Development 124, 2463-2476 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV8418

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

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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 germline 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 germline 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 germline 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 germline cells, which in many species are specified early during embryogenesis by the localized germline 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). *pum* is widely expressed and has effects outside the gonads, as *pum* 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 germline 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 germline 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 germarium, producing new egg chambers that leave the germarium 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 germarium, 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 embyros 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 Bam protein required for cystoblast differentiation (McKearin 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 containing a copy of the wild-type *y* gene (Timmons et al., 1993). The *ovt*, *piwi* and hts^1 (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*(*ry*) gene, and contains a *lacZ* gene fused to the P element promoter for enhancer trapping. The pum^{680}, pum^{TE3}, pum^{ET1}, pum^{FC8} and pum^{Msc} 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 $CaCl_2$, 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 (Pesacreta 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 (46F11) against *Drosophila* Vasa protein was described by Hay et al. (1988) and was a gift from Dr Yuh Nung Jan. Mouse anti-Hts polyclonal 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-Hts 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 Axiophot 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 *hts*^{1} mutant ovary

To compare the rate of germline stem cell division between wild-type and *hts*^{1} mutant ovaries, *hts*^{1}/*CyO* and *hts*^{1}/*hts*^{1} 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 *hts*^{1}/*CyO* and *hts*^{1}/*hts*^{1} 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. *hts*^{1}/*CyO* and *hts*^{1}/*hts*^{1} 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. Polytene squashes were prepared and hybridized to the biotinylated probe according to the method of Engels 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; ry*^{506} parent), the mutant females were exposed to the P-transposase by mating them with *Sp/CyO; SbΔ2-3/TM6* males (Robertson et al., 1988). The resulting *ry*$^-$ *Sb*$^+$ (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*^{1} excision lines, 41 completely restored the fertility and viability. Out of 20 independent *piwi*^{1} 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: *pum*^{680}, *pum*^{TE3}, *pum*^{ET1}, *pum*^{FC8}, and *pum*^{Msc} (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 cells, 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 germarial somatic cells are weakly stained (Fig. 1A, also see Materials and Methods). We analyzed the 3-D arrangement of apical germarial 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). These 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 (Wieshcuas 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 $\pm 30^o$), 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.

hts^{1} 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 *hts*^{1} 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 *hts*^{1} 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 *hts*^{1}*/CyO* control ovaries. However, germ cells in the *hts*^{1} ovaries are no longer confined to the middle region (Fig. 4, compare with 2J). 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 *hts*^{1} mutant germaria with *hts*^{1}*/Cyo* heterozygous sibling females as controls (see Materials and Methods). Both *hts*^{1} and *hts*^{1}*/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

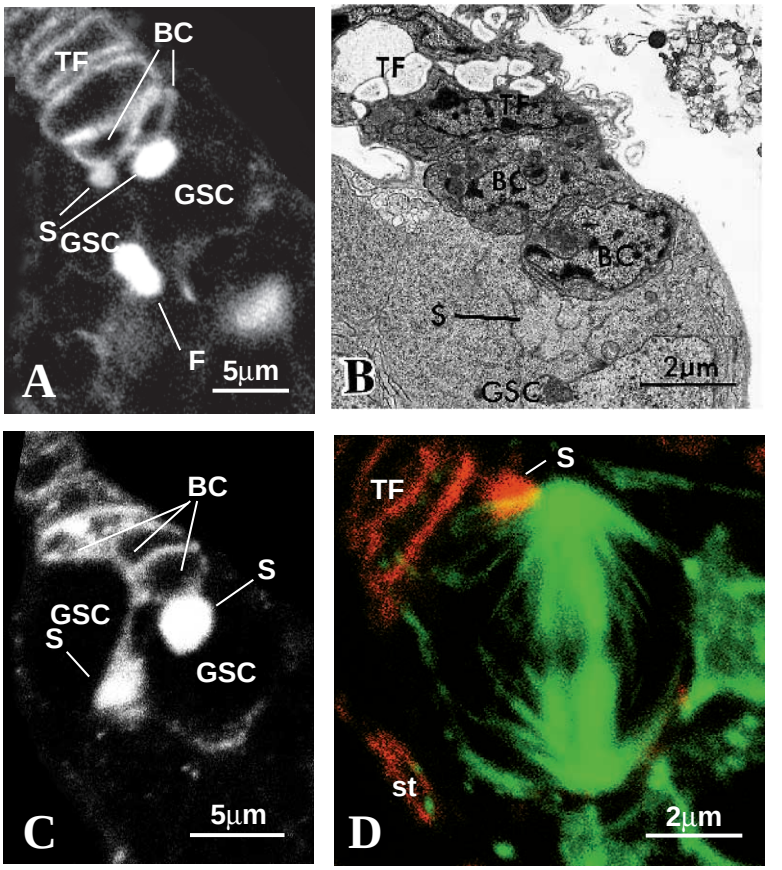

Fig. 1. The location and asymmetric division of germline stem cells. (A) Confocal image of the apical tip of a wild-type germarium visualized by an anti-α-spectrin antibody (see Materials and Methods). Note that the terminal filament (TF) contains two cells (basal cells, BC) at its base. In this case, 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 a single BC in different fashions. The left spectrosome is associated with the BC via a thin tail, while the right one isclosely 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.

mutagenesis screens involving more than 8,500 chromosomes (Karpen and Spradling, 1992; Zhang and Spradling, 1994). Females bearing mutations in stem cell function would be expected to be weakly fertile or sterile and contain small ovaries in which the 2-3 stem cells in each ovariole had differentiated into egg chambers (Lin and Spradling, 1993). 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

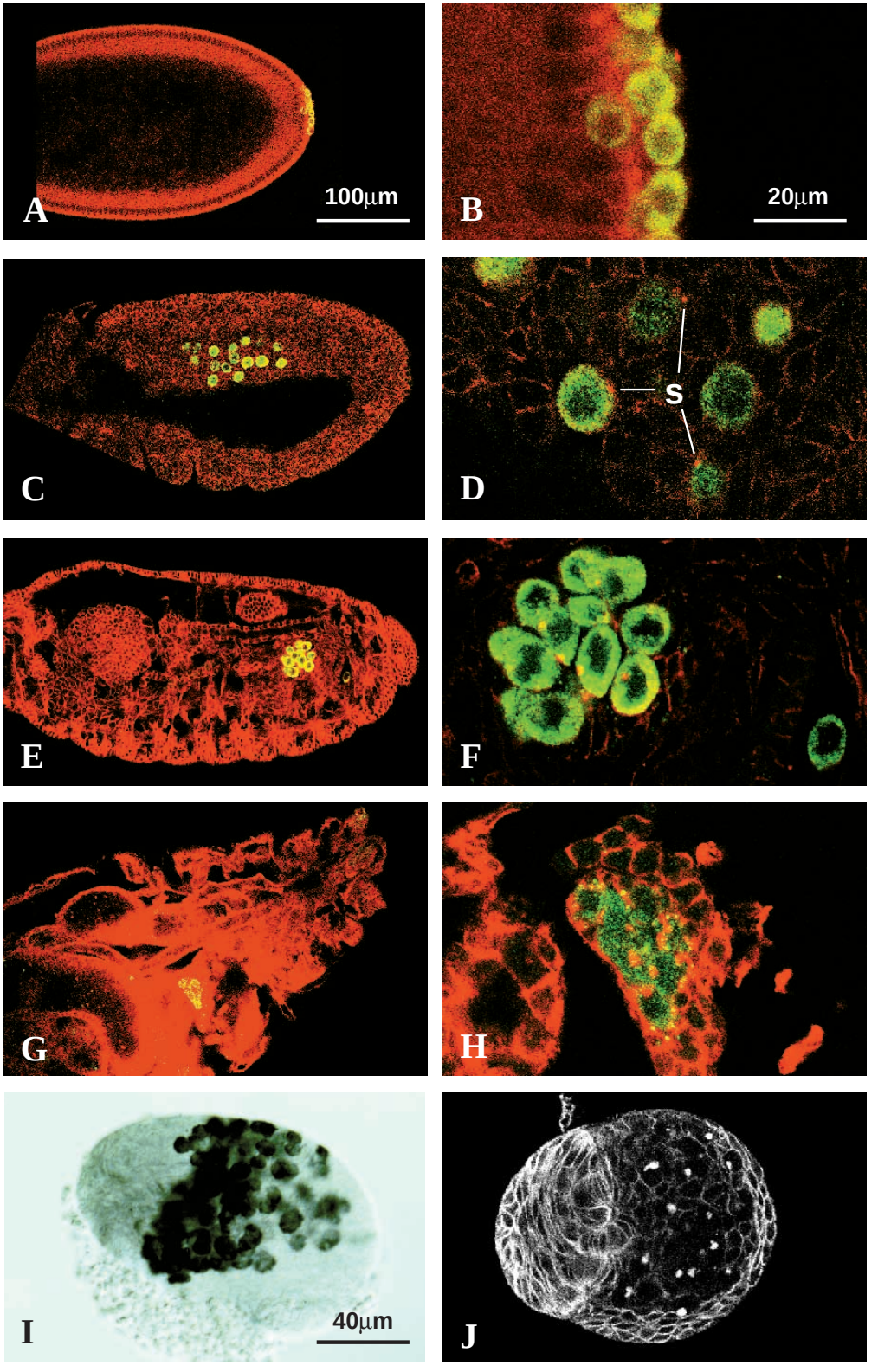

Fig. 2. Ontogeny of the spectrosome. Embryonic stages are according to Campos-Ortega and Hartensein (1985). A,C,E and G are confocal images of a cellular blastula (stage 5), a gastrula at late germband extension stage (stage 11), a gastrula just completed germband shortening (stage 14), and a first instar 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.

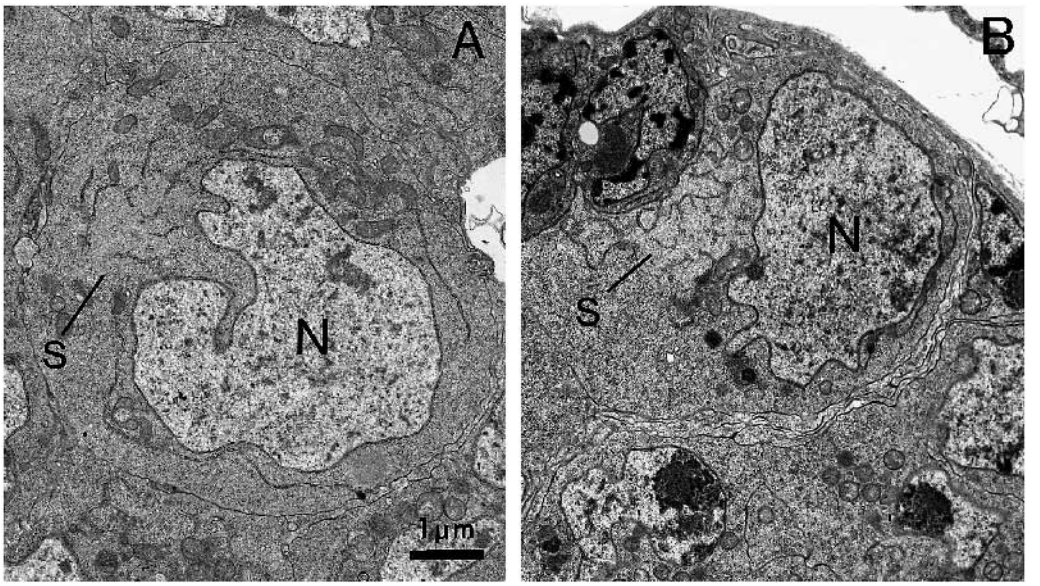

Fig. 3. The ultrastructure of spectrosomes in the third instar larval (A) and adult (B) ovaries. S, spectrosome; N, nucleus. The bar in A denotes the magnification for both panels.

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 $pum^{ovarette}$ mutations.

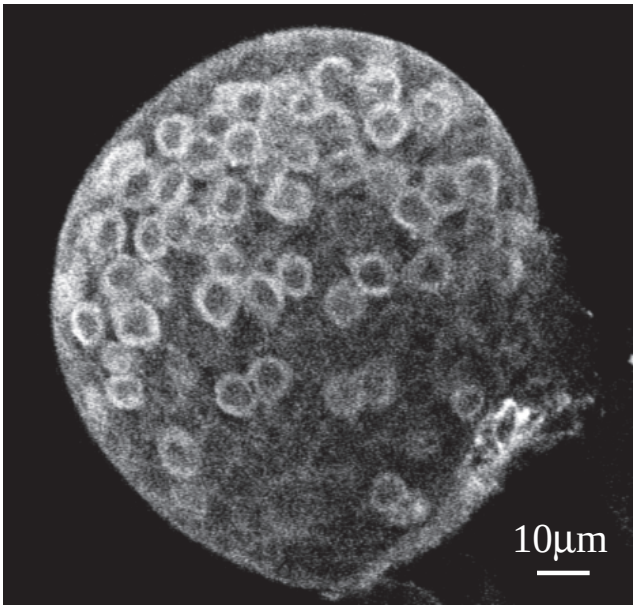

Fig. 4. Confocal image of a homozygous hts^1 third instar larval ovary as visualized by anti-spectrin staining. Note that spectrin is dispersed throughout the cytoplasm (Compare with Fig. 2J).

To examine whether the small ovary phenotype was the result of stem cell dysfunction, we examined the germaria of females bearing a strong *ovt* class mutation, pum^{2003}. None of the mutant germ cells in pum^{2003} 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 1. *ovarette* mutations in the *pumilio* locus and *piwi* mutations

Mutations	Old names	% viability*	% sterility†	source‡
pum^{404}	*l(3)RK404*	2.6	100	a
pum^{4277}	*fs(3)04277*	8.5	100	b
pum^{4806}	*fs(3)04806*	8.6	100	b
pum^{2003}	ovt^{1}, *fs(3)02003*	19	100	b
pum^{7098}	*fs(3)07098*	19	100	b
pum^{1688}	*l(3)01688*	25	100	b
pum^{3203}	*l(3)03203*	25	100	b
pum^{6897}	*fs(3)06897*	32	100	b
$piwi^{1}$	*fs(2)P-1*	36	100	c
$piwi^{2}$	*fs(2)ry5*	50	100	a
$piwi^{6843}$	*fs(3)06843*	67	100	b

*Viability = 2×(no. of homozygotes)/no. of heterozygotes. For pum^{ovt} mutants, at least 300 flies were counted for each allele except for pum^{4806} 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 homozygous females 3- to 5-day old were tested over a period of 5 days. No eggs were observed from any of the homozygous 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).

Table 2. Complementation behavior of *pum* alleles*

	Known alleles†					New alleles‡		
New alleles‡	Msc	FC8	ET1	680	TE3	1688	3203	RK404
2003	mel	mel	mel	mel	wmel	l	oog. def.	mel
4277	l	wmel	mel	mel	wmel	l	mel	wmel
4806	l	wmel	wmel	mel	wmel	l	oog. def.	wmel
6897	oog. def.	oog. def.	oog. def.	mel	mel	l	oog. def.	mel
7098	l	oog. def.	mel	mel	wmel	oog. def.	oog. def.	oog. def.
1688	l	wmel	wmel	mel	mel			
3203	oog. def.	oog. def.	oog. def.	mel	mel			
RK404	mel	wmel	wmel	mel	wmel			

*The defects of the *pum* transheterozygotes are classified into four phenotypic classes: (1) lethal (l), which indicates that, among at least 50 F_1 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.

contain a spectrosome detectable using anti-α-spectrin antibodies or by the electron microscopy (Fig. 5D). This defect suggests that the 2-3 mutant germline stem cells have undergone symmetric divisions to produce the clusters of un- (or ill-) differentiated germline cells. The cell clusters eventually degenerate and become undetectable in aged females. As a result, most aged mutant females are completely devoid of germline cells.

Some ovaries in newly eclosed pum^{2003} mutant females completely lack germline cells, though individual ovarioles are clearly visible (Fig. 5E,F). This suggests that the mutation affects the ability of germline cells to be maintained prior to ovary differentiation. Other germlineless 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 pum^{2003} 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 germline cells in the adult ovary (Fig. 5L). This suggests that the *ovt* mutation abolishes the ability of the germline stem cells in these ovarioles to maintain themselves but not to differentiate. Other *ovt* class alleles also showed both types of ovariole, but the proportion of the two types varied between the alleles.

The two alternative fates for *ovt* stem cells can sometimes be observed in the same ovariole or even the same egg chamber. Approximately 2% of the pum^{2003} ovarioles contain 1-2 undifferentiated germ cell clusters as well as a developing egg chamber (Fig. 5J), suggesting that pum^{2003} causes different defects to different germline 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 pum^{2003} affects proliferation of germline stem cells prior to oogenesis, we traced germline development in the mutant from the beginning of embryogenesis to the late third instar larval stage when germline stem cells are formed. Homozygous pum^{2003} 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). Germline cells in the homozygous mutant embryos migrate and proliferate normally during embryogenesis (Fig. 6A-D). They eventually form a normal number of germline stem cells by the late third instar larval stage (Fig. 6E). This suggest that the pum^{2003} mutation does not affect the proliferation of most germline cells prior to oogenesis.

Although mutant third instar larval ovaries contain a normal number of germline 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 germline stem cells

The second gene identified in the screen was named *piwi* (for *P*-element *i*nduced *wi*mpy testis). The *piwi* gene was defined by two independent, non-complementing P-element insertional mutations, $piwi^{1}$ and $piwi^{6843}$ (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)ry5* (Berg and Spradling, 1991), also failed to complement the other *piwi* alleles and was re-named $piwi^{2}$. We verified that the *piwi* mutant phenotype is caused by the *piwi* insertions since excising the P element from $piwi^{1}$ and $piwi^{6843}$ 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, $piwi^{1}$ and $piwi^{2}$ show the strongest oogenic phenotype. Moreover, $piwi^{1}$ also causes male sterility and mutant males show severe defects in spermatogenesis.

We analyzed the effects of the $piwi^{1}$ mutation on germ line

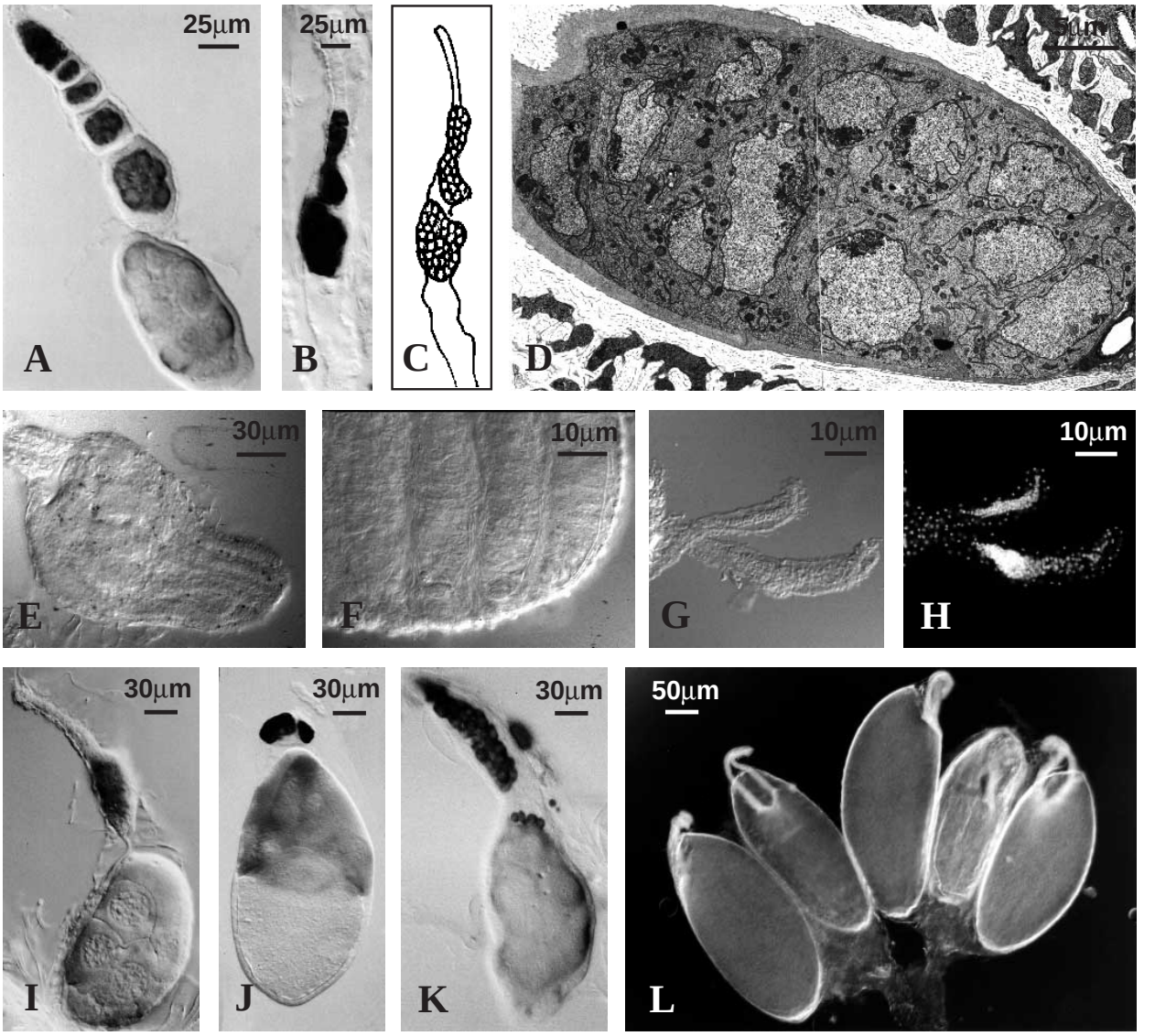

Fig. 5. The *pum*^{2003} mutation abolishes the asymmetry of germline stem cell division. In all panels (except for D) the samples were stained with anti-Vasa antibodies for germ cells. (A,B) A wild-type and an *pum*^{2003} mutant ovariole, respectively. Note that in B, the entire ovariole contains only 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*^{2003} ovary in which ovarioles lack germ cells. (G,H) Nomarski and DAPI images of a pair of *pum*^{2003} ovaries lacking individual ovarioles and germ cells. (I) A *pum*^{2003} mutant ovariole containing only two developing egg chambers. (J) A *pum*^{2003} mutant ovariole containing two germ cell clusters and a developing egg chamber. (K) A *pum*^{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*^{2003} mutant ovary containing only mature eggs.

stem cell behavior by analyzing mutant germaria labeled with various antisera and by electron microscopy. The ovarioles of *piwi*^{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*^{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*^{1} mutant testes

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 *piwi*^{1} 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 *piwi*^{1} 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 *piwi*^{1} 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 *piwi*^{1} 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 then 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 *pum*^{ovt} and *piwi* mutations were induced by enhancer trap P elements, we examined the pattern of *lacZ* expression in these lines. *pum*^{2003}, 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 *pum*^{ovt} 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

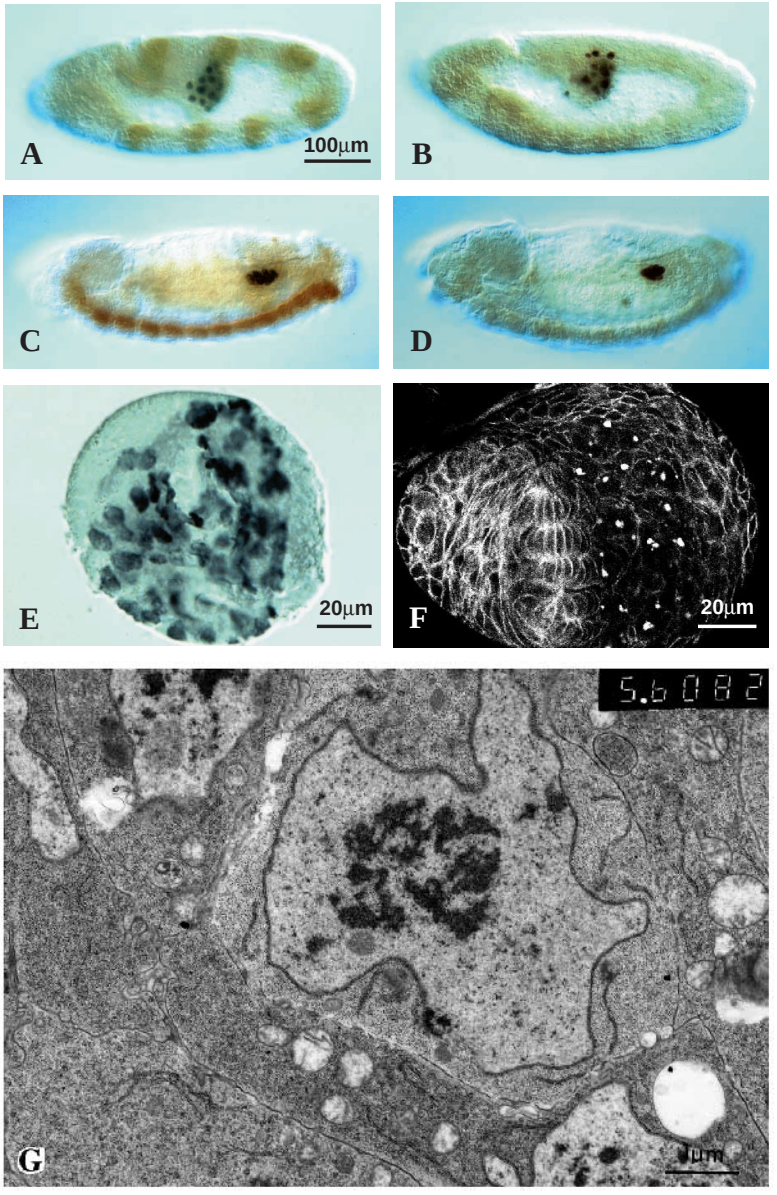

Fig. 6. Pre-oogenic germline development in *pum*^{2003} mutant. Germ cells in A-E are stained dark brown by anti-Vasa antibody. (A,C) Wild-type embryos during germ cell migration (stage 11) and embryonic gonad formation (stage 14), respectively. The wild-type embryos are marked by the expression (in stripes) of a *ftz-lacZ* transgene on the *TM3* balancer chromosome. (B,D) Mutant embryos at stages 11 and 14, respectively. (E,F) A mutant late third instar larval ovary stained for Vasa and spectrin, respectively. (G) Electron micrograph of a germline stem cell in the mutant late third instar larval ovary with no obvious spectrosome organization. This stem cell was subjected to complete serial EM section analysis. The bar in A denotes the magnification in A-D.

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*^{ovt} 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. Spectrosome-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 spectrosome 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 spectrosome failed to reveal an essential role for these structures in stem cell maintenance or division rate. In *hts*^{1} females lacking a spectrosome, cysts continue to be produced at essentially normal rates. However, *hts*^{1} 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*^{1} 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

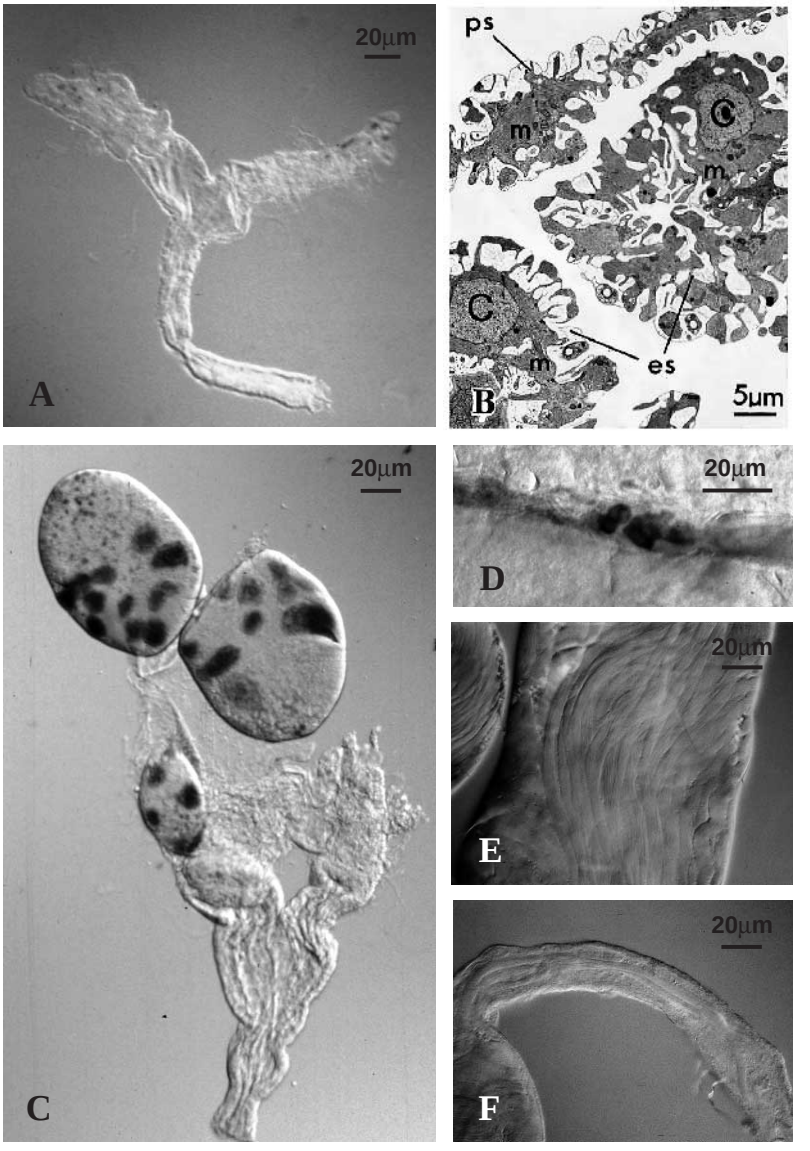

Fig. 7. The *piwi*^{1} mutation affects the maintenance of both male and female germline stem cells. (A) A typical pair of germlineless *piwi*^{1} ovaries. (B) An EM cross-section image of a part of a typical *piwi*^{1} ovary, showing two adjacent ovarioles, each containing a single cell (C) enveloped by the collapsed epithelial sheath (es) rich in musculature fibers (m). The ovarioles are encased by a peritoneal sheath (ps) which is also rich in musculature fibers. (C) A *piwi*^{1} mutant ovary containing several poorly-developed egg chambers. (D) A *piwi*^{1} ovariole containing only two small clusters of germ cells, in contrast to the large germ cell clusters in *ovt*^{1} mutant. (E) A section of a wild-type testis containing numerous bundles of sperm. Panel F, a typical *piwi*^{1} mutant testis with only a few bundles of sperm.

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.

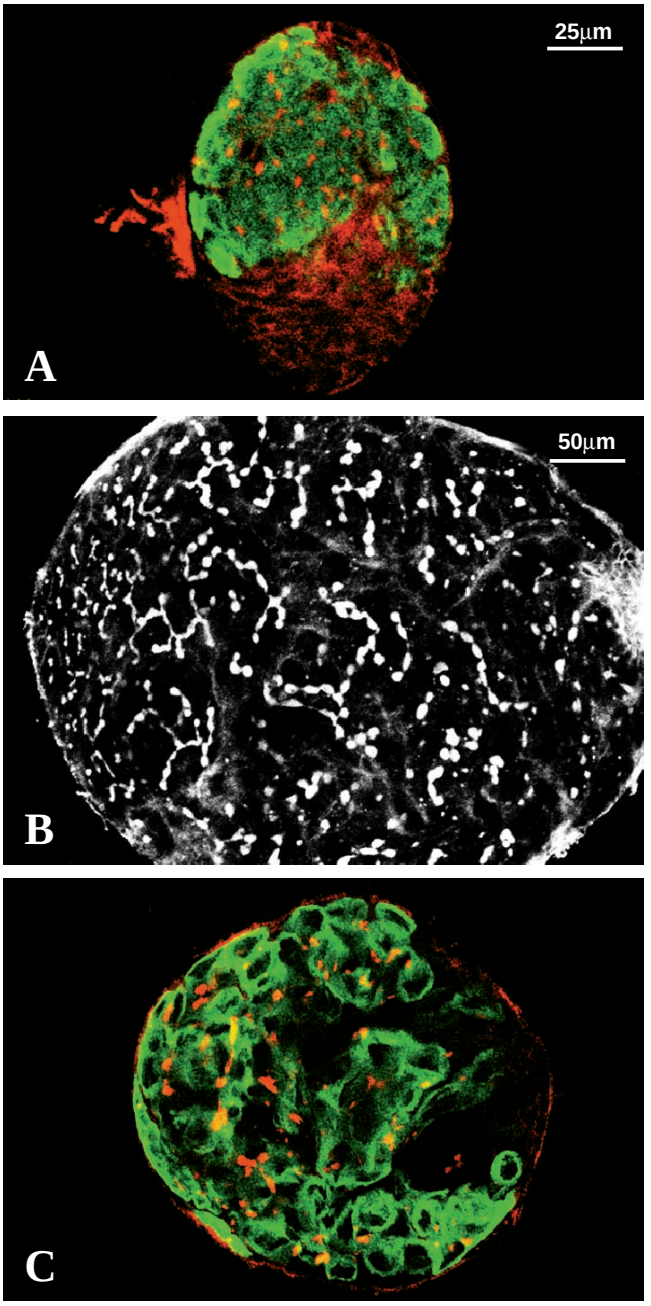

Fig. 8. *piwi*^{1} mutant larval gonads. (A,C) Confocal images of a *piwi*^{1} mutant ovary and testis, respectively, with Vasa stained in green and spectrin stained in red. (B) A wild-type third instar larval testis stained for spectrin. B and C are at the same magnification.

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,

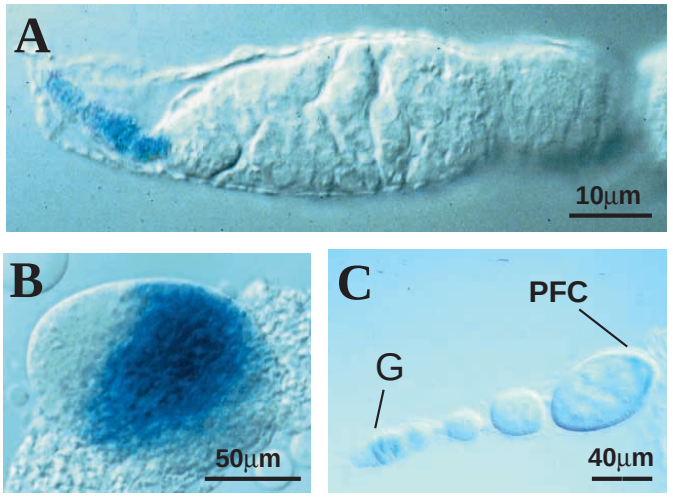

Fig. 9. The enhancer trap pattern of *ovt*^{1} and *piwi*^{1} mutations. (A) An *ovt*^{1}*/TM3* germarium, showing *lacZ* staining specific to the terminal filament. (B) A *piwi*^{1}*/TM3* third instar larval ovary, showing *lacZ* staining in germline stem cells which reside in the equatorial region of the ovary. (C) An adult *piwi*^{1}*/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 *pum^{ovt}* 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 *pum^{ovt}* 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 *pum^{ovt}* 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 germarium. 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 *piwi^{1}* mutation, Mr Alexander Urioste for assistance with *ovt/pum* complementation analysis, and Dr Michael Parisi for quantifying the lethality of *ovt* 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

Barker, D. D., Wang, C., Moore, J., Dickinson, L. K. and Lehmann, R. (1992). Pumilio is essential for function but not for distribution of the Drosophila abdominal determinant Nanos. *Genes Dev.* **6**, 2312-2326.

Beklemishev, W. N. (1969). *Principals of Comparative Anatomy of Invertebrates*. Edinburgh, Oliver and Boyd.

Berg, C. and Spradling, A. C. (1991). Studies on the rate and site-specificity of P element transposition. *Genetics* **127**, 515-524.

Blose, S. H., Meltzer, D. I., and Feramisco, J. R. (1984). 10-nm filaments are induced to collapse in living cells microinjected with monoclonal and polyclonal antibodies against tubulin. *J. Cell Biol.* **98**, 847-858.

Boswell, R. E. and Mahowald, A. P. (1985). *Tudor*, a gene required for the proper assembly of the germ plasm of *Drosophila melanogaster*. *Cell* **43**, 97-104.

Boswell, R. E., Prout, M. E. and Steichen, J. C. (1991). Mutations in a newly identified *Drosophila melanogaster* gene, *mago nashi*, disrupt germ cell formation and result in the formation of mirror-image symmetrical double abdomen embryos. *Development* **113**, 373-384.

Browder, L. W. (1984). *Developmental Biology*. pp. 497-516. Philadelphia, Saunders College Publishing.

Brown, E. H. and King, R. C. (1962). Oogonial and spermatogonial differentiation within a mosaic gonad of *Drosophila melanogaster*. *Growth* **26**, 53-70.

Brown, E. H. and King, R. C. (1964). Studies on the events resulting in the formation of an egg chamber in *Drosophila melanogaster*. *Growth* **28,** 41-81.

Bruno, E., Luikart, S. D. L., M. W. and Hoffman, R. (1995). Marrow-derived heparan sulfate proteoglycan mediates the adhesion of hematopoietic progenitor cells to cytokines. *Exp. Hematol.* **23**, 1212-1217.

Buder, J. E. (1917). Die Spermatogenese von Deilephele euphorbiae. *L. Arch. Zellforsch.* **14**, 26-78.
Buning, J. (1994). *The Insect Ovary*. Chapman and Hall, London.
Byskov, A. G. (1974). Does the rate ovarii act as a trigger for the onset of meiosis? *Nature* **252**, 396-397.
Campos-Ortega, J. A. and Hartensein, V. (1985). *The Embryonic Development of* Drosophila melanogaster. Berlin, Springer-Verlag.
Canfield, V., West, A. B., Goldenring, J. R. and Levenson, R. (1996). Genetic ablation of parietal cells in transgenic mice: a new model for analyzing cell lineage relationships in the gastric mucosa. *Proc. Natl. Acad. Sci. USA* **93**, 2431-2435.
Chenn, A. and McConnell, S. K. (1995). Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. *Cell* **82**, 631-641.
Cline, T. W. (1983). Functioning of the genes *daughterless* (*da*) and *Sex-lethal* (*Sxl*) in Drosophila germ cells. *Genetics* **104**, 16-17.
Cooley, L., Kelly, R. and Spradling, A. (1988). Insertional mutagenesis of the Drosophila genome with single P elements. *Science* **239**, 1121-1128.
Cooper, K. W. (1950). Normal spermatogenesis in *Drosophila*. In *Biology of Drosophila*. New York, John Wiley and Sons, Inc. 1-61.
de Cuevas, M., Lee, J. and Spradling, A C. (1996) α-spectrin is required for germline cell division and differentiation in the *Drosophila* ovary. *Development* **122**, 3959-3968.
Ding, D., Parkhurst, S. M., Halsell, S. R. and Lipshitz, H. D. (1993). Dynamic Hsp83 RNA localization during Drosophila oogenesis and embryogenesis. *Mol. Cell Biol.* **13**, 3773-3781.
Engels, W. R., Preston, C. R., Thompson, P. and Eggleston, W. B. (1986). *In situ* hybridization to Drosophila salivary chromosomes with biotinylated DNA probes and alkaline phosphatase. *Focus* **8**, 6-8.
Eddy, E. M. (1975). Germ plasm and the differentiation of the germ cell line. *Int. Rev. Cytol.* **43**, 229.
Ellis, R. E. and Kimble, J. (1994). Control of germ cell differentiation in *Caenorhabditis elegans*. *Ciba Found Symp.* **82**, 179-88; discussion 189-92.
Feinberg, A. P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132,** 6-13.
Fleischman, R. A. (1993). From white spots to stem cells: the role of the kit receptor in mammalian development. *Trends Genet.* **9**, 285-90.
Flickinger, T. W. and Salz, H. K. (1994) The Drosophila sex determination gene *snf* encodes a nuclear protein with sequence and functional similarity to the mammalian U1A snRNP protein. *Genes Dev.* **8,** 914-925.
Forbes, A. J., Lin, H., Ingham, P. W. and Spradling, A. C. (1996a). *hedgehog* is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in *Drosophila*. *Development* **122**, 1125-1135.
Forbes, A. J., Spradling, A. C., Ingham, P. W. and Lin, H. (1996b). The role of segment polarity genes during early oogenesis in *Drosophila*. *Development* **122**, 3283-3294.
Fuller, M. T. (1993). Spermatogenesis. In *The Development of Drosophila*. Cold Spring Harbor, NY, Cold Spring Harbor Press. 71-147.
Gateff, E. (1982). *Gonial cell neoplasm of genetic origin affecting both sexes of* Drosophila melanogaster. New York: Alan R. Liss.
Gilbert, S. F. (1994). Autonomous cell specification by cytoplasmic determinants. In *Developmental Biology*. Sunderland, Massachusetts, Sinauer Associates, Inc. 4th, ed. 493-530.
Godt, D. and Laski, F. A. (1995). Mechanisms of cell rearrangement and cell recruitment in *Drosophila* ovary morphogenesis and the requirement of *bric a brac*. *Development* **121**, 173-187.
Goodner, B. and Quatrano, R. S. (1993). Fucus embryogenesis: a model to study the establishment of polarity. *The Plant Cell* **5**, 1471-1481.
Gregorio, C. C., Kubo, R. T., Bankert, R. B. and Repasky, E. A. (1992). Translocation of spectrin and protein kinase C to a cytoplasmic aggregate upon lymphocyte activation. *Proc. Natl. Acad. Sci. USA* **89**, 4947-4951.
Guo, S. and Kemphues, K. J. (1996). A non-muscle myosin required for embryonic polarity in *Caenorhabditis elegans*. *Nature* **382**, 455-458.
Hardy, C. L. and Megason, G. C. (1996). Specificity of hematopoietic stem cell homing. *Hematol Oncol.* **14**, 17-27.
Hay, B., Ackerman, L., Barbel, S., Jan, L.Y., and Jan, Y.N. (1988). Identification of a component of Drosophila polar granules. *Development* **103**, 625-640.
Hay, B., Jan, L.Y., and Jan, Y.N. (1990) Localization of *vasa*, a component of *Drosophila* polar granules, in maternal-effect mutants that alter embryonic anteroposterior polarity. *Development*, **109**, 425-433.
Howard, K., Jaglarz, M., Zhang, N., Shah, J. and Warrior, R. (1993). Migration of *Drosophila* germ cells: analysis using enhancer trap lines. *Development* **Supplement** 213-218.
Illmensee, K. and Mahowald, A. P. (1974). Transplantation of posterior polar plasm in Drosophila: Induction of germ cells at the anterior pole of the egg. *Proc. Natl. Acad. Sci. USA* **71**, 1016-1020.
Illmensee, K. and Mahowald, A. P. (1976). The autonomous function of germ plasm in a somatic region of Drosophila egg. *Exp. Cell Res.* **97**, 127-140.
Jones, P. H., Harper, S. and Watt, F. M. (1995). Stem cell patterning and fate in human epidermis. *Cell* **80**, 83-93.
Jongens, T. A., Ackerman, L. D., Swedlow, J. R., Jan, L. Y. and Jan, Y. N. (1994). *Germ cell-less* encodes a cell type-specific nuclear pore-associated protein and functions early in the germ-cell specification pathway of *Drosophila*. *Genes Dev.* **8**, 2123-2136.
Jongens, T. A., Hay, B., Jan, L. Y. and Jan, Y. N. (1992). The *germ cell-less* gene product: A posteriorly localized component necessary for germ cell development in Drosophila. *Cell* **66**, 23-35.
Karpen, G. H. and Spradling, A. C. (1992). Analysis of subtelomeric heterochromatin in a Drosophila minichromosome by single P element insertional mutagenesis. *Genetics* **133**, 737-53.
Kerr, J. B. and Dixon, K. E. (1974). An ultrastructural study of germ plasm in spermatogenesis of *Xenopus laevis*. *J. Embryol. Exp. Morphol.* **32**, 573.
Kimble, J. and White, J. (1981). On the control of germ cell development in *Caenorhabditis elegans*. *Dev. Biol.* **81**, 208-19.
King, R. C. (1970). *Ovarian development in* Drosophila melanogaster. New York, Academic Press.
Kobayashi, S., Yamada, M., Asaoka, M. and Kitamura, T. (1996). Essential role of the posterior morphogen Nanos for germline development in Drosophila. *Nature* **380**, 708-711.
Kraut, R., Chia, W., Jan, L. Y., Jan, Y. N. and Knoblich, J. A. (1996). Role of *inscuteable* in orienting asymmetric cell divisions in *Drosophila*. *Nature* **383**, 50-55.
Lantz, V., Ambrosio, L. and Schedl, P. (1992). The Drosophila *orb* gene is predicted to encode sex-specific germline RNA-binding proteins and has localized transcripts in ovaries and early embryos. *Development* **115**, 75-88.
Lehmann, R. and Nusslein-Volhard, C. (1986). Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of oskar, a maternal gene in Drosophila. *Cell* **47**, 141-52.
Lehmann, R. and Nusslein-Volhard, C. (1987). Involvement of the *pumilio* gene in the transport of an abdominal signal in the Drosophila embryo. *Nature* **329**, 167-170.
Lehmann, R. and Nusslein-Volhard, C. (1991). The maternal gene *nanos* has a central role in posterior pattern formation of the *Drosophila* embryo. *Development* **112**, 679-693.
Li, Q., Karam, S. M. and Gordon, J. I. (1996). Diphtheria toxin-mediated ablation of parietal cells in the stomach of transgenic mice. *J. Biol. Chem.* **271**, 3671-3676.
Lin, H. and Schagat, T. (1997). Neuroblasts: a model for asymmetric division of stem cells. *Trends Genet.* **13**, 33-39.
Lin, H. and Spradling, A. (1993). Germline stem cell division and egg chamber development in transplanted *Drosophila* germaria. *Dev. Biol.* **159**, 140-152.
Lin, H. and Spradling, A. (1995). Fusome asymmetry and oocyte determination in *Drosophila*. *Dev. Genet.* **16**, 6-12.
Lin, H., Yue, L. and Spradling, A. S. (1994). The *Drosophila* fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development* **120**, 947-956.
Lindsley, D. L. and Zimm, G. (1992). The genome of *Drosophila melanogaster*. Academic Press.
MacDonald, P. M. (1992). The *Drosophila pumilio* gene: an unusually long transcription unit and an unusual protein. *Development* **114**, 221-232.
Mahowald, A. P. (1962). Fine structure of pole cells and polar granules in *Drosophila melanogaster*. *J. Exp. Zool.* **151**, 201-216.
Mahowald, A. P. (1971). Polar granules of *Drosophila* III. The continuity of polar granules during the life cycle of *Drosophila*. *J. Exp. Zool.* **176**, 329-344.
Mahowald, A. P. (1977). The germ plasm of *Drosophila*: a model system for the study of embryonic determination. *Am. Zool.* **177**, 551.
Manseau, L. and Schupbach, T. (1989b). *cappuccino* and *spire*: two unique maternal-effect loci required for both the anteroposterior and dorsoventral patterns of the *Drosophila* embryo. *Genes Dev.* **3**, 1437-1452.
Margolis, J. and Spradling, A. C. (1995). Identification and behavior of epithelial stem cells in the *Drosophila* ovary. *Development* **121**, 3797-3807.
McKearin, D. and Ohlstein, B. (1995). A role for the *Drosophila bag-of-marbles* protein in the differentiation of cystoblasts from germline stem cells. *Development* **121**, 2937-47.

McKearin, D. M. and Spradling, A. C. (1990). *Bag-of-marbles*: a *Drosophila* gene required to initiate both male and female gametogenesis. *Genes Dev.* **4**, 2242-2251.

Mlodzik, M., and Hiromi, Y. (1992). The enhancer trap method in *Drosophila* and its application to neurobiology. In *Methods in Neuroscience*, vol. 9, Academic Press, New York. 397-414.

Morrison S. J., Shah, N. M., and Anderson, D. J. (1997) Regulatory mechanisms in stem cell biology. *Cell* **88**, 287-298.

Muller-Sieburg, C. E. and Deryugina, E. (1995). The stromal cells' guide to the stem cell universe. *Stem Cells* **13**, 477-86.

Murata, Y. and Wharton, R. P. (1995). Binding of Pumilio to maternal *hunchback* mRNA is required for posterior patterning in *Drosophila* embryos. *Cell* **80**, 747-756.

Nakamura, A., Amikura, R., Mukai, M., Kobayashi, S. and Lasko, P. F. (1996). Requirement for a noncoding RNA in *Drosophila* polar granules for germ cell establishment. *Science* **274**, 2075-2079.

Palmer, R. E., Sullivan, D. S., Huffaker, T. and Koshland, D. (1992). Role of astral microtubules and actin in spindle orientation and migration in the budding yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* **119**, 583-593.

Perrimon, N. and Gans, M. (1983). Clonal analysis of the tissue specificity of recessive female-sterile mutations of *Drosophila melanogaster* using a dominant female-sterile mutation Fs(1)K1237. *Dev. Biol.* **100**, 365-373.

Pesacreta, T. C., Byers, T. J., Dubreuil, R., Kiehart, D. P. and Branton, D. (1989). *Drosophila* spectrin: the membrane skeleton during embryogenesis. *J. Cell Biol.* **108**, 1697-1709.

Patel, N.H., Martin-Blanco, E., Coleman, K.G., Poole, S.J., Ellis, M.C., Kornberg, T.B. and Goodman, C.S. (1989). Expression of engrailed proteins in arthropods, annelids and chordates. *Cell* **58**, 955-968.

Pringle, J. R., Bi, E., Harkins, H. A., Zahner, J. E., De Virgilio, C., Chant, J., Corrado, K., and Fares, H. (1995). Establishment of cell polarity in yeast. *Cold Spring Harbor Symp. Quant. Biol.* **60**, 729-744.

Raff, J. W., Whitfield, W. G. F. and Glover, D. M. (1990). Two distinct mechanisms localize cyclin B transcripts in syncytial *Drosophila* embryos. *Development* **110**, 1249-1261.

Robertson, H. M., Preston, C. R., Phillis, R. W., Johnson-Schlitz, D. M., Benz, W. K. and Engles, W. R. (1988). A stable genomic source of P element transposase in *Drosophila melanogaster*. *Genetics* **118**, 461-470.

Sahut-Barnola, I., Godt, D., Laski, F. A. and Couderc, J.-L. (1995). *Drosophila* ovary morphogenesis: Analysis of terminal filament formation and identification of a gene required for this process. *Dev. Biol.* **170**, 127-135.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning.* Cold Spring Harbor, NY, Cold Spring Harbor Press.

Schüpbach, T. and Wieschaus, E. (1986). Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo. *Wilhelm Roux Arch. Dev. Biol.* **195**, 302-317.

Smithwick, E. B. and Young, L. G. (1996). Germ cell maturation and cellular associations in the seminiferous epithelial cycle of the chimpanzee. *Tissue Cell* **28**, 137-148.

Stewart, F. M. (1995). Cytokines in stem cell transplantation. *Ann. N Y Acad. Sci. USA* **770**, 53-69.

Storto, P. D. and King, R. C. (1989). The role of polyfusomes in generating branched chains of cystocytes during *Drosophila* oogenesis. *Dev. Genet.* **10**, 70-86.

Strome, S. (1993). Determination of cleavage planes. *Cell* **72**, 3-6.

Strome, S. and Wood, W. B. (1982). Immunofluorescence visualization of germ line-specific cytoplasmic granules in embryos, larvae and adults of *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **79**, 1558-1562.

Telfer, W. H. (1975). Development and physiology of the oocyte-nurse cell syncytium. *Adv. Insect Physiol.* **11**, 223-319.

Timmons, L., Hersperger, E., Woodhouse, E. Xu, J., Liu, L.-Z., and Shearn, A. (1993) The expression of the Drosophila *awd* gene during normal development and in neoplastic brain tumors caused by *lgl* mutations. *Dev. Biol.* **158**, 364-379.

Warrior, R. (1994). Primordial germ cell migration and the assembly of the *Drosophila* embryonic gonad. *Dev. Biol.* **166**, 180-194.

Weiler, S. R. Mou, S., DeBerry, C. S., Keller, J. R., Ruscetti, F. W., Ferris, D. K., Longo, D. L., Linnekin, D. (1996). JAK2 is associated with the *c-kit* proto-oncogene product and is phosphorylated in response to stem cell factor. *Blood* **87**, 3688-93.

Wieschaus, E. and Szabad, J. (1979). The development and function of the female germline in *Drosophila melanogaster*, a cell lineage study. *Dev. Biol.* **68**, 29-46.

Yue, L. and Spradling, A. (1992). *hu-li tai shao*, a gene required for ring canal formation during *Drosophila* oogenesis, encodes a homolog of adducin. *Genes Dev.* **6**, 2443-2454.

Zhang, P. and Spradling, A. C. (1994). Insertional mutagenesis of *Drosophila* heterochromatin with single P elements. *Proc. Natl. Acad. Sci. USA* **91**, 3539-3543.

Zhong, W., Feder, J. N., Jiang, M.-M., Jan, L. Y. and Jan, Y. N. (1996). Asymmetric localization of a mammalian Numb homologue during mouse cortical neurogenesis. *Neuron* **17**, 43-53.

Zick, K. (1911). Die post-embryonale Entwicklung des mannlichen Geschlectsapparat der Schmetterlinge. *Z. Wiss. Zool.* **98**, 430-477.

(Accepted 9 April 1997)