Aberrantly Segregating Centromeres Activate the Spindle Assembly Checkpoint in Budding Yeast

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Abstract. The spindle assembly checkpoint is the mechanism or set of mechanisms that prevents cells with defects in chromosome alignment or spindle assembly from passing through mitosis. We have investigated the effects of mini-chromosomes on this checkpoint in budding yeast by performing pedigree analysis. This method allowed us to observe the frequency and duration of cell cycle delays in individual cells. Short, centromeric linear mini-chromosomes, which have a low fidelity of segregation, cause frequent delays in mitosis. Their circular counterparts and longer linear mini-chromosomes, which segregate more efficiently, show a much lower frequency of mitotic delays, but these delays occur much more frequently in divisions where the mini-chromosome segregates to only one of the two daughter cells. Using a conditional centromere to increase the copy number of a circular mini-chromosome greatly increases the frequency of delayed divisions. In all cases the division delays are completely abolished by the *mad* mutants that inactivate the spindle assembly checkpoint, demonstrating that the Mad gene products are required to detect the subtle defects in chromosome behavior that have been observed to arrest higher eukaryotic cells in mitosis.

Progress through the cell cycle depends on specific transitions caused by the activation and inactivation of cyclin-dependent kinases. Activating the kinase activity of $p34^{cdc2/CDC28}$-cyclin B complexes induces entry into mitosis. Ubiquitin-mediated proteolysis of cyclin B (Glotzer et al., 1991; Hershko et al., 1991) and other proteins (Holloway et al., 1993) is required to inactivate the kinase and allow anaphase and cytokinesis. To ensure the fidelity of chromosome segregation, anaphase can be delayed if the cell detects a problem in assembling the spindle or aligning the chromosomes on it. This control is an example of the growing number of cell cycle checkpoints that have been identified by mutations that can bypass cell cycle arrests induced by defects in the chromosome replication and segregation cycle (for reviews see Hartwell and Weinert, 1989; Murray, 1994, 1995; Hartwell and Kastan, 1994).

The prototypic checkpoint mutant is the budding yeast *rad9* mutant, which prevents cells from delaying their cell cycle in response to DNA damage (Weinert and Hartwell, 1988). The budding yeast *mad* (mitotic arrest deficient) (Li and Murray, 1991) and *bub* (budding uninhibited by benzimidazole) (Hoyt et al., 1991; Roberts et al., 1994) mutants define the spindle assembly checkpoint that monitors chromosome alignment and the structure of the mitotic spindle. In the presence of benomyl, a microtubule-depolymerizing drug which prevents proper spindle assembly, these mutants fail to arrest in mitosis and thus suffer lethal damage. They also suffer increased rates of chromosome loss during normal vegetative growth, presumably due to a failure to detect occasional defects in chromosome attachment.

How does the spindle assembly checkpoint detect defects in the chromosome segregation machinery? One possibility is that the checkpoint monitors the interaction between microtubules and kinetochores, the protein complexes assembled on the centromeric DNA. In this scenario, chromosomes whose kinetochores are not attached to microtubules would generate the signal that the cell is not ready to exit mitosis. The observation that mammalian cells do not initiate anaphase until the last free kinetochore in the cell attaches to the spindle (Rieder et al., 1994) supports this hypothesis, as do the mitotic delays caused by the injection of antibodies to kinetochore proteins (Bernat et al., 1990; Tomkiel et al., 1994).

Although the small size of budding yeast precludes the direct observation of individual chromosomes in living cells, the ability to manipulate chromosome structure in defined ways provides a powerful tool for analyzing mitosis. The short region of DNA which is necessary and sufficient for centromere function has been defined (Clarke and Carbon, 1980). Circular and linear mini-chromosomes can be created by combining centromeric sequences with DNA segments that act as origins of replication, telomeres, and encode selectable markers (Clarke and Carbon, 1980; Dani and Zakian, 1983; Murray and Szostak,

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1983*a*). Using such artificial chromosomes, we can perturb the process of chromosome segregation without the use of drugs or mutations and ask whether defects in the ability of mini-chromosomes to attach to the spindle engage the spindle assembly checkpoint. For example, dicentric chromosomes induce a cell cycle delay that depends on both the G2 DNA damage and spindle assembly checkpoints (Neff and Burke, 1992), and the presence of certain mutant centromeric sequences induces mitotic delays (Spencer and Hieter, 1992).

Pedigree analysis in budding yeast allows the segregation of mini-chromosomes to be followed in defined cell lineages and correlated with defects in cell division (Murray and Szostak, 1983*b*). We have used pedigree analysis to follow the segregation of a variety of mini-chromosomes which carry a single wild-type centromere, but whose segregation is compromised by their small size. We show that monocentric mini-chromosomes that carry wild-type centromeres can induce mitotic delays, that these delays are correlated with errors in mini-chromosome segregation, and that the severity of the delay increases with the copy number of the mini-chromosomes. These mitotic delays are completely abolished by *mad* mutations, strongly suggesting that the spindle assembly checkpoint monitors kinetochore-microtubule interactions.

Materials and Methods

Yeast Strains and Media

Table V lists the strains used in this work. The original *mad* mutants were isolated in the A364A background (Li and Murray, 1991). Media were prepared and genetic manipulations were performed as described (Sherman et al., 1974).

Pedigree Analysis

Pedigree analysis was performed as described (Murray and Szostak, 1983*b*). All pedigrees were started with unbudded cells, and the interval between observing cells in a given pedigree never exceeded 90 min. Pedigrees involving transfer from galactose- (YPGalactose) to glucose-containing medium (YPD)[1] were performed on plates containing two segments of agar, YPGalactose and YPD, separated by a gap of 2–5 mm. Unbudded cells from stationary cultures grown in glucose-containing medium were placed on the YPGalactose segment of the plate, allowed to undergo from one to three divisions, and then transferred to the YPD segment of the plate and followed for an additional two to three divisions. For these experiments, cells were transferred from YPGalactose to the adjacent YPD slab as soon as possible after their last division. Incubation was at 30°C, except for the time taken to inspect the cells when they were at room temperature.

During pedigree analysis we scored divisions as delayed if they satisfied three criteria: the mother cell was unusually large at the time of cell division, the mother and daughter cells were of similar size at the time of division, and the division of the affected cell was delayed relative to other cells in the same pedigree. Although these criteria are somewhat subjective, the almost perfect correlation between delayed divisions and cells that are subsequently shown to contain the mini-chromosome argues that they are robust and reliable.

Immunofluorescence

Logarithmically growing cells were treated with 10% formaldehyde for 1 h at 22°, and then washed twice with 0.7 M sorbitol, 0.1 M KPO_4, pH 7.5. The cell walls were digested with zymolyase in 0.7 M sorbitol, 0.1 M KPO_4, pH 7.5, for 30–40 min at 37°C, before being attached to polylysine-coated multi-well microscope slides. The slides were plunged into methanol (−20°C) for 5 min, and acetone (−20°C) for 30 s, and then allowed to air-dry. After washing with PBS, cells were blocked for 30 min with PBS with 4% milk and 0.1% Tween 20 (PBSTM) and washed with PBSTM. Staining was overnight at 4°C with primary rat anti-tubulin antibody diluted 1:200 in PBSTM. Cells were washed several times with PBSTM, and then incubated for 1 h at room temperature with FITC-labeled anti-rat secondary antibody at 1:50 dilution. After washing with PBSTM and then PBS, the DNA was stained with 1 mg/ml DAPI in PBS and cells were mounted in 90% glycerol, 1 mg/ml phenylenediamine, pH 9.0. Coverslips were sealed with clear nail polish and slides stored at −20°C.

Results

Short Linear Mini-Chromosomes Cause a mad-dependent Mitotic Delay

We investigated the relationship between the spindle assembly checkpoint and the segregation of linear mini-chromosomes. Although these small artificial chromosomes contain all of the elements required for normal chromosome function, their distribution is much less faithful than that of natural chromosomes (Murray and Szostak, 1983*a*; Dani and Zakian, 1983). Natural budding yeast chromosomes are present in a single copy in haploid cells and are lost at a frequency of approximately one division in 10^5, but linear mini-chromosomes have an average copy number of 15 and are lost at a frequency of 0.1 (Murray and Szostak, 1983*a*). The high copy number of short linear mini-chromosomes is likely to be a direct consequence of their poor segregation: a high frequency of segregation errors will create cells with increased copy numbers, and these cells will be favored by growth in selective medium since the probability of generating two mini-chromosome bearing progeny increases with increasing copy number.

We asked if the presence of such poorly segregating linear mini-chromosomes perturbed passage through mitosis. These experiments were performed by pedigree analysis, in which a small lineage of cells is produced by separating the progeny of an individual cell, and each member of the pedigree is allowed to grow up into a colony of cells that can be tested for the presence of the selectable marker that the mini-chromosome carries. We followed cells harboring either the 16-kb linear mini-chromosome pVL106 (Lunblad, V., personal communication) or the 11-kb linear mini-chromosome YLp4 (Murray and Szostak, 1983*a*) (Fig. 1). At each cell division, the size of the cell, the size of the bud (if any), the time of division, and the relationship (mother vs daughter) of the cells were recorded. We scored divisions as delayed if they satisfied three criteria: the mother cell was unusually large at the time of cell division, the mother and daughter cells were of similar size at the time of division, and the division of the affected cell was delayed relative to other cells in the same pedigree.

Cells lacking the mini-chromosome can arise by loss of the mini-chromosome in the cell divisions either before or during pedigree analysis. Since the pattern of cell divisions was observed before the pattern of chromosome segregation was determined, these experiments are intrinsically double blind. In cells lacking the mini-chromosomes, the division time was quite uniform (~90 min), cell division occurred before the bud grew as large as the mother cell,

1. *Abbreviations used in this paper*: YPD, glucose-containing medium; YPGalactose, galactose-containing medium.

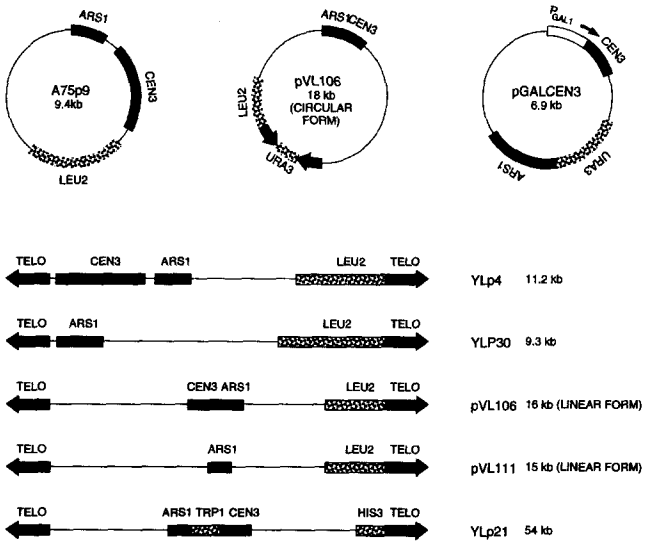

Figure 1. Maps of the plasmids used for pedigree analysis. Schematic views of the linear and circular mini-chromosomes used in this work. The linear forms of pVL106, pVL111, and YLp30 are obtained by digestion of plasmid DNA with BamHI, which removes a stuffer fragment between two *Tetrahymena* telomeric fragments, followed by transformation of yeast with the linear DNA. The details of mini-chromosome construction can be found in the following references: YLp4 and YLp21 (Murray and Szostak, 1983*a*); and YLp30 (Murray and Szostak, 1983*b*); pGALCEN3 (Hill and Bloom, 1987); pVL106 and pVL111 (Lunblad, V., personal communication).

and mother cells always completed cytokinesis before the daughter they had produced in the preceding division had divided (Fig. 2 *a*).

In contrast to the mini-chromosome–free divisions, 52–61% of divisions of the cells containing linear mini-chromosomes were delayed (Table I). Fig. 2 *b* shows a representative pedigree in which cell division was delayed and the cells could not be separated even after the daughter cell had grown to be as large as the mother cell and both cells had rebudded. Such delays depend on the presence of the centromere of the mini-chromosomes. The divisions of cells containing the related acentric plasmids, pVL111 (Lunblad, V., personal communication) and YLp30 (Murray and Szostak, 1983*a*), are not delayed (Table I). Acentric linear mini-chromosomes are present at roughly three times the copy number of centromeric linear chromosomes (Murray and Szostak, 1983*a*). Thus, the division delays in cells containing centromeric mini-chromosomes are not due to the presence of excess telomeres or any other noncentromere element on the linear mini-chromosomes.

We determined whether the delay due to linear mini-chromosomes was due to the spindle assembly checkpoint by performing pedigree analysis on the *mad* mutants, which lack this control. When pedigrees were performed in *mad1*, *mad2*, and *mad3* strains almost no delays were seen (0–4%, Table I). Thus, the division delay is induced by the engagement of the spindle assembly checkpoint, despite the presence of an apparently normal spindle as assessed by anti-tubulin immunofluorescence (data not shown).

To see where in the cell cycle the short linear mini-chro-

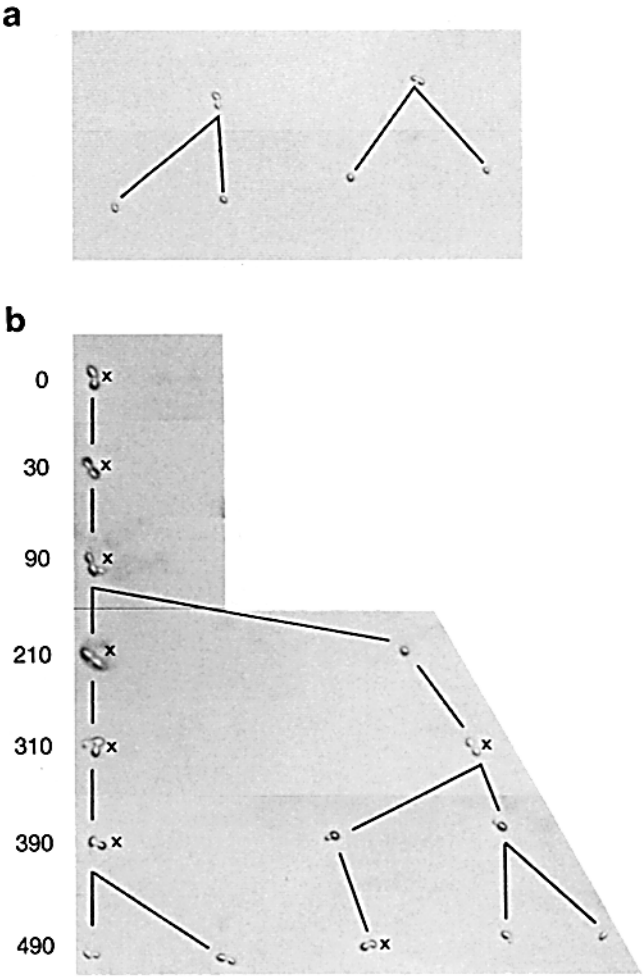

Figure 2. Pedigree analysis of yeast cells containing the short linear artificial chromosome YLp4. (*a*) Two cell pairs from a section of a pedigree that does not contain the mini-chromosome. The cells are shown before and immediately after the mother and daughter cells were separated by micromanipulation. This pedigree came from the same experiment as the pedigree shown in *b*. Note that daughters can be separated when they are smaller than their mothers and that new buds are formed near the point at which mother and daughter cells were joined. (*b*) The figure shows photomicrographs of a pedigree starting from a single cell containing YLp4. The time in minutes after the start of the pedigree is shown in the left margin and the lines indicate the cell lineage. *X*, indicates a cell pair where the daughter has reached a size at which it would normally be possible to separate it from the mother cell. This pedigree contains several aberrant cell divisions: the initial cell pair failed to divide even after 90 min when both mother and daughter cells had budded again. The two new buds on this cell were formed at opposite ends of the cell pair, rather than at the junction between mother and daughter; at 210 min only one of the new buds was separable and between this time and 310 min one member of the original cell pair died as indicated by a loss of refractility (more clearly visible at 390 min). Because of the aberrations in the pattern of cell divisions, no attempt was made to distinguish mother and daughter cells at the time of cell separation. After the last time point, the cells were more widely separated, allowed to grow up into colonies, and then tested for the inheritance of YLp4. All the cells in this pedigree inherited YLp4.

Table I. Cell Division Delay by Linear Centromeric Plasmids

Checkpoint genotype	Plasmid	Plasmid-bearing cells: Percent delayed divisions	Plasmid-bearing cells: No. divisions observed	Plasmid-free cells: Percent delayed divisions	Plasmid-free cells: No. divisions observed
MAD	pVL106 (CEN-linear)	61	147	1	233
mad1	pVL106 (CEN-linear)	2	330	3	38
mad2	pVL106 (CEN-linear)	1	284	2	54
mad3	pVL106 (CEN-linear)	4	400	0	42
MAD	pVL111 (acentric linear)	1	89	0	74
MAD	YLp4 (CEN-linear)	52	138	0	116
MAD	YLp30 (acentric linear)	0	80	0	25

Pedigree analysis of linear plasmids was performed on YPD plates and scored as described in Materials and Methods. Strains used were TBW2 (*MAD*, pVL106), TBW40 (*mad1*, pVL106), TBW18 (*mad2*, pVL106), TBW67 (*mad3*, pVL106), Ta1165 (*MAD*, YLp4), and TA1186 (*MAD*, YLp30).

Table II. Distribution of Spindle Morphologies in Cultures Containing Short Linear Plasmids

Checkpoint genotype	Mini-chromosome	No spindle	Short spindle	Long spindle
		%	%	%
MAD	No mini-chromosome	60	25	15
MAD	pVL106 (CEN-linear)	44	49	7
MAD	pVL106 (CEN-circle)	63	24	13
MAD	pVL111 (acentric linear)	59	27	14
mad1	pVL106 (CEN-linear)	58	31	11
mad1	pVL106 (CEN-circle)	55	30	15

Asynchronous cultures of wild-type or *mad1* mutant cells harboring circular or linear derivatives of the plasmid pVL106 (or an acentric linear derivative, pVL111) were fixed and stained with anti-tubulin antibodies. Two hundred cells from each culture were scored as being in one of three categories: no spindle, short spindle, or long spindle. Linear and circular mini-chromosomes were produced as described in Fig. 1. Applying the χ^2 test shows that the distribution of morphologies in the *MAD* strain containing the linear form of pVL106 is statistically significant from all other strains ($P < 0.002$ for all pairwise comparisons) but that none of the other strains show a statistically significant difference from each other ($P > 0.25$ for all pairwise comparisons). Strains used were BW30 (*MAD*), TBW1 (*MAD*, pVL106 circular), TBW2 (*MAD*, pVL106 linear), TBW40 (*mad1*, pVL106 linear), and TBW39 (*mad1*, pVL106 circular).

mosomes are causing a delay, wild-type or *mad1* cultures containing various mini-chromosomes were fixed and stained with anti-tubulin antibodies. The presence of the linear form of pVL106 in wild-type cells results in a marked increase in the proportion of cells with short spindles as compared to cells that lack mini-chromosomes (Table II). This observation indicates that the cell cycle delay induced by linear mini-chromosomes occurs after the onset of spindle assembly. FACS analysis also indicates an increase in cells with a 2C DNA content (data not shown). Control cultures with an acentric linear mini-chromosome (pVL111) or *mad1* cells carrying a centromeric mini-chromosome show the same distribution of spindle morphologies as mini-chromosome–free cells. These comparisons show that the mitotic delay depends on the presence of the centromere and a functional spindle assembly checkpoint.

Rare Errors in Segregation Correlate with a mad-dependent Cell Cycle Delay

We wanted to know if the delay induced by the short linear mini-chromosomes is peculiar to these molecules. Circular mini-chromosomes segregate more faithfully than linear mini-chromosomes, but less faithfully than natural chromosomes: circular mini-chromosomes are present at 1–3 copies per cell and are lost at a frequency of about 10^{-2}. Examining the spindle morphology of cultures containing the circular form of the centromeric mini-chromosome pVL106 failed to reveal a consistent mitotic delay (Table II). Because this analysis only provides information about a population of cells, we turned to pedigree analysis which follows the properties of individual cells. We followed the segregation of cells harboring the circular mini-chromosomes pGALCEN3 or A75p9 (whose structures are shown in Fig. 1) as they divided on rich medium. A75p9 differs from YLp4 only by the absence of telomeric sequences allowing a direct comparison of linear and circular plasmids. In both cases division delays were observed in some cells that contained the mini-chromo-

Table III. Rare Division Delay by Low Copy Centromeric Plasmids

Checkpoint genotype	Plasmid	Plasmid-bearing cells: Percent delayed divisions	Plasmid-bearing cells: No. divisions observed	Plasmid-free cells: Percent delayed divisions	Plasmid-free cells: No. divisions observed	Segregation Frequency
						%
MAD	pGALCEN3 (CEN circle)	9.2	523	0.6	162	0.054
mad1	pGALCEN3 (CEN circle)	0.4	566	0	171	0.034
mad2	pGALCEN3 (CEN circle)	0	427	0	47	0.044
mad3	pGALCEN3 (CEN circle)	0.4	520	0	107	0.027
MAD	A75p9 (CEN circle)	6.3	397	0	105	0.013
MAD	YLp21 (50 kb linear YAC)	2.6	723	0	505	0.017

Pedigree analysis was performed on YPD plates and scored as described in Materials and Methods. The strains used were TBW10 (*MAD*, pGALCEN3); TBW28 (*mad1*, pGALCEN3); TBW29 (*mad2*, pGALCEN3); TBW65 (*mad3*, pGALCEN3), TA624 (*MAD*, A75p9), DA248, DA249, DA250, and DA251 (all *MAD*, YLp21). Segregation frequency is the fraction of cell divisions containing the mini-chromosome in which only one for the two progeny cells inherits the mini-chromosome.

somes, but not in mini-chromosome–free cells. The frequency of cell cycle delays (6–9%) was much lower than that observed for cells containing linear mini-chromosomes (Table III).

To ask whether the division delays reflected problems in segregating the mini-chromosomes, we correlated the occurrence of division delays with errors in chromosome segregation. A much larger proportion of the divisions which resulted in errors were delayed (50–90%; Fig. 3) as compared to divisions in which both mother and daughter inherited the mini-chromosome (5–7%). This difference is highly statistically significant ($P < 10^{-10}$ by the χ^2 test). The observation that the cells that delay still mis-segregate mini-chromosomes suggests that the checkpoint can only produce a transient block to cell division. Like linear mini-chromosomes, the ability of circular molecules to delay the cell cycle depends on the spindle assembly checkpoint as *mad1*, *mad2*, or *mad3* cells show delays in only 0–0.4% of divisions. No correlation between cell division delays and segregation errors occurred in cells containing short linear mini-chromosomes (data not shown), probably because the presence of multiple copies of these molecules in a cell obscures segregation defects for any given pair of sister chromatids.

We quantified the fidelity of mini-chromosome segregation as the segregation frequency, the fraction of cell divisions in which only one of the two progeny receives the mini-chromosome (Table III). The absence of cell cycle delays in the *mad* mutants does not improve circular mini-chromosome segregation. This strongly suggests that the segregation difficulties of the mini-chromosomes lead to delays rather than that cell cycle delays lead to aberrant mini-chromosome segregation.

The inability of *mad* mutants to delay in mitosis does not increase the frequency of errors in mini-chromosome segregation. The difficulty of measuring the distribution of mini-chromosome copy numbers within a population of cells makes rigorous interpretation of this finding impossible. One attractive explanation, however, is that many of the errors in segregation are due to defects that cannot be corrected once cells have entered mitosis. A mitotic delay would not be expected to reduce the frequency of this type of error. The observation that circular centromeric mini-chromosomes show roughly five times as many 1:0 as they do 2:0 loss events (Hieter et al., 1985) is consistent with this possibility.

Linear mini-chromosomes segregate more faithfully and decrease in copy number as their length increases (Murray et al., 1986; Hieter et al., 1985). YLp21 is a 55-kb linear mini-chromosome (Murray and Szostak, 1983*a*) which is present at single copy and segregates correctly in 98–99% of mitoses. In pedigrees, 2.6% of divisions with this artificial chromosome exhibited a delay (Table III). As with the circular mini-chromosomes, a large number of divisions which resulted in errors in segregation showed a delay (10 of 11) whereas only 1% of symmetric divisions were delayed (Fig. 3). Since YLp21 is maintained at a stable copy number and segregates normally in meiosis, we could determine the copy number of the artificial chromosome by producing diploid cells and examining the segregation of YLp21 in meiosis. Using this method we examined the copy number of cells whose sisters had failed to inherit the artificial chromosome to distinguish between chromosome

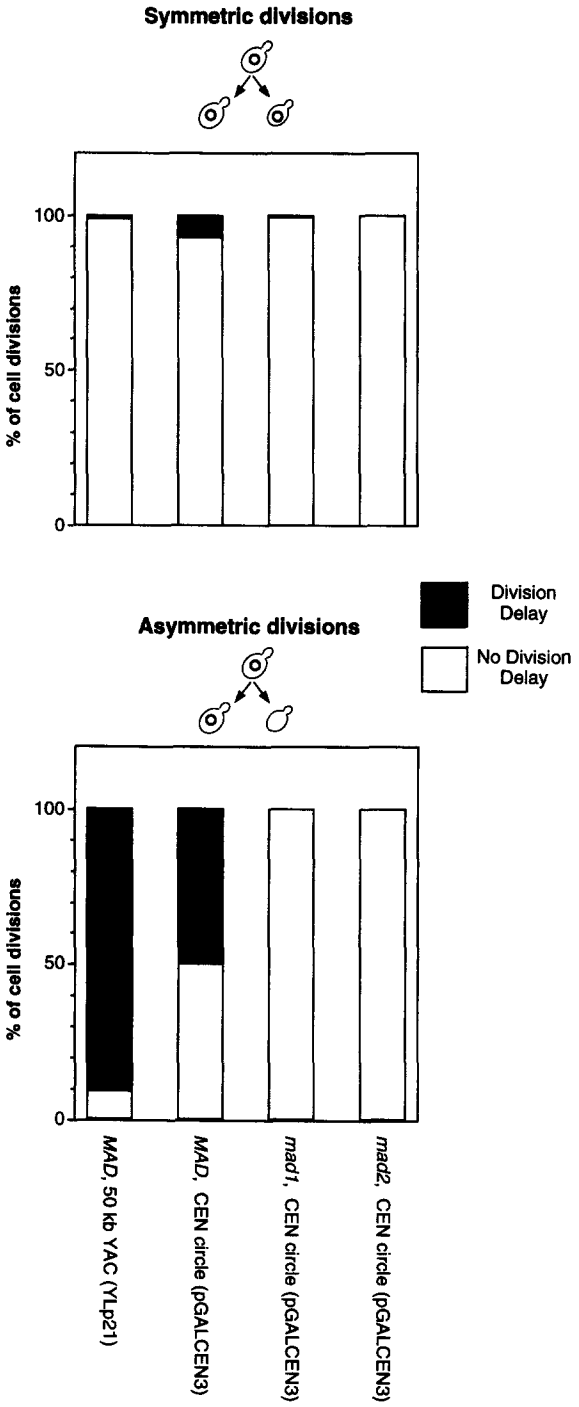

Figure 3. Errors in mini-chrome segregation correlate with mitotic delays. Divisions were scored as normal or delayed during pedigree analysis on glucose-containing medium for strains containing the indicated mini-chromosomes, and the pattern of mini-chromosome segregation was determined after the cells had grown into colonies. The null hypothesis that the frequency of delayed divisions is identical in divisions in which both progeny received the mini-chromosome compared to those divisions where only one cell received the mini-chromosome is rejected with a probability of less than 10^{-10} for both the 50-kb linear (YLp21) and the circular mini-chromosome (pGALCEN3). Strains used were TBW10 (MAD, pGALCEN3); TBW28 (*mad1*, pGALCEN3); TBW29 (*mad2*, pGALCEN3); DA248, DA249, DA250, and DA251 (MAD, YLp21). The fractions of cell divisions that show asymmetric plasmid segregation are listed in Table III as the segregation frequency.

loss (1:0 segregation) or nondisjunction (2:0 segregation) events. This analysis revealed that 10 of the 11 segregation errors events for YLp21 were 1:0 segregation events, suggesting that the primary cause of errors in segregation for this molecule is failure of replication or loss of a chromosome from the nucleus, rather than nondisjunction. Thus most dividing cells harbor two copies of the artificial chromosome and show no division delay, whereas most cells that have a single copy do delay. This observation rules out the possibility that the division delays observed with artificial chromosomes are simply due to the presence of extra kinetochores in the cell.

Excess Circular Centromeric Mini-Chromosomes Cause a mad-dependent Cell Cycle Delay

Previous attempts to determine the effect of multiple copies of circular mini-chromosomes on the cell cycle have yielded conflicting results. By simultaneously selecting for several different mini-chromosomes bearing different selectable markers, Futcher and Carbon (1986) concluded that the presence of excess kinetochores greatly delayed the cell cycle. An alternative method of raising mini-chromosome copy number uses the activation and inactivation of conditional centromeres, created by placing a strong, regulated promoter adjacent to the centromere. Transcription from the galactose (Hill and Bloom, 1987) or alcohol dehydrogenase II (Chelbowicz-Sledziewska and Sledziewska, 1985) promoters towards the centromere renders it incapable of directing segregation. This results in an increase in mini-chromosome copy number due to the bias of acentric plasmids to segregate to mother cells (Murray and Szostak, 1983*b*). Shutting off the promoter then reactivates the centromeres which are now present in multiple copies in a subset of the cells. Increasing copy number by this method has been variously reported as either toxic to the cell (Runge et al., 1991), possibly toxic (Hill and Bloom, 1987; Smith et al., 1990), or nontoxic (Chelbowicz-Sledziewska and Sledziewska, 1985).

All of the previous experiments examining the effect of excess centromeres suffer from the disadvantage that their conclusions are derived from analyzing populations of cells where the only known parameter is the average copy number. In addition, the selection of multiple differently marked mini-chromosomes in the same cell could lead to recombination events between the mini-chromosomes, resulting in the formation of dicentric molecules which are known to cause cell cycle delays (Koshland et al., 1987). To avoid these difficulties we exploited the combination of conditional centromeres, pedigree analysis, and the bias of acentric plasmids to segregate to the mother to create cells in which we could deduce the factor by which the mini-chromosome copy number has increased. We used pGALCEN3 (Hill and Bloom, 1987), a circular mini-chromosome with a conditional centromere. Due to transcription of the galactose promoter into the centromeric region, this plasmid segregates like an acentric ARS plasmid in cells growing on galactose, but has a fully functional centromere when cells are exposed to glucose, which inhibits transcription from the *GAL1* promoter. We placed glucose-grown cells on galactose-containing medium, and performed pedigree analysis for three generations before transferring the cells to glucose-containing medium and continuing pedigree analysis for one to three additional generations, as shown in Fig. 4. Since pGALCEN3 behaves as if it is acentric in cells on galactose, the mother cells will accumulate multiple copies of the plasmid. In particular, in those pedigrees where only the original mother cell inherits the plasmid we can estimate its minimum copy number. For example, a pedigree of three divisions on galactose that produces only one cell containing pGALCEN3 will have undergone three rounds of DNA replication without segregating the plasmid and the mother cell will contain $2^3 = 8$ times the number of mini-chromosomes as it did at the start of the experiment. The predicted increase in the copy number could be reduced by 1:0 segregation events; divisions in which one progeny cell lacks the mini-chromosome and the other contains only a single copy. For a circular centromeric mini-chromosome this type of error occurs in only 2% of cell divisions (Hieter et al., 1985). Thus, unless the frequency of 1:0 events is increased by the absence of a functional centromere, the effect of 1:0 events on our estimate of mini-chromosome copy number is small.

We analyzed the segregation of the mini-chromosome and the fraction of cell division delays in these pedigrees. As expected, exposure to galactose caused rapid inactivation of the centromere leading to asymmetric segregation of the mini-chromosome in 45% of the cells undergoing their first division on galactose-containing plates. Centromere reactivation after transfer to glucose-containing medium was efficient as mini-chromosome loss was reduced to 13% in the first division and 3% in the second di-

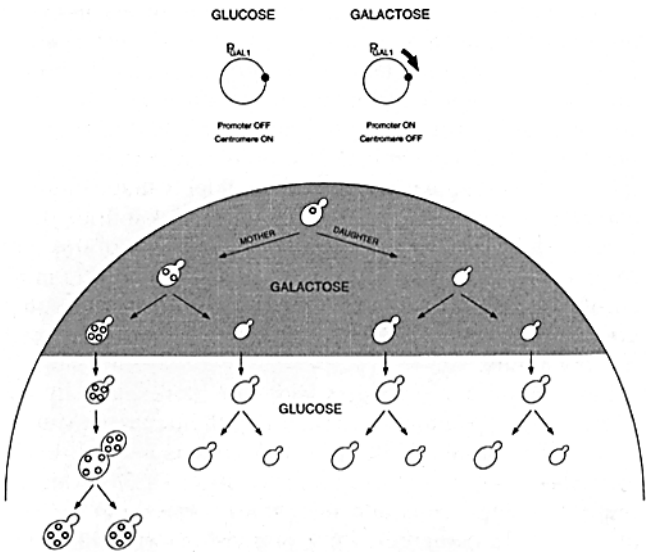

Figure 4. Use of a conditional centromere to increase the copy number of mini-chromosomes. On galactose-containing medium the transcriptional activity of the *GAL1* promoter inactivates the centromere of pGALCEN3, and such functionally acentric mini-chromosomes are biased to segregate to the mother cell at cell division. Transferring cells to glucose-containing medium leads to inactivation of the *GAL1* promoter and restoration of centromere activity. In pedigrees, such as the one illustrated, where the plasmid segregates exclusively to the original mother cell, the copy number of the plasmid can be deduced.

Table IV. Division Delays Induced by High Copy Circular Centromeric Plasmids Are MAD Dependent

Checkpoint genotype	Plasmid	Plasmid-bearing cells		Plasmid-free cells	
		Percent delayed divisions	No. divisions observed	Percent delayed divisions	No. divisions observed
MAD	pGALCEN3 (CEN circle)	39	502	0.1	905
mad1	pGALCEN3 (CEN circle)	0	247	0.3	330
mad2	pGALCEN3 (CEN circle)	0.3	285	0	130
mad3	pGALCEN3 (CEN circle)	0	195	0	231

Pedigree analysis was performed for three divisions on YPGalactose plates followed by three divisions on YPD plates and scored as described in Materials and Methods. The strains used were TBW10 (*MAD*, pGALCEN3); TBW28 (*mad1*, pGALCEN3); TBW29 (*mad2*, pGALCEN3); and TBW65 (*mad3*, pGALCEN3).

vision on glucose (numbers derived from the pedigrees whose results appear in Table IV and Fig. 5). Mini-chromosome–free cells showed a very low frequency (<0.1%) of division delays, whether they arose from pedigrees started from a mini-chromosome–free cell or by segregation in a mini-chromosome–containing pedigree. Cells plated directly on glucose-containing medium without exposure to galactose showed a low level of delays (see previous data in Table III). Mini-chromosome–containing cells which had been on galactose for two or three generations, however, showed a high incidence of delays which was absent in *mad1*, *mad2*, or *mad3* cells (Table IV).

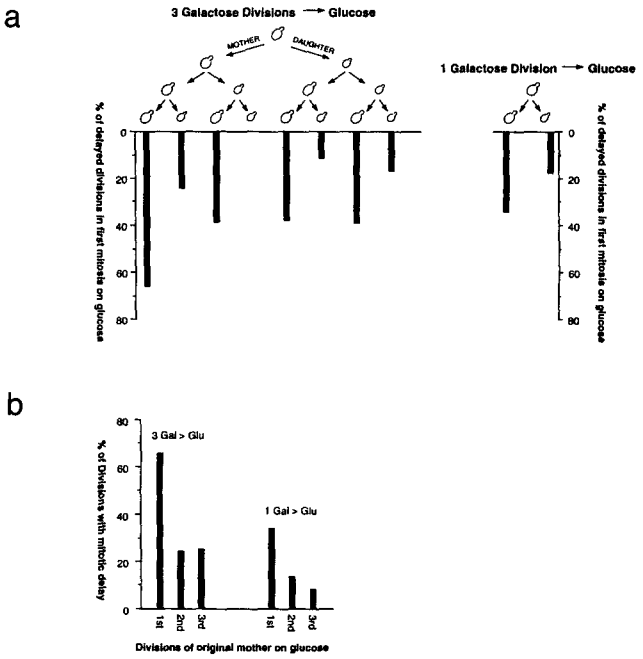

Figure 5. Mitotic delays induced by multiple copies of circular mini-chromosomes. (*a*) The fraction of delayed cell divisions of pGALCEN3-containing cells in the first division on glucose-containing medium after transfer from galactose-containing medium. The figure shows this value for cells at each of the positions of the cell lineages derived from either three or one division on galactose-containing medium. The data is from multiple experiments involving 80 pedigrees with three divisions, and 181 pedigrees with one division on galactose-containing medium. (*b*) The fractions of delayed division in each of the first three divisions following transfer from galactose- to glucose-containing medium. The figure shows this value for cells from either three or one division on galactose-containing medium.

The effects of altering mini-chromosome copy number on cell division delays are shown in Fig. 5. The distribution of the delays is predicted by the bias of acentric mini-chromosomes to segregate to the mother cell. For each mother-daughter pair the mother is at least twice as likely as the daughter to show a division delay on glucose-containing medium. In addition, the initial cell used to start the pedigree, which has been a mother for three divisions on galactose, and thus should have a higher number of mini-chromosomes than any other cell in the pedigree, has a substantially higher frequency of mitotic delays than any other mother cell after transfer to glucose-containing medium (Fig. 5 *a*). The χ^2 test demonstrates that both of these differences have a probability of less than 0.001 of occurring by chance, strongly suggesting that the frequency of division delays increases as the mini-chromosome number rises.

We performed two tests to determine whether the divi-

Table V. Yeast Strains

Strain name		Genotype
BW30	a	*ade3, ura3-52, leu2,3-112, his3-D200, met2*
TBW1	a	*ade3, ura3-52, leu2,3-112, his3-D200, met2, pVL106*
TBW2	a	*ade3, ura3-52, leu2,3-112, his3-D200, met2, pVL106* (linearized)
TBW6	a	*ade3, ura3-52, leu2,3-112, his 3-D200, met2, pVL111* (linearized)
TBW40	a	*ura3-52, leu2,3-112, his3-D200, trp1, mad1-1, pVL106* (linearized)
TBW18	a	*ura3-52, leu2,3-112, his3-D200, trp1, mad2-1, pVL106* (linearized)
TBW67	a	*ade2, ura3-52, leu2,3-112, his3-D200, trp1, mad3-1, pVL106* (linearized)
A281	α	*cir°, leu2-3,112, his3-11,15, can1*
TA1165	α	*cir°, leu2-3,112, his3-11,15, can1, YLp4*
YA1186	α	*cir°, leu2-3,112, his3-11,15, can1, YLp30*
TBW10	a	*ura3, leu2,3-112, his3-D200, trp1, pGALCEN3*
TBW28	a	*ura3-52, leu2,3-112, his3-D200, trpl, mad1-1, pGALCEN3*
TBW29	a	*ura3-52, leu2,3-112, his3-D200, trp1, mad2-1, pGALCEN3*
TBW65	a	*ade2, ura3-52, leu2,3-112, his3-D200, trp1, mad3-1, pGALCEN3*
TA624	α	*cir°, leu2-3,112, his3-11,15, can1, A75p9*
DA248	a/α	*leu2-3,112/leu2-3,112, his3-11,15/his3-11,15, trp1/trp1,ura3/ura3, ade1/ADE1, arg4/ARG4,YLp21*
DA249		same genotype as DA248
DA250		same genotype as DA248
DA251		same genotype as DA248
BW172	a	*ura3-52, trp1-D63, leu2-D1, lys2-D202, his3-D200*

sion delays are due to slow or partial reactivation of the centromeres on glucose. First, in some experiments, we followed the cells for three divisions after transfer from galactose to glucose-containing medium (Fig. 5 *b*). There is still a substantial fraction of division delays in the third division on glucose-containing medium, when the fidelity of mini-chromosome segregation demonstrates that normal centromere function has been fully restored. Second, we tested the effects of allowing only a single division on galactose before transfer to glucose. These cells exhibited half the frequency of division delays on glucose-containing medium, compared to cells allowed to divide three times on galactose. Both of these tests strongly suggest that the strong mitotic delays after multiple divisions on galactose are due to the accumulation of excess centromeres over several generations.

Futcher and Carbon (1986) suggested that the division delay induced by excess centromeres was due to the titration of a factor required for centromere function. If this explanation is correct, eliminating the division delay would be expected to induce anaphase before kinetochore assembly was complete on some chromosomes, leading to loss of both natural and artificial chromosomes, and cell death. To test this possibility, we followed the fate of *mad1*, *mad2*, and *mad3* cells containing pGALCEN3 after transferring cells from galactose to glucose. As stated above, the inactivation of the spindle assembly checkpoint eliminated the cell cycle delay seen after transferring cells from galactose to glucose, but led to no increase (*mad3*, 2% of divisions) or only a small increase (*mad1*, 11% of divisions; *mad2*, 4% of divisions) in the amount of cell death of mini-chromosome–bearing cells on glucose. This result strongly suggests that the presence of excess mini-chromosomes delays mitosis because the mini-chromosomes themselves have difficulty interacting with the spindle, rather than because they interfere with the behavior of the natural chromosomes.

Discussion

We have used pedigree analysis to investigate the interaction between the spindle assembly checkpoint and the segregation of mini-chromosomes in budding yeast. Short linear mini-chromosomes, which segregate poorly and are present in multiple copies per cell, induce a high frequency of mitotic delays which are dependent on the presence of a functional centromere on the mini-chromosome. Circular and long linear mini-chromosomes, which segregate more faithfully and are present in 1–3 copies per cell, induce a lower frequency of mitotic delays and these are seen preferentially in cells which show aberrant segregation of the mini-chromosome. Increasing the copy number of circular mini-chromosomes increases the frequency of mitotic delays toward the value seen in cells with short linear mini-chromosomes. Finally, all of these delays are eliminated by mutations that abolish the spindle assembly checkpoint.

Although *mad* mutants abolish the cell cycle delays caused by circular mini-chromosomes, they do not increase the fidelity of plasmid segregation. This observation suggests that it is the defects in mini-chromosome interactions with the spindle that cause the delays, rather than delays induced by some other mechanism leading to defects in mini-chromosome segregation. When the copy number of a circular mini-chromosome was increased by centromere inactivation and reactivation, the frequency of delays (66% of divisions of original mother cells, Fig. 5) increased to a value similar to that seen in strains containing linear mini-chromosomes (61% of divisions, Table I). The simplest interpretation of this experiment is that each plasmid molecule in the cell behaves independently. Increasing the number of plasmids per cell raises the probability that at least one plasmid will experience problems in attaching correctly to the mitotic spindle, activate the checkpoint, and thus delay the onset of anaphase. The toxicity of excess centromeres seen in earlier experiments (Futcher and Carbon, 1986; Runge et al., 1991) is therefore most likely due to the ability of these poorly segregating molecules to activate the checkpoint, rather than their ability to compete with natural chromosomes for limiting quantities of kinetochore components.

All of the delays described above are absent in *mad1*, *mad2*, and *mad3* mutants. Therefore, the defect in the association of these plasmids with the spindle is being detected by the spindle assembly checkpoint that was previously defined by the failure of mutants to arrest in response to microtubule depolymerization (Li and Murray, 1991). It is clear from our results and those of others (Neff and Burke, 1992; Spencer and Hieter, 1992) that defects that have no effect on overall spindle structure can activate the spindle assembly checkpoint. Observations of a wide range of cells in both mitosis and meiosis reveal that the most frequent spontaneous spindle defects are in the attachments of individual chromosomes to the spindle rather than in the overall morphology of the spindle. In particular, experiments on newt lung cells (Rieder et al., 1994) and insect spermatocytes (Li and Nicklas, 1995) demonstrate that the misorientation of a single chromosome is sufficient to delay cells in mitosis or meiosis, presumably via the spindle assembly checkpoint.

How would a checkpoint that monitored kinetochore-microtubule interactions detect other mitotic defects? Suppression of microtubule dynamics might produce unoccupied microtubule-binding sites at the kinetochores or affect the tension exerted by microtubules on the kinetochore, whereas failures in spindle pole duplication will prevent up to half of the kinetochores from attaching to microtubules at all. Experiments in sea urchin eggs suggest that other mechanisms of detecting spindle defects must exist since although these cells cannot delay in mitosis in response to unattached chromosomes (Sluder et al., 1994), they can still respond to spindle depolymerization (Sluder et al., 1986).

Most delayed cells do eventually divide, even though they mis-segregate mini-chromosomes. This suggests that cells can eventually override the checkpoint signal and proceed into anaphase. This interpretation is consistent with the observation that budding yeast cells containing damaged DNA eventually override the Rad9-dependent checkpoint and pass through mitosis (Sandell and Zakian, 1993) and that many plant and animal cells treated with anti-microtubule drugs only arrest transiently in mitosis (Kung et al., 1990; Rieder and Palazzo, 1992; Rieder et al., 1994).

Why do linear mini-chromosomes segregate poorly and

activate the spindle assembly checkpoint at a high frequency? Understanding these problems should yield valuable clues about the mechanism of chromosome segregation and the signal that activates the checkpoint. The possible explanations for the poor segregation of linear mini-chromosomes relative to their circular counterparts fall into two classes: those based on topological differences between linear and circular molecules (Murray and Szostak, 1985) and those based on effects of the nearby telomeres which have been observed to silence nearby genes (Aparicio et al., 1991) and delay the firing of nearby replication origins (Ferguson and Fangman, 1992; Raghuraman et al., 1994). Two observations suggest that the aberrant behavior of linear mini-chromosomes is due to topology rather than telomeres. First, manipulated versions of natural chromosomes that have one centromere very close to a telomere segregate normally (Murray and Szostak, 1986). Second, cells with mutations in the *SIR2* gene, whose product plays an essential role in telomeric silencing (Aparicio et al., 1991), show the same frequency of errors in mini-chromosome segregation and mitotic delays as wild-type cells (data not shown).

Is there a common defect in the interaction of linear and circular mini-chromosomes with the spindle that explains why high copy numbers of both can activate the spindle assembly checkpoint? Both precocious sister chromatid separation and failures in chromosome replication produce kinetochores that are not linked to a partner that can attach to the opposite spindle pole. Experiments in mantid spermatocytes show that even though an unpaired meiotic chromosome remains attached to microtubules, it activates the spindle assembly checkpoint. Applying tension to this chromosome (which mimics the opposing force normally generated by a paired chromosome) allows the cell to enter anaphase, demonstrating that cells can monitor tension at the kinetochore (Li and Nicklas, 1995). This observation suggests that premature separation of sister chromatids could induce mitotic delays either by removing tension at the kinetochore or by destabilizing kinetochore-microtubule interactions (Nicklas and Koch, 1969; Nicklas et al., 1995; Rieder et al., 1995). In situ hybridization shows that the two sister chromatids of a circular mini-chromosome remain associated with each other in cells arrested in mitosis (Guacci et al., 1994), although various technical limitations could conceal a small fraction of the population where the sisters had separated precociously. Because of their heterogeneous and high copy number, in situ hybridization of fixed cells cannot easily monitor when the sister chromatids of short linear mini-chromosomes separate. Thus precocious sister separation remains a possible mechanism for activating the spindle assembly checkpoint.

Small chromosomes, both circular and linear, may have problems capturing microtubules and aligning correctly on the spindle. In animal cells sister chromatid pairs move rapidly towards the pole that first captures them (Rieder and Alexander, 1990), but their approach is limited by a microtubule-dependent repulsive force referred to as the polar wind or astral exclusion force (Rieder et al., 1986; Rieder and Salmon, 1994; Cassimeris et al., 1994). Laser ablation of the chromosome arms allows them to be expelled from the spindle, while the remaining kinetochore fragment moves closer to the spindle pole (Rieder et al., 1986). If yeast spindle pole bodies exert a polar wind, mini-chromosomes would be largely immune to it and could approach very close to the pole they initially attached to. Such a position would constrain a mini-chromosome to be far from the opposite pole and would make it hard for the unattached kinetochore to capture microtubules from the distant pole. Polar wind forces in animal cells appear to play a role in restricting the oscillation of sister chromatid pairs that are attached to both poles to positions that are near the midpoint of the spindle (Skibbens et al., 1993). If such forces were ineffective on mini-chromosomes these molecules might engage in longer range oscillations that would destabilize their attachment to microtubules, and in situ hybridization should reveal that mini-chromosomes show a broader distribution along the length of the spindle than natural chromosomes.

Regardless of the nature of the errors in mini-chromosome–spindle interactions, we observe a strong correlation between divisions in which delays are induced and divisions in which segregation errors occur. This correlation strengthens the assertion that the spindle assembly checkpoint detects nascent errors in chromosome segregation and delays cell cycle progression to give the cell a chance to correct the mistake. Increasing the copy number of a small circular plasmid presents the cell with more possible defects and so a delay is induced more often. The failure of *mad* mutants to delay in these circumstances provides an alternative screen for further *mad* mutants, and will allow us to test specific centromere elements and kinetochore proteins for their role in the spindle assembly checkpoint.

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