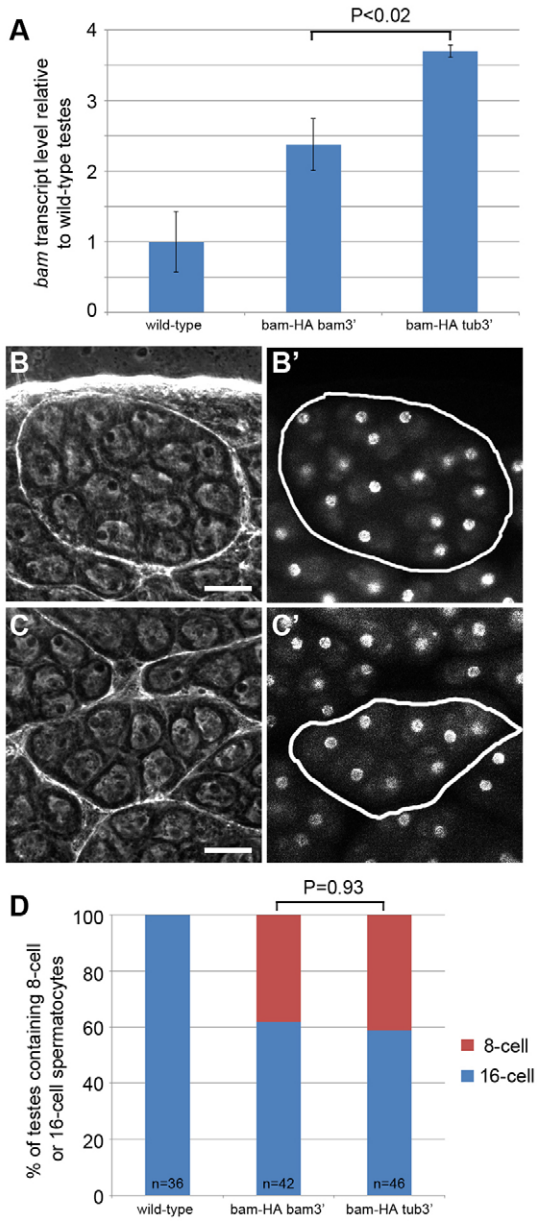

Fig. 3. The endogenous *bam* 3′UTR may destabilize mRNA, but replacement of the *bam* 3′UTR does not affect spermatogonia-to-spermatocyte transition. (**A**) Quantitative RT-PCR of *bam* mRNA in testes from wild-type, *bam-HA bam3′* and *bam-HA tub3′* transgenic males. The *bam* transcript level in wild-type testes was set to 1. Error bars indicate s.d. based on three independent biological replicates. *P* values were calculated using Student's *t*-test. (**B**) Phase-contrast image of a 16-cell spermatocyte cyst (**B′**), all spermatocytes have distinct nucleolar Sa-GFP. (**C**) Phase-contrast image of an eight-cell spermatocyte cyst (**C′**), all have nucleolar Sa-GFP. Scale bars: 20 μm. (**D**) Percentage of testes containing at least one eight-cell spermatocyte cyst (red) versus testes containing only 16-cell spermatocyte cysts (blue). The *P* value was calculated using the χ^2 test.

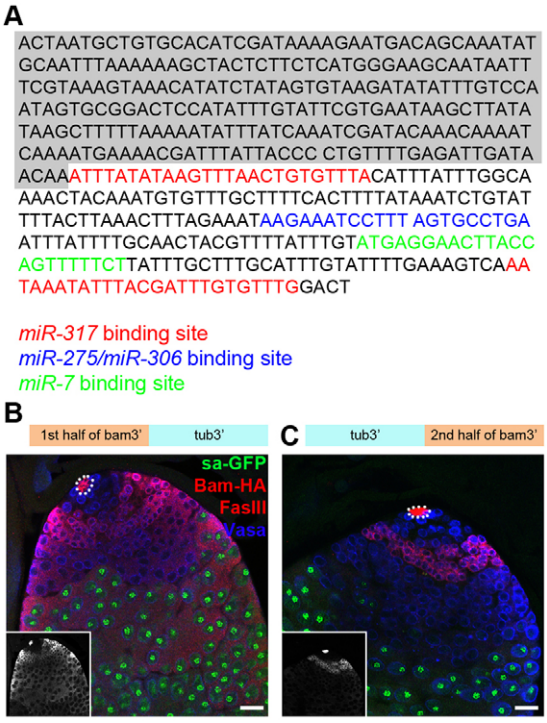

Fig. 4. The second half of the *bam* 3′UTR containing putative microRNA-binding sites is both necessary and sufficient for endogenous *bam* expression. (**A**) Sequence of the *bam* 3′UTR. The first half of *bam* 3′UTR does not contain any putative microRNA-binding site (gray). The second half contains four putative microRNA-binding sites, labeled with different colors. (**B**,**C**) Top: structure of the 3′UTR of the transgene. Bottom: immunofluorescence images of testis containing the *sa-GFP* (green) transgene stained using anti-Fas3 (red), anti-HA (red) and anti-Vasa (blue). The hub area is outlined. Insets show only red channel representing HA and Fas3. Scale bars: 20 μm.

Hannon, 2004). Bioinformatics analysis was performed to screen for putative microRNA recognition sites at the *bam* 3′UTR sequences (Fig. 4A). Four putative microRNA-binding sites were identified: miR-7, miR-275, miR-306 and miR-317, including one site shared by miR-275 and miR-306, and two sites recognized by miR-317 (Fig. 4A). Furthermore, all four sites were located at the second half of the *bam* 3′UTR (sequences not highlighted in Fig. 4A). By contrast, no microRNA-binding site was found at the *α-tubulin84E* 3′UTR.

To investigate whether the second half of the *bam* 3′UTR containing all putative microRNA recognition sites is required for downregulation of Bam protein in spermatocytes, *α-tubulin84E* 3′UTR was used to specifically replace the second half of *bam* 3′UTR so that the entire 3′UTR length would be similar to the endogenous *bam* 3′UTR (Fig. 4B). With this substitution, Bam expression was detectable in spermatocytes (Fig. 4B), indicating that the second half of *bam* 3′UTR was necessary for downregulation of Bam in spermatocytes. By contrast, when only the first half of the *bam* 3′UTR was replaced by *α-tubulin84E* 3′UTR, the *bam* expression pattern was unchanged (Fig. 4C), suggesting that the second half of *bam* 3′UTR was sufficient to downregulate Bam in spermatocytes. In summary, these results suggest that the downregulation of Bam in spermatocytes most probably occurs through microRNAs.

miR-275 and miR306 act through their common binding site at the *bam* 3′UTR to repress Bam accumulation

In animals, microRNAs usually bind to their target mRNAs through seed sequences, which are 2-7 nucleotide mRNA sequences complementary to the 5′ sequences of microRNAs (He

and Hannon, 2004; Hesketh, 2004). Mutation of seed sequences can abolish the binding between microRNAs and their target mRNAs (Bartel, 2009). To pinpoint which putative microRNA-binding site is necessary for post-transcriptional regulation of Bam, seed sequences of each putative microRNA-binding site (Fig. 4A) were replaced with a 6 bp restriction enzyme site. We found that mutation of the seed sequence shared by miR-275 and miR-306 led to abundant Bam accumulation in spermatocytes (Fig. 5A). By contrast, mutations of seed sequences of either the miR-7- or the miR-317-binding site did not change Bam endogenous pattern (Fig. 5B,C). These results suggest that miR-275 and/or miR-306 are the potential *trans*-regulatory factors that downregulate Bam in spermatocytes.

Next, in order to examine whether miR-275 and/or miR-306 represses Bam accumulation, we used a sensitive assay for Bam accumulation. Each of the microRNAs was overexpressed using an early germ cell-specific *nos-Gal4* driver (Van Doren et al., 1998). Thus, if overexpression of microRNAs is sufficient to block Bam protein accumulation, a delayed transition from spermatogonia to spermatocytes would be observed using a single spermatocyte cyst-counting assay. Indeed, we found that overexpression of either *miR-275* or *miR-306* resulted in testes containing 32-cell spermatocyte cysts (Fig. 5D,E), suggesting an extra spermatogonial division before switching to spermatocytes (Insco et al., 2009). By contrast, neither *miR-7* nor *miR-317*, when overexpressed using the same *nos-Gal4* driver, led to testes containing 32-cell spermatocyte cysts (Fig. 5E). Moreover, addition of one copy of *bam-HA* transgene fully rescued the 32-cell spermatocyte cysts phenotype in *miR-275*- or *miR-306*-overexpressing testes (Fig. 5E), confirming that this phenotype was caused by reduced Bam levels. In addition, we found that Bam was downregulated in *miR-275*- or *miR-306*-overexpressing spermatogonial cells compared with the neighboring wild-type cells (supplementary material Fig. S2). In summary, these results demonstrate that overexpression of *miR-275* or *miR-306*, but not *miR-7* or *miR-317*, is sufficient to repress Bam protein accumulation.

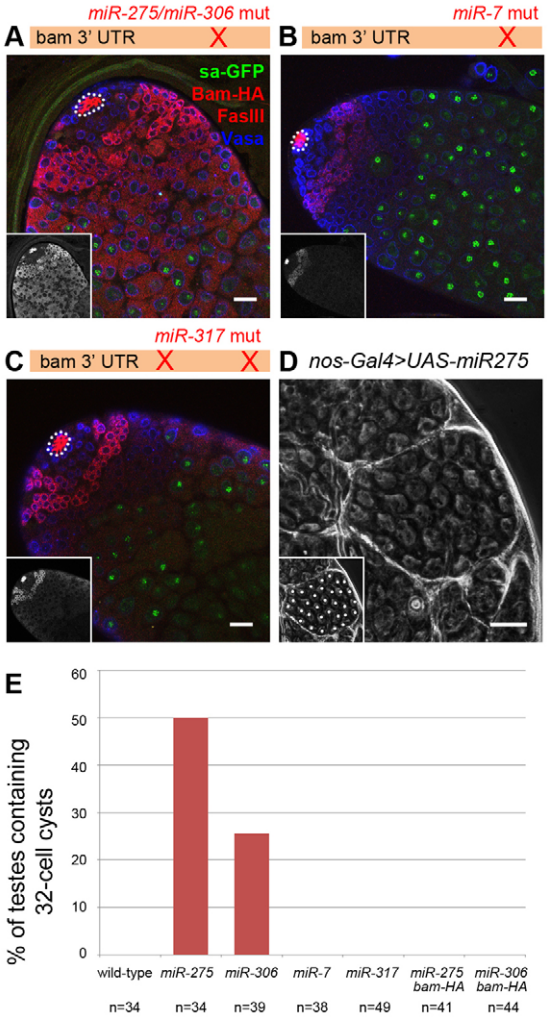

Fig. 5. Mutation of the *miR-275/miR-306*-binding site at the *bam* 3′UTR or overexpression of *miR-275* or *miR-306* affect Bam accumulation. (**A-C**) Immunofluorescence images of testis containing the *sa-GFP* (green) transgene, stained with anti-Fas3 (red), anti-HA (red) and anti-Vasa (blue). The hub area is outlined. Insets show only HA (left) and only Fas3 (right) staining (**D**) Phase-contrast image of a 32-cell spermatocyte cyst. (Insets) Each cell was labeled with a white dot. Scale bars: 20 µm. (**E**) Percentage of testes containing at least one 32-cell cyst when *UAS-microRNAs* were driven by *nos-Gal4*. Addition of a *bam-HA* transgene in testes overexpressing either *miR-275* or *miR-306* completely rescued the 32-cell spermatocyte cyst phenotype.

Consistent with the function of miR-275 in downregulating Bam protein in spermatocytes, *in situ* hybridization using miR-275 probe showed enriched miR-275 expression in early spermatocytes (supplementary material Fig. S3A). Although miR-306 expression pattern shown by *in situ* was broader, it was also detectable in spermatocytes (supplementary material Fig. S3B). These data suggest that both miR-275 and miR-306 could cooperate in downregulating Bam protein in spermatocytes.

It has been shown that a tandem-repeat sequence of a microRNA binding site called sponge (SP) sequence can downregulate microRNAs (Ebert et al., 2007; Loya et al., 2009). In order to investigate whether knockdown of miR-275 and/or miR-306 in spermatocytes stabilizes Bam, we expressed *UAS-GFP-miR275SP* or *UAS-GFP-miR306SP* using the *bam-Gal4*; *UAS-Gal4* drivers to achieve a high expression in 4-16 spermatogonia and all spermatocytes (supplementary material Fig. S4A). To enhance SP knockdown efficiency, we removed one copy of the *dicer-1* (*dcr-1*) gene using a null allele $dcr\text{-}1^{Q1147X}$ to further suppress microRNA biogenesis (Lee et al., 2004). However, neither the expression of *miR-275SP* or *miR-306SP* alone (supplementary material Fig. S4B,C) nor the co-expression of *miR-275SP* and *miR-306SP* (supplementary material Fig. S4D) stabilized Bam in spermatocytes (supplementary material Fig. S4B,C). It is possible that expression of these SP sequences was insufficient to knock down robust expression of microRNAs in spermatocytes. Alternatively, it is possible that the Gal4; UAS system does not work effectively in spermatocytes and the sustained GFP reporter in spermatocytes (supplementary material Fig. S4A) is due to perdurance of the GFP protein. Last, it is possible that some RNA-binding proteins act through the same 3′UTR sequences to regulate Bam accumulation, a mechanism that we did not examine in this study.

Accumulation of Bam in spermatocytes leads to spermiogenesis defects and increased male sterility

The failure to downregulate Bam in spermatocytes could have biological consequences. To understand the scope of such consequences, we performed a fertility assay of *bam-HA bam3′* and *bam-HA tub3′* transgenic males. Interestingly, we found a significant increase ($P<0.05$) of male sterility in *bam-HA tub3′* transgenic males, in which Bam persisted in spermatocytes.

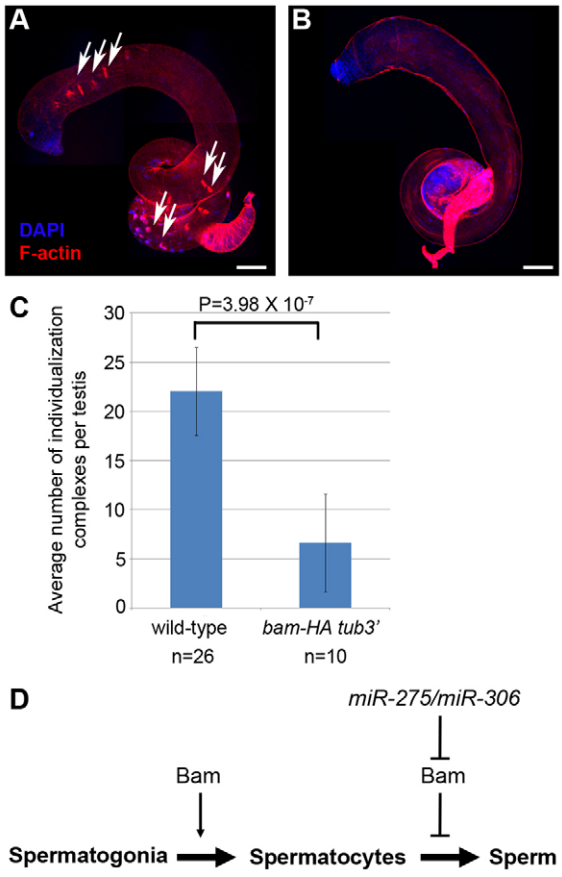

Fig. 6. Prolonged Bam in spermatocytes leads to increased male sterility. (**A**,**B**) Testes stained with DAPI (blue) and phalloidin (red) of wild-type testes (A) and sterile *bam-HA tub3′* transgenic testis (B); individualization complexes are indicated (arrows). Scale bars: 100 μm. (**C**) The number of individualization complexes per testis in wild-type and *bam-HA tub3′* males. Error bars indicate s.d. *P* values were calculated using Student's *t*-test. (**D**) The distinct roles of Bam during spermatogenesis.

Approximately 13% of *bam-HA tub3′* transgenic males were sterile (*n*=149), whereas only 3% of *bam-HA bam3′* transgenic males were sterile (*n*=99). To further investigate the defects leading to sterility of *bam-HA tub3′* transgenic males, we dissected seminal vesicles, where mature sperm are stored, from sterile males and examined sperm motility using phase-contrast microscopy. Although all seminal vesicles from wild-type males contained motile sperm (*n*=30), 90% of the seminal vesicles (*n*=20) from *bam-HA tub3′* sterile males had no motile sperm.

During *Drosophila* spermiogenesis, interconnected spermatids descending from germline syncytia undergo a process called individualization (Fuller, 1993; Lindsley and Tokuyasa, 1980). During this process, individualization complexes containing 64 actin cones move along the sperm axoneme to generate individual sperm (Fabrizio et al., 1998; Noguchi and Miller, 2003). Using phalloidin staining, we quantified these actin cone bundles or individualization complexes. On average, 22.0 individualization complexes per testis were detected in wild-type testes (Fig. 6A,C), whereas only 6.6 individualization complexes per testis were detected in testes from *bam-HA tub3′* sterile males (Fig. 6B,C). These results demonstrate that failure in downregulation of Bam in spermatocytes leads to spermiogenesis defects and increased male sterility.

DISCUSSION

Distinct roles of Bam in regulating cellular differentiation of male germ cells

In the male germline, it has been shown that ectopic expression of Bam in male GSCs induces their loss, probably by precocious differentiation (Schulz et al., 2004; Sheng et al., 2009). Our data showed that Bam expression must be repressed in spermatocytes for proper terminal differentiation during spermiogenesis (Fig. 6D). Therefore, although Bam functions as a differentiation gene for GSCs, it must be downregulated in spermatocytes for proper terminal differentiation. These results suggest that Bam plays distinct roles at different stages of spermatogenesis and that the microRNA-mediated post-transcriptional regulation of Bam is specific for differentiating spermatocytes, but not for GSCs. Previous studies have focused on the molecular mechanisms that prevent ectopic Bam expression in GSCs. Our data reveal a novel regulatory mechanism that prevents Bam accumulation in differentiating spermatocytes, which is crucial for their proper terminal differentiation into functional gametes. Therefore, our discoveries highlight the specificity of developmentally programmed cellular differentiation pathways.

In the male GSC lineage, Bam is required for the transition from proliferative stage to differentiation stage. Previously, it was shown that accumulation of Bam to the threshold level is important to induce the transition from spermatogonia to spermatocytes (Insco et al., 2009). Indeed, either *bamHA bam3′* or *bamHA tub3′* transgene, on top of the endogenous Bam, induced early transition from spermatogonia to spermatocytes, which resulted in increased testes containing at least one eight-cell spermatocyte cyst. However, in the previous study (Insco et al., 2009), a transgene that is almost identical to the *bam HA bam3′* we used in this report did not lead to premature spermatogonia-to-spermatocyte transition. This discrepancy could have resulted from the different expression level of transgenes. In our assay, site-directed incorporation was used for all transgenes. The insertion site attP40 has been optimized for high expression (Markstein et al., 2008), whereas in the previous assay, P-element-mediated random incorporation was used, which may not lead to high expression of transgenes.

However, the observation that bam mRNA is present while Bam is absent in early spermatocytes provokes another possibility: bam mRNA may have novel functions in early spermatocytes that are independent of Bam. Such functions remain unexplored in bam mutant testes because germ cells are arrested at the spermatogonial stage and cannot differentiate into early spermatocytes. Further studies will be carried out to knock down bam mRNA specifically in early spermatocytes and address this possibility. Alternatively, the previous study (Insco et al., 2009) directly quantified the percentage of eight-cell spermatocyte cysts, whereas we quantified the percentage of testes containing at least one eight-cell spermatocyte cyst (see Materials and methods). It is possible that our method produces a higher ratio compared with the previous study.

Distinct functions of Bam in different stem cell lineages

Bam is an essential differentiation factor in multiple stem cell lineages, including male and female GSC lineages (Gönczy et al., 1997; McKearin and Spradling, 1990). In both male and female GSCs, TGFβ signaling activated by ligands emanated from somatic cells in the niche represses *bam* transcription (Kawase et al., 2004; Schulz et al., 2004; Shivdasani and Ingham, 2003). In both male and female gonads, ectopic

expression of Bam in GSCs induces differentiation and results in loss of GSCs (Ohlstein and McKearin, 1997; Sheng et al., 2009). Despite a high degree of similarity between male and female GSC lineages and Bam expression pattern, our data demonstrate that the microRNA-controlled post-transcriptional regulation of Bam is specific for male, but not for female, gametogenesis. Therefore, our data provide a new line of evidence showing distinct molecular mechanisms employed in male versus female germline stem cell differentiation pathways.

However, in the lymph gland, Bam maintains hematopoietic progenitors at an undifferentiated state and *bam* mutation results in a reduction of hematopoietic progenitors, but an increase in differentiated hemocytes (Tokusumi et al., 2011). Therefore, in different tissues, Bam acts differently to either block or induce differentiation.

Post-transcriptional regulation of Bam by microRNAs

Previously, it has been suggested that miR-7 binds to *bam* 3′UTR and that overexpression of *miR-7* in early germ cells increases the number of spermatogonial cysts (Pek et al., 2009). However, overexpression of *miR-7* did not affect Bam expression in our analysis. We speculate two possible explanations for this discrepancy. First, in our study, miR-7 might not be expressed at a high enough level to change Bam expression. Second, we used a different reporter in our assay. We used the *bam-HA* transgene as the reporter, which has endogenous promoter, *bam*-coding sequence and *bam* 3′UTR in germ cells, whereas the previous study used the reporter with *tubulin* promoter, *GFP* coding sequence and *bam* 3′UTR in somatic cells. In addition, as readout for decreased Bam protein levels, we used the percentage of testes containing at least one 32-spermatocyte cyst, which reflects Bam protein levels precisely (Insco et al., 2009); the previous study used nuclear morphology (Pek et al., 2009). In hematopoietic progenitors, Bam and miR-7 function cooperatively, but *bam* is not a target of miR-7 (Tokusumi et al., 2011).

Tight regulation of adult stem cell differentiation in various lineages is vital because disruption of such regulation can lead to tumorigenesis (Clarke and Fuller, 2006). MicroRNAs have been reported to play an essential role in regulating stem cell self-renewal and differentiation (Gangaraju and Lin, 2009). It has also been shown that microRNA expression is misregulated in many cancers (Lu et al., 2005; Volinia et al., 2006). Although it has been proposed that microRNAs contribute to stem cell maintenance and proper differentiation, most of the evidence in support of this has been based on the phenotypes caused by mutating the enzymes responsible for the biogenesis or stabilization of microRNAs. For example, mutations of the microRNA biogenesis components *dcr-1*, *loquacious*, *argonaut 1* and *mei-P26* lead to female GSC loss (Förstemann et al., 2005; Jin and Xie, 2007; Li et al., 2012; Park et al., 2007; Yang et al., 2007). It is much less understood how individual microRNAs regulate their endogenous target genes during stem cell differentiation programs *in vivo*. Our data provide a key insight into how microRNAs regulate *Drosophila* male germline stem cell lineage. Delay of the proliferation-to-differentiation transition upon overexpression of *miR-275* or *miR-306* in testes indicates that microRNA expression also needs to be tightly controlled. Interestingly, either miR-275 or miR-306 can affect the Bam expression levels. Therefore, a single microRNA can have multiple targets; in addition, a single gene can be regulated by multiple microRNAs. Tight regulation of gene expression can be partially achieved by spatial- and temporal-specific expression of microRNAs.

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The authors declare no competing financial interests.

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References

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215-233.
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, X., Hiller, M., Sancak, Y. and Fuller, M. T. (2005). Tissue-specific TAFs counteract Polycomb to turn on terminal differentiation. *Science* **310**, 869-872.
Chen, X., Lu, C., Prado, J. R., Eun, S. H. and Fuller, M. T. (2011). Sequential changes at differentiation gene promoters as they become active in a stem cell lineage. *Development* **138**, 2441-2450.
Clarke, M. F. and Fuller, M. (2006). Stem cells and cancer: two faces of eve. *Cell* **124**, 1111-1115.
Ebert, M. S., Neilson, J. R. and Sharp, P. A. (2007). MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat. Methods* **4**, 721-726.
Fabrizio, J. J., Hime, G., Lemmon, S. K. and Bazinet, C. (1998). Genetic dissection of sperm individualization in Drosophila melanogaster. *Development* **125**, 1833-1843.
Förstemann, 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.
Fuller, M. T. (1993). Spermatogenesis. In *The Development of Drosophila melanogaster*. Vol. I (ed. M. Bate and A. Martinez-Arias), pp. 71-147. Cold Spring Harbor, New York: Cold Spring Harbor Press.
Gan, Q., Chepelev, I., Wei, G., Tarayrah, L., Cui, K., Zhao, K. and Chen, X. (2010). Dynamic regulation of alternative splicing and chromatin structure in Drosophila gonads revealed by RNA-seq. *Cell Res.* **20**, 763-783.
Gangaraju, V. K. and Lin, H. (2009). MicroRNAs: key regulators of stem cells. *Nat. Rev. Mol. Cell Biol.* **10**, 116-125.
Gönczy, P., Matunis, E. and DiNardo, S. (1997). bag-of-marbles and benign gonial cell neoplasm act in the germline to restrict proliferation during Drosophila spermatogenesis. *Development* **124**, 4361-4371.
Groth, A. C., Fish, M., Nusse, R. and Calos, M. P. (2004). Construction of transgenic Drosophila by using the site-specific integrase from phage phiC31. *Genetics* **166**, 1775-1782.
He, L. and Hannon, G. J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. *Nat. Rev. Genet.* **5**, 522-531.
Hesketh, J. (2004). 3′-Untranslated regions are important in mRNA localization and translation: lessons from selenium and metallothionein. *Biochem. Soc. Trans.* **32**, 990-993.
Insco, M. L., Leon, A., Tam, C. H., McKearin, D. M. and Fuller, M. T. (2009). Accumulation of a differentiation regulator specifies transit amplifying division number in an adult stem cell lineage. *Proc. Natl. Acad. Sci. USA* **106**, 22311-22316.
Jin, Z. and Xie, T. (2007). Dcr-1 maintains Drosophila ovarian stem cells. *Curr. Biol.* **17**, 539-544.
Kawase, E., Wong, M. D., Ding, B. C. and Xie, T. (2004). Gbb/Bmp signaling is essential for maintaining germline stem cells and for repressing bam transcription in the Drosophila testis. *Development* **131**, 1365-1375.

Kiger, A. A., Jones, D. L., Schulz, C., Rogers, M. B. and Fuller, M. T. (2001). Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. *Science* **294**, 2542-2545.
Knoblich, J. A. (2008). Mechanisms of asymmetric stem cell division. *Cell* **132**, 583-597.
Lai, E. C. (2002). Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat. Genet.* **30**, 363-364.
Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J. and Carthew, R. W. (2004). Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* **117**, 69-81.
Li, Y., Minor, N. T., Park, J. K., McKearin, D. M. and Maines, J. Z. (2009). Bam and Bgcn antagonize Nanos-dependent germ-line stem cell maintenance. *Proc. Natl. Acad. Sci. USA* **106**, 9304-9309.
Li, Y., Maines, J. Z., Tastan, O. Y., McKearin, D. M. and Buszczak, M. (2012). Mei-P26 regulates the maintenance of ovarian germline stem cells by promoting BMP signaling. *Development* **139**, 1547-1556.
Lindsley, D. L. and Tokyuasu, K. T. (1980). Spermatogenesis. In *Genetics and Biology of Drosophila* (ed. M. Ashburner and. T. R. Wright), pp. 225-294. New York, NY: Academic Press.
Loya, C. M., Lu, C. S., Van Vactor, D. and Fulga, T. A. (2009). Transgenic microRNA inhibition with spatiotemporal specificity in intact organisms. *Nat. Methods* **6**, 897-903.
Lu, J., Getz, G., Miska, E. A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B. L., Mak, R. H., Ferrando, A. A. et al. (2005). MicroRNA expression profiles classify human cancers. *Nature* **435**, 834-838.
Markstein, M., Pitsouli, C., Villalta, C., Celniker, S. E. and Perrimon, N. (2008). Exploiting position effects and the gypsy retrovirus insulator to engineer precisely expressed transgenes. *Nat. Genet.* **40**, 476-483.
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.
McKearin, D. M. and Spradling, A. C. (1990). bag-of-marbles: a Drosophila gene required to initiate both male and female gametogenesis. *Genes Dev.* **4**, 2242-2251.
Monk, A. C., Siddall, N. A., Volk, T., Fraser, B., Quinn, L. M., McLaughlin, E. A. and Hime, G. R. (2010). HOW is required for stem cell maintenance in the Drosophila testis and for the onset of transit-amplifying divisions. *Cell Stem Cell* **6**, 348-360.
Morris, C. A., Benson, E. and White-Cooper, H. (2009). Determination of gene expression patterns using in situ hybridization to Drosophila testes. *Nat. Protoc.* **4**, 1807-1819.
Morrison, S. J. and Spradling, A. C. (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell* **132**, 598-611.
Noguchi, T. and Miller, K. G. (2003). A role for actin dynamics in individualization during spermatogenesis in Drosophila melanogaster. *Development* **130**, 1805-1816.
Ohlstein, B. and McKearin, D. (1997). Ectopic expression of the Drosophila Bam protein eliminates oogenic germline stem cells. *Development* **124**, 3651-3662.
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.
Pek, J. W., Lim, A. K. and Kai, T. (2009). Drosophila maelstrom ensures proper germline stem cell lineage differentiation by repressing microRNA-7. *Dev. Cell* **17**, 417-424.
Schulz, C., Kiger, A. A., Tazuke, S. I., Yamashita, Y. M., Pantalena-Filho, L. C., Jones, D. L., Wood, C. G. and Fuller, M. T. (2004). A misexpression screen reveals effects of bag-of-marbles and TGF beta class signaling on the Drosophila male germ-line stem cell lineage. *Genetics* **167**, 707-723.
Secombe, J., Li, L., Carlos, L. and Eisenman, R. N. (2007). The Trithorax group protein Lid is a trimethyl histone H3K4 demethylase required for dMyc-induced cell growth. *Genes Dev.* **21**, 537-551.
Sheng, X. R., Brawley, C. M. and Matunis, E. L. (2009). Dedifferentiating spermatogonia outcompete somatic stem cells for niche occupancy in the Drosophila testis. *Cell Stem Cell* **5**, 191-203.
Shivdasani, A. A. and Ingham, P. W. (2003). Regulation of stem cell maintenance and transit amplifying cell proliferation by tgf-beta signaling in Drosophila spermatogenesis. *Curr. Biol.* **13**, 2065-2072.
Stark, A., Brennecke, J., Bushati, N., Russell, R. B. and Cohen, S. M. (2005). Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3′UTR evolution. *Cell* **123**, 1133-1146.
Tokusumi, T., Tokusumi, Y., Hopkins, D. W., Shoue, D. A., Corona, L. and Schulz, R. A. (2011). Germ line differentiation factor Bag of Marbles is a regulator of hematopoietic progenitor maintenance during Drosophila hematopoiesis. *Development* **138**, 3879-3884.
Van Doren, M., Williamson, A. L. and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. *Curr. Biol.* **8**, 243-246.
Volinia, S., Calin, G. A., Liu, C. G., Ambs, S., Cimmino, A., Petrocca, F., Visone, R., Iorio, M., Roldo, C., Ferracin, M. et al. (2006). A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc. Natl. Acad. Sci. USA* **103**, 2257-2261.
White-Cooper, H. (2010). Molecular mechanisms of gene regulation during Drosophila spermatogenesis. *Reproduction* **139**, 11-21.
Yang, L., Chen, D., Duan, R., Xia, L., Wang, J., Qurashi, A., Jin, P. and Chen, D. (2007). Argonaute 1 regulates the fate of germline stem cells in Drosophila. *Development* **134**, 4265-4272.