Bam-dependent deubiquitinase complex can disrupt germ-line stem cell maintenance by targeting cyclin A

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Edited by Allan C. Spradling, Carnegie Institution of Science, Baltimore, MD, and approved April 20, 2017 (received for review November 21, 2016)

Drosophila **germ-line stem cells (GSCs) provide an excellent model to study the regulatory mechanisms of stem cells in vivo. *Bag of marbles* (*bam*) has been demonstrated to be necessary and sufficient to promote GSC and cystoblast differentiation. Despite extensive investigation of its regulation and genetic functions, the biochemical nature of the Bam protein has been unknown. Here, we report that Bam is an ubiquitin-associated protein and controls the turnover of cyclin A (CycA). Mechanistically, we found that Bam associated with Otu to form a deubiquitinase complex that stabilized CycA by deubiquitination, thus providing a mechanism to explain how ectopic expression of Bam in GSCs promotes differentiation. Collectively, our findings not only identify a biochemical function of Bam, which contributes to GSC fate determination, but also emphasizes the critical role of proper expression of cyclin proteins mediated by both ubiquitination and deubiquitination pathways in balancing stem cell self-renewal and differentiation.**

germ-line stem cells | bag of marbles | ubiquitin-binding protein | cyclin A deubiquitination

The *Drosophila* ovary provides an excellent model to study the regulatory mechanisms of how the fate of stem cell self-renewal and differentiation is determined and balanced (1, 2). In adult females, asymmetric division of germ-line stem cells (GSCs) occurs in the anterior region of the germarium to produce two daughter cells. Whereas one daughter cell remains attached to the somatic cap cell for GSC self-renewal, the other becomes a cystoblast (CB). The CB continues to divide four times with incomplete cytokinesis at each division to produce a 16-cell cyst that sustains *Drosophila* oogenesis (Fig. 1*A*) (1).

Early genetic studies have demonstrated that the *bag of marbles* (*bam*) gene is necessary and sufficient for GSC and CB differentiation, because mutation of *bam* blocks germ-cell differentiation causing GSC hyperplasty, whereas ectopic expression of *bam* in GSCs results in their precocious differentiation (3–5). Importantly, previous studies have identified *bam* as a key gene that responds to niche bone morphogenetic protein (BMP) signaling via the interaction of Smad proteins with a discrete DNA-silencing element in the *bam* 5′ untranslated region (UTR) (6–8). Thus, transcriptional silencing of *bam* that directly establishes a link between GSCs and their associated stromal cells (stem cell niche) is essential for GSC fate determination. Blockage of the *bam* silencing pathway leads to ectopic expression of Bam and loss of GSCs (7, 9–11). However, the molecular mechanism underlying the action of ectopic Bam in GSCs has remained unexplored. In addition to transcriptional control through the niche–stem cell interaction, genetic studies have suggested that the maintenance of GSCs is cell-autonomously regulated by several translational repressor complexes such as Nos–Pum and Ago1/Dcr/Loq–microRNA (miRNA) complexes (12–16). It has been suggested that Bam functions in concert with Bgcn, a DExH box-containing protein, to antagonize the function of Nos/Pum and Ago1/miRNA translational complexes, thus allowing CB differentiation (12, 14).

Despite these significant advances in understanding the genetic roles of *bam* in regulating GSC fate, the biochemical nature of the Bam protein remains elusive.

Cell fate changes (e.g., cell differentiation or regeneration) commonly dictate a change in the cell cycle of daughter cells (17, 18). In *Drosophila*, it has been proposed that GSC fate determination is tightly regulated by a specific cell cycle program (19, 20). Previous studies have revealed that mutation or dysregulated expression of cyclin proteins, such as cyclin A (CycA), cyclin B (CycB), and cyclin E (CycE), result in defects of GSC maintenance, differentiation, and/or an abnormal cell number of (32- or 8-cell) cysts (19–23). Thus, proper expression of cyclin proteins is critical for GSC fate determination and/or normal germ-line development. We have previously shown that the E2 ubiquitin-conjugating enzyme Effete regulates GSC maintenance by controlling turnover of CycA in GSCs, suggesting that ubiquitin-mediated regulation of CycA plays pivotal roles in determining the fate of GSCs (22). Notably, ectopic expression of the stable form of CycA in germ cells causes loss of GSCs, which is similar to the phenotype resulting from ectopic expression of *bam* in GSCs. These lines of evidence prompted us to investigate a potential regulatory link between CycA and Bam proteins. In this study, we report that Bam directly associates with the ubiquitin protein and forms a complex with Otu, a putative deubiquitinase, to promote deubiquitination and stabilization of CycA. We further show that this biochemical pathway can explain the precocious GSC differentiation resulting from ectopic expression of Bam in GSCs.

Significance

Previous studies have demonstrated that Bam protein plays a critical role promoting early germ-line cell differentiation in the *Drosophila* ovary. Although its regulation and genetic functions have been extensively investigated over the last 20 years, the biochemical nature of Bam has still remained elusive. Here, we show that Bam functions as an ubiquitin-associated protein and regulates the stability of CycA. Our study uncovers a mechanism by which Bam functions as an ubiquitin-associated protein, and cooperates with Otu to deubiquitinate and stabilize CycA, thereby balancing GSC self-renewal and differentiation.

Author contributions: S.J., Q.S., and D.C. designed research; S.J., C.L., L.H., K.L., J.M., Y.L., Y.T., and Z.X. performed research; S.J., C.L., Q.S., and D.C. analyzed data; and S.J., Q.S., and D.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

See Commentary on page 6154.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1619188114/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1619188114

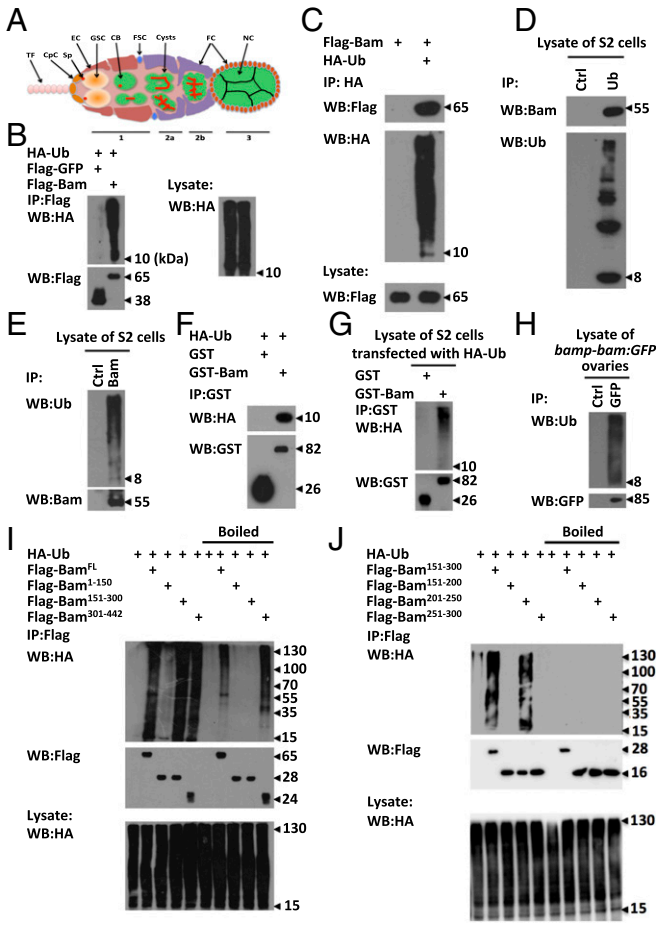

Fig. 1. Bam is an ubiquitin-association protein. (*A*) Schematic diagram displaying the ovarian germarium with various cell types and organelles including the terminal filament (TF), cap cells (CpC), spectrosome (Sp), escort cells (EC), germ-line stem cells (GSC), cystoblasts (CB), follicle stem cells (FSC), cysts, follicle cells (FC), and Nurse cells (NC). (*B* and *C*) S2 cells were transfected with indicated plasmids. Cell lysates were subjected to immunoprecipitation assays using Flag beads (*B*) or anti-HA antibody (*C*), followed by Western blot assays. (*D* and *E*) Lysates of S2 cells were immunoprecipitated with anti-ubiquitin (*D*) or anti-Bam (*E*) or mouse IgG (control) antibody, followed by Western blot assays. (*F* and *G*) Purified HA-ubiquitin proteins (*F*) or lysates of S2 cells transfected with HA-Ub plasmids (*G*) were mixed with GST or GST-Bam, followed by immunoprecipitation and Western blot assays. (*H*) Ovaries of P{*bamp-bam:GFP*} were dissected and lysed, followed by immunoprecipitation using anti-GFP or mouse IgG (control) antibody. Western blot assays were performed to detect the levels of indicated proteins. (*I* and *J*) S2 cells were transfected with indicated plasmids. Cell lysates were subjected to immunoprecipitation by using Flag beads with or without boiling pretreatment as indicated. Western blot assays were performed to detect the HA signal. All of the biochemical experiments were performed at least three times.

Results

Bam Associates with Ubiquitin. To explore the biochemical function of Bam in the regulation of germ-cell differentiation, we sought to search for Bam-associated partners. According to our described method (11), we expressed Flag epitope-tagged Bam in S2 cells and then performed coimmunoprecipitation experiments followed by mass spectrometric analysis. From this assay, we identified a number of proteins in the Bam immunoprecipitants. In addition to the known Bam-associated partners, Ter94 (24) and eIF4A (25), observed in Bam immunoprecipitants (Fig. S1*A*), we also found that ubiquitin was highly enriched in Bam complexes in our assays (Fig. S1*A*). To confirm this finding, we performed independent coimmunoprecipitation experiments in S2 cells, and found that in addition to high molecular weight ubiquitin signal, the free HA-tagged ubiquitin was also present in the Flag-tagged Bam immunoprecipitants (Fig. 1*B*). Moreover, the unubiquitinated form of Flag-Bam was observed in the ubiquitin immunoprecipitants (Fig. 1*C*), suggesting that Bam associates with ubiquitin in a noncovalently modified manner. Consistently, we found that endogenous Bam formed a complex with free ubiquitin protein (Fig. 1 *D* and *E*). To test whether ubiquitin directly binds Bam, we then performed a GST pull-down assay. As shown in Fig. 1 *F* and *G*, the free HA-ubiquitin directly bound to GST-Bam. To examine whether Bam interacts with ubiquitin in germ cells, we performed coimmunoprecipitation experiments in *Drosophila* ovaries and observed complex formation between Bam and ubiquitin (Fig. 1*H*).

Bam Interacts with Ubiquitin in a Domain-Specific Manner. To determine the specific domains of Bam essential for the Bam–ubiquitin interaction, we generated a series of truncated Bam fragments, including the N terminus (amino acids 1–150), center (amino acids 151–300), and C terminus (amino acids 301–442), and then performed coimmunoprecipitation assays. As shown in Fig. 1*I*, HA-ubiquitin signals were easily detected in immunoprecipitants of Flag-tagged full-length, central, and C-terminal Bam. However, the signals of HA-ubiquitin were dramatically reduced in central Bam when the cell lysates were preboiled before immunoprecipitation assays. Of note, strong HA-ubiquitin signals were still observed in immunoprecipitants of full-length and C-terminal Bam, even with boiling pretreatment (26). The preboiling treatment impaired the physical association of Bam with ubiquitin rather than affecting conjugated binding of ubiquitin with Bam. Therefore, these findings suggest that, whereas the C-terminal region of Bam is modified by ubiquitin, the central region (amino acids 151–300) is necessary for the interaction of Bam with free ubiquitin proteins. To narrow down the region of Bam, which interacts with ubiquitin, we further divided central Bam into smaller fragments including amino acids 151–200, 201–250, and 251–300. Immunoprecipitation assays revealed that a fragment of Bam (amino acids 201–250) is necessary for the Bam–ubiquitin interaction (Fig. 1*J*). Consistent results were obtained in further GST-pull down assays (Fig. S1*B*).

Bam Forms a Complex with CycA. Several lines of evidence promoted us to propose the hypothesis that Bam controls GSC functions by regulating CycA turnover through the ubiquitin-mediated pathway. First, ubiquitin-mediated degradation of CycA is essential for GSC maintenance (22). Second, ectopic expression of either Bam or CycA leads to a GSC-loss phenotype (5, 22). Third, *cycA* has been shown to genetically interact with *bam* to control germ cell cyst division (21). To explore the biochemical relationship between Bam and CycA, we first determined whether Bam and CycA form a complex by performing immunoprecipitation assays. As shown in Fig. 2 *A* and *B*, Bam and CycA were reciprocally coimmunoprecipitated with each other in transfected S2 cells. CycA is a highly conserved protein and contains multiple domains including D-box and extended D-box domains at its N-terminal region and the cyclin box at its C-terminal region (27). To map the specific region of CycA required for the Bam-CycA association, we generated both N terminus- and C terminus-truncated CycA constructs (Fig. 2*C*). As shown in a coimmunoprecipitation assay, the C-terminal CycA was sufficient to form a complex with Bam, whereas the N-terminal CycA failed to associate with Bam (Fig. 2*D*). To test whether endogenous CycA associates with Bam in germ cells, we performed immunoprecipitation experiments in ovaries and obtained similar results (Fig. 2*E*).

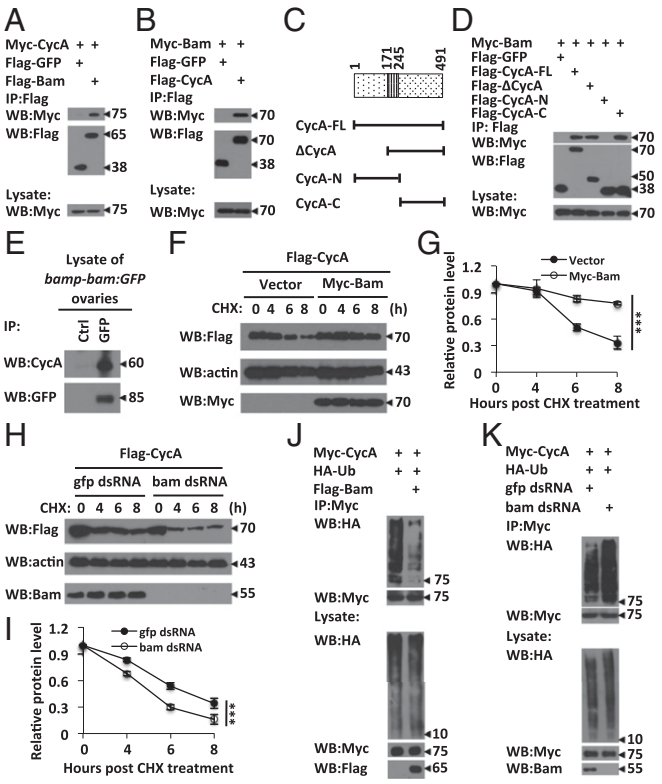

Fig. 2. Bam associates with CycA and regulates its turnover. (*A* and *B*) S2 cells were transfected with indicated plasmids. Cells lysates were subjected to immunoprecipitation assays with Flag beads, followed by Western blot assays. (*C*) Schematic diagrams of CycA and truncated mutants. (*D*) S2 cells were transfected with indicated plasmids. Cell lysates were subjected to immunoprecipitation and Western blot assays. (*E*) Lysates of P{*bamp-bam:GFP*} ovaries were immunoprecipitated with anti-GFP or mouse IgG (control) antibody, followed by Western blot assays. (*F–I*) S2 cells were transfected with expression constructs (*F*) or pretreated with *gfp* or *bam* dsRNAs (*H*). After 48 h, cells were treated with CHX (50 ng·mL^{-1}) for various times, followed by immunoblotting to examine CycA levels. Densitometric analyses to quantify CycA expression in *F* and *H* are shown in *G* and *I*, respectively. Error bars represent SD ($n = 3$). (*J* and *K*) S2 cells were transfected with combinations of plasmids (*J*) or pretreated with *gfp* or *bam* dsRNAs for 48 h (*K*). Cell lysates were immunoprecipitated with Myc beads, followed by Western blot assays. All of the biochemical experiments were performed at least three times. In *G* and *I*, the log rank test was used to analyze statistical variance. ***$P < 0.001$.

Bam Stabilizes CycA by Negatively Regulating Its Ubiquitination. Expressional fluctuation at the protein level is a hallmark of cyclin proteins during the cell cycle (27). Considering that Bam interacts with CycA, we next investigated whether Bam affects the fluctuation of CycA expression. We determined the half-life of Flag-tagged CycA in S2 cells with or without cotransfected Myc-tagged Bam by performing pulse–chase experiments. As shown in Fig. 2 *F* and *G*, the half-life of CycA was measured at approximately 6 h in the control experiments, whereas CycA exhibited a much longer half-life when the cells were coexpressed with Myc-Bam, suggesting that Bam is potentially involved in controlling the process of CycA turnover. To confirm this inference, we next tested whether knockdown of *bam* influences the stability of CycA. As shown in pulse–chase experiments (Fig. 2 *H* and *I*), knockdown of *bam* by dsRNA in S2 cells significantly reduced the half-life of CycA, compared with the control. Collectively, our findings identified a role of Bam in stabilizing CycA in S2 cells. To test whether Bam has the same role in early germ cells, we collected the ovaries from P{*hs-bam*};*bam*$^{-/-}$ females at the time point of 6 h after heat-shock treatment to perform Western blot assays. In this assay, P{*hs-bam*};*bam*$^{-/-}$ females without heat-shock treatment were used as control. Western blot analysis revealed that overexpression of Bam appeared to increase levels of CycA protein in *bam* mutant germ cells (Fig. S2 *A* and *C*).

We next determined whether Bam controls the ubiquitination status of CycA by performing ubiquitination assays in S2 cells. As shown in Fig. 2*J*, a strong signal of ubiquitinated CycA was detected when S2 cells were transfected with epitope-tagged CycA and ubiquitin. However, levels of ubiquitinated CycA were significantly reduced when the cells were cotransfected with Bam (Fig. 2*J*). Consistently, the conjugation of ubiquitin to CycA was evidently increased when S2 cells were treated with dsRNA against *bam* (Fig. 2*K*), suggesting that Bam negatively regulates CycA ubiquitination.

***CycA* Is a Genetic Target of *bam* in Early Germ Cells.** Considering that Bam stabilizes CycA in S2 cells, we hypothesized that ectopic expression of Bam in GSCs would increase the stability of CycA, which may at least in part account for the phenotype of GSC loss induced by Bam. To test this hypothesis, we determined whether down-regulation of CycA antagonized the function of ectopic Bam in GSCs. We first generated two miRNA-based *cycA* knockdown flies, P{*uasp-art-miRcycA*} and P{*uasp-art-miRcycA3′utr*}, according to our described method (28). As shown in Fig. S3*A*, expression of artificial miRNAs that targeted the coding region or 3′ UTR of *cycA* with the *nanos* promoter significantly reduced the levels of *cycA* transcripts in ovaries. However, knockdown of *cycA* did not affect the GSC number and allowed normal germ-line development in most ovarioles (Fig. S3*B*). Additionally, an EdU (5-ethynyl-2′-deoxyuridine)-labeling assay suggested that knockdown of *cycA* did not obviously affect early germ cells dividing, because wild-type control and *cycA* knockdown flies showed no significant difference in the number of EdU-positive cells in germaria (Fig. S3 *C* and *D*). Previous studies have shown that ectopic expression of Bam in germ cells with the *nos* promoter by generating P{*nosp-gal4:vp16*}/P{*uasp-bam:GFP*} leads to a strong GSC-loss phenotype (6). To establish a robust "read-out" system in our genetic test, we generated a germ cell driver, P{*nosp-gal4:vp16*}^{second}, which is located on the second chromosome. The transgene combination, P{*nosp-gal4:vp16*}^{second};P{*uasp-bam:GFP*}, resulted in a GSC-loss phenotype in most germaria, but a proportion of germaria (10–20%) containing a normal number of GSCs (Fig. 3 *B* and *F*). We next generated P{*nosp-gal4:vp16*}^{second};P{*uasp-bam:GFP*}/P{*uasp-cycA*} flies, and found that the female flies displayed a stronger GSC-loss phenotype compared with ovaries from P{*nosp-gal4:vp16*}^{second};P{*uasp-bam:GFP*} flies (Fig. 3 *A–C* and *F*), suggesting that expression of wild-type CycA enhances Bam function in early germ cells. Of note, P{*nosp-gal4:vp16*}^{second};P{*uasp-cycA*} flies showed no apparent defect in GSCs (Fig. S3*E*). We next tested whether *cycA* knockdown antagonizes the function of ectopic Bam in GSCs. As shown in Fig. 3 *A*, *B*, and *D–F*, knockdown of *cycA* significantly suppressed the GSC-loss phenotype induced by ectopic expression of *bam*, suggesting that down-regulation of *cycA* antagonizes the ectopic *bam*-induced GSC-loss phenotype.

We have previously shown that removal of *bam* silencer element (SE) leads to ectopic expression of *bam* gene at native levels in GSCs and GSC-loss phenotype (6). To test whether GSC loss caused by derepressed expression of *bam* in GSCs can be rescued by loss of CycA, we used the transgene, P{*bampdeltaSE-bam:GFP*}, in which the Bam:GFP conding sequences are under the control of the *bam* promoter without *bam* SE. As shown in Fig. 3 *G* and *K*, absence of *bam* silence element (SE) resulted in GSC-loss phenotype induced by ectopic Bam:GFP in GSCs, compared with control flies carrying P{*bamp-bam:GFP*} (Fig. 3 *I* and *I′*). Notably, low levels of ectopic Bam:GFP expression were detected in GSCs (Fig. 3 *H* and *H′*). To validate our

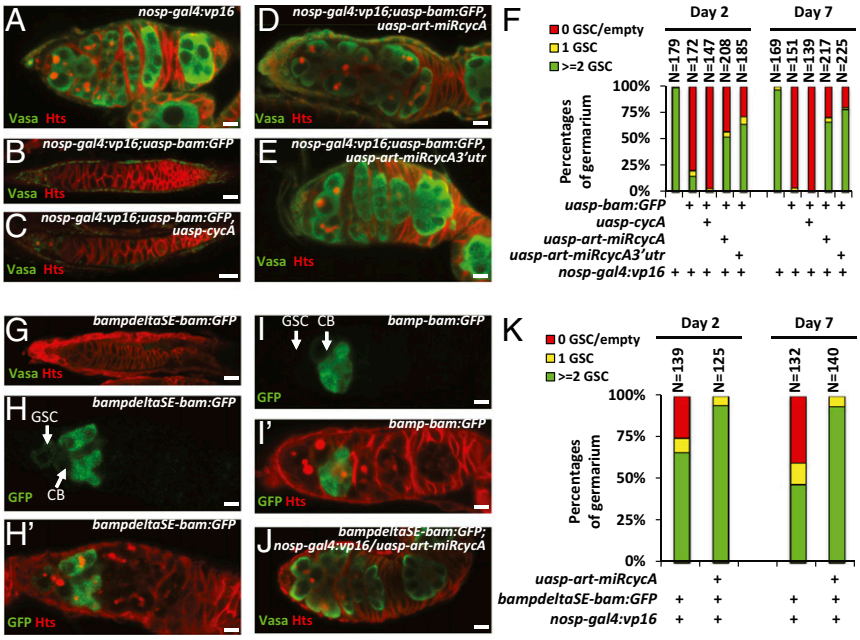

Fig. 3. CycA is a downstream target of Bam. (*A–E*) Ovaries from P{nosp-gal4:vp16} (*A*, wild-type control), P{nosp-gal4:vp16}; P{uasp-bam:GFP} (*B*), P{nosp-gal4:vp16};P{uasp-bam:GFP}/P{uasp-cycA} (*C*), P{nosp-gal4:vp16};P{uasp-bam:GFP}/P{uasp-art-miRcycA} (*D*), and P{nosp-gal4:vp16};P{uasp-bam:GFP}/P{uasp-art-miRcycA3′utr} (*E*) were stained with anti-Vasa (green) and anti-Hts (red) antibodies. Vasa was used to visualize germ cells. Hts was used to outline the germarium and morphology of fusomes. (*F*) Quantification of the germarium phenotypes of ovaries in *A–E*. (*G* and *J*) Ovaries of P{bampdeltaSE-bam:GFP} (*G*) and P{nosp-gal4:vp16}; P{bampdeltaSE-bam:GFP}/P{uasp-art-miRcycA} (*J*) were stained with anti-Vasa (green) and anti-Hts (red) antibodies. (*H–I′*) Ovaries of P{bampdeltaSE-bam:GFP} (*H* and *H′*) and P{bamp-bam:GFP} (*I* and *I′*) were stained with anti-GFP (green) and anti-Hts (red) antibodies. (Scale bars: 10 μm.) (*K*) Statistical quantification of the germarium phenotypes of ovaries in *G* and *J*.

argument, we performed further genetic experiments. As shown in Fig. 3 *G*, *J*, and *K*, knockdown of *cycA* significantly suppressed the GSC-loss phenotype induced by removal of *bam SE*. Taken together, our genetic findings suggest that *cycA* is one of the downstream targets of *bam* in GSCs.

Identification of Otu as a Bam Cofactor in the Deubiquitination Complex. Considering that Bam is an ubiquitin-interacting protein and its role in negatively regulating CycA ubiquitination, we examined whether Bam has an intrinsic deubiquitinating enzymatic activity. To this end, we carried out deubiquitination assays by using eukaryotic-expressed Flag-tagged Bam and found that the purified Flag-Bam had no deubiquitinase activity (Fig. S4 *A* and *B*). Based on these observations, we reasoned that Bam might form a complex with other cofactors, likely deubiquitinating enzymes, to negatively regulate CycA ubiquitination. To test this possibility, we used a dsRNA library that targets 41 deubiquitinating enzymes in *Drosophila* and performed screening to search for a deubiquitinating enzyme involved in regulating CycA deubiquitination (Table S1). In this screening, we identified a pair of dsRNA that target *Drosophila* Otu. As shown in Fig. 4*A* and Fig. S4*C*, knockdown of *otu* significantly increased the abundance of ubiquitin conjugating to CycA. A recent study reported that *Drosophila* Otu has no detectable deubiquitinating enzyme (Dub) activity (29). However, given the fact that *Drosophila* Otu is required for inhibiting the CycA ubiquitination in S2 cells, we sought to determine the issue of whether *Drosophila* Otu is a Dub, and used Ub-Rhodamine110 as a substrate to perform quantitative deubiquitinating enzyme assays (30). As shown in Fig. 4*B* and Fig. S4*A*, although it exhibited much lower Dub activity compared with the positive control vOtu, *Drosophila* Otu was able to cleave the amide bond between the C-terminal glycine of ubiquitin and rhodamine in a dose-dependent manner, suggesting that *Drosophila* Otu possesses a Dub enzyme activity. We next asked whether *Drosophila* Otu regulates CycA protein turnover by designing another pair of *otu* dsRNA (Fig. S4 *D* and *E*) and found that knockdown of *otu* led to destabilization of the CycA protein (Fig. 4 *C* and *D*). These findings suggest that *Drosophila* Otu negatively regulates CycA ubiquitination and, thus, stabilizes CycA.

Bam Acts in Concert with Otu To Promote CycA Deubiquitination. We next sought to investigate whether Otu form a complex to regulate CycA deubiquitination. As shown by immunoprecipitation assays (Fig. 4*E*), a Bam-Otu protein association was detected in S2 cells cotransfected with both Flag-Bam and Myc-Otu. GST-pull down experiments confirmed that Bam and Otu proteins directly bound to each other (Fig. 4*F*). Because CycA associated with Otu in coimmunoprecipitation assays (Fig. 4*G*), and both Otu and Bam caused CycA deubiquitination, we next asked whether CycA, Bam, and Otu formed a trimeric complex by performing two-step immunoprecipitation assays in S2 cells. As shown in Fig. 4*H*, after two-step immunoprecipitations, both HA-Bam and Flag-CycA were present in the Myc–Otu complex, suggesting that CycA, Bam, and Otu form a trimeric complex. Of note, knockdown or mutation of *bam* significantly impaired the association between Otu and CycA, suggesting that Bam is required for Otu to target CycA both in S2 cells and in germ cells (Fig. 4*I* and Fig. S4*F*). Next, we evaluated whether Otu participates in Bam-mediated CycA deubiquitination in S2 cells. As shown in Fig. 4*J*, overexpression of Bam reduced the conjugation of ubiquitin to CycA compared with the control. However, the deubiquitination of CycA induced by overexpression of Bam was significantly suppressed when *otu* was knocked down in S2 cells, suggesting that Bam stabilizes CycA by promoting its deubiquitination in a manner that depends on the activity of Otu.

Otu and Bam Cooperatively Control Early Germ Cell Development. Previous studies have shown that the loss of *otu* results in severe germ cell hyperplasty (31), similar to the tumorous phenotype

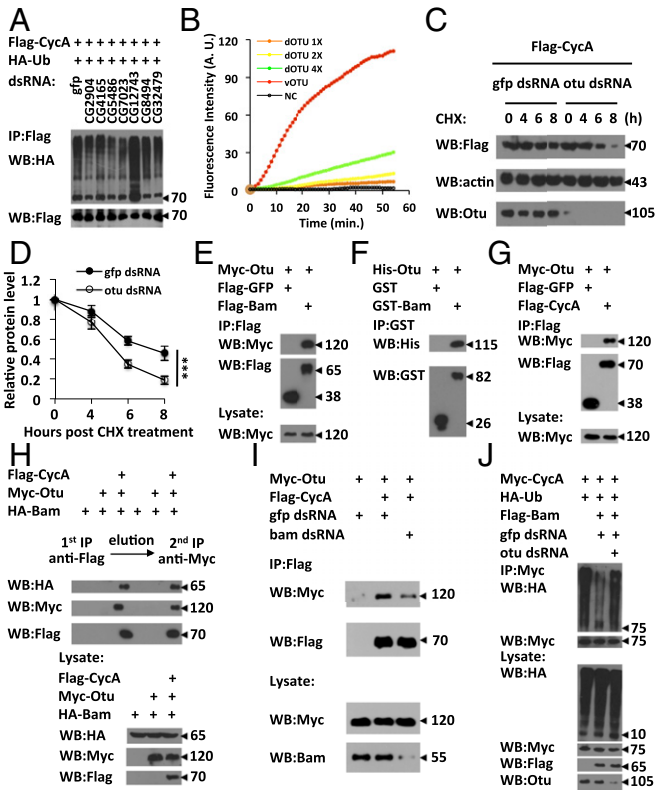

Fig. 4. Bam targets CycA in an Otu-dependent manner. (*A*) S2 cells were treated with dsRNAs targeting deubiquitination genes for 48 h, followed by plasmid transfections. Cells were lysed for immunoprecipitation and Western blot assays. (*B*) In vitro Dub assays showed the enzymatic activity of *Drosophila* Otu (the detailed information was shown in *SI Text*). (*C* and *D*) S2 cells were pretreated with *gfp* or *otu* dsRNAs for 48 h and then transfected with indicated plasmids (*C*). Cells were treated with CHX (50 ng·mL^{-1}) for various time points as indicated, followed by immunoblotting to measure expression levels of CycA. Densitometric analysis for quantifying CycA expression in *C* is shown in *D*. Error bars represent SD ($n = 3$). (*E*) S2 cells were transfected with indicated plasmids. Cell lysates were prepared and immunoprecipitated, followed by Western blot assays. (*F*) His-tagged Otu protein purified from *Escherichia coli* was coimmunoprecipitated with GST (control) or GST-Bam, followed by Western blot assays. (*G*) Transfection and coimmunoprecipitation experiments were performed as in *E*, except that the Flag-CycA plasmid was used instead of Flag-Bam. (*H*) S2 cells were transfected with indicated plasmids. Cell lysates were prepared and subjected to a two-step immunoprecipitation method by using Flag and Myc beads successively, followed by Western blot assays. (*I*) S2 cells were treated with *gfp* or *bam* dsRNAs for 48 h, followed by transfection with indicated plasmids. Cells lysates were subjected to immunoprecipitation and Western blot assays. (*J*) S2 cells were treated with dsRNA targeting *gfp* or *otu* for 48 h and then transfected with indicated expression vectors. Cells were lysed and subjected to immunoprecipitation assays, followed by Western blot assays. All of the biochemical experiments were performed at least three times. In *D*, the log rank test was used to analyze statistical variance. ***$P < 0.001$.

caused by mutation of the *bam* gene. To elucidate the genetic relationships between *bam* and *otu* in regulating ovarian germ cell development, we generated *otu*^{1};P{*hs-bam*} flies and examined whether Otu is required for the Bam function in promoting precocious differentiation of GSCs. As shown in Fig. 5 *A*–*C* and *F*, ectopic expression of Bam in wild-type background resulted in a strong GSC-loss phenotype; by contrast, ectopic Bam in the *otu* mutant had no effect on GSC loss. Immunostaining showed that *otu*^{1};P{*hs-bam*} ovaries still displayed tumorous germarium phenotypes that were similar to those in females carrying the *otu* mutant alone (Fig. 5 *C* and *F*). To confirm these observations, we generated the transgenic line P{*uasp-art-miRotu*} that carried artificial miRNAs targeting the *otu* gene (28). As shown in Fig. 5*D*, knockdown of *otu* by the nos promoter effectively reduced the level of *otu*, and accordingly caused the tumorous germarium phenotype (Fig. S5*A*). Importantly, further genetic experiments revealed that knockdown of *otu* efficiently suppressed the GSC-loss phenotype induced by overexpression of Bam (Fig. 5 *E* and *F*). Additionally, we did not observe any detectable phenotypes in ovaries from P{*nosp-gal4:vp16*}/P{*uasp-otu*} or P{*nosp-gal4:vp16*}/P{*uasp-otu*};*bam*^{86} flies (Fig. S5 *B* and *C*). One explanation is that the levels of endogenous Otu could be saturated in germ cells. Of note, the *otu* transgene could fully rescue *otu* mutant phenotype in germ cells (Fig. S5*D*). Collectively, these findings suggest that Otu is critical for the function of ectopic Bam in GSCs.

Discussion

Previous studies have shown that Bam plays an essential role in promoting early germ cell differentiation in *Drosophila* ovaries. To protect GSCs from this powerful differentiation inducer, the expression of *bam* is inhibited in GSCs by the BMP/Dpp signaling pathway. Disrupting the BMP/Dpp pathway, or ectopically inducing bam expression in GSCs, causes them to differentiate, resulting in a loss of the germ line (5, 7–9). However, little is known about how ectopic expression of Bam drives GSC differentiation. In this study, we showed that knockdown of CycA significantly suppressed the GSC-loss phenotype induced by

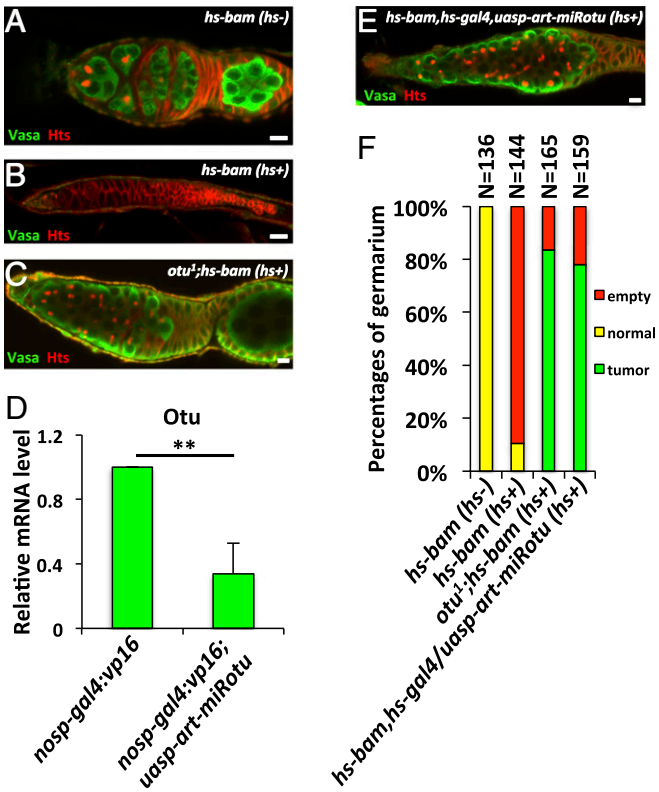

Fig. 5. *Otu* genetically interacts with *bam*. (*A*–*C*) Ovaries from P{*hs-bam*} (hs-) (*A*, wild-type control), P{*hs-bam*} (hs+) (*B*), and *otu*^{1};P{*hs-bam*} (hs+) (*C*) flies were stained with anti-Vasa (green) and anti-Hts (red) antibodies. (*D*) Ovaries from P{*nosp-gal4:vp16*} (wild-type control) and P{*nosp-gal4:vp16*};P{*uasp-art-miRotu*} were dissected and subjected to quantitative RT-PCR (qRT-PCR) assays. Error bars represent SD ($n = 3$). (*E*) The staining was performed as in *C*, except that flies used were P{*uasp-art-miRotu*}/P{*hs-bam, hs-gal4*} (hs+). (Scale bars: 10 μm.) (*F*) Quantification of the germarium phenotypes of ovaries in *A*–*C* and *E*. In *D*, the Student's *t* test was used to analyze statistical variance. **$P < 0.01$.

ectopic Bam in GSCs. Importantly, we found that Bam acts in concert with Otu to negatively regulate CycA ubiquitination. Thus, our findings suggest ectopically expressed Bam acts as a component of an Otu-containing Dub complex that promotes accumulation of deubiquitinated CycA in GSCs, and CycA-dependent differentiation.

A hallmark of early germ cell development is that each round of cyst division is accompanied by a morphological change of a germ cell-specific organelle called the fusome (32). While it is observed as a spherical dot in GSCs/CBs, the fusome progressively grows upon cell division to become elongated and finally a branched structure in matured cysts. Because a significant proportion of CycA is associated with the fusome, regulation of CycA at the fusome likely plays important roles in controlling asymmetric division of GSCs and germ cell-specific mitosis (21). Interestingly, previous and current studies have shown that Bam is partially associated with the fusome and required to promote proper incomplete cytokinesis (detailed information shown in *SI Text* and Fig. S6). In particular, the BamF protein is detectable in the fusome at a low level in GSCs (4). Of note, loss of function of the *otu* gene encoding a Bam cofactor leads to fragmented fusomes in tumorous egg chambers (31). Based on previous and current findings, we propose that the turnover of CycA mediated by Bam/Otu-dependent deubiquitination is likely processed in a fusome-dependent manner. Indeed, considering that Cul1 and 19S-S1 proteins are also present in the fusome, previous studies suggest that the ubiquitin-based proteolysis machinery is localized at the fusome and regulates the turnover of cyclins (e.g., CycA and CycE) (20). Thus, our findings emphasize that proper balancing of CycA turnover either by ubiquitination or deubiquitination processes is critical for GSC asymmetric division.

Bam has been shown to form a complex with its obligate cofactor Bgcn to regulate CB differentiation (33). Previous studies show that the Bam/Bgcn complex antagonizes Nos expression likely via the *nos* 3′ UTR, thus derepressing CB-promoting factors (12). However, the molecular basis of how Bam regulates translational repression of *nos* has remained poorly understood. Nevertheless, the current findings suggest that Bam plays multiple roles in regulating early germ cell differentiation through both translational repression and posttranslational control. Recent studies have suggested that ubiquitin, a regulator of protein degradation, also contributes to regulation of mRNA turnover through the action of a family of RNA-binding proteins that also have an E3 ubiquitin ligase activity, thus establishing a link between translational repression and posttranslational control (34, 35). Considering that Bam is an ubiquitin-associated protein, it would be interesting to explore the detailed function of Bam in the future.

Materials and Methods

All fly strains were maintained under standard culture conditions. The w^{1118} strain was used as the host for all P-element–mediated transformation. Fly strains, including P{*hs-bam*}, P{*uasp-bam:GFP*}, P{*uasp-cycA*}, P{*nosp-gal4:vp16*}, and P{*bamp-bam:GFP*} have been described (5, 6, 21, 22). Knockdown transgenic flies, including P{*uasp-art-miRcycA*}, P{*uasp-art-miRcycA3′utr*}, and P{*uasp-art-miRotu*}, were generated according to methods that we have described (28). For the P{*bampdeltaSE-bam:GFP*} transgene, the coding sequences of Bam and GFP were successively inserted after the *bam* promoter without silence element (SE). The otu^1 was obtained from the Bloomington stock center. Additional materials and methods are available in *SI Materials and Methods*.

ACKNOWLEDGMENTS. This work was supported by the National Basic Research Program of China Grant 2013CB945002; Natural Science Foundation of China Grants 3159830021, 91640204, and 31130036; and Strategic Priority Research Program of the Chinese Academy of Sciences Grant XDB19000000.

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