Mutations in *mákos*, a *Drosophila* gene encoding the Cdc27 subunit of the anaphase promoting complex, enhance centrosomal defects in *polo* and are suppressed by mutations in *twins/aar*, which encodes a regulatory subunit of PP2A

Péter Deák*, Mary Donaldson‡ and David M. Glover§

Cancer Research UK, Cell Cycle Genetics Research Group, University of Cambridge, Department of Genetics, Downing Street, Cambridge CB2 3EH, UK

*Present address: Institute of Biochemistry, Biological Research Center, H-6726 Szeged, Hungary
‡Present address: The Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Wilmslow Road, Manchester M20 4BX, UK
§Author for correspondence (e-mail: dmg25@mole.bio.cam.ac.uk)

Accepted 20 June 2003
Journal of Cell Science 116, 4147-4158 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00722

Summary

The gene *mákos* (*mks*) encodes the *Drosophila* counterpart of the Cdc27 subunit of the anaphase promoting complex (APC/C). Neuroblasts from third-larval-instar *mks* mutants arrest mitosis in a metaphase-like state but show some separation of sister chromatids. In contrast to metaphase-checkpoint-arrested cells, such mutant neuroblasts contain elevated levels not only of cyclin B but also of cyclin A. Mutations in *mks* enhance the reduced ability of hypomorphic *polo* mutant alleles to recruit and/or maintain the centrosomal antigens γ-tubulin and CP190 at the spindle poles. Absence of the MPM2 epitope from the spindle poles in such double mutants suggests Polo kinase is not fully activated at this location. Thus, it appears that spindle pole functions of Polo kinase require the degradation of early mitotic targets of the APC/C, such as cyclin A, or other specific proteins. The metaphase-like arrest of *mks* mutants cannot be overcome by mutations in the spindle integrity checkpoint gene *bub1*, confirming this surveillance pathway has to operate through the APC/C. However, mutations in the *twins/aar* gene, which encodes the 55kDa regulatory subunit of PP2A, do suppress the *mks* metaphase arrest and so permit an alternative means of initiating anaphase. Thus the APC/C might normally be required to inactivate wild-type *twins/aar* gene product.

Key words: APC/C, Cdc27, Polo kinase, 55kDa regulatory subunit of PP2A, Mitosis

Introduction

Progression through mitosis requires the ubiquitin-mediated proteolysis of several regulatory proteins (Morgan, 1999; Zachariae and Nasmyth, 1999). A large multisubunit complex known as the anaphase-promoting complex or cyclosome (APC/C) plays a key role as an E3 ubiquitin-protein ligase in this process (Peters, 1999). The APC/C adds chains of ubiquitin to substrate proteins, targeting them for proteolysis by the 26S proteasome. It is evolutionarily conserved and comprises a 20S complex containing ten or more subunits. Substrates of the APC/C include the mitotic cyclins A, B and B3, which are degraded during prometaphase and the early and late stages of anaphase, respectively (Sigrist et al., 1995; Parry and O'Farrell, 2001). Separation of sister chromatids also depends upon APC/C-mediated proteolysis of the Securin inhibitor proteins Pds1 and Cut2 in the budding and fission yeasts, respectively (Cohen-Fix et al., 1996; Funabiki et al., 1997) and their counterpart Pimples in *Drosophila* (Leismann et al., 2000).

There have been few studies of the roles of individual APC/C components. The core catalytic activity of the APC/C appears to reside with the Zn^{2+}-chelating APC11 subunit, which appears able to provide ubiquitin ligase activity alone in vitro in conjunction with Ubc4 as an E2 ubiquitin transfer enzyme (Gmachl et al., 2000; Leverson et al., 2000). However, in order to achieve ubiquitination in vitro using UbcH10 as an E2 enzyme, APC11 has to be partnered by APC2 (Tang et al., 2001).

In *Drosophila*, studies directed at the genes for four APC/C components have suggested different functions. The APC2 subunit is required not only to mediate progression through metaphase in mitotically cycling cells but also to repress mitotic functions in the endoreduplicating cells of the female germ line, which have repeated S and G phases (Kashevsky et al., 2002). Characterization of the phenotype of *ida* mutants indicated that the APC5 subunit encoded by this gene was not required for sister chromatid separation, leading the authors to suggest that other APC/C subunits met this requirement (Bentley et al., 2002). The functions of the APC/C components Cdc27 and Cdc16 of *Drosophila* have

been tested individually by RNA interference (RNAi) in cultured *Drosophila* S2 cells (Huang and Raff, 2002), suggesting that the two molecules might modulate the activity of the complex in different ways at different sites. Their experiments indicated a tendency for sister centromeres to separate following *cdc27* RNAi but not *cdc16* RNAi, with chromatids appearing more scattered on the spindle. However, there was also a greater requirement for *cdc27* function for chromosome-associated cyclin A degradation. One difficulty in interpreting RNAi experiments with S2 cells is their ability, under some growth conditions, to continue cycling. We therefore chose in this work to examine the phenotypes of mutations in *mákos* (*mks*), a *Drosophila* gene encoding the counterpart of Cdc27, in larval neuroblasts. Such mutant cells show a robust mitotic arrest with chromosomes in a metaphase-like arrangement and a failure to degrade mitotic cyclins. The finding of some degree of sister chromatid separation in *mks* supports the growing evidence for differential roles for APC subunits for different mitotic functions.

The APC/C is activated upon entry into mitosis and its substrate specificity is thought to be regulated mainly by the transient association of co-factors such as Cdc20/Fizzy and Cdh1/Hct1/Fzr (Dawson et al., 1993; Sigrist and Lehner, 1997; Schwab et al., 1997; Visintin et al., 1997; Fang et al., 1998; Kramer et al., 1998; Kramer et al., 2000). APC/C function is further known to be regulated by phosphorylation, for which Cdk1/cyclin-B, Polo-like kinase 1 (Plk1) and cAMP-dependent protein kinases have all been implicated as playing major roles (Kotani et al., 1998; Kotani et al., 1999). Plk1 appears to facilitate APC/C activation by preferentially phosphorylating Cdc16 and Cdc23 (Golan et al., 2002), and indeed its fission yeast counterpart, Plo1, binds specifically to the Cdc23 (APC8) subunit (May et al., 2002). Although Polo-like kinases have been reported to be required for APC/C activation, we report here the unexpected finding that *mks* mutants enhance the mutant phenotype of an hypomorphic allele of *polo* with respect to its ability to orchestrate one known function of Polo-like kinases: the recruitment of γ-tubulin and associated molecules to the centrosome (Sunkel and Glover, 1988; Lane and Nigg, 1996; Glover et al., 1998; Donaldson et al., 2001; do Carmo Avides et al., 2001).

Should the spindle function aberrantly, activity of the APC/C is prevented by the spindle integrity checkpoint until chromosomes are correctly aligned at metaphase. One of the spindle integrity checkpoint proteins, Mad2, also binds the APC/C as part of the mechanism that prevents its activity until chromosomes are correctly aligned on a functional spindle (Li et al., 1997; Fang et al., 1998). Here, we show that, in *mks* mutants, the spindle checkpoint protein Bub1 is present at the kinetochores of prometaphase and metaphase chromosomes but the metaphase arrest of *mks* mutants cannot be over-run by mutation in *bub1*. The 55 kDa regulatory subunit of protein phosphatase 2A (PP2A) has also been implicated in regulating Cdk1 activity and the APC/C regulated process of sister chromatid separation at the metaphase-anaphase transition in budding yeast and has been postulated to have a spindle checkpoint function (Minshull et al., 1996; Wang and Burke, 1997). In this study, we find that *mks* induced metaphase arrest can be overcome by mutations in the 55 kDa regulatory subunit of PP2A, identifying an alternative means of initiating anaphase. We discuss the possibility that inactivation of the 55 kDa regulatory subunit of PP2A is one target of the APC/C in relation to the regulatory networks governing mitotic progression.

Materials and Methods

Fly stocks and P element manipulation

Wild-type and mutant strains were maintained and mated on standard yeast-cornmeal-agar medium and all experiments were performed at 25°C. All genetic markers used are described in Lindsley and Zimm (Lindsley and Zimm, 1992). The *mks*^{1} insertion was originally designated 923/9 in the collection of third chromosomal P element insertion mutants (Deak et al., 1997). The *mks*^{2} allele was originally designated l(3)L7123 in the Berkeley *Drosophila* Genome Project gene disruption project (Spradling et al., 1999).

Molecular analysis

Genomic DNA was isolated from 150-200 flies by the method of Jowett (Jowett, 1986). Plasmid rescue from genomic DNA was performed according to Pirrotta (Pirrotta, 1986). Other molecular techniques were performed by standard procedures as in Sambrook et al. (Sambrook et al., 1989). Oligonucleotides complementary to sequences close to the 3′ (5′-TCACTCGCACTTATTGCAA-GCATACG-3′) and 5′ ends (5′-GCTATCGACGGGACCACCTT-3′) of the P element were used to obtain sequences of the insertion site using an ABI PRISM 377 sequencer. DNA was sequenced using double stranded templates and dye terminator cycle sequencing as described in the Perkin Elmer ABI PRISM sequencing kit.

RT-PCR total RNA was isolated from wandering third instar larvae using Tri Reagent extraction (Sigma). cDNA was synthesized from 5 μg total RNA as template with M-MuLV reverse transcriptase (Fermentas) and random hexanucleotide primers (Pharmacia). Two primers complementary to the first and third exons of *mks* (Rtupper, 5′-AGAGCGCCCCTAGAAAGTCG-3′; Rtlower, 5′-ACGGGCGT-GGTCAGAATGTAGTT-3′) were used in PCR amplifications. A control PCR was performed using *rpL17A* primers (*rpL17A* upper, 5′-GTGATGAACTGTGCCGACAA-3′; *rpL17A* lower, 5′-CCTT-CATTTCGCCCTTGTTG-3′) on the same cDNA templates. RT-PCR products were separated by agarose gel electrophoresis.

P-element-mediated transformation

A 6.2 kb genomic fragment containing the entire *mks* gene was subcloned into pCasper4 (Thummel and Pirrotta, 1992). The resulting plasmid (pR6.2) was used for germ-line transformation of *yw* flies by injection with the transposase coding plasmid, pΠ25.1, into embryos. Several X and 2nd chromosomal homozygous viable transgenic lines were established and tested for rescue of the *mks*^{1} mutation. All rescued the lethality and the mitotic defects of *mks*^{1} homozygotes. A control plasmid (pR6.2ΔBam) lacking a 1 kb *Bam*HI fragment of pR6.2 was also tested for rescue of *mks*^{1} by the same procedure. The small deletion in this construct removes most of the tetratricopeptide (TPR) motifs of the Mks protein, so that it failed to rescue *mks*^{1}.

Antibodies

The primary antibodies were the rat monoclonal anti-α-tubulin antibody, YL1/2 (Kilmartin et al., 1982), rabbit anti-Centrosomin antibody (Heuer et al., 1995), rabbit anti-Prod antibody (Török et al., 1997), the Rb666 rabbit anti-Bub1 antibody (Basu et al., 1999), the Rb270 rabbit anti-cyclin-A antibody (Whitfield et al., 1990), the Rb271 rabbit anti-cyclin-B antibody (Whitfield et al., 1990) and the mouse monoclonal antibody MPM2 (Davis et al., 1983) and the rabbit anti-CP190 antibody (Rb188) (Whitfield et al., 1988).

Cytological analysis

Brains were dissected from larvae in PBS, fixed in 3.7% formaldehyde for 20 minutes at room temperature, washed in PBS and then incubated in PBS containing 0.1% Triton X-100 for 10 minutes at room temperature. The fixative was then blocked by incubating with PBS containing 10% foetal calf serum (FCS) for 40 minutes at room temperature. The brains were incubated with primary antibodies diluted to an appropriate concentration in PBS containing 0.1% Tween 20 (PBST) overnight at 4°C. They were then washed four times for 15 minutes each with PBST before being incubated with appropriate secondary antibodies (Jackson) diluted in PBST for 4 hours at room temperature in the dark. The preparations were finally washed three times for 15 minutes in PBST and a further 15 minutes in PBS. DNA was stained with TOTO-3 (Molecular Probes, Eugene, OR) before the specimens were mounted in Vectashield.

Variations from this protocol were made for staining with antibodies to cyclin A or B, in which case the formaldehyde fix was substituted by incubation for 20 minutes in a dry ice bath with methanol that had been prechilled to –20°C. For brains that were to be stained to reveal MPM2 phosphoepitopes, microcystin was added to the dissecting buffer and formaldehyde fix at concentrations of 1 mM and 10 mM, respectively.

Immunoblotting

Extracts of larval brains were prepared for immunoblotting according to Scaerou et al. (Scaerou et al., 1999). In order to compensate for differing brains sizes in the different genotypes, four brains were used per lane for wild-type and *polo*^{1}, six for *mákos*^{1} and *polo*^{1}*mákos*^{1}, and five for all other genotypes.

Table 1. Mitotic phenotypes of *mks* in combination with *polo*^{1}, *bub1* and *twins*$^{618/5}$

Genotype	Total number of cells	Number of cells in mitosis	MI	Meta:anaphase ratio	% Polyploids
A					
Canton S	1878	26	1.4	2.8	0
mks^{1}	2304	198	8.6	8.2	3.0
mks^{2}	2019	126	6.8	8.7	3.9
B					
polo^{1}	2025	77	3.8	4.7	2.5
mks^{1} *polo*^{1}	3058	221	7.2	8.0	4.0
C					
bub^{1}	2684	38	1.4	0.9	0
bub^{1}*; mks*^{2}	2097	176	8.4	7.4	5.4
D					
twins$^{618/5}$	1217	92	7.5	1.4	3.6
mks^{1}*;twins*$^{618/5}$	1330	140	10.5	1.0	9.8

(A) Analysis of squashed larval neuroblast preparations of the *mákos*^{1} and *mákos*^{2} alleles compared with those of wild-type. In both mutant alleles, the mitotic index is raised considerably over that of the wild-type and the metaphase to anaphase ratio indicates that the majority of mitotic cells are arrested or delayed in metaphase.

(B) Comparable analysis indicating that *mks*^{1} is epistatic to *polo*^{1} for these parameters.

(C) *mks*^{2} is epistatic to *bub1*. The squashed preparations of this double mutant combination are indistinguishable in appearance from *mks*^{2} alone.

(D) The metaphase:anaphase ratio of *twins*$^{618/5}$ is considerably lower than that of wild-type, which indicates premature progression into anaphase. A similar ratio is seen in *mks*^{1}*:twins*$^{618/5}$ indicating that *twins/aar* is epistatic to *mks*.

Results

mákos mutants show a metaphase-like delay with highly condensed chromosomes

In the course of screening a collection of P-element insertion mutants (Deak et al., 1997) for those showing mitotic defects, we discovered a gene that we called *mákos* (*mks*). We adopted this term because the cells of the larval central nervous system in the mutant exhibited a high mitotic index and contained highly condensed mitotic chromosomes that resembled the poppy seeds in the Hungarian cake of this name. Both the original mutant allele, *mks*^{1}, and a second allele *mks*^{2} that we identified in the Bloomington stock collection (see Materials and Methods) showed recessive pharate adult lethal and semilethal female sterile phenotypes, respectively. The extent of hypercondensation of mitotic chromosomes in *mks*^{1} can be seen in Fig. 1. Although the arms of mitotic chromosomes in wild-type cells are clearly evident in both metaphase (Fig. 1A) and anaphase (Fig. 1E), it is difficult to discern the arms of the dot-like chromosomes in *mks* (Fig. 1B-D,F-H). The high proportion of cells in a metaphase-like state and the low frequency of anaphases suggested that *mks* cells arrest or delay in metaphase (Fig. 1B,C; Table 1A). When anaphase-like cells were observed, they were frequently disorganized, with chromosomes lagging along the spindle (Fig. 1F,G, arrowheads). We also observed a low frequency of polyploid cells (~3% of mitoses; Fig. 1D,H; Table 1A). The extent of chromosome condensation indicates that cells have been delayed in metaphase for a period of time. It suggested a role for the *mákos* gene product either in some aspect of spindle structure (failure of which would trigger the spindle integrity checkpoint) or in the mechanism regulating the metaphase-anaphase transition itself.

The *mákos* gene is located at 65E and encodes a homologue of the APC subunit Cdc27

We mapped the P element insertions of *mks*^{1}and *mks*^{2} to the left arm of chromosome 3, to location 65E7-12 (Fig. 1I, inset). The elements appeared to be directly responsible for the observed phenotypes, because mutants could revert to wild type following P element remobilization. Molecular cloning of genomic DNA flanking the P element insertion sites enabled us to isolate embryonic cDNA clones from the libraries of Brown and Kafatos (Brown and Kafatos, 1988). An additional full-length cDNA clone was subsequently obtained from the Berkeley *Drosophila* Genome Project (Rubin et al., 2000). Sequence analysis of the *mákos* locus and its corresponding cDNAs identified an open reading frame of ~3 kb interrupted by two small introns. The P element insertion of *mks*^{1} was located within the 5′-untranslated region 253 bp upstream of the ATG initiator codon. The *mks*^{2} P-element insertion was 532 bp upstream of the ATG. RT-PCR experiments revealed a dramatic reduction of the *mks* transcript in both alleles, with *mks*^{1} being a strong hypomorph (Fig. 1J). A genomic DNA fragment containing the transcription unit was cloned into a P-element germ-line transformation vector (pR6.2, Fig. 1I) and was found to be able to rescue *mks* mutants (see Materials and Methods). Moreover, a variant of this DNA segment in which ~1 kb of

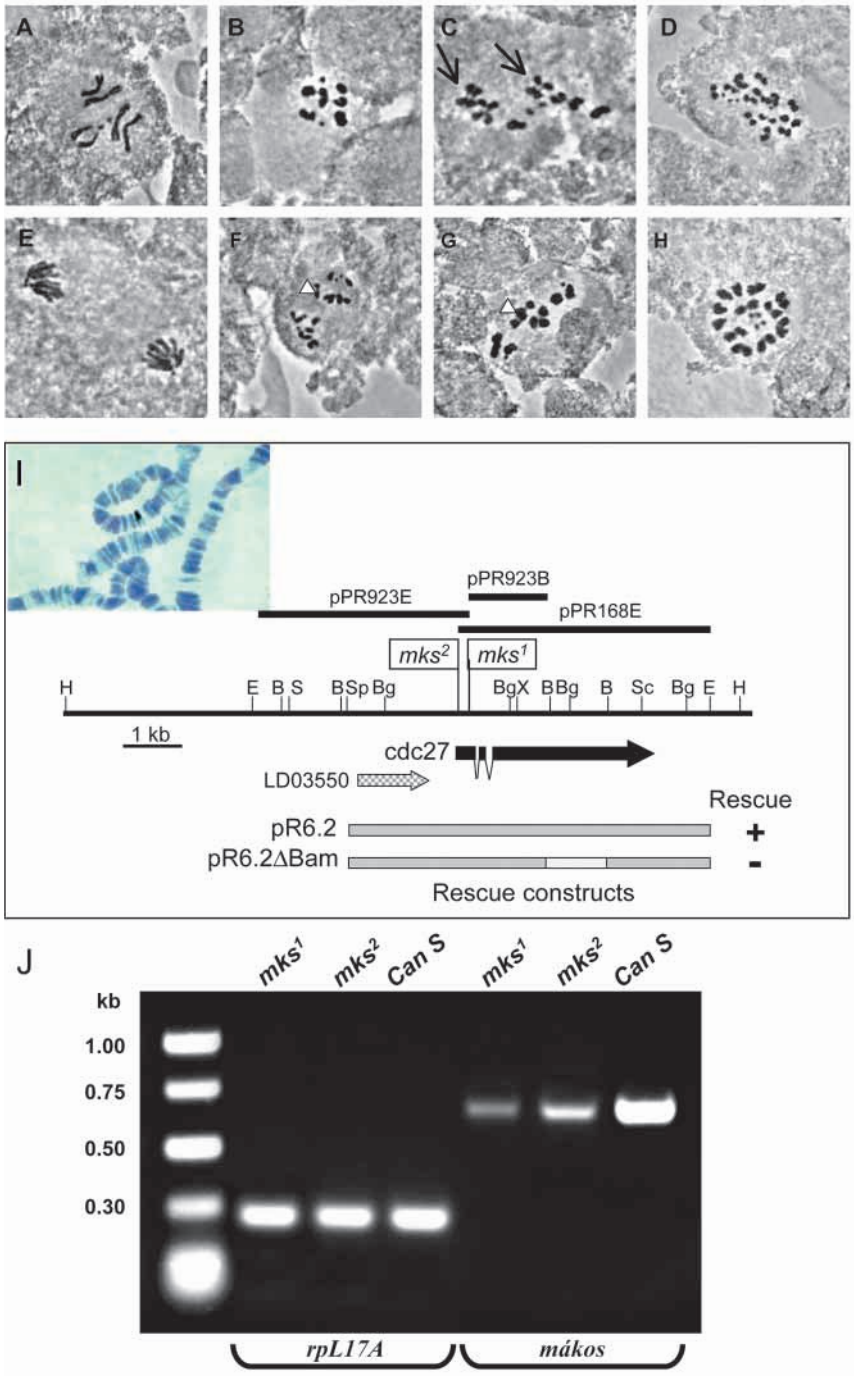

Fig. 1. Mutations in *mks*, a Cdc27 APC/C subunit gene at 65E, result in a metaphase arrest with overcondensed chromosomes. (A,E) Squashed preparations of wild-type larval neuroblasts in metaphase and late anaphase, respectively. (B-D,F-H) Overcondensed mitotic chromosomes in preparations of mks^1 cells. Most cells are diploid (B,C,F,G), although 1-2% are tetraploid (D,H). (C, arrows) A diploid cell in which sister chromatids are well separated. Anaphase cells were classified as cells that were always elongated and in which the chromosome distribution followed the direction of the elongation. They were distinguished from prometaphase cells, which did not show such elongation and had chromosomes in a random position. The proportion of anaphases is reduced compared to the wild type (Table 1A) and, when these do occur, they show lagging chromatids (F,G, arrowheads). (I) A molecular map of the 65E region indicating the sites of the P-insertions in mks^1 and mks^2. In situ hybridization shows the P-element responsible for the mks^1 mutation is inserted at 65E. Restriction endonuclease cleavage sites for *Hin*dIII (H), *Eco*RI (E), *Bam*HI (B), *Bgl*II (Bg), *Sac*II (S), *Spe*I (Sp), *Sca*I (Sc) and *Xmn*I (X) are indicated. Segments of chromosomal DNA inserted into the rescuing transformation plasmid, pR6.2 and its negative control, p76.2ΔBam (see Materials and Methods) are indicated below the map. (J) Estimation of *mks* transcripts relative to those of *rpL17A* in third instar larvae by RT-PCR (see Materials and Methods). Canton S (Can S) larvae were used as the wild type.

internal sequence was deleted (pR6.2ΔBam; Fig. 1I) failed to rescue the mutant.

The sequence of *mks* reveals that it encodes the *Drosophila* homologue of the budding yeast gene *CDC27*, a subunit of the APC/C. The MKS protein contains ten tandemly arranged 34 amino acid repeat units termed TPR motifs, nine of which are located in a single block at the carboxy-terminal with a single disconnected unit closer to the N-terminal end. This arrangement is conserved in CDC27 homologues from the yeasts to humans (Tugendreich et al., 1995). Such TPR repeats have been implicated in facilitating protein-protein interactions between APC/C subunits.

mákos mutants undergo metaphase-like arrest but with separated sister chromatids

A major role of the APC/C is to mediate the metaphase-anaphase transition by initiating events that lead to the separation of sister chromatids. We therefore examined *mks* cells to determine which, if any, of these events had taken place. Surprisingly, careful observation of orcein stained preparations of mitotic cells in *mks* larval brains revealed that the sister chromatids of at least one chromosome had separated in 20% of all mitotic figures. A clear example of this can be seen in Fig. 1C (arrows). However, in this particular image, it is difficult to know whether the thin threads of chromatin between the highly condensed chromosome arms represent different condensation or stretching of centromeric regions of the chromosomes. To help address this question, we carried out a further series of experiments. First, to confirm that sister chromatid separation could occur in the *mks* mutant, we chose to examine the spatial distribution of the dodecasatellite, a repetitive sequence element associated with the pericentromeric heterochromatin of chromosome 3 (Carmena et al., 1993). In situ hybridization of the dodecasatellite probe on wild-type larval brain cells reveals these sequences at two sites on 4*N* mitotic chromosomes corresponding to the connected sister centromeres of the homologous third

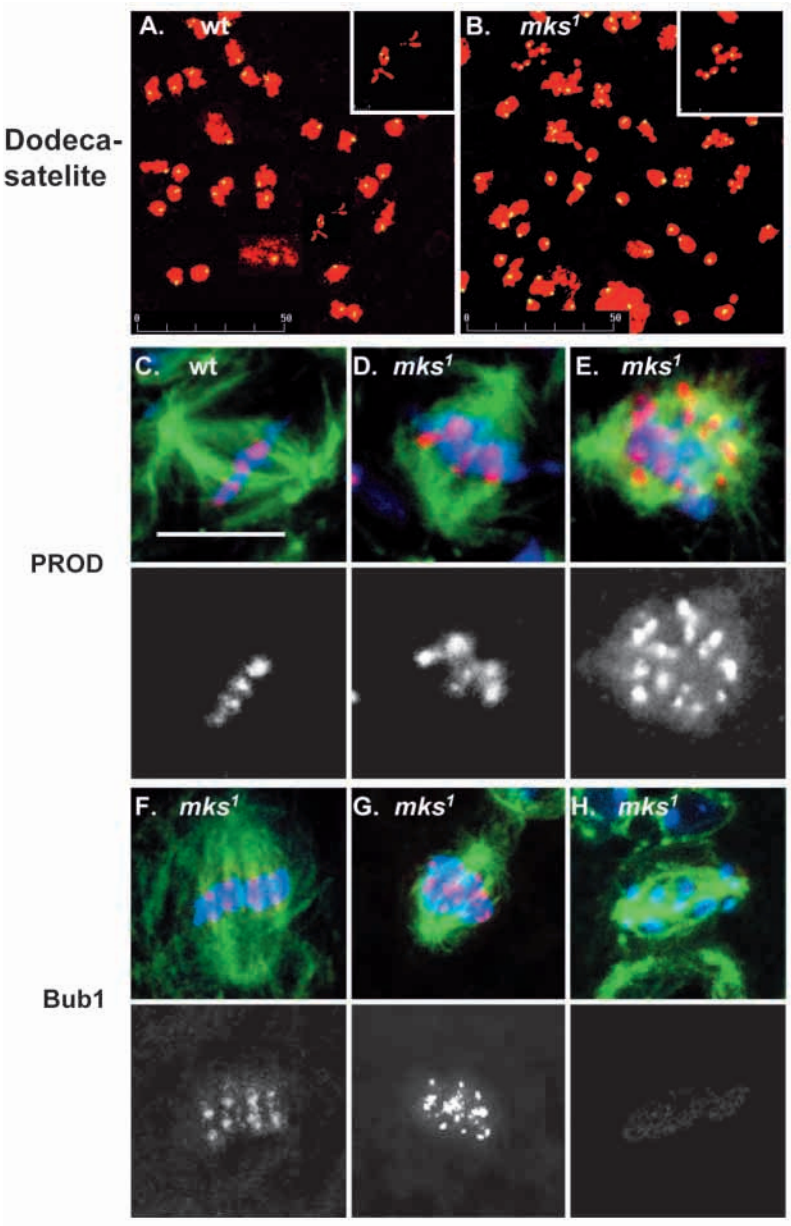

Fig. 2. Sister centromere separation occurs in *mks*^{1} neuroblasts. (A,B) Hybridization of a dodecasatellite probe to squashed preparations of wild-type and *mks*^{1} cells, respectively. Two dots of signal can be detected in wild-type cells (mitotic chromosomes in inset), corresponding to the centromeric regions of the maternal and paternal third chromosome. *mks*^{1} cells show four dots of signal, indicating that the sister centromeric regions have separated. DNA is shown in red, hybridization signal in yellow. Scale bar, 50 μm. (C-H) Immunolocalization of Prod (red, C-E) or Bub1 (red, F-H) on condensed chromosomes (blue) associated with mitotic spindles (green) in wild-type (C,F) and *mks*^{1} mutant cells (D,E,G,H). The paired monochromatic images show Prod (C-E) or Bub1 (F-H) staining alone. Prod is present at four punctate sites on chromosomes 2 and 3 in wild-type cells (C) and four pairs of sites, separated centromeres, in *mks*^{1} (D: one pair is out of the focal plane). (E) A polyploid cell. Bub1 is present on separated kinetochores in *mks*^{1} cells (F, 4*N*; G, 8*N*; not all kinetochores are in the focal plane). The *mks*^{1} cell in (H) has progressed into anaphase and the Bub1 signal can no longer be detected. Scale bars, 10 μm.

chromosome pair (Fig. 2A). In interphase cells, the signal was often seen as two closely paired dots. In *mks* cells, we found cells containing a 4*N* complement of chromosomes in which there were four sites of hybridization indicating that sister chromatids had separated in 19% of mitotic figures (Fig. 2B). This compared with a frequency of 0.4% metaphase figures showing clear separation of chromatids to give four sites of hybridization in wild-type cells. That the greater majority of cells that show four dots of hybridization are diploid cells with separated chromatids rather than tetraploid cells whose chromosomes have connected sister chromatids is confirmed from the frequency of their occurrence. We are able to estimate the frequency of tetraploid cells both by counting the numbers of condensed chromosomes in squashed preparations or by counting the number of centromeres (revealed by staining for the Prod antigen on chromosomes 2 and 3) or kinetochores [revealed by staining for the Bub1 antigen (see below)]. The proportion of polyploid cells (3-4%) was consistent using any of these criteria and was much lower than the proportion of cells showing four sites of dodecasatellite hybridization that we take as indicative of sister separation.

We then sought to examine whole-mount preparations of *mks* larval neuroblasts by immunofluorescence microscopy to determine whether we were able to observe the separation of sister chromatids on the mitotic spindle. To this end, we carried out immunostaining to reveal the distribution of the product of the gene *proliferation disrupter* (*prod*), a pericentromeric protein found on chromosomes 2 and 3 (Török et al., 1998). In wild-type neuroblasts at metaphase, the Prod protein can be seen to be present in four punctate regions of staining corresponding to the adjoined centromeric regions of these two pairs of chromosomes (Fig. 2C). We found that, in mitotic *mks* cells, the chromosomes gave a broad band of DNA staining on the metaphase plate. However, in all *mks* cells, the centromeric regions of the two pairs of major autosomes revealed by Prod staining were present as a series of four separate pairs of dots indicative of chromatid separation (Fig. 2D). A consistent small proportion of such *mks* cells contained a replicated tetraploid set of chromosomes as expected from observations of the stained squashed preparations (Table 1A). Such cells showed 16 dots of Prod staining, also indicating separation of the centromeric regions in these tetraploid cells (Fig. 2E). Thus, these observations extended the conclusion from examining the distribution of dodecasatellite that, although APC/C function is likely to be compromised in the *mks* mutant, the centromeric regions of sister chromatids appeared to have separated in all metaphase-like cells and there had been separation of chromatid arms in some 20% of cells.

Finally, we wished to see whether the separated centromeric regions were associated with spindle integrity checkpoint proteins. These are generally believed to associate with the

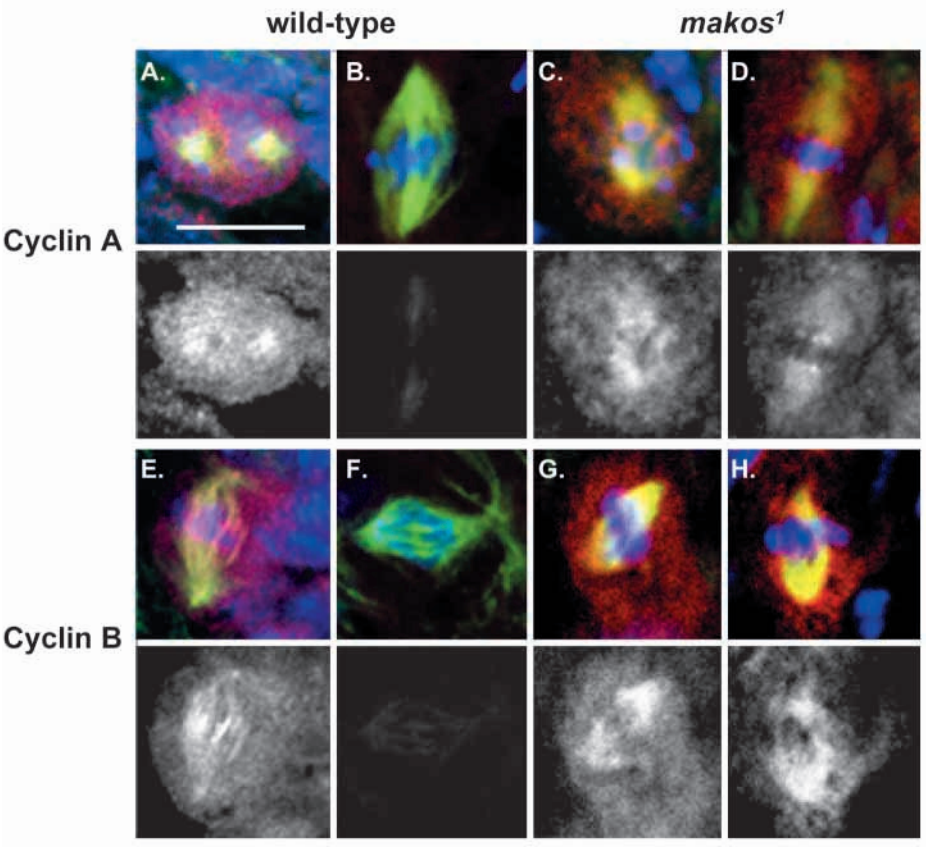

Fig. 3. Cyclins A and B are not degraded in *mks*^{1} cells. Paired images of mitotic cells showing tubulin (green), DNA (blue) and cyclin A (red and monochrome; A-D) or cyclin B (red and monochrome; E-H). (A) Wild-type cell in prophase showing cyclin A staining. (B) Wild-type cell at onset of metaphase in which cyclin A has been degraded. (C,D) Metaphase-like *mks*^{1} cells in which cyclin A is not degraded. (E) Wild-type cell at metaphase showing cyclin B staining. (F) Wild-type cell at anaphase in which cyclin B has been degraded. (G) Metaphase-like *mks*^{1} cells in which cyclin B is not degraded. Scale bar, 10 μm.

kinetochores of chromosomes when the sisters are under unequal tension. Once chromosomes are aligned at metaphase and the sisters are under equal tension from both poles, the proteins are released allowing activation of the APC/C functions that mediate sister separation. Thus, localization of Bub1, a component of the spindle integrity checkpoint complex (Basu et al., 1998), would serve the two purposes of permitting visualization of kinetochores of non-aligned chromosomes and determining the state of the mitotic checkpoint when *mks* (APC/C) function was compromised. We again found that chromatin in metaphase-like mutant *mks* cells was distributed in a broad band, and were able to recognize up to 16 dots of Bub1 staining, corresponding to each of the separated kinetochores of sister chromatids (Fig. 2F,G). This finding is similar to that reported for strong hypomorphic alleles of *polo*, which arrest in a metaphase-like state but with centromeric regions of the sisters separated and yet still with the Bub1 checkpoint protein associated with the kinetochore (Donaldson et al., 2001). This is unusual, in that all kinetochores might be expected to be under equal tension, and yet the checkpoint proteins would appear still to be present. Nevertheless, in those rare *mks* cells that undertake anaphase and show full separation of sister chromatids, Bub1 staining was not seen (Fig. 2H).

mákos mutants arrest with high levels of mitotic cyclins A and B

A second major function of the APC is to promote the degradation of mitotic cyclins, so we monitored the presence of A- and B-type cyclins in wild-type and *mks* neuroblasts by immunostaining. In wild-type cells, cyclin A was present at high levels at prometaphase (Fig. 3A). It was reduced at metaphase and was absent from cells in early anaphase (Fig. 3B). In *mks* neuroblasts, we found that cyclin A was maintained at high levels in all cells arrested in a metaphase-like state (Fig. 3C,D). Cyclin B was present in all wild-type metaphase cells and absent from anaphase cells (Fig. 3E,F). It accumulated to high levels in all metaphase arrested *mks* cells (Fig. 3G,H). Thus, the metaphase arrest seen in *mks* cells differs from the checkpoint arrest seen in mutants such as *dd4* (Barbosa et al., 2000) or following depolymerization of microtubules with colchicine (Whitfield et al., 1990). In these circumstances, cyclin A is degraded whereas cyclin B accumulates to high levels. The high levels of both A- and B-type cyclins in *mks* mutants indicates that the Cdc27 component of the APC/C is required for their degradation.

mákos enhances centrosomal defects in the *polo*^{1} hypomorph

Evidence from studies in several organisms indicates that the Polo-like kinases can regulate APC/C function. We therefore considered whether mutations in *polo* would show genetic interactions with the *mks*^{1} mutant allele. To this end, we constructed double mutants between *mks* and hypomorphic alleles of *polo*. A comparison of viability shown by flies with these genotypes indicates that the lethal phase of *mks*^{1} is advanced to an earlier developmental stage when in combination with either a weak or strong hypomorphic allele of *polo* (*polo*^{1} or *polo*^{9} respectively). The stage of death is also earlier than with either *polo* allele alone (Table 2).

The strongest enhancement of phenotype was seen in the *mks*^{1} *polo*^{1} combination, so we examined squashed preparations of nervous systems from larvae of this genotype (Fig. 4A-D). We found that such cells displayed highly condensed chromosomes and had a mitotic index comparable to those of *mks*^{1} larvae, although a proportion of the mitotic figures were circular (Fig. 4A,B), as is also seen in the *polo*^{1} single mutant. The ratio of metaphase:anaphase figures in the

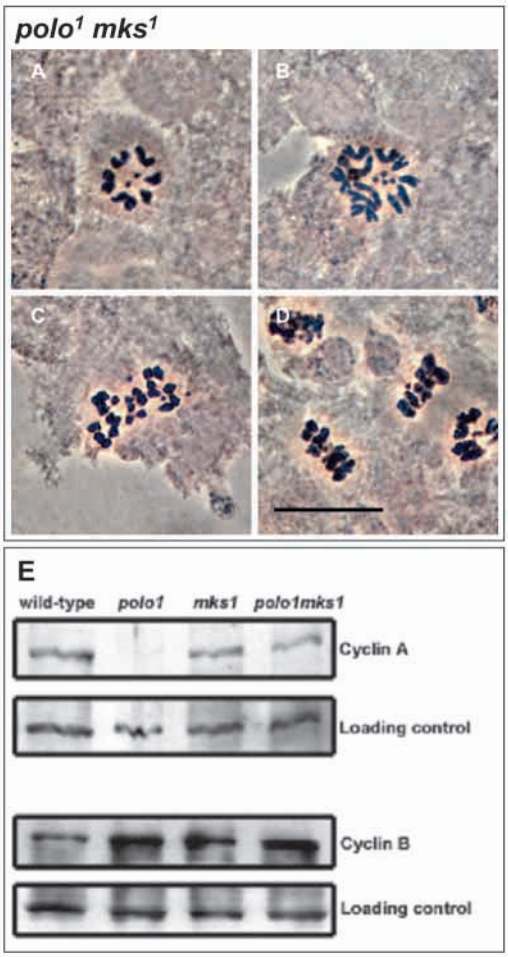

Fig. 4. *mks[1]* is epistatic to *polo[1]* in respect of mitotic progression and cyclin degradation. (A-D) Orcein-stained squashed preparations of *polo[1] mks[1]* cells that display overcondensed chromosomes (A,C,D), polyploidy (B,C) and circular (A,B) or bar-like figures (D). The mitotic index, proportions of metaphase:anaphase cells and proportion of polyploids are similar to that in the *mks[1]* mutant alone (Table 1B). (E) Western blots showing levels of cyclins A and B in wild-type, *polo[1]*, *mks[1]* and *polo[1] mks[1]* brains. This reflects cyclin levels in the cycling cells of this tissue. Cyclin A is substantially reduced in, but not absent from, *polo[1]* cells, suggesting that the APC/C is functional. Cyclin B is still present, indicative of checkpoint delay to mitosis. Both mitotic cyclins are present at equivalent levels in the double mutant combination, indicating loss of APC/C function. Actin was used as a protein-loading control.

double mutant was similar to that seen in *mks[1]* alone (Table 1B). When we examined extracts of the central nervous system from *polo[1]* larvae for the presence of A- and B-type mitotic cyclins, we found elevated levels of cyclin B and reduced levels of cyclin A, typical of some delay at the spindle integrity checkpoint. By contrast, both *mks[1]* and *polo[1] mks[1]* brains showed high levels of both A- and B-type cyclins, consistent with the block to APC/C function imposed by the *mks[1]* mutation (Fig. 4E). Thus, in terms of the mitotic index, the metaphase:anaphase ratio, the condensation state of the chromosomes and the levels of mitotic cyclins, the phenotype of *mks[1] polo[1]* cells did not differ significantly from that of the *mks[1]* single mutant.

Table 2. Lethal phases of *mks* in combination with *polo*, *bub1* and *twins/aar*

Gene	Lethal phase of single mutant	Lethal phase of double mutant with *mks**
mks[1]	Pharate adult	
mks[2]	Semilethal	
polo[1]	Semilethal	Pupal
polo[9]	Pupal	Larval-pupal
bub1	Pupal	Larval
twins[618/5]	Pharate adult	Pharate adult

*Double mutants were with *mks[1]* except for the case of *bub1*, where the double mutant examined was with *mks[2]*

When, however, we examined the appearance of the arrested mitotic spindles by immunostaining for features typical of each mutant, it was evident that centrosomal antigens showed differences in their patterns of distribution. The core centrosomal antigen Centrosomin (CNN) (Heuer et al., 1995) was present in distinct bodies at the spindle poles of the two single mutants and the double mutant combination. However, although both γ-tubulin and the centrosomal antigen CP190 were present at the spindle poles of the individual *mks[1]* or *polo[1]* mutants, these proteins were both dispersed throughout *mks[1] polo[1]* mitotically arrested cells (Fig. 5). This dispersal of γ-tubulin and CP190 is also seen in strong *polo* hypomorphs such as *polo[9]* (Donaldson et al., 2001). Thus, rather unexpectedly, we found that the *polo[1]* phenotype with respect to spindle pole organization is enhanced by the *mks[1]* mutation.

Phosphorylation of a substrate by Polo-like kinases has been reported to confer immunoreactivity to the monoclonal antibody against mitotic phosphoepitopes MPM2 (Davis et al., 1983; Logarinho and Sunkel, 1998). It is specifically known that Polo kinase can phosphorylate Asp, a *Drosophila* spindle pole component, to make it reactive to the MPM2 antibody and to activate its ability to nucleate asters of microtubules (do Carmo Avides et al., 2001). When we stained either *polo[1]* or *mks[1]* cells with MPM2, we were able to detect the phosphoepitope throughout the cell and also in distinct bodies corresponding to centrosomes and kinetochores (Fig. 5). By contrast, the mitotically arrested *mks[1] polo[1]* cells lacked the MPM2 epitope at their spindle poles. These observations are consistent with diminished levels of Polo kinase activity at the spindle poles in the double mutant. The continued presence of MPM2 epitopes elsewhere in the cell including the kinetochores of the double mutant suggests either that Polo kinase activity is not affected at these other sites or that they result from phosphorylation catalysed by one of the other mitotic kinases known to generate MPM2 epitopes.

Mitotic phenotype of mákos is suppressed by mutation in a regulatory subunit of PP2A

To confirm that the metaphase block in *mks* was a result of failure of APC/C function rather than a consequence of checkpoint activation, we constructed a double mutant between *mks[2]* and *bub1*. Examination of the larval central nervous system from such double mutants showed cells arrested at metaphase at a comparable frequency to the *mks2* mutant alone (Table 1C). Moreover, the high frequency of anaphases seen in

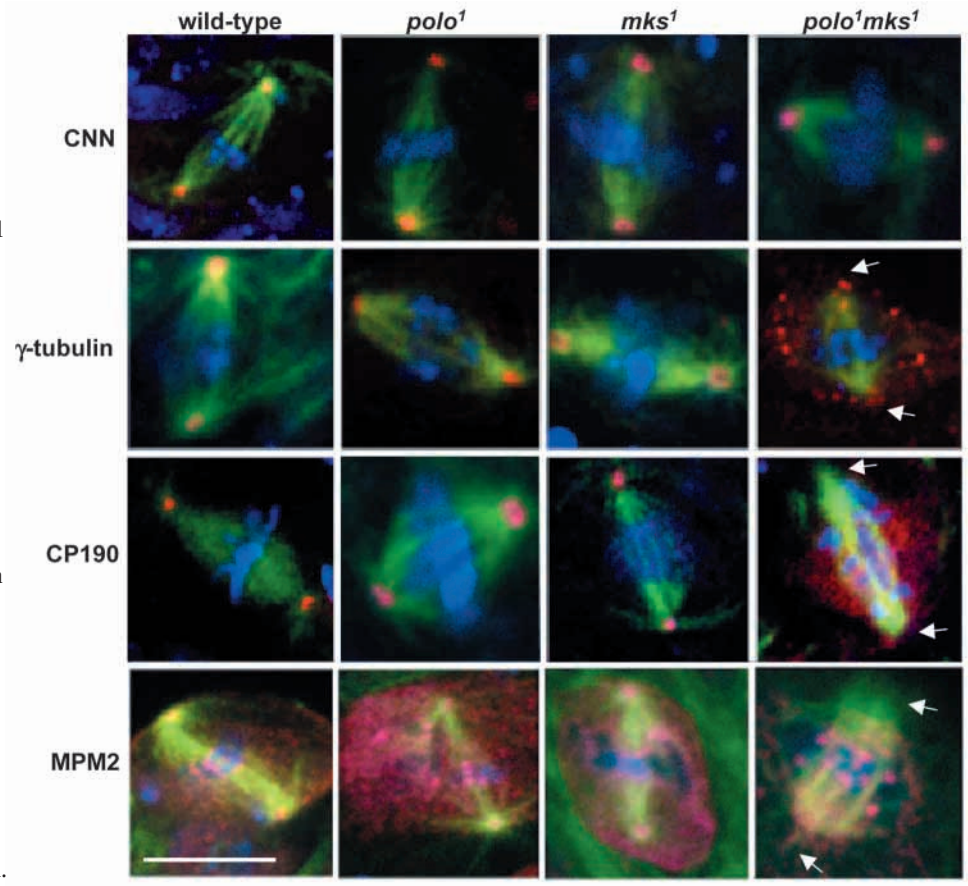

Fig. 5. *mks*^{1} enhances *polo*^{1} centrosomal defects. The columns display mitotic spindles from neuroblasts of the respective genotypes wild-type, *polo*^{1}, *mks*^{1} and *polo*^{1} *mks*^{1} stained to reveal DNA (blue) and microtubules (green). The first three rows show immunostaining to reveal the indicated centrosomal antigens CNN, γ-tubulin and CP190 (all in red). The final row displays immunostaining of the mitotic phosphoepitope MPM2 (red). In all the indicated genotypes, the core centrosomal antigen CNN is localized in a wild-type manner to the centrosomes. γ-Tubulin and CP190 are localized to centrosomes and have a wild-type pattern of localization in both *polo*^{1} and *mks*^{1}. However, in the double mutant, they are dispersed throughout the cell. The MPM2 epitope is present throughout the cell, at kinetochores and spindle poles in all indicated genotypes except *polo*^{1} *mks*^{1}, in which it is absent from the spindle poles. Scale bar, 10 μm.

the *bub1* mutant as a result of the malfunctioning checkpoint is replaced in the double mutant by an elevated frequency of metaphase-like figures typical of *mks*. The appearance of mitotic figures in squashed preparations of *mks*^{2} *bub1* brains was indistinguishable from that seen in the *mks*^{2} mutant alone (data not shown). Thus, the *mks*^{1} arrest cannot be overcome by removing this checkpoint function.

An alternative checkpoint like function has been ascribed to *CDC55* in budding yeast (Minshull et al., 1996; Wang and Burke, 1997; Yang et al., 2000). *CDC55* encodes the 55 kDa regulatory subunit of PP2A and has its counterpart in *Drosophila* in the gene *twins/aar* (Mayer-Jaekel et al., 1993; Uemura et al., 1993). Mutations in *twins/aar* result in an elevated proportion of anaphase figures that are highly disorganized and show many lagging and bridging chromatids (Meyer-Jaekel et al., 1993). The protein phosphatase is likely to have pleiotropic function. The disorganised nature of these mutant anaphases suggests a role for this PP2A subunit in regulation of the metaphase-anaphase transition. However, the accumulation of anaphase figures might be due to a failure to exit mitosis because *aar* mutants have been previously shown to have reduced ability to dephosphorylate substrates of Cdk1 (Mayer-Jaekel et al., 1994). To determine whether mutations in *tws/aar* could overcome the metaphase arrest of *mks*^{1}, we constructed the double mutant strain. Although there was no apparent shift in lethal phase in the *mks tws*$^{618/5}$ double mutant (Table 1D), cytological analysis of neuroblasts showed an elevation of mitotic index and a dramatic reduction of metaphase figures replaced by an elevated frequency of anaphase-telophase figures equivalent to the *tws*$^{618/5}$ mutant alone

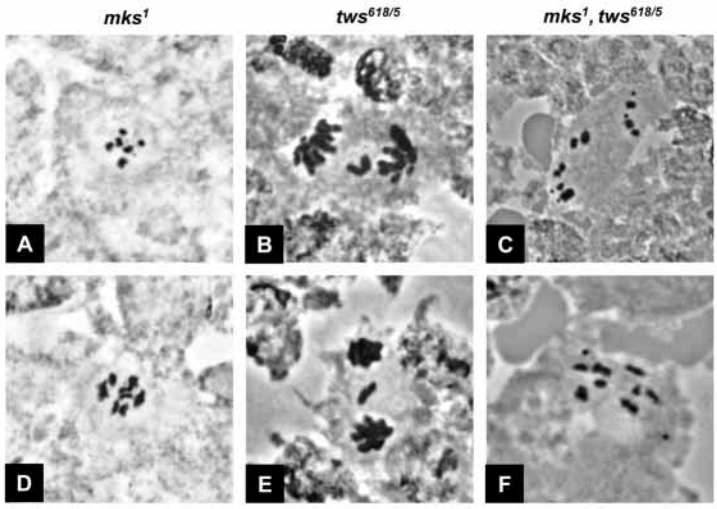

Fig. 6. *tws*$^{618/5}$ *mks*^{1} cells show abnormal anaphases. Orcein stained squashed preparations of *mks*^{1}(A,D), *tws*$^{618/5}$ (B,E) and *tws*$^{618/5}$ *mks*^{1} (C,F) cells show that *tws*$^{618/5}$ is epistatic to *mks*^{1} with respect to abnormal anaphase. The proportion of anaphase figures is similar in *tws*$^{618/5}$ *mks*^{1} and *tws*$^{618/5}$ cells (Table 1C). However, *mks*^{1} is epistatic to *tws*$^{618/5}$ with respect to chromosome condensation.

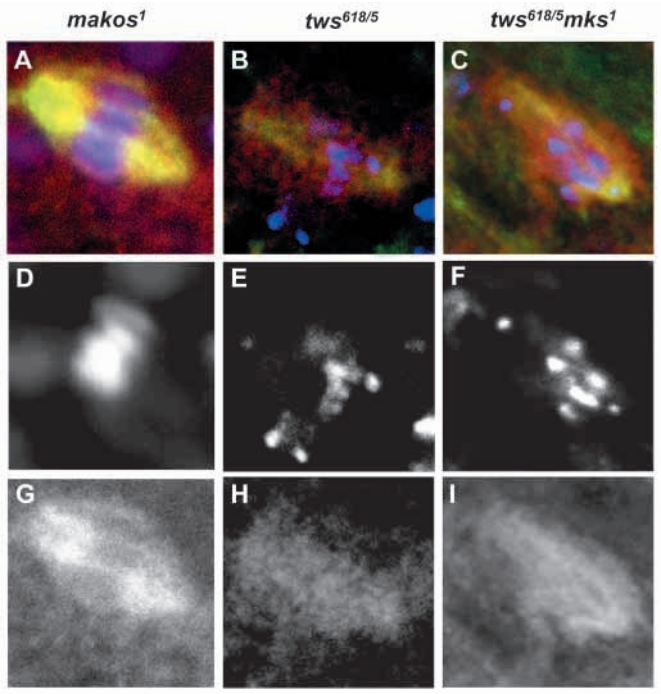

Fig. 7. Cyclin B is not degraded in the abnormal anaphase *tws*$^{618/5}$ *mks*^{1} cells. Typical mitotic figures from *mks*^{1} (A,D,G), *tws*$^{618/5}$ (B,E,H) and *tws*$^{618/5}$*mks*^{1} (C,F,I) cells stained to reveal microtubules (green), DNA (blue) and cyclin B (red). The panels of monochrome staining show chromosomes (D-F) and cyclin B (G-I) alone. Cyclin B is not degraded in *mks*^{1}, *tws*$^{618/5}$ or *tws*$^{618/5}$ *mks*^{1} double mutant cells. We use the same criteria to define the abnormally resolved anaphases of the *twins/aar* mutants as Gomes et al. (Gomes et al., 1993) and Mayer-Jaekel et al. (Mayer-Jaekel et al., 1993).

(Table 1D; Fig. 6). Thus, the most striking feature of the double mutant is the ability to overcome the metaphase arrest caused by the *mks* mutation. Nevertheless, the degree of chromosome condensation in the double mutant was more comparable to *mks*, suggesting that the cells were delayed in a mitotic state, possibly through the prevention of cyclin B degradation. To confirm that cyclin proteolysis was prevented by the *mks*^{1} mutation in the double mutant, we stained mutant neuroblasts to reveal cyclin B and compared such cells with each single mutant. Residual cyclin B can be seen associated with the abnormal anaphase figures in the *tws*$^{618/5}$ mutant and in *mks tws*$^{618/5}$ at a level not seen in wild-type anaphases (Fig. 7). It would thus appear that *mks tws*$^{618/5}$ cells are capable of entering anaphase without activation of the APC/C. However, under these conditions, anaphase is highly abnormal.

Discussion

Sister chromatid separation in *mks* mutants

We describe phenotypes resulting from mutations in *mákos*, a gene encoding a subunit of the APC/C homologous to CDC27 of budding yeast. The mitotic arrest in the mutant neuroblasts appears to be more robust than the effects seen by Huang and Raff (Huang and Raff, 2002), who interfered with *Drosophila cdc27* function in cultured cells by RNAi. Thus, our observations strengthen and extend several findings from this previous study. Although the *mks* mutant cells accumulate both A- and B-type mitotic cyclins, several lines of evidence indicate that their sister chromatids have undergone separation. These include in situ hybridization studies with a centromeric satellite sequence, and immunostaining experiments to reveal a centromeric antigen and the checkpoint protein Bub1 curiously at separated kinetochores. Several aspects of the *mks* phenotype thus resemble those seen in mutations in *ida*, the *Drosophila* gene encoding the APC5 homologue (Bentley et al., 2002). Both mutants show an elevated mitotic index with cells arrested in a metaphase-like state yet with separated sister chromatids in the continued presence of Bub1 at kinetochores and elevated levels of B-type cyclin. The observation of sister chromatid separation in *ida* mutants led to the suggestion that removal of the single APC5 subunit might only affect degradation of a subset of APC/C substrates. In other words, APC5 would not be necessary for the degradation of securin, the release of separase and the cleavage of cohesins, and so chromatid separation would be unaffected. If, as has been suggested by King et al. (King et al., 1995), Cdc27 is a core component of the APC/C, our finding of sister separation in *mks* would suggest lack of a widespread requirement for the APC/C for sister chromatid separation in *Drosophila*, at least to the extent that it is observed in *mks* and *ida* mutants. We cannot exclude the possibility that it occurs as a result of residual function of either of these gene products because no null alleles of *ida* or *mks* have been studied. A similar argument can be applied to the RNAi experiments, in which, although the knock-down is impressive, there is some residual protein following the treatment. Alternatively, the minimal catalytic activity of the APC/C, thought to be mediated by the direct ubiquitination of substrates by Apc11 acting alone or together with Apc2 depending upon the E2 enzyme (Gmachl et al., 2000), could be sufficient for securin degradation. We note, however, that there are subtle differences between the phenotypes of *mks* and *ida*. The proportion of anaphase figures is higher in *ida* cells and these also exhibit a level of aneuploidy that is not detected in *mks*. At present, it remains unclear whether this reflects specific characteristics of the mutant alleles studied or whether the block to chromosome movement at anaphase is stronger following perturbation of Cdc27 function.

APC/C and the activation of Polo kinase

The motivation for studying genetic interactions between *mks* and *polo* arose from evidence pointing to a role for Polo kinase in APC/C activation. Depleting Plx1 in *Xenopus* extracts blocks degradation of cyclin B (Descombes and Nigg, 1998). In budding yeast, the Polo-like kinase encoded by *CDC5* has also been implicated in activation of the APC/C (Charles et al., 1998; Shirayama et al., 1998). Moreover, Kotani and colleagues (Kotani et al., 1998; Kotani et al., 1999) have shown that mammalian APC/C can be activated by Plk1 phosphorylation in vitro. The phosphorylation patterns of APC/C components have been carefully studied by Golan and colleagues (Golan et al., 2002), who have showed that Plk1 and Cdk1/cyclin-B have additive effects in phosphorylating and activating the APC/C; the former preferentially phosphorylates Cdc16 and Cdc23, and the latter preferentially phosphorylates Cdc27. It was also recently shown that the fission yeast Polo-

like kinase Plo1 interacts physically with the Cut23 component of the APC/C (May et al., 2002). Nevertheless, it appears that the APC/C is active in the *polo*^{1} because cyclin A levels are reduced and cyclin B remains, as would be seen following checkpoint delay at metaphase. We were able to see only a slightly earlier lethal phase of *mks* when in combination with different *polo* alleles. By contrast, we made the unexpected observation that the weakly hypomorphic phenotype of *polo*^{1} in larval neuroblasts was enhanced by *mks* in respect to its effects upon centrosome structure and function.

The original *polo*^{1} allele encodes an apparently full-length protein that is incompletely phosphorylated and has low catalytic activity (Tavares et al., 1996). Thus, *polo*^{1} is a weak hypomorph and, although embryos derived from homozygous *polo*^{1} mothers show defects in the recruitment of the centrosomal antigen CP190 (Sunkel and Glover, 1988), those mothers would appear to have been able to develop to adulthood in part because of the lack of any obvious centrosomal defect in mitotically dividing cells of their larval central nervous systems (this paper). This is in contrast to brains of the strong hypomorphic alleles *polo*^{9} or *polo*^{10}, in which neuroblasts arrest in a metaphase-like state, with spindles that have core centrosomal components such as CNN at their spindle poles but lack γ-tubulin and CP190 (Donaldson et al., 2001). This phenotype echoes the requirement for Polo kinase in recruiting γ-tubulin to the centrosome upon mitotic entry (Lane and Nigg, 1996) following experiments in which antibodies against Plk1 were microinjected into HeLa cells.

Our finding in this paper that *mks* enhances the centrosomal phenotype of *polo*^{1} such that it resembles the stronger *polo* hypomorphs suggests that the APC/C is required either directly or indirectly to fully activate Polo kinase function. It is possible to gain a measure of the activity of Polo kinase by assessing the presence of the MPM2 epitope (Logarinho and Sunkel, 1998). Indeed, at least one *Drosophila* centrosomal protein (Asp) has been shown to acquire an MPM2 epitope following phosphorylation by Polo (do Carmo Avides et al., 2001). Our finding of the absence of MPM2 reactivity at the centrosome in *mks*^{1} *polo*^{1} mutants is therefore consistent with reduced Polo kinase activity. A direct functional interaction between the APC/C and Polo cannot be excluded because Polo kinase has been shown to bind the APC/C, at least in fission yeast, and both the APC/C and Polo kinase are present on the centrosome. However, we favour the idea that an early mitotic function of the APC/C might be required for the full activation of Polo kinase and the recruitment of the γ tubulin ring complex (γ-TuRC) to the centrosome. Indeed, cyclin A is normally degraded ahead of cyclin B and continues to be degraded when cells are arrested at the spindle checkpoint by microtubule depolymerising drugs (Whitfield et al., 1990), but remains present in both *mks*^{1} and *mks*^{1} *polo*^{1} cells. Normally, the degradation of cyclin A takes place from the beginning of mitosis in an APC/C-dependent manner (Geley et al., 2001). Thus, we postulate that, as APC/C is normally active from the very beginning of mitosis, one of its functions might be to facilitate the full activation of Polo kinase at the centrosome by mediating the degradation either of cyclin A or some other crucial inhibitory molecule. This would constitute a new cell cycle autoregulatory loop whereby the early mitotic functions of the APC/C are required for the activation of an enzyme itself implicated in activating later functions of the APC/C.

APC/C requirement for the metaphase-anaphase transition suppressed by mutants in the regulatory subunit of PP2A

The metaphase-like arrest of *mks* cells cannot be overcome by the *bub1* mutation. This is consistent with known functions of the APC/C downstream of the spindle integrity checkpoint. However, the ability of mutants in the *aar/twins* gene to overcome the metaphase arrest of *mks* is suggestive of an alternate mechanism for regulating the transition. In fact, the *aar/twins* mutant appears to be totally epistatic to *mks*. Thus, the *mks aar/twins* double mutant shows a similar proportion of anaphase figures to the *aar/twins* mutant alone, and this is higher than the frequency of anaphases seen in wild-type cells. These observations cast some light on the possible multiple functions of the regulatory subunit of PP2A encoded by *aar/twins* in regulating anaphase and mitotic exit. It suggests that APC/C function might normally be required to inactivate the wild-type 55 kDa PP2A subunit that, in turn, negatively regulates sister chromatid separation. Thus, in the absence of *aar/twins* function, this aspect of APC/C involvement would not be required for anaphase, thus accounting for the epistasis of *aar/twins* to *mks*. The mutant *aar/twins* phenotype that then develops is akin to that observed following the expression of non-degradable forms of cyclin B, in which mitosis proceeds into anaphase (Rimmington et al., 1994; Sigrist et al., 1997; Parry and O'Farrell, 2001). This outcome would be reinforced by a failure to exit mitosis as a result of the reduced ability of *aar/twins* mutants to dephosphorylate substrates of Cdk1 (Mayer-Jaekel et al., 1994).

The anaphases in *aar/twins* and in the double mutant are highly abnormal, as previously observed (Meyer-Jaeckel et al., 1993), indicating that the checkpoint pathway that monitors chromosome alignment at metaphase and works through regulation of the APC/C is being circumvented. Consequently, there are many bridging and lagging chromatids in both *aar/twins* and *mks aar/twins* anaphase figures. This phenotype bears a striking resemblance to that seen in mutants of the *CDC55* gene of budding yeast that encodes the orthologous regulatory subunit of PP2A. Cells with a *cdc55* mutation have also been shown to leave mitosis without B-type cyclin destruction, in this case apparently owing to inhibitory tyrosine phosphorylation (Minshull et al., 1996). However, it is also postulated in budding yeast that Cdc55p function is required for the kinetochore/spindle checkpoint. Such *cdc55* mutants are sensitive to nocodazole and, in contrast to the situation we here report for *Drosophila* cells, *cdc55* mutations do not overcome the arrest imposed by mutation in an APC/C protein, in this case Cdc23p (Wang and Burke, 1997). Nevertheless, the abnormal morphology of *cdc55* mutants and their conditional lethality is suppressed by a *cdc28F19* mutation that encodes a variant kinase not susceptible to inhibitory phosphorylation. By contrast, nocodazole sensitivity cannot be suppressed by *cdc28F19*. This suggests that, in yeast, Cdc55p might have a checkpoint role that is independent of Cdc28/Cdk1 and a second role in regulating Cdc28 phosphorylation. The cause of the elevated phosphorylation levels of Cdc28 in *cdc55* mutants has been addressed by Yang et al. (Yang et al., 2000), who found elevated levels of the Cdc28 inhibitory kinase Swe1 in the mutant cells. Unfortunately, we have not been able to monitor the levels of Dwee1, the *Drosophila* homologue of Swe1, in *mks aar/twins* mutants with the antibodies that are

currently available against this protein. We have examined extracts of larval brains for histone H1 kinase activity, most of which is attributable to Cdk1 (data not shown). The histone H1 kinase activity was high in the metaphase arrested *mks*^{1} brains and reduced by approximately three times in *mks tws*$^{618/5}$ mutant brains, but was still significantly higher that in the *twins/aar* mutant alone. This suggests that Cdk1 might be partially inactivated in the presence of persisting cyclin B as a result of the *aar/twins* mutation.

At the present time, we cannot account for how the APC/C might regulate the function of the 55kDa PP2A subunit although one possibility is through its direct proteolysis. It appears that this regulatory subunit of PP2A must participate in regulating the metaphase-anaphase transition, in controlling the activity of Cdk1 and in dephosphorylating Cdk1 substrates. It therefore remains a question of considerable future interest to determine exactly how these activities are co-ordinated.

We are grateful to Cancer Research UK for supporting this work. We thank R. Giet for carrying out H1 kinase assays and for valuable discussions.

References

Amon, A. (1999). The spindle checkpoint. *Curr. Opin. Genes Dev.* **9**, 69-75.

Avides, M. and Glover, D. M. (1999). Abnormal spindle protein Asp, and the integrity of mitotic centrosomal microtubule organizing centres. *Science* **283**, 1733-1735.

Barbosa, V., Yamamoto, R. R., Henderson, D. S. and Glover, D. M. (2000). Mutation of a *Drosophila* γ tubulin ring complex subunit encoded by *discs degenerate-4* differentially disrupts centrosomal protein localization. *Genes Dev.* **14**, 3126-3139.

Basu, J., Logarinho, E., Herrmann, S., Bousbaa, H., Li, Z., Chan, G. K., Yen, T. J., Sunkel, C. E. and Goldberg, M. L. (1998). Localization of the *Drosophila* checkpoint control protein Bub3 to the kinetochore requires Bub1 but not Zw10 or Rod. *Chromosoma* **107**, 376-385.

Basu, J., Bousbaa, H., Logarinho, E., Li, Z., Williams, B. C., Lopes, C., Sunkel, C. E. and Goldberg, M. L. (1999). Mutations in the essential spindle checkpoint gene *bub1* cause chromosome missegregation and fail to block apoptosis in *Drosophila*. *J. Cell Biol.* **146**, 13-28.

Bentley, A. M., Williams, B. C., Goldberg, M. L. and Andres, A. J. (2002). Phenotypic characterization of *Drosophila ida* mutants: defining the role of APC5 in cell cycle progression *J. Cell Sci.* **115**, 949-961.

Brown, N. H. and Kafatos, F. (1988). Functional cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.* **203**, 425-437.

Carmena, M., Abad, J. P., Villasante, A. and Gonzalez, C. (1993). The *Drosophila melanogaster* dodecasatellite sequence is closely linked to the centromere and can form connections between sister chromatids during mitosis. *J. Cell Sci*. **105**, 41-50.

Charles, J. F., Jaspersen, S. L., Tinker-Kulberg, R. L., Hwang, L., Szidon, A. and Morgan, D. O. (1998). The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in *S. cerevisiae*. *Curr. Biol.* **8**, 497-507.

Cohen-Fix, O., Peters, J. M., Kirshner, M. W. and Koshland, D. (1996). Anaphase initiation in *Saccharomyces cerevisiae* is controlled be the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev.* **10**, 3081-3093.

Davis, F. M., Tsao, T. Y., Fowler, S. K. and Rao, P. (1983). Monoclonal antibodies to mitotic cells *Proc. Natl. Acad. Sci. USA* **80**, 2926-2930.

Dawson, I. A., Roth, S., Akam, M. and Artavanis-Tsakonas, S. (1993). Mutations of the *fizzy* locus cause metaphase arrest in *Drosophila melanogaster* embryos. *Development* **117**, 359-376.

Deak, P., Omar, M. M., Saunders, R. D., Pal, M., Komonyi, O., Szidonya, J., Maroy, P., Zhang, Y., Ashburner, M., Benos, P. et al. (1997). P-element insertion alleles of essential genes on the third chromosome of *Drosophila melanogaster*: correlation of physical and cytogenic maps in chromosomal region 86E to 87F. *Genes Dev.* **147**, 1697-1722.

Descombes, P. and Nigg, E. A. (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.

do Carmo Avides, M., Tavares, A. and Glover, D. M. (2001). Polo kinase and Asp are needed to promote the mitotic organizing activity of centrosomes. *Nat. Cell Biol.* **3**, 421-424.

Donaldson, M. M., Tavares, A. A., Ohkura, H., Deak, P. and Glover, D. M. (2001). Metaphase arrest with centromere separation in polo mutants of *Drosophila*. *J. Cell Biol.* **153**, 663-676.

Fang, G., Yu, H. and Kirschner, M. W. (1998). The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev.* **12**, 1871-1883.

Funabiki, H., Kumada, K. and Yanagida, M. (1996). Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. *EMBO J.* **15**, 6617-6628.

Geley, S., Kramer, E., Gieffers, C., Gannon, J., Peters, J. M. and Hunt, T. (2001). Anaphase-promoting complex/cyclosome-dependent proteolysis of human cyclin a starts at the beginning of mitosis and is not subject to the spindle assembly checkpoint *J. Cell Biol.* **153**, 137-147.

Glover, D. M., Hagan, I. M. and Tavares, A. A. M. (1998). Polo-like kinases: a team that plays throughout mitosis. *Genes Dev.* **12**, 3777-3787.

Gmachl, M., Gieffers, C., Podtelejnikov, A. V., Mann, M. and Peters, J. M. (2000). The RING-H2 finger protein APC11 and the E2 enzyme UBC4 are sufficient to ubiquitinate substrates of the anaphase-promoting complex. *Proc. Natl. Acad. Sci. USA* **97**, 8973-8978.

Golan, A., Yudkovsky, Y. and Hershko, A. (2002). The cyclin-ubiquitin ligase activity of cyclosome/APC is jointly activated by protein kinases Cdk1-cyclin B and Plk. *J. Biol. Chem.* **277**, 15552-15557.

Gomes, R., Karess, R. E., Ohkura, H., Glover, D. M. and Sunkel, C. E. (1993). *abnormal anaphase resolution* (*aar*): a locus required for progression through mitosis. *J. Cell Sci.* **104**, 583-593.

Heuer, J. G., Li, K. and Kaufman, T. C. (1995). The *Drosophila* homeotic target gene *centrosomin* (*cnn*) encodes a novel centrosomal protein with leucine zippers and maps to a genomic region required for midgut morphogenesis. *Development* **121**, 3861-3876.

Jowett, T. (1986). Preparation of nucleic acids. In *Drosophila*, A Practical Approach (ed. D. B. Roberts), pp. 275-286. Oxford: IRL Press.

Huang, J. and Raff, J. W. (2002). The dynamic localisation of the *Drosophila* APC/C: evidence for the existence of multiple complexes that perform distinct functions and are differentially localised *J. Cell Sci*. **115**, 2847-2856.

Kashevsky, H., Wallace, J. A., Reed, B. H., Lai, C., Hayashi-Hagihara, A. and Orr-Weaver, T. L. (2002). The anaphase promoting complex/cyclosome is required during development for modified cell cycles. *Proc. Natl. Acad. Sci. USA* **99**, 11217-11222.

Kilmartin, J. V., Wright, B. and Milstein, C. (1982). Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* **93**, 576-582.

King, R. W., Peters, J. M., Tugendreich, S., Rolfe, M., Hieter, P. and Kirschner, M. W. (1995). A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* **81**, 279-288.

Kotani, S., Tanaka, H., Yasuda, H. and Todokoro, K. (1999). Regulation of APC activity by phosphorylation and regulatory factors. *J. Cell Biol.* **146**, 791-800.

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.

Kramer, E. R., Gieffers, C., Holzl, G., Hengstschlager, M. and Peters, J. M. (1998). Activation of the human anaphase-promoting complex by proteins of the CDC20/Fizzy family. *Curr. Biol.* **8**, 1207-1210.

Kramer, E. R., Scheuringer, N., Podtelejnikov, A. V., Mann, M. and Peters, J. M. (2000). Mitotic regulation of the APC activator proteins CDC20 and CDH1. *Mol. Biol. Cell* **11**, 1555-1569.

Lane, H. A. and Nigg, E. A. (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.

Leismann, O., Herzig, A., Heidmann, S. and Lehner, C. F. (2000). Degradation of *Drosophila* PIM regulates sister chromatid separation during mitosis. *Genes Dev.* **14**, 2192-2205.

Leverson, J. D., Joazeiro, C. A., Page, A. M., Huang, H., Hieter, P. and Hunter, T. (2000). The APC11 RING-H2 finger mediates E2-dependent ubiquitination. *Mol. Biol. Cell* **11**, 2315-2325.

Li, Y., Gorbea, C., Mahaffey, D., Rechsteiner, M. and Benezra, R. (1997). MAD2 associates with the cyclosome/anaphase-promoting complex and inhibits its activity. *Proc. Natl. Acad. Sci. USA* **23**, 12431-12436.

Lindsley, D. L. and Zimm, G. G. (1992). *The Genome of* Drosophila melanogaster. San Diego: Academic Press.

Logarinho, E. and Sunkel, C. E. (1998). The *Drosophila* POLO kinase localises to multiple compartments of the mitotic apparatus and is required for the phosphorylation of MPM2 reactive epitopes. *J. Cell Sci.* **111**, 2897-2909.

May, K. M., Reynolds, N., Cullen, C. F. and Ohkura, H. (2002). Polo boxes and Cut23 (Apc8) mediate an interaction between polo kinase and the anaphase-promoting complex for fission yeast mitosis. *J. Cell Biol.* **156**, 23-28.

Mayer-Jaekel, R. E., Ohkura, H., Gomes, R., Sunkel, C. E., Baumgartner, S., Hemmings, B. A. and Glover, D. M. (1993). The 55 kD regulatory subunit of *Drosophila* protein phosphatase 2A is required for anaphase. *Cell* **72**, 621-633.

Mayer-Jaekel, R. E., Ohkura, H., Ferrigno, P., Andjelkovic, N., Shiomi, K., Uemura, T., Glover, D. M. and Hemmings, B. A. (1994). *Drosophila* mutants in the 55 kDa regulatory subunit of protein phosphatase 2A show strongly reduced ability to dephosphorylate substrates of $p34^{cdc2}$. *J. Cell Sci.* **107**, 2609-2616.

Minshull, J., Straight, A., Rudner, A. D., Dernburg, A. F., Belmont, A. and Murray, A. W. (1996). Protein phosphatase 2A regulates MPF activity and sister chromatid cohesion in budding yeast. *Curr. Biol.* **6**, 1609-1620

Morgan, D. O. (1999). Regulation of the APC and the exit from mitosis. *Nat. Cell Biol.* **1**, E47-E53.

Parry, D. H. and O'Farrell, P. H. (2001). The schedule of destruction of three mitotic cyclins can dictate the timing of events during exit from mitosis. *Curr. Biol.* **11**, 671-683.

Peters, J. M. (1999). Subunits and substrates of the anaphase-promoting complex. *Exp. Cell Res.* **248**, 339–349.

Pirrotta, V. (1986). Cloning *Drosophila* genes. In Drosophila, *A Practical Approach* (ed. D. B. Roberts), pp. 83-110. Oxford: IRL Press.

Rimmington, G., Dalby, B. and Glover, D. M. (1994). Expression of N-terminally truncated cyclin B in the *Drosophila* larval brain leads to mitotic delay at late anaphase. *J. Cell Sci.* **107**, 2729-2738.

Rubin, G. M., Hong, L., Brokstein, P., Evans-Holm, M., Frise, E., Stapleton, M. and Harvey, D. A. (2000). A *Drosophila* complementary DNA resource. *Science* **287**, 2222-2224.

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

Scaerou, F., Aguilera, I., Saunders, R., Kane, N., Blottiere, L. and Karess, R. (1999). The *rough deal* protein is a new kinetochore component required for accurate chromosome segregation in *Drosophila*. *J. Cell Sci.* **12**, 3757-3768.

Schwab, M., Lutum, A. S. and Seufert, W. (1997). Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. *Cell* **90**, 683-693.

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 cerevisisae*. *EMBO J.* **17**, 1336-1349.

Sigrist, S. J. and Lehner, C. F. (1997). *Drosophila fizzy-related* down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. *Cell* **90**, 671-681.

Sigrist, S. J., Jacobs, H., Stratmann, R. and Lehner, C. F. (1995). Exit from mitosis is regulated by *Drosophila fizzy* and the sequential destruction of cyclins A, B and B3. *EMBO J.* **14**, 4827-4838.

Spradling, A. C., Stern, D., Beaton, A., Rhem, E. J., Laverty, T., Mozden, N., Misra, S. and Rubin, G. M. (1999). The Berkeley *Drosophila* genome project gene disruption project. Single P-element insertions mutating 25% of vital *Drosophila* genes. *Genetics* **153**, 135-177.

Sunkel, C. E. and Glover, D. M. (1988). *polo*, a mitotic mutant of *Drosophila* displaying abnormal spindle poles. *J. Cell Sci.* **89**, 25-38.

Tang, Z., Li, B., Bharadwaj, R., Zhu, H., Ozkan, E., Hakala, K., Deisenhofer, J. and Yu, H. (2001). APC2 Cullin protein and APC11 RING protein comprise the minimal ubiquitin ligase module of the anaphase-promoting complex. *Mol. Biol. Cell* **12**, 3839-3851.

Tavares, A., Glover, D. M. and Sunkel, C. E. (1996). The conserved mitotic kinase polo is regulated by phosphorylation and has preferred microtubule associated substrates in *Drosophila* embryo extracts. *EMBO J.* **15**, 4873-4883.

Thummel, C. S. and Pirrotta, V. (1992). Technical notes: new pCasper P-element vectors. *Drosoph. Inf. Serv.* **71**, 150.

Török, T., Harvie, P. D., Buratovich, M. and Bryant, P. J. (1997). The product of *proliferation disrupter* is concentrated at centromeres and required for mitotic chromosome condensation and cell proliferation in *Drosophila*. *Genes Dev.* **11**, 213-225.

Tugendreich, S., Tomkiel, J., Earnshaw, W. and Hieter, P. (1995). CDC27Hs colocalizes with CDC16Hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. *Cell* **81**, 261-268.

Uemura, T., Shiomi, K., Togashi, S. and Takeichi, M. (1993). Mutation of twins encoding a regulator of protein phosphatase 2A leads to pattern duplication in *Drosophila* imaginal discs *Genes Dev.* **7**, 429-440.

Visintin, R., Prinz, S. and Amon, A. (1997). CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. *Science* **278**, 460-463.

Wang, Y. and Burke, D. J. (1997) Cdc55p, the B-type regulatory subunit of protein phosphatase 2A, has multiple functions in mitosis and is required for the kinetochore/spindle checkpoint in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **17**, 620-626.

Whitfield, W. G. F., Millar, S. E., Saumweber, H., Frasch, M. and Glover, D. M. (1988). Cloning of a gene encoding an antigen associated with the centrosome in *Drosophila*. *J. Cell Sci.* **89**, 467-480.

Whitfield, W. G. F., Gonzalez, C., Maldanado-Codina, G. and Glover, D. M. (1990). The A- and B-type cyclins of *Drosophila* are accumulated and destroyed in temporally distinct events that define separable phases of the G2-M transition. *EMBO J.* **9**, 2563-2572.

Yang, H., Jiang, W., Gentry, M. and Hallberg, R. L. (2000). Loss of a protein phosphatase 2A regulatory subunit (Cdc55p) elicits improper regulation of Swe1p degradation. *Mol. Cell. Biol.* **20,** 8143-8156.

Zachariae, W. and Nasmyth, K. (1999). Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev.* **13**, 2039-2058.