Drosophila Polo Kinase Is Required for Cytokinesis

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Abstract. A number of lines of evidence point to a predominance of cytokinesis defects in spermatogenesis in hypomorphic alleles of the *Drosophila polo* gene. In the pre-meiotic mitoses, cytokinesis defects result in cysts of primary spermatocytes with reduced numbers of cells that can contain multiple centrosomes. These are connected by a correspondingly reduced number of ring canals, structures formed by the stabilization of the cleavage furrow. The earliest defects during the meiotic divisions are a failure to form the correct mid-zone and mid-body structures at telophase. This is accompanied by a failure to correctly localize the Pavarotti kinesin-like protein that functions in cytokinesis, and of the septin Peanut and of actin to be incorporated into a contractile ring. In spite of these defects, cyclin B is degraded and the cells exit M phase. The resulting spermatids are frequently binuclear or tetranuclear, in which case they develop either two or four axonemes, respectively. A significant proportion of spermatids in which cytokinesis has failed may also show the segregation defects previously ascribed to *polo*^{1} mutants. We discuss these findings in respect to conserved functions for the Polo-like kinases in regulating progression through M phase, including the earliest events of cytokinesis.

Key words: *Drosophila* • Polo • cytokinesis • spermatogenesis • meiosis

Attention has recently focused upon the Polo-like family of protein kinases (PLKs)[1] as regulators of the cellular architecture in the passage through mitosis (for reviews see Glover et al., 1996; Lane and Nigg, 1997). The family is named after its founding member encoded by the *polo* gene of *Drosophila*. The original *polo*^{1} allele is a hypomorphic mutation that allows homozygotes to survive to adulthood, but results in poor male fertility and a maternal effect leading to embryonic lethality (Sunkel and Glover, 1988). During larval development, homozygotes display both mis-shaped and monopolar mitotic spindles in the developing central nervous system (Sunkel and Glover, 1988; Llamazares et al., 1991). In addition, non-disjunction takes place in both meiotic divisions in the male, and multipolar meiotic spindles can be seen. The centrosomes of *polo*^{1}-derived embryos fail to assemble correctly resulting in multiply branched arrays of mitotic microtubules (Sunkel and Glover, 1988). These embryos contain Polo kinase that is inactive as a result of its failure to become phosphorylated (Tavares et al., 1996). Stronger hypomorphic and amorphic alleles have also been isolated that show lethality in pupal and larval stages and display mitotic arrest in cells of the central nervous system (White-Cooper et al., 1996; Tavares, A.M., H. Ohkura, and D.M. Glover, unpublished data).

A number of functional studies in a variety of other eukaryotes have also pointed to key roles for the gene family in several stages of mitosis. A *Xenopus* PLK copurifies with and can activate cdc25, and thus may play a role in the positive feedback loop that operates during p34^{cdc2} activation (Kumagai and Dunphy, 1996; Abrieu et al., 1998; Qian et al., 1998). Micro-injection of anti–Polo-like kinase antibodies into human cells prevents the separation of centrosomes which remain small and show reduced immunoreactivity to anti-tubulin antibodies (Lane and Nigg, 1996). A role for the enzyme in activating the anaphase-promoting complex (APC) has been suggested in budding yeast and vertebrate cells (Descombes and Nigg, 1998; Kotani et al., 1998; Shirayama et al., 1998). Mutations in the budding yeast gene *CDC5* (Hartwell et al., 1973; Byers and Goetsch, 1974) that encodes a Polo-like kinase (Kitada et al., 1993) result in a late mitotic arrest in which

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1. *Abbreviations used in this paper*: APC, anaphase-promoting complex; PLK, Polo-like kinase;

DNA has segregated upon an elongated spindle. Two phenotypes predominate in disruptants of the fission yeast Polo-like kinase gene plo1, namely the formation of monopolar spindles as a consequence of the failure of the spindle pole bodies to separate, and a failure to form either an actin ring or septum before cytokinesis. On the other hand, the overexpression of *plo1*$^+$ in fission yeast leads to the formation of multiple septa. Strikingly, septation can be driven by *plo1*$^+$ overexpression in cells blocked at any stage of the cell cycle indicating the potential of the enzyme to overcome the dependence of this process upon the completion of mitosis (Ohkura et al., 1995). Expression of an activated form of mammalian PLK in budding yeast has also been found to drive the formation of multiple septa (Lee and Erikson, 1997).

In contrast to the yeasts, no direct evidence has yet been presented to support a role for the Polo-like kinases in cytokinesis in animal cells. However, the subcellular distribution of the enzymes immediately before and during cytokinesis in fly, amphibian, and mammalian cells suggests this as a possibility. Early in mitosis, Polo-like kinases are associated with the centrosome and kinetochore regions of chromosomes, but at anaphase they become concentrated in the central part of the mitotic spindle (Goldsteyn et al., 1995; Logarinho and Sunkel, 1998; Wianny et al., 1998). They associate with the mid-body during telophase and are lost from the cell along with the remnants of this structure during cytokinesis (Goldsteyn et al., 1995). The main features of this pattern of distribution are shared in mammalian cells with the kinesin-like protein MKLP1 (Nislow et al., 1990), and in *Drosophila* with the homologous protein Pav-KLP (Adams et al., 1998). These motor proteins have been shown to be physically associated with the respective Plk and Polo kinases. Mutations in *pavarotti*, the gene encoding Pav-KLP, result in abnormal morphology of the central spindle and a failure to localize Polo kinase correctly, culminating in a failure of cytokinesis (Adams et al., 1998).

The original observations of the cytological phenotype of *polo*^{1} mutations in male meiosis were carried out solely by phase-contrast microscopy. It is possible to observe the meiotic spindle in this way as contrast is provided by mitochondria that are also partitioned along its microtubules. However, the development of immunolabeling techniques for spermatocytes, and the recent availability of antibodies against many essential components of the mitotic apparatus and the contractile ring have led us to re-examine the meiotic phenotype. We now show that in addition to defects in spindle pole behavior and chromosome non-disjunction previously reported (Sunkel and Glover, 1988), failure of cytokinesis can be seen throughout spermatogenesis. We discuss the implications of this finding in terms of a unifying role for the Polo-like kinases in eukaryotic cells.

Materials and Methods

Reagents

The following primary antibodies and dilutions were used: a 1:20 dilution of rat anti–α-tubulin YL1/2 mAb (Harlan Sera-Lab Ltd., Leicestershire, England), or a 1:50 dilution of mouse anti–α-tubulin N356 mAb (Amersham Life Science, Inc., Arlington Heights, IL), a 1:200 dilution of mouse anti–β-tubulin KMX-1 mAb (Boehringer Mannheim UK, East Sussex, UK), a 1:4 dilution of mouse anti-peanut 4C9H4 mAb (developed by G. Rubin, Drosophila Genome Center, Berkeley, CA, and obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA), a 1:100 dilution of rabbit anti–Pav-KLP polyclonal Rb3301 (Adams et al., 1998), or a 1:500 dilution of rabbit anti–Pav-KLP polyclonal GM2. FITC, Texas red, Cy3, and Cy5 secondary antibodies were purchased from Jackson Immunochemicals or Cappel (West Grove, PA or Malvern, PA, respectively), Alexa 488 secondary antibodies were purchased from Molecular Probes Europe BV (Leiden, The Netherlands). DNA was visualized with propidium iodide, Hoechst 33258 (Sigma, St. Louis, MO) or TOTO-3 iodide (Molecular Probes Europe BV). Vectashield mounting medium H-1000 was purchased from Vector Laboratories (Burlingame, CA). BSA was obtained from Sigma.

Fluorescence Microscopy

Immunostaining of testes from pharate adults or young (0–1-d-old) flies was performed either by the methanol/acetone fixation method as described by Glover and Gonzalez (1993) or by the ethanol/formaldehyde fixation method as described by Hime et al. (1996). After counterstaining of DNA with either Hoechst 33258, propidium iodide (1 mg/ml) or TOTO-3 iodide (1:200), the samples were rinsed in PBS and mounted in either 90% glycerol containing 2.5% *N*-propyl gallate (Giloh and Sedat, 1982) or in Vectashield mounting medium H-1000. Fluorescence observations were made on a Leitz Aristoplan microscope equipped with FITC, TRITC, and UV filters, or on a MRC 1024 Confocal Imaging Head (Bio-Rad Laboratories, Hercules, CA) on a Nikon Optiphot microscope. Photomicrographs were taken with Kodak Tri-X 400 Pro and developed in Kodak HC110 developer for 7 min at 20°C. Confocal images were processed using Photoshop (Adobe Systems, Mountain View, CA) and printed using an Epson Stylus Photo color printer.

Electron Microscopy

Testes from pupae and adult flies were fixed in the trialdehyde solution of Kalt and Tandler (1971) for 2 h at room temperature or overnight at 4°C. After rinsing in 0.1 M cacodylate buffer, pH 7.2, the samples were post-fixed in 1% osmium tetroxide for 2–3 h, bulk stained in 1% uranyl acetate in distilled water, dehydrated in successively increasing concentrations of ethanol, treated with propylene oxide, embedded in an Epon-Araldite mixture, and then polymerized at 60°C for 48 h. Sections cut using an LKB Nova ultramicrotome and a diamond knife (Diatome Ltd., Biel, Switzerland) were collected on copper grids and stained with uranyl acetate and lead citrate. Sections were observed with a Philips CM10 electron microscope (Philips Electron Optics, Mahwah, NJ) at 80 kV.

Results

polo^1 Mutants Show Different Phenotypes in Mitosis and Meiosis

Previous studies of *polo*^{1} mutants have revealed a variety of spindle pole defects at different developmental stages that have been interpreted as a consequence of abnormal centrosome behavior (Sunkel and Glover, 1988; Llamazares et al., 1991; and summarized in the Introduction). The most dramatic mitotic defects are seen in *polo*^{1}-derived syncytial embryos, where the centrosomal antigen CP190 fails to associate with any microtubule-organizing centers (Sunkel and Glover, 1988). In *polo*^{1} brains, the finding of angled bipolar spindles and monopolar spindles (Sunkel and Glover, 1988; Llamazares et al., 1991), suggests that the centrosomes are failing to separate properly. Cells having more than two MTOCs are extremely rare in the *polo*^{1} larval central nervous system. In contrast, giant cells with multiple γ-tubulin–containing MTOCs can readily be observed in testes from homozygous *polo*^{1} males (Fig. 1).

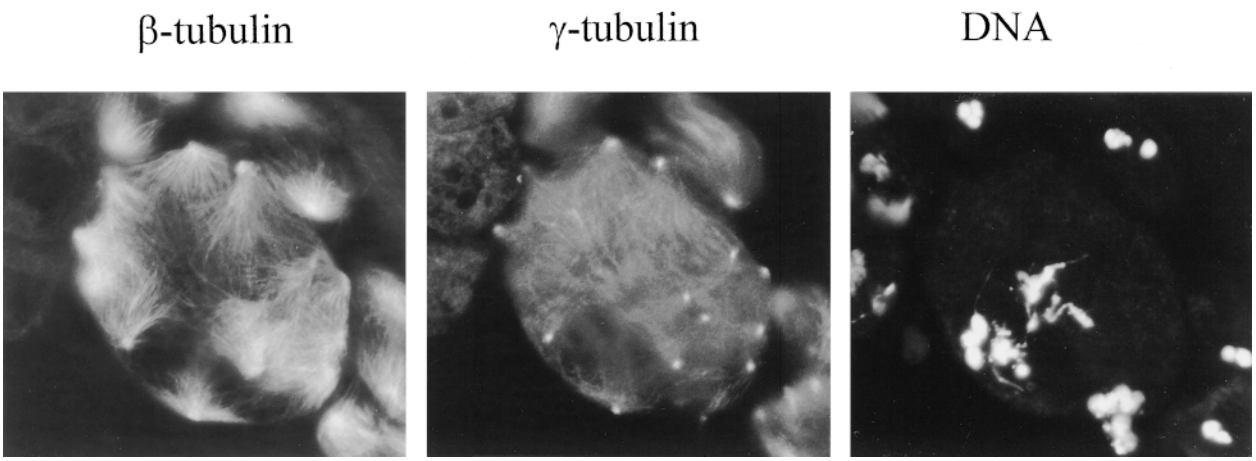

Figure 1. A giant gonial cell resulting from failure of pre-meiotic cytokinesis in testes from homozygous *polo*^{1} males. Staining reveals microtubules (*left*), γ-tubulin distribution revealing centrosomes (*middle*), and chromatin (*right*). Multiple monopolar spindles originate from as many γ-tubulin–containing foci, suggesting that centrosome duplication and separation continue in the absence of cytokinesis.

Thus, whereas the mutant shows a block in centrosome duplication and separation in the mitotic cells of the central nervous system, it seems that in the male germ-line many rounds of centrosome duplication can occur.

Ring Canal Formation Is Defective in Polo^{1} *Spermatocytes*

In both male and female gametogenesis in *Drosophila* a founder cell, produced by the asymmetric division of a germ line stem cell, first undertakes four rounds of mitosis to produce a cell of either 16 spermatocytes or 15 nurse cells and a single oocyte (for review see Fuller, 1993; Spradling, 1993). Cytokinesis is incomplete in these divisions and the cells remain connected by ring canals. Additional ring canals are formed in the male at each meiotic division such that the resulting cyst contains 64 spermatids connected by 63 cytoplasmic bridges. A phospho-tyrosine epitope has been described to accumulate early in ring canal formation in both males and females (Robinson et al., 1994; Hime et al., 1996). The ring canals in the male have been shown to contain three septins, polypeptides first identified as being required to form filaments in the *Saccharomyces cerevisiae* bud neck, one of which is encoded by the gene *peanut*, a gene essential for cytokinesis (Neufeld and Rubin, 1994). These proteins are indeed also present during cytokinesis in the contractile ring, from which the ring canals are derived (Hime et al., 1996). The

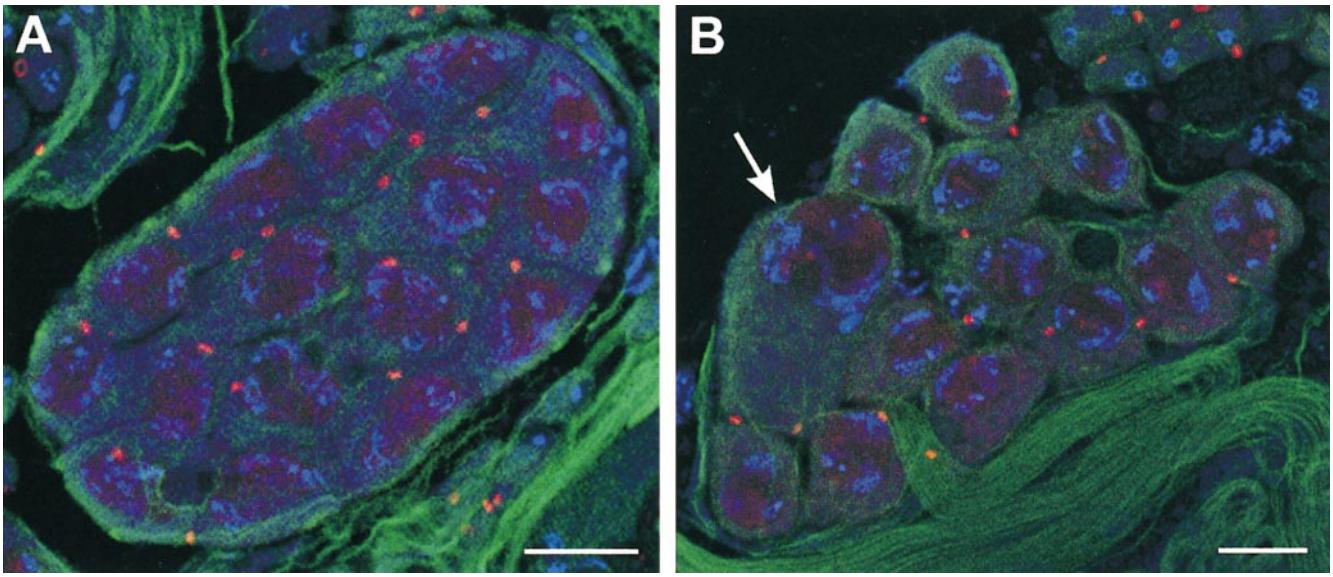

Figure 2. Cytokinesis defects in the pre-meiotic divisions are indicated by reduced numbers of ring canals, and enlarged cells in cysts of primary spermatocytes. Optical sections of a cyst of (*A*) wild-type and (*B*) *polo*^{1} mutant primary spermatocytes. DNA is stained blue, microtubules in green and Pav-KLP in red. The wild-type cyst contains 16 cells inter-connected through 15 ring canals that contain Pav-KLP. The *polo*^{1} mutant cyst contains 13 cells connected by 12 ring canals. One cell (*arrow*) is considerably larger than its neighbors. Bars, 20 μm.

normal developmental progression of spermatogenesis is diagrammed in Fig. 9.

The appearance of the cell displayed in Fig. 1 would suggest that it has undergone the pre-meiotic cell cycles in which the γ-tubulin–containing MTOCs have increased geometrically in number in the absence of cytokinesis. It has the characteristics of a multinuclear cell that has entered meiosis. Examination of cysts of primary spermatocytes revealed them to vary in appearance from those containing single enlarged cells to others of wild-type appearance. Many cysts were intermediate between these extremes and contained both normal and polyploid cells. One such cyst is shown in comparison with a wild-type cyst in Fig. 2. Although the gonial mitoses are difficult to study because these cells are very small, the process of cytokinesis leaves its "footprint" on these cells in the form of ring canals. Cytokinesis is normally incomplete in the gonial cysts and the 16 discrete primary spermatocytes are connected by 15 cytoplasmic bridges. The ring canals are structures formed by stabilization of contractile rings following cytokinesis through which these cytoplasmic bridges pass. The kinesin-like protein encoded by *pava-*

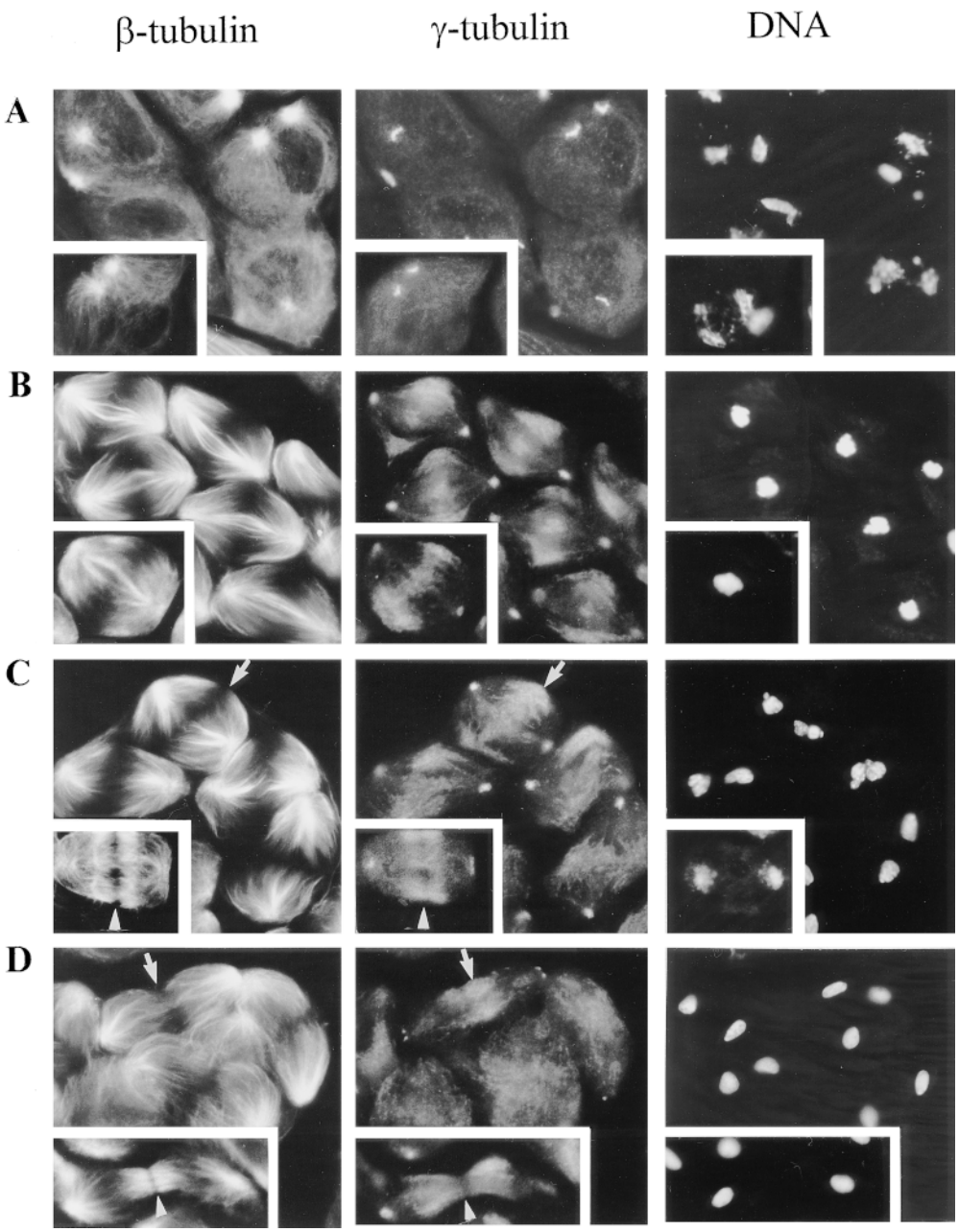

Figure 3. polo[1] spermatocytes show spindle mid-zone defects at late anaphase. Progression through the first meiotic division in *polo[1]* is shown in the main panels, and in wild-type in the insets. Testes preparations have been stained with antibodies against β-tubulin (*left-hand panels*), γ-tubulin (*middle panels*), and Hoechst to reveal DNA (*right-hand panels*) (see Materials and Methods). (*A*) Mature spermatocytes. Note the orthogonal rod-like γ-tubulin structures at the focus of the cytoplasmic microtubules. (*B*) Metaphase spermatocytes. γ-Tubulin is transformed into more diffuse bodies at the poles of the spindles. Bivalents congregate in single masses. (*C*) Late anaphase spermatocytes. A prominent mid-zone structure strongly stained for β-tubulin (*arrowhead* in *insets*) is present in wild-type anaphase spindles, whereas no such structure is found in *polo[1]* spindles (*arrows*). γ-Tubulin is present in this region in both mutant and wild-type. (*D*) Telophase spermatocytes. Wild-type spermatocytes show characteristic mid-bodies with a distinct gap in β-tubulin staining between the two halves of the spindles (*arrowheads* in *insets*); in contrast the spindle microtubules of *polo[1]* mutant spermatocytes fail to organize similar structures during telophase (*arrows*). Whereas in the wild-type, the γ-tubulin in the central region of the spindle begins to redistribute towards the poles, in the mutant cell it remains in the central region.

rotti (Pav-KLP) is essential for cytokinesis, and associates with the central region of the spindle at telophase. In the embryonic mitoses it is discarded from the cell as part of the mid-body upon the completion of cell division (Adams et al., 1998). In male meiosis it persists to become incorporated into the 15 ring canals of the primary spermatocyte cyst (Fig. 2 *A*, *red stain*). The *polo*^{1} mutant cyst (Fig. 2 *B*) has just 12 ring canals connecting 13 cells, one of which (*arrow*) is considerably larger than the others. The reduction in the total number of ring canals in this particular cyst indicates that the large cell has failed to undertake the previous two rounds of cytokinesis.

The polo^{1} *Mutation Prevents Formation of the Spindle Mid-zone at Late Anaphase*

To look directly for defects in centrosome behavior or cytokinesis during cell division, we studied the intracellular distribution of both γ- and β-tubulins. During the transition into the first meiotic division, γ-tubulin becomes organized as a compact body at the centrioles where it remains until completion of meiosis II (Wilson et al., 1997). The majority of *polo*^{1} spermatocytes showed a pattern of γ-tubulin localization at the spindle poles indistinguishable from wild-type during meiosis I (Fig. 3). As previously described, the γ-tubulin condenses into orthogonal rod-like structures during prophase I (Fig. 3 *A*), which transforms into more diffuse bodies during metaphase and anaphase (Fig. 3, *B* and *C*). At metaphase, γ-tubulin also becomes localized perpendicular to the axis of the meiotic spindle, delimiting the central spindle region. In late anaphase–telophase, however, many *polo*^{1} mutant cells show defects in the appearance of the central region of the spindle (Fig. 3, *C* and *D*). Whereas in wild-type, a distinct mid-zone structure develops in late anaphase that shows pronounced staining for β-tubulin and moderate staining for γ-tubulin (Fig. 3 *C*, *inset*, *arrowheads*), no such γ-tubulin structure is seen at this stage in bipolar spindles in the *polo*^{1} mutant. However, γ-tubulin still localizes to the mid-zone in the absence of this central spindle structure (Fig. 3 *C*, *arrows*). Many bipolar spindles fail to constrict at telophase in *polo*^{1} mutants (Fig. 3 *D*, *arrows*) to form the characteristic mid-bodies seen in wild-type meiocytes (Fig. 3 *D*, *insets*, *arrows*). The localization of γ-tubulin must be dependent upon correct mid-body formation at this stage since we observe it to have an abnormal distribution, remaining in the middle region instead of "migrating" polewards to the dividing nuclei as in the wild type. Mutations in genes encoding two kinesin-like motor proteins, *Klp3A* and *pavarotti*, show similar defects in the structure of the mid-zone region of the spindle in late anaphase–telophase and also ultimately result in a failure of cytokinesis (Williams et al., 1995; Adams et al., 1998).

Secondary spermatocytes that have failed to undergo cytokinesis in the first division enter the second meiotic cycle and undergo centrosome separation. Such cells typically contain four MTOCs (Fig. 4, *arrows*). Chromosomes frequently appear to undergo non-disjunction on such tetra-polar structures (Fig. 4, *arrowheads*). This is consistent with the diverse sizes of spermatid nuclei (see also Table I), and the non-disjunction seen by following the segregation of marked chromosomes (Sunkel and Glover, 1988).

In testes from *polo*^{1} mutants we typically found 3–4 cysts of cells undergoing meiosis, in comparison with wild-type testes where there are 5–6 meiotic cysts. We scored 1,965 meiotic figures from 20 homozygous *polo*^{1} mutant testes, and found 69% to show normal meioses. Meiotic abnormalities were very rare at metaphase of the first division, but defects of the types illustrated in Figs. 4–6 were found in a total of 31% of cells from anaphase I onwards.

The polo^{1} *Mutation Affects the Relocalization of the Pav-KLP to the Mid-zone of the Spindle and Prevents Contractile Ring Formation*

To correlate the spindle defects described above with abnormal cytokinesis, we studied the distribution of a number of proteins that associate with the contractile furrow. We have previously shown that the kinesin-like protein encoded by *pavarotti* can associate with Polo kinase. In mitotically dividing cells, both proteins can be found at spindle poles until anaphase, but become associated with the most central part of the spindle in the region of the cleav-

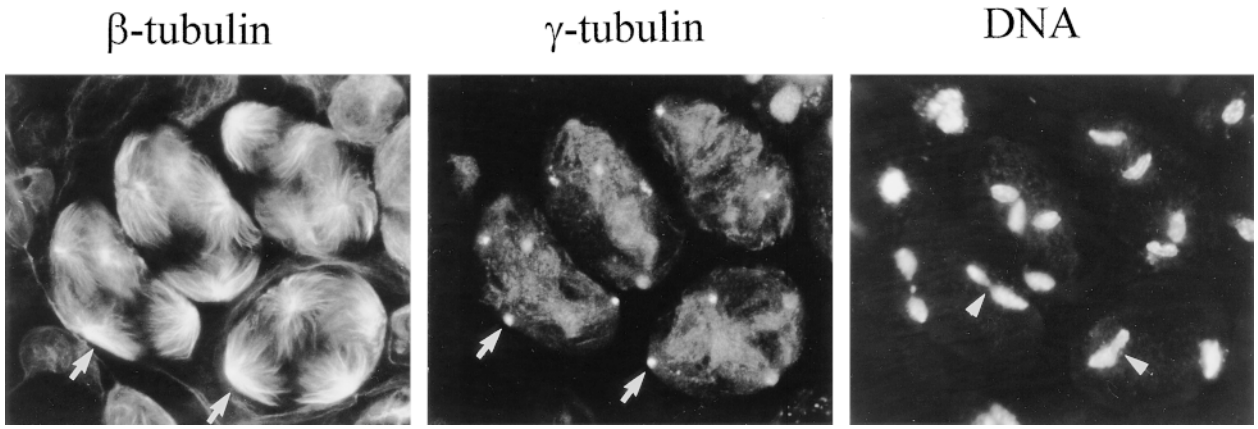

Figure 4. Tetrapolar cells in which cytokinesis has failed in both meiotic divisions in the *polo*^{1} mutant. Testes have been stained to reveal microtubules (*left*), γ-tubulin (*middle*), and chromatin (*right*). Each cell contains four astral microtubules and four foci of γ-tubulin (*arrows*). Chromosomes frequently appear to undergo non-disjunction (*arrowheads*).

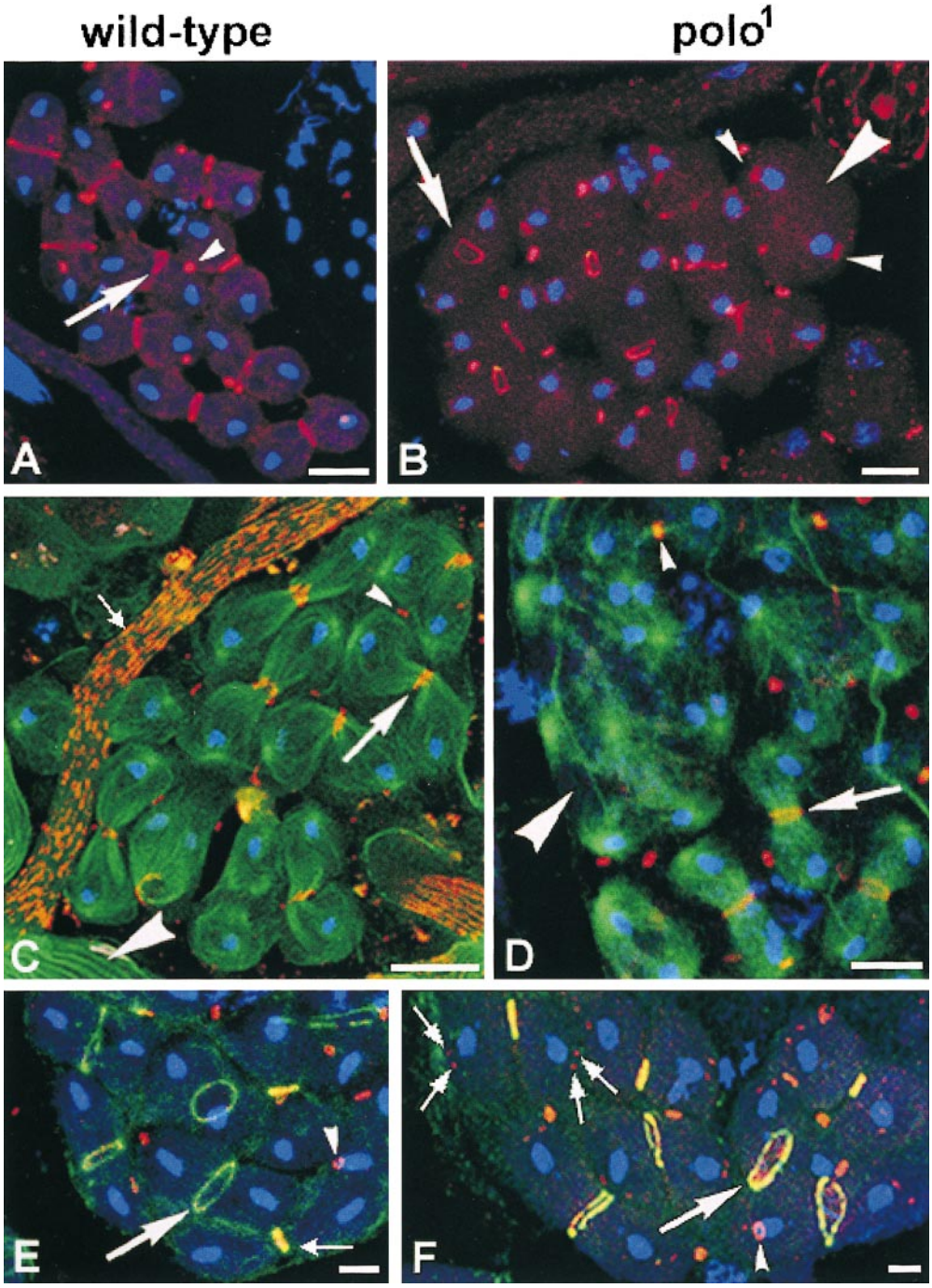

Figure 5. Mislocalization of Pav-KLP and Peanut at telophase in *polo*^{1} mutants. In all of these panels, DNA is stained blue, and Pav-KLP red. The third immunostain (*green*) in *C* and *D* reveals microtubules, and in *E* and *F*, the septin Peanut. (*A*) A wild-type cyst at late anaphase/early telophase I in which Pav-KLP can be seen to be present in the ring canals (*arrowhead*) and the contractile rings (*arrow*). (*B*) In an equivalent *polo*^{1} mutant cyst, Pav-KLP (*red*) is localized in the contractile ring (*arrow*) and remains in the ring canal in normal cells. In spermatocytes showing the mutant phenotype, Pav-KLP accumulates in the polar regions (*small arrowhead*) and does not redistribute to the midbody. (*C*) A telophase I cyst showing the distribution of Pav-KLP (*red*) relative to microtubules (*green*). *Large arrow*, a cleavage ring; and *small arrowhead*, a ring canal. In addition, Pav-KLP is associated with some (putatively immature) axonemal microtubules (*small arrow*), but not with others (*large arrowhead*). (*D*) Telophase figures in *polo*^{1} in which Pav-KLP is associated with early (*arrow*) and late (*small arrowhead*) cleavage rings in cells undergoing apparently normal late meiotic events. Cleavage rings fail to form correctly in the multipolar figure (*large arrow*). (*E*) Immunolocalization of Pav-KLP (*red*) and Peanut (*green*) in a wild-type late anaphase/early telophase I cyst. The large arrow indicates an early cleavage ring in which the staining for Peanut is more pronounced than for Pav-KLP. The two proteins colocalize in the later cleavage ring (*small arrow*) with Pav-KLP being more concentrated towards the inner side. Ring canals appear to contain Pav-KLP and not Peanut (*small arrow*). (*F*) *polo*^{1} mutant cyst with Pav-KLP and Peanut colocalizing in both early (*large arrow*) and late cleavage rings. Punctate staining of Pav-KLP is also seen in the regions of the spindle poles (*small arrows*).

age furrow at telophase. This pattern of subcellular localization of both proteins is disrupted in *pavarotti* mutants (Adams et al., 1998). We therefore decided to examine the distribution of Pav-KLP in meiosis in both wild-type and *polo*^{1} mutant males. We found it difficult to visualize Pav-KLP at the spindle poles in wild-type meiosis, although we could readily observe the protein in the cleavage furrows at the telophase of both meiotic divisions (Fig. 5 *A*, *large arrow*), and to the ring canals (Fig. 5 *A*, *arrowhead*). *polo*^{1} mutant cysts contain ~30% of cells of mutant phenotype alongside cells of wild-type appearance. In such a mutant cyst displayed in Fig. 5 *B*, Pav-KLP can be seen associated with the cleavage furrow of the wild-type appearing cell indicated by the arrow. In contrast, the protein is not found between the telophase nuclei in the cell showing mutant phenotype indicated by the large arrowhead, but

instead appears to accumulate in the region of the poles (Fig. 5, *small arrowheads*).

The other striking feature of cysts from *polo*^{1} mutant testes is the loss of synchrony of the meiotic divisions in comparison with wild-type meiosis. All of the cells in the wild-type cyst in Fig. 5 *C*, for example, have progressed to approximately the same stage of telophase. Pav-KLP is present in the rings of the cleavage furrows (Fig. 5 *C*, *large arrow*) and in the ring canals (Fig. 5 *C*, *small arrowhead*). In addition, Pav-KLP is found associated with some (putative immature) axonemes (Fig. 5 *C*, *small arrow*), but not with others (Fig. 5 *C*, *large arrowhead*). We speculate that this points to another function of this motor protein in transporting flagellar components to its growing distal tip, or to facilitate the elongation of organelles such as mitochondria in the sperm tail. On the other hand, the *polo*^{1} mutant cyst (Fig. 5 *D*) contains cells at a variety of late meiotic stages. Many cells have a wild-type appearance in which case Pav-KLP may be seen in large cleavage rings at late anaphase-early telophase (Fig. 5 *D*, *arrow*), or more compact rings at late telophase (Fig. 5 *D*, *arrowhead*). In other cells such rings of Pav-KLP never seem to form even though the nuclei have moved to the poles of the spindle. This is frequently the case with multipolar cells (Fig. 5 *D*, *large arrowhead*). The *polo*^{1} mutation does not affect the association of Pav-KLP with the flagellar axonemes (not shown).

As septins are known to become incorporated into the contractile ring, we attempted to localize the *Drosophila* septin Peanut in telophase cells from *polo*^{1} mutant cysts during meiosis (Fig. 5 *F*). In wild-type cysts, Peanut appears in the cleavage furrow rings at anaphase (Fig. 5 *E*, *green stain*, *large arrow*), slightly ahead of Pav-KLP (*red stain*). The two proteins colocalize in the furrow at telophase (Fig. 5 *E*, overlap shown by *yellow staining*, *small arrow*), with Pav-KLP appearing more concentrated on the inner side of the ring. The ring canals appear to contain Pav-KLP but Peanut is less easily detected (Fig. 5 *E*, *small arrowhead*). It is our impression that in mutant cysts, there is a tendency for both proteins to accumulate in the larger cleavage furrow rings (Fig. 5 *F*, *large arrow*). This particular mutant cyst, which has a reduced number of nuclei, has paired dots of Pav-KLP staining that has accumulated at the spindle pole regions (Fig. 5 *F*, *small arrows*) even though the cleavage furrows are forming.

The loss of synchrony of the meiotic divisions within a cyst is also apparent in the late meiotic cyst shown in Fig. 6, that illustrates the formation of actin rings at the cleavage furrow. In the wild-type cyst shown in this Fig. 6, cells are uniformly at telophase, and show actin rings of comparable sizes, in contrast to the cells of the mutant cyst that are at a variety of meiotic stages. Late telophase cells can be seen in which the spindle mid-body is well formed and is associated with a compact actin ring (Fig. 6, *small arrow*). Other bipolar cells in which the chromatin has migrated fully to the poles have no mid-zone structure to the spindle microtubules, and are lacking any actin ring (Fig. 6, *large arrow*). This directly links the defect in the central spindle to a failure to establish a contractile ring. A number of cells with tripolar spindles are also seen in this particular cyst. In some of these cells there is no indication of any actin ring formation (Fig. 6, *large arrowhead*), whereas in others a large somewhat misshapen actin ring has formed (*small arrowhead*). Such cells could arise as a result of cytokinesis failure in the first meiotic division, and failure of one of the centrosomes to separate in the second division.

Cyclin B Is Degraded in polo^{1} *Mutant Meioses*

A number of recent papers have reported that polo-like kinases are required for mitotic exit and cyclin destruction, functions that are mediated through activation of the anaphase-promoting complex (APC) (Charles et al., 1998; Descombes and Nigg, 1998; Kotani et al., 1998; Shirayama et al., 1998). Chromatid separation at anaphase is also thought to be under control of the APC. Thus our observations that anaphase could occur in *polo*^{1} mutant cells did not seem to accord with these results. We therefore examined whether cyclin B degradation could take place in *polo*^{1} mutant testes. In wild-type meiotic cysts, a gradient of cyclin B degradation can be seen as cells progress

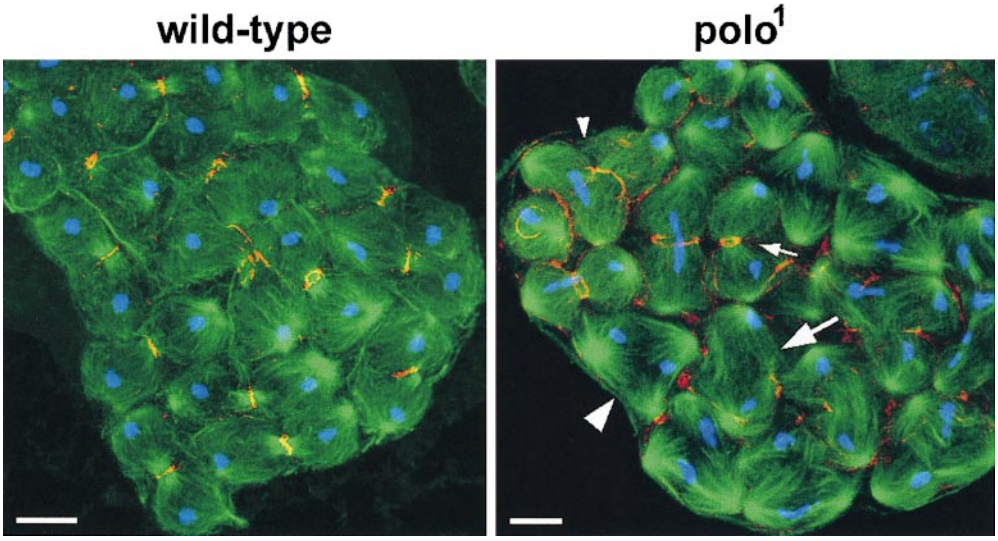

Figure 6. Abnormal actin ring formation during cytokinesis in *polo*^{1} mutants. DNA is stained blue; microtubules are stained green; and actin is stained red. Actin rings at equivalent stages can be seen in the telophase figures from the wild-type cyst. In the *polo*^{1} mutant cyst, actin forms a ring in late telophase cells with a normal spindle mid-body (*small arrow*), but not in cells in which the mid-body has not formed (*large arrow*). Ring-like structures of actin may (*small arrowhead*) or may not (*large arrowhead*) form in cells with tripolar spindles. Bars, 10 μm.

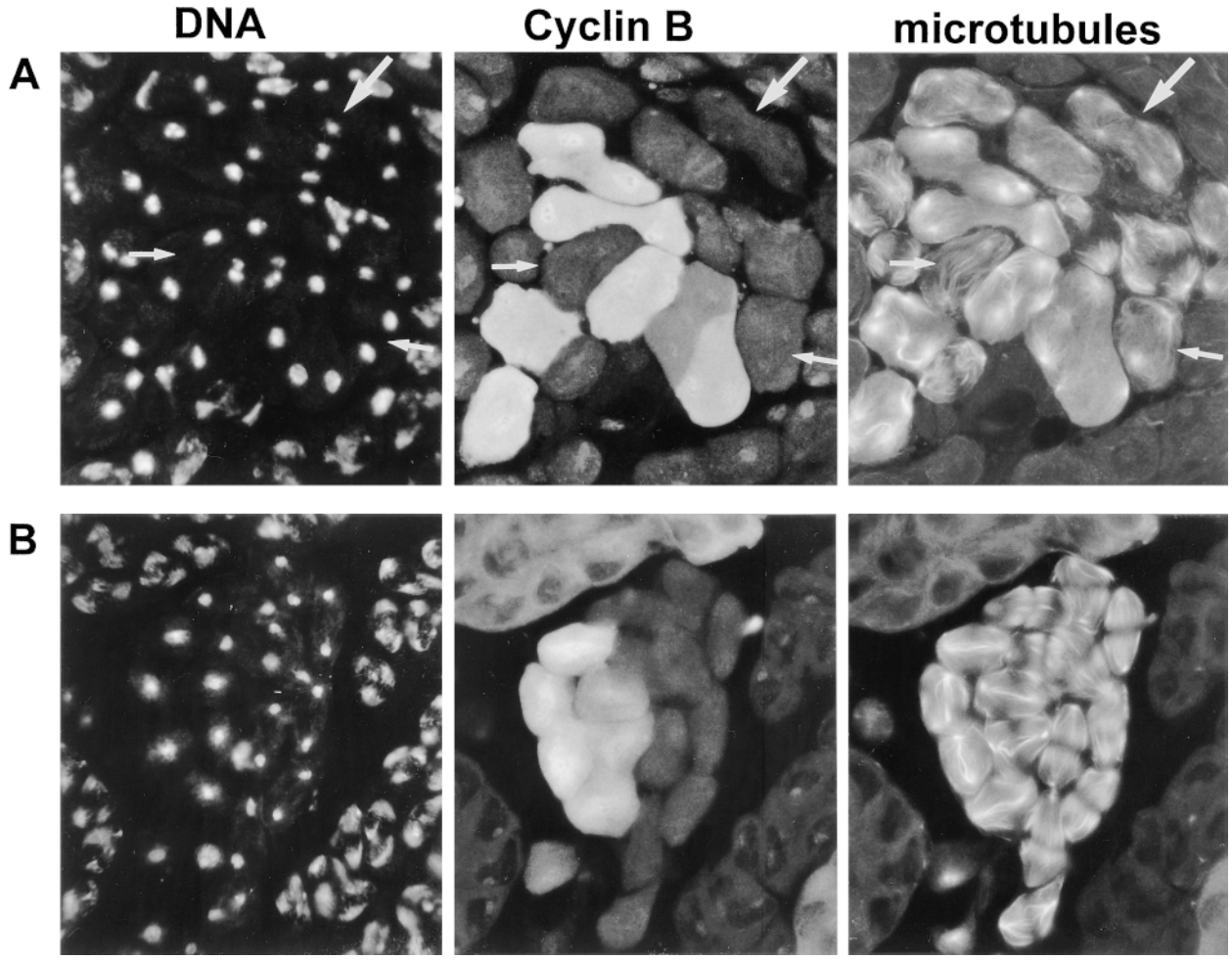

Figure 7. Cyclin B is degraded upon meiotic exit in *polo*[1] mutants. Metaphase–telophase mutant figures in the *polo*[1] mutant. This cyst has several multipolar cells at stages both before and after cyclin B degradation. The large and small arrows indicate cells with a tetrapolar and abnormal bipolar spindles respectively in which cyclin B has undergone degradation. Wild-type cyst with cells showing progressive cyclin B degradation at various stages of the metaphase-anaphase transition.

through the late stages of meiotic division (Fig. 7 *B*). Cyclin B degradation is also seen late in the meiotic cycle in *polo*[1] cells that display either multipolar spindles (Fig. 7 *A*, tetrapolar spindle indicated by the *large arrow*), or bipolar spindles in which the central spindle is abnormal (Fig. 7 *A*, *small arrow*). We therefore conclude either that polo kinase is not required to activate the APC to mediate cyclin B degradation in these cells even though cytokinesis has been affected, or that these processes (together with chromosome disjunction) are differentially sensitive to the *polo*[1] mutation.

Tetranuclear Spermatids Arising from a Failure of Cytokinesis Can Undergo Differentiation

The spermatids resulting from meiosis pass through a stage of development known as the onion stage, in which mitochondria aggregate into a large phase dark body known as the "Nebenkern." Within this structure the mitochondrial membranes are wrapped around each other like the layers of an onion. Wild-type spermatids contain one nucleus and one Nebenkern, and these bodies are of equal size within the cyst of 64 cells. In the *polo*[1] mutants, the majority (82.8%) of onion stage spermatids appear normal (Fig. 8 *A*; Table I). Alternatively they may have varying numbers of nuclei of which binucleate cells (13.1%; Fig. 8 *A*, *arrowhead*) and tetranucleate cells (2.7%; large cell in Fig. 8 *C*) are the most frequent. These cells have a single enlarged Nebenkern, and indicate a failure of cytokinesis in either one or both meiotic divisions. 79% of nuclei in the binucleate cells and 48% in the tetranucleate cells are of uniform diameter suggesting that the major defect has been solely in cytokinesis. The remaining cells have nuclei that show a variation in their diameter indicative of additional non-disjunction (Sunkel and Glover, 1988). Most of the non-disjunction observed cytologically accompanies cytokinesis defects (Table I). As spermatids initiate their differentiation into mature sperm, the mitochondria first undergo elongation. Elongating spermatids can be seen at this stage in *polo*[1] testes that have two or more nuclei at the base of the sperm tail, indicative of a failure of cytokinesis in one of the meiotic divisions (Fig. 8 *D*).

Elongation of the mitochondria is shortly followed by

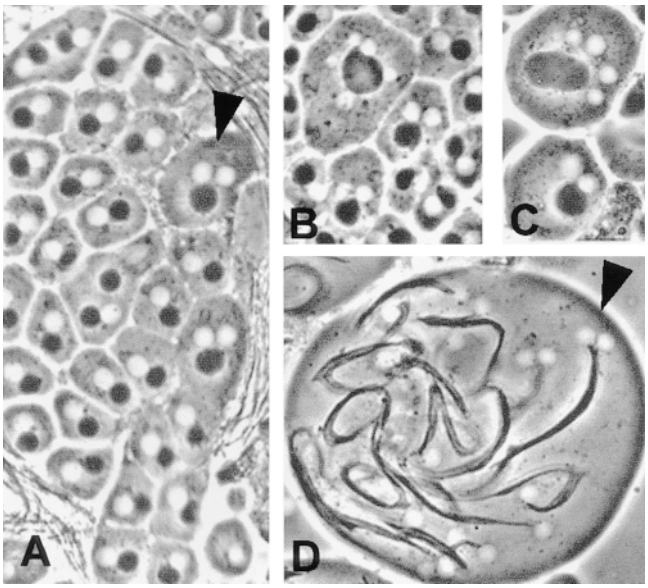

Figure 8. Multinuclear spermatids in *polo*^{1}. (*A* and *B*) Squashed preparations of onion stage spermatids. The clear spherical structure is the nucleus, and the dark spherical structure the mitochondrial derivative or Nebenkern. A binucleate cell with a single Nebenkern is indicated by the arrowhead in *A*. *C* and *D* show larger cells that have 5 or 4 nuclei, respectively and a single Nebenkern. *D* shows spermatids at an early stage of elongation. The arrowhead points to a binucleate cell.

elongation of the nuclei accompanied by growth of the flagellar axoneme from the basal body. Sections of sperm tails at sequential stages of elongation are compared between wild-type and the *polo*^{1} mutant in Fig. 9. Occasional spermatids in the *polo*^{1} mutant show defective axonemes (Fig. 9 *A*). However, whereas at all stages only a single axoneme can be seen in wild-type sperm tails (Fig. 9, *B*, *D*, and *F*), the mutant sperm tails frequently contain either two or four axonemes indicative of a failure of cytokinesis in either one (most likely the second) or both meiotic divisions, respectively. The observed frequency of multiple axonemes in elongating spermatids and sperm is consistent with the frequency of multinucleate spermatids at the onion stage (Table I).

Finally we wished to ensure that the cytokinesis defects we describe here for *polo*^{1} were in fact due to mutations at the *polo* locus, and not some other mutation carried on the chromosome. We selected two hypomorphic mutants, *polo*^{3} and *polo*^{8}, from an allelic series of ems induced *polo* mutations since we had previously determined them to be similar in strength to *polo*^{1} (White-Cooper et al., 1996). We then made heterozygotes between these mutations and *polo*^{1} and examined testes preparations from such males by phase contrast microscopy. In both cases we were able to detect similar defects throughout spermatogenesis to those seen in *polo*^{1} homozygotes, although quantitation of defects in onion stage spermatids indicated that *polo*^{8} is a stronger allele (Table II). An apparently greater frequency of defects consistent with cytokinesis failure before meiosis was seen in other stronger alleles in the series (see Discussion).

Table I. Spermatid Abnormalities in polo^{1} *Homozygotes*

(A)					
Nuclei per onion stage spermatid	1	2	3	4	>4
Percent spermatids	82.8	13.1	0.5	2.7	0.6
Percent spermatids with variation in nuclear size	0.2	2.8	0.5	1.4	0.6
(B)					
Axonemes per elongated spermatid	1	2	3	4	0
Percent spermatids	81.8	11.3	1.3	5.6	1

Discussion

Cytokinesis Can Fail at All Stages of Spermatogenesis in polo^{1} Mutants

The data that we present can best be interpreted by assuming that the *polo*^{1} mutation provides a low level of residual function that permits some cells to pass through both mitosis and meiosis in the developing fly. However, a considerable proportion of cells fail to do so, and in the developing central nervous system they become arrested or delayed in a metaphase-like state in mitosis. In the testes, on the other hand, while there are defects in centrosome behavior and chromosome disjunction as previously reported (Sunkel and Glover, 1988) cytokinesis can also be seen to fail at any of the developmental stages of spermatogenesis (see Fig. 10). Several pieces of evidence point to cytokinesis defects occurring in the pre-meiotic divisions. Many cysts of primary spermatocytes have a reduced number of ring canals, the structures normally formed by stabilization of the cleavage furrow. Moreover, cells in these cysts may become enlarged and polyploid, and contain multiple centrosomes nucleating multipolar spindles. Such a phenotype can be explained by a failure of cell division coupled with ongoing rounds of DNA replication and centrosome duplication. Cytokinesis can also fail in either (or both) of the meiotic divisions. As a result of cytokinesis failing in meiosis I, many secondary spermatocytes develop tetrapolar spindles and subsequently following a second failure of cytokinesis produce tetranuclear spermatids with a single large mitochondrial aggregate. The sperm that develop from such cells can be seen, upon elongation, to have four axonemes. In addition, there are also both binucleate spermatids, and elongating sperm containing two axonemes, indicative of a failure of cytokinesis in the second meiotic division.

Most of the multinuclear spermatids have nuclei of a uniform size suggesting that cytokinesis has been the only abnormal meiotic event in these cells. However, when chromosome non-disjunction has occurred, as indicated by spermatid nuclei of heterodispersed size, this is usually associated with failure of cytokinesis. This suggests that multiple defects can arise as a result of partial loss of *polo* function. The segregation defect may be related to the observed association of polo kinase with the centromeric regions of chromosomes (Logarinho and Sunkel, 1998; our own unpublished observations).

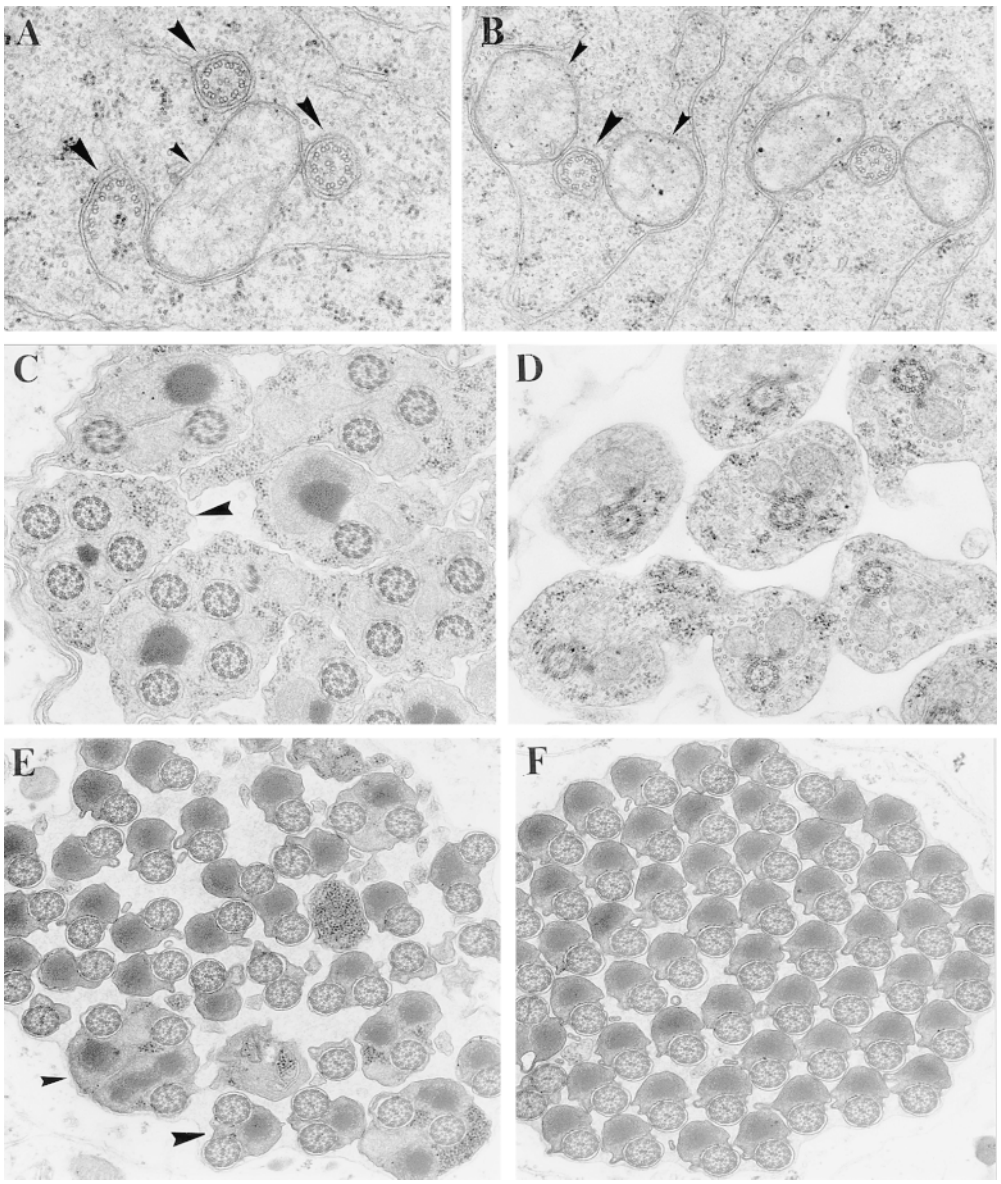

Figure 9. Transmission electron micrographs of *polo*^{1} (*A*, *C*, *E*) and wild-type (*B*, *D*, *F*) cysts of spermatids at sequential stages of elongation. (*A*) Cross section of an early elongating *polo*^{1} spermatid showing three axonemes (*large arrowheads*) associated with only one mitochondrial derivative (*small arrowhead*). The structure of one axoneme appears abnormal (*large arrowhead*). A fourth axoneme is outside the frame of the picture. (*B*) In contrast, in wild-type early elongating spermatids one axoneme (*large arrowhead*) is normally associated with a pair of mitochondrial derivatives (*small arrowheads*). (*C*) Cross section of elongating cysts at a later stage shows that spermatids in *polo*^{1} males may contain four axonemes (cell indicated by *arrowhead*). (*D*) Wild-type spermatids at a similar stage contain single axonemes. (*E*) Section of mature sperm from *polo*^{1} males showing irregular numbers of axonemes (two in the cell indicated by the *large arrowhead*, three in the cell indicated by the *small arrowhead*). (*F*) Mature wild-type sperm contain single axonemes. Although the occasional abnormal axoneme is seen in the *polo*^{1} mutant (*A*), the majority of axonemes from *polo*^{1} and wild-type males have an identical structure throughout the sequential stages of elongation: they comprise one pair of central tubules surrounded by nine doublets, and nine accessory tubules in later stages.

The Earliest Defects Before Cytokinesis Are in the Structure of the Central Spindle

It seems likely that defects in the organization of the central region of the spindle at anaphase anticipate the failure of the contractile actin ring to form and the failure of cytokinesis in *polo*^{1} meiosis. The central spindle shows an abnormal distribution of mid-zone microtubules from late anaphase–telophase. This region normally shows some staining of γ-tubulin that recedes polewards as the mid-body structure matures. In the *polo*^{1} mutant a mid-body does not form and some γ-tubulin remains in the mid-zone region. γ-Tubulin at the central region of the spindle has previously been suggested to be required to form the mid-body, and for the process of cytokinesis (Julian et al., 1993; Shu et al., 1995). Thus a common function of γ-tubulin in both the centrosome and spindle mid-zone might be to act as a microtubule organizing center, a property that might require to be activated by the Polo-like kinases.

The central spindle defects are accompanied by a failure to correctly localize the kinesin-like protein encoded by *pavarotti*, a gene shown to be required for cytokinesis in developing embryos (Adams et al., 1998). Moreover, rings of the septin Peanut and of actin fail to form in the mid-

Table II. Spermatid Abnormalities in polo Transheterozygotes

Nuclei per onion stage spermatid	1	2	3	4	>4
Percent spermatids - *polo*^{1}/*polo*^{3}	88.2	11.3	0	0.3	0
Percent spermatids - *polo*^{1}/*polo*^{8}	79.0	16.2	0	3.2	0.4

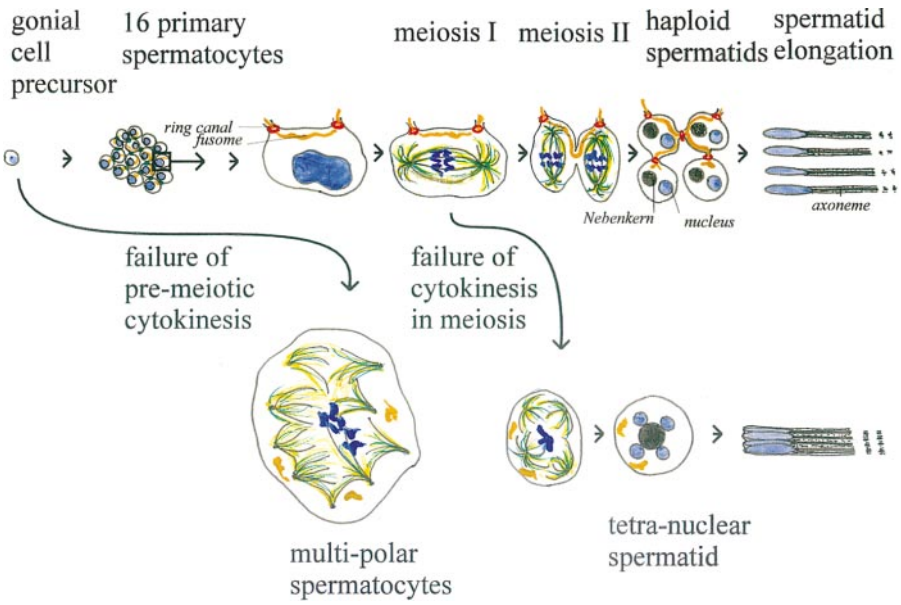

Figure 10. A schematic illustration of spermatogenesis in *Drosophila* illustrating some of the defects that arise as a result of the failure of cytokinesis. Color is used to depict DNA (*blue*); ring canals (*red*); the fusome (*ochre*), a structure rich in membranes and spectrin that passes through the ring canals; microtubules (*green*); and mitochondrial derivatives (*black*).

region of the cell. These defects are similar to those seen in other cytokinesis mutants. The formation of a defective spindle mid-zone at late anaphase and failure to form a mid-body is seen with mutations in *Klp3A* and *pavarotti* (Williams et al., 1995; Adams et al., 1998). These genes each encode kinesin-like proteins, KLP3A and Pav-KLP, respectively, that have been postulated to have a direct role in organizing the central spindle in preparation for cytokinesis.

An additional role has been suggested for Pav-KLP by its association with Polo kinase, namely that it might also serve as a means of localizing this enzyme first to the centrosomes, and subsequently at the central spindle to choreograph formation of the bipolar spindle and then cytokinesis (Adams et al., 1998). The two proteins also appear to be mutually dependent for their correct localization. Polo kinase fails to localize to spindle poles or the spindle mid-zone in *pavarotti* mutants (Adams et al., 1998) and we now show that Pav-KLP accumulates at the spindle poles in meiosis in *polo*^{1} males and often fails to become associated with the spindle mid-zone. This suggests a model whereby the motor properties of Pav-KLP might be changed as a consequence of phosphorylation by Polo kinase, a hypothesis that is currently under investigation.

Polo kinase could also phosphorylate other cytoskeletal components, such as septins or actin-associated proteins to directly modify the structure of the contractile apparatus. Such modifications may in turn lead to changes in the structure of the central spindle. It appears that cooperative interactions occur between the central spindle and the acto–myosin ring to effect cytokinesis. The acto–myosin contractile ring fails to form following mutation of the *chickadee* gene, which encodes the actin binding protein profilin (Giansanti et al., 1998), and consequently the morphology of the central spindle is affected during late meiosis. Thus it would seen that there is a mutual dependence for the correct formation of the cleavage ring and the central spindle region.

Why Should It Be Easier to Observe Cytokinesis Defects in polo Mutants during Spermatogenesis?

One possible answer to this question could be that there are different demands upon *polo* function in mitosis and meiosis. In such a case one might imagine that Polo kinase is less important for correct centrosome behavior in the meiotic cycle, so emphasizing the requirement for cytokinesis. This may be accentuated by the hypomorphic nature of the *polo*^{1} mutation, the residual function being sufficient to form a functional meiotic spindle, but insufficient for its subsequent role. Examination of spermatogenesis in an allelic series of *polo* mutants (White-Cooper et al., 1996) reveals a range of meiotic defects consistent with the strength of the mutant allele. The large primary spermatocytes we previously reported in the stronger alleles (*polo*^{4}, *polo*^{6}, *polo*^{7}) are very similar to those we now describe in detail for *polo*^{1}, but occur at a higher frequency. However, the asynchrony in the passage through spermatogenesis within a cyst is much more pronounced in these strong alleles. Few cells develop to spermatids suggesting a block to pre-meiotic or meiotic divisions. The spermatids that are formed show multiple nuclei and enlarged Nebenkern indicative of cytokinesis defects.

It is also possible that the cytokinesis defects seen in male meiosis in *polo*^{1} could reflect the absence of the checkpoint controls that monitor the structure of the spindle during the male meiotic divisions. The continuation of the meiotic cycle in the absence of a functional spindle is reported for the $\beta 2t^{n}$ mutant that lacks a functional male specific tubulin isoform. The gene for this isoform is expressed at high levels in the growing stage of primary spermatocytes, and the protein is the major γ-tubulin present in the microtubules of the meiotic spindle, and subsequently in the flagellar axoneme. Although these mutants lack a meiotic spindle, their chromosomes are described to undergo normal meiotic condensation–decondensation cy-

cles, and although homologues separate, they fail to segregate resulting ultimately in the formation of tetraploid spermatids that begin to differentiate (Fuller, 1993). Thus in the absence of a spindle integrity checkpoint in male meiosis, if meiotic spindle defects develop in *polo* mutants, chromosome segregation may be able to continue. However, meiosis may fail subsequently at cytokinesis. In contrast, if spindle defects occur in the mitotic divisions of larval neuroblasts, then the cycle is arrested at metaphase by checkpoint controls and so cytokinesis is never attempted.

A Unifying Role for polo-like Kinases in the Early Events of Cytokinesis

The central importance of our present findings is that they clearly establish a role for Polo kinase in the early events of cytokinesis in animal cells, as has previously been established for its fission yeast counterpart *plo1* (Ohkura et al., 1995). Thus there appears to be a unifying role for the Polo-like kinases in both establishing the bipolar spindle and in regulating late mitotic events from the yeasts to the metazoans. It is of interest that the Polo-like kinases of fission yeast and animal cells show some common aspects of localization. In flies and mammals, Polo-like kinases are found associated with centrosomes and centromeres early in mitosis and then subsequently at the spindle mid-zone and cleavage furrow before cytokinesis (Goldsteyn, 1995; Adams et al., 1998). The fission yeast Plo1p like its animal cell counterparts localizes to the spindle poles at the onset of mitosis, but structures analogous to the central spindle and cleavage furrow are not found in fission yeast cytokinesis (Mulvihill, D., H. Ohkura, I. Hagan, and D.M. Glover, manuscript in preparation). Indeed, other proteins required for septation such as the protein kinase encoded by *cdc7*, that lies at the head of the hierarchy regulating septum formation, have also been found to accumulate at the spindle poles (Sohrmann et al., 1998). This establishes the spindle pole bodies as a potential source for signaling molecules that regulate the onset of cytokinesis in the fission yeast. Animal cells may show a variation in this theme by which the Polo-like kinases become redistributed from the spindle poles and centromeres to the central region of the spindle. This could be an evolutionary adaptation that has paralleled the increase in size and complexity of the metazoan mitotic spindle in comparison with its yeast counterpart. Thus the association of Polo kinase with a kinesin-like protein, recently shown to be essential for cytokinesis (Lee et al., 1995; Adams et al., 1998), may be required both to regulate the change in structure of the central spindle immediately before cytokinesis and to correctly localize Polo kinase to its site of action late in cell division. Significantly mutations in Polo and in the associated Pav-KLP both lead to defects in the structure of the central spindle in late anaphase and telophase, and the two proteins appear to be mutually dependent upon each other for their correct subcellular localization.

Our findings have implications towards the existing models for the origins of the signals for cytokinesis in animal cells. Rappaport (1961) has provided compelling evidence that in Echinoderm embryos asters can dictate the position of the cleavage furrow. On the other hand, Wheatley and Wang (1996) found that when tripolar cells were induced to undergo mitosis, the position of cytokinesis was determined by the position of mid-zone microtubules. Moreover, Cao and Wang (1996) found that cytokinesis could be blocked by placing a barrier between the central spindle and the cell cortex suggesting a signal for cytokinesis emanates from the central spindle. These two general hypotheses could be reconciled if the signaling molecule(s), for which Polo-like kinase could be one prime candidate, were initially localized at the poles, and subsequently at the central spindle anticipating the position of the cleavage furrow, and if the extent of this relocalization were to vary between different cell types.

Can Late M-phase Events Be Distinguished from Early Cytokinesis?

It might be argued both for the *S. pombe plo1* disruptant and *Drosophila* hypomorphic *polo* meiotic phenotypes that defects are not in cytokinesis per se, but rather in the events that immediately precede it. Is this more than a question of semantics? In the animal cell the distinction is difficult, as the processes that lead to the formation of the central anaphase–telophase spindle cannot be unraveled from those resulting in formation of the actin ring. In the fission yeast, cytokinesis fails in *plo1* disruptants, but as a consequence of a failure to assemble the actin ring and the septum. Conversely, it should be noted that overexpression of $plo1^{+}$ drives the formation of multiple septa, and not the whole of the process of cytokinesis, suggesting it might be reasonable to make a distinction between these events.

The late mitotic phenotypes of *S. pombe plo1* and its *S. cerevisiae* counterpart *cdc5* differ; failure of septum deposition in fission yeast and a late nuclear division arrest in budding yeast. A role for *CDC5* specifically in mediating late mitotic events is further suggested by the recent demonstration that it is required to activate the cyclin destruction machinery (Charles et al., 1998; Shirayama et al., 1998). A generality for this role is supported by findings that mouse polo-like kinase can phosphorylate and activate components of the APC (Kotani et al., 1998), and that the *Xenopus* homologue is required for mitotic exit and cyclin destruction (Descombes and Nigg, 1998). In $polo^{1}$ mutant testes, however, although a proportion of cells display defects in chromosome segregation, anaphase seems to take place normally in the majority of $polo^{1}$ mutant meiocytes that will fail cytokinesis. Moreover, cyclin B is degraded within these cells that then exit the meiotic division. These observations that suggest the regulation of the APC is not affected in $polo^{1}$. Nevertheless, it is possible either that anaphase, cyclin B degradation, and early cytokinesis may be differentially sensitive to polo kinase function, or alternatively, that the meiotic divisions have their own specific regulatory system for these events (see also Discussion above). We have recently characterized two new strong hypomorphic mutant alleles of *polo* that show a high mitotic index in the larval central nervous system. This phenotype would be consistent with a role for the enzyme in regulating the activity of the anaphase promoting complex (Tavares, A.M., H. Ohkura, and D.M. Glover, unpublished data). It will be of considerable future interest to study the behavior of components of the *Drosophila*

APC in these and other mutant *polo* alleles that differentially affect mitosis and meiosis.

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