A novel group of *pumilio* mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary

Haifan Lin* and Allan C. Spradling
Howard Hughes Medical Institute Research Laboratories, Department of Embryology, Carnegie Institution of Washington, 115 W. University Parkway, Baltimore, MD 21210, USA

*Author for correspondence at present address: Department of Cell Biology, Box 3709, 412 Nanaline Duke Building, Duke University Medical School, Durham, NC 27710, USA (e-mail: h.lin@cellbio.duke.edu)

SUMMARY

Germline stem cells play a pivotal role in gametogenesis; yet little is known about how they are formed, how they divide to self-renew, and how these processes are genetically controlled. Here we describe the self-renewing asymmetric division of germine stem cells in the *Drosophila* ovarian germline, as marked by the spectrosome, a cytoplasmic structure rich in membrane skeletal proteins. The ontogeny of the spectrosome marks the lineage of germine stem cells. We identified two new groups of mutations in which the divisional asymmetry is disrupted. The first, which we refer to as *ovarette (ovt)* mutations, was shown to correspond to a novel class of mutations in the *pumilio* locus. Since *pumilio* is known to posttranscriptionally repress the expression of target genes at earlier stages of germ cell development, our results suggest that a similar activity is needed to maintain germ line stem cells. We have also identified a second and novel gene, *piwi*, whose mutations abolish germine stem cell division.

Key words: *Drosophila*, stem cell, asymmetric division, germline, *pumilio*

INTRODUCTION

Germline stem cells provide a steady and continuous source of germ cells for the production of gametes with remarkable totipotency to generate new individuals. These stem cells are derived from primordial germ cells, which in many species are specified early during embryogenesis by the localized germine determinants, as implicated by the presence of cytoplasmic structures specific to germ plasm in the egg, such as polar granules in *Drosophila* (Mahowald, 1962), P granules in *C. elegans* (Strome and Wood, 1982), and similar structures in other insects and amphibians (Browder, 1984). The distinctive properties of germ cells in a variety of organisms are also marked later during their development by cytoplasmic structures such as nuages (Kerr and Dixon, 1974; Eddy, 1975; Mahowald, 1977) and fusomes (Telfer, 1975; Storto and King, 1989; Lin et al., 1994).

In *Drosophila*, the germ plasm (i.e., pole plasm) is both necessary and sufficient for the formation of pole cells, the primordial germ cells (Illmensee and Mahowald, 1974, 1976). The formation of polar granules and pole cells requires eight of the ten ‘posterior group genes’, *tudor, staufen, valois, vasa, oskar, cappuccino, spire* and *mago nashi* (Boswell and Mahowald, 1985; Lehmann and Nusslein-Volhard, 1986; Schüpbach and Wieschaus, 1986; Manseau and Schupbach, 1989; Boswell et al., 1991). Two remaining genes, *pumilio* (*pum*) and *nanos* (*nos*), also encode RNAs as components of the pole plasm (Barker et al., 1992; MacDonald, 1992), even though they are not required for pole cell formation but only for abdominal patterning (Lehmann and Nusslein-Volhard, 1987, 1991). However, *nos* is crucial for the maturation and migration of pole cells (Kobayashi et al., 1996) and for germline stem cell maintenance (Lehmann and Nusslein-Volhard, 1991). *pumilio* is widely expressed and has effects outside the gonads, as *pumilio* mutations are semi-lethal and result in abnormal bristles (Lehmann and Nusslein-Volhard, 1987). Other known components of the pole plasm include RNAs encoded by *cyclin B, orb, germ cell less (gcl)*, *hsp83* and *Polar granule component (Pgc)* (Raff et al., 1990; Lantz et al., 1992; Jongens et al., 1992, 1994; Ding et al., 1993; Nakamura et al., 1996). Anti-sense suppression experiments suggest that *gcl* and *Pgc* are required specifically for pole cell formation (Jongens et al., 1992; Nakamura et al., 1996).

Although germline establishment in *Drosophila* is relatively well understood, very little is known about subsequent development that leads to germline stem cell formation. Pole cells, once formed, migrate during gastrulation to reach the gonadal region where they become surrounded by somatic mesodermal cells to form embryonic gonads (Howard et al., 1993; Warrior, 1994). In females, the gonads remain in this undifferentiated organization until the larval-pupal transition, during which gonadal somatic cells begin to differentiate, partitioning germine cells into ovarian functional units called ovarioles (King, 1970). As ovarioles form, a continuous production of new egg chambers starts (King, 1970), indicating that the germine cells at this stage have acquired stem cell properties.

Stem cells are characterized by their ability to produce a large number of differentiated progeny while capable of self-renewal.
In mammals, stem cells play a central role in the formation and/or maintenance of most tissues (for reviews, see Lin and Schagat, 1997; Morrison et al., 1997). However, little is known about how mammalian stem cells form and how they divide due to their low abundance, lack of specific markers, and high sensitivity to manipulation. In the *Drosophila* ovary, germline stem cells have long been proposed to exist at the very apical tip of the ovariole in a region called the gerarium, producing new egg chambers that leave the gerarium and move towards the oviduct as they develop (Brown and King, 1962, 1964). This stem cell hypothesis has been strongly supported by genetic analysis (Wieschaus and Szabad, 1979; Margolis and Spradling, 1995) and by laser ablation experiments (Lin and Spradling, 1993). Recently, somatic stem cells have also been located within the gerarium, responsible for producing follicle cells that envelop the egg chambers (Margolis and Spradling, 1995).

The division of germline stem cells and other stem cells is expected to be controlled by intra- or inter-cellular mechanisms, or both. Intracellular cell-fate determinants have been discovered in early embryos of many organisms (for review see Gilbert, 1994) as well as in neural stem cells (for review see Lin and Schagat, 1997). However, such a mechanism has not been identified in adult stem cells capable of many self-renewing asymmetric divisions. Intercellular signaling mechanisms have been shown to regulate germ cell proliferation, such as in the germline of *C. elegans* and mammals, where mitosis and meiosis are regulated by signals from the neighboring somatic cells, as mediated by molecules such as the EGF-repeat-containing receptor at the surface of germline cells (Zick, 1911; Buder, 1917; Beklemishev, 1969; Byskov, 1974; Kimble and White, 1981; Ellis and Kimble, 1994). Intercellular signaling also regulates stem cell proliferation, as mediated by molecules such as oncoproteins c-KIT and JAK-2 (Fleischman, 1993; Weiler, et al., 1996), cytokines (Stewart, 1995), and extracellular matrix molecules (Bruno et al., 1995; Jones et al., 1995). Despite this progress, it is unknown whether these factors regulate the formation or maintenance of the stem cells, or certain aspects of their division, since little is known about how, or even if, stem cells indeed divide asymmetrically to generate a daughter stem cell and a differentiated daughter cell. Nor is it known whether some of these regulating factors play the same role in vivo as they do in vitro.

Germline stem cells in the *Drosophila* ovary provide an excellent model for studying the development and division of stem cells. The asymmetric division of these stem cells has long been proposed (Brown and King, 1962, 1964), even though it has not been observed. Recently, a novel cytoplasmic structure, the spectrosome, rich in membrane skeletal proteins such as spectrins, the adducin-like Hts protein and ankyrin, was identified in the germline stem cells and their differentiated daughter cells, the cystoblast (Lin et al., 1994; Lin and Spradling, 1995; de Cuevas and Spradling, 1996; also see Results). The spectrosome also contains the Bar protein required for cystoblast differentiation (Mckean and Ohlstein, 1995) and segregates asymmetrically during cystoblast division (Lin and Spradling, 1995). Here we report the asymmetric division of the germline stem cells as marked by the spectrosome. We also describe the ontogeny of the spectrosome during germline development, which may reflect the making of an intrinsic mechanism for asymmetric stem cell division. Moreover, we identify mutations in two genes that abolish the ability of germ line stem cells to divide asymmetrically. Mutations in one of these genes are allelic to *pumilio*, and their phenotype suggests a novel requirement for stem cell maintenance.

**MATERIALS AND METHODS**

*Drosophila* strains and cultures

Flies were grown at 25°C on yeast-containing corn meal/agar medium. Genetic markers have been described by Lindsley and Zimm (1992). The *CyO elav-lacZ* balancer chromosome contains a transgenic *lacZ* gene under the control of the *elav* promoter. The *TM3 ftz-lacZ* balancer contains a transgenic *lacZ* gene under the control of the *ftz* promoter. The *CyO y* balancer is a *CyO* chromosome a copy of the wild-type *y* gene (Timmons et al., 1993). The *ott, pwi* and *hts* (Yue and Spradling, 1992) mutations were induced by single P element insertion. The generation of the single P element lines used in this study was described previously (Karpen and Spradling, 1992; Zhang and Spradling, 1994). These lines carry single insertions of the ‘PZ’ element (Mlodzik and Hiromi, 1992) which is marked with the *rosy* gene, and contains a *lacZ* gene fused to the P element promoter for enhancer trapping. The *pum*680, *pum*TE3, *pum*FT1, *pum*FC8 and *pum*MC alleles have been reported previously (Lehmann and Nuslein-Volhard, 1987; Barker et al., 1992; McDonald, 1992).

Histochemical and immunofluorescence microscopy

β-galactosidase activity staining

Ovaries were dissected in Ringer’s solution (EBR: 130 mM NaCl, 5 mM KCl, 2 mM CaCl2, and 10 mM Hepes at pH 6.9), fixed, and stained as described by Lin and Spradling (1993).

Antisera

The anti-*Drosophila* α-spectrin polyclonal rabbit sera were described previously (Pescarreta et al., 1989) and were a gift from Dr Daniel Kiehart. The monoclonal antibodies against *Drosophila* α-tubulin were described by Blose et al. (1984) and were a gift from Dr Nipam Patel. A monoclonal antibody (4DF11) against *Drosophila* Vasa protein was described by Hay et al. (1988) and was a gift from Dr Yuh Nung Jan. Mouse anti-β-tubulin monoclonal antisera were generated and provided by K. Cant and L. Cooley, as described by Lin et al. (1994). The AffilPure™ donkey anti-rabbit or anti-mouse antibodies conjugated with TRITC, or FITC, or horseradish peroxidase (HRP) were purchased from Jackson ImmunoResearch Laboratories, Inc.

Immunostaining and confocal microscopy

For immunofluorescence staining, the anti-α-spectrin antibody was used at 1:200 dilution, the anti-β-tubulin antibody at 1:100 dilution, and the anti-α-tubulin antibody at 1:150 dilution. All the secondary antibodies were used at 1:200 dilution. Adult and larval ovaries were dissected and fixed as described previously (Lin and Spradling, 1993) and immunologically stained according to Lin et al. (1994). HRP histochemical staining and embryo fixation were according to Patel et al. (1989). Immunofluorescently labeled samples were also counterstained with DNA-specific dye DAPI as detailed by Lin and Spradling (1993). All the immunologically stained samples were examined using Nomarski and epifluorescence microscopy under a Zeiss Axioshot microscope. Fluorescently labeled samples were further subjected to confocal microscopic analysis using a Zeiss Laser Scanning Microscope. The Zeiss software was used for image collection and stacking of z-sections. Superimposition of double-labeled images was carried out using the Image-1 program (version 4.0, Universal Imaging Corporation, West Chester, PA). All the images were prepared for publication using Adobe Photoshop™ and Aldus Pagemaker™ programs.

Electron microscopy

Whole ovaries from either adult or third instar larvae were dissected
and processed for transmission electron microscopic analysis as described by Yue and Spradling (1992).

Determining the rate of germline stem cell division in hts1 mutant ovary
To compare the rate of germline stem cell division between wild-type and hts1 mutant ovaries, hts1/Cyo and hts1/Cyo sibling flies were dissected within 2 hours of eclosion so that all the egg chambers in the wild-type ovaries would be at early oogenic stages, up to stage 8, and, thus, still remain in the ovary (King, 1970). The wild-type and mutant ovarioles were dissected and stained with the anti-Vasa antibody to visualize germline cells in the germaria for easy counting of cysts. Twenty ovarioles from at least five females were counted for both hts1/Cyo and hts1/Cyo flies. The number of germline stem cell divisions equals the number of germline cysts in a germarium plus the number of postgermarial egg chambers in the same ovariole. hts1/Cyo and hts1/Cyo flies produced the same number of germline cysts and egg chambers (see Results).

Cytogenetic mapping of ovt and piwi mutations
To map P-induced ovt and piwi mutations, a biotinylated PZ DNA probe was prepared by the random hexamer extension method of Feinberg and Vogelstein (1983), except that dTTP was substituted by c-11 biotinylated dUTP. Polytenic squashes were prepared and hybridized to the biotinylated probe according to the method of Engel et al. (1986). The hybridized probe was detected by color reaction mediated by avidin-conjugated alkaline phosphatase.

P-element excision analysis
To induce the excision of the PZ element in piwi and ovt mutants (derived from the Canton S; ry506 parent), the mutant females were exposed to the P-transposase by mating them with Sp/Cyo; ShΔ2-3/TM6 males (Robertson et al., 1988). The resulting rySh+ (indicating P excision and free of P-transposase, respectively) flies were mated to flies carrying the original P alleles to test for the rescue of fertility, and whenever applicable, viability. Out of 50 independent ovt excision lines, 41 completely restored the fertility and viability. Out of 20 independent piwi excision lines, 13 completely restored female and male fertility. Genomic Southern blotting analysis, as based on standard protocols (Sambrook et al., 1989), verified that these revertant lines have restored the wild-type restriction pattern of the genomic DNA in the loci, while other non-revertant lines show various types of excision defect at the original P-insertion sites.

Complementation test between ovt and pum alleles
To determine whether ovt or piwi mutations belong to any known complementation groups in their corresponding cytogenetic region, inter se complementations were carried out between ovt alleles and all known mutants in 85C-D region (see Encyclopedia of Drosophila Release 2.0, September, 1995 by Berkeley Drosophila Genome Center). Complementation tests were also conducted for piwi alleles and the known mutants in the 32A-F region, piwi alleles complemented all the known EMS and single P mutations (data not shown). However, ovt alleles fail to complement or only partially complement the following five pumilio (pum) mutations: pum565, pumTE3, pumET1, pumFC8, and pumMic (Lehmann and Nusslein-Volhard, 1987; Barker et al., 1992; MacDonald, 1992). The results of the ovt complementation tests are summarized in Table 2.

RESULTS

Germline stem cells, contacting basal terminal filament cells, undergo self-renewing asymmetric divisions
In the Drosophila ovary, germline stem cells are among the 3-5 most apically located germ cells in the germarium (Brown and King, 1964; Wieschaus and Szabad, 1979; Lin and Spradling, 1993; also see Fig. 1A). To discriminate stem cells from cystoblasts and to observe their division pattern, we first correlated the position of these germ cells to their surrounding somatic cells. Wild-type germaria were immunofluorescently stained with the anti-α-spectrin antibody (Pesacreta et al., 1989) to outline somatic lines, to mark spectrosomes in germline stem cells and cystoblasts, and to label fusomes in the germline cysts (Lin et al., 1994; Lin and Spradling, 1995). The terminal filament cells show uniformly strong staining while the rest of germlarial somatic cells are weakly stained (Fig. 1A, also see Materials and Methods). We analyzed the 3-D arrangement of apical germlarial cells in 10 samples by serial confocal sectioning (see Materials and Methods). In all 10 germaria, the base of the terminal filament contains two to three squamous somatic cells (Fig. 1A-C) rather than a single basal cell (King, 1970; Godt and Laski, 1995; Sahut-Barnola et al., 1995). The basal terminal filament cells retain strong anti-spectrin staining (Fig. 1A,C) and share ultra-structural features with the rest of the terminal filament cells (Fig. 1B).

The basal cells are in contact with two to three, instead of a single, underlying germ cell. In these germ cells, the spectrosome is usually apically located in the cytoplasm, closely apposed to the basal terminal filament cells (Fig. 1A). Occasionally, the spectrosome is not in contact with the basal cells. Even so, it is still tethered to the basal cell by a thin tail (Fig. 1C).

The 2-3 most apical germ cells contacting the basal terminal filament cells are likely stem cells, given the expected number and location based on clonal analysis (Wieschaus and Szabad, 1979), laser ablation (Lin and Spradling, 1993), and mitotic analysis of cystoblasts (Lin and Spradling, 1995). To verify this, we analyzed the mitotic behavior of these cells by staining germaria with the anti-α-spectrin antibody to label spectrosomes, the anti-α-tubulin antibody to label mitotic spindles, and DAPI to label chromosomes (see Materials and Methods). The 2-3 apical germ cells showed striking asymmetry in the behavior of the spectrosome during mitosis. First, one pole of their mitotic spindles is always associated with the spectrosome and the terminal filament (Fig. 1D), clearly marking a cytological asymmetry of the division. Second, the mitotic spindles are oriented along the apico-basal axis of the germarium (within 30°), generating a daughter cell in contact with the basal cell and another daughter cell that is one cell away from the basal cell (Fig. 1D). This distal daughter cell, as reported previously (Lin and Spradling, 1995), undergoes incomplete divisions to form germline cysts. These observations strongly suggest that the 2-3 apical germline stem cells are stem cells, and that they divide asymmetrically with respect to basal terminal filament and the spectrosome to generate a daughter stem cell and a cystoblast.

Spectrosome formation correlates with major stages of germline stem cell development
The spectrosome might reflect an intracellular machinery for asymmetric division inherited from the stem cell lineage. To examine the ontogeny of the spectrosome, we used anti-Vasa antibodies to label germline cells and anti-α-spectrin and anti-Hts antibodies to monitor the dynamics of membrane skeletal proteins during germline development (see Materials and Methods). During early embryogenesis, both spectrin and Hts proteins in pole cells are localized at the cell periphery and are
present in the cytoplasm (Fig. 2A,B). However, at the germband extension stage, when the pole cells have migrated to the posterior midgut, a spectrin spot starts to appear in the cytoplasm of each pole cell (Fig. 2C,D). This appearance coincides with previously observed ultra-structural changes occurring at this stage, such as the fragmentation of polar bodies and the formation of fibrous bodies near the nuclear membrane (Mahowald, 1971). Subsequently, the spectrin spots increase in size, so that by the end of gastrulation when pole cells have congregated in the embryonic gonads, the spectrin sphere becomes easily detectable (Fig. 2E,F). During subsequent embryonic and larval development, the spectrin sphere continues to grow in size. In the first instar larval gonad, the spectrin spheres become more prominent (Fig. 2G,H), so that by the late third instar larval stage, when the ovary starts to differentiate, the spectrin sphere has reached its final size (Fig. 2I,J). Under the electron microscope (see Materials and Methods), the spectrin sphere at this stage has acquired all the characteristics of a spectrosome in the adult germline stem cells, such as a dearth of ribosomes, exclusion of other organelles, and enrichment of membranous vesicular materials (Fig. 3).

The above pattern of spectrosome development was confirmed by anti-Hts staining (data not shown). The only observed difference was that anti-Hts antibody first detects the cytoplasmic sphere in pole cells at the time they reach the ventral mesoderm, somewhat later than with the anti-spectrin antibody. This delayed detection by the anti-HTS antibody could either be due to the lower sensitivity of the anti-HTS antibody or the later incorporation of HTS into the spectrosome.

**hts₁** mutation abolishes the spectrosome but does not affect the rate of stem cell division

To investigate the possible role of the spectrosome in the stem cell division, we examined whether the germline can proliferate normally in the absence of the spectrosome. The **hts₁** mutation, which eliminates the expression of Hts in the germline (Yue and Spradling, 1992), abolishes spectrosomes in the adult ovary (Lin et al., 1994). To see whether the spectrosome is abolished prior to oogenesis, we stained homozygous **hts₁** mutant third instar larval ovaries with the anti-spectrin antibody (see Materials and Methods). In these ovaries, the spectrin molecules in germline cells are completely dispersed throughout the cytoplasm (Fig. 4). 65±12 (n=10) germ cells are present in each mutant ovary, a number similar to 63±10 (n=10) germ cells in the **hts₁/CyoO** control ovaries. However, germ cells in the **hts₁** ovaries are no longer confined to the middle region (Fig. 4, compare with 2I). Instead, they become scattered throughout the ovary. These results suggest that the lack of the spectrosome does not affect the proliferation of the germline primordial cells but indicates that hts function is required to correctly position germline stem cells.

To test whether the rate of stem cell division is affected in the absence of the spectrosome, we examined the number of germline cysts produced by **hts₁** mutant germaria with **hts₁/Cyo** heterozygous sibling females as controls (see Materials and Methods). Both **hts₁** and **hts₁/Cyo** ovaries contain similar number of ovarioles. The mutant ovarioles each contain 11.2±0.8 germline cysts and egg chambers while the wild-type controls each contain 11.7±0.5 cysts and egg chambers at the time of eclosion. Thus, the spectrosome does not regulate the rate of germline stem cell division.

**ovarette** mutations in the *pumilio* locus affect the asymmetric division of germline stem cells

To identify genes that control the asymmetric divisions, we searched for mutations that affect germline stem cell division by screening a collection of single P element enhancer-trap female sterile mutants using anti-vasa immunostaining and electron microscopic analyses (see Materials and Methods, and see below). This collection of mutants was generated in two

![Image 1](https://via.placeholder.com/150)

**Fig. 1.** The location and asymmetric division of germline stem cells. (A) Confocal image of the apical tip of a wild-type germarium visualized by an anti-α-spectrin antibody (see Materials and Methods). Note that the terminal filament (TF) contains two cells (basal cells, BC) at its base. In this case, each BC contacts a underlying germline stem cell (GSC) with a spectrosome in the stem cell associated with each BC. However, this one-to-one correspondence does not always exist (see C). (B) Electron micrograph of the germarial region corresponding to A. (C) Confocal image of a wild-type germarium stained with an anti-α-spectrin antibody, showing that two spectrosomes in two GSCs are associated with the BC via a thin tail, while the right one is closely apposed to the BC. (D) Confocal image of an asymmetrically dividing wild-type germline stem cell, with spectrin labeled in red and tubulin labeled in green.
Asymmetric germline stem cell division

Several mutations with such effects were identified in the two screens and were found to fall into two complementation groups. The first locus is represented by eight female sterile mutations and was named ovarette (ovt, see Table 1). We verified that the ovt mutant phenotype is caused by the inserted P element by showing that the sterile phenotype reverted to

Fig. 2. Ontogeny of the spectrosome. Embryonic stages are according to Campos-Ortega and Hartenstein (1985). A, C, E and G are confocal images of a cellular blastula (stage 5), a gastrula at late germ band extension stage (stage 11), a gastrula just completed germ band shortening (stage 14), and a first instar larva, respectively, with spectrin in red and Vasa in green. B, D, F and H are close-up views of germ cells in A, C, E and G, respectively. Note that the cytoplasmic spectrin spheres start to appear at stage 14 and continue to grow during subsequent development so that in a third instar larval ovary, where the newly formed germline stem cells reside in the medial region (I, with germ cells stained in black by anti-Vasa antibody), the spectrosomes in the germ cells have reached the final size (J). The bar in A denotes the magnification in A, C, E, and G; the bar in B denotes magnification in B, D, F and H; and the bar in I denotes magnification in I and J.
wild type at high frequency following P element excision (see Materials and Methods). The P element insertions in all these independently derived lines mapped at the same site, 85D1-2, by in situ hybridization (data not shown). Complementation analysis with previously defined genes in this region revealed that ovarette mutations are alleles of the pumilio(pum) locus (Lehmann and Nusslein-Volhard, 1987; Barker et al., 1992; MacDonald, 1992; see Materials and Methods; Table 2). Several lethal alleles of pumilio were also recovered in the P screens. They also fail to complement the pum mutations (Table 2). Because this class of female sterile mutations differ from previously described pumilio alleles, we refer to them as pum<sup>ovarette</sup> mutations.

To examine whether the small ovary phenotype was the result of stem cell dysfunction, we examined the germaria of females bearing a strong ov<sup>vt</sup> class mutation, pum<sup>2003</sup>. None of the mutant germ cells in pum<sup>2003</sup> germaria appear to undergo asymmetric divisions. Instead, anti-Vasa antibody staining shows that, in the newly eclosed mutant females, the great majority of ovarioles contain only two (or, less frequently, three) clusters of apparently undifferentiated germline cells (Fig. 5B,C). Under the electron microscope, these cell clusters do not show obvious signs of differentiation, such as dispersed nucleoli and the presence of ring canals (Fig. 5D). They appear to be diploid by DAPI staining, stain positively with anti-Vasa antibody, but do not

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Old names</th>
<th>% viability*</th>
<th>% sterility†</th>
<th>source‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>pum&lt;sup&gt;404&lt;/sup&gt;</td>
<td>l(3)RK404</td>
<td>2.6</td>
<td>100</td>
<td>a</td>
</tr>
<tr>
<td>pum&lt;sup&gt;277&lt;/sup&gt;</td>
<td>fs(3)04277</td>
<td>8.5</td>
<td>100</td>
<td>b</td>
</tr>
<tr>
<td>pum&lt;sup&gt;806&lt;/sup&gt;</td>
<td>fs(3)04806</td>
<td>8.6</td>
<td>100</td>
<td>b</td>
</tr>
<tr>
<td>pum&lt;sup&gt;2003&lt;/sup&gt;</td>
<td>ov&lt;sup&gt;vt&lt;/sup&gt; fs(3)02003</td>
<td>19</td>
<td>100</td>
<td>b</td>
</tr>
<tr>
<td>pum&lt;sup&gt;7098&lt;/sup&gt;</td>
<td>fs(3)07098</td>
<td>19</td>
<td>100</td>
<td>b</td>
</tr>
<tr>
<td>pum&lt;sup&gt;1688&lt;/sup&gt;</td>
<td>l(3)01688</td>
<td>25</td>
<td>100</td>
<td>b</td>
</tr>
<tr>
<td>pum&lt;sup&gt;2003&lt;/sup&gt;</td>
<td>l(3)03203</td>
<td>25</td>
<td>100</td>
<td>b</td>
</tr>
<tr>
<td>pum&lt;sup&gt;897&lt;/sup&gt;</td>
<td>fs(3)06897</td>
<td>32</td>
<td>100</td>
<td>b</td>
</tr>
<tr>
<td>piwi&lt;sup&gt;1&lt;/sup&gt;</td>
<td>fs(2)P-1</td>
<td>36</td>
<td>100</td>
<td>c</td>
</tr>
<tr>
<td>piwi&lt;sup&gt;2&lt;/sup&gt;</td>
<td>fs(2)ry5</td>
<td>50</td>
<td>100</td>
<td>a</td>
</tr>
<tr>
<td>piwi&lt;sup&gt;6843&lt;/sup&gt;</td>
<td>fs(3)06843</td>
<td>67</td>
<td>100</td>
<td>b</td>
</tr>
</tbody>
</table>

*Viability = 2×(no. of homozygotes)/no. of heterozygotes. For pum<sup>ovt</sup> mutants, at least 300 flies were counted for each allele except for pum<sup>406</sup> whose viability is based on counting 150 flies. For piwi mutants, at least 150 flies were counted for each allele.

†Sterility = 1–(no. of eggs laid per mutant female/no. of eggs laid per wild-type sibling). For each mutant, at least 10 homzygous females 3- to 5-day old were tested over a period of 5 days. No eggs were observed from any of the homzygous mutant females.

‡The above alleles were identified from the following single P element insertional mutagenesis screens: a, a screen by Cooley et al. (1988) that generated 1300 single P insertional mutants; b, a 25,000-chromosome screen by the Spradling lab and collaborators (Karpen and Spradling, 1992); c, a 1,000-chromosome screen by Zhang and Spradling (1994). Also see FlyBase (1994).
contain a spectrosome detectable using anti-α-spectrin antibodies or by the electron microscopy (Fig. 5D). This defect suggests that the 2-3 mutant germ line stem cells have undergone symmetric divisions to produce the clusters of un- (or ill-) differentiated germ line cells. The cell clusters eventually degenerate and become undetectable in aged females. As a result, most aged mutant females are completely devoid of germ line cells.

Some ovaries in newly eclosed pum2003 mutant females completely lack germ line cells, though individual ovarioles are clearly visible (Fig. 5E,F). This suggests that the mutation affects the ability of germ line cells to be maintained prior to ovary differentiation. Other germ lineless ovaries do not contain recognizable ovarioles (Fig. 5G,H), suggesting that the mutation may also affect the differentiation of the ovarian somatic cells.

A small number (approximately 2%) of the pum2003 mutant ovarioles, however, contain only 2-3 normally developing egg chambers instead of the undifferentiated cell clusters (Fig. 5I). These egg chambers eventually develop to morphologically normal mature oocyte which become the only germ line cells in the adult ovary (Fig. 5L). This suggests that the ovt mutation abolishes the ability of the germ line stem cells in these ovaries to maintain themselves but not to differentiate. Other ovit class alleles also showed both types of ovariole, but the proportion of the two types varied between the alleles.

The two alternative fates for ovit stem cells can sometimes be observed in the same ovariole or even the same egg chamber. Approximately 2% of the pum2003 ovarioles contain 1-2 undifferentiated germ cell clusters as well as a developing egg chamber (Fig. 5J), suggesting that pum2003 causes different defects to different germ line stem cells in the same ovarioles. Occasionally, a developing egg chamber also contains a small number of undifferentiated germ cells (Fig. 5K). These egg chambers eventually develop into normal mature oocytes or degenerate, since mature egg chambers associated with undifferentiated cells were not observed.

To examine whether pum2003 affects proliferation of germ line stem cells prior to oogenesis, we traced germ line development in the mutant from the beginning of embryogenesis to the late third instar larval stage when germ line stem cells are formed. Homozygous pum2003 embryos and larvae were stained with the anti-Vasa antibody to label the germ cells and they were compared to their phenotypically wild-type heterozygous siblings (see Materials and Methods). Germ line cells in the homozygous mutant embryos migrate and proliferate normally during embryogenesis (Fig. 6A-D). They eventually form a normal number of germ line stem cells by the late third instar larval stage (Fig. 6E). This suggest that the pum2003 mutation does not affect the proliferation of most germ line cells prior to oogenesis.

Although mutant third instar larval ovaries contain a normal number of germ line stem cells, the morphology of these stem cells is abnormal. An interesting defect is seen in the spectrosome in these stem cells (Fig. 6F,G). Anti-spectrin staining revealed that the spectrosomes in the mutant stem cells are often reduced in size or altered in shape, with the abnormalities varying from stem cell to stem cell within a single ovary (Fig. 6F, see Materials and Methods). Electron microscopy (see Materials and methods) reveals that, in extreme cases, the spectrosome is completely abolished, and membrane components in these cells are found dispersed throughout the cytoplasm (compare Fig. 6G with 3A).

**piwi mutations abolish the proliferation ability of both female and male germ line stem cells**

The second gene identified in the screen was named *piwi* (for *P*-element induced wimpy testis). The *piwi* gene was defined by two independent, non-complementing *P*-element insertional mutations, *piwi* and *piwi* (Table 1). The insertion in each allele was shown to reside at 32C on the second chromosome as determined by polytene in situ hybridization (data not shown, see Materials and Methods). A third insertion in the 32C region, originally called *fs(2)ny5* (Berg and Spradling, 1991), also failed to complement the other *piwi* alleles and was re-named *piwi*.

We verified that the *piwi* mutant phenotype is caused by the *piwi* insertions since excising the *P* element from *piwi* and *piwi* rescued the mutant defects (data not shown, see Materials and Methods). *piwi* appears to be a new gene based on complementation tests with known mutations in the region (data not shown). Among the three alleles, *piwi* and *piwi* show the strongest oogenetic phenotype. Moreover, *piwi* also causes male sterility and mutant males show severe defects in spermatogenesis.

We analyzed the effects of the *piwi* mutation on germ line

Table 2. Complementation behavior of *pum* alleles*

<table>
<thead>
<tr>
<th>New alleles‡</th>
<th>Known alleles†</th>
<th>New alleles‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>2003</td>
<td>mel</td>
<td>1688</td>
</tr>
<tr>
<td>4277</td>
<td>mel</td>
<td>3203</td>
</tr>
<tr>
<td>4806</td>
<td>mel</td>
<td>RK404</td>
</tr>
<tr>
<td>6897</td>
<td>oog. def.</td>
<td>1</td>
</tr>
<tr>
<td>7098</td>
<td>mel</td>
<td>oog. def.</td>
</tr>
<tr>
<td>1688</td>
<td>mel</td>
<td>mel</td>
</tr>
<tr>
<td>3203</td>
<td>mel</td>
<td>mel</td>
</tr>
<tr>
<td>RK404</td>
<td>mel</td>
<td>mel</td>
</tr>
</tbody>
</table>

*The defects of the *pum* transheterozygotes are classified into four phenotypic classes: (1) lethal (l), which indicates that, among at least 50 F1 flies that were examined, none was transheterozygous for the two involved *pum* alleles. (2) Oogenic defects (oog. def.), transheterozygous females are defective in oogenesis so that no eggs were produced. (3) Maternal effect lethal (mel), transheterozygous females produced morphologically normal eggs that fail to develop. (4) Weakly maternal effect lethal (wmel), the transheterozygous females produced eggs some of which developed into adulthood, reflecting partial complementation between the two involved *pum* alleles.

†Isolated by Lehmann and Nusslein-Volhard (1987), also see FlyBase (1994)

‡From this study. For details, see Table 1 footnote.
stem cell behavior by analyzing mutant germaria labeled with various antisera and by electron microscopy. The ovarioles of piwi\textsuperscript{1} females are most commonly devoid of germline cells, as indicated by Nomarski, DAPI, anti-Vasa and electron microscopic imaging analyses (Fig. 7A,B). Although individual ovarioles are present as revealed by electron microscopic analysis (Fig. 7B), they are often difficult to recognize. At low frequency, some ovarioles contain a few developing egg chambers or mature eggs (Fig. 7C). A small number of ovarioles contain 2-3 small clusters of germ cells, with each cluster composed of only a few germ cells (Fig. 7D). Given that the mutant ovaries initially contain normal number of germline stem cells (see below), this oogenic phenotype suggests that most of these stem cells either die or differentiate into an egg chamber, and thus fail to maintain themselves. The few surviving egg chambers in the mutant ovaries often show a variety of defects, including abnormal egg chamber polarity and reduced nurse cell number (Fig. 7C).

The spermatogenic defects of piwi\textsuperscript{1} mutants parallel its oogenic defects. Wild-type testes contain germline cells at all stages of spermatogenesis, including 5-8 germline stem cells at the apical tip as well as numerous bundles of mature sperm that occupy most of the lumenal space in the testis (Cooper, 1950; Fuller, 1993; Fig. 8E). In contrast, the piwi\textsuperscript{1} mutant testes

Fig. 5. The pum\textsuperscript{2003} mutation abolishes the asymmetry of germline stem cell division. In all panels (except for D) the samples were stained with anti-Vasa antibodies for germ cells. (A,B) A wild-type and an pum\textsuperscript{2003} mutant ovariole, respectively. Note that in B, the entire ovariole contains only two clusters of small germ cells. C is a line tracing of B, while D is an electron micrograph of a germ cell cluster similar to those in B in which the morphologically similar germ cells lack spectrosomes (based on examining over 1,000 such germ cells by EM). E and F are low and high magnification views of a pum\textsuperscript{2003} ovary in which ovarioles lack germ cells. (G,H) Nomarski and DAPI images of a pair of pum\textsuperscript{2003} ovaries lacking individual ovarioles and germline cells. (I) A pum\textsuperscript{2003} mutant ovariole containing only two developing egg chambers. (J) A pum\textsuperscript{2003} mutant ovariole containing two germ cell clusters and a developing egg chamber. (K) A pum\textsuperscript{2003} mutant ovariole containing a germ cell cluster and a developing egg chamber containing a small number of small germ cells. (L) An aged pum\textsuperscript{2003} mutant ovary containing only mature eggs.
only contain 1-6 bundles of mature sperm but no other germline cells (Fig. 7F). Since each sperm bundle derives from a single product of a stem cell division, this defect suggests that germline stem cells in the mutant testes either die or differentiate to found a spermatogenic cyst.

To analyze whether the mutant gonads initially contain a normal complement of germline cells, we examined the germline in the piwi1 mutant during embryonic and larval development using the anti-Vasa antibody to label germ cells and the anti-spectrin antibody to visualize spectrosomes and fusomes. Germ cells in mutant embryos developed normally during embryogenesis. The number of germline stem cells in the third instar larval ovaries was also found to be normal, and the spectrosomes in these cells appeared normal following staining with anti-spectrin antibodies (Fig. 8A). However, the stem cells often do not reside in the middle of the ovary. These analyses suggest that the piwi1 mutation does not affect the initial proliferation of germline stem cell population but acts later to disrupt stem cell division or maintenance during gametogenesis in both sexes.

To confirm this conclusion, we further examined the developing germline cysts in piwi1 mutant third instar testes. In wild-type third instar larval testes, the oldest cysts have progressed into meiosis (Fig. 8B). Premeiotic cysts contain an elaborate fusome connecting the 16 primary spermatocytes. These cysts grow in size as they leave the apical stem cell region and eventually enter meiosis. The mutant piwi1 testes at this stage show four defects (Fig. 8C). First, they often contain fewer growing cysts, suggesting a defect in germline stem cell division; second, the premeiotic cysts frequently contain fewer than 16 spermatocytes, suggesting a defect in spermatocyte division; third, the germline cysts often appear to develop aberrantly, as indicated by defective fusome morphology, suggesting a defect in spermatocyte differentiation; fourth, presumably because of the above defects, the mutant testes often are smaller than the wild-type testes. As with the defects seen in females, these results suggest that piwi function is required both to maintain germline stem cells and subsequently for the division and differentiation of the stem cell progeny in both sexes.

**pum and piwi may act in different cells**

Since the pum004 and piwi mutations were induced by enhancer trap P elements, we examined the pattern of lacZ expression in these lines. pum003, as well as four other ovt alleles, show lacZ expression specifically in the terminal filament (Fig. 9A), a group of apical somatic cells involved in regulating germline stem cell division (Lin and Spradling, 1993). No expression in the germline stem cells or other germ line cells was detected. In contrast, the piwi elements caused lacZ to be expressed in the germline both in the third instar larval ovary (Fig. 9B) and in the germarium in the adult ovary (Fig. 9C). These observations raise the prospect that pum004 and piwi mutations may act in different cells. Studies of the cellular autonomy and molecular biology of these loci will be presented elsewhere.

**DISCUSSION**

The molecular mechanisms that govern the asymmetric division of stem cells largely remain to be elucidated. We have
shown that the germline cells of the *Drosophila* ovary provide a valuable system for analyzing the molecular basis of stem cell function. While the asymmetric nature of germline stem cell divisions has been consistent with clonal analysis (Wieschaus and Szabad, 1979), no physical asymmetry in the divisions of presumptive stem cells had been observed previously in either the male or female germline. Moreover, no defined molecular components that segregated asymmetrically had been previously identified. While disrupting spectrosomes had little effect on the rate of stem cell division, our studies of *pum* and *piwi* mutations indicate that it is possible to perturb stem cell function genetically and suggest novel mechanisms that may operate in stem cells undergoing asymmetric division.

**The spectrosome segregates asymmetrically but is not required to maintain stem cells**

Spectrosomes are structurally related to fusomes, unusual cytoplasmic structures present during the production of germline cysts (Lin et al., 1994; Büning, 1994; Lin and Spradling, 1995). It has long been known that the fusome segregates asymmetrically during the last cystocyte division (Storto and King, 1989), and recently a similar asymmetry was observed during the first three divisions as well (Lin and Spradling, 1995). However, it was surprising to find a similar asymmetry in the stem cell. All of the aforementioned divisions with fusomal asymmetry give rise to daughter cells that remain connected by a ring canal. In contrast, the separation between stem cell and cystoblast is complete, and these daughter cells move far apart where such a connection could not be easily maintained. Nonetheless, most of the known molecular components of the fusome are also present in the spectrosome (Lin et al., 1994; McKearin and Ohlstein, 1995; de Cuevas et al., 1996; this study), and the behavior of these materials during metaphase appears very similar in stem cells as in divisions producing a cytoplasmic bridge. Spectosome-like structures are present in embryonic germ cells that will complete divisions and are also present in activated B lymphocytes (Gregorio et al., 1992). Consequently, the presence of fusome-like structures may not correlate with the production of interconnected daughters or daughters differing in developmental potential.

Asymmetric spectosome positioning during metaphase ultimately leads to the inheritance by the daughter cystoblast of a reduced amount of spectrosomal material (H. Lin, unpublished; M. de Cuevas and A. Spradling, unpublished) and provides a marker of asymmetry. However, disrupting the spectosome failed to reveal an essential role for these structures in stem cell maintenance or division rate. In *hts* females lacking a spectosome, cysts continue to be produced at essentially normal rates. However, *hts* cystoblasts undergo a drastically modified process of cyst formation and are unable to differentiate an oocyte (Yue and Spradling, 1992), presumably due to their inability to support a fusome (Lin et al., 1994). This suggests that the asymmetric segregation of the spectrosome in stem cells may only become important in subsequent divisions. Alternatively, *hts* cystoblasts may already contain defects that are not manifested until cysts begin to form.

The actin-based cytoskeleton is known to play an important role in defining the spindle orientation to establish a mitotic asymmetry during yeast budding (Palmer et al., 1992; Pringle et al., 1994).
et al., 1995), during specific embryonic divisions of *C. elegans* (Strome, 1993; Guo and Kemphues, 1996), and in *Fucus* zygotes (Goodner and Quatrano, 1993). In *Drosophila* neuroblasts, *inscuteable* mutations, which affect a protein with weak homology to ankyrin, disrupt the normal spindle orientation, the position of localized determinants, and abolish divisional asymmetry (Kraut et al., 1996). The association of the spectrosome with the basal terminal filament cells and the apical pole of the mitotic spindle suggests that the spectrosome may anchor one pole of the mitotic spindle so that only one of the two daughter cells will remain in contact with these specific somatic cells. Since the spectrosome is rich in membrane vesicles, it may also serve as a localization mechanism to retain factors important for maintaining stem cells. These observations make it plausible that spectrosomes play a dispensable role in the stem cell, possibly by defining divisional orientation or retaining stem cell determinants.

**Pum is required to maintain functional germline stem cells**

The isolation of a novel class of *pum* mutations that fail to maintain ovarian germline stem cells reveals a previously unknown role for this gene in the adult ovary. In females bearing *pum*/*ovt* mutations, most germline stem cells appear to divide symmetrically to produce small aggregates of apparently undifferentiated cells that remain in the ovarioles. These cell clusters differ from ovarian tumors since they are discrete clusters of cells unsurrounded by follicle cells. Moreover, unlike most analyzed ovarian tumors, the cells lack ring canals and appear to remain diploid (see Results). These germ cells, however, are not normal stem cells since they do not contain spectrosomes and do not undergo asymmetric divisions. At a frequency that depends on the particular allele, some stem cells are able to follow a different pathway. They differentiate into normal egg chambers that can complete oogenesis. This implies that the stem cell or a daughter was able to acquire the identity of a normal cystoblast and successfully execute cyst formation and all subsequent steps. The relative specificity of the effects to germline stem cells we observed suggest that these cells may be more sensitive to perturbations of *pum* regulation than other germline cells. Clonal analysis using null *pum* alleles will be required to reveal the role of *pum* during earlier stages of germline development.

A variety of mutations have been described previously that produce ovarian tumors, including *Sex-lethal* (Cline, 1983), *sans fille* (Flickinger and Salz, 1994), *ovarian tumor* (Storto and King, 1989), *bag-of-marbles* (McKearin and Spradling, 1993), and *piwi* (Xue et al., 1995).

---

**Fig. 8.** *piwi*/*mutant larval gonads. (A,C) Confocal images of a *piwi*/*mutant ovary and testis, respectively, with *Vasa* stained in green and *spectrin* stained in red. (B) A wild-type third instar larval testis stained for spectrin. B and C are at the same magnification.

**Fig. 9.** The enhancer trap pattern of *ovt* and *piwi*/*mutant mutations. (A) An *ovt*/*TM3* germarium, showing *lacZ* staining specific to the terminal filament. (B) A *piwi*/*TM3* third instar larval ovary, showing *lacZ* staining in germline stem cells which reside in the equatorial region of the ovary. (C) An adult *piwi*/*TM3* ovariole, showing *lacZ* staining in germline cells in the germarium.
1990), benign gonial cell neoplasm (Gateff, 1982), and fused (King, 1970). However, these genes produce quite different effects from pumovt lesions. Except in strong otu alleles, which affect germ cell survival even prior to oogenesis, these tumors contain spectrosomes, fusomes, and often polyploid ‘pseudo-nurse’ cells as well as diploid cells (Lin et al., 1994; McKearin and Olstein, 1995). Undifferentiated cell clusters are rarely found along with mature eggs in the same ovariole. Consequently, pum has very different effects on stem cells than previously described mutations.

Previous studies of pum provide a simple model for its potential role in maintaining stem cells. pum was initially identified as a maternal effect gene required for establishing the posterior identity of the embryo (Lehmann and Nusslein-Volhard, 1987; Barker et al., 1992; MacDonald, 1992). Pum acts in concert with Nos to posttranscriptionally block the utilization of maternal hunchback mRNA stored in the posterior of the Drosophila egg. Pum and Nos probably suppress translation by directly binding to nos-regulatory elements (NREs) located in the 3’ untranslated region of hunchback mRNA and other target molecules (Murata and Wharton, 1995). Although it was previously thought that Nos and Pum functioned primarily to allow abdomen formation, Nos is now also known to be required for embryonic germ cell migration (Kobayashi et al., 1996). In these nos mutant pole cells, several mRNAs are translated prematurely (Kobayashi et al., 1996). This inappropriate expression may precociously initiate germ cell differentiation and block their ability to migrate properly.

A similar mechanism may explain the effect of pumovt mutations on germline stem cells. The simplest interpretation of our results would be for Pum and likely Nos to act in the stem cell itself where they might act in concert with germ cell-specific molecules such as Vasa to participate in translational suppression, which keeps certain genes inactive until specific times in development or in the cell cycle. Inappropriate expression of the suppressed genes would cause the stem cells to assume a cystoblast identity or to proceed down an abnormal developmental pathway leading to the undifferentiated cell clusters. In support of this mechanism, Lehmann and Nusslein-Volhard (1991) reported that certain strong alleles of nos also appear to be required to maintain germline stem cells, although the cellular defects in these alleles have not been reported in detail.

However, the pum and nos regulatory mechanism does not appear to be confined to the germline. Strong pum alleles are lethal and the pum mRNA and protein are widely distributed at all stages of development (Barker et al., 1992; MacDonald, 1992). Moreover, most pumovt alleles show the lacZ enhancer trap expression specifically in the terminal filament. Thus, it remains possible that pum acts in somatic cells to block a mechanism that suppresses the terminal filament signaling required for stem cell maintenance or asymmetric division.

Several previous observations on the Drosophila ovary make such a role for the terminal filament likely. A signal from somatic cells adjacent to the stem cells could explain the effects of terminal filament ablation on stem cell activity (Lin and Spradling, 1993). Terminal filament cells are known to express hedgehog and basal terminal filament cells express wingless (Forbes et al., 1996a,b). Perturbations of hedgehog signaling had little effect on germline stem cells but strongly influence the proliferation of ovarian somatic cells (Forbes et al., 1996a,b). There is extensive precedence for the regulation of stem cell activity by adjacent cells; for example, in epithelia (Jones et al., 1995; Canfield et al., 1996; Li et al., 1996), the male germline (Smithwick and Young, 1996), the developing cerebrum (Chenn and McConnell, 1995; Zhong et al., 1996), and the hematopoietic system (Muller-Sieburg and Deryugina, 1995; Hardy and Megason, 1996).

**piwi is required to maintain germline stem cells**

A newly formed ovary contains at least two stem cells per ovariole while each newly differentiated testis contains 5-8 stem cells. piwi mutant gonads contain fewer than this number of egg chambers or sperm bundles, respectively. Since a normal number of germline stem cells are present at the onset of oogenesis and spermatogenesis, piwi may prevent the differentiation of germ line stem cells in both sexes. However, our results are equally consistent with a role for piwi in maintaining the viability of germline cells during pupal and adult life. Germ cells that had developed past the stem cell stage showed diverse defects, including the formation of cysts with a reduced number of nurse cells, mis-positioned oocytes, and degenerating chambers. The gene may function in the germline itself since an enhancer-trap allele is expressed in the germline cells in the gerarium. Molecular studies of this gene will reveal more about its mechanisms of function.

We are grateful to Mr Mike Sepanski for valuable assistance with electron microscopy, Dr Ping Zhang for the piwi1 mutation, Mr Alexander Urioste for assistance with ovtrpum complementation analysis, and Dr Michael Parisi for quantifying the lethality of ovtr mutations. We thank Dr Lynn Cooley for the anti-Hts antibody, Dr Daniel Kiehart for the anti-spectrin antibody, Dr Yuh Nung Jan for the anti-Vasa antibody, Dr Nipam Patel for the anti-tubulin antibody and lacZ marked CyO and TM3 chromosomes, and Dr Allan Shearn for the CyO y+ chromosome. This research was supported by Howard Hughes Medical Institute. H. L. was a Jane Coffin Childs Fellow for Medical Research.

**REFERENCES**


