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piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells

Daniel N. Cox, Anna Chao and Haifan Lin*

Department of Cell Biology, Box 3709, Duke University Medical Center, Durham, NC 27710, USA
*Author for correspondence (e-mail: h.lin@cellbio.duke.edu)

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SUMMARY

***piwi* represents the first class of genes known to be required for stem cell self-renewal in diverse organisms. In the *Drosophila* ovary, *piwi* is required in somatic signaling cells to maintain germline stem cells. Here we show that *piwi* encodes a novel nucleoplasmic protein present in both somatic and germline cells, with the highly conserved C-terminal region essential for its function. Removing PIWI protein from single germline stem cells significantly decreases the rate of their division. This suggests that PIWI has a second role as a cell-autonomous promoter of germline stem cell division. Consistent with its dual function, over-expression of *piwi* in somatic cells causes an increase both in the number of germline stem cells and the rate of their division. Thus, PIWI is a key regulator of stem cell division – its somatic expression modulates the number of germline stem cells and the rate of their division, while its germline expression also contributes to promoting stem cell division in a cell-autonomous manner.**

Key words: Stem cell, Germline, *piwi*, Cell fate, Asymmetric division, *Drosophila melanogaster*

INTRODUCTION

Stem cells are self-renewing tissue progenitor cells responsible for generating and replenishing many tissues that undergo continuous cell turnover, such as the epithethial, hematopoietic, nervous and germline systems (reviewed by Potten, 1997; Lin, 1997; Morrison et al., 1997). Perturbation of stem cell function can have drastic effects on growth, leading to neoplasia or tissue dystrophy.

The self-renewing division of stem cells is controlled by both cell-autonomous and extrinsic signaling mechanisms. The cell-autonomous mechanism, involving localized cell fate regulators, has been identified in *Drosophila* neural and germline stem cells (reviewed by Lin and Schagat, 1997; Lin, 1998), while the extrinsic mechanism, mediated by cytokines and cell-surface ligands, exists in various types of stem cells in mammals, *Drosophila* and *C. elegans* (reviewed by Morrison et al., 1997; Potten, 1997; Lin, 1997, 1998).

The *Drosophila* ovary provides an effective model for analyzing mechanisms underlying the self-renewing division of stem cells. Germline stem cells (GSC) of the *Drosophila* ovary, like other stem cells, have the ability to self-renew and to produce a large number of differentiated progeny (reviewed by Lin, 1997, 1998). The *Drosophila* ovary is composed of functional units termed ovarioles that are assembly lines of developing ovarian follicles. At the apical tip of each ovariole is a specialized structure termed the germarium in which two to three GSCs reside (Brown and King 1962, 1964; Schupbach, et al., 1978; Wieschaus and Szabad, 1979; Lin and Spradling, 1993, see Fig. 1). GSCs divide asymmetrically with respect to a cytoplasmic organelle termed the spectrosome, producing a daughter stem cell and a differentiated cell called the cystoblast (Lin and Spradling, 1997; Deng and Lin, 1997). The daughter GSC remains in contact with the basal terminal filament cells (a.k.a. cap cells) while the cystoblast is positioned one cell away from the terminal filament. The cystoblast then undergoes four rounds of incomplete divisions to form a 16-cell germline cyst interconnected by a spectrosome-derived organelle called the fusome. The 16-cell cyst, now residing in the middle (region 2) of the germarium, becomes enveloped by follicle cells produced by somatic stem cells in this region to form an egg chamber (Margolis and Spradling, 1995), which then leaves the germarium and develops into a mature egg (reviewed by Spradling, 1993).

As with other stem cell systems, the self-renewing ability of GSCs in the *Drosophila* ovary is regulated both by an intracellular mechanism and by intercellular signaling (reviewed by Lin, 1998). The intracellular mechanism involves the spectrosome that controls the divisional orientation of GSCs (Deng and Lin, 1997). It also requires *pumilio* (*pum*) which acts cell-autonomously to maintain the stem cells (Lin and Spradling, 1997; Forbes and Lehmann, 1998; Parisi and Lin, 1999). The PUM proteins are accumulated at a high level in the stem cells but low level in the cystoblast (Forbes and Lehmann, 1998; Parisi and Lin, 1999). In contrast, a cytoplasmic protein product of *bag-of-marbles* (*bam*), a gene required for the proper differentiation of the cystoblast (McKearin and Spradling, 1990), is frequently present in

cystoblasts but not in the stem cells (McKearin and Ohlstein, 1995). This also appears to be the case for the NANOS (NOS) protein, which may be required for cystoblast differentiation (Forbes and Lehmann, 1998). Since PUM and NOS are translational suppressors in the *Drosophila* embryo (Barker et al., 1992; Murata and Wharton, 1995), the translational regulation mechanism may also play an important role in the asymmetric division of GSCs. In addition to the intracellular mechanism, recent studies have revealed the critical role of somatic signaling in maintaining GSCs. Laser ablation studies indicated that the terminal filament acts as a somatic signaling center that regulates GSC division (Lin and Spradling, 1993). This signaling center expresses *piwi* and *fs(1)Yb*, whose somatic function is essential for GSC maintenance (Cox et al., 1998; King and Lin, 1999). Moreover, the *decapentaplegic* (*dpp*) signaling pathway has been implicated in the regulation of GSC division via intercellular signaling (Xie and Spradling, 1998).

Among these genes, *piwi* defines a novel class of evolutionarily conserved genes required for stem cell self-renewal (Cox et al., 1998). Homologs of *piwi* have been identified in *C. elegans* (*prg-1* and *prg-2*) and human (*hiwi*) (Cox et al., 1998). In *Drosophila*, GSCs in *piwi* mutants differentiate without self-renewing divisions, leading to their depletion in both males and females (Lin and Spradling,1997; Cox et al., 1998). This essential function of *piwi* in GSC maintenance resides in the apical somatic cells of the germarium, which include the main terminal filament cells, the cap cells, and the inner sheath cells (Cox et al., 1998; Fig. 1). In *C. elegans*, a decreased *prg-1* and *prg-2* activity leads to drastic reduction of germline proliferation (Cox et al., 1998). In *Arabidopsis thaliana,* two *piwi*-like genes, *zwille* and *argonaute*, are known to be required for meristem cell maintenance (Bohmert et al., 1998; Moussian et al., 1998). All these observations suggest that *piwi* represents the first class of stem cell genes known to exist in diverse organisms in both animal and plant kingdoms (Benfey, 1999).

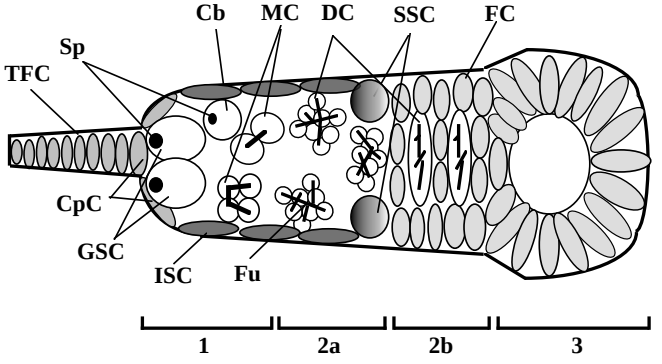

Fig. 1. Schematic diagram of a *Drosophila* germarium. The schematized germarium is oriented with the apical end toward the left. The numbers below the germarium in the brackets denote distinct regions within the germarium. Germarial region 1 extends from the apical terminal filament cells (TFC, including cap cells, CpC) to the zone of mitotically active cysts. Germarial regions 2a and 2b include differentiating 16-cell cysts. Two somatic stem cells (SSC) are located at the border between regions 2a and 2b. Germarial region 3 contains a newly formed egg chamber that is preparing to bud off from the germarium and contribute to the developing string of egg chambers which comprise the rest of the ovariole. GSC, germline stem cell; Sp, spectrosome; ISC, inner sheath cell; Cb, cystoblast; Fu, fusome; MC, mitotically active cysts; DC, differentiating 16-cell cysts; FC, follicle cell.

To investigate further the mechanisms by which *piwi* exerts its effect in the regulation of GSC division, we have systematically analyzed the expression and function of the PIWI protein during oogenesis. We report here that PIWI is localized in the nucleoplasm in both somatic and germline cells in both sexes, with the highly conserved C-terminal region essential for its function. Our clonal analysis of individual stem cells reveals that the PIWI protein confers a cell-autonomous function in promoting stem cell division. Overexpression of PIWI in the soma increases both the number of GSCs and their rate of division. These results suggest that *piwi* regulates GSC division through both inter- and intracellular mechanisms.

MATERIALS AND METHODS

Drosophila strains and culture

All *Drosophila* strains were grown at 25˚C on yeast/molasses-containing agar medium. The following fly strains were used in this study: *piwi*^{1} and *piwi*^{2} mutant chromosomes, generated in different genetic screens (Lin and Spradling, 1997), were dominantly marked with *Irregular facets (If)* (Lindsley and Zimm, 1992); *ep(2)1024* is an EP element insertion (Rorth, 1996; Rorth et al., 1998) in the *piwi* 5′ UTR; *piwi*^{1}FRT40A/*CyO* and *piwi*^{2}FRT40A/*CyO* (Cox et al., 1998); hsFlp; *piwi*$^{+}$FRT40A/*CyO* (Xu and Rubin, 1993); hsFlp; *armadillo-lacZ* FRT40A (*armlacZ* FRT40A, Lecuit and Cohen, 1997); *hsGal4* (Brand and Perrimon, 1993) are as described previously (Cox et al., 1998).

Generation of mutant germline stem cell clones

piwi mutant GSC clones were generated by Flp-mediated recombination as described previously (Xu and Rubin, 1993). To generate wild-type and mutant GSC clones for analyses, *piwi*$^{+}$ FRT40A/*CyO*, *piwi*^{1}FRT40A/*CyO* and *piwi*^{2}FRT40A/*CyO* males were mated to *w* hsFlp1; *armlacZ* FRT40A virgin females, respectively, to produce *w hsFlp1/+; piwi** FRT40A/ *armlacZ* FRT40A progeny, where * denotes the wild-type or mutant alleles. The parental adults were transferred to fresh vials after 2 days. Larvae from the original vials were heat-shocked once daily for 1 hour in a 37°C water bath on days 3 and 4 to induce mitotic recombination immediately prior to the onset of oogenesis at the late third instar stage (Cox et al., 1998). After eclosion, adult *w hsFlp1/+; piwi** FRT40A/*armlacZ* FRT40A females were transferred to fresh food at room temperature and ovaries were removed 1 week, 2 weeks, and 3 weeks after the last heat shock treatment and processed for anti-β-gal antibody staining to look for lacZ-negative clones (see below).

Heat-shock induced PIWI overexpression

To construct a *piwi*-overexpressing stock, *ep(2)1024/CyO* virgin females were crossed to males homozygous for a *hsGal4* transgene on chromosome 3. Females carrying both the *ep(2)1024* chromosome and the *hsGal4* transgene were heat shocked at 37˚C for 1 hour each time with an interval of 12 hours for 3-4 days. At the end of the heat shock regime, ovaries were dissected and processed for antibody staining.

To analyze the functionality of the PIWI protein produced by *hsGal4*-induced overexpression of *ep(2)1024*, *ep(2)1024/CyO; hsGal4/hsGal4* virgin females were crossed to *piwi*^{1} *If/CyO; +/+* males to produce the *ep(2)1024/piwi*^{1}*If; hsGal4/+* transheterozygous progeny. After 2 days, parental adults were transferred to fresh vials. Larvae were heat shocked daily for 1 hour at 37˚C starting at day 3

and continued to 3 days after eclosion. The transheterozygous progeny were subsequently dissected and processed for antibody staining (see below). The transheterozygous progeny that were not subjected to the heat shock regime served as controls.

Immunofluorescence microscopy and BrdU labeling

Ovaries and testes were dissected, fixed and stained as described by Lin et al. (1994). For immunofluorescence staining the following antisera were used: polyclonal anti-Vasa antibody (1:2000; Hay et al., 1990), monoclonal anti-Hts antibody 1B1 (1:1; Zaccai and Lipshitz, 1996), polyclonal anti-α-spectrin antibody (1:200; Byer et al., 1987), monoclonal anti-myc epitope antibody 1-9E10.2 (1:50; Evan et al., 1985), monoclonal anti-BamC antibody (1:1000; McKearin and Ohlstein, 1995), polyclonal anti-β-galactosidase antibody (1:600; Cappel), monoclonal anti-BrdU antibody (1:50; Becton-Dickinson). All the fluorescence-conjugated secondary antibodies were from Jackson Immunoresearch Laboratory and were used at 1:200 dilution. Immunofluorescently labeled samples were also counter-stained with DAPI as described previously (Lin and Spradling, 1993). Micrographs were taken using either a Zeiss Axioplan microscope or a Zeiss LSM410 confocal microscope as described by Cox et al. (1998). BrdU labeling was performed essentially as described by Gonczy and DiNardo (1996). Briefly, on day 3 of the heat shock regime, *ep(2)1024; hsGAL4* females were transferred to an Eppendorf tube that was held horizontally and contained 100 μl of 100 mM BrdU in grape juice. The flies were fed (pulse) with BrdU for 1 hour at room temperature. After the pulse, flies were returned to fresh vials without BrdU for 12 hours at room temperature (chase). These females were subsequently dissected and fixed for immunofluorescence labeling with anti-BrdU and anti-α-spectrin antibodies.

Construction of transgenes encoding N- and C-terminal-tagged PIWI proteins

Synthetic oligonucleotides (Gibco BRL) encoding a myc-epitope were cloned into unique sites at the N and C terminus of the *piwi* ORF in a 6.8 kb *piwi* genomic construct (pRc12) that fully rescues the *piwi* activity (Cox et al., 1998) as follows: For the N-terminal myc insertion, the following primers were used: (*Bcl/myc:* 5′-GAT CAT ATG GAG CAA AAG CTT ATT AGC GAG GAA GAT CTG AAT-3′ and *Bcl/antisense myc:* 5′-GAT CAT TCA GAT CTT CCT CGC TAA TAA GCT TTT GCT CCA TAT-3′). The primers were annealed according to the manufacturer's recommendations and cloned into a unique *BclI* recognition site in a Bluescript clone containing *piwi* genomic DNA. The resulting recombinant *myc-piwi* gene was cloned into pCasper 4 (Pirrotta, 1988) to produce the plasmid pPMB1-6 in which the *myc* sequence is inserted between PIWI amino acid residues 3 and 4. For the C-terminal *myc* insertion, the following primers were used: (*Nhe/myc:* 5′-CTA GCA TAT GAG CAA AAG CTT ATT AGC GAG GAA GAT CTG AAT AAG-3′ and *Nhe/antisense myc:* 5′-CTA GCT TAT TCA GAT CTT CCT CGC TAA TAA GCT TTT GCT CAT ATG-3′). Primers were annealed as described above and cloned into a unique *NheI* recognition site in a Bluescript clone containing part of *piwi* genomic DNA. The recombinant insert was cloned into pCasper 4 (Pirrotta, 1988) to produce the plasmid pPMN in which *myc* is inserted between PIWI amino acid residues 795 and 796. The insertions were confirmed by DNA sequencing.

P-element mediated germline transformation

Transgenic flies were produced according to the method of Spradling and Rubin (1982), using *w; Δ2-3 Sb e/TM6 e* (Robertson et al., 1988) embryos as recipients. Eight independent pPMB1-6 lines and twelve independent pPMN lines were recovered. The myc-tagged transgenes were then separated from the *Δ2-3* transposase and introduced into the homozygous *piwi*^{1} background for fertility tests by genetic crosses. Rescue crosses were carried out at 25°C. Each transgene was further tested for Myc expression by whole-mount immunofluorescence (see above).

Immunoblotting analysis and in vitro translation

To prepare total protein extracts from pPMB1-6 transgenic flies, ovaries were dissected in 1× EBR (130 mM NaCl; 5 mM KCl; 2 mM $CaCl_2$; 10 mM Hepes pH 6.9) and homogenized in 1× sample buffer (5% 2-mercaptoethanol, 3% SDS, 10% glycerol, 62.5 mM TrisCl pH 6.8, 0.1% bromophenol blue). Protein samples were boiled for 5 minutes at 95°C and then loaded on 10% SDS-PAGE gels. After electrophoresis, gels were electro-transferred to Genescreen (NEN Research Products, DuPont) in a BioRad Trans-Blot apparatus. Blots were blocked for 1 hour in Blotto (5% non-fat dry milk in 1× PBS) and primary antibodies were subsequently diluted in Blotto followed by an incubation overnight at 4°C. The monoclonal 9E10 anti-myc antibody (Evan et al., 1985) was used at a dilution of 1:50. HRP-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch Laboratory) was used at 1:4000. Blots were washed and processed for detection using enhanced chemiluminescent (ECL) reagents as recommended by the manufacturer (Amersham). in vitro translation analysis was performed on the plasmid pDC2 (Cox et al., 1998) to produce ^{35}S-labeled PIWI protein according to the manufacturer's recommendations (Promega). The translation products were separated on a 10% SDS-PAGE gel and images were collected by exposing the gel to X-ray film.

Quantitation of germline stem cell division rate

Relative division rate of *piwi*$^{-}$ GSCs was determined as follows: The number of marked *piwi*$^{-}$ cysts in 30 germaria was counted to obtain the average number of the *piwi*$^{-}$ cysts per germarium. This number was then divided by the average number of marked wild-type cysts per germarium as obtained from counting 20 germaria. The relative division rate of GSCs in the *piwi*-overexpressing germaria and that in the wild-type and un-induced *ep(2)1024* control germaria was determined by counting the frequency of telophase spectrosomes contacting the cap cells in the 2-3 GSCs in the most apical region of the germarium.

RESULTS

PIWI is a nuclear protein present in both the soma and germline in both sexes

Previous investigation predicted that *piwi* encodes a 97.2 kDa novel protein required for the self-renewing division of GSCs in both males and females (Lin and Spradling, 1997; Cox et al., 1998). To elucidate further the function of *piwi* in regulating GSC division, we studied the expression and subcellular behavior of the PIWI protein in vivo. The PIWI protein was tagged by inserting a sequence encoding a myc epitope (Evan et al., 1985) into the *piwi* gene, at the 5′ end of the *piwi* open reading frame (Fig. 2A, see Materials and Methods). The p[5′-*myc-piwi*] transgene (denoted as *myc-piwi*) was introduced into *Drosophila* via P-element mediated germline transformation (Spradling and Rubin, 1982). *myc-piwi* fully restores the fertility and gametogenesis of *piwi*^{1} mutant males and females (Table 1). Thus, the myc-PIWI protein confers wild-type PIWI function. In contrast, a p[*piwi*-3′-*myc*] transgene (denoted as *piwi-myc*) with the *myc* sequence inserted in the highly conserved C-terminal region (Fig. 2A, see Materials and Methods) fails to rescue any *piwi* mutant phenotype (Table 1). The PIWI-myc protein was not detectable either by Western blotting analysis (data not shown) or by immunostaining of the transgenic ovaries (Table 1), indicating that this region is essential at least for the stability of the PIWI protein.

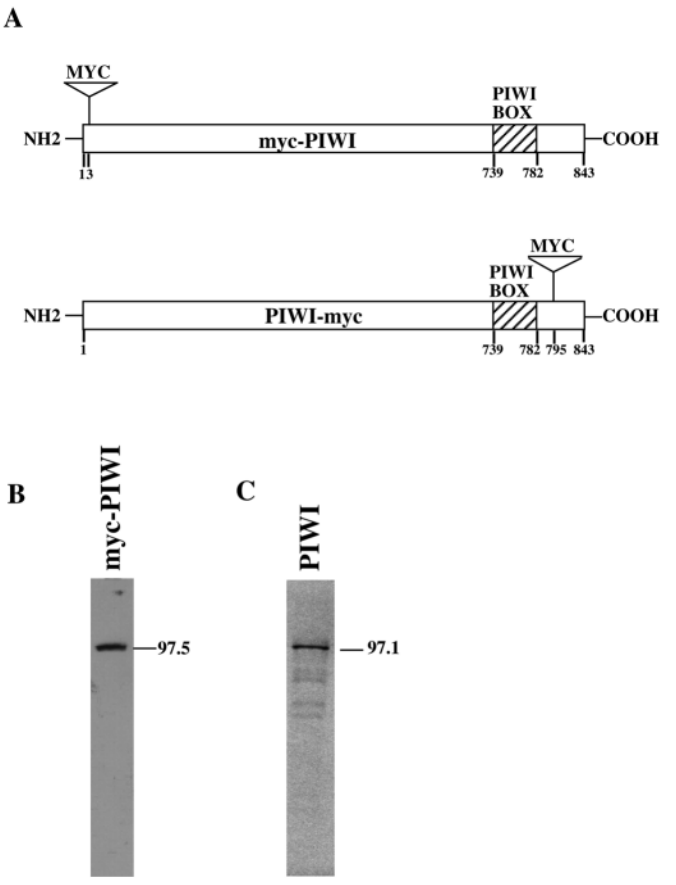

Fig. 2. The *myc-piwi* transgene encodes a 97.5 kDa protein. (A) A schematic diagram of the myc-PIWI and PIWI-myc constructs. In the myc-PIWI construct, the myc epitope is inserted after the third amino acid residue of PIWI. In the PIWI-myc construct, the epitope is inserted 13 amino acids downstream of the highly conserved PIWI box (hatched), 48 residues away from the C terminus. (B) Western analysis using an anti-Myc antibody reveals a single 97.5 kDa protein in ovarian extracts of females carrying a myc-PIWI transgene. This size agrees well with in vitro translation of the full-length *piwi* cDNA which produces a predominant protein product of 97.1 kDa (C). NH2, the amino terminus; COOH, the carboxy terminus.

The expression of myc-PIWI was confirmed by immunoblot analysis using a monoclonal anti-myc antibody as a probe, which reveals a single 97.5 kDa band in the ovarian extract of *myc-piwi* flies (Fig. 2B). This agrees well with both the 97.2 kDa predicted size of PIWI and the 97.1 kDa observed size of PIWI when a full-length *piwi* cDNA was translated in vitro (Fig. 2C). Furthermore, eight independent *myc-piwi* transgenic lines share the identical subcellular localization pattern throughout gametogenesis (see below). Thus, the myc-PIWI protein reflects the function and distribution of endogenous PIWI.

We examined the expression and localization pattern of myc-PIWI during gametogenesis by immunofluorescence microscopy. Whole-mount ovaries and testes isolated from wild-type flies carrying a *myc-piwi* transgene were stained with the anti-myc antibody to visualize myc-PIWI; this antibody did not detect any signal above the background in ovaries and testes from siblings without *myc-piwi* (Fig. 3H; see Materials and Methods). The samples were also stained with anti-VASA antibody to highlight germ cells, and DAPI to label nuclear DNA. In the third instar larval ovary, where GSCs begin their asymmetric divisions, myc-PIWI was found in the nucleus of all germ cells of the ovary (Fig. 3A). In addition, myc-PIWI was also present in the nuclei of the forming terminal filament cells (Fig. 3A). In adult ovaries, myc-PIWI is present in the nuclei of both the somatic and germline cells (Fig. 3B-E), as predicted by *piwi* RNA in situ analyses (Cox et al., 1998). Specifically, in the germarium, myc-PIWI is expressed in all the somatic cells, including the main terminal filament cells, the cap cells and the inner sheath cells (Fig. 3B,C), consistent with the previous genetic clonal analyses which suggested that *piwi* is required in these cells to maintain GSCs (Cox et al., 1998). PIWI is also expressed in somatic stem cells and the follicle cells in the germarium (Fig. 3C). In the germline, myc-PIWI shows a dynamic nuclear expression pattern: it is present abundantly in GSCs, but is down-regulated in cystoblasts and developing cysts (Fig. 3C,D). In 16-cell cysts in regions 2 and 3, the myc-PIWI regains its high level expression (Fig. 3B,C), and remains so in nurse cells and oocytes in post-germarial egg chambers throughout oogenesis (Fig. 3D,E). This expression is consistent with previous clonal and RNA in situ analyses which indicate that PIWI is also loaded in eggs as a maternal component essential for embryogenesis (Cox et al., 1998). In all post-germarial egg chambers, myc-PIWI is also specifically expressed in the anterior polar follicle cells (Fig. 3E). The consistent nuclear localization of myc-PIWI in various types of somatic and germline cells during oogenesis indicates that PIWI is a nuclear protein.

PIWI is also expressed as a nuclear protein during spermatogenesis. In the third instar larval testes which contain mostly premeiotic germ cells (reviewed by Fuller, 1993), myc-PIWI is localized to the nuclei of apical somatic cells, including the hub cells, which are the testicular equivalent of terminal filament cells. myc-PIWI is also present in somatic stem cells and their progeny, the cyst progenitor (Fig. 3F). At the apical germline, PIWI is present in the nuclei of GSCs and their immediate daughter cells (Fig. 3F). In newly formed 16-cell cysts of primary spermatocytes somewhat away from the apex, the myc-PIWI staining is sharply reduced in the germline. The staining is only present in the cyst progenitor cells, which are equivalent to follicle cells in the ovary. Once the developing cyst enters the spermatocyte growth phase, myc-PIWI expression is completely undetectable (Fig. 3F). This pattern of expression is maintained in the adult testis which displays the same apical-distal organization but now contains more differentiated post-meiotic germ cells in the basal region of the testis (Fig. 3G). Given the essential role of *piwi* in testicular germline stem cell maintenance (Lin and Spradling, 1997), the nuclear localization of PIWI in the testis should also be functionally important.

PIWI is localized in the nucleoplasm

To investigate the role of PIWI in the nucleus, we further examined the subnuclear localization of the myc-PIWI protein. In interphase GSCs, myc-PIWI is present in subnuclear areas where DNA staining is the weakest (Fig. 4A). The myc-PIWI staining appears as numerous bright foci in DNA-deficient areas that are sometimes connected into a network, complementing the DNA staining pattern. The DNA-bright

Table 1. Rescue of the *piwi[1]* phenotype and myc expression by myc-PIWI and PIWI-myc*

	No. of independent transgenic lines	Staining for myc	Rescue of *piwi[1]* fertility‡					
			female			male		
			piwi[1]	*myc;piwi[1]*	*piwi[1]/CyO*	*piwi[1]*	*myc;piwi[1]*	*piwi[1]/CyO*
myc-PIWI	8	100% (8)	0	34.9±6.8	30.9±8.7	0	49.8±6.7	49.8±0.6
PIWI-myc	12	0% (7)	0	0	30.9±8.7	0	0	49.8±0.6

*The number of independent transformant lines tested for myc staining and for fertility is indicated in parentheses.

‡The fertility rescue is measured by counting the number of F_1 adult eclosed from the tester or control parents. The data are summarized from three independent transgenic lines of myc-PIWI and two independent transgenic lines of PIWI-myc. *piwi[1]* denotes homozygous *piwi[1]*, *myc;piwi[1]* denotes either *myc-piwi; piwi[1]/piwi[1]* or *piwi-myc; piwi[1]/piwi[1]* transgenic lines. Homozygous *piwi[1]* females not only show zero fertility as indicated, but also fail to lay eggs. The small number of eggs produced by these females are retained in the ovary and eventually undergo atrophy.

area is completely devoid of myc-PIWI. In interphase cap cells, myc-PIWI shows a similar staining pattern (Fig. 4A). This staining pattern suggests that, in interphase somatic and germline nuclei, myc-PIWI is not associated with the chromatin or the nuclear envelope, but is localized in the nucleoplasm.

The nucleoplasmic localization of myc-PIWI is even more evident during GSC division. At metaphase, myc-PIWI is not associated with mitotic chromosomes or the nuclear envelope area, but becomes scattered throughout the cytoplasm, forming concentrated foci at the cell cortex (Fig. 4B). The localization pattern remains so throughout anaphase (Fig. 4C). This dynamic localization pattern of myc-PIWI verifies that PIWI is not associated with chromosomes or the nuclear envelope, but is a nucleoplasmic factor that is dispersed into the cytoplasm during mitosis.

The myc-PIWI protein also shows clear nucleoplasmic localization in the nuclei of nurse cells, oocytes, and follicle cells in developing egg chambers. In nurse cell nuclei, myc-PIWI is accumulated in subnuclear areas devoid of DNA staining (Fig. 4D, F), complementing the DNA localization pattern. This complementary relationship is most clearly illustrated in the oocyte nucleus (germinal vesicle), in which chromatin forms a discrete subnuclear structure called the karyosome that occupies a spherical area in the nucleus (King, 1970; see Fig. 4D,E). The myc-PIWI staining is not detectable in the karyosome, but is conspicuously present in the nucleoplasm that surrounds the karyosome (Fig. 4D, E). In follicle cells, myc-PIWI is also localized in the nucleoplasm in a punctate fashion (Fig. 4D,E). Thus, PIWI is a nucleoplasmic factor in all types of ovarian cells.

PIWI is cell-autonomously required in germline stem cells to promote their division

Previous clonal analyses indicated that *piwi* functions in the apical somatic cells of the germarium to regulate GSC maintenance (Cox et al., 1998). However, as PIWI is also present in the nuclei of GSCs, we sought to test the potential function of PIWI in GSCs by removing PIWI from a single stem cell using the FLP/FRT-mediated clonal technique (Xu and Rubin, 1993; see Materials and Methods). *piwi[1]* and *piwi[2]* mutations were used for generating *piwi*$^-$-deficient clones because they are both strong mutations (Cox et al., 1998). The *piwi*$^-$ stem cell clones were induced in *piwi* FRT/*armlacZ* FRT transheterozygous flies. The *armlacZ* transgene (Lecuit and Cohen, 1997) is expressed in all somatic and germline cells in the germarium (Fig. 5A); *piwi*-deficient clones created by a mitotic recombination no longer contain the *armlacZ* gene and can thus be identified by the absence of the β-gal protein (Fig. 5A). The control β-gal-negative *piwi*$^+$ germline clones were induced in *piwi*$^+$ FRT/*armlacZ* FRT transheterozygous flies for comparison. Since it takes 4 days for a cystoblast to develop into an egg chamber that buds off the germarium (Margolis and Spradling, 1995), among *piwi*-deficient clones, only GSC clones can persist over more than 5 days (Fig. 5B). If no *piwi*$^-$ GSC is observed 5 days after induced mitotic recombination, this would clearly indicate the failure of *piwi*$^-$ stem cells to maintain themselves. Because recombination events can only occur in mitotically active cells yet all somatic signaling cells are postmitotic, this approach allows the analysis of the cell-autonomous function of *piwi* in single GSCs without the complication of generating mutant clones in the signaling cells. However, even if any somatic clones are induced, they can be readily identified. Furthermore, by marking individual mutant and wild-type GSCs, we can determine the relative division rate and maintenance ability of each stem cell by counting the number of its cyst progeny within the germarium (also see Xie and Spradling, 1998; Fig. 5B). Finally, we can also assess the cell-autonomous function of *piwi* in the germline at all stages of oogenesis by examining the development of the individually marked *piwi*$^-$ germline cysts and egg chambers.

We induced mitotic recombination immediately prior to oogenesis and looked for *piwi*$^-$ GSC clones 1 week, 2 weeks, and 3 weeks following oogenesis (see Materials and Methods). *piwi*$^-$ GSCs were present even three weeks following oogenesis, consistent with our previous observation that PIWI in GSCs is not required for their maintenance (Cox et al., 1998).

We then examined the number of marked *piwi*$^-$ and *piwi*$^+$ germline cysts in tester and control germaria to compare the division rate between the *piwi*$^-$ and *piwi*$^+$ GSCs within the germarium. For accurate comparison, we always examined germaria that contained only two GSCs, one marked and one unmarked. In wild-type control germaria, marked wild-type stem cells gave rise to approximately 50% of the cysts present within the germarium. This indicates that the *lacZ*-marked and unmarked wild-type stem cells divide at the same rate (Fig. 5C). However, in tester germaria, the number of the marked *piwi*$^-$ germline cysts is consistently only 25% of marked wild-type cysts (Fig. 5C). This analysis reveals that *piwi*$^-$ GSCs divided four-fold slower than wild-type GSCs. Thus, in addition to its somatic function, PIWI acts cell-autonomously in the stem cells to facilitate their division.

We also examined the *piwi*$^-$ cysts and postgermarial *piwi*$^-$ egg chambers by DAPI staining and by Nomarski microscopy for potential developmental defects. They usually developed

normally (data not shown), suggesting that *piwi* does not play an important cell-autonomous role in germline cyst development and subsequent stages of oogenesis.

Overexpression of PIWI increases the number of germline stem cells

Previous investigations established that the somatic expression of PIWI is essential for GSC maintenance (Cox et al., 1998). This somatic induction can be achieved via a threshold mechanism, in which a certain level of somatic PIWI activity is required to establish or maintain the stem cell identity, but a higher level of somatic PIWI expression will not increase the number of GSCs. Alternatively, the PIWI-mediated somatic induction could act via a dosage-dependent mechanism, in which the somatic level of PIWI dictates the number of GSCs. To test these possibilities, we overexpressed PIWI in the somatic cells of the adult ovary via heat shock induction using *hsp70-Gal4* (*hsGal4*) and *ep(2)1024*, an EP element inserted in the 5′ UTR of the *piwi* locus. EP is a modified P element carrying an UAS_t promoter oriented to transcribe the flanking genomic sequence upon the activation of the yeast Gal4 protein (Rorth, 1996). Specifically, *ep(2)1024* in the 5′UTR of *piwi* is oriented in the same direction as *piwi* transcription so that it can produce a *piwi* mRNA containing the complete PIWI open reading frame (data not shown). The UAS_t promoter is active in the soma but not in the germline (Rorth, 1998). We have confirmed its somatic specificity by examining the ovarian expression of the UAS_t-GFP reporter gene under our experimental conditions (data not shown). Because endogenous PIWI is expressed in all somatic cells in the germarium, by constructing a *Drosophila* strain that contains *hsGal4* and *ep(2)1024*, we were able to over express, but not ectopically express, *piwi* specifically in the somatic cells by heat shock induction.

To verify that *ep(2)1024* can express a functional PIWI protein, we overexpressed *ep(2)1024* by heat shocking *hsGal4* in an *ep(2)1024*/*piwi*^{1} mutant background. Because *ep(2)1024* is a strong loss-of-function mutation of *piwi*, *ep(2)1024*/*piwi*^{1}*; hsGal4* females and males in the absence of heat shock display typical *piwi* phenotype (Fig. 6A,C). In contrast, the same mutants, after receiving daily heat shock treatment from the second instar larvae to 3 days after eclosion, produced significantly restored fertility in both *ep(2)1024*/*piwi*^{1} females and males. The morphology of both the ovaioles and testes in the heatshocked mutants are indistinguishable from their wild-type siblings (Fig. 6B,D). In addition, the fertility of the rescued mutants is comparable to their wild-type siblings (data not shown). Heatshock starting at the pupal stage does not rescue the fertility. Therefore, *ep(2)1024* can produce a functional PIWI protein

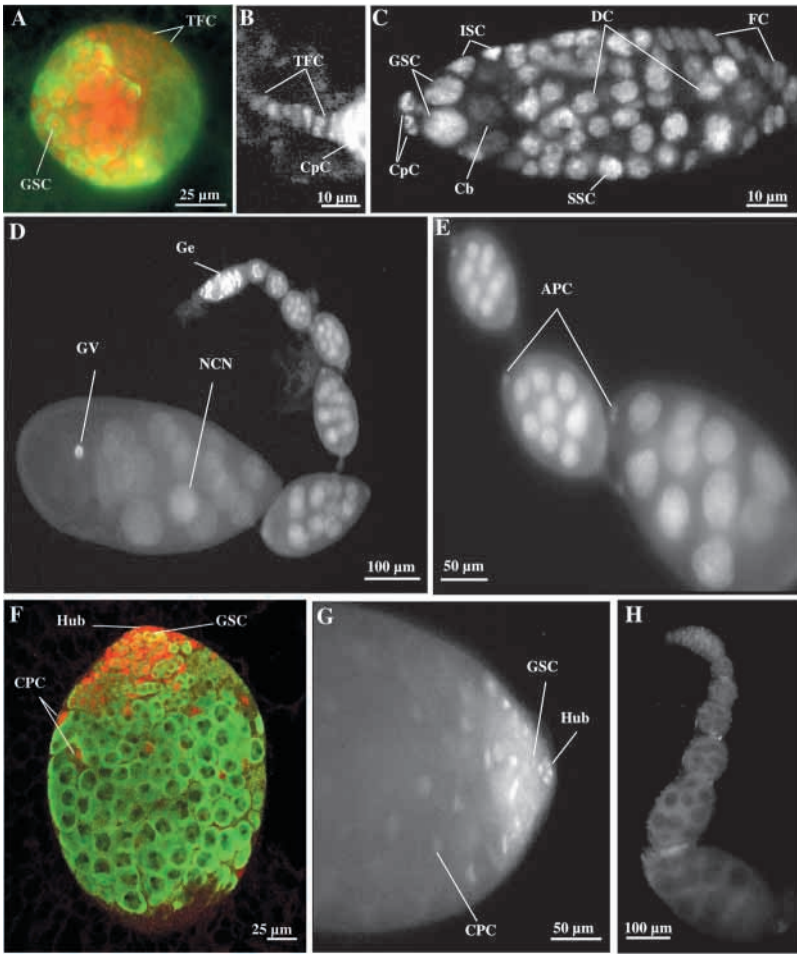

Fig. 3. PIWI is a nuclear protein present in the soma and germline of both sexes. (A) In the third instar larval ovaries, PIWI is present in the nucleus of all germline cells (GSC) as well as in forming terminal filament cells (TFC) as revealed by myc-PIWI (red) and by VASA, a germline-specific marker (green). DAPI staining is not shown in this figure. (B-D) PIWI is expressed in a dynamic pattern in the adult germarium (Ge in D). (B) PIWI is weakly expressed in main terminal filament cells and strongly expressed in cap cells (CpC). (C) PIWI expression in the germarium proper. In the soma, PIWI is strongly expressed in the cap cells, inner sheath cells (ISC), somatic stem cells (SSC), and follicle cells (FC). In the germline, PIWI is expressed in germline stem cells (GSC) and is downregulated in cystoblasts (Cb) and early mitotic cysts. PIWI regains its high level expression in differentiating 16-cell cysts (DC) in regions 2b and 3. (D,E) In post-germarial egg chambers, PIWI is expressed strongly in all nurse cell nuclei (NCN) as well as in the nucleus (germinal vesicle, GV) of the developing oocyte. In the somatic follicle cell layer around each egg chamber, PIWI is expressed in anterior polar follicle cells (APC). Note that the sharp down regulation of PIWI in cystoblast is also evident in the germarium in D, in which the three PIWI-bright small nuclei at the germarial apex are cap cell nuclei. The two larger nuclei in the next layer are GSC nuclei. Other small PIWI-bright nuclei in region 1 are inner sheath nuclei. The PIWI-negative region starts with a cystoblast positioned in the third layer. (F) In the third instar larval testis, PIWI, shown in red, is present in the nuclei of both somatic and germline cells. In the soma, PIWI is expressed in the nuclei of hub cells (Hub) apical to GSCs and in cyst progenitor cells (CPC). In the germline (labeled in green), PIWI is present in the nuclei of GSCs and their immediate daughters. (G) The apical region of the adult testis showing the same apical-distal organization as the third instar larval testis. PIWI expression pattern in the adult testis is identical to that in the third instar larval testis. PIWI is expressed in hub cells, GSC and CPC. (H) Ovaries from sibling females that do not carry the *myc-piwi* transgene show no specific staining with the anti-Myc antibody.

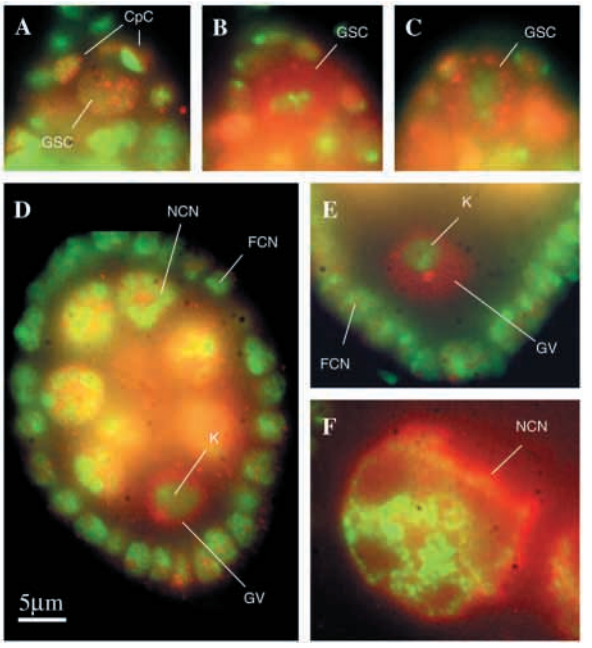

Fig. 4. PIWI is localized in the nucleoplasm. In all the panels, myc-PIWI and DNA are pseudo-colored in red and green, respectively. All images, shown at the same magnification (see the bar in D), are oriented with the apical end of the germarium or egg chambers up and basal side down. (A) In the interphase germline stem cell nucleus, myc-PIWI mostly accumulates as bright foci in DNA-deficient areas within the nucleus. Note that DNA-bright spots are completely devoid of myc-PIWI. This complementary staining pattern is also observed in cap cells (CpC). (B,C) In metaphase and anaphase germline stem cells, the myc-PIWI staining is dispersed throughout the cytoplasm, forming numerous foci at the cortical region of the cell. (D) In a stage 4 egg chamber (for staging, see King, 1970), myc-PIWI is accumulated in regions of nurse cell nuclei (NCN) and follicle cell nuclei (FCN) devoid of DNA staining. In the germinal vesicle (GV), the myc-PIWI staining is absent from the karyosome (K) but present in the nucleoplasm that surrounds it. (E) The oocyte region of a stage 6 egg chamber showing the complementary myc-PIWI and DNA staining in the germinal vesicle (GV) and follicle cell nuclei (FCN). (F) A magnified view of a nurse cell nuclei (NCN) in a stage 6 egg chamber, showing the largely non-overlapping staining pattern of myc-PIWI and DNA.

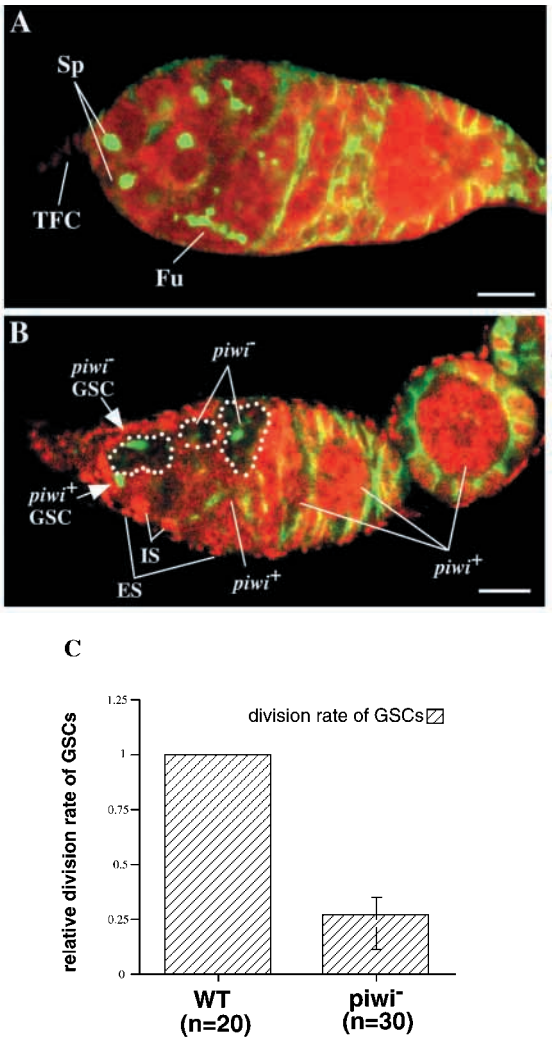

Fig. 5. PIWI acts cell-autonomously in GSCs to promote their division. (A,B) Germaria were labeled with anti-β-gal antibody (red) for *piwi*$^+$ cells and anti-1B1 antibody (green) for spectrosomes and fusomes. Marked stem cells and cysts developed from *piwi*$^-$ germline stem cells (GSCs) are recognized by the absence of *lacZ* expression. (A) A *piwi*$^-$ *lacZ*-FRT/*piwi*$^+$ *lacZ*$^+$ FRT germarium in the absence of heat shock. No *piwi*$^-$ *lacZ*$^-$FRT/*piwi*$^-$ *lacZ*$^-$FRT clones were induced. GSCs are distinguished by the presence of the spectrosome (Sp) in direct contact with the terminal filament cells (TFC). (B) A *piwi*$^-$ *lacZ*$^-$FRT/*piwi*$^+$ *lacZ*$^+$ FRT germarium stained 1 week after heat shock, showing a wild-type GSC and a mutant GSC, as well as three *piwi*$^-$ cysts (dotted outline) derived from the mutant stem cell. The entire ovariole in B is enveloped by the epithelial sheath (ES), which is different from the inner sheath cells (IS). (C) The graph displays the relative division rates (see Materials and Methods) of wild-type and *piwi*$^-$ GSCs. The number of germaria counted is given in parentheses. In both B and C, only germaria containing one marked and one unmarked GSC were examined. Bars in A and B, 10 μm.

capable of rescuing the *piwi* mutant phenotype when expressed in somatic cells.

We then used *ep(2)1024* to overexpress PIWI in wild-type adult females (see Materials and Methods) and analyzed its effects on GSCs. The females were heat-shocked at a 12-hour interval for 3 days and then dissected. The ovaries were stained with anti-Vasa antibody (Hay et al., 1990) to highlight germ cells and anti-1B1 antibody (Zaccai and Lipshitz, 1996) to outline somatic cells as well as to label spectrosomes and fusomes that mark individual stages of germline development in the germarium (Lin and Spradling, 1995). Wild-type germaria typically have 2-3 spectrosome-containing cells, with one often being a cystoblast that is not associated with the terminal filament. This is also the case for uninduced *ep(2)1024*/+ germaria (Fig. 7A). Interestingly, the number of the spectrosome-containing germ cells increased to an average of 7.5 cells per *ep(2)1024* /+ germarium following heat shock induction (Fig. 7B,E). In the most extreme case, up to 15 spectrosome-containing cells were observed in a single germarium. This increase is not due to the expression of *hsGal4* alone, since ovaries from *hsGal4* females subjected to the same heat shock regime were indistinguishable from wild-type (data not shown). Thus, overexpression of PIWI in the soma leads to a 3- to 4-fold increase in the number of GSCs and/or cystoblasts.

To distinguish whether the spectrosome-containing cells are stem cells or cystoblasts, we stained the germaria with anti-cytoplasmic BAM (BAM-C) antibody, since BAM-C is only expressed in cystoblasts and early mitotic cysts but not in GSCs (McKearin and Ohlstein, 1995). In *piwi*-overexpressed germaria, BAM-C staining is strongly present in early cysts (Fig. 7B). However, BAM-C staining is conspicuously absent from all the spectrosome-containing cells (Fig. 7B). This observation suggests that the ectopically induced spectrosome-containing cells are GSC-like cells.

The ectopic GSC-like cells appear to be functional GSCs. First, they incorporate BrdU, an indicator of DNA replication, at a level similar to the wild-type GSCs (Fig. 7C; see Materials and Methods). This suggests that they are not arrested in the cell cycle. Second, these GSC-like cells can all differentiate within 4 days following the withdrawal of heat shock; leaving the germaria with only 2-3 GSCs at their normal locale (Fig. 4D). No signs of cell death, such as pycnotic nuclei or apoptotic bodies, were detected by DAPI staining and Nomarski optics. This suggests that the GSC-like cells are capable of oogenesis. Thus, the dependence of GSC number on the PIWI level reveals that *piwi*-mediated somatic signaling controls the number of GSCs via a dosage dependent mechanism.

The ectopically induced GSCs show an even distribution pattern along the germarial axis. In wild-type germaria, spectrosome-containing cells are exclusively located in the apical area of region 1, either in direct contact with or one-cell away from the terminal filament (for stem cells and cystoblasts, respectively; Fig. 7A). However, following PIWI overexpression, spectrosome-containing cells were evenly expanded along the somatic germarial wall to both regions 1 and 2a (Fig. 7B). All the spectrosome-containing cells appear to be in contact with the somatic cells; there is no apparent gradient of the stem-like cell distribution towards the terminal filament. In wild-type germaria, inner sheath cells express as much PIWI as cap cells, and much more than the main terminal filament cells, yet there are no GSCs located near them in posterior region 1 or region 2a (Fig. 3B). Therefore, the appearance of the ectopic stem-like cells in the entire regions 1 and 2a following *piwi* overexpression suggests that a very high level of PIWI in the inner sheath cells expands the stem cell domain to region 2 (see Discussion).

In regions 2b and 3 of the germarium, where somatic cells are composed of somatic stem cells and their progeny (prefollicle and follicle cells), spectrososome-containing cells are never observed (Fig. 7B,C). Instead, 16-cell cysts developed normally to form egg chambers (Fig. 7B,C). This may indicate the following possibilities: (1) the somatic stem cells and their progeny are not competent to support PIWI-mediated somatic signaling; (2) the differentiated 16-cell germline cysts are no longer capable of returning to the stem cell state; and (3) there may be additional somatic signal(s) emanating from the apical somatic cells that are required to maintain the GSC fate. These signal(s) may not reach regions 2b and 3 of the germariaum.

Overexpression of PIWI increases the rate of germline stem cell division

Since over-expression of *piwi* in somatic cells increases the number of GSCs (see above), yet loss of *piwi* function from the soma and germline in *piwi* mutants abolishes stem cell division and thus their maintenance (Cox et al., 1998), one might expect that over-expression of *piwi* in the soma may also increase the rate of GSC division. To address this possibility, we directly quantified the mitotic index of GSCs in *piwi*-overexpressed females. GSC division is unique in that it has an unusually long telophase-to-interphase period characterized by the presence of a dumb bell-shaped telophase spectrosome spanning the two daughter cells (Deng and Lin, 1997; de Cuevas and Spradling, 1998; see Figs 7D, 8). The morphologically distinctive telophase spectrosome provides an easy marker for the accurate measurement of the telophase/early interphase index, which is a direct quantitation of the frequency of GSC division. To avoid a bias in measuring the division frequency due to the presence of an increased number of GSC-like cells in *piwi*-overexpressed germaria, we quantified only the 2-3 anterior-most GSCs that contact the terminal filament (Fig. 8), and we quantified the same number of stem cells in the wild type and uninduced *ep(2)1024* controls. In the wild type and uninduced *ep(2)1024* controls, 20-25% of germaria contain a single GSC with a telophase spectrosome; only 2-3% of germaria contain two GSCs with telophase spectrosomes (Fig. 8). These frequencies are increased to 31% and 13% in *piwi*-overexpressed germaria. Moreover, 3% of the *piwi*-overexpressed germaria contain three GSCs with a telophase spectrosome, a situation we did not observe in the control germaria (Fig. 8). These results indicate that in addition to an increase in the number of stem cells, *piwi* overexpression also increases the rate of GSC division. Because the GSCs under examination are located in their normal niche, the increase in their mitotic frequency should be mostly, if not all, due to the increased PIWI expression in the terminal filament.

DISCUSSION

Stem cells are characterized by two common properties: the capacity for self-renewal and the ability to propagate numerous progeny fated for further differentiation (for review, see Morrison et al., 1997; Lin, 1997). The unique self-renewing division of stem cells is controlled by both intracellular and extrinsic signaling mechanisms (Knoblich, 1997; Lin, 1997). In this report, we have shown that *piwi* encodes a novel nucleoplasmic protein present in both somatic and germline cells of both sexes. Moreover, our single stem cell clonal analyses and *piwi* overexpression experiments establish the important function of *piwi* in both the intracellular and the cell-cell signaling mechanisms. This work reveals a novel nuclear factor whose activity modulates the number of GSCs and the rate of their division.

The role of PIWI as a nucleoplasmic factor in somatic signaling

Our immunofluorescence analysis of the fully functional myc-PIWI protein reveals that PIWI is a nucleoplasmic protein. Consistent with RNA in situ analyses (Cox et al., 1998), PIWI is present in both the soma and the germline during oogenesis. Previous clonal analysis suggests that *piwi* expression in the apical somatic cells in the germarium is essential for GSC maintenance. In this report, we further revealed the cell-

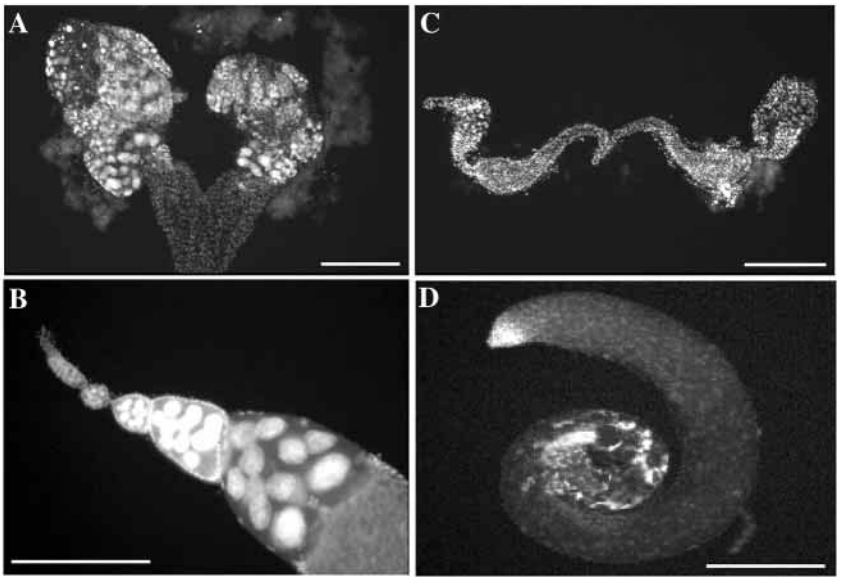

Fig. 6. EP(2)1024 expression rescues the *piwi*^{1} mutant phenotype. Shown are gonads from *ep(2)1024/piwi*^{1}; *hsGAL4* females (A,B) and males (C,D) either without (A,C) or with (B,D) heat shock, all stained with DAPI. In the absence of the heat shock, the ovaries (A) and testes (C) display the typical *piwi*^{1} mutant phenotype. Following *hsGal4*-induced over-expression of the *ep(2)1024* in the soma, significant rescue of the mutant phenotype was observed in both females (B) and males (D). Specifically, in females, germline stem cells are maintained and a string of developing egg chambers was observed as shown in this single ovariole (B). Similarly, in males, germline stem cells are also rescued, and spermatogenesis is fully restored (D). Bars in all panels, 200 μm.

autonomous function of PIWI as a mitotic promoter in GSCs. Consistent with this dual role, PIWI is localized in the nucleoplasm in both the apical somatic cells and GSCs. Hence, the nucleoplasm is probably the site of PIWI function. A number of transcriptional factors, signaling molecules, their receptors and their cytoplasmic effectors have been reported to regulate stem cell division in various systems (reviewed by Potten, 1997, Morrison et al., 1997; Lin, 1997; Parisi and Lin,

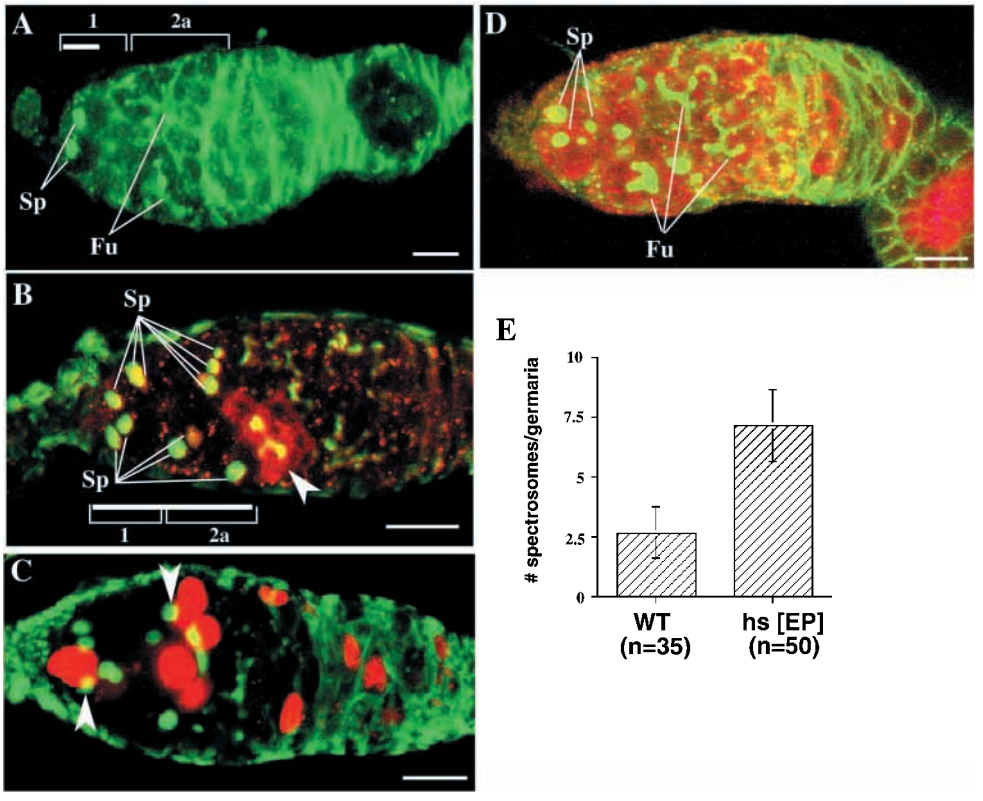

Fig. 7. Somatic PIWI over-expression increases the number of germline stem cells. (A) A wild-type germarium immunofluorescently labeled with anti-1B1 antibody (green). At the apical tip of region 1 in direct contact with the terminal filament are two germline stem cells containing spectrosomes (Sp). The stem cell region is denoted by a bar within the region 1 bracket. Posterior to germline stem cells are developing germline cysts containing branched fusomes (Fu). (B) A germarium from a *ep(2)1024; hsGal4* female displays many germline stem-like cells following 4 days of heat shock treatment, with 12 shown in this focal plane (Sp; stained with anti-1B1 antibody in green) and the absence of the BAM-C protein (red). The stem-like cells are evenly distributed between regions 1 and 2a, as indicated by an expanded bar that spans region 1 and 2a. The BAM-C protein is only present in a cyst containing a branched fusome in region 2a (arrowhead). (C) A germarium containing ectopic stem-like cells (eight shown) stained with anti-BrdU (red) and anti-α-spectrin (green) antibodies following BrdU incorporation (see Materials and Methods). The arrowheads show some mitotically active germline cells which have incorporated BrdU in their nuclei. Note also that a fusome-containing germline cyst is present in region 1 but not in contact with somatic cells. (D) A germarium from an *ep(2)1024; hsGal4* female subjected to 4 days of heat shock treatments followed by 4-5 days of culture at room temperature. The red and green stainings indicate anti-VASA and anti-1B1 antibody signals, respectively. This germarium, like a typical wild-type germarium, contains two germline stem cells contacting the apical somatic cells and a cystoblast that is one cell away from the terminal filament. In addition, the number of fusome-containing cysts (Fu) has increased to a normal level. (E) A comparison of the number of spectrosomes present per wild-type germarium versus per *piwi*-overexpressed germarium. The number of germaria examined (n) is in parentheses. All germaria are oriented with anterior toward the left. B-D are 10 μm stacks of confocal z-series. Bars in all panels, 10 μm.

1998). To our knowledge, PIWI is the first nucleoplasmic factor known to play an essential role in controlling GSC division.

As a nucleoplasmic factor in the apical somatic cells, PIWI is expressed in three types of post-mitotic cells that form a somatic cap surrounding GSCs and their immediate daughter cells: the main terminal filament cells, the cap cells, and the inner sheath cells (Fig. 3B). Among these cell types, the main terminal filament cells express a very low level of PIWI while cap cells and inner sheath cells express a very high level of PIWI. Since cap cells are in direct contact with GSCs, PIWI expression in cap cells may play an immediate role in somatic signaling (also see below).

Since PIWI is a nucleoplasmic protein, it is unlikely to be a somatic signal itself, but rather an essential component of the somatic signaling machinery responsible for producing the signal. Given its nucleoplasmic localization, PIWI may be involved in post-transcriptional mRNA processing in the nucleus. Alternatively, it may be involved in nuclear functions indirectly related to gene expression. In either case, the somatic activity of PIWI appears to act via a dosage-dependent mechanism to control the number of GSCs in the germline. Therefore, our results show that *piwi* may help to define a stem cell niche in the germarium for GSC maintenance, with the size of the niche corresponding to the level of PIWI activity.

A number of genes, including *fs(1)Yb* (King and Lin, 1999) and *dpp* (Xie and Spradling, 1998), have recently been shown to play essential roles in the cell-cell signaling mechanism in maintaining GSCs. Among these genes, *dpp* is the only known signaling molecule. DPP overexpression dramatically expands the number of GSCs and prevents their differentiation, leading to tumorous germaria filled with undifferentiated GSC-like cells (Xie and Spradling, 1998). This overexpression phenotype, in sharp contrast to that of *piwi*, suggests that DPP is not the downstream signal of the *piwi*-mediated mechanism, even though it may be one of the signals. *fs(1)Yb*, on the other hand, shares a very similar loss-of-function phenotype with *piwi*. These two genes are more likely to be involved in the same somatic signaling pathway.

Despite the presence of PIWI in somatic and germline cells during most oogenic stages, egg chambers with either somatic or germline cells mutant for $piwi^1$ do not show any detectable morphological or patterning defects (see Results). This suggests that either PIWI does not play an essential role in post-GSC stages of oogenesis or that such a role requires only an extremely low level of PIWI that is not affected by the strong $piwi^1$mutantion. Further molecular characterization of the $piwi^1$ mutation will distinguish between the two possibilities.

The role of PIWI as a nucleoplasmic factor in germline stem cells

PIWI is expressed dynamically in the germline of the germarium. In region 1, PIWI is present in the nucleus of GSCs at a high level but is sharply down regulated in cystoblasts and early mitotic cysts. This expression pattern correlates well with the mitosis-promoting function of PIWI in GSCs as revealed by single stem cell clonal analysis. The differential distribution of proteins between the stem cell and the cystoblast has also been observed for several other factors known to be important for GSC division or differentiation. For example, PUM is required for GSC maintenance (Lin and Spradling, 1997; Forbes and Lehmann, 1998) and is accumulated at a high level in GSCs but a low level in the cystoblasts (Forbes and Lehmann, 1998; Parisi and Lin, 1999). On the other hand, NOS and BAM-C are required for cystoblast development and are localized either preferentially or exclusively in the cystoblast. All of these molecules, except for BAM-C, are known to play important roles in other developmental processes (Cox et al., 1998; Parisi and Lin, 1999). For example, the germline expression of PIWI during oogenesis also provides PIWI as a maternal component

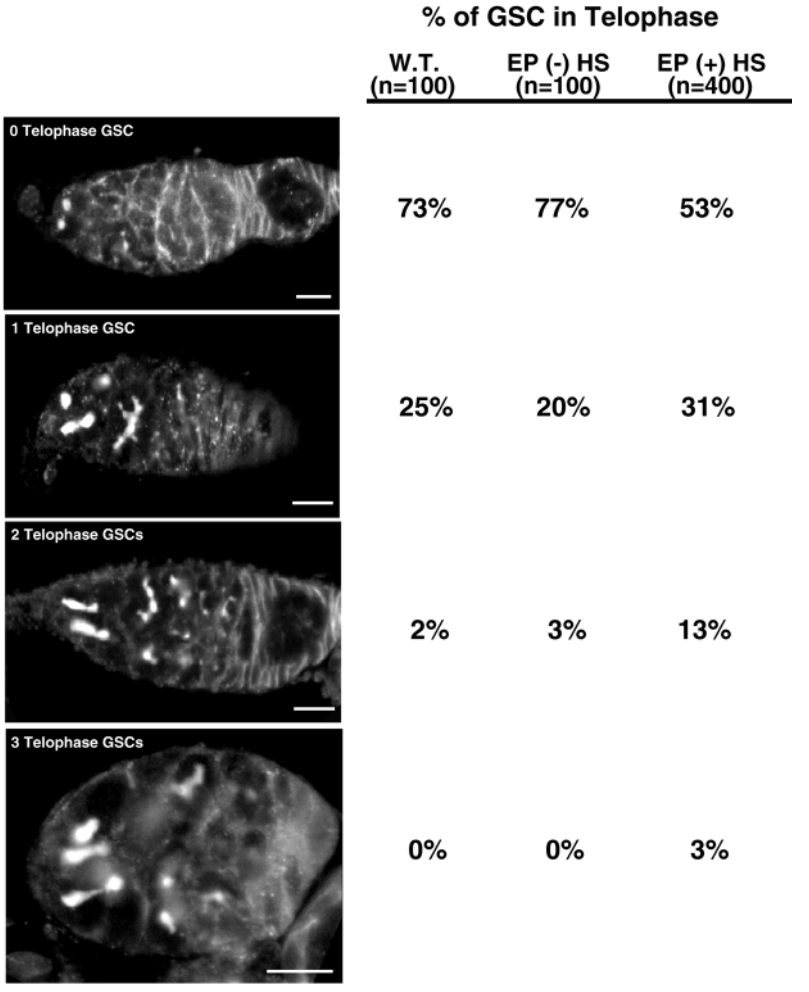

Fig. 8. PIWI overexpression in somatic signaling cells increases the rate of germline stem cell division. The four micrographic panels on the left are anti-1B1 images to illustrate germaria containing 0, 1, 2 and 3 telophase spectrosome-containing germline stem cells. The frequency of observing such germaria in the wild-type control, the uninduced *ep(2)1024* control, and the *piwi*-overexpressed germaria is tabulated to the right. The number of germaria counted for each genotype (n) is given in parentheses. Bars in all panels, 10 μm.

essential for embryogenesis (Cox et al., 1998). Therefore, the asymmetric fates of GSCs and cystoblasts may not be determined by unique “stem cell factors” or “cystoblast factors”. It, instead, may be created by unique combinations of various regulatory molecules that are repeatedly used throughout development.

This pleiotropic function of stem cell genes is also manifested within the stem cell mechanisms, in which *piwi* is involved in both somatic signaling and in cell-autonomous mitotic functions. How can *piwi* acts in two distinct cell types and in two different mechanisms? This is probably because the PIWI protein directly or indirectly mediates gene expression in the nucleus either at the transcriptional or post-transcriptional level. The different *piwi* target genes or their products in different cell types then lead to the distinctly different cellular function of *piwi*.

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