Somatic signaling mediated by \textit{fs(1)Yb} is essential for germline stem cell maintenance during \textit{Drosophila} oogenesis

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**SUMMARY**

\textit{Drosophila} oogenesis starts when a germline stem cell divides asymmetrically to generate a daughter germline stem cell and a cystoblast that will develop into a mature egg. We show that the \textit{fs(1)Yb} gene is essential for the maintenance of germline stem cells during oogenesis. We delineate \textit{fs(1)Yb} within a 6.4 kb genomic region by transgenic rescue experiments. \textit{fs(1)Yb} encodes a 4.1 kb RNA that is present in the third instar larval, pupal and adult stages, consistent with its role in regulating germline stem cells during oogenesis. Germline clonal analysis shows that all \textit{fs(1)Yb} mutations are soma-dependent. In the adult ovary, \textit{fs(1)Yb} is specifically expressed in the terminal filament cells, suggesting that \textit{fs(1)Yb} acts in these signaling cells to maintain germline stem cells. \textit{fs(1)Yb} encodes a novel hydrophilic protein with no potential signal peptide or transmembrane domains, suggesting that this protein is not itself a signal but a key component of the signaling machinery for germline stem cell maintenance.

Key words: Stem cell, Germline, Somatic signaling, \textit{fs(1)Yb}, Self-renewal, Asymmetric division

**INTRODUCTION**

Stem cells possess the unique ability to self-renew and to generate many differentiated daughter cells. Stem cells exist in a wide range of organisms, from hydra and insects to mammals and plants (reviewed in Lin, 1997, 1998). In mammals, stem cells play a central role in the formation and maintenance of many organs and tissues, such as epithelia, blood, the nervous system and the germline (reviewed in Potten, 1997; Morrison et al., 1997; Lin and Schagat, 1997; Lin, 1997). However, stem cells exist in low abundance, lack specific markers, and are extremely sensitive to manipulations in vitro. As a result, their identity and their self-renewing division mechanism remains largely unknown.

A well-defined model for studying stem cell function is the \textit{Drosophila} ovary, an organ composed of approximately 17 repeating units called ovarioles (Fig. 1A). At the apical tip of each ovariole resides 2-3 germline stem cells (GSCs) in a specialized structure called the germarium. GSCs were first proposed by Brown and King (1962, 1964), later confirmed by germline clonal analysis (Schüpbach et al., 1978; Wieschaus and Szabad, 1979), and directly identified by laser ablation and cytological analyses (Lin and Spradling, 1993, 1995, 1997; see Fig. 1A,B). The GSC divides asymmetrically in a self-renewing fashion to produce a daughter stem cell and a differentiated daughter called the cystoblast (Lin and Spradling, 1997; Deng and Lin, 1997; Fig. 1B,C). The cystoblast undergoes four more divisions with incomplete cytokinesis to generate a 16-cell cyst. Concurrently, the somatic stem cells (SSC) in region 2a (Margolis and Spradling, 1995) divide to produce follicle cells, which invaginate and surround each cyst to form an egg chamber. The egg chamber then buds off the germarium, joining other developing egg chambers in the ovariole (reviewed in King, 1970; Mahowald and Kambysellis, 1980; Spradling, 1993).

GSCs originate from embryonic germline precursor cells called pole cells (reviewed in King, 1970). Pole cells undergo complicated migration during embryogenesis (Warrior, 1994; Jaglarz and Howard, 1994). Approximately 12 pole cells reach each of the two gonadal sites in the abdominal region (segment A5), where they join the somatic gonadal cells to form an embryonic gonad. During larval development, both germline and somatic cells in the female gonad proliferate without substantial differentiation, so that by the third instar larval stage there are approximately 60 pre-GSCs in each larval ovary, occupying the medial portion of the gonadal disc, flanked by apical and basal somatic cells. Ovary differentiation begins at the third instar larval stage. At this time, one group of the anterior somatic cells differentiate into terminal filament cells, suggesting that \textit{fs(1)Yb} acts in these signaling cells to maintain germline stem cells. Another group migrates between terminal filament cells, partitioning pre-GSCs into approximately 17 ovarioles and establishing SSCs, while a third group migrates to the posterior to form the basal stalk cells of the ovariole (King, 1970; Godt and Laski, 1995). Concomitant to ovarian differentiation, cystoblasts and early cysts are produced, implying that these pre-GSCs have become mature stem cells capable of self-renewing asymmetric divisions (King, 1970).

The self-renewing division of stem cells requires both intracellular mechanisms and cell-cell communications. Although the intracellular mechanisms are not well characterized...
in mammalian stem cells, they have been illustrated in Drosophila neuroblasts and ovarian GSCs (reviewed in Knoblich, 1997; Lin and Schagat, 1997; Lin, 1997). In Drosophila neuroblasts, differentially segregated intracellular determinants, such as the Numb and Prospero proteins, play a key role in stem-like asymmetric divisions (Rhyu et al., 1994; Hirata et al., 1995; Knoblich et al., 1995; Doe, 1996). In Drosophila ovarian GSCs, the intracellular mechanism involves a cytoplasmic organelle termed the spectrosome that is located in the apical cytoplasm of GSCs adjacent to the terminal filament cells (Lin et al., 1994; Lin and Spradling, 1995). The spectrosome anchors one pole of the mitotic spindle to define the orientation of GSC division (Lin and Spradling, 1997; Deng and Lin, 1997). Meanwhile, it may also serve as a localization mechanism for the differential segregation of cell fate determinants (Gregorio et al., 1992; McKearin and Ohlstein, 1995). The GSC intracellular mechanism also involves the Pumilio (Pum) protein, which acts cell-autonomously to maintain GSCs during oogenesis (Lin and Spradling, 1997; Forbes and Lehmann, 1998; M. Parisi and H. Lin, unpublished data), while the BAM protein appears to be required in the cystoblast for its further differentiation (McKearin and Spradling, 1990; McKearin and Ohlstein, 1995; Ohlstein and McKearin, 1997).

Considerable progress has also been made towards understanding cell-cell interaction mechanisms involved in regulating stem cell division. In mammalian systems, the proliferation and differentiation of hematopoietic, epidermal and neurogenic stem cells requires extrinsic signals that act on specific stem cell receptors (Metcalf, 1989; Jones et al., 1995; Morrison et al., 1997). In both invertebrates and vertebrates, the proliferation of germline cells during gametogenesis is controlled by adjacent somatic cells (for reviews, see Lin, 1997, 1998). Some of these germline cells possess stem cell properties. In the Drosophila ovary, the dpp signaling pathway is required for GSC division and maintenance (Xie and Spradling, 1998). Although it is not known whether dpp acts as a paracrine in the somatic or germline cells or as an autocrine in GSCs to control GSC division, the terminal filament has been implicated in regulating the division of both GSCs and SSCs (Lin and Spradling, 1993; Forbes et al., 1996a,b). Recently, the terminal filament has been further shown to control GSC maintenance (Cox et al., 1998).

This paper reports that somatic signaling mediated by the fs(1)Yb (Yb) gene is essential for the maintenance of GSCs during oogenesis. Yb was originally identified as a female sterile complementation group in the zeste-white region of the X chromosome (Young and Judd, 1978; Mohler and Carroll, 1984). Johnson et al. (1995) showed that Yb mutations affect the proliferation and differentiation of follicle cells in a soma-dependent manner. Here we report that Yb plays an essential role in GSC self-renewal; loss of Yb function leads to failure in GSC maintenance. Our germline clonal analysis suggests that this function of Yb is soma-dependent. We have cloned the Yb gene; our molecular characterization further reveals that Yb is specifically expressed in terminal filament cells to control GSC maintenance. This study reveals the essential role of the somatic signaling mechanism in the maintenance of stem cells in the germline.

MATERIALS AND METHODS

Drosophila strains and culture

All fly strains were raised at room temperature on standard yeast-containing molasses/agar medium. The Yb1 mutation was initially generated in an X chromosome marked by y2 w67 spl and sn1 (Young and Judd, 1978). Given that spl is an allele of Notch (N), and that Notch interacts with Yb (Johnson et al., 1995), we generated a new y2 fs(1)Yb1 w67 chromosome by replacing spl and sn1 mutations with wild-type alleles. This chromosome, denoted as the Yb3co (co, cross-out) chromosome, was used for all the analyses reported here. The y2 Yb3co chromosome and the y2 Yb6 cv v f chromosome (Mohler and Carroll, 1984; Johnson et al., 1995) were kindly provided by Dr Rodney Nagoshi for our analysis. The w; A2-3 Sb eatM6 e embryos were used for P-element-mediated transformation (Roberson et al., 1988). The ovoD1 FRT101; hsFLP strain was obtained from Dr N. Perrimon (Chou and Perrimon, 1992).Oregon R (Ore-R) served as the wild-type strain for all experiments. Sex-specific populations of larvae, pupae and adults were generated from a cin y w strain for northern blotting analysis as described in DiBenedetto et al. (1987).

Immunocytochemistry and confocal microscopy

The Yb mutant larval ovaries and their wild-type control ovaries were dissected en masse from late third instar larvae produced by a cross between Yb/FM6 females and Yb3 males, so that the mutant ovaries contribute to 50% of total ovaries. The larval and adult ovaries were

Fig. 1. The Drosophila ovary and asymmetric GSC division. (A) A pair of Drosophila ovaries. Each ovary is composed of 16-20 ovarioles with germa ria at their apical tips. (B) A germarium with a germline stem cell (gsc) at the apical end, contacting the terminal filament (tf) and the basal cells (bc). Basal to the stem cell is a cystoblast (cb). The cystoblast undergoes four rounds of incomplete mitosis to generate a 16-cell cyst. These mitotic events occur in the apical portion of the germarium, designated region 1. The somatic stem cells (ssc) produce daughters which envelope the cyst in region 2 to form a stage-1 egg chamber that defines region 3. This egg chamber is composed of cl umber epithelial follicle cells (fc), 15 nurse cells (nc) and a posteriorly located oocyte (o). The germarium is covered by the epithelial sheath (sh). (C) Self-renewing asymmetric division of a GSC generates a daughter GSC and a cystoblast. A and B are modified from King (1970).
dissected, fixed and stained according to Lin et al. (1994). Polyclonal rabbit anti-VASA antisera (Hay et al., 1990) was used at 1:2000 dilution to mark germline cells. Monoclonal mouse anti-1B1 (Zaccaci and Lipshitz, 1996) was used at 1:1 dilution to outline somatic cells and to mark spectrosomes and fusomes. Polyclonal rabbit anti-ARM N2 antibody (Riggleman et al., 1990) was used at 1:50 dilution to label basal terminal filament cells. Polyclonal rat anti-BAB2 antibody (Godd et al., 1993) and anti-EN monoclonal antibody mAb49 (Patel et al., 1989) were used at a dilution of 1:1600 and 1:1, respectively, to label terminal filament cells. In addition, ovaries were stained with the DNA-specific dye DAPI to reveal nuclei. For primary antibody detection, FITC-conjugated Affipure™ donkey anti-rabbit antibody and LSRC-conjugated goat anti-mouse secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Inc., and used at 1:200 dilution. Epifluorescence and Nomarski microscopy was performed on a Zeiss Axioplan microscope using appropriate filter sets. Images from the Zeiss Axioplan were captured using a Star-l cooled CCD camera (Photometrics, Inc.), collected using IP Lab software, and processed using the Adobe Photoshop™ program.

P-element-mediated germline transformation rescue experiments

Genomic constructs were generated in pW5 and pCaSpeR4 germline transformation vectors (Klemenz et al., 1987; Pirrotta, 1988). w; Δ2-3, se/TM6 embryos were injected with 400-500 ng of DNA per embryo by the standard germline transformation method (Spradling and Rubin, 1982). Red-eyed transformants were identified by crossing the adults developed from the injected embryos to w1118 flies. The transgenic germline was then introduced into Yb and Ya backgrounds as described by Lin and Wolfrer (1991). The resulting y2 Yb1 w+/y2 Yb1 w/+; P+ females were used for fertility tests, where P+ designates the presence of the transgenic construct on an autosome. Their y2 Yb1 w+/y2 Yb1 w/; P+ or P− and y2 Yb1 w+/y2 Yb1 w/; P− siblings were used as positive and negative controls, respectively. The fertility test is carried out as follows. Females from each class were collected over several days and individually mated to y2 Yb1 w/y2 Yb1 w/Y males. The use of the y2 Yb1 w/Y males allowed further confirmation of the predicted genotype. 3 days following the mating, the flies were removed from the original vial. 11 days following the removal, each of the vials were counted for the number of F1 progeny produced by the mating. Meanwhile, females from each class were pooled together, their DNA was extracted and a genomic Southern blot was probed to confirm the genotype.

Molecular biology techniques

DNA isolation, cloning, analysis and cDNA library screening are performed according to standard protocols (Sambrook et al., 1989) unless otherwise described. For transcription II in the Yb region, an EST is also present in the Drosophila Genome Project database as LD024566. This transcript is expressed in a non-stage-specific and non-sex-specific manner (Lin and Wolfrer, 1989). For northern analysis, 10 μg poly(A)+ RNA was isolated from various stage flies (see Results). The RNA was size-fractionated on a 1.5% formaldehyde/agarose gel, transferred to Genescreen™ (NEN Research Products) and hybridized using a 32P-labeled random hexamer-primed DNA probe (see below). To sequence Yb1, Yb2 and Yb4 mutant alleles and Yb9 allele in the Ya9 flies, genomic DNA was isolated from the above strains. Overlapping PCR fragments covering the entire Yb locus were generated from the isolated genomic DNA and sequenced. 

Yb cDNA isolation

To isolate Yb cDNAs, we screened Drosophila cDNA libraries using a 32P-labeled random primed 4.6 kb EcoRI-BamHI genomic DNA fragment from the Yb region as a probe. A 1.2 kb cDNA was isolated by screening 3.4×106 clones from Dr Steve Elledge’s third instar larval cDNA library using the 1.2 kb cDNA as a probe. These frequencies are in agreement with the previous estimate that the Yb transcript, if it exists, should be less than 0.01% of poly(A)+ RNA (Lin and Wolfrer, 1989).

DNA sequence analysis

DNA sequence was determined using an automated ABI dye-terminator sequencing machine at the Duke Sequencing Facility and analyzed using the DNASTar software package. Kyte-Doolittle hydrophathy profile and average charge density distribution was generated using Protein software program from the DNASTar package. Gapped Blast searches of the YB protein against the non-redundant protein database were performed on the NCBI web page (Altschul et al., 1997).

RNA tissue in situ analysis

Tissue in situ hybridization was performed on ovaries from adult Oregon R females using a modified version of the protocol of Tautz and Pfeifle (1989). Ovaries were dissected in EBR (130 mM NaCl, 5 mM KC1, 2 mM CaCl2 and 10 mM Heps, pH 6.9), rinsed once, fixed in heptane-saturated 4% paraformaldehyde for 20 minutes, and then placed in methanol. Ovaries were then rehydrated through a methanol/EGTA/paraformaldehyde series, washed three times for 5 minutes each in PBT. Following the wash, the samples were treated with 50 μg/ml Proteinase K in PBT for 5 minutes to detect RNA in germlarial cells and 15 minutes to detect RNA in inner germlarial cells (Cox et al., 1998). The pBluescript sequence and oskar RNA were used for non-specific and positive controls, respectively. Proteinase K treatment was stopped by incubating ovaries in 2 mg/ml glycine in PBT. Ovaries were then washed in PBT, and refixed in heptane-saturated 4% paraformaldehyde. Ovaries were prehybridized for 1 hour at 43°C in hybridization solution (50% formamide, 5×SSC, 0.1% Tween 20). Digoxigenin-labeled DNA probes were prepared using the Genius™ DIG-labeling and detection kit (Boehringer) and were added to the hybridization buffer at an approximate concentration of 10 μg/ml. Probe hybridization was carried out overnight at 43°C in hybridization solution. After hybridization, the ovaries were washed in hybridization solution, hybridization solution/PBT, and then in PBT. Probe was detected using a 1:2000 dilution of anti-DIG antibody preabsorbed against antisequences. Antibody hybridization was carried out for 1 hour at room temperature. Probe was detected using Genius DIG-labeling and detection kit (Boehringer).

FRT-mediated dominant female sterile germline clonal analysis

y2 Yb1 w/Yb, y Yb6 f and y Yb6 cv y f were recombined onto a w FRT19A chromosome to generate y2 Yb1 w/y2 FRT19A, y Yb1 w FRT19A and y Yb6 cv y FRT19A (Chou and Perrimon, 1992). The presence of the FRT sequence was verified by genomic Southern analysis. Dominant female sterile analysis was conducted by mating Yb FRT19A/FM6 females to ovoD2 FRT19A; hsFLP, Sb/TM6, Tb males (kindly provided by Dr Richard Fehon). Progeny were collected for 24 hours and then heat shocked once for 2 hours at 37°C, 48 hours following egg collection. 10 Yb FRT19A/ovoD2 FRT19A; hsFLP, Sb females for each Yb allele were crossed to males individually, and allowed to lay eggs for 3 days. The number of progeny produced by these crosses were counted 14 days following mating. The same number of Yb FRT19A/ovoD2 FRT19A; hsFLP, Sb without heat shock were used as negative controls for heat shock. The same number of Yb FRT19A/ovoD2 FRT19A females treated with the above heat shock regime were used as negative controls for FLP activity. Adult progeny were counted on day 14 to determine female fertility.
γ-ray induced dominant female sterile germline clonal analysis

To generate flies bearing Yb\textsuperscript{1} and Yb\textsuperscript{6} mutant germlines, γ-ray induced germline clonal analysis was performed according to Perrimon and Gans (1983). γ\textsuperscript{2} Yb\textsuperscript{1} w\textsuperscript{D}FM6 and γ\textsuperscript{2} Yb\textsuperscript{6} cv v ff FM6 heterozygous females were crossed to ovo\textsuperscript{D1} FRT\textsuperscript{101}; F38 males (Chou and Perrimon, 1992). This ovo\textsuperscript{D1} line blocks oogenesis at a much earlier stage than the original ovo\textsuperscript{D1} stock (see Fig. 7B,D). Embryos were collected for 12 hours on grape juice/agar plates. Between 34 and 46 hours after egg laying, second instar larvae were exposed to 1000 rads from a \textsuperscript{137}Cs γ-ray source, and then transferred to vials containing standard cornmeal molasses medium. Adult females were dissected 6-8 days after eclosion. Their ovaries were fixed, stained for immunofluorescence, mounted and examined for the presence of germline clones.

RESULTS

Yb mutants fail to maintain germline stem cells during oogenesis

The Yb mutations were isolated by Young and Judd (1978; Yb\textsuperscript{1}) and by Mohler and Carroll (1984; Yb\textsuperscript{4} and Yb\textsuperscript{6}) as recessive female sterile mutations that produce few or no eggs. We first examined whether the sterility of Yb mutants is due to defects in ovarian development prior to the onset of oogenesis by immunofluorescence and confocal microscopy. Third instar larval ovaries from homozygous Yb\textsuperscript{1} and Yb\textsuperscript{4} mutants and their phenotypically wild-type siblings were isolated and triple-stained with DAPI, anti-VASA antibodies (Hay et al., 1990) and anti-1B1 antibodies (Zaccai and Lipshitz, 1996) to label DNA, germline cells, and spectrosomes and fusomes, respectively (see Materials and methods). Spectrosomes are present only in GSCs and cystoblasts while fusomes are intercellular cytoplasmic structures that mark differentiating stages of germline cysts (Lin and Spradling, 1995; Deng and Lin, 1997; see Fig. 6). Both Yb\textsuperscript{1} and Yb\textsuperscript{4} third instar mutant ovaries show normal number and location of GSCs as well as normal overall ovarian morphology (Fig. 2G). The mutant

Fig. 2. Yb mutations affect GSC maintenance. Confocal images of 0- to 1-day-old wild-type (A,A') and homozygous Yb\textsuperscript{4} mutant ovarioles (B-E') with VASA labeled in green to mark germline cells and the 1B1 antigen labeled in red to mark spectrin-rich structures. Each pair of images shows a low magnification view of an ovariole and a corresponding high magnification view of its germarium (g). The wild-type ovariole (A) contains five egg chambers (ec) and a progression of developing germline cells in the germarium, including GSCs and cystoblasts containing spectrosomes (sp) and germline cysts containing fusomes (fu in A'). The terminal filament (tf) marks the apical tip of the germarium. Yb\textsuperscript{4} ovarioles usually have two (B,D,E) or one (C) egg chambers and often lack the germline in the germaria (B',C'). When germline cells are present in the germaria, they are present either as ill-differentiated cell clusters (D') or as different stages of germline cysts (E'). In this situation, ovarioles still typically contain one or two egg chambers (D,E). Some Yb\textsuperscript{6} mutant ovarioles (F) are very small, and primarily consist of clusters of ill-differentiated germline cells. (G) A morphologically normal third instar larval ovary which could be either homozygous or heterozygous for Yb\textsuperscript{4} (see Materials and methods). These third instar ovarioles contain a wild-type number of GSCs occupying the medial portion of the ovary and morphologically normal spectrosomes. The apical somatic cells have started to form terminal filaments. Bars, 30 μm (A-G), 10 μm (A'-E').
Ovaries are thus indistinguishable from their sibling heterozygous ovaries. This analysis suggests that the Yb<sup>i</sup> and Yb<sup>d</sup> mutations do not affect germline and ovarian development prior to the onset of oogenesis.

We then analyzed the oogenic defects of Yb mutants by examining the adult mutant ovaries using the same methods. In newly eclosed (0-24 hour old) heterozygous sibling females that are phenotypically wild type, ovarioles usually contain five egg chambers (Fig. 2A). Moreover, the germarium contains a full complement of germline cells, with 2-3 GSCs and a cystoblast containing spectrosomes and 7-8 developing germline cysts containing fusomes (Fig. 2A'). These observations suggest that GSCs have divided approximately 13-14 times following the onset of oogenesis at the third instar larval stage.

In contrast, Yb mutant ovarioles show severe defects in GSC division and maintenance. The predominant defect is that mutant ovarioles contain only two (Fig. 2B), or occasionally one (Fig. 2C), egg chambers, with no other germline cells present in the germarium (Fig. 2B', C', also see Table 1, Type 1). This defect clearly indicates the failure of GSC maintenance in these mutant ovarioles, likely due to the differentiation of GSCs without a self-renewing division.

The second defect is that ovarioles contain two (sometimes one or three) egg chambers and a small number of germline cells in the germarium (Fig. 2D, D', Table 1, Type 2). These germline cells contain spectrosomes and 2-cell fusomes. However, they do not increase in number as females increase in age. Hence, they are not GSCs but are ill-fated germline cells. This defect again indicates the failure of GSC maintenance in these mutant ovarioles, possibly due to a defect in the asymmetry of GSC division following one round of normal division. As a milder manifestation of the second defect, some mutant ovarioles contain two egg chambers and a complement of germline cells and cysts in the germarium (Fig. 2E, E', Table 1, Type 2). Several different stages of germline cysts were evident by the presence of fusomes (Fig. 2E'). However, these ovarioles usually contain only 1-3 egg chambers (e.g. Fig. 2E), suggesting that even if the GSC can self-renew in these ovarioles, this ability is severely affected.

The third defect is that ovarioles in a mutant ovary contain no egg chambers. There are only a small number of differentiating germline cells arrested in the germarium, resulting in a very small ovary (Fig. 2F, Table 1, Type 3).

Occasionally, some ovarioles are completely devoid of germline cells (Table 1 legend). This defect indicates failure in maintaining asymmetrically dividing GSCs in these mutant ovaries. The above three types of defects reveal the essential role of Yb for the maintenance of GSCs during oogenesis.

In all the above three scenarios, egg chambers can develop into mature eggs. In Yb<sup>i</sup> mutants, these eggs are occasionally laid. The laid eggs can develop into adults. These observations, together the strong or possibly null nature of the Yb mutations (see below), suggest that the Yb function is specifically required for GSC divisions in the female.

Given that the terminal filament plays a crucial role in GSC maintenance (Cox et al., 1998) and that Yb is expressed specifically in the terminal filament (see below), we further investigated whether the above GSC maintenance defects are an indirect consequence of the absence or ill-development of the terminal filament. The morphology of all three Yb mutant germaria was first examined by Nomarski optics and by anti-B1 antibody staining, which outlines the somatic cytoskeletal system. The terminal filaments show normal morphology both at the initial formation stage (Fig. 2G) and in the adult ovaries (Fig. 2H, I, C', D', E'), including the presence of morphologically normal basal cells. We then examined the expression of brica-brac (bab) and engrailed (en), two genes specifically expressed in the terminal filament and important for its function (Godt and Laski, 1995; Forbes et al., 1996a), as well as armadillo (arm), whose expression specifically highlights the basal cells (Forbes et al., 1996b). Immunofluorescence microscopy (see Materials and methods) revealed that both the morphology of the terminal filament and the localization of the BAB, EN and ARM proteins in the terminal filament cells are normal (Fig. 3). These analyses suggest that Yb is not involved in terminal filament development but directly involved in GSC maintenance.

Johnson et al. (1995) have suggested a role for Yb in somatic cell proliferation and patterning based on their phenotypic analysis of 3- to 5-day-old mutant females. We have also observed similar somatic defects in Yb mutants in our 0- to 24-hour-old mutant females, including germainium-egg chamber fusion, improper partitioning of germline cysts by follicle cells, and missing inter-follicular stalk cells (data not shown). These somatic defects, however, occur at a low frequency and thus do not appear to be predominant defects during early oogenesis. Meanwhile, we observed clear GSC division defects

### Table 1. Summary of the oogenic defects of Yb mutants

<table>
<thead>
<tr>
<th>GSC defects</th>
<th>Yb&lt;sup&gt;i&lt;/sup&gt;/Yb&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>Yb&lt;sup&gt;i&lt;/sup&gt;/Df (%)</th>
<th>Yb&lt;sup&gt;i&lt;/sup&gt;/Yb&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>Yb&lt;sup&gt;i&lt;/sup&gt;/Yb&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>Yb&lt;sup&gt;i&lt;/sup&gt;/Yb&lt;sup&gt;d&lt;/sup&gt; (%)</th>
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<td>42</td>
<td>8</td>
<td>50</td>
<td>15</td>
<td>12</td>
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<tr>
<td>No GCs in germarium, 1-3 ec (Fig. 2B-C')</td>
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<tr>
<td>Type 2</td>
<td>39</td>
<td>5</td>
<td>13</td>
<td>43</td>
<td>19</td>
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<tr>
<td>Undif. GCs in germarium, 1-3 ec (Fig. 2D,D')</td>
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<tr>
<td>Dif. GCs in germarium, 1-3 ec (Fig. 2E,E')</td>
<td>17</td>
<td>88</td>
<td>28</td>
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<tr>
<td>Type 3</td>
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<td>0</td>
<td>4</td>
<td>15</td>
<td>7</td>
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<td>Undif. GCs in germarium, no ec (Fig. 2F)</td>
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<tr>
<td>Dif. GCs in germarium, no ec (Fig. 2F)</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>No. of ovarioles examined</td>
<td>230</td>
<td>128</td>
<td>454</td>
<td>206</td>
<td>73</td>
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</table>

The differentiation state of germline cells (GCs) in the germarium was determined based on the morphology of these cells as outlined by anti-VASA staining, as well as on the presence and morphology of spectrosomes and fusomes. ec, egg chambers; Undif., undifferentiated germ cells (contain spectrosomes); Dif., differentiated germline cysts (contain spectrosomes and different stages of fusome containing cysts); Df, Df(1)w<sup>258-45</sup>.

For a detailed description of the three types of defects, see text.

*Ovaries completely lacking germline cells were also observed at low frequency in Yb<sup>i</sup> (3%) and Yb<sup>d</sup> (10%).*
in the data presented previously (e.g. Fig. 4A in Johnson et al., 1995), even though 3- to 5-day-old females are generally too old for deciphering GSC defects. The somatic phenotype may suggest an additional role of Yb in follicle cell proliferation and differentiation, as proposed by Johnson et al. (1995).

Molecular cloning of the Yb gene

Yb and its neighboring complementation group, fs(1)Ya (Ya), have previously been localized to an 8.1 kb genomic DNA isolated from the zeste white region of the X chromosome (Lin and Wolfner, 1989; see Fig. 4). However, only the 2.35 kb female-specific Ya transcript was identified within this region (Fig. 4). Thus, Yb either shares a common transcript with Ya or encodes a yet to be identified separate transcript (Lin and Wolfner, 1989). To distinguish between these two possibilities, given that an essential region of Ya is distal to Ya in the 8.1 kb fragment (Lin and Wolfner, 1991; see Fig. 4, deletion clone f), we constructed serial deletions of the 8.1 kb fragment (Lin and Wolfner, 1991; see Fig. 4, deletion clone f), progressing from the proximal to distal end and introduced them to homozygous Yb flies to test their ability to rescue the female sterility (Fig. 4; see Materials and methods). A 6.4 kb SalI-Stul fragment, truncating 1.25 kb of the Ya transcript, rescues the sterility of Yb, but not Ya, suggesting that Yb does not share the Ya transcript (Fig. 4, deletion clone b). Other deletions that further define the Yb boundaries within the 6.4 kb region are summarized in Fig. 4.

To search for the Yb transcript, we isolated a 3.5 kb cDNA representing a novel transcript which spans Yb mutations (see below) and is the only RNA completely encoded within the 6.4 kb Yb region, thus representing the Yb transcript (Fig. 4; also see Materials and methods). We have also isolated a 2.2 kb cDNA representing transcript II. The position and splicing pattern of the cDNAs were precisely mapped to the genome by sequencing the 6.5 kb genomic DNA and the cDNAs. Although the 3.5 kb cDNA is smaller than the 4.1 kb Yb RNA as determined by the northern blot analysis (see below), sequence analysis reveals that this cDNA is missing only part of the 5’ untranslated region (UTR; see below). The Yb cDNA is only 176 bp away from transcript II at its 5’ side and 127 bp away from the start of Ya transcript at its 3’ end, reflecting the unusually high gene density of this region.

The Yb cDNAs were recovered at a frequency of 7×10−7 from adult and larval cDNA libraries (see Materials and methods), suggesting that Yb is present at extremely low abundance in multiple stages of the Drosophila life cycle.

Structural features of the 117 kDa YB protein

The 3507 bp Yb cDNA has a 110-bp 5’ UTR, which contains multiple in-frame stop codons upstream of the start of a 3129-bp large open reading frame (ORF; Fig. 5). It also contains a 268-bp 3’ UTR with a typical polyadenylation site 13 bp upstream of the poly(A)+ tail. The large ORF encodes a predicted 117,478 Da novel protein with a pI of 6.851. Searches against the Prosise database (Bairoch et al., 1997) predict that this protein contains an ATP/GTP-binding site motif A; blast searches reveal limited homology between YB and p68 family RNA helicases (30% over 70 residues) around this predicted ATP/GTP binding site. However, the homologous region contains no DEAD boxes or other known RNA binding motifs, thus YB is unlikely to be an RNA helicase. YB contains 17 predicted protein kinase C phosphorylation sites and 20 predicted Casein Kinase II phosphorylation sites, suggesting that YB function might be regulated by phosphorylation. In addition, YB contains 3 potential asparagine glycosylation sites, 8 myristoylation sites and a leucine zipper, according to a Prosise database search.

The primary sequence and the hydrophobicity profile of the predicted YB protein reveals no signal peptides or potential transmembrane domains (data not shown). An average charge plot shows that YB has charged residues distributed fairly evenly over its length. These features are in agreement with the PSORT II analysis (Nakia and Kanehisa, 1992), which suggests that YB is a cytoplasmic protein.

Yb mRNA is expressed in multiple developmental stages in both females and males

To examine the expression of Yb during development, we conducted northern blot analysis with RNA isolated from third instar larvae, pupae and adults (see Materials and methods). A 4.1 kb Yb mRNA was detected on northern blots in both males and females to test their ability to rescue the female sterility (Fig. 4; also see Materials and methods). A 6.4 kb SalI-Stul fragment, truncating 1.25 kb of the Ya transcript, rescues the sterility of Yb, but not Ya, suggesting that Yb does not share the Ya transcript (Fig. 4, deletion clone b). Other deletions that further define the Yb boundaries within the 6.4 kb region are summarized in Fig. 4.

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**Fig. 3.** Yb mutants contain normal terminal filaments. The BAB, ARM and EN proteins are localized properly in the terminal filament (tf), including basal terminal filament cells (bc) in all three Yb mutants. All panels are shown at the same magnification.
and females at these stages (Fig. 6A). In females, Yb is weakly detectable at the third instar larval stage, becomes more abundant at the pupal stage and decreases at the adult stage. This expression pattern is consistent with Yb being required for germline maintenance starting at the onset of oogenesis. In males, the Yb expression is at the highest level at the third instar larval stage, decreases during pupal development, and is present at an even lower level in adults. Given that Yb mutants are all female sterile and show no effect on male fertility, the Yb expression in males may have a functional implication that has yet to be identified by genetic analysis.

**Yb¹, Yb⁴ and Yb⁶ are genetically null mutations**

Johnson et al. (1995) have shown that the Yb⁴ mutation is not required in the germline for fertility by γ-ray-induced clonal analysis. To determine whether this indicates that the Yb gene is dispensable in the germline for fertility, we first examined whether Yb⁴ or any other existing Yb alleles are null alleles. Genetic tests by Johnson et al. (1995) suggest that Yb⁴ behaves like a null mutation with regard to viability and potential somatic phenotype. Since Yb⁴ shares very similar homozygous phenotype with Yb⁴ and Yb⁶ (Fig. 2, Table 1), we tested whether Yb⁴ and Yb⁶ are also genetically null by examining the phenotype of these two alleles over the deficiency Df(1) J6092, which completely uncovers Yb (Young and Judd, 1978). Both hemizygous Yb⁴ and Yb⁶ flies show defects similar to that of homozygous Yb¹, Yb⁴ and Yb⁶ (Table 1), suggesting that all three alleles are strong or possibly null alleles.

To investigate the nature of these genetically null mutations, we sequenced the 4.1 kb Yb genomic DNA from the Yb¹, Yb⁴ and Yb⁶ mutants (see Materials and methods). The sequence of the Yb¹ allele, as compared to the wild-type Yb sequence from its phylogenetic sibling Ya² flies, showed only a single nucleotide difference at position +2916, changing an arginine residue to a glutamine residue (Fig. 5). Thus, this change is the Yb¹ mutation. Comparison between the sibling Yb⁴ and Yb⁶ sequences again showed that each allele has only one nucleotide polymorphism that deviates from the wild-type sequence. The Yb⁴ mutation occurs at the +2239 site, changing an aspartic acid residue to an asparagine residue, while the Yb⁶ mutation occurs at +2573, changing a cysteine residue to a tyrosine residue. The localization of these three mutations at the C-terminal third of YB suggests that this part of the protein is an essential functional domain.

**Yb is dispensable in the germline for fertility**

Since Yb¹, Yb⁴ and Yb⁶ are genetically null mutations, we conducted germline clonal analysis in all three alleles using FRT-mediated dominant female sterile technique to confirm the clonal analysis of Yb⁴ by Johnson et al. (1995) (Chou and Perrimon, 1992; see Materials and methods). Homozygous Yb⁻ germline clones were generated in Yb⁻ ovo⁺ FRT/Yb⁻ ovoD2 FRT; hsFLP females, where Yb⁻ designates a Yb allele and FRT designates the FRT recombination site. Since ovoD2 dominantly blocks oogenesis in a cell-autonomous manner, these females are completely sterile (Table 2). Heat-shocking

![Diagram of Genomic Region](image)

**Table 2. FLP-DFS germline clonal analysis of fs(1)Yb alleles**

<table>
<thead>
<tr>
<th>Yb mutants</th>
<th>Yb¹/FRT/ovoD2 FRT</th>
<th>Yb⁴/FRT/ovoD2 FRT</th>
<th>Yb⁶/FRT/ovoD2 FRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsFLP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Heat shock</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>% fertile females</td>
<td>30</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Number of progeny</td>
<td>5.7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>per fertile female</td>
<td>21.1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

10 females of each class were tested for fertility. The progeny from each fertile female was quantified, and the average number of progeny per fertile female is shown. Note that only heat-shocked Yb⁻ ovo⁺ FRT/Yb⁺ ovoD2 FRT; hsFLP females restores their fertility. Parallel heat-shock treatment to Yb⁻ ovo⁺ FRT/Yb⁺ ovoD2 FRT females without hsFLP did not restore their fertility. Moreover, Yb⁻ ovo⁺ FRT/Yb⁺ ovoD2 FRT; hsFLP females without heat shock are all sterile except for one of the Yb⁴ females, which produced one egg, a frequency expected for the leakiness of Yb¹ (see Fig. 4).
these females at the early third instar larval stage induced the formation of homozygous Yb	extsuperscript{+} ovo	extsuperscript{D2} FRT clones, which are blocked in oogenesis, and homozygous Yb	extsuperscript{+} ovo	extsuperscript{+} FRT clones, which restored oogenesis in all three mutant alleles, showing that Yb is not required in the germine for fertility (Table 2).

**Yb is dispensable in the germine for GSC function**

The restoration of the fertility in the Yb	extsuperscript{-} germine suggests the possibility that the self-renewing division of GSCs is restored. To test this, we first examined the morphology of the ovaries of the fertile tester and control females by DAPI staining to determine that Yb clones were not becoming depleted in the ovari of the fertile tester females. Since ovo	extsuperscript{D2} ovaries contain full germaria and multiple egg chambers, if Yb	extsuperscript{-} germine clones become depleted of GSCs, we would expect to see some ovarioles with few egg chambers and small germaria without germ cells, which we did not detect (data not shown). These results suggest that Yb	extsuperscript{-} GSCs are capable of supporting oogenesis.

To identify unambiguously Yb	extsuperscript{-} germine clones, we used an ovo	extsuperscript{D1} mutation that affects oogenesis very early, creating rudimentary germaria containing only a few germ cells that do not detect (data not shown).

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**Fig. 5.** The cDNA and predicted amino acid sequences of Yb. The 3507 bp Yb cDNA and intronic sequences are shown, with the predicted amino acid sequence shown below the corresponding triplet.

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**Table 2.**

| Allele | Description | Number of GSCs | Germaria

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**Fig. 7A.** The cDNA and predicted amino acid sequences of Yb. The 3507 bp Yb cDNA and intronic sequences are shown, with the predicted amino acid sequence shown below the corresponding triplet.

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**Fig. 7B.** The cDNA and predicted amino acid sequences of Yb. The 3507 bp Yb cDNA and intronic sequences are shown, with the predicted amino acid sequence shown below the corresponding triplet.
Yb mRNA is expressed specifically in the terminal filament in the germarium

To further identify somatic cells required for Yb function, we conducted RNA in situ analysis to ovaries isolated from the wild-type adult females (see Materials and methods). In the ovariole, Yb is consistently detected specifically in the terminal filament and the apical epithelial sheath that envelopes the terminal filament (Fig. 5B). However, we did not detect Yb staining in germline cells at any stages of oogenesis. The specific expression of Yb in the terminal filament and its surrounding sheath is particularly interesting. The epithelial sheath has no effect on GSC division, since transplanted germaria with or without the sheath produce the same number of egg chambers (Lin and Spradling, 1993). Hence, Yb appears to acts in the terminal filament cells to ensure the self-renewal of GSCs.

DISCUSSION

Cell-cell interactions have been implicated in regulating stem cell division and differentiation (reviewed in Lin, 1997; Potten, 1997). In this paper, our phenotypic and molecular analyses reveal a somatic mechanism mediated by Yb that is important for the self-renewal of stem cells in the Drosophila ovarian germ line.

Yb-mediated somatic signaling mechanism is essential for GSC maintenance during oogenesis

Previous analyses of Yb mutants suggested a role for Yb in somatic cell proliferation and patterning during Drosophila oogenesis (Johnson et al., 1995). Here, we have shown that Yb mutants have major defects in the maintenance of GSCs. Females lacking Yb function lose all their GSCs. Often the mutant ovarioles contain only two normally or aberrantly developed egg chambers, suggesting that GSCs in these ovarioles have directly entered the cystoblast differentiation.
Signaling pathways control the division of GSCs, with the source of dpp signaling being connected by fusomes (shaded ovoid). The incomplete division of the cystoblast. Individual cells within a germarium produce follicle cells to envelope germline cysts (CT) resulting from perpendicularly to the apico-basal axis of the germarium. As a result, the pole of the spindle so that the divisional plan is approximately perpendicular to the apico-basal axis of the germarium. In this case, some GSCs may have undergone several aberrant divisions, generating ill-fated daughter cells. The consequence of all of these defects is the loss of functional GSCs. Thus, both types of Yb germline defects reflect the requirement of Yb for GSC maintenance.

In parallel to Yb, piwi and dpp signaling pathways have also been shown to be required for GSC maintenance (Lin and Spradling, 1997; Xie and Spradling, 1998; Cox et al., 1998). Loss of piwi or dpp function causes Yb-like germline maintenance defects, while ectopically over-expressing dpp produces GSC tumors. These observations reveal the essential role of these genes in regulating GSC self-renewal. Genetic clonal analysis further suggests that the dpp signaling pathway requires direct interaction between the signal and its receptors on the GSC surface (Xie and Spradling, 1998). Given that the source of dpp signal has yet to be identified, it is possible that DPP is produced as a paracrine in certain sets of somatic or germline cells, or even as an autocrine in GSCs themselves. In mouse testes, BMP8B, a dpp homolog, is expressed only in germline cells, including spermatogonia, for the initiation and maintenance of spermatogenesis (Zhao et al., 1996). Complementary to the understanding of the dpp signaling pathway, genetic and molecular analyses have revealed Yb and piwi signaling pathways as somatic signaling mechanisms residing in the terminal filament cells to regulate GSC division. The molecular characteristics of YB suggest that it is unlikely to be a signal itself, but probably a protein in the terminal filament cells involved in the production of a somatic signal(s). PIWI is also a component for somatic signaling in the terminal filament (Cox et al., 1998). Thus, studies of Yb and piwi have revealed an essential somatic signaling source that controls GSC maintenance, while the study of dpp has identified the first signal required for GSC division and maintenance in Drosophila.

Interactions between germline cells and their adjacent non-mitotic somatic cells have been implicated in the control of germline cell proliferation or meiosis in organisms ranging from invertebrates to mammals (reviewed in Lin, 1997). An illuminating example is in C. elegans, where the interaction between the LAG-2 signal on the surface of the distal tip cell (DTC) at the gonad tip and its GLP-1 receptor on the surface of the germline syncytium is essential for the division and maintenance of a population of syncytial mitotic germline nuclei (Austin and Kimble, 1987; reviewed in Berry et al., 1997). However, the C. elegans germline does not possess stereotypic stem cells that are individual cells undergoing self-renewing asymmetric divisions. All C. elegans mitotic nuclei in the germline share a common cytoplasm that is partially partitioned. The LAG2/GLP-1-equivalent pathway in Drosophila, i.e. the Notch (N) signaling pathway, appears to have no detectable effect on GSC division (Ruohola et al., 1991; Xu et al., 1992). This may imply that syncytial ‘stem cells’ have a unique regulatory mechanism. In contrast to C. elegans, the somatic signaling mechanisms represented by Yb and piwi and the signaling mechanism mediated by dpp may be required for the self-renewal of GSCs in diverse organisms. Consistent with this, piwi-like genes have been identified in C. elegans, human and plants (Cox et al., 1998). In C. elegans and Arabidopsis, piwi-like genes are important for germline and meristem cell maintenance, respectively (Cox et al., 1998; Bohmert et al., 1998; Moussian et al., 1998). Similarly, dpp homologs in mammals have been shown to be involved in germline maintenance during spermatogenesis (Zhao et al., 1996). Yb-like genes do not exist in C. elegans or yeast, suggesting the Yb-mediated mechanism is not involved in yeast budding-like asymmetric divisions or in the maintenance of syncytial ‘stem cells’. The search for Yb homologs in higher organisms should address whether a Yb-mediated mechanism is general for all stereotypic stem cells or specific for a subtype of them that divide like GSCs in Drosophila.

The terminal filament is a signaling center that controls the division of both somatic and germline stem cells

Terminal filament cells have been shown to be a signaling source to regulate the division and differentiation of somatic stem cells and/or their immediate daughters in the germarium via the hh signaling pathway (Forbes et al., 1996a,b). Partial ablation of the terminal filament increases the rate of GSC division, suggesting its involvement in GSC division (Lin and Spradling, 1993). However, the importance and the exact role

![Fig. 8. Terminal filament signaling controls the coordinated division of both germline and somatic stem cells during oogenesis. A schematic view of the apical region of the germarium, with germline cells lightly shaded. There are usually two GSCs in the germarium, contacting apical somatic cells expressing Yb, piwi and hh. In GSCs, spectrosomes (shaded spheres) reside in the apical region of the cytoplasm both at interphase (left) and during mitosis (right), apposed to the signaling somatic cells. During mitosis, it anchors one pole of the spindle so that the divisional plan is approximately perpendicular to the apico-basal axis of the germarium. As a result, the daughter GSC remains in contact with the apical somatic cells while the cystoblast (CB) becomes one cell away from the somatic cells. The somatic stem cells (SSC) are located in the middle region of the germarium, 2-5 cells away from the signaling cells. They produce follicle cells to envelope germline cysts (CT) resulting from the incomplete division of the cystoblast. Individual cells within a cyst are connected by fusomes (shaded ovoid). The hh signaling pathway controls the division of SSCs, while the Yb, piwi and dpp signaling pathways control the division of GSCs, with the source of dpp unidentified.](image-url)
of the terminal filament in GSC division remains speculative. The study presented here and a similar study on pivi (Cox et al., 1998) define the essential role of the terminal filament in GSC maintenance during oogenesis.

The regulation of somatic and germline stem cell division appears to be independent (Fig. 8). Somatic cells continue to divide to a certain extent in the absence of the germline (Margolis and Spradling, 1995), while the germline overproliferates in benign gonial cell neoplasms and bag of marbles mutants without accompanying somatic overproliferation (Gateff, 1982; McKearin and Spradling, 1990). Furthermore, the regulation of somatic stem cell division involves the hh signaling pathway, but hh does not affect GSC division (Forbes et al., 1996a). Conversely, the dpp signaling pathway plays an important role for GSC division and maintenance, but it does not appear to control somatic stem cell division, since overexpressing dpp causes overproliferation of GSCs but not somatic cells in the germarium (Xie and Spradling, 1998). Under normal circumstances, oogenesis in Drosophila requires coordinated division of germline and somatic stem cells, so that each germline cyst will be enveloped by an appropriate number of follicle cells to form an egg chamber (see Introduction; also see Fig. 2A.A”). What coordinates these two different types of stem cells remains a mystery. In Yb mutants, not only do GSCs fail to be maintained, but the somatic cell division is also correspondingly reduced to match the reduced number of germline cysts (see Results). Thus, Yb may be required for SSC division as well. Further analysis of Yb might reveal the mechanism underlying the coordinated division of two distinctive types of stem cells within the germarium.

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