

Failure of pronuclear migration and repeated divisions of polar body nuclei associated with MTOC defects in *polo* eggs of *Drosophila*

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SUMMARY

The meiotic spindle of *Drosophila* oocytes is acentriolar but develops an unusual central microtubule organising centre (MTOC) at the end of meiosis I. In *polo* oocytes, this common central pole for the two tandem spindles of meiosis II was poorly organised and in contrast to wild-type failed to maintain its associated Pav-KLP motor protein. Furthermore, the polar body nuclei failed to arrest at metaphase, and the four products of female meiosis all underwent repeated haploid division cycles on anastral spindles. This was linked to a failure to form the astral array of microtubules with which the polar body

chromosomes are normally associated. The MTOC associated with the male pronucleus was also defective in *polo* eggs, and the sperm aster did not grow. Migration of the female pronucleus did not take place and so a gonameric spindle could not form. We discuss these findings in relation to the known roles of polo like kinases in regulating the behaviour of MTOCs.

Key words: *Drosophila*, *polo* oocyte, Meiotic spindle, Sperm aster, Polar body

INTRODUCTION

The Polo-like family of protein kinases (plks) plays a variety of roles in the passage of cells through M phase (for reviews see Glover et al., 1998; Nigg, 1998). The plks have been implicated in at least two roles on mitotic entry; the activation of cdk1 and the organisation of the mitotic centrosome. The *Xenopus* enzyme, Plx, copurifies with and can activate cdc25, and may thus play a role in the positive feedback loop that operates during p34^{cdc2} activation at the G₂-M transition (Kumagai and Dunphy, 1996; Abrieu et al., 1998; Qian et al., 1998). Interfering with enzyme activity either by mutation or using antibodies can lead to the formation of monopolar spindles in fission yeast, *Drosophila*, *Xenopus*, or human cells (Ohkura et al., 1995; Sunkel and Glover, 1988; Qian et al., 1998; Lane and Nigg, 1996) suggesting a common function in organising the poles of the mitotic spindle. Later in mitosis, a role is indicated for the enzyme in activating some functions of the anaphase promoting complex (APC) both in budding yeast and vertebrate cells. Mutants of the *Saccharomyces cerevisiae* plk gene, *CDC5*, fail to degrade the mitotic cyclin Clb2, and yet do mediate the degradation of another APC substrate, Pds1 required for sister chromatid cohesion (Shirayama et al., 1998). Conversely, overexpression of *CDC5* results in proteolysis of Clb2 but not Pds1 (Charles et al., 1998). In a *Xenopus* egg extract, Plx is required for proteolysis of mitotic proteins upon release from cytostatic factor arrest (Descombes and Nigg, 1998) and the purified mammalian plk1 can phosphorylate subunits of the APC in vitro (Kotani et al.,

1998). Finally, the polo like kinases have been implicated in the late mitotic pathway that establishes conditions for correct cytokinesis. Two phenotypes predominate in disruptants of the fission yeast Plk gene *plol*, namely monopolar mitotic spindles resulting from a failure of spindle pole bodies to correctly duplicate or separate, and multinucleate cells in which neither an actin ring or septum has been formed. Over-expression of *plol*⁺ in fission yeast, on the other hand, leads to the formation of multiple septa 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). Moreover, a study of ts alleles of *plol* revealed a joint requirement for the enzyme and the product of *dmf1* to position the septum correctly (Bahler et al., 1998). Expression of an activated form of mammalian plk in budding yeast has also been found to drive the formation of multiple septa suggesting this is a conserved property of the enzyme (Lee and Erikson, 1997). In animal cells, an involvement of a plk in cytokinesis is most clearly demonstrated throughout male meiosis in *Drosophila polo* mutants where the formation of spermatocyte cysts with reduced numbers of cells and multinucleate spermatids has been described by Carmena and colleagues (1998). In these cells, failure of cytokinesis appears to be a downstream consequence of a failure to correctly organise the central region of the meiotic spindle in late M-phase. This is a common feature of a number of mutants defective in cytokinesis. The Pavarotti kinesin-like protein, one motor protein known also to be required to organise the central spindle microtubules, has been shown to associate with polo

kinase. In fact, the two proteins appear mutually dependent for their correct localisation to the central region of the late M-phase spindle (Adams et al., 1998; Carmena et al., 1998).

As the plks influence the function of microtubule associated structures at the spindle poles at the onset of mitosis, and at the central spindle late in mitosis, it is of considerable interest to examine female meiosis in *polo* mutants. Not only does the female meiotic spindle of *Drosophila* show unusual structure of its poles and central regions, but also the meiotic divisions are immediately followed by a dynamic microtubule mediated process that sets up the first gonameric spindle upon which segregate the chromosomes of the male and female pronuclei. The first meiotic spindle is acentriolar and appears not to contain any centrosomal proteins such as γ -tubulin and CP190 (Theurkauf and Hawley, 1992). The spindle microtubules are initially nucleated from chromatin and require the minus end motor Ncd to focus the poles (Hatsumi and Endow, 1992). Meiosis then becomes arrested at metaphase I until the completion of meiotic exchange (McKim et al., 1993). A single chiasma is enough to bring about this metaphase arrest, which does not occur in mutants in which crossing over is suppressed. The second meiotic division follows when the egg is activated on its passage through the oviduct. The second meiotic spindles are tandemly arranged and are connected at a common central pole. The two distal acentriolar poles correspond to those of the first meiotic spindle (Endow and Komma, 1997), whereas the shared central spindle pole comprises a disc-like body containing centrosomal antigens from which radiate an array of microtubules (Riparbelli and Callaini, 1996). The Ncd motor is required for γ -tubulin to localise to this central body and for the reorganisation of spindle microtubules in this central region (Endow and Komma, 1998). At the end of meiosis II, the three outer most nuclei become the polar body nuclei. Their chromosomes condense after telophase, but they do not participate in further nuclear division cycles, and persist in this state until blastoderm stages. The inner most female pronucleus migrates towards its male partner, now associated with a prominent sperm aster. Neither sperm aster formation nor pronuclear migration occur in dominant *Tomai* mutants of the maternal α -tubulin gene or in mutants for the Klp3A motor protein (Mathe et al., 1998; Williams et al., 1997).

In this paper we now report that females homozygous for hypomorphic mutations of *polo* show defects both in the organisation of the unusual central microtubule organising centre (MTOC) at meiosis II, and in sperm aster formation. We discuss these findings with respect to the broader role for the *polo* kinase in regulating the integrity of microtubule organising centres and M-phase progression.

MATERIALS AND METHODS

Reagents

A mouse monoclonal anti- β -tubulin (Boehringer, Mannheim UK) was used at a 1:200 dilution; a rabbit anti-Pav-KLP polyclonal Rb3301 (Adams et al., 1998) at 1:100; and a rabbit anti- γ -tubulin polyclonal Rbcs1 at 1:100. Goat anti-mouse or anti-rabbit secondary antibodies coupled to fluorescein or rhodamine (Cappel, West Chester, PA) were used at 1:600 dilution. DNA was visualized with propidium iodide (Sigma, St Louis, MO). Bovine serum albumin (BSA) and Ribonuclease A (RNase) were obtained from Sigma.

Fluorescence microscopy and confocal images

Eggs were obtained from 5- to 7-day-old females after allowing a short pre-collection period. They were dechorionated in a 50% bleach solution for 2-3 minutes, rinsed in distilled water and fixed in a cold methanol solution to remove the vitelline envelope as described by Gonzalez and Glover (1993). Eggs were fixed at 20 minutes to obtain meiotic stages or held for 35 minutes or two hours before fixation to examine later developmental stages. After fixation the eggs were washed in phosphate buffered saline (PBS) and incubated for one hour in PBS containing 0.1% bovine serum albumin (BSA). For double staining the eggs were first incubated in Rb3301 or Rbcs1 antisera overnight at 4°C and then in anti- β -tubulin antibody for 4-5 hours at room temperature. After washing in PBS-BSA the samples were incubated for one hour with the appropriate secondary antibodies. For simultaneous tubulin and DNA staining, the eggs were incubated for 4-5 hours at room temperature in the anti- β -tubulin antibody. After washing in PBS-BSA, the eggs were then incubated in the goat anti-mouse antibody to which 1 mg/ml RNase was added. After washing in PBS the eggs were incubated 30 minutes in 1 μ g/ml propidium iodide. Confocal images were obtained using a Leica TCS4D confocal microscope equipped with a Krypton/Argon laser (Leica Microsystems, Eidelberg). Images were collected using low laser emission to attenuate photobleaching and 8 frame-averaged scans per image to improve the signal/noise ratio. Images of chromosomes and microtubules collected at several focal planes were superimposed and merged into a single file and imported into Adobe Photoshop to adjust the size and contrast. Prints were made using a Epson Stylus Photo color printer.

RESULTS

The central spindle body of meiosis II is defective in *polo*¹ oocytes

As *polo* is known to be required for the correct organisation and function of the spindle poles, we wished to study effects of the *polo*¹ mutation upon the unusual spindle poles and microtubule organising centres in the newly fertilised *Drosophila* egg. Resumption of female meiosis that occurs during the passage through the oviduct triggers dynamic changes in the orientation and architecture of the meiotic spindle. In the wild-type oocyte the anastral metaphase I spindle rotates from its initial position parallel to the oocyte surface into a perpendicular orientation (Endow and Komma, 1997). The spindle then elongates and reorganises into two tandemly aligned meiosis II spindles linked to the oocyte surface through the pole of the proximal spindle (Huettner, 1924; Fig. 1A). The outer most poles of the tandem spindles are anastral but a characteristic aster-like structure nucleated by a central acentriolar microtubule organising center (MTOC) forms to connect the two spindles (Riparbelli and Callaini, 1996). During anaphase the sister chromatids separate and move to the opposite poles of the twin spindles that appear to loose contact with the central aster (Fig. 1B). At the beginning of telophase the spindle microtubules reduce in length and the four haploid sets of maternally derived chromosomes are positioned at the opposite extremities of the spindles (Fig. 1C). The central aster of microtubules persists beyond this stage to late telophase when small midbodies are visible among the four meiotic products (Fig. 1D).

We were unable to see any appreciable differences between the organisation of the first female meiotic spindle in oocytes produced by homozygous *polo*¹ females and wild-type oocytes.

In contrast, eggs derived from such *polo*¹ mothers showed a number of atypical features that appeared to be linked to a delay in progression through the second meiosis (Table 1). The metaphase II spindles appeared abnormal in two respects. First of all, the meiotic figures were not aligned particularly well perpendicular to the egg surface. Secondly, although the microtubule array of the central spindle pole body was well developed, the microtubules did not have a well organised focus (Fig. 2A). It appeared that as metaphase progressed in the *polo*¹-derived eggs, the twin spindles moved away from each other, and the central aster expanded as a disorganised array of microtubules (Fig. 2B). This pattern of spindle organisation appeared to persist through early anaphase as the sister chromatids separated and migrated to the poles of the two meiotic spindles (Fig. 2C). The central aster was reduced in size to become barely detectable from late anaphase onwards (Fig. 2D). At this time, the meiotic spindles appeared elongated and narrowed in their midzone. During telophase the central aster was no longer visible and the two minispindles appeared connected by a thin bridge of microtubules (Fig. 2E). These spindle microtubules appeared to have regressed by the end of telophase such that the

haploid complements of chromosomes were misaligned (Fig. 2F). Midbody microtubules were detected in only two of 38 telophase figures examined.

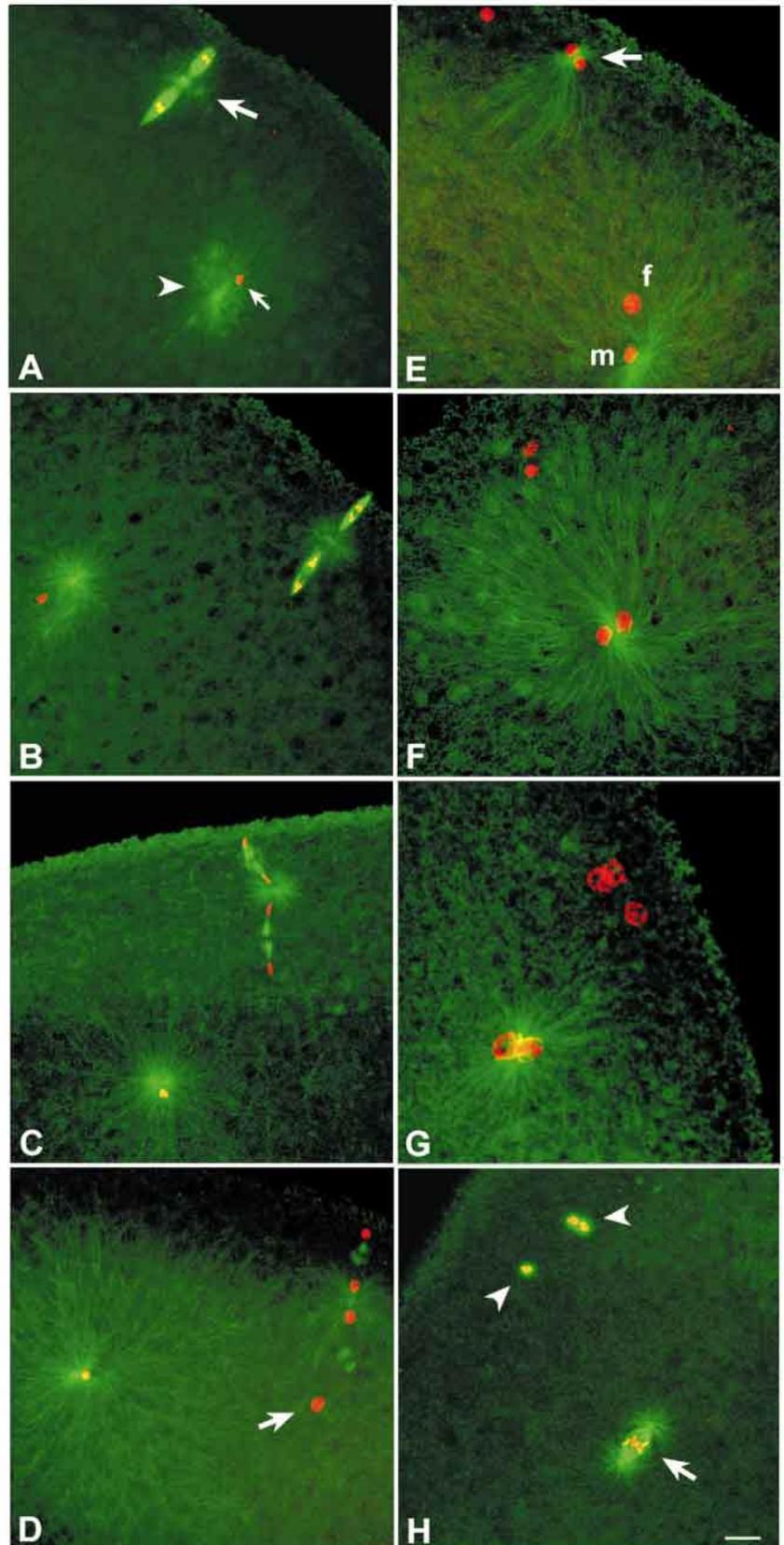


Fig. 1. Meiosis and syngamy in wild-type eggs. Projected series of optical sections of eggs stained with antibodies against β -tubulin (green) and propidium iodide (red). The metaphase II oocyte (A) shows two anastral tandemly aligned meiotic spindles separated by a central spindle pole body (large arrow) and one large microtubule aster (arrowhead) associated with the sperm head (small arrow). During anaphase (B) and early telophase (C) II the sister chromatids separate and migrate to the opposite poles of the meiotic spindles to form four haploid complements; the microtubules of the sperm aster focus near the sperm head. Starting from late telophase II (D) the meiotic spindles become barely detectable among the four decondensing haploid female complements: the more centrally located complement (arrow) becomes the female pronucleus, whereas the outermost complements will be the polar body nuclei; at this time the microtubules of the sperm aster grow to reach the egg cortex. After the completion of meiosis (E,F) the distance between male (m) and female (f) pronuclei gradually decreases; remnant microtubules of the spindle pole body (arrow) are visible between the haploid complements that retreat to the egg periphery. Once male and female pronuclei are in contact (G), their chromatin condenses and a bipolar array of microtubules organise among them at the beginning of the first prophase; the condensation of the polar body chromatin is in phase with that of the pronuclei. During metaphase of the first mitosis (H) the parental complements congress separately at the midzone of the biastral gonameric spindle (arrow); polar body chromosomes are surrounded by thick shells of short microtubules (arrowheads). Bar, 15 μ m.

Mutations in the genes *pavarotti* (*pav*) and *Klp3A* result in defects in the structure of the mid-zone region of the late spindle leading to a failure of cytokinesis during mitosis and male meiosis (Adams et al., 1998; Williams et al., 1995). The formation of the central pole body at the end of meiosis I in oocytes occurs at a comparable stage in M-phase progression to the formation of the mid-zone on other types of spindles. As plks have been shown to associate with Pav-KLP or the mammalian counterpart, *CHO1* /*MKLPI* and to co-localize to the central spindle region (Adams et al., 1998; Carmena et al., 1998; Lee et al., 1998), it was of interest to determine whether the motor protein was associated with the central pole body in female meiosis. We therefore examined the localisation of Pav-KLP in female meiosis using an antibody specific for this motor protein (Adams et al., 1988). We found that in wild-type female meiosis Pav-KLP localised in a ring-shaped structure to the central aster of microtubules that appears during anaphase of meiosis I (Fig. 3A). This remains prominent becoming more compacted as the second meiotic division proceeds (Fig. 3C,E). Pav-KLP did associate to the central spindle pole body during anaphase I in oocytes derived from *polo¹* homozygous females (Fig. 3B), but as this central region lost its structure so the Pav-KLP appeared to become dispersed. Subsequently,

the motor protein could not be detected between the tandem meiosis II spindles (Fig. 3D,F). Thus it would appear that the correct assembly of the central spindle pole body of the female

Fig. 2. Meiosis and the first mitosis in eggs derived from *polo¹* homozygous females. Microtubules are revealed by staining with antibodies against β -tubulin (green) and DNA is stained with propidium iodide (red). The metaphase II oocyte (A) shows misaligned twin meiotic spindles and unfocused spindle pole body microtubules (arrow) and a large sperm aster (arrowhead). The microtubules of the central spindle pole body (large arrow) expand during late metaphase (B) and early anaphase (C) II, but remain unfocused between the twin meiotic spindles; the single sperm aster transforms into two small astral arrays of microtubules (arrowheads) that lose contact with the sperm nucleus (small arrow). Starting from late anaphase (D) the unfocused spindle pole body becomes barely detectable and the sperm asters move away from each other. During early telophase (E) the female chromosomes are held by misaligned minispindles, that disappear during late telophase (F) leaving the haploid female complements not aligned as in wild-type eggs (compare with Fig. 1D); the astral microtubules increase in length, but do not reach the egg cortex. At the beginning of prophase (G) the condensation of male (m) and female (f) chromatin is synchronous even if the parental pronuclei do not come in contact. The first metaphase spindle (H) organises around the male pronucleus (arrowhead), whereas the female complements (arrows) retreat to the egg surface; the spindle associated with the male pronucleus shows defects in centrosome attachment (small arrowhead). The spindles associated with the female meiotic products are anastral. Bar, 15 μ m.

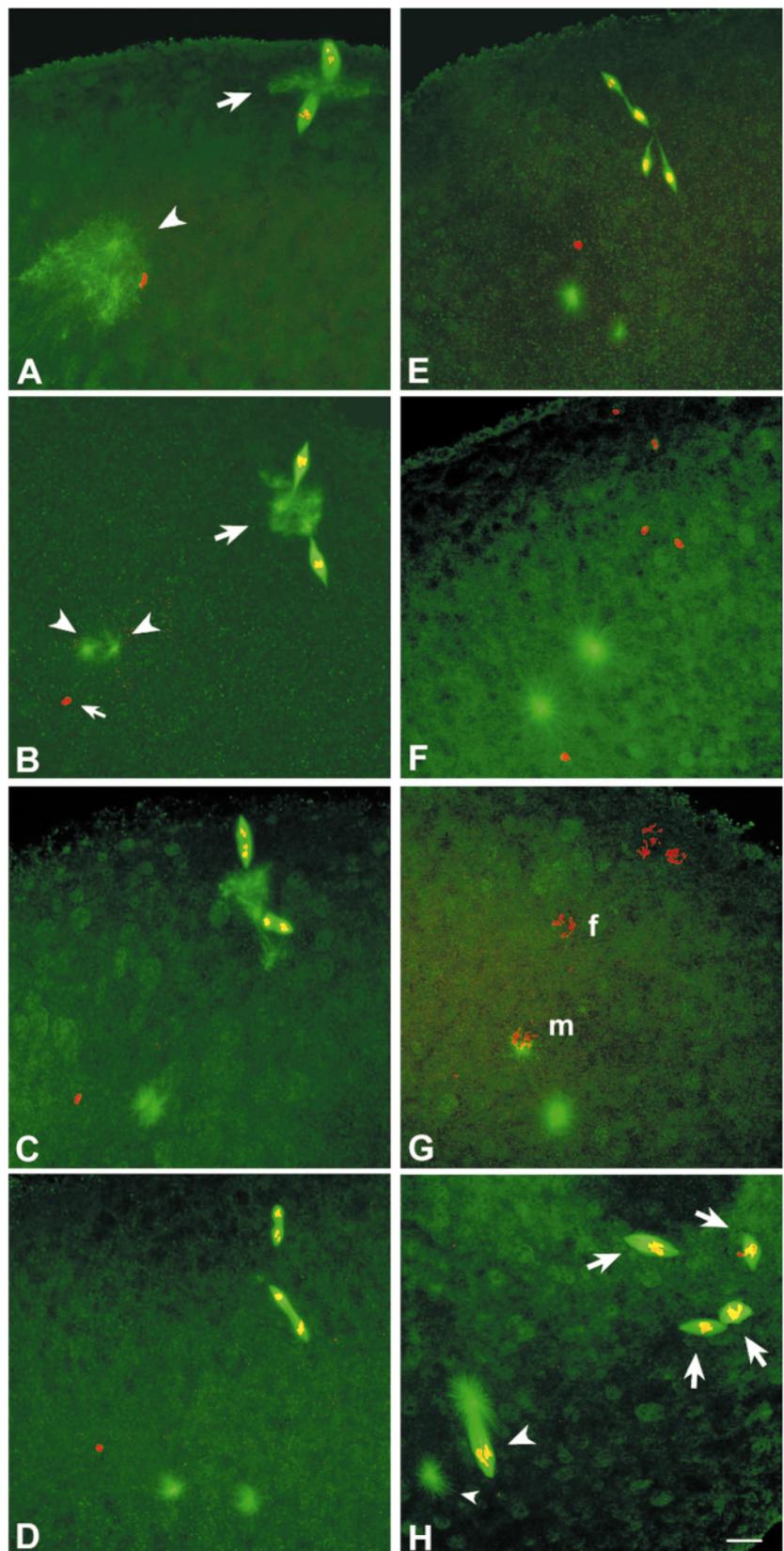


Table 1. Meiotic Progression in *polo*¹ and wild-type (Oregon R) eggs 20 minutes after oviposition

Genotype	Eggs scored	Anaphase I (%)	Metaphase II (%)	Anaphase II (%)	Telophase II (%)	Subsequent stages (%)
<i>polo</i> ¹	267	5 (1.9)	111 (41.6)	84 (31.4)	38 (14.2)	29 (10.9)
Oregon R	374	1 (0.3)	33 (8.8)	54 (14.5)	97 (25.9)	189 (50.5)

Eggs were collected 15 minutes after egg deposition, fixed for 5 minutes, and stained with anti-tubulin antibody and Hoechst dye to score meiotic and mitotic figures.

meiosis II spindle is likely to require the functions of polo kinase to maintain the distribution of Pav-KLP.

Microtubules of the sperm aster fail to grow in *polo*¹-derived eggs

As the sperm enters the egg some centrosomal proteins such as γ -tubulin and CP-190 are recruited from the egg cytoplasm around the sperm basal body which nucleates a small aster of microtubules (Riparbelli et al., 1997). This sperm aster is irregularly shaped and comprises short microtubules until metaphase II (Fig. 1A). The microtubules then begin to grow and by the anaphase of the second meiotic division, they are tightly focused in the proximity of the decondensing sperm head (Fig. 1B). As telophase progresses (Fig. 1C,D), microtubules of the sperm aster grow to reach their maximum length and make contact with the anterior cortex of the egg (Fig. 1D).

The sperm aster in *polo*¹-derived eggs was comparable in size and organisation to that seen in wild-type towards the end of meiosis I and the onset of meiosis II (Fig. 2A). Astral microtubules were short and focused toward several points. By the end of metaphase II the sperm aster was often seen to have divided to give two reduced size astral arrays of microtubules several microns distant from the male pronucleus. Optical sectioning indicated that whereas the distance between the sperm aster and sperm head at the metaphase of meiosis II in wild-type was 3.5 ± 1.21 ($n=31$) μm , in *polo*¹-derived eggs they were widely separated by 21.2 ± 1.16 ($n=23$) μm . The sperm aster increased in dimensions from telophase onwards (Fig. 2F), but the microtubules never extended to the egg cortex and did not make interaction with the putative female pronucleus.

To determine if the abnormal behaviour of the sperm aster during meiosis in *polo*¹ eggs could be affected by the paternal genotype, we examined such eggs that had been fertilised by either wild-type or *polo*¹ sperm. 85% of *polo*¹-derived eggs could be fertilized by *polo*¹ males. These eggs developed two small sperm asters around late metaphase of meiosis II, and pronuclear migration failed to take place (see also below). When crossed to wild-type males, 98% of *polo*¹-derived eggs were fertilised, but all

developed exactly the same phenotype as described above. In contrast, when wild-type females were crossed to *polo*¹ homozygous males, 88% of the eggs were fertilised, and all showed a wild-type meiotic phenotype with a prominent sperm aster that duplicated during telophase of the second meiosis, and nucleated large microtubular bundles able to drive pronuclear migration. These results show that maternally provided polo kinase is required for the correct specification and function of the sperm centrosome.

Female meiosis and sperm centrosome cycle are uncoupled in *polo*¹ mutants

Soon after the male gamete enters the wild-type egg, several centrosomal proteins accumulate around the sperm basal body

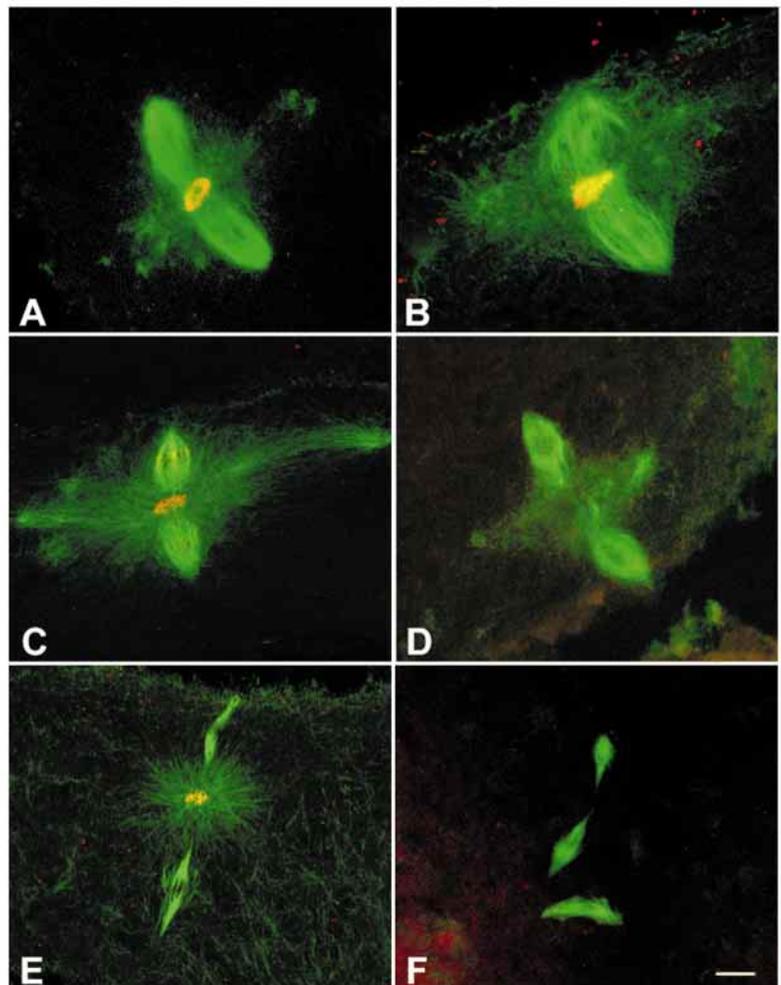
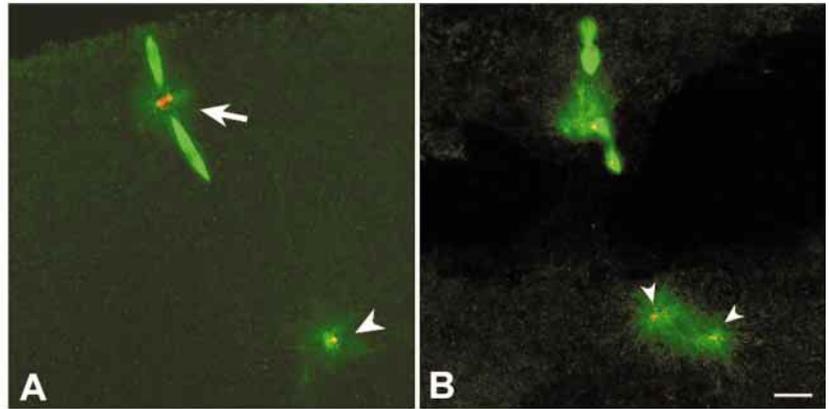


Fig. 3. Localisation of PAV-KLP in wild-type (A,C,E) and *polo*¹ (B,D,F) spindles from late anaphase in meiosis I (A,B), metaphase in meiosis II (C,D), and anaphase of meiosis II (E,F). Pav-KLP is stained red and microtubules, green. Bar, 10 μm .

Fig. 4. γ -tubulin fails to localise to the abnormal central meiotic spindle pole body in *polo*¹. Wild-type (A) and *polo*¹ (B) oocytes stained with antibodies against γ - (red) and β - (green) tubulin during anaphase of the second female meiosis. Note that γ -tubulin colocalises with the spindle pole body (arrow) and with the focus of the sperm aster (arrowhead) in the wild-type. In the mutant oocytes γ -tubulin is greatly diminished in the female meiotic apparatus whereas it can be seen in two distinct foci of the sperm aster (small arrowheads). Bar, 10 μ m.



to form the functional zygotic centrosome (Riparbelli et al., 1997). This centrosome nucleates an aster of microtubules that increases in dimension as meiosis progresses. The sperm centrosome shows one focus of γ -tubulin during anaphase (Fig. 4A) and appears to duplicate during telophase of meiosis II since by this time the sperm aster contains two distinct γ -tubulin and CP-190 foci. In eggs derived from homozygous *polo*¹ females, we saw two small asters near the sperm nucleus from late metaphase II onwards. Each aster contained a γ -tubulin focus (Fig. 4B). We therefore conclude that in *polo*¹-derived eggs either the duplication of the paternally provided centrosome occurs prematurely, or that the timing of female meiosis in *polo*¹ is delayed beyond that in wild-type eggs. These observations do not allow us to distinguish categorically between these hypotheses. However, comparative analysis of meiosis in eggs of wild-type or *polo*¹ females 20 minutes after oviposition suggests that this process takes longer in *polo*¹ mutants (Table 1).

Gonameric spindle formation is prevented in *polo*¹-derived eggs

Completion of meiosis in wild-type eggs leads to the formation of four haploid chromosome complements perpendicularly aligned to the surface: the innermost complement of chromosomes becomes the female pronucleus, and the others form polar body nuclei. During postmeiotic interphase the chromatin of the male and female pronuclei begins to decondense and the distance between the pronuclei decreases until they are juxtaposed in the center of the egg, the polar body nuclei remaining at the cortex (Fig. 1E,F). Once the pronuclei are in contact, their chromatin condenses and a bipolar array of microtubules becomes organised from the duplicated centrosome (Fig. 1G). The maternal and paternal chromosomes then congress as individual groups at the equatorial region of the gonameric spindle (Fig. 1H).

In contrast, at completion of meiosis in *polo*¹-derived eggs, the four haploid nuclei resulting from female meiosis were not as a rule arranged in line. Progression from postmeiotic interphase to prophase could be inferred by the degree of condensation of the parental chromatin (Fig. 2G). In contrast to wild-type fertilised eggs where a prominent aster with long microtubules is associated with the male pronucleus, two small asters with short microtubules were found in *polo*¹-derived eggs. We observed close juxtaposition or contact of the pronuclei in only 2.1% of eggs examined 35 minutes after

oviposition, and were never able to see the formation of a gonameric spindle. Instead, a bipolar spindle was established around the haploid paternal complement (Fig. 2H). In the majority (87%) of cases, only one centrosome was found in association with a single pole of this spindle, whereas the other centrosome remained free at a slight distance.

'Polar body nuclei' undertake repeated division cycles in *polo*¹ eggs

In wild-type eggs, three of the four haploid nuclei resulting from female meiosis behave differently from the innermost female pronucleus which migrates towards the male pronucleus. These remaining three haploid nuclei retreat to the egg periphery to form the polar body nuclei (Fig. 1E,F) whose chromosomes then condense and aggregate in a focused microtubule associated array (Fig. 1H). Their chromosomes remain in this configuration until the syncytial mitotic cycles 8-10, whereupon they become dispersed.

In embryos derived from *polo*¹ homozygous females, pronuclear fusion did not take place and all four haploid complements retreated to a position immediately below the embryo surface (Fig. 2H). Unlike wild-type eggs where the polar body chromosomes stop cycling during the first metaphase, all four haploid complements of maternally derived chromosomes continued to undergo division cycles in *polo*¹ eggs. The chromosomes became associated with anastral bipolar spindles strongly resembling meiotic spindles (Fig. 5) and with poles that lack centrosomal components such as γ -tubulin and CP190 (not shown). The nuclei associated with these anastral spindles underwent several division cycles and populated the embryo surface, while the spindle organized around the male complement was as a rule unable to divide, although its centrosomes replicated (Fig. 5C,D). Only those male derived nuclei associated with spindles retaining both centrosomes were able to divide several times in which case we could find embryos in which anastral and biastral spindles populated the same cytoplasm (Fig. 5E). Nevertheless, these biastral spindles appeared to behave abnormally and give rise to occasional syncytial blastoderm embryos with scattered centrosomes and irregularly sized nuclei (Fig. 5F).

Although sister chromatids successfully segregated during anaphase of these haploid divisions in *polo*¹-derived eggs, we frequently observed errors in chromosome inheritance in the form of spindles with abnormal chromosome content. This assists in understanding the previously reported terminal

phenotype of *polo*¹-derived embryos which is characterized by large chromatin clusters irregularly distributed through the cytoplasm presumably resulting from the collision of neighboring nuclei (Sunkel and Glover, 1988).

Wild-type unfertilised eggs can complete meiosis, after passage through the oviduct (Doane, 1960) but do not develop beyond metaphase of cycle I (Foe et al., 1993). In such circumstances, chromosomes of the haploid nuclei condense and form a radial array resembling the polar bodies in fertilised eggs. As with fertilised eggs, those derived from *polo*¹ homozygous virgin females underwent meiosis but did not form polar bodies. Rather, optical sections of eggs fixed within 30 minutes after oviposition revealed that about 60% of eggs contained many anastral bipolar spindles that were similar to spindles observed in fertilized *polo*¹-derived eggs (data not shown). The remaining eggs contained figures at different stages of meiosis. We did not see any free cytoplasmic asters associated with γ -tubulin and CP190 antigens.

Stronger *polo* hypomorphs arrest in meiosis II

We wished to ensure that meiotic defects observed here for *polo*¹ were only due to mutations at the *polo* locus, and also to observe the effects of more severe hypomorphic mutant combinations upon meiotic progression. To this end, we produced heterozygous females carrying *polo*¹ and one of two hypomorphic mutations *polo*³ and *polo*⁸. These alleles are respectively a weak and a strong hypomorph selected from an allelic series of EMS induced *polo* mutations (White-Cooper et al., 1996). In both cases we were able to detect defects in the organisation of the female meiotic apparatus similar to those seen in *polo*¹ homozygotes. The twin spindles were not aligned during metaphase II and the aster between them was unfocused and irregular (Fig. 6A). Eggs derived from *polo*³/*polo*¹ females completed meiosis

and showed a phenotype similar to that of *polo*¹: they contained many bipolar anastral spindles but lacked structures resembling polar bodies. On the other hand, the greater proportion of eggs from *polo*⁸/*polo*¹ females were arrested in meiosis as indicated by the presence of two major bipolar anastral spindles in 56.7% of fertilised mutant eggs fixed within 30 minutes after oviposition (Fig. 6B). A proportion of eggs had three (15.3%) or four (11.3%) spindles suggesting some progression through anaphase/telophase of the second meiosis. In these cases, chromosomes could be found at the equator of spindles or scattered within them. In contrast to *polo*¹-derived eggs where many anastral spindles populated the egg cytoplasm, we were never able to observe more than five anastral spindles in *polo*⁸-derived embryos. As we observed in *polo*¹-derived eggs, the sperm aster split in two during late metaphase II, but its centrosome was unable to drive the formation of a biastral spindle. Consequently, the haploid male complement was also

Fig. 5. Mitotic spindles in *polo*¹ embryos.

(A) Metaphase of the second meiosis. (B) Female-derived chromosomes and associated post-meiotic (mitosis I) anastral spindles. (C) Second mitosis: the female complements have replicated (large arrow), in contrast to the male pronucleus (small arrow) which has not undergone division and has lost its associated centrosome (arrowhead). (D) Low magnification of the surface of two hour old embryo showing many anastral spindles. (E) Second round of mitosis in an embryo in which the haploid male complement has replicated (arrows). (F) Surface view of a two hour old embryo in which the male complement has undergone many replication cycles; note the scattered asters (arrowheads). In 118 eggs scored at two hours of development, 34.7% showed predominantly tapered anastral spindles; 29.7% showed both anastral and biastral spindles; 26.3% showed irregular fragmented nuclei; and 9.3% could not be easily categorised into this simple classification. The eggs are stained with antibodies against β -tubulin (green) and propidium iodide (red). Bars: 5 μ m (A, B); 15 μ m (C-F).

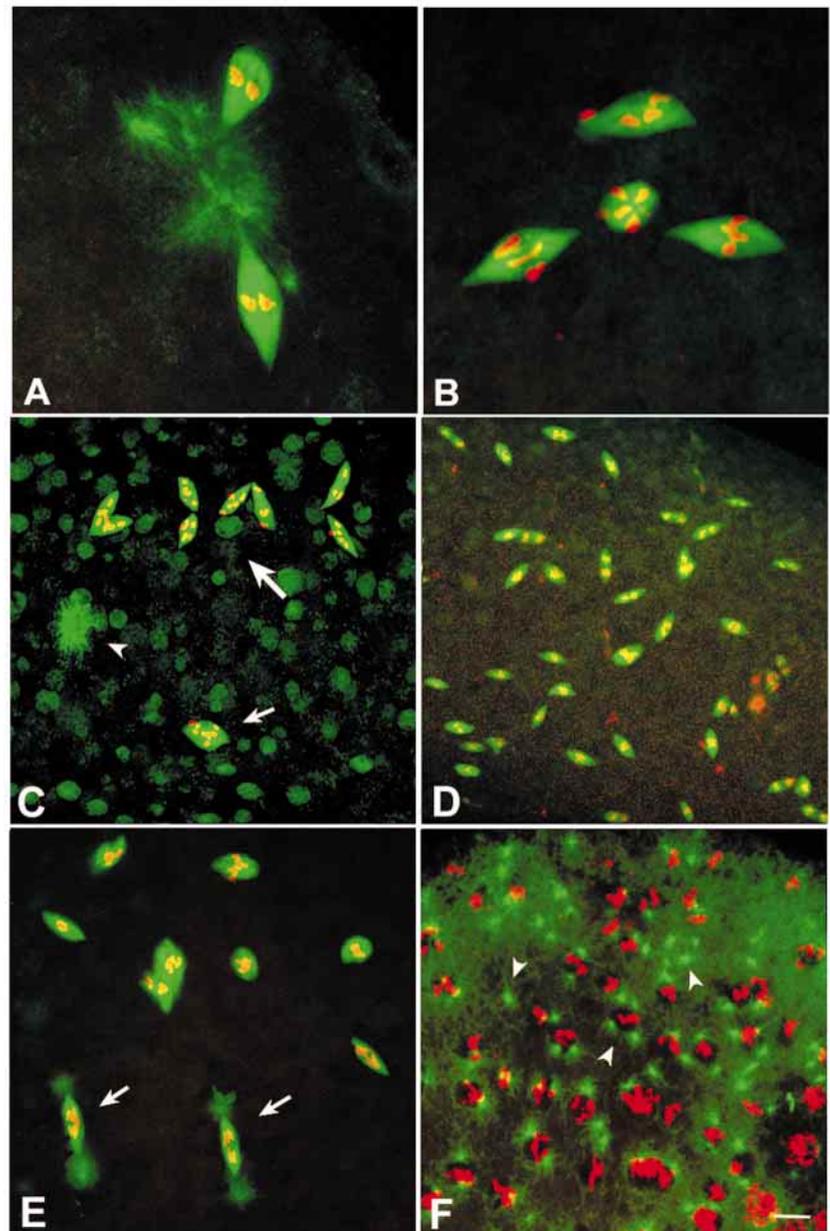
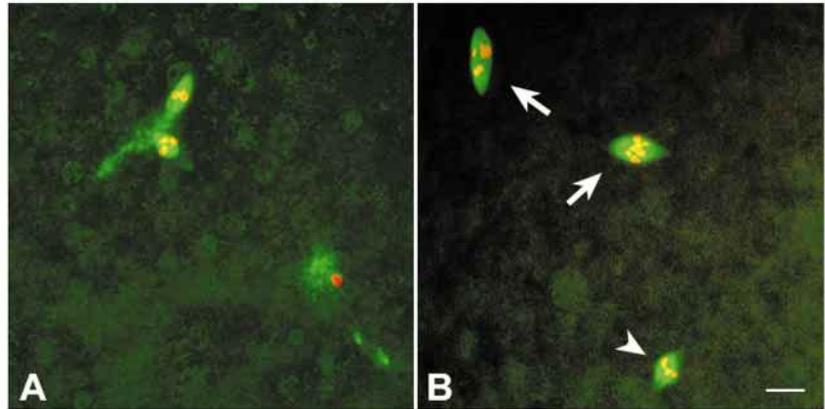


Fig. 6. Eggs derived from *polo⁸/polo¹* females. Microtubules are stained green, and DNA, red. (A) Spindle organisation during metaphase of the second female meiosis. (B) 30 minutes old embryo with two spindles associated with female derived chromosomes (arrows) and one anastral spindle associated with a male derived nucleus (arrowhead). In 150 eggs scored at this stage of development, 56.7% had this appearance indicating arrest at meiosis II with well separated spindles. A further 14.7% had spindles that appeared to be at metaphase II with poorly connected spindles and 2% had progressed to telophase. A small proportion of eggs had four (15.3%) or five (11.3%) anastral spindles suggesting some division of polar body nuclei. Bar, 15 μ m.



associated with a bipolar anastral spindle. These findings are consistent with previous work that showed *polo⁸* to be a stronger allele than *polo¹* or *polo³* and indicate that *polo* is essential for the metaphase-anaphase transition during the second female meiosis.

DISCUSSION

Our detailed observations of meiosis in *polo* mutant females point towards a requirement for polo kinase in the second meiotic division. Two lines of evidence support this assertion. First of all, in females homozygous for the weak hypomorphic allele *polo¹* or heterozygous for *polo¹* and another weak allele *polo³*, the structure of the microtubule organising centre (MTOC) that forms the common pole for the second mitotic spindles is defective. Secondly, in females transheterozygous for *polo¹* and the strong hypomorphic allele *polo⁸*, the majority of eggs are blocked at the second meiotic metaphase. It is possible that these phenotypes reflect two aspects of the known roles for polo-like kinases in late M-phase. The metaphase arrest of the stronger hypomorphic combination may well indicate a requirement for polo kinase in activating the anaphase promoting complex (APC) as proposed in other organisms (see Introduction).

The defective central spindle pole in meiosis II indicates a role for polo kinase in regulating MTOC formation that has similarities to the reported function of the enzyme both in organising or maintaining the structure of the central spindle late in M-phase, and the spindle poles at the onset of M-phase. This appears to be a conserved function of the polo like kinases as demonstrated by failure of bipolar spindle formation in fission yeast *plp1* disruptants (Ohkura et al., 1995), and by the formation of monoastral structures around centrosomes that failed to recruit γ -tubulin following the injection of anti-polo antibodies into HeLa cells (Lane and Nigg, 1996). In fact, this central meiotic MTOC has some features in common with each of these mitotic structures. Like the mitotic centrosome, it contains the centrosomal antigens CP190 and γ -tubulin, and it nucleates an aster of microtubules. Indeed just as the structure and function of the mitotic centrosome is affected in *polo* mutants, we find that the central MTOC and its associated microtubules are diminished in *polo* eggs. Like the central or mid-zone spindle structure that anticipates mid-body formation, the central MTOC forms at late anaphase, and also

contains the Pavarotti kinesin-like protein, a motor protein that is required for the formation of the central spindle region of the mitotic spindle (Adams et al., 1998). In *polo* females Pav-KLP localises to the central MTOC of the meiotic spindle as it forms during anaphase I, but fails to be maintained within this structure. This is consistent with previous observations that Polo and Pav-KLP have a mutual dependency for their correct pattern of localisation (Adams et al., 1998; Carmena et al., 1998), and the findings that the two proteins associate as do their mammalian counterparts plk1 and CHO1/MKLP1 (Lee et al., 1998). Not only does the central MTOC fail to form correctly at late anaphase of meiosis I in *polo* eggs, but mid-bodies also fail to form in the central spindle regions towards the end of the second meiotic division. By this time, the central MTOC has become completely dispersed in the mutant eggs. This is in contrast to wild-type where the central MTOC appears to hold the two central haploid nuclei together and eventually contribute to the microtubular array maintaining the condensed polar body chromosomes in their radial array that remains until the syncytial blastoderm stages of development.

It is not understood how mitotic arrest of the polar body nuclei is maintained while other nuclei sharing the same cytoplasm undergo repeated cycles of mitosis. In *polo*-derived eggs, however, the polar body nuclei are not maintained in this arrested state. Instead they may undergo multiple rounds of divisions on anastral spindles that strongly resemble those seen in meiosis. We entertain several possible interpretations of this phenotype. On one hand *polo* kinase could be directly responsible for establishing and/or maintaining the metaphase arrest of the polar body nuclei, or it is required for the correct exit from the meiotic divisions. On the other, the effect could be indirect and failure to arrest may be a consequence of an inability to sequester the condensed polar body chromosomes into the astral array that usually persists throughout the syncytial mitotic cycles. We favour the latter explanation, as continued abnormal divisions of the female meiotic products have been described in *ncd* mutants (Hatsumi and Endow, 1992; Endow and Komma, 1997). The chromosomes and acentriolar spindles can spread down the dorsal surface of the oocyte in such mutants (S. Endow, personal communication). It is possible that some of the phenotypes seen with mutant alleles of the maternally expressed α -tubulin gene could also be explained in this way (see for example Matthews et al. (1993) Figs 2C-E, 4 and 5). However, continued replication cycles of the polar body nuclei were not seen either in

dominant α -tubulin mutants and in eggs lacking the KLP3A motor (Mathe et al., 1998; Williams et al., 1997). Thus the effect could be allele specific with respect to tubulin mutations, and we feel it is likely to be due to a requirement for specific motor proteins to set up the polar body associated aster.

The failure of pronuclear migration in *polo*-derived eggs could be a consequence of its failure to separate from the other three meiotic products. This could be further accentuated by the delay in progressing through meiosis, and the loss of synchrony with the splitting of centrosomes derived from the male pronucleus. However, a major factor in preventing migration of the female pronucleus is likely to be the poor development of the sperm aster. Failure of pronuclear migration associated with a diminutive sperm aster is also seen in dominant α -tubulin mutants and in eggs lacking the KLP3A motor where the three polar body nuclei do arrest and remain clustered together (Mathe et al., 1998; Williams et al., 1997). As in many organisms, the sperm contributes a centriole and organises an aster of microtubules that is required for the migration of the male and female pronuclei. In the wild-type *Drosophila* egg, this aster undergoes dramatic growth so that it extends from the interior of the egg to the cortex. Polo kinase activity would appear to be required for the growth of this sperm aster, since in *polo*¹-derived eggs, the aster forms but its microtubules fail to grow, and the female pronucleus fails to migrate. Consequently it remains at the cortex with the other 'polar body' nuclei, and continues to undertake the unusual haploid nuclear division cycles.

The separation of the diminutive sperm aster in the *polo*-derived egg to give two small MTOCs out of synchrony with the meiotic divisions could be occurring prematurely perhaps as a secondary consequence of the failure of the sperm aster to grow to establish its correct structure. Alternatively it could reflect a delay to the meiotic divisions, the timing of duplication of the sperm aster being unaffected. The consequence is that usually, one of the two sperm derived sister asters appears to become separated from the male pronucleus to the extent that when a spindle eventually forms it is most frequently bipolar, but monoastal. Such spindles do not appear able to support mitosis. On occasion, however, bipolar biastral spindles are formed that permit haploid mitoses of paternally derived chromosomes, whereas the maternally derived chromosomes undergo some rounds of haploid mitoses on anastral spindles at the cortex of the embryo. These observations point to different roles of paternal and maternal chromosomes in spindle organization and function. The spindles organized around the products of female meiosis appear able to support chromosome segregation, whereas the spindles associated with the paternal chromosomes appear not to have this function unless they are biastral.

In conclusion, the major defects that we have observed in female meiosis and syngamy of *polo* hypomorphic mutants suggest primary roles for the polo kinase in mediating the reorganisation and function of four different types of microtubule organising centre: the unusual central MTOC of the tandemly arranged second meiotic spindle; the mid-zone (central spindle) structures of each of the two late second meiotic spindles; the microtubule array that maintains the condensed oocyte chromosomes as polar bodies; and the sperm aster. The failure to correctly form these structures has a number of consequences upon the completion of female

meiosis, the arrest of the division cycles of the polar body nuclei, the establishment of the gonameric spindle, and the onset of zygotic nuclear divisions in the *polo*-derived egg. We cannot at present rule out multiple roles for polo kinase in these processes, and indeed these might be expected from the known functions of the enzyme throughout M-phase.

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REFERENCES

- Abrieu, A., Brassac, T., Galas, S., Fisher, D., Labbe, J.-C. and Doree, M. (1998). The polo-like kinase Plx1 is a component of the MPF amplification loop at the G₂/M phase transition of the cell cycle in *Xenopus* eggs. *J. Cell Sci.* **111**, 1751-1757.
- Adams, R., Tavares, A., Salzberg, A., Bellen, H. and Glover, D. (1998). *pavarotti* encodes a kinesis-like protein required to organize the central spindle and contractile ring for cytokinesis. *Genes Dev.* **12**, 1483-1494.
- Bahler, J., Steever, A. B., Wheatley, S., Wang, Y. L., Pringle, J. R., Gould, K. L. and McCollum, D. (1998) Role of polo kinase and Mid1p in determining the site of cell division in fission yeast *J. Cell Biol.* **143**, 1603-1616.
- Carmena, M., Riparbelli, M. G., Ministrini, G., Tavares, A., Adams, R., Callaini, G. and Glover, D. (1998). *Drosophila* polo kinase is required for cytokinesis. *J. Cell Biol.* **143**, 659-671.
- Charles, J., Jaspersen, S., Tinker-Kulberg, R., Hwang, L., Szidon, A. and Morgan, D. (1998). The Polo-related kinase Cdc5 activates and is destroyed by mitotic cyclin destruction machinery in *S. cerevisiae*. *Curr. Biol.* **8**, 497-507.
- Descombes, P. and Nigg, E. (1998). The polo-like kinase Plx1 is required for M phase exit and destruction of mitotic regulators in *Xenopus* egg extracts. *EMBO J.* **17**, 1328-1335.
- Doane, W. W. (1960). Completion of meiosis in unseminated eggs of *Drosophila melanogaster*. *Science* **132**, 677-678.
- Foe, V. E., Odell, G. M. and Edgar, B. A. (1993). Mitosis and morphogenesis in the *Drosophila* embryo: point and counterpoint. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez Arias), pp. 149-300. Cold Spring Harbor Laboratory Press.
- Endow, S. A. and Komma, D. J. (1997). Spindle dynamics during meiosis in *Drosophila* oocytes. *J. Cell Biol.* **137**, 1321-1336.
- Endow, S. A. and Komma, D. J. (1998). Assembly and dynamics of an anastral:astral spindle: the meiosis II spindle of *Drosophila* oocytes. *J. Cell Sci.* **111**, 2487-2495.
- Glover, D. M., Hagan, I. M. and Tavares, A. M. (1998). Polo-like kinases: a team that plays throughout mitosis. *Genes Dev.* **12**, 3777-3787.
- Gonzalez, C. and Glover, D. M. (1993). Methods for studying mitosis in *Drosophila*. In *The Cell Cycle: A Practical Approach* (ed. P. Fantes and R. Brookes), pp. ??-??. IRL Press at OUP, Oxford.
- Hatsumi, M. and Endow, S. A. (1992) Mutants of the microtubule motor protein, nonclaret disjunctional, affect spindle structure and chromosome movement in meiosis and mitosis. *J. Cell Sci.* **101**, 547-559.
- Huettnner, A. F. (1924). Maturation and fertilization in *Drosophila melanogaster*. *J. Morphol. Physiol.* **39**, 249-265.
- Kotani, S., Tugendreich, S., Fujii, M., Jorgensen, P.-M., Watanabe, N., Hoog, C., Hieter, P. and Todokoro, K. (1998). PKA and MPF-activated Polo-like kinase regulate anaphase-promoting complex activity and mitosis progression. *Mol. Cell* **1**, 371-380.
- Kumagai, A. and Dunphy, W. (1996) Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from *Xenopus* egg extracts. *Science* **273**, 1377-1380.
- Lane, H. and Nigg, E. (1996). Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. *J. Cell Biol.* **135**, 1701-1713.
- Lee, K. and Erikson, R. (1997). Plk is a functional homolog of *Saccharomyces cerevisiae* cdc5, and elevated Plk activity induces multiple septation structures. *Mol. Cell Biol.* **17**, 3408-3417.
- Lee, K., Greenfell, T., Yarm, F. and Erikson, R. (1998). Mutations of the

- polo-box disrupts localization and mitotic functions of the mammalian polo kinase Plk. *Proc. Nat. Acad. Sci. USA* **95**, 9301-9306.
- Mathe, E., Boros, I., Josvay, K., Li, K. J., Puro, J., Kaufman, T. C. and Szabad, J.** (1998). The Tomaj mutant alleles of alpha Tubulin 67C reveal a requirement for the encoded maternal specific tubulin isoform in the sperm aster, the cleavage spindle apparatus and neurogenesis during embryonic development in *Drosophila*. *J. Cell Sci.* **111**, 887-896.
- Matthews, K. A., Rees, D. and Kaufman, T. C.** (1993) A Functionally Specialized Alpha-Tubulin is Required for Oocyte Meiosis and Cleavage Mitoses in *Drosophila*. *Development* **117**, 977-991
- McKim, K. S., Jang, J. K., Theurkauf, W. E. and Hawley, R. S.** (1993). Mechanical basis of meiotic metaphase arrest. *Nature* **362**, 364-366.
- Nigg, E. A.** (1998). Polo-like kinases: positive regulators of cell division from start to finish. *Curr. Opin. Cell Biol.* **10**, 776-783.
- Ohkura, H., Hagan, I. and Glover, D.** (1995). The conserved *Shizosaccharomyces pombe* kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G₁ and G₂ cells. *Genes Dev.* **9**, 1059-1073.
- Qian, Y.-W., Erikson, E., Li, C. and Maller, J.** (1998). Activated polo-like kinase Plx1 is required at multiple points during mitosis in *Xenopus laevis*. *Mol. Cell Biol.* **18**, 4262-4271.
- Riparbelli, M. G. and Callaini, G.** (1996). Meiotic spindle organization in fertilized *Drosophila* oocyte: presence of centrosomal components in the meiotic apparatus. *J. Cell Sci.* **109**, 911-918.
- Riparbelli, M. G., Whitfield, W. G. F., Dallai, R. and Callaini, G.** (1997). Assembly of the zygotic centrosome in the fertilized *Drosophila* egg. *Mech. Dev.* **65**, 135-144.
- Shirayama, M., Zachariae, W., Ciosk, R. and Nasmyth, K.** (1998). The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. *EMBO J.* **17**, 1336-1349.
- Sunkel, C. E. and Glover, D. M.** (1998). polo, a mitotic mutant of *Drosophila* displaying abnormal spindle poles. *J. Cell Sci.* **89**, 25-38.
- Theurkauf, W. E. and Hawley, R. S.** (1992). Meiotic spindle assembly in *Drosophila* females-behaviour of nonexchange chromosomes and the effects of mutations in the nod kinesin-like protein. *J. Cell Biol.* **116**, 1167-1180.
- White-Cooper, H., Carmena, M., Gonzalez, C. and Glover, D. M.** (1996). Mutations in new cell cycle genes that fail to complement a multiply mutant third chromosome of *Drosophila*. *Genetics* **144**, 1097-1111.
- Williams, B. C., Riedy, M. F., Williams, E. V., Gatti, M. and Goldberg, M. L.** (1995). The *Drosophila* kinesin-like protein KLP3A is a midbody component required for central spindle assembly and initiation of cytokinesis. *J. Cell Biol.* **129**, 709-723.
- Williams, B. C., Dernburg, A. F., Puro, J., Nokkala, S. and Goldberg, M. L.** (1997). The *Drosophila* kinesin-like protein KLP3A is required for proper behaviour of male and female pronuclei at fertilization. *Development* **124**, 2365-2376.