

Effete-mediated degradation of Cyclin A is essential for the maintenance of germline stem cells in *Drosophila*

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Increasing evidence supports the idea that the regulation of stem cells requires both extrinsic and intrinsic mechanisms. However, much less is known about how intrinsic signals regulate the fate of stem cells. Studies on germline stem cells (GSCs) in the *Drosophila* ovary have provided novel insights into the regulatory mechanisms of stem cell maintenance. In this study, we demonstrate that a ubiquitin-dependent pathway mediated by the *Drosophila eff* gene, which encodes the E2 ubiquitin-conjugating enzyme Effete (Eff), plays an essential role in GSC maintenance. We show that Eff both physically and genetically interacts with dAPC2, a key component of the anaphase-promoting complex (APC), which acts as a multisubunit E3 ligase and plays an essential role in targeting mitotic regulators for degradation during exit from mitosis. This interaction indicates that Eff regulates the APC/C-mediated proteolysis pathway in GSCs. Moreover, we show that expression of a stable form of Cyclin A, but not full-length Cyclin A, results in GSC loss. Finally we show that, in common with APC2, Eff is required for the ubiquitylation of Cyclin A, and overexpression of full-length Cyclin A accelerates the loss of GSCs in the *eff* mutant background. Collectively, our data support the idea that Effete/APC-mediated degradation of Cyclin A is essential for the maintenance of germline stem cells in *Drosophila*. Given that the regulation of mitotic Cyclins is evolutionarily conserved between flies and mammals, our study also implies that a similar mechanism may be conserved in mammals.

KEY WORDS: APC/C complexes, Cyclin A, Stem cell fate determination, Ubiquitin-conjugating enzymes

INTRODUCTION

The germline stem cells (GSCs) of the *Drosophila* ovary provide an excellent model system for studying the molecular mechanisms of stem cell regulation in vivo. In adult *Drosophila* females, two to three GSCs are easily recognized by their molecular markers (either a spherical spectrosome, or an extending fusome when GSCs are dividing) and their location at the apical region of the germarium in close contact with surrounding somatic cells, the terminal filament and cap cells, which together generate a specific micro-environment, or niche, for GSC regulation (Fig. 1A). The GSC divisions take place along the anteroposterior axis of the ovary to produce an anterior GSC, which remains attached to the niche cells, and a posterior cystoblast (Cb). The Cb divides precisely four times by incomplete cytokinesis to generate 16 interconnected cells that form the germline cyst of the follicle and sustain oogenesis.

Genetic analyses have revealed that the stem cell state of GSCs is maintained by both extrinsic and intrinsic mechanisms that repress their differentiation (Spradling et al., 2001). BMP ligands (Dpp, Gbb) from the niche cells (Song et al., 2004) maintain GSCs by suppressing Cb differentiation in the anteriormost cells. This is achieved by silencing the transcription of the *bam* gene, which encodes a GSC/Cb differentiation-promoting factor (Chen and McKearin, 2003a; Song et al., 2004). In the GSCs, BMP signaling activates cytoplasmic Mad and Medea, the *Drosophila* Smads, and promotes their nuclear translocation. In the nucleus, the Smads complex physically interacts with both the *bam* silencer element and nuclear lamin-associated protein (Ote), resulting in *bam*

transcriptional silencing (Chen and McKearin, 2003a; Jiang et al., 2008; Song et al., 2004). Thus, BMP/Dpp-dependent *bam* transcriptional control serves as the primary pathway for the regulation of GSC fate. Independent of the niche-based regulation of the *bam* silencing mechanism, the fate of GSCs is also intrinsically controlled by other GSC maintenance factors that repress their differentiation. It has been demonstrated that Pum/Nos-mediated and microRNA-mediated translational repression pathways are not required for *bam* silencing, suggesting that these pathways act either downstream of or parallel to *bam* action (Chen and McKearin, 2005; Jin and Xie, 2007; Park et al., 2007; Szakmary et al., 2005; Yang et al., 2007). Although it is proposed that the Pum/Nos-mediated and microRNA-mediated pathways repress the translation of key differentiation factors to prevent GSC differentiation, the targets of these translational pathways in GSCs are not identified. Thus, the intrinsic mechanisms that repress GSC differentiation are still poorly understood. In addition, as loss of function of the components in these translational repression pathways is not sufficient to completely cause *bam* mutant germ-cell differentiation (Chen and McKearin, 2005), we speculate that the repression of GSC differentiation may also be controlled by other unknown intrinsic mechanisms.

Ubiquitin-mediated protein degradation plays a variety of roles in the regulation of many developmental processes (Glickman and Ciechanover, 2002). The enzymatic reaction of protein ubiquitylation is a coordinated three-step process involving three classes of enzymes known as E1 (Uba1 – FlyBase), E2 (UbcD4 – FlyBase) and E3. Firstly, E1 (Ubiquitin activating enzyme 1) catalyzes the formation of a thiolester bond linkage between the active-site cysteine residue on E1 and the C terminus of ubiquitin. Secondly, the activated ubiquitin (E1-Ub) is then transferred to E2 (Ubiquitin conjugating enzyme 4) via formation of an E2-Ub thiolester. Thirdly, E3 (ubiquitin ligase) promotes the transfer of the ubiquitin from E2-Ub to a lysine residue of the target protein through an isopeptide bond. Repeated cycles of this reaction can result in

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polyubiquitylation of the target protein, which is finally targeted for degradation by the 26S proteasome (Glickman and Ciechanover, 2002). The *Drosophila effete* (*eff*) gene encodes a class I ubiquitin-conjugating enzyme that was first shown to be required for proper telomere behavior (Cenci et al., 1997). Early studies also showed that *eff* is required for proper cyst formation in ovary (Lilly et al., 2000). However, whether *eff* is involved in the regulation of GSC fate remains unknown.

Mitotic cyclins, in concert with their Cyclin-dependent kinase (CDK) partners, play important roles in the promotion of mitosis, such as entry into mitosis and the alignment of chromosomes at metaphase (Murray, 2004). The degradation of mitotic cyclins by the anaphase-promoting complex/cyclosome (APC/C), a large multisubunit E3 ligase, leads to inactivation of CDKs during the metaphase-anaphase transition and is essential for the completion of mitosis in all eukaryotes (Peters, 2002). In *Drosophila*, it has been shown that induction of stable versions of the three mitotic cyclins, Cyclin A, B and B3, arrests mitosis with different phenotypes, suggesting that degradation of distinct mitotic cyclins is required for specific mitotic events (Parry and O'Farrell, 2001). It has been shown that the asymmetric division of GSCs requires the activity of Cyclin B (Wang and Lin, 2005); however, it remains unknown whether the regulation of mitotic cyclins also contributes to fate determination in GSCs. In this study, we demonstrate that the *eff* gene, which encodes an E2 ubiquitin-conjugating enzyme (Eff), is intrinsic to and essential for *Drosophila* GSC maintenance. Moreover, we demonstrate that Eff both genetically and physically interacts with dAPC2, a key component of APC/C complexes. We also show that dCDC20/Fzy, a key regulator of APC/C complexes, is involved in GSC regulation. Finally, we show that constant expression of a stable form of Cyclin A results in the loss of GSCs. Notably, we show that in common with APC2, Eff is required for the ubiquitylation of Cyclin A, and overexpression of full-length Cyclin A accelerates the loss of GSCs in the *eff* mutant background. Thus, our data support the idea that Effete/APC-mediated degradation of Cyclin A is essential for the maintenance of GSCs in *Drosophila*.

MATERIALS AND METHODS

Drosophila strains

Fly stocks used in this study were maintained under standard culture conditions. The *w¹¹¹⁸* strain was used as the host for all P-element-mediated transformations (Rubin and Spradling, 1982). The following strains were also used for experimentation: (1) *bam⁸⁶*, a null *bam* allele (McKearin and Ohlstein, 1995); (2) P{*bamP-GFP*}, which was described previously (Chen and McKearin, 2003b); (3) *eff⁸*, *eff¹⁷⁸²*, *eff^{mer1}* and *eff^{mer4}*, which are strong alleles for the *eff* gene; (4) *mr¹* and *mr²*, which are weak alleles for the *dAPC2/mr* gene; (5) *fzy¹*, which is a null allele for the *fzy* gene (from Bloomington Stock Center); (6) P{*nosP-gal4:vp16*}, which was described previously (Van Doren et al., 1998).

Anti-Eff and anti-BamC antibodies

Anti-Eff and anti-BamC antibodies were generated by immunizing rabbits and mice with His6-Eff (full-length) and His6-Bam (full-length) recombinant proteins, respectively, produced in *Escherichia coli*.

Immunohistochemistry

Ovaries were prepared for immunohistochemistry as described previously (Chen and McKearin, 2005). The following primary antibody dilutions were used: rabbit anti-GFP (1:5000, Invitrogen); mouse anti-Hts (1:500, DSHB); rabbit anti-Vasa (1:1000, Santa Cruz); mouse anti-Eff (1:1000); mouse anti-BamC (1:2000); rabbit anti-BamC (1:2000). The following secondary antibodies were used at a 1:200 dilution: goat anti-mouse Alexa fluor 568, goat anti-rabbit Alexa fluor 488 and goat anti-rat CY3 (Molecular Probes).

Germline clonal analysis

The FLP/FRT-mediated recombination method was used to generate *eff* and other mutant GSC clones (Xie and Spradling, 1998; Yang et al., 2007). For example, to generate *eff* GSC clones, 3-day-old female flies with the genotype *hs-flp/+; FRT82B, ubi-gfp/FRT82B, eff (hs-flp/+; FRT82B, ubi-gfp/FRT82B* as the *FRT* control) underwent heat-shock treatment at 37°C for 60 minutes twice per day. After 5 days of heat-shock treatment, ovaries were dissected for quantification of GSC clones at days 2, 6, 10, 14 and 18 following post-clonal induction. GSC clones were identified by the lack of GFP expression, as well as their attachment position to the base cells of the terminal filament or to cap cells.

Cell culture, immunoprecipitation and western blot analysis

S2 cells were cultured in Schneider's *Drosophila* medium (Sigma). DNA construct transfection was performed using the Calcium Phosphate Transfection Kit (Specialty Media) according to the manufacturer's instructions. Immunoprecipitation and western blot were performed using previously described protocols (Jiang et al., 2008; Zhang et al., 2006). The following antibodies were used: mouse anti-Myc (Santa Cruz); rabbit anti-Myc (Santa Cruz); mouse anti-Flag (Sigma). Anti-Flag M2 affinity gel (Sigma) was used to purify anti-Flag antibody-antigen complexes.

dsRNAs synthesis

DNA fragments corresponding to *eff* and *morula* mRNAs were synthesized in PCR reactions. The fragments were then fused to the T7 RNA polymerase-binding site at both 5' and 3' ends, which were used to generate the double-stranded RNAs (dsRNAs) in vitro by using the RiboMAX Large Scale RNA Production Systems (Promega). The primers used for the generation of dsRNAs are: *eff*, GCCGGACAGCCCTTATC (forward) CTGCCGTTGCTCTTGGT (reverse); *mr*, TGTTGGGCAGCATAGAC (forward) CATATCCGTCAGCCTTT (reverse).

In vivo ubiquitylation assay

For the in vivo ubiquitylation assay, S2 cells were treated with dsRNA and transfected according to standard protocols, 40 hours after transfection, 50 μM MG132 (final concentration) was added into the media. Cells were harvested after 4-hour treatment of MG132 and lysed with lysis buffer (50 mM Tris, pH 7.5, 120 mM NaCl, 0.5% NP40). After immunoprecipitation with anti-Flag M2 affinity gel (Sigma), the immunoprecipitate was washed with lysis buffer containing 0.1% SDS and then subjected to immunoblot analysis. The following antibodies were used: mouse anti-Myc (Santa Cruz); rabbit anti-HA (Santa Cruz); anti-Ub (Santa Cruz); mouse anti-flag antibody and M2 affinity Gel (Sigma).

In vitro ubiquitylation assay for Cyclin A

His-Eff was purified from BL21 *E. coli*. Myc-APC2, Myc-APC11 and Flag-Cyclin A proteins were purified from S2 cell cultures by the immunoprecipitating method. To test whether the ubiquitylation of Cyclin A was supported by Eff in vitro, E1, E2 (His-Eff), E3 (Myc-APC2 and Myc-APC11), substrate (Flag-Cyclin A) and Ub were then incubated at 30°C for 2 hours in a 40 μl ubiquitylation reaction (50 mM Tris-HCl pH 7.5, 2 mM dithiothreitol, 50 mM NaCl, 5 mM MgCl₂, 2 mM ATP), with 0.2 μg of E1, 10 μg of ubiquitin (both from Upstate, USA), 0 or 0.4 μg of His-Eff (E2), and 0 or 1.5 μg of Flag-Cyclin A. Reactions were terminated with loading buffer and analyzed by western blotting with anti-Ub and anti-Flag antibodies.

RESULTS

Identification of Eff, a *Drosophila* E2 ubiquitin-conjugating enzyme, as a GSC maintenance factor

To identify new factors that regulate the self-renewal or differentiation of GSCs in the *Drosophila* ovary, we screened female sterile lines, or weak fertile lines with P-element insertion, available from Bloomington Stock Center. As described in previous studies, the typical characteristic of GSC maintenance defects is a reduction in germ-cell number. This eventually results in an empty germline lacking germ cells, and a decline in the production of egg chambers (Forbes and Lehmann, 1998; Lin and

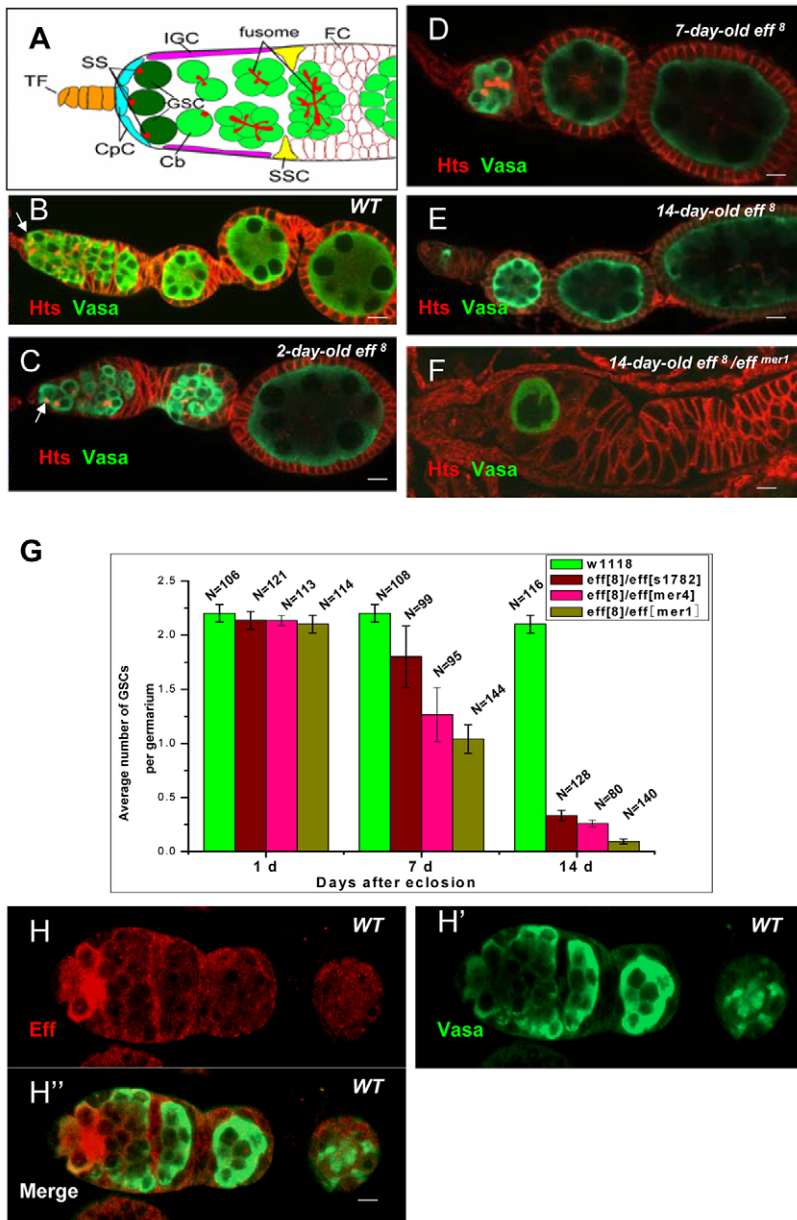


Fig. 1. Identification of an *eff* mutant with defects in germ-cell development. (A) Schematic diagram of the ovarium, with different cell types and organelles indicated as follows: cap cells (CpC), cystoblast cells (Cb), follicle cells (FC), fusome, germline stem cells (GSC), inner germarium sheath cells (IGC), spectrosome (SS) and terminal filament (TF).

(B-F) Ovaries collected from wild-type *w*¹¹¹⁸ (B), *eff* (C-F) flies at the different ages indicated were stained with anti-Vasa (green) and anti-Hts (red) antibodies. Anti-Hts was used to outline the germarium and the morphology of the fusome, whereas the staining of anti-Vasa was used to visualize all germ cells in the germarium and egg chambers. GSCs were identified by germ cells with anterior-positioned spectrosomes (indicated by arrows). (G) Quantitative analysis of the number of GSCs in *eff* mutant; the x-axis shows the day of examination post-eclosion, and the y-axis shows the average number of GSCs per ovarium in different allelic combination of *eff* mutant and wild-type control. (H-H'') Ovaries from wild type were stained with anti-Vasa (green) and anti-Eff (red) antibodies as indicated. Scale bars: 10 μ m.

Spradling, 1997; Maines et al., 2007). Based on these criteria, we identified a line with a P-element insertion in the third chromosome, P{PZ}*eff*⁸ (Castrillon et al., 1993), which exhibits severe defects in germline development, including the loss of germ cells. To systematically study the behavior of GSCs and early germ cells in the *eff*⁸ mutant, we used anti-Vasa and anti-Hts antibodies to visualize germ cells (Lasko and Ashburner, 1990) and fusomes (Zaccari and Lipshitz, 1996), respectively. In the tip of wild-type ovarium, two or three GSCs were readily recognized by anti-Vasa antibody, and fusomes were morphologically spherical and anchored between the GSCs and cap cells (Fig. 1A,B). In addition, a normal germline lineage with sequentially differentiated cells marked by branched fusomes was also observed. However, in the 7-day-old ovaries from *eff*⁸ homozygous females, about 30% of mutant ovarioles ($n > 100$) contained either empty or abnormal germaria (Fig. 1D). Furthermore, the germ-cell defect phenotype became much more severe with age (Fig. 1E-G). These findings suggest that the loss of *eff* may affect the maintenance of GSCs. To

determine whether the GSC maintenance defect associated with *eff*⁸ was indeed due to the loss of *eff* function rather than other genetic backgrounds, we next analyzed the phenotypes resulting from removal of *eff* in several allelic combinations, *eff*⁸/*eff*¹⁷⁸², *eff*⁸/*eff*^{mer1} and *eff*⁸/*eff*^{mer4}. According to methods described in the previous study (Cox et al., 2000) (and see Fig. S1 in the supplementary material), we quantified the number of GSCs in the available *eff* allelic combinations at days 1, 7 and 14 after eclosion. As shown in Fig. 1G, compared to wild type, the average number of GSCs in all *eff* mutants either rapidly or progressively declined during the testing period, indicating that the loss of *eff* resulted in the loss of GSCs. To further confirm this observation, we generated a transgene, P{*effP-eff*}, in which an *eff* cDNA was placed under the control of a 5.8 kb *eff* promoter. The GSC loss phenotype in different *eff* allelic backgrounds was fully rescued by the transgene line, P{*effP-eff*} (see Fig. S2 in the supplementary material). Taken together, our findings indicate that the *eff* gene plays an essential role in the maintenance of GSCs.

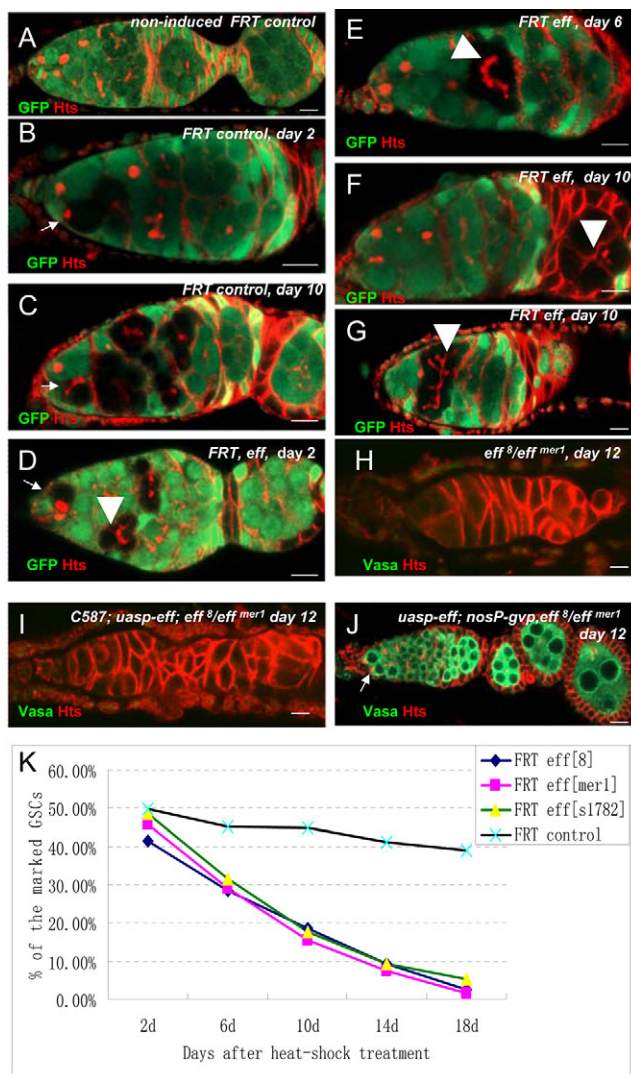


Fig. 2. Eff is required intrinsically for GSC maintenance. GSC clones were induced by FLP/FRT-mediated mitotic recombination in adult female flies. (A–G) Ovaries from FRT control flies (A–C) and FRT, *eff* flies (D–G) were collected at the indicated times following heat-shock treatment and stained with anti-GFP (green) and anti-Hts (red) antibodies; GSC clones (indicated by arrows) and cyst clones (indicated by arrowheads) were identified by the lack of GFP expression. (H–J) Ovaries from *eff* mutant (H), C587/*UASp-eff*; *eff^β/eff^{mer1}* (I) and *UASp-eff*; *nosP-gal4:vp16*; *eff^β/eff^{mer1}* (J) female flies were stained with anti-Vasa (green) and anti-Hts (red) antibodies. Scale bars: 10 μm. (K) Percentage of negatively GFP-marked GSC clones in FRT control and three FRT, *eff* null alleles at days 2, 6, 10, 14 and 18 following post-clonal induction. In contrast to wild-type-marked control GSCs (GFP⁺), the percentage of marked GSCs (GFP[−]) that lacked *eff* was reduced dramatically.

The *eff* gene is intrinsically required for GSC maintenance

The maintenance of GSCs requires both intrinsic and extrinsic signaling pathways. To characterize the role of *eff* in GSC maintenance, we first examined the pattern of Eff protein in germ cells. As shown in Fig. 1H, Eff was highly present in early germ cells including GSCs, which is consistent with the data in the previous study (Buszczak et al., 2007). We then used the FLP/FRT-mediated mitotic recombination technique (Xie and Spradling, 1998; Xu and

Rubin, 1993) to generate marked *eff* mutant GSC clones, and examined the role of *eff* in GSC maintenance by calculating the loss rates of the marked GSC clones as described previously (Yang et al., 2007). The loss rates of the marked GSCs were measured from the genotypes of female flies, *hs-flp*; *FRT82B*, *eff*/*FRT82B*, *ubi-gfp* and *hs-flp*; *FRT82B*/*FRT82B*, *ubi-gfp*, at days 2, 6, 10, 14 and 18 after heat-shock treatments (AHST). The marked FRT control and *eff* mutant GSCs were recognized by a lack of *gfp* expression in nuclei and also spectrosomes, which are anchored between GSCs and cap cells. As shown in Fig. 2, in the FRT control, the initial rate of the marked GSC clones was measured as 49.7% ($n=167$) from tested samples at day 2 AHST, and 39.1% ($n=120$) of the marked control GSCs were present at day 18 AHST. This suggested that only 21.3% of the marked GSCs were lost during the 18 day ASHT period. In contrast, under the same experimental conditions, 41.4% ($n=152$) of the marked *eff^β* mutant GSCs were measured at day 2 AHST, but only 2.5% ($n=97$) of the marked *eff^β* mutant GSC clones were detectable at day 18 AHST. This suggested that 93.6% of marked *eff^β* mutant GSC clones were lost during the 18-day AHST period. Similar results were obtained from the clonal analysis of the other two alleles, *eff^{s1782}* and *eff^{mer1}* (Fig. 2K). Taken together, these findings demonstrated that *eff* plays an essential and intrinsic role in GSC maintenance.

The loss of GSCs in *eff* mutants could be a result of either their death or their premature differentiation. To distinguish these two possibilities, we examined the rate of apoptosis in the *eff* mutant GSC clones by TUNEL assay as described in our previous study (Yang et al., 2007). We found that there was no evidence for increased apoptosis with *eff* mutant GSCs (see Fig. S3 in the supplementary material). We conclude that intrinsic activity of the *eff* gene is essential for GSC self-renewal.

To further determine whether extrinsic activity of *eff* is also required for GSC self-renewal, we generated a new transgene, P{*UASp-eff*}, in which expression of an *eff* cDNA was driven by the *UASp* promoter (Rorth, 1998). Using the Gal4-UAS system, we expressed Eff protein specifically in germ cells by combining P{*UASp-eff*} with somatic promoters, C587 or P{*ptc-gal4*}. As shown in Fig. 2I, we observed that the *eff* phenotype could not be rescued in *eff* mutant flies carrying the C587 and P{*UASp-eff*} transgenes. Similar results were observed in P{*ptc-gal4*}; *eff* ovaries, indicating that somatic expression of Eff could not rescue the loss of GSCs in *eff* mutants. However, when Eff protein was expressed specifically in germ cells by combining P{*UASp-eff*} with P{*nosP-gal4:vp16*}, we found that this combination of P{*nosP-gal4:vp16*} and P{*UASp-eff*} was sufficient to fully rescue the GSC loss in the *eff* mutant ovaries (Fig. 2J). Taken together, these data indicate that *eff* is an intrinsic, rather than extrinsic, factor for the control of GSC self-renewal.

eff is not required for *bam* transcriptional silencing

To determine whether *eff* is involved in BMP/Dpp-dependent *bam* silencing, we examined *bam* expression pattern in *eff* ovaries by anti-*bamC* antibody staining, and by scoring the expression of *bam* transcriptional reporter-P{*bamP-GFP*}. We noted that most germaria from the newly closed *eff* mutants contained a normal number (two or three) of GSCs carrying spectrosomes, which anchored between the GSCs and cap cells. The putative GSCs in the *eff* mutants were all, in common with the wild-type control, BamC and GFP negative (see Fig. S4A–D in the supplementary material), suggesting that *eff* is dispensable for *bam* silencing.

To test if the *eff* gene functions in a *bam*-independent manner, we generated *eff* and *bam* double-mutant flies. In *bam* single-mutant ovaries from 10-day-old flies we observed that all germ

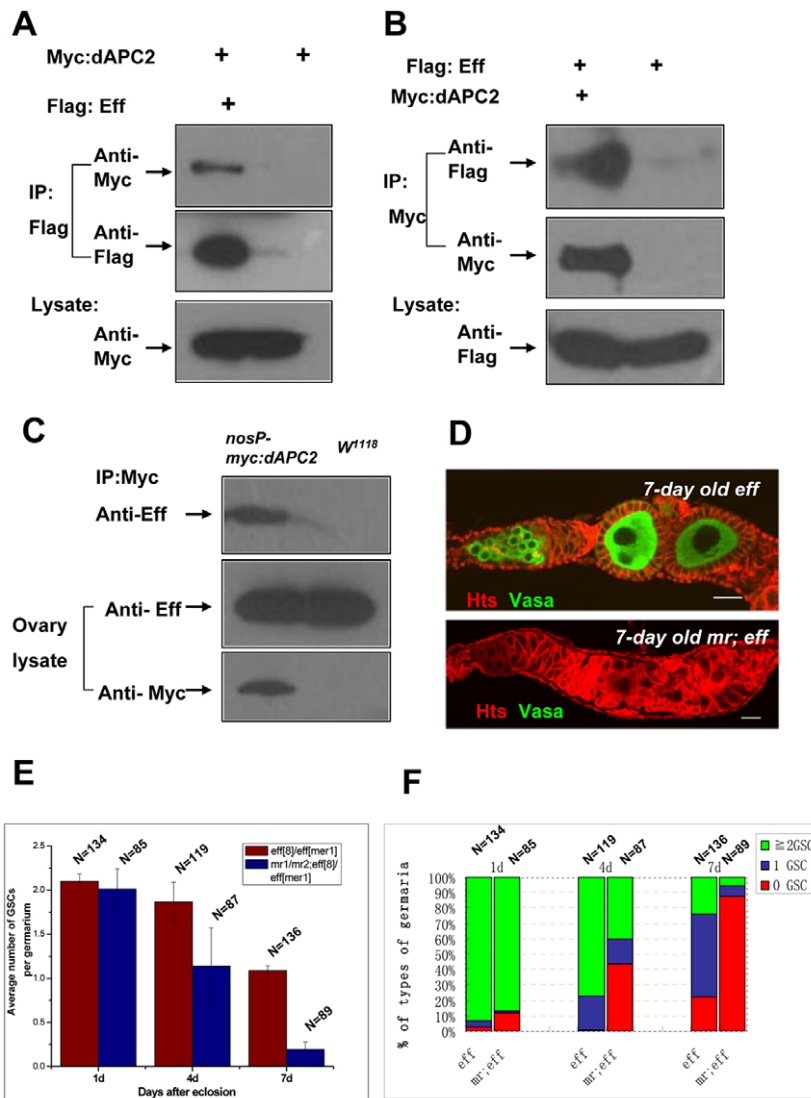


Fig. 3. Eff interacts with dAPC2 both in vitro and in vivo. (A) S2 cells were transfected with a combination of Myc epitope-tagged dAPC2 and Flag epitope-tagged Eff, or with Myc epitope-tagged dAPC2 alone as indicated. Forty-eight hours after transfection, lysates from transfected S2 cells were immunoprecipitated with anti-Flag M2 affinity gel, and western blots were performed to analyze the presence of Myc epitope-tagged dAPC2 as indicated. The results showed that dAPC2 specifically associated with Eff. (B) S2 cells were transfected with a combination of Flag epitope-tagged Eff and Myc epitope-tagged dAPC2, or with Flag epitope-tagged Eff alone. Forty-eight hours after transfection, lysates from transfected S2 cells were immunoprecipitated with anti-Myc antibody. Western blots were performed to analyze the presence of Flag epitope-tagged Eff as indicated. The results showed that Eff specifically associated with dAPC2. (C) Ovarian extracts from transgene P[*nosP-myc:dAPC2*] and *w¹¹¹⁸* flies were immunoprecipitated with anti-Myc antibody. Western blots were performed by anti-Eff and anti-Myc antibodies to analyze the presence of Eff and Myc:dAPC2 proteins, respectively, as indicated. (D) Ovaries dissected from 7-day-old *eff* single-mutant and *mr; eff* double-mutant females were stained with anti-Vasa and anti-Hts antibodies. Scale bars: 20 μ m. (E) A quantitative analysis of the number of GSCs in *eff* single-mutant or *mr; eff* double-mutant ovaries. The x-axis shows the day of examination post-eclosion, and the y-axis shows the average number of GSCs per germarium in different genotypes. (F) The percentage of germarium types that carried two or three GSCs, 1 GSC or 0 GSC (see Fig. S1 in the supplementary material) were measured in *eff* single-mutant and *mr; eff* double-mutant ovaries.

cells carried either spherical spectrosomes or associated spherical fusomes between two germ cells (see Fig. S4E in the supplementary material). By contrast, *bam, eff* double-mutant ovaries of the same age produced a distinct phenotype. Even though most of the germaria were morphologically tumorous, more than 90% ($n > 100$) of the germaria contained many germ cell clusters with highly branched fusomes (see Fig. S4F in the supplementary material), suggesting that these cells were undergoing differentiation. Thus, *eff* function is positioned downstream of or parallel to *bam* action.

Eff interacts both physically and genetically with dAPC2, a core component of APC/C complexes

Next, we attempted to understand the molecular mechanism underlying the action of Eff in GSCs by searching for Eff-interacting partners. Given that Eff functions as an E2 ubiquitin-conjugating enzyme, we carried out an E2/E3-based small-scale candidate screen by performing yeast two-hybrid experiments in which Eff was used as the bait to screen Eff-interacting E3 (data not shown). Notably, we found that, among the candidates, a protein named dAPC2, which is encoded by the *Drosophila morula* (*mr*) gene, could strongly interact with Eff protein (see Fig. S5A in the supplementary material). To confirm this yeast two-hybrid interaction, we

investigated whether Eff interacts with dAPC2 in *Drosophila* S2 cells by performing immunoprecipitation experiments. As shown in Fig. 3A,B, Eff and dAPC2 could co-immunoprecipitate each other in transfected S2 cells, suggesting that Eff and dAPC2 are physically associated. To test whether dAPC2 physically associates with endogenous Eff in germ cells, we generated a transgene, P[*nosP-myc:dAPC2*]. Results from co-immunoprecipitation showed that endogenous Eff physically associated with Myc:dAPC2 (Fig. 3C), supporting further the argument that Eff interacts with dAPC2 in germ cells. In mitosis, the anaphase-promoting complex/cyclosome (APC/C), a multisubunit complex that functions as an E3 ligase, plays important roles in ubiquitylating mitotic regulators such as mitotic cyclins and thus targets them for degradation by 26S proteasome. During this process, APC2, a cullin domain-containing protein, has been shown to function as a key mediator of APC/C complex activity (Tang et al., 2001). To test whether *eff* genetically interacts with *mr* (*dAPC2*) in the regulation of GSCs, we quantified the number of GSCs in both *eff* single-mutant and *mr; eff* double-mutant backgrounds at different time points (Fig. 3E,F). A weak allelic combination of *mr* (*mr¹/mr²*) (Kashevsky et al., 2002) exhibited no apparent defect in GSC maintenance (data not shown). However, as shown in Fig. 3D-F, *mr; eff* double-mutant ovaries showed more rapid GSC loss than the *eff* mutant alone, suggesting

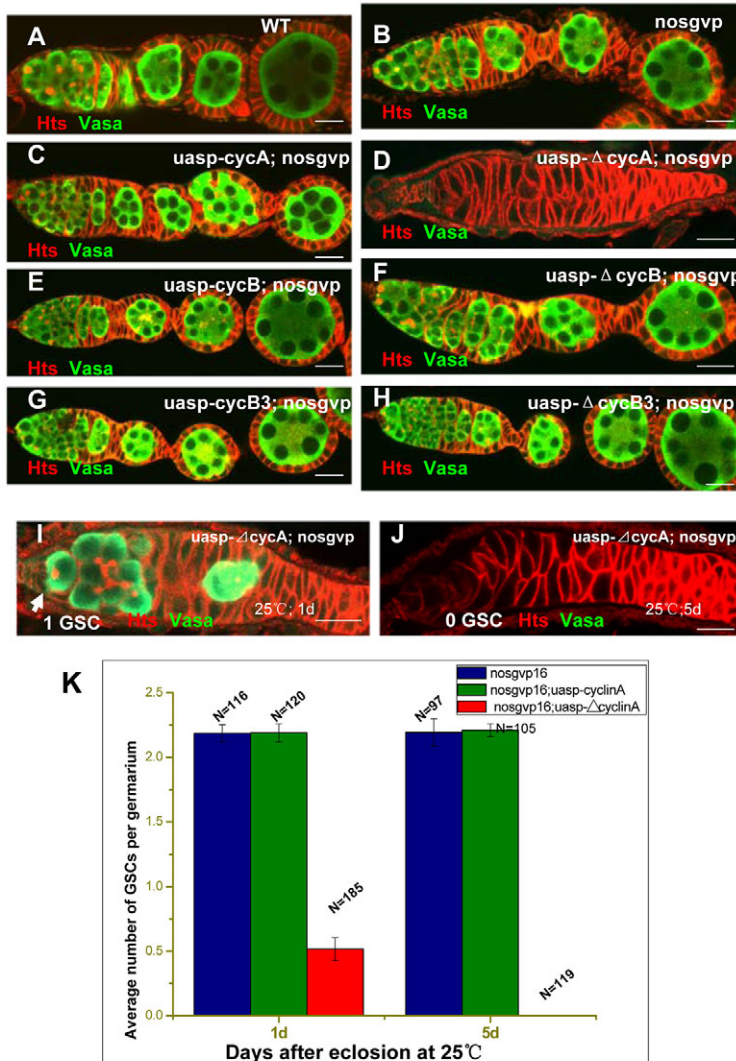


Fig. 4. Overexpression of a stable form of Cyclin A in germ cells leads to GSC loss. (A-H) Ovaries from wild type (A), nosP-Gal4:Vp16 (abbreviated as nosgvp here, B), nosgvp, UASp-cycA (C), nosgvp, UASp-ΔcycA (D), nosgvp, UASp-cycB (E), nosgvp, UASp-ΔcycB (F), nosgvp, UASp-cycB3 (G) and nosgvp, UASp-ΔcycB3 (H) were dissected and stained with anti-Vasa and anti-Hts antibodies. (I,J) Ovaries from flies overexpressing a stable form Cyclin A (nosgvp, UASp-ΔcycA) at day 1 (I) and day 5 (J) post-eclosion were stained with anti-Vasa and anti-Hts antibodies. Scale bars: 20 μm. (K) Quantitative analysis of the number of GSCs in ovaries overexpressing a stable form Cyclin A (ΔcycA). The x-axis shows the day of examination post-eclosion, and the y-axis shows the average number of GSCs per germarium at different days post-eclosion. Flies analyzed in I-K were shifted from 18°C to 25°C following eclosion.

that *dAPC2/mr* enhances the phenotype of GSC loss in *eff*. Together, our results demonstrate that Eff interacts both physically and genetically with dAPC2.

Ectopic expression of a stable form of Cyclin A leads to loss of GSCs

It has been shown previously that *eff* and mitotic *cyclins* (e.g. *cyclin A* and *cyclin B*) play opposite roles in the formation of the germline cyst (Lilly et al., 2000). Given that the APC/C complex is required for exit from mitosis by degradation of mitotic cyclins, and that Eff interacts with dAPC2 both in vitro and in vivo in the maintenance of GSCs, we hypothesized that Eff may act in concert with the APC/C complex to regulate GSC fate through controlling the proper turnover of cyclins. To test this hypothesis, we generated a series of transgenes in which different forms of *cyclin A*, *cyclin B* and *cyclin B3* were under the control of the UASp promoter. P{UASp-cycA}, P{UASp-cycB} and P{UASp-cycB3} encoded full-length Cyclin A, Cyclin B and Cyclin B3, respectively, whereas P{UASp-ΔcycA}, P{UASp-ΔcycB} and P{UASp-ΔcycB3} encoded truncated forms of Cyclin A, Cyclin B and Cyclin B3, respectively, which lacked the destruction box (D-box) at their N-terminus (Glotzer et al., 1991). The deletion of the D-box significantly increases the stability of the ΔCyclin proteins relative to the full-length cyclins (Sigrist et al., 1995). Using the UAS-GAL4 system, we used the germ-cell-specific promoter, P{nosP-gal4-

Vp16}, to overexpress each full-length and truncated cyclin in germ cells under standard culture conditions. As shown in Fig. 4A-H, overexpression of *cycA*, *cycB*, *cycB3*, Δ*cycB* or Δ*cycB3* exhibited no apparent defect in GSCs. However, overexpression of Δ*cycA* led to severe defects in germ-cell maintenance, in which most germaria were either empty or filled with very few differentiated germ cells (Fig. 4D). These observations indicated that proper turnover of Cyclin A may be important for GSC establishment and maintenance. To further confirm that overexpression of Δ*cycA* does indeed cause GSC loss, we took advantage of the temperature-dependent activity of Gal4:Vp16 to control the expression level of Δ*cycA*. Crosses were performed and the resulting progeny were initially raised at 18°C until adult eclosion, when they were shifted to the standard culture temperature (25°C). The number of GSCs per germarium from the control and overexpressing Δ*cycA* females was quantified at days 1 and 5 after eclosion. As shown in Fig. 4I-K, in contrast to controls, the number of GSCs per germarium decreased in the ovaries from the overexpressing Δ*cycA* females during the testing period. In addition, when we overexpressed Δ*cycA* in *bam* mutant background, we found that overexpression of Δ*cycA* did indeed lead to *bam* cell differentiation, which was similar to the phenotype of overexpression of Δ*cycA* in wild type (data not shown). Collectively, these data strongly support the argument that proper turnover of Cyclin A is important for GSC maintenance.

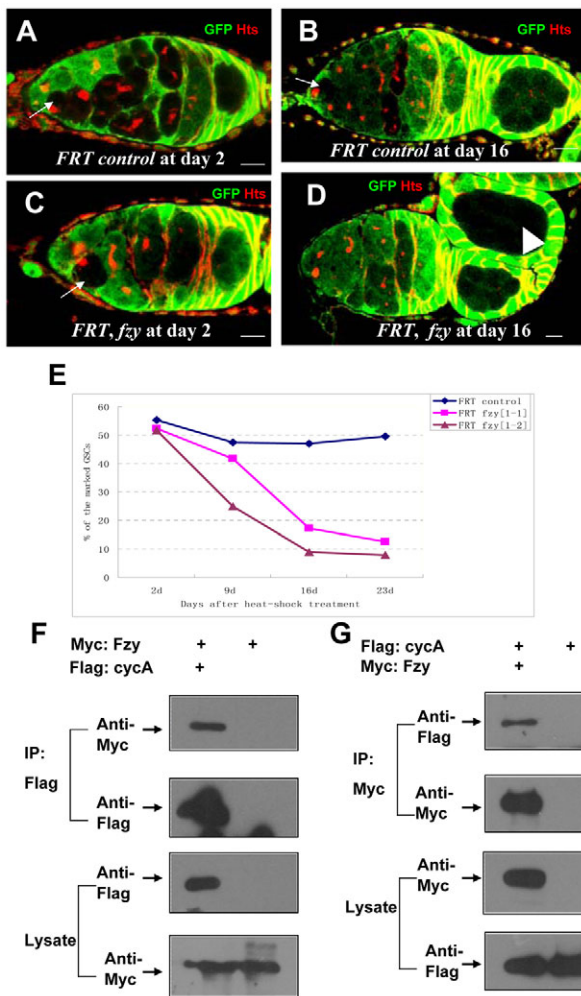


Fig. 5. The *cdc20/fzy* gene is essential for GSC maintenance. (A–D) GSC clones were induced by heat-shock treatment in adult female flies. Ovaries from FRT control flies (A,B) and FRT, *fzy* flies (C,D) were dissected at day 2 and day 16 following heat-shock treatment and stained with anti-GFP (green) and anti-Hts (red) antibodies; GSC clones (indicated by arrows) and cyst clones (indicated by arrowheads) were identified by the absence of GFP expression. Scale bar: 10 μ m. (E) Percentage of negatively GFP-marked GSC clones in FRT control and FRT, *fzy* null alleles at days 2, 9, 16 and 23 after heat-shock treatment. (F) S2 cells were transfected with a combination of Myc epitope-tagged Fzy and Flag epitope-tagged CycA, or with Myc epitope-tagged Fzy alone as indicated. Forty-eight hours after transfection, lysates from transfected S2 cells were immunoprecipitated with anti-Flag M2 affinity gel, and western blots were performed to analyze the presence of Myc epitope-tagged Fzy as indicated. The results showed that Fzy specifically associated with CycA. (G) S2 cells were transfected with a combination of Flag epitope-tagged CycA and Myc epitope-tagged Fzy, or with Flag epitope-tagged CycA alone. Forty-eight hours after transfection, lysates from transfected S2 cells were immunoprecipitated with anti-Myc antibody. Western blots were performed to analyze the presence of Flag epitope-tagged CycA as indicated. The results showed that CycA specifically associated with Fzy.

The key regulator of APC/C complexes, dCDC20, is essential for GSC maintenance

It has been shown that, in mitosis, mitotic cyclins are recognized by APC/C E3 ligase, which is activated by CDC20 protein (Zachariae and Nasmyth, 1999). In *Drosophila*, mutations in the *fizzy* gene, which

encodes the *Drosophila* CDC20 protein (dCDC20), lead to high-level expression of Cyclin A (Wolthuis et al., 2008). To assay whether dCDC20 forms a complex with Cyclin A, we performed co-immunoprecipitation experiments in S2 cells. As shown in Fig. 5F,G, in agreement with previous findings (Wolthuis et al., 2008), dCDC20 forms a complex with Cyclin A. However, no apparent association was observed between Δ Cyclin A and dCDC20 proteins (data not shown), suggesting that dCDC20 associates with full-length Cyclin A through the D-box of Cyclin A. To further explore the biological function of *dCDC20/fzy* in GSCs, we used the FLP/FRT method to generate *dCDC20/fzy* mutant GSC clones, and traced the rates of GFP-negative *dCDC20/fzy*^{-/-} GSC clones for 3 weeks AHST. As shown in Fig. 5A–E, for FRT controls, the rates of wild-type GSC clones declined slightly from 55.4 to 47.1% during the testing period. By contrast, the rate of *dCDC20/fzy*^{-/-} GSC clones dramatically reduced from 51.8 to 9.0% under the same conditions, suggesting that, in addition to Eff being an important regulator of the APC/C complex, dCDC20 is also essential for the maintenance of GSCs.

Eff is required for the ubiquitylation and degradation of Cyclin A

Based on the observations described above, we hypothesized that Eff acts in conjunction with the APC/C complex to regulate the proper turnover of Cyclin A in GSCs. To test this hypothesis, we first tested whether Eff is sufficient for the ubiquitylation of Cyclin A in vitro. As shown in Fig. 6A,B, a ubiquitylation reaction in which Eff functioned as E2 in concert with the APC2 and APC11 complex, which has been shown to represent the minimal E3 ligase activity of APC (Tang et al., 2001), was sufficient to catalyze the ubiquitylation of Cyclin A. In the absence of Eff, the APC2 and APC11 complex alone showed the background activity. We then investigated whether Eff is involved in the ubiquitylation of Cyclin A in vivo. As shown in Fig. 6C,D, strong ubiquitylation of Cyclin A was observed when S2 cells were transfected with epitope-tagged Cyclin A and ubiquitin. However, the conjugation of HA-ubiquitin to Cyclin A was markedly reduced when the cells were also treated with the dsRNA of either *eff* (Fig. 6D) or *dAPC2* (see Fig. S5B in the supplementary material), suggesting that, as with *dAPC2*, *eff* is also important for the ubiquitylation of Cyclin A. To test whether *eff* is involved in the stability of Cyclin A, we compared the expression of Cyclin A in the *bam* single-mutant ovaries with that in *eff*, *bam* double-mutant ovaries by a western blot assay. As shown in Fig. 6E, the expression of Cyclin A in *eff*, *bam* mutant ovaries was higher than that in *bam* single-mutant ovaries. We then performed immunostaining experiments to examine the expression of Cyclin A in dividing *eff* GSC clones (4 days after clonal induction), which carried short-bar fusomes. As shown in Fig. 6F, 64.3% ($n=28$) of dividing *eff* GSC clones have strong Cyclin A expression, whereas only 39.5% ($n=38$) of dividing control GSC clones expressing Cyclin A were observed. Taken together, these results support the view that *eff* plays a role in the regulation of Cyclin A stability.

Overexpression of full-length Cyclin A accelerates GSC loss in *eff* mutant ovaries

Given that Cyclin A is a substrate of the Eff protein, and that the fate of GSCs is sensitive to expression of the stable form of Cyclin A (Δ CycA) rather than the corresponding full-length Cyclin A, we proposed that GSCs should be sensitive to full-length Cyclin A when *eff* activity is compromised or reduced. To substantiate this hypothesis, we quantified the number of GSCs in *eff* ovaries overexpressing full-length Cyclin A. As shown in Fig. 7, overexpression of Cyclin A in wild-type ovaries resulted in no apparent defect in GSC maintenance; the average number of GSCs per germarium was 2.2 ($n>100$), which

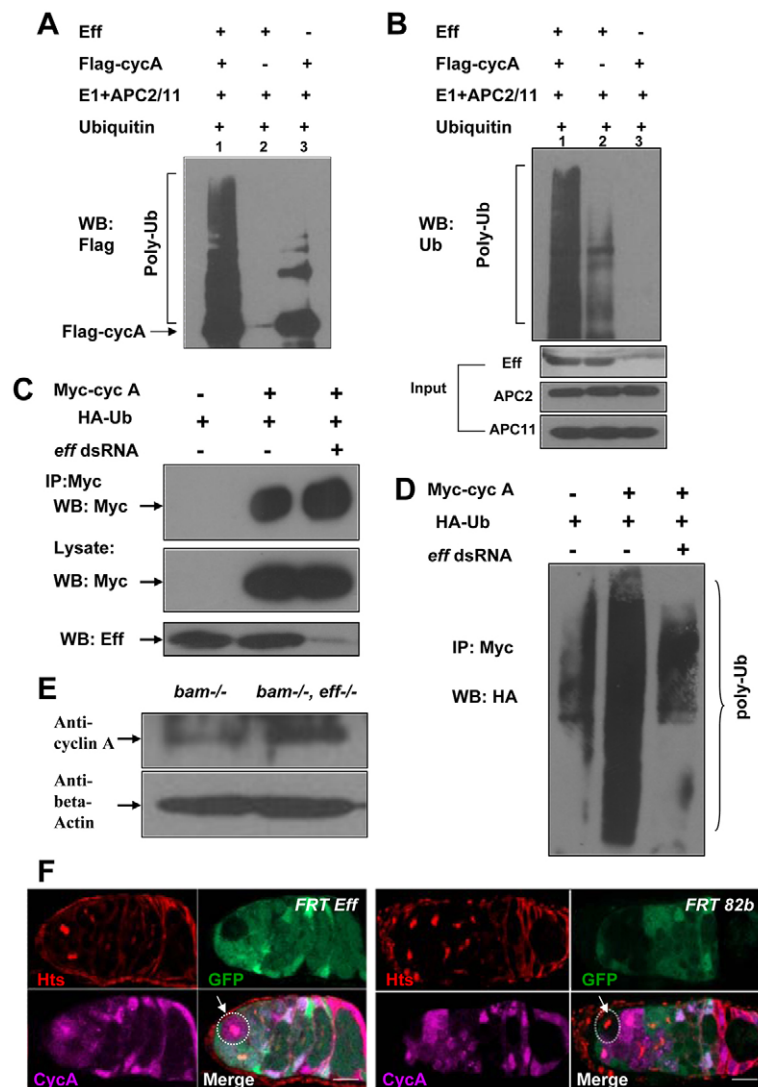


Fig. 6. Eff is required for Cyclin A ubiquitylation and stability. (A,B) An in vitro ubiquitylation reaction was reconstituted with a complex that contained E1, Eff, APC11, APC2 and the putative substrate, Cyclin A, as indicated (lane 1). In lane 2 and lane 3 the ubiquitylation reaction lacked Cyclin A or Eff, respectively. Western blots were performed to analyze ubiquitylation products using the antibodies indicated. (C,D) For in vivo ubiquitylation assay, S2 cells were transfected with DNA combinations including Myc epitope-tagged Cyclin A and/or HA epitope-tagged Ub (Ubiquitin) with or without *eff* dsRNA treatment as indicated. Western blots were performed to analyze the ubiquitylation product of Cyclin A. (E) Ovarian extracts were probed with anti-Cyclin A antibodies. The left lane contains an extract from *bam* mutant ovaries and Cyclin A protein is indicated by an arrow. The right lane contains an extract from *bam* and *eff* double-mutant ovaries and shows a significant increase in the Cyclin A protein levels. Equal loading of the extracts was confirmed by western blotting with anti-beta actin antibody as a control. (F) GSC clones were induced by heat-shock treatment, and ovaries from FRT control flies and FRT, *eff* flies (as indicated) were dissected at day 4 following heat-shock treatment and stained with anti-Cyclin A (pink) and anti-Hts (red) antibodies. Dividing GSC clones (indicated by arrows) were identified by the absence of GFP expression and carrying short-bar fusomes. Scale bars: 10 μ m.

was similar to that in wild-type ovaries. However, we found that the overexpression of full-length Cyclin A strongly enhanced the *eff* phenotype in GSCs. For example, in 6-day-old *eff* single-mutant ovaries, about 27.9 and 31.4% of germaria contained 1 GSC or no GSCs, respectively, and the average number of GSCs per germarium was 1.2 ($n=204$). However, for age-matched *eff* mutant overexpressing full-length Cyclin A, about 93.3% of germaria did not possess GSCs, and the average number of GSCs was only 0.08 ($n=104$). These data suggest that GSCs are much more sensitive to overexpression of full-length Cyclin A in the *eff* background than in the wild-type background, indicating that *eff* interacts with *cyclin A* in vivo. These findings are consistent with our hypothesis that Eff functions in concert with the APC/CDC20 complexes to regulate GSC fate, probably through controlling the proper degradation of Cyclin A.

DISCUSSION

In this study, by using a combination of genetic and biochemical strategies, we demonstrate that the Eff/APC-mediated ubiquitylation pathway plays an important role in the regulation of GSCs in *Drosophila* ovary, probably through controlling the degradation of mitotic Cyclin A. Our results reveal a new role for the tight regulation of mitotic cyclins in the fate determination of stem cells, implicating that a similar mechanism may be conserved in mammals.

The Eff-mediated ubiquitylation pathway represses GSC differentiation in a Bam-independent manner

The ubiquitin-mediated proteolysis mechanism, which is evolutionarily conserved for the regulation of protein turnover, has been shown to play important roles in numerous biological processes, such as the cell cycle, pattern formation and tissue homeostasis (Harper et al., 2002; Tang et al., 1997; Zachariae and Nasmyth, 1999). *Drosophila* Eff, which was initially identified as a class I E2 ubiquitin-conjugating enzyme encoded by the *eff* gene, has been shown to be involved in several cellular and developmental processes, including chromosome segregation, chromatin remodeling and protection against cell death (Cenci et al., 1997; Ryoo et al., 2002). In this study, we found that loss of *eff* function results in the depletion of GSCs, revealing a new role for *eff* in the regulation of GSC fate. Using germline clonal analysis and rescue experiments, we further defined that the *eff* gene is an intrinsic, rather than extrinsic, factor for the maintenance of GSCs. Previous studies have shown that Eff is involved in the *rpr*-induced apoptosis pathway through a physical interaction with DIAP1 that stimulates DIAP1 auto-ubiquitylation (Ryoo et al., 2002). Therefore, it is possible that loss of GSCs in *eff* mutants may be due to reduced viability of GSCs. Our results

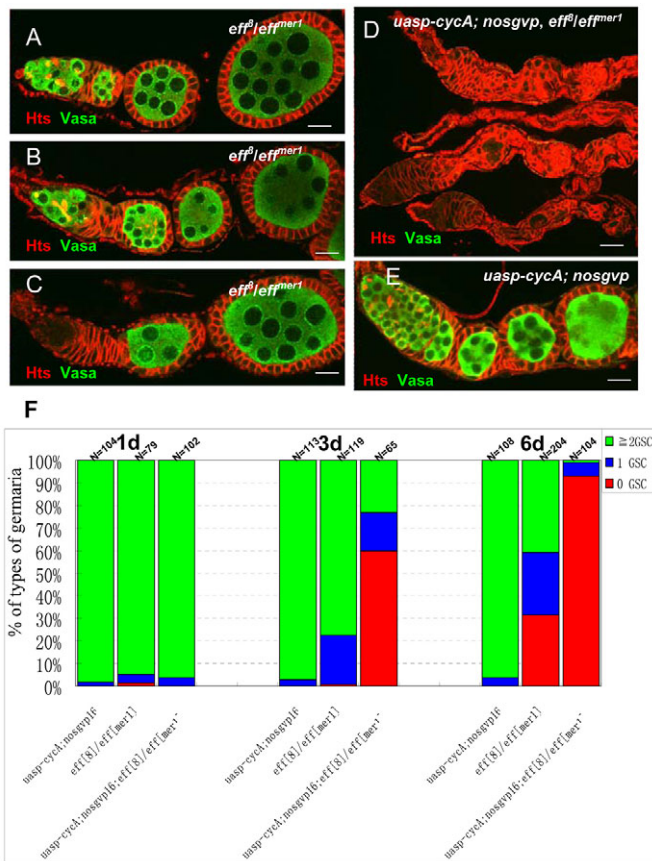


Fig. 7. Overexpression of the full-length version of Cyclin A accelerates GSC loss in *eff* mutant ovaries. (A–E) Ovaries from 6-day-old *eff^Δ/eff^{mer1}* mutant (A–C), UASp-*cycA*; *nosgvp*, *eff^Δ/eff^{mer1}* (D) and UASp-*cycA*; *nosgvp* (E) female flies were stained with anti-Vasa and anti-Hts antibodies. Scale bars: 20 μ m. (F) Quantitative analysis of the percentage of germaria types in *eff* mutants, and overexpression of full-length Cyclin A in wild-type or the *eff* mutant background are indicated. The x-axis shows the day of examination post-eclosion, and the y-axis shows the percentage of types of germaria in different genotypes.

clearly show that *eff^{-/-}* GSCs undergo differentiation rather than apoptosis, thus supporting the idea that the role of *eff* is to repress the premature differentiation of GSCs.

The biochemical role of Eff in the regulation of GSCs

In the ubiquitin pathway, E2 conjugating enzymes have much lower specificity compared with E3 ligases. Certain E2s are known to function together with distinct type E3 ligases for substrate ubiquitylation and degradation. Eff is involved in protein degradation mediated by various RING finger-containing E3 ligases [e.g. Sina, Neur and DIAP (Iap2 – FlyBase)], and regulates several signaling transduction pathways (Kuo et al., 2006; Ryoo et al., 2002; Tang et al., 1997). As Eff plays a role downstream of, or parallel to, *bam* function, it is important to know what biochemical functions Eff performs in the regulation of GSCs. In this work, we provided biochemical evidence showing that Eff not only physically interacts with the dAPC2 protein, but is also crucial for the ubiquitylation and degradation of Cyclin A. Moreover, our genetic analyses revealed that *eff* interacts with both *dAPC2* and *cyclin A* with respect to the

regulation of GSCs. In addition, we also show that dCDC20/Fzy, a key regulator of APC/C complexes, is involved in GSC regulation. Together, these data strongly support our model in which Eff facilitates the E3 ligase function of APC/CDC20 to ensure the self-renewal of GSCs.

Early studies in *Xenopus* and clam extracts demonstrated that both UBC4, a homolog of Eff, and UBCx/E2-C equally supported APC-mediated ubiquitylation reactions in vitro (Aristarkhov et al., 1996; King et al., 1995; Yu et al., 1996). However, an in vivo study showed that these two classes of E2 are not functionally equivalent but exhibit distinct functions in mitotic cyclin degradation, suggesting that different E2 family members probably execute distinct functions (Seino et al., 2003). The *Drosophila* ortholog of UBCx/E2-C, Vihar E2, has been reported to be involved in Cyclin B degradation during the metaphase-anaphase transition (Mathe et al., 2004). In this work, we present both genetic and biochemical evidence that Eff, the *Drosophila* homolog of UBC4, is essential for Cyclin A degradation in GSCs. Because the mitosis-related ubiquitin-conjugating enzyme, Vihar E2, is involved in APC/C-mediated ubiquitylation that potentially regulates Cyclin A degradation, it would be interesting to determine whether and/or how different E2 family members (e.g. Eff and Vihar E2) coordinately support specific APC-mediated mitotic cyclin destruction with respect to GSC regulation.

The relationship between the proper cell mitosis mediated by mitotic cyclins and the maintenance of stem cells

It has been shown that APC/C activity is required for the asymmetric localization of Miranda and its cargo proteins during neuroblast division (Slack et al., 2007). In *Drosophila* ovary, previous studies have demonstrated that Cyclin B plays important roles in GSC division and is essential role for GSC maintenance (Hsu et al., 2008; Wang and Lin, 2005). However, it still remains unexplored whether the tight regulation of cyclins is also required for the fate determination of GSCs. The regulatory roles of mitotic cyclins at the cellular level during mitosis have been explored in detail (Parry and O'Farrell, 2001). It has been reported that the sequential degradation of Cyclin A, Cyclin B and Cyclin B3 completes mitotic exit, which is mediated by APC/CDC20 in early M phase and by APC/Cdh1 during late M phase (Zachariae and Nasmyth, 1999). Interestingly, the expression of stable forms of each cyclin leads to distinct mitosis defects, suggesting that the degradation of distinct mitotic cyclins is responsible for specific steps of mitosis. However, the biological basis for the control of the cyclin destruction remains elusive. Given that the APC-mediated pathway plays important roles in the proper cell mitosis, as loss of function of components in the pathway results in upregulation of mitotic cyclins that cause mitosis delay/or arrest (Dawson et al., 1995; Harper et al., 2002; Sigris et al., 1995), the question becomes whether the maintenance of GSCs requires the proper cell mitosis mediated by the regulation of mitotic cyclins. In this study, we showed that the forced expression of a stable form of Cyclin A leads to defects in GSC maintenance, suggesting that blocking mitotic progression may force germline stem cells to precociously differentiate, essentially altering their fate. Although the forced expression of a stable form of Cyclin B or Cyclin B3 does not give rise to any apparent defect in GSCs, one explanation is that stabilized Cyclin A may block cell-cycle progression more severely than the other stabilized Cyclins and prolonged M phase might be unfavorable for stem cell maintenance.

Taken together, our findings support a mechanism underlying the fate determination of stem cells that is linked to the control of the proper cell mitosis. As the control of degradation of mitotic cyclins

is evolutionarily conserved between flies and mammals, it would be interesting to also determine whether the control of proper cell mitosis is important for the maintenance of stem cells from other organisms, including mammals.

Acknowledgements

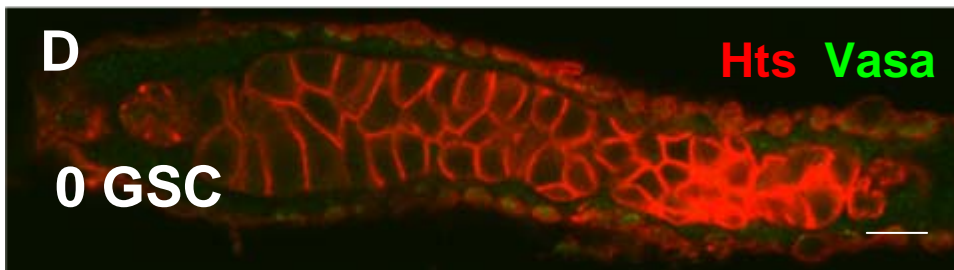
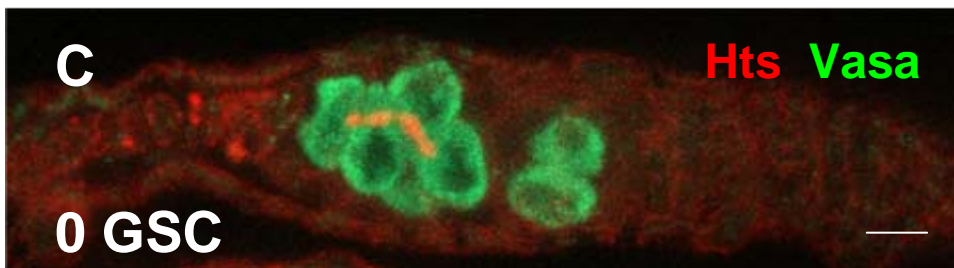
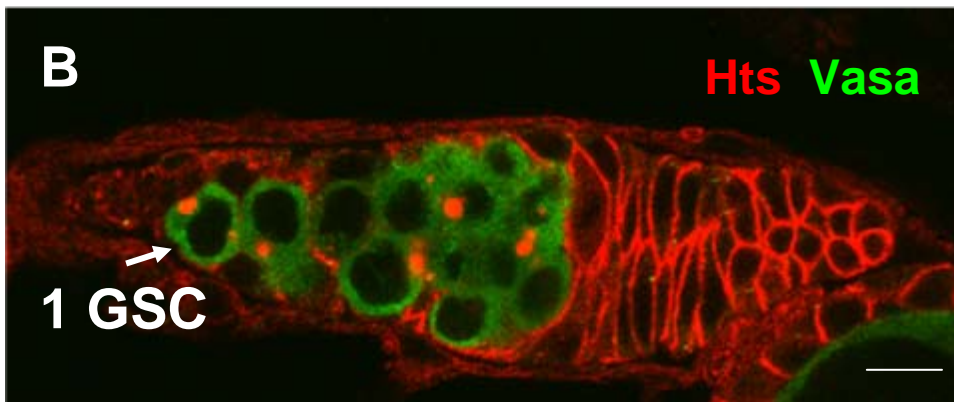
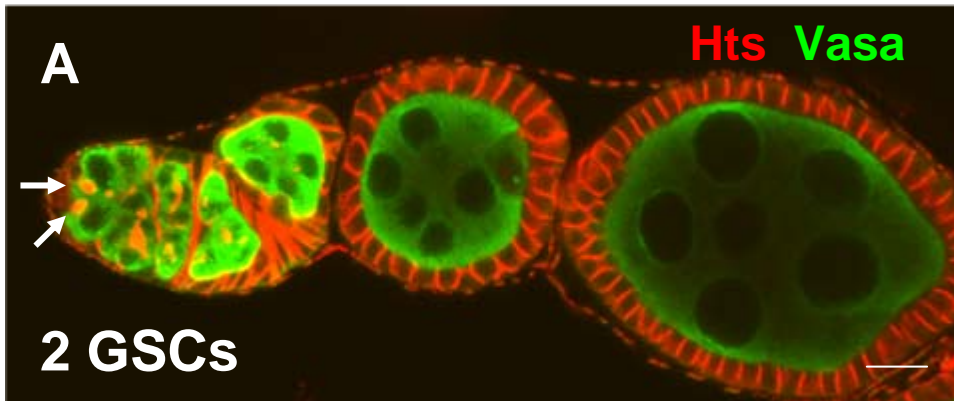
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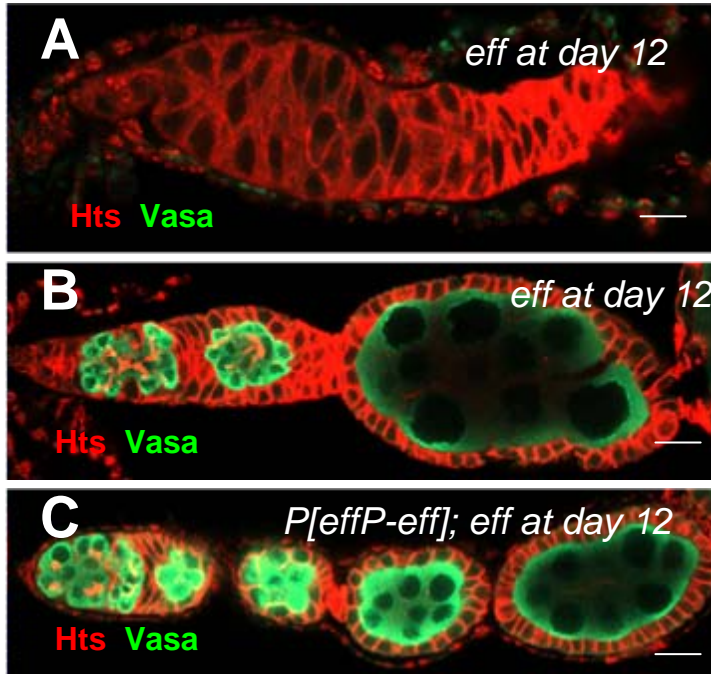
Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/24/4133/DC1>

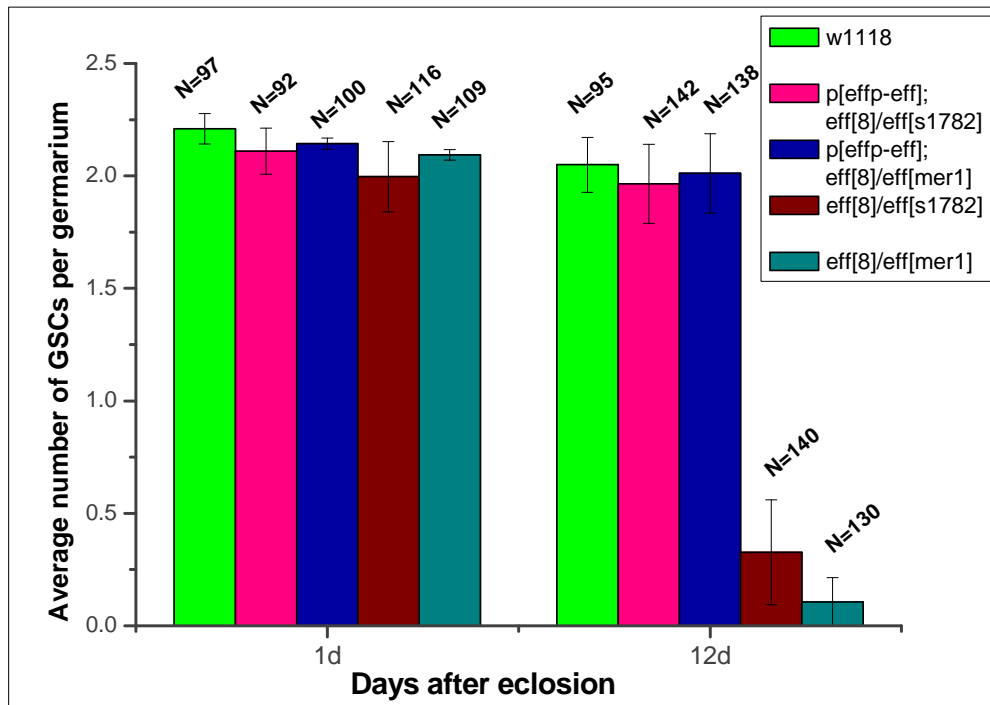
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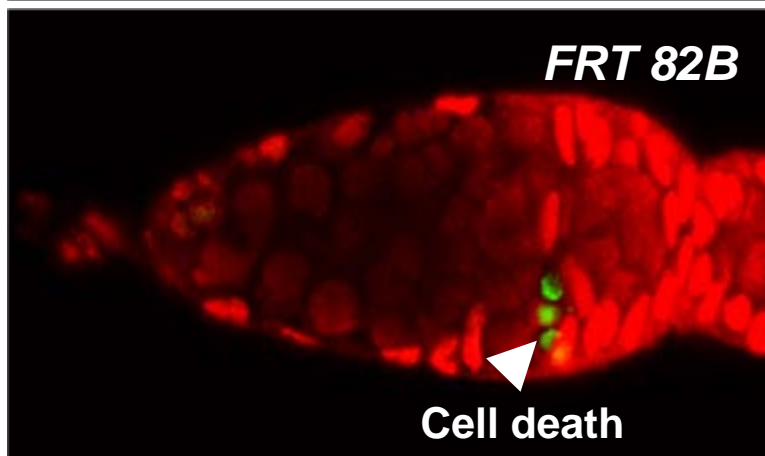
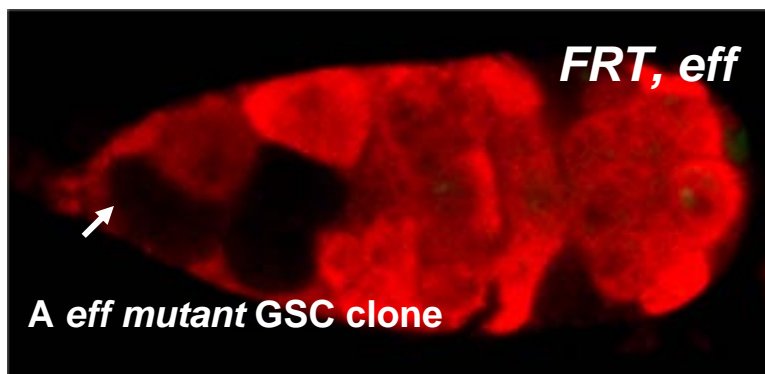
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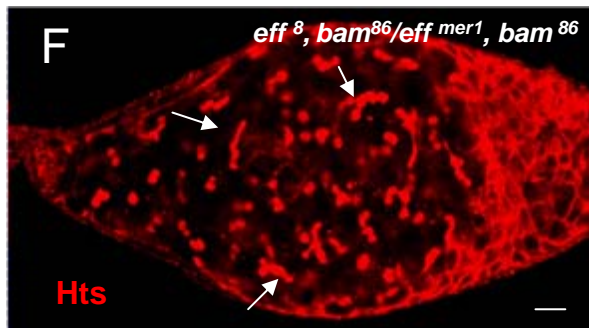
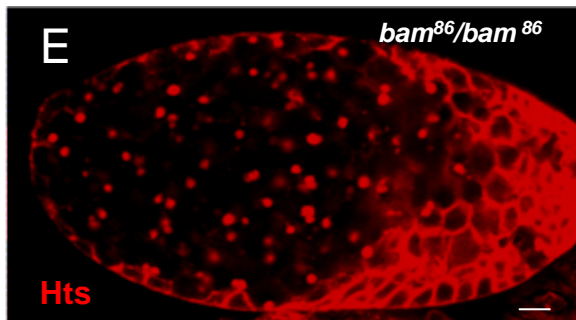
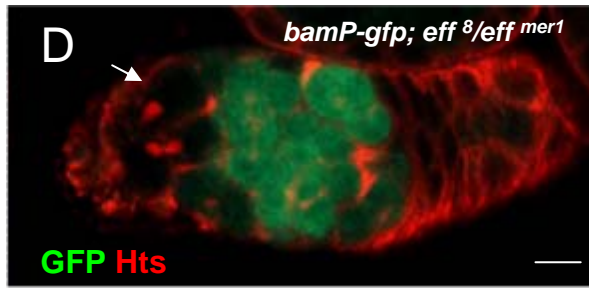
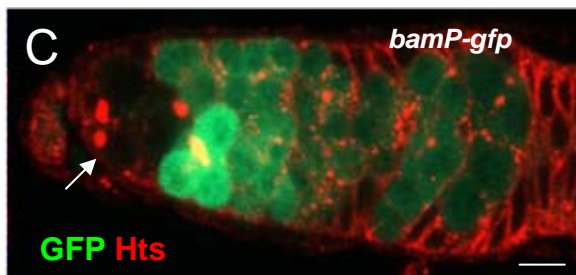
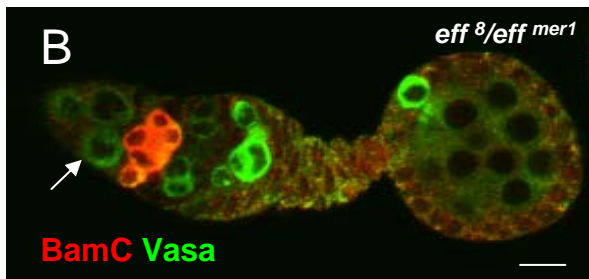
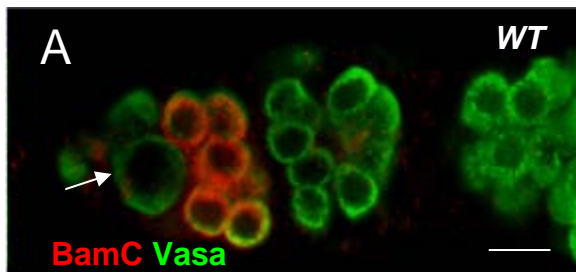




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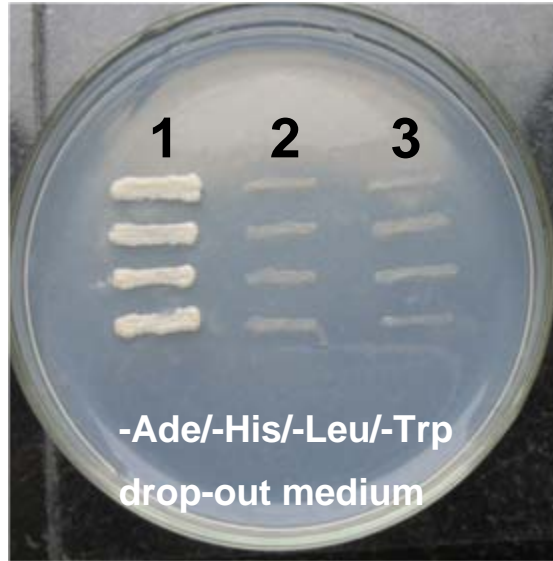






A

pGBKT7-Eff	+	+	-
pGBKT7	-	-	+
pACT2-Morula	+	-	+
pACT2	-	+	-

**B**

HA-Ub	+	+	+
Flag-cycA	-	+	+
mr dsRNA	-	-	+

IP: flag
WB: HA



IP: flag
WB: flag →

