The Spindle Checkpoint of the Yeast *Saccharomyces cerevisiae* Requires Kinetochore Function and Maps to the CBF3 Domain

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ABSTRACT

We have measured the activity of the spindle checkpoint in null mutants lacking kinetochore activity in the yeast *Saccharomyces cerevisiae*. We constructed deletion mutants for nonessential genes by one-step gene replacements. We constructed heterozygous deletions of one copy of essential genes in diploid cells and purified spores containing the deletion allele. In addition, we made gene fusions for three essential genes to target the encoded proteins for proteolysis (degron alleles). We determined that Ndc10p, Ctf13p, and Cep3p are required for checkpoint activity. In contrast, cells lacking Cbf1p, Ctf19p, Mcm21p, Slk19p, Cse4p, Mif2p, Mck1p, and Kar3p are checkpoint proficient. We conclude that the kinetochore plays a critical role in checkpoint signaling in *S. cerevisiae*. Spindle checkpoint activity maps to a discreet domain within the kinetochore and depends on the CBF3 protein complex.

THE spindle checkpoint is a regulatory system that controls the onset of anaphase in response to spindle malfunction (Amon 1999; Burke 2000; Gardner and Burke 2000). We identified seven mutants of the yeast *Saccharomyces cerevisiae* that could not arrest in the cell cycle in response to benzimidazoles (such as nocodazole and benomyl) that inhibit microtubule assembly. Homologs of the genes have been identified in a number of different organisms, suggesting that the checkpoint is conserved from yeast to humans (Taylor and McKeon 1997; Bernard *et al.* 1998; Cahill *et al.* 1998; Chen *et al.* 1998; Basu *et al.* 1999; Chan *et al.* 1999; Yu *et al.* 1999). Mutants affecting microtubule assembly, microtubule-based motors, spindle pole body components, and kinetochore proteins induce checkpoint activity and inhibit anaphase (Wang and Burke 1995; Pangilinan and Spencer 1996; Skibbens and Hieter 1998; Hardwick *et al.* 1999). The kinetochore is a complex of proteins bound to centromere DNA (Choo 1997; Espelin *et al.* 1997; Meluh and Koshland 1997; Skibbens and Hieter 1998). Antibodies that recognize homologs of yeast checkpoint proteins stain kinetochores in a variety of cells (Gorbsky *et al.* 1998). This staining occurs at the time of checkpoint activity, suggesting that kinetochore localization of checkpoint proteins is an important part of checkpoint function (Taylor and McKeon 1997; Gorbsky *et al.* 1998; Kallio *et al.* 1998; Basu *et al.* 1999; Yu *et al.* 1999).

Formal proof that the kinetochore plays a role in checkpoint activity comes from analysis of kinetochore mutants in *S. cerevisiae* (Tavormina and Burke 1998; Sassoon *et al.* 1999). The core of the kinetochore in yeast is a protein complex, called CBF3, bound to a conserved centromere DNA element (CDEIII) that contains four essential proteins: Ndc10p, Cep3p, Ctf13p, and Skp1p (Russell *et al.* 1999). A number of other proteins are also associated with CBF3, and at least three of them are essential (Brown *et al.* 1993; Stoler *et al.* 1995; Meluh and Koshland 1997; Meluh *et al.* 1998; Ortiz *et al.* 1999). Temperature sensitive (Ts^-) mutants defining these genes have two different phenotypes. Most of the Ts^- mutants arrest or show a pronounced delay in the cell cycle at the restrictive temperature (Brown *et al.* 1993; Doheny *et al.* 1993; Lechner 1994; Stoler *et al.* 1995; Strunnikov *et al.* 1995; Connelly and Hieter 1996; Ortiz *et al.* 1999). Ts^- *ndc10* mutants have a different phenotype. The cells, when grown at the restrictive temperature, are unable to attach chromosomes to the spindle microtubules and complete anaphase with massive nondisjunction of the chromosomes (Goh and Kilmartin 1993). This catastrophic missegregation of chromosomes does not cause cells to arrest in the cell cycle. Instead, they divide the DNA asymmetrically and replicate the unbalanced genomes in the subsequent cell cycle, resulting in genomic instability. *ndc10-1* mutants cannot maintain an arrest in the presence of nocodazole; therefore, *ndc10-1* mutants lack

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the spindle checkpoint (Tavormina and Burke 1998). The two different phenotypes associated with kinetochore mutants reflect the presence or absence of the spindle checkpoint. One explanation for the different phenotypes associated with Ts^- mutants affecting kinetochore activity is that there is reduced activity of the gene products (hypomorphs) for some of the mutants. Perhaps more kinetochore mutants would show a lack of spindle checkpoint activity if null alleles could be used.

We have used a variety of techniques to produce null alleles of genes encoding kinetochore proteins. We used targeted insertion to generate deletion mutations of nonessential kinetochore genes. We developed a method to recover null mutants after sporulation and germination from diploids that were heterozygous for a targeted deletion mutation. We also constructed "degron-tagged" alleles of many of the essential genes encoding kinetochore proteins. The degron targets the proteins for proteolysis by the proteasome and results in temperature-sensitive null mutants. We show that null alleles of *NDC10, CEP3,* and *CTF13*, which encode components of CBF3, do not arrest the cell cycle in the absence of the proteins. Null alleles of *CSE4, MIF2, CBF1, CTF19, MCM21, SLK19, MCK1,* and *KAR3* are checkpoint proficient. We conclude that spindle checkpoint activity, within the kinetochore, is dependent on CBF3.

MATERIALS AND METHODS

Strains and media: Strains are listed in Table 1. Cells were grown in YM-1 medium (Hartwell 1967) and synthetic complete medium lacking essential nutrients as required (Burke *et al.* 2000). Meiosis was induced in diploid cells by incubation in 1% potassium acetate (Burke *et al.* 2000). Benomyl [Sigma (St. Louis) and a gift from Dupont] was added to a final concentration of 15 μg/ml in agar-containing plates. Nocodazole (Sigma) was used in liquid medium at 15 μg/ml when cells were grown at a temperature <36°. A mixture of nocodazole and benomyl (20 μg/ml of nocodazole and 30 μg/ml of benomyl) was used when incubating cells at temperatures >36° (Tavormina *et al.* 1997). A stock solution of 1 mg/ml α-factor (Sigma) was diluted 1:20,000 for *bar1* strains. Cycloheximide (Sigma) was used at a final concentration of 10 μg/ml.

Plasmid and strain construction: Plasmids are listed in Table 1. Degron alleles were constructed and integrated based on the method of Dohmen *et al.* (1994). The first 200–500 bp of the gene were amplified by PCR (sequences of all primers are available upon request). PCR products were subcloned into the PCR2.1 plasmid (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. All plasmids were sequenced (University of Virginia DNA Core Facility) to confirm the wild-type sequence. The PCR fragments were excised from PCR2.1 and subcloned into the YIp degron vector pPW66R (Dohmen *et al.* 1994). The correct sequence of the degron fusion was confirmed by sequencing to assure that the products were cloned in-frame. The degron is a copper-regulated promoter driving expression of an amino terminal fusion protein that targets the fusion protein for degradation by the 26S proteasome in a temperature-dependent fashion (Dohmen *et al.* 1994). The degron also contains an epitope derived from haemagglutinnin protein (HA) so that the fusion protein can be detected by Western blots. The degron fusion was targeted to the appropriate genomic locus by cutting at a unique restriction site within the targeted gene.

Precise deletions of open reading frames were constructed by integrating fragments derived by PCR (Wach *et al.* 1994). The fragments contained 40 bp of homology to the 5′ and 3′ ends of the target genes flanking a fusion gene that expresses aminoglycoside (kanamycin) resistance. Deletion alleles constructed in this manner were designated ::*KAN* (see Table 1). After transformation, cells were plated onto YPD agar plates and grown overnight, and transformants were selected by transferring cells, using sterile velveteen pads, onto YPD agar plates containing 500 μg/ml Geneticin (G418, Life Technologies). All integrations were confirmed by PCR. *SLK19* was deleted by integrating a *Pvu*II-*Spe*I restriction fragment of plasmid pΔsdb24:*URA3*, selecting for integration on SC − ura plates. Integrants were confirmed on Southern blots.

A YIp plasmid (pDB110) containing *LEU2* and *CYH2* was constructed by subcloning the *LEU2* and *CYH2* genes, contained on a *Bst*XI fragment of pRS318, into the *Bst*XI sites of pRS305. Approximately 500 base pairs from the 3′ ends of *MIF2* and *CTF13* were amplified by PCR and subcloned into PCR2.1 as described above. The fragments were excised from PCR2.1 and subcloned into pDB110 to produce plasmids pDB133 and pDB112, respectively. The plasmids pDB133 and pDB112 were targeted to their respective genomic loci, *MIF2* and *CTF13*, by cutting with *Bgl*II (pDB133) and *Sph*I (pDB112). Transformants were selected on SC − leu plates.

Flow cytometry: Cells were prepared for flow cytometry using propidium iodide (Sigma) as described previously (Smith 1991).

Spore enrichment: Spores were enriched using a modified version of the protocol described by Rockmill *et al.* in Guthrie and Fink (1991). Briefly, cells were pelleted, washed once in a large volume of water (at least 40 ml), resuspended in 1 ml of water, and transferred to a 15-ml conical polypropylene tube. Asci were digested by adding 100 μl zymolyase (10 mg/ml; Seikagaku, Rockville, MD) and incubating at 30° for 30 min to 1 hr. Spores were concentrated by centrifugation, washed in 5 ml of water, and resuspended in 500 μl of water by stirring with a sterile wooden dowel. The spores adhered to the surface of the tube after suspending with the aid of a vortex mixer. The remaining liquid was transferred to a second tube for further dispersion onto the tube walls to recover additional spores. Both tubes were gently washed twice with 500 μl of water. Spores were recovered by adding 10 ml of water containing 1 μl Nonidet P40 and sonicated on ice for 90 sec. Spores were transferred to a glass tube, concentrated by centrifugation, resuspended in ~500 μl, and sonicated. The recovery was $\sim 1.5 \times 10^9$ spores per 25 ml of sporulated cells.

Spore germination: Spores were inoculated into 25 ml of YM-1 containing 2% glucose + 10 μg/ml cycloheximide and incubated at 37°. Budding of cycloheximide-resistant cells was monitored by microscopy and usually began at 5–6 hr after inoculation. Cycloheximide-resistant cells were purified from the cycloheximide-sensitive spores after most had entered the cell cycle. Cells were concentrated by centrifugation in a polypropylene tube. Many of the cells and spores adhered to the walls. Spores that retained hydrophobicity could be removed by gentle washing. The cells were resuspended in 500 μl of water and applied to the tube walls by use of a vortex mixer machine twice more and each time the centrifuge tube was gently washed. Efficiency of initial spore enrichment was determined by testing viability after plating on YPD plates. If the purification were completely successful, none of the cells would be viable since they contain a deletion of an essential gene. Viability was typically ~10%; therefore 90% of the cells

TABLE 1

Strains and plasmids

Strain	Genotype	Source
1293-R	*MAT***a** *ade2-101 his3Δ200 leu2Δ1 lys2-801 ura3-52 trp1Δ63 ctf13-30*	P. Hieter
2114	*MAT***a** *cep3-2 ade2-101 his3Δ200 leu2Δ1 lys2-801 trp1Δ1 ura3-52 mad2::HIS3*	This study
2124	*MAT***a** *his3Δ1 ade2-1 ade6-1 cyh2 can1 hom3-H1 trp1-289 leu2-3,112 ura3-52 bar1::KAN*	This study
2145	*MAT***a** *his3Δ1 ade2-1 ade6-1 cyh2 can1 hom3-H1 trp1-289 leu2-3,112 ura3-52 bar1::KAN (YIp URA3 cep3td)*	This study
2156	*MAT***a** *his3Δ1 ade2-1 ade6-1 cyh2 can1 hom3-H1 trp1-289 leu2-3,112 ura3-52 bar1::KAN (YIp URA3 skp1td)*	This study
2158	*MAT***a** *his3Δ1 ade2-1 ade6-1 cyh2 can1 hom3-H1 trp1-289 leu2-3,112 ura3-52 bar1::KAN (YIp URA3 ndc10td)*	This study
2160	*MAT***a** *his3Δ1 ade2-1 ade6-1 cyh2 can1 hom3-H1 trp1-289 leu2-3,112 ura3-52 bar1::KAN (YIp URA3 ctf13td)*	This study
2162	*MAT***a** *his3Δ1 ade2-1 ade6-1 cyh2 can1 hom3-H1 trp1-289 leu2-3,112 ura3-52 bar1::KAN (YIp URA3 cse4td)*	This study
2165	*MAT***a** *his3Δ1 ade2-1 ade6-1 cyh2 can1 hom3-H1 trp1-289 leu2-3,112 ura3-52 bar1::KAN (YIp URA3 ctf13td) GAL10-UBR1:HIS3*	This study
2185	*MAT***a**/*MAT*α *mif2::KAN/MIF2:LEU2 CYH2 ste5-1/ste5-1 ade5-1/+ ura3-52/+ leu2-3,112/leu2-3,112 cyh2/cyh2*	This study
2215	*MAT***a**/*MAT*α *MIF2:LEU2 CYH2/+ ste5-1/ste5-1 ade5-1/+ ura3-52/+ leu2-3,112/ leu2-3,112 cyh2/cyh2*	This study
2252	*MAT***a**/*MAT*α *ctf13::KAN/CTF13:LEU2 CYH2 ste5-1/ste5-1 ade2-1/ lys2-1/+ ura3-52/ ura3-52 leu2-3,112/ leu2-3,112 cyh2/cyh2*	This study
2254	*MAT***a**/*MAT*α *CTF13:LEU2 CYH2/+ ste5-1/ste5-1 ade5-1/+ ura3-52/+ leu2-3,112/ leu2-3,112 cyh2/cyh2*	This study
2268	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ctf19::KAN*	This study
2269	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mcm21::KAN*	This study
2272	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 slk19::URA3*	This study
2288	*MAT***a** *cbf1 ura3-53 leu2-3 ade2-1 ade3-1 cyh2 his4 mad2::URA3*	This study
2289	*MAT***a** *ade2-101 his3Δ200 leu2Δ1 lys2-801 ura3-52 trp1Δ63 mck1::HIS3 mad2::URA3*	This study
2290-5-1	*MAT***a** *ade2-1 can1-100 his3 leu2 trp1 ura3 lys5 slk19::URA3 mad2::KAN1*	This study
2291-22-2	*MAT***a** *ade2-1 can1 lys5 leu2 trp1 hom3-H1 his3 ura3 mad2::KAN1 (*Yip *URA3 cse4td)*	This study
2407-5-3	*MAT***a** *ade2-1 hom3-H1 his3Δ1 leu2-2,113 trp1-289 ura3-52 lys2-1 ndc10-1bar1::KAN*	This study
2419	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 skp1-1::TRP1 skp1-4::LEU2 mad2::HIS3*	This study
2bAS282	*MAT***a** *ade2-101 his3Δ200 leu2Δ1 lys2-801 trp1Δ1 ura3-52 cep3-2*	A. Strunnikov
792-1-1	*MAT***a** *ade2-1 his3Δ1 leu2-3,112 trp1-289 ura3-52 ctf13-30 mad2-1*	This study
A364A	*MAT***a** *ade1-1 ade2-1 tyr1-H1 lys2-1 his7-H1 ura1-H1*	L. Hartwell
MS1357	*MAT*α *trp1Δ1 ura3-52 leu2-3,112 kar3-1*	M. Rose
R95-1-1-1	*MAT***a** *cbf1Δ ura3-52 leu2-3,112 ade2-1 ade3-1 cyh2 his4*	R. Baker
W303a	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
YPH1161	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 skp1-1::TRP1 skp1-4::LEU2* CFIII (CEN3.L.YPH983)	P. Hieter
YPH636	*MAT***a** *ade2-101 his3Δ200 leu2Δ1 lys2-801 ura3-52 trp1Δ63 mck1::HIS3*	P. Hieter

Plasmid	Purpose	Source
pΔsdb24:URA3	*slk19::URA3*	Joe Donovan
pDB110	YIp *LEU2 CYH2*	This study
pDB112	YIp *CTF13 LEU2 CYH2*	This study
pDB117	*mif2* 3′Δ in pPW66R	This study
pDB118	*cep3* 3′Δ in pPW66R	This study
pDB119	*cse4* 3′Δ in pPW66R	This study
pDB120	*skp1* 3′Δ in pPW66R	This study
pDB130	*ndc10* 3′Δ in pPW66R	This study
pDB131	*ctf13* 3′Δ in pPW66R	This study
pDB133	YIp*MIF2 LEU2 CYH2*	This study
pPW66R	YIp *URA3* degron vector plasmid	A. Varshavsky
pRC10.1	*mad2*Δ in Bluescript	R. Li

contained the deletion allele. Viable cells were contaminating wild-type spores or diploid cells, both of which were cycloheximide sensitive. The germinated cells were grown in YM-1 medium containing cycloheximide and any contaminating wild-type cells could not enter the cell cycle and did not add significantly to the population of cells in the analysis. Germinating the spores in rich medium resulted in reliable synchrony and the *ste5-1* mutation prevented mating so that we could monitor changes in ploidy in haploid cells.

Protein transfer: Protein transfer and detection with antibodies was performed as described previously (Tavormina and Burke 1998). A mouse monoclonal anti-HA antibody 12CA5 (Babco) was used for detection in combination with enhanced chemilumiscence (Amersham, Buckinghamshire, UK).

Immunofluorescence: Antitubulin immunofluorescence was performed essentially as described previously (Adams and Pringle 1984). The antitubulin antibody YOL3/4 (Serotec, Oxford, UK) was cultured supernatant and was used undiluted. The monoclonal antibody was detected using a CY3 conjugated rabbit anti-rat antibody (Molecular Probes, Eugene, OR). Cells were mounted with Vecta-Shield (Vector Technologies) to prevent photobleaching during examination.

Degron experiments: Cells were grown to 1×10^7 cells/ml at 23° in YM-1 medium containing 2% glucose and 100 μM $CuSO_4$. The mating pheromone α-factor was added and cells were incubated for 3 hr, washed three times in 50 ml of water to remove any residual copper, and resuspended in SC medium containing 2% glucose plus α-factor. Cells were incubated at 37° for 90 min to destroy the degron-tagged protein and were released into the cell cycle at 37° by adding pronase to 50 μg/ml. Samples were taken at 30-min intervals and processed for flow cytometry.

RESULTS

Temperature-sensitive *ctf13-30* mutants delay in the cell cycle because of the spindle checkpoint (Wang and Burke 1995; Pangilinan and Spencer 1996). The activity of the spindle checkpoint in kinetochore mutants is easily assayed by constructing double mutants that lack one of the checkpoint genes, such as *MAD2*, and by growing the double mutants under conditions where they are limited for kinetochore protein function. The double mutants die because the spindle checkpoint is required for viability under these conditions. We confirmed that the spindle checkpoint is active in temperature-sensitive kinetochore mutants by assaying double mutants constructed by targeted deletion of *MAD2*. We grew cells at a semipermissive temperature and determined the viability by spotting serial dilutions of cells onto plates. Figure 1 shows the growth of the CBF3 mutants *cep3-2*, *skp1-4*, and *ctf13-30*. In each case the double mutants were compromised for growth compared to the single mutant. By examining budding morphology, we confirmed that the accumulation of large budded cells that is evident in all of the single mutants is eliminated in the double mutants (not shown). These data show that the kinetochore mutants activate the spindle checkpoint and that checkpoint activity is required for maximal viability.

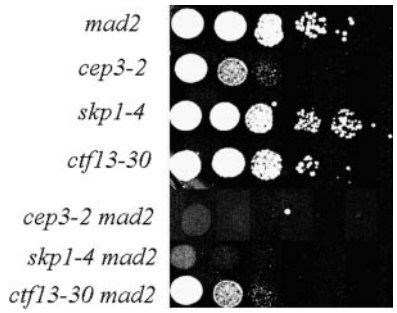

Figure 1.—CBF3 mutants interact with the spindle checkpoint. Tenfold serial dilutions of cells were spotted onto YPD plates and grown at 32°. In each case, the double mutant is compromised for growth compared to the single mutants.

The checkpoint is unaffected when nonessential kinetochore genes are deleted: We assayed the presence of the checkpoint in deletion mutants for all of the nonessential genes that encode kinetochore proteins. Deleting most of the nonessential genes that encode kinetochore proteins results in sensitivity to the drug benomyl that causes microtubule depolymerization (Foreman and Davis 1993; Hyland *et al.* 1999; Poddar *et al.* 1999). If the benomyl sensitivity is due to the loss of the spindle checkpoint, then mutants should be unable to arrest in the cell cycle in response to nocodazole, a similar microtubule inhibitor. We grew cells from the *cbf1*, *mcm21*, and *ctf19* deletion mutants, treated them with nocodazole, and analyzed them by flow cytometry. In these and other experiments we also compared, where possible, the percentage of arrested cells (large buds and undivided nuclei) from each mutant to the percentage of arrested cells of each mutant in a *mad2* background, to demonstrate that any arrest observed was checkpoint dependent. The majority of cells accumulated with a 2C content of DNA, suggesting that the mutant arrested in the cell cycle and that the spindle checkpoint was intact (Figure 2, A–C; 68% of *cbf1* cells were arrested *vs.* 20% of *cbf1 mad2* cells (data not shown). Double mutants were not possible for *mcm21* and *ctf19* because the double mutants are inviable (data not shown). In addition, we tested deletion mutants for nonessential genes that have been implicated in kinetochore function. *SLK19* encodes a protein that associates with the kinetochore and is required for spindle integrity (Zeng *et al.* 1999). *MCK1* encodes a protein kinase that was identified as a multicopy suppressor of the *ndc10-42* mutant (Jiang *et al.* 1995). *KAR3* encodes a kinesin-like protein that has been implicated in kinetochore function (Middleton and Carbon 1994). The spindle checkpoint was unaffected by deleting *SLK19*, *MCK1*, or *KAR3* (Figure 2, D–F. *slk19* cells showed a 91% arrest *vs.* 22% in *slk19 mad2* cells (data not shown). *mck1* cells showed a 65% arrest *vs.* an 18% arrest in *mck1 mad2* cells (data not shown). Double mutants were not possible for *kar3* because they are inviable. None of the six nonessential genes that encode either kinetochore

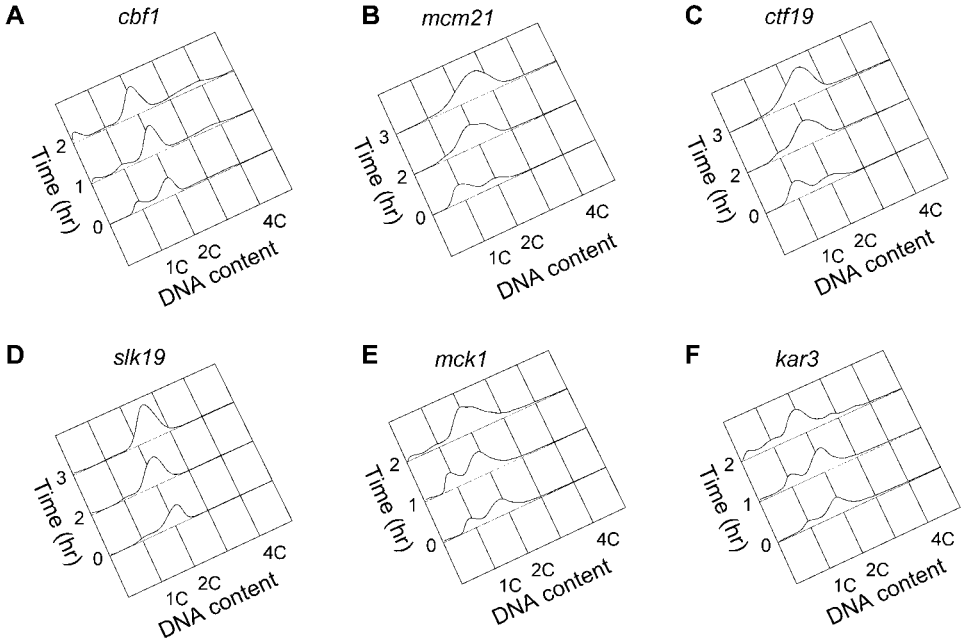

FIGURE 2.—Response of deletion mutants to checkpoint activation. Strains with deletion mutations (A) *cbf1* (2264), (B) *mcm21* (2269), (C) *ctf19* (2268), (D) *slk19* (2272), (E) *mck1* (YPH636), and (F) *kar3* (MS1353) were grown to midlog (time 0) at 30° and nocodazole was added to 15 μg/ml. Cells were collected at the indicated times (hours), stained with propidium iodide, and analyzed by flow cytometry.

proteins or proteins that interact with kinetochores is required for the activity of the spindle checkpoint.

The checkpoint is eliminated when Cep3p is missing: We constructed degron-tagged alleles of three of the essential kinetochore genes. The efficiency of protein degradation in the three different degron mutants is shown in Figure 3. The degron-tagged protein fusion is detected by anti-HA Western blots when grown at the permissive temperature in the presence of copper. When grown in the absence of copper at the restrictive temperature, the fusion proteins are degraded within 90 min.

We adopted a standard assay to determine if the degron mutants lacked checkpoint activity. We synchronized cells by arresting them with the mating pheromone and then induced protein degradation for 90 min. We released the cells into the cell cycle in the absence of the kinetochore protein and assayed cell cycle progression by flow cytometry. An example for the degron-tagged *ndc10* mutant is shown in Figure 4B. The DNA is asymmetrically distributed to the daughter cells when the kinetochores do not attach and the spindle checkpoint does not restrain mitosis. The cells divide and enter the subsequent cell cycle to re-replicate the DNA to produce some cells with greater than 2C content of DNA and most of the remaining cells have a less than 1C content of DNA. We interpret the changes in ploidy to mean that the kinetochores are unable to attach to the mitotic spindle and the chromosomes are asymmetrically distributed to the daughter cells. In addition, the spindle checkpoint is inactive and fails to respond to the unattached chromosomes, resulting in re-replication of the genome producing some cells with a greater than 2C content of DNA. Furthermore, the phenotype of the degron-tagged *ndc10* allele is indistinguishable from the *ndc10-1* mutant, showing that the missense mutant has the null phenotype.

We tested other kinetochore components with similar expectations. The missense temperature-sensitive mutations (for example, *cep3-2*) result in cell cycle arrest due to checkpoint activation (STRUNNIKOV *et al.* 1995; WANG and BURKE 1995; PANGILINAN and SPENCER 1996; this study). If this were the null phenotype, we would expect that the degron mutants should similarly arrest in the cell cycle. The data for the degron-tagged *cep3* mutant are shown in Figure 4C. The mutant has a phenotype

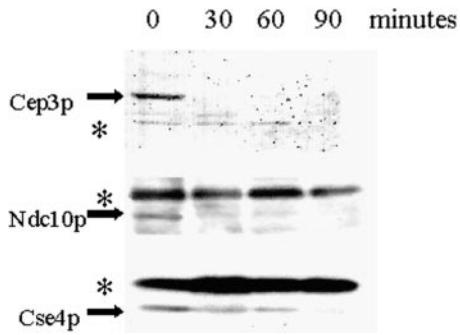

FIGURE 3.—Stability of Cep3p, Ndc10p, and Cse4p fusion proteins in their respective degron mutants. Cells from degron strains 2145 (*cep3*), 2174 (*ndc10*), and 2162 (*cse4*) were grown in YM-1 medium containing copper at 23° to 10^7 cells per milliliter. A protein sample was prepared from cells expressing the fusion protein at 23° (0 min). The cells were washed three times and resuspended in SC-ura lacking copper and cells were incubated at 37° to initiate proteolysis of the fusion protein. Protein samples were prepared from cells taken at 30, 60, and 90 min. The fusion protein was identified using a monoclonal anti-HA antibody after PAGE and transferring the proteins to a membrane. Asterisks indicate nonspecific bands of similar size that interact with the anti-HA antibody.

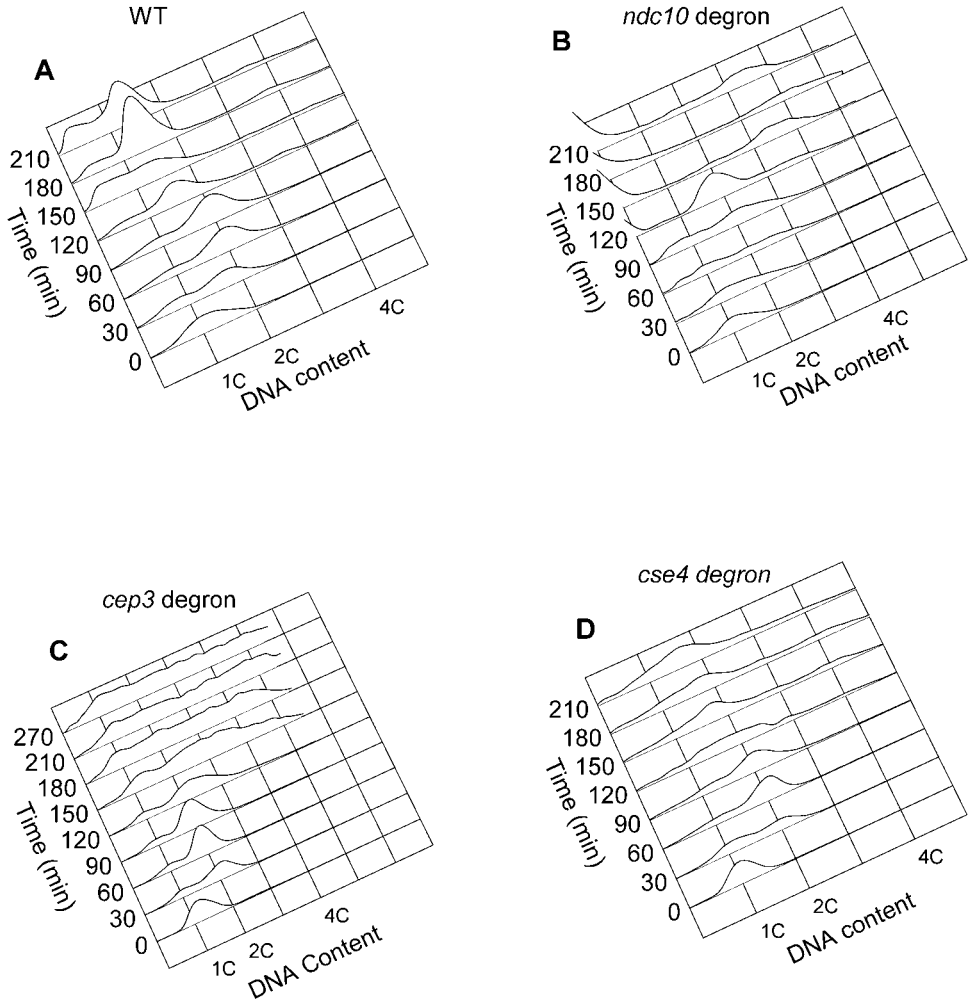

FIGURE 4.—The spindle checkpoint is inactive in the *ndc10* and *cep3* degron mutants, but is active in the *cse4* degron mutant. Wild-type cells from strain 2124 or mutant cells from degron strains 2158 (*ndc10*^{td}), 2145 (*cep3*^{td}), or 2162 (*cse4*^{td}) were grown in YM-1 medium containing copper at 23° to 10^7 cells per ml and α-factor was added to arrest cells. The cells were washed three times in water and resuspended in SC-ura lacking copper and α-factor and cells were incubated at 37°. After 90 min ($t = 0$), pronase was added to the medium to inactivate the α-factor and cells were allowed to re-enter the cell cycle. Samples were taken at the indicated times (minutes) and DNA content in the cells was determined by flow cytometry. (A) Wild-type. (B) *ndc10*^{td}. (C) *cep3*^{td}. (D) *cse4*^{td}.

similar to the *ndc10* degron allele. Cells are not restrained in the cell cycle but divide and some cells rereplicate the DNA in the subsequent cell cycle. The *cep3* degron mutant does not produce a phenotype as severe as the *ndc10-1* or the *ndc10* degron allele. There is a broader distribution of cells with increased ploidy and fewer aploid cells. The low degree of aploidy was confirmed by examining the DNA distribution in dividing cells by microscopy (data not shown). We interpret this to mean that the *cep3* degron fails to destroy all of the Cep3 fusion protein. The result is that the degree of nondisjunction is less severe than in the *ndc10* mutants. Regardless, kinetochores lacking Cep3p do not induce a spindle checkpoint arrest. Checkpoint activity within the kinetochore depends on both Ndc10p and Cep3p.

The checkpoint is intact when Cse4p is eliminated: A histone H3 variant, Cse4p, is present at the kinetochores of yeast and is thought to form a specialized nucleosome required for kinetochore function (MELUH *et al.* 1998). Missense Ts$^-$ mutants show a prominent cell cycle delay at the restrictive temperature and the delay is dependent on the spindle checkpoint. We constructed a degron-tagged allele of *CSE4* and determined whether the missense mutants had the null phenotype and whether the checkpoint was intact in the absence of Cse4p. The data in Figure 4D show that the majority of cells from the *cse4* degron mutant accumulate with a 2C content of DNA. More than 80% of the cells in each sample, beginning at 60 min ($n = 500$ for each sample), were large budded with a single undivided nucleus. A *mad2* deletion in the degron-tagged *cse4* strain resulted in only 6% of the cells accumulating with a large bud and an undivided nucleus. We conclude that the missense *cse4* mutants have the null phenotype and that the spindle checkpoint is intact in the absence of Cse4p.

We could not produce degron-fusion proteins, useful for these studies, using two of the kinetochore genes. The amino terminal fusion of the degron to Mif2p results in lethality. The degron fusion to Ctf13p did not result in temperature sensitivity. Excess expression of Ubr1p, a ubiquitin ligase, can improve the efficiency of proteolysis of degron-tagged proteins (LABIB *et al.* 1999). We found that excess expression of Ubr1p in the strain containing the Ctf13p degron caused the cells to be Ts$^-$. However, the cells could form small microcolonies of ~50 cells at the restrictive temperature. Therefore, we could not use the Ctf13 degron-tagged allele for cell synchrony experiments.

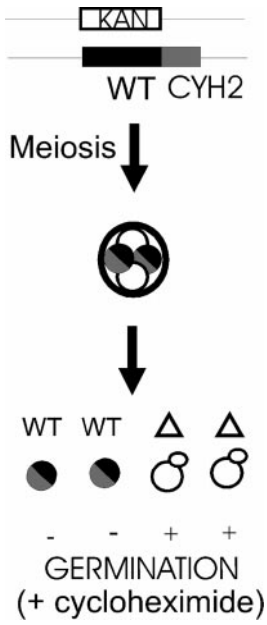

FIGURE 5.—Isolating deletion mutants. Deletion mutations were constructed by targeted integration of the kanamycin resistance gene by PCR amplification from pFA6a-KanMX. The deletions were constructed in diploid strain (2239) that was homozygous *leu2*, *cyh2*, and *ste5*. A fragment of the 3′ end of the target gene was generated by PCR and cloned into plasmid pDB110, a YIp plasmid containing *CYH2* and *LEU2*. The plasmid was targeted to integrate into the heterozygous diploid cell to integrate into the wild-type allele. The resulting diploid was sporulated and germinated in YM-1 medium containing glucose and cycloheximide. The only cells that germinate are the deletion mutants. These cells can be purified away from the wild-type spores.

An alternative method for constructing null mutants: We developed a new method to recover null mutants (Figure 5). In this method, we modify "your favorite gene" (*YFG1*) to create and sporulate a heterozygous diploid strain Δ*yfg1/YFG1-CYH2* (see MATERIALS AND METHODS). The diploid is also homozygous for *cyh2* and the temperature-sensitive *ste5-1*. The strain is sporulated and two types of spores are produced. The spores containing the wild-type allele of the gene of interest are *CYH2* and therefore cycloheximide sensitive. The spores containing the deletion allele are cycloheximide resistant. Germination requires protein synthesis (HERMAN and RINE 1997). Therefore only the spores containing the deletion allele can germinate in the presence of cycloheximide. Spores are hydrophobic and can be purified away from cells on the basis of their hydrophobicity. The purified germinated spores were grown at 36° to inactivate Ste5p and prevent mating. The resulting haploid cells were analyzed by flow cytometry to determine the effect of the null allele on cell cycling and to determine if there is a change of ploidy in cells that lack the protein.

We purified spores with a null allele of *MIF2* and analyzed the mutant cells by flow cytometry. The data in Figure 6B show that the *mif2::KAN* mutant arrests in the cell cycle with a 2C content of DNA. The cells had an undivided nucleus, suggesting that the cells were arrested in mitosis. We stained cells with antitubulin antibodies and found that the arrested cells had typical-looking mitotic spindles with a mean length of 1.9 ± 0.4 μm ($n = 50$). Therefore cells arrest prior to anaphase in a *mif2* null mutant.

We also used the strategy to construct a null allele of *CTF13* and recovered mutant cells for flow cytometry (Figure 6D). The cells were clearly cycling as approximately half of the cells passed through the G2/M stage of the cell cycle (9 hr). We spread unpurified spores from the diploid onto a YPD plate containing cycloheximide and determined the fate of the cells after 12 hr of incubation (Figure 6E). Half of the spores (wild type) did not germinate as expected and produced cells with one cell body. The *ctf13::KAN* cells divided 2–3 times after germinating before finally ceasing cell division. The cells did not uniformly accumulate at a stage in the cell cycle as determined by bud morphology. Therefore, the cells were inviable due to a loss of Ctf13p and therefore ceased dividing, but the cells did not arrest homogenously in the cell cycle. We conclude that Ctf13p, like Ndc10p and Cep3p, two other proteins in the CBF3 complex, is required for spindle checkpoint activity.

DISCUSSION

CBF3 is required for spindle checkpoint activity: We have analyzed null mutants to determine which kinetochore proteins are required for spindle checkpoint activity. *NDC10*, *CEP3*, and *CTF13* are required for the spindle checkpoint. *CSE4*, *MIF2*, *CBF1*, *MCM21*, *CTF19*, *SLK19*, *MCK1*, and *KAR3* are not required for checkpoint function. We conclude that the kinetochore plays an active role in the spindle checkpoint in *S. cerevisiae* and that the activity is dependent on the CBF3 complex within the kinetochore.

Our data show that the *ndc10* degron-tagged allele has the same phenotype as *ndc10-1*, a temperature-sensitive missense mutant. The spindle checkpoint is eliminated in cells lacking Ndc10p. Both the *ndc10* and *cep3* degron-tagged mutants lacked the spindle checkpoint and showed dramatic genetic instability as assayed by flow cytometry. Therefore eliminating Cep3p has the same effect as eliminating Ndc10p: the spindle checkpoint is nonfunctional. We constructed a degron-tagged allele of the fourth CBF3 gene, *SKP1,* but the experiments were complicated to execute and interpret because of the dual requirement of Skp1p in the cell cycle (BAI *et al.* 1996; CONNELLY and HIETER 1996). We conclude that the CBF3 components Ndc10p and Cep3p are required for spindle checkpoint activity in *S. cerevisiae.*

We obtained a different phenotype with the *ctf13* null mutant compared to *ndc10* and *cep3* null alleles. Cells lacking Ctf13p were isolated after sporulating a hetero-

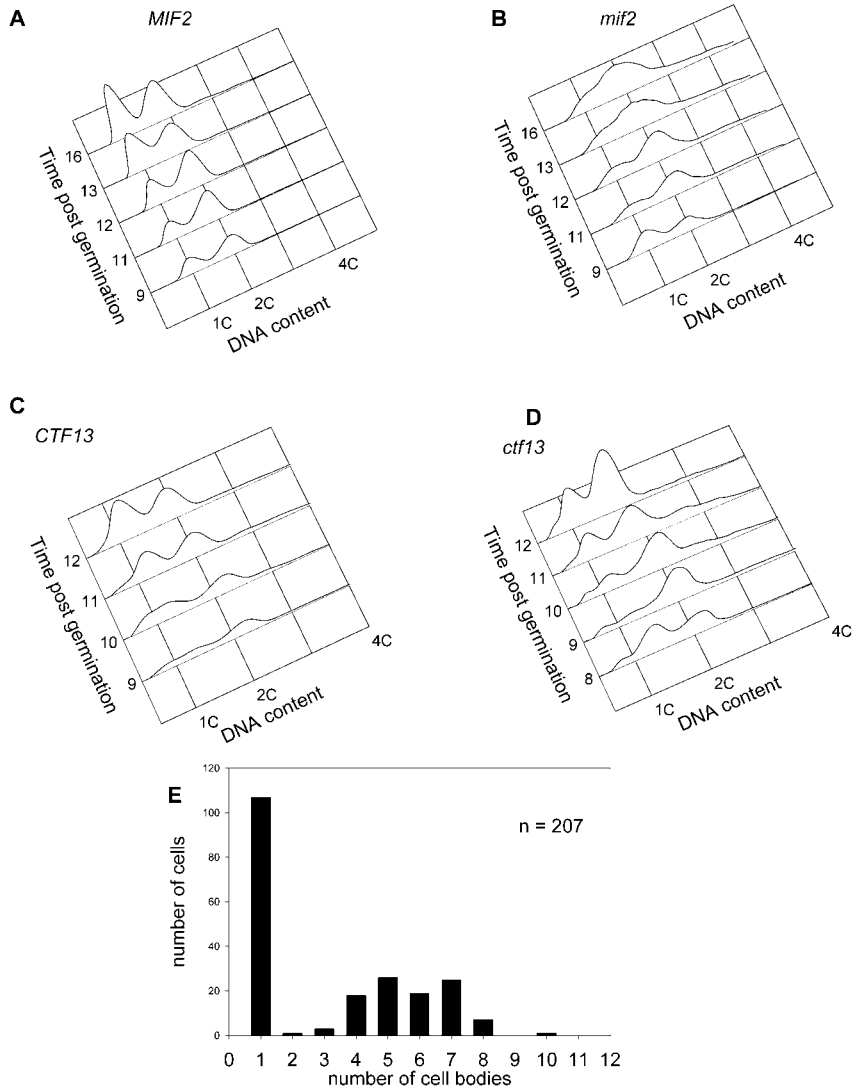

FIGURE 6.—Analysis of deletion mutants lacking *MIF2* and *CTF13*. Cells containing a *mif2* deletion mutation were purified after sporulating strain 2185 (B) and cells containing a *ctf13* deletion mutation were purified after sporulating strain 2252 (D). Wild-type cells were identified by sporulating isogenic diploids that did not have the deletion mutations (A and C). Cells were grown in YM-1 medium containing glucose plus cycloheximide for the indicated times (hours) and the DNA content was determined by flow cytomtery. Spores from the sporulated diploid strain 2252 (*ctf13/+*) were spread onto a YPD plate containing cycloheximide and incubated at 30° for 24 hr (E). The number of cell bodies was determined by direct observation on the surface of the agar. Approximately one-half of the cells were small, had a single cell body, and were highly refractile, suggesting that they were ungerminated spores.

zygous diploid. The spores containing the deletion allele divided two to three times and did not arrest at mitosis. We interpret the multiple divisions to mean that there is some carryover of the Ctf13p from the heterozygous diploid parent. However, the absence of a homogeneous mitotic arrest indicates that the spindle checkpoint is unaffected by the loss of Ctf13p.

Previous data have also suggested that Ctf13p is required for spindle checkpoint activity. Cells that progress through the cell cycle in the absence of DNA replication have an intact spindle checkpoint that is dependent on Ctf13p (TAVORMINA *et al.* 1997). The data are also consistent with kinetochore assembly experiments. The loss of Ndc10p has a drastic effect on kinetochore structure *in vivo*. Kinetochore proteins, including those in the CBF3 complex, do not localize with centromeric DNA in a *ndc10-1* mutant (ORTIZ *et al.* 1999). This agrees with the results from experiments showing that all of the CBF3 proteins must be present to assemble a stable kinetochore complex *in vitro* (RUSSELL *et al.* 1999). The *in vivo* and the *in vitro* data predict that eliminating one of the CBF3 proteins should eliminate the entire complex. Our data give the expected result; mutations that eliminate CBF3 proteins result in similar phenotypes with respect to checkpoint activity.

The relationship between CBF3 and the spindle checkpoint: What role does CBF3 play in the spindle checkpoint? There is abundant evidence, from a variety of organisms, showing that checkpoint proteins localize to the kinetochore when the spindle checkpoint is active (SKIBBENS and HIETER 1998). The simple explanation is that at least one kinetochore protein interacts with one of the spindle checkpoint proteins and the interaction is required to signal that the chromosome is misaligned. The consequence is that the cell arrests in the cell cycle. Preliminary data from our lab have shown that the checkpoint protein Mad2p associates with the kinetochore and the association is CDEIII dependent (data not shown). This suggests that a CBF3-dependent Mad2p kinetochore interaction is required for spindle checkpoint activity.

We propose three possible roles for CBF3 in the pro-

cess on the basis of the hypothesis that kinetochore association of checkpoint proteins is required for checkpoint activity. The simplest is that one of the CBF3 proteins directly interacts with Mad2p (or other checkpoint proteins) and when CBF3 is missing the interaction cannot occur. The second possibility is that the loss of CBF3 from the kinetochore indirectly affects the interaction of the kinetochore with checkpoint proteins. Mif2p, Cse4p, Ctf19p, Mcm21p, and Okp1p do not localize to the kinetochores in a *ndc10-1* mutant, suggesting that kinetochore structure is severely impaired (Meluh and Koshland 1997; Ortiz *et al.* 1999). A loss of any CBF3 component probably eliminates many proteins from the kinetochore. If there is a kinetochore protein that interacts with the spindle checkpoint and is dissociated by a loss of CBF3, then it is not encoded by any of the known kinetochore genes. Null mutations that eliminate each of the other known kinetochore proteins do not eliminate the spindle checkpoint. Therefore, if checkpoint proteins interact with some kinetochore protein, other than a CBF3 component, it has not been identified yet. This is an appealing possibility because the spindle checkpoint is conserved from yeast to humans and the expectation is that a conserved protein that is at the kinetochore plays a role in the spindle checkpoint. Two kinetochore proteins that have obvious mammalian homologs (Mif2p and Cse4p) are not required for checkpoint signaling. The third possibility is that the spindle checkpoint signal depends on combinations of the kinetochore proteins that are already known. This model predicts that there must be some redundancy in function to allow checkpoint activity when one protein is absent.

The robust phenotype of checkpoint mutants overcomes limitations in the analysis of null alleles: Both methods that we have chosen to produce null alleles of essential genes have limitations. Deletion mutants isolated after sporulation inherit an unknown amount of wild-type protein from the heterozygous diploid into the mutant spores. The phenotype of cells having a *mif2* deletion mutation is the same as the Ts^- mutants; cells arrest uniformly in the cell cycle. Therefore the Ts^- allele results in the null phenotype and this validates the data produced from analyzing null mutants produced by this method. The important difference in phenotypes between the spores from the *ctf13* null mutant and the spores from the *mif2* null mutant is that the lack of Ctf13p did not result in a uniform cell cycle arrest. A uniform arrest is expected if the mutant cells with defective kinetochores were checkpoint proficient. The different phenotypes associated with deletion mutations allow us to come to opposite conclusions with respect to functions of the two genes in the spindle checkpoint.

The degron-tagged alleles contain an epitope tag to provide an assay for the presence of the protein. However, there are also limitations in this approach. Following the degradation of the protein by Western blots does not assure that 100% of the protein is eliminated. Proteins may assemble only one molecule per kinetochore and it is possible that we could not detect one or two functional kinetochores. Therefore we cannot say with certainty that every kinetochore is affected in a degron-tagged mutant. It is possible that only a few kinetochores are affected in the *cse4* mutant. The mutant arrests in the cell cycle but we cannot say with certainty how many kinetochores lack Cse4p. Cells adopt a uniform cell cycle arrest in a checkpoint-dependent manner when only a single kinetochore is affected (Skibbens and Hieter 1998; Gardner and Burke 2000). In the cases of *cep3* and *ndc10* degron-tagged alleles, the phenotypes are quite different. The effects on chromosome stability are drastic so that we assume a large number of kinetochores are affected in each mutant. The important observation is that the cells fail to arrest uniformly in the cell cycle and therefore the dysfunctional kinetochores fail to induce the spindle checkpoint. The robust nature of the phenotypes has allowed us to assign a role for each kinetochore protein in the spindle checkpoint.

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LITERATURE CITED

Adams, A. E., and J. R. Pringle, 1984 Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. J. Cell Biol. **98:** 934–945.

Amon, A., 1999 The spindle checkpoint. Curr. Opin. Genet. Dev. **9:** 69–75.

Bai, C., P. Sen, K. Hofmann, L. Ma, M. Goebl *et al.*, 1996 SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell **86:** 263–274.

Basu, J., H. Bousbaa, E. Logarinho, Z. Li, B. C. Williams *et al.*, 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.

Bernard, P., K. Hardwick and J. P. Javerzat, 1998 Fission yeast *bub1* is a mitotic centromere protein essential for the spindle checkpoint and the preservation of correct ploidy through mitosis. J. Cell Biol. **143:** 1775–1787.

Brown, M. T., L. Goetsch and L. H. Hartwell, 1993 *MIF2* is required for mitotic spindle integrity during anaphase spindle elongation in *Saccharomyces cerevisiae*. J. Cell Biol. **123:** 387–403.

Burke, D. J., 2000 Complexity in the spindle checkpoint. Curr. Opin. Genet. Dev. **10:** 26–31.

Burke, D., D. Dawson, and T. Stearns, 2000 *Methods in Yeast Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Cahill, D. P., C. Lengauer, J. Yu, G. J. Riggins, J. K. Willson *et al.*, 1998 Mutations of mitotic checkpoint genes in human cancers. Nature **392:** 300–303.

Chan, G. K., S. A. Jablonski, V. Sudakin, J. C. Hittle and T. J. Yen, 1999 Human BUBR1 is a mitotic checkpoint kinase that

monitors CENP-E functions at kinetochores and binds the cyclosome/APC. J. Cell Biol. **146:** 941–954.

Chen, R. H., A. Shevchenko, M. Mann and A. W. Murray, 1998 Spindle checkpoint protein Xmad1 recruits Xmad2 to unattached kinetochores. J. Cell Biol. **143:** 283–295.

Choo, K. H. A., 1997 *The Centromere*. Oxford University Press, Oxford.

Connelly, C., and P. Hieter, 1996 Budding yeast *SKP1* encodes an evolutionarily conserved kinetochore protein required for cell cycle progression. Cell **86:** 275–285.

Doheny, K. F., P. K. Sorger, A. A. Hyman, S. Tugendreich, F. Spencer *et al.*, 1993 Identification of essential components of the *S. cerevisiae* kinetochore. Cell **73:** 761–774.

Dohmen, R. J., P. Wu and A. Varshavsky, 1994 Heat-inducible degron: a method for constructing temperature-sensitive mutants. Science **263:** 1273–1276.

Espelin, C. W., K. B. Kaplan and P. K. Sorger, 1997 Probing the architecture of a simple kinetochore using DNA-protein crosslinking. J. Cell Biol. **139:** 1383–1396.

Foreman, P. K., and R. W. Davis, 1993 Point mutations that separate the role of *Saccharomyces cerevisiae* centromere binding factor 1 in chromosome segregation from its role in transcriptional activation. Genetics **135:** 287–296.

Gardner, R. D., and D. J. Burke, 2000 The spindle checkpoint: two transitions, two pathways. Trends Cell Biol. **10:** 154–158.

Goh, P. Y., and J. V. Kilmartin, 1993 *NDC10*: a gene involved in chromosome segregation in *Saccharomyces cerevisiae*. J. Cell Biol. **121:** 503–512.

Gorbsky, G. J., R. H. Chen and A. W. Murray, 1998 Microinjection of antibody to Mad2 protein into mammalian cells in mitosis induces premature anaphase. J. Cell Biol. **141:** 1193–1205.

Guthrie, C., and G. R. Fink (Editors), 1991 *Guide to Yeast Genetics and Molecular Biology*. Academic Press, San Diego.

Hardwick, K. G., R. Li, C. Mistrot, R. H. Chen, P. Dann *et al.*, 1999 Lesions in many different spindle components activate the spindle checkpoint in the budding yeast *Saccharomyces cerevisiae*. Genetics **152:** 509–518.

Hartwell, L. H., 1967 Macromolecule synthesis in temperature-sensitive mutants of yeast. J. Bacteriol. **93:** 1662–1670.

Herman, P. K., and J. Rine, 1997 Yeast spore germination: a requirement for Ras protein activity during re-entry into the cell cycle. EMBO J. **16:** 6171–6181.

Hyland, K. M., J. Kingsbury, D. Koshland and P. Hieter, 1999 Ctf19p: a novel kinetochore protein in *Saccharomyces cerevisiae* and a potential link between the kinetochore and mitotic spindle. J. Cell Biol. **145:** 15–28.

Jiang, W., M. Y. Lim, H. J. Yoon, J. Thorner, G. S. Martin *et al.*, 1995 Overexpression of the yeast *MCK1* protein kinase suppresses conditional mutations in centromere-binding protein genes *CBF2* and *CBF5*. Mol. Gen. Genet. **246:** 360–366.

Kallio, M., J. Weinstein, J. R. Daum, D. J. Burke and G. J. Gorbsky, 1998 Mammalian p55CDC mediates association of the spindle checkpoint protein Mad2 with the cyclosome/anaphase-promoting complex, and is involved in regulating anaphase onset and late mitotic events. J. Cell Biol. **141:** 1393–1406.

Labib, K., J. F. Diffley and S. E. Kearsey, 1999 G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus. Nat. Cell. Biol. **1:** 415–422.

Lechner, J., 1994 A zinc finger protein, essential for chromosome segregation, constitutes a putative DNA binding subunit of the *Saccharomyces cerevisiae* kinetochore complex, Cbf3. EMBO J. **13:** 5203–5211.

Meluh, P. B., and D. Koshland, 1997 Budding yeast centromere composition and assembly as revealed by in vivo cross-linking. Genes Dev. **11:** 3401–3412.

Meluh, P. B., P. Yang, L. Glowczewski, D. Koshland and M. M. Smith, 1998 Cse4p is a component of the core centromere of *Saccharomyces cerevisiae*. Cell **94:** 607–613.

Middleton, K., and J. Carbon, 1994 KAR3-encoded kinesin is a minus-end-directed motor that functions with centromere binding proteins (CBF3) on an *in vitro* yeast kinetochore. Proc. Natl. Acad. Sci. USA **91:** 7212–7216.

Ortiz, J., O. Stemmann, S. Rank and J. Lechner, 1999 A putative protein complex consisting of Ctf19, Mcm21, and Okp1 represents a missing link in the budding yeast kinetochore. Genes Dev. **13:** 1140–1155.

Pangilinan, F., and F. Spencer, 1996 Abnormal kinetochore structure activates the spindle assembly checkpoint in budding yeast. Mol. Biol. Cell **7:** 1195–1208.

Poddar, A., N. Roy and P. Sinha, 1999 *MCM21* and *MCM22*, two novel genes of the yeast *Saccharomyces cerevisiae* are required for chromosome transmission. Mol. Microbiol. **31:** 349–360.

Russell, I. D., A. S. Grancell and P. K. Sorger, 1999 The unstable F-box protein p58-Ctf13 forms the structural core of the CBF3 kinetochore complex. J. Cell Biol. **145:** 933–950.

Sassoon, I., F. F. Severin, P. D. Andrews, M. R. Taba, K. B. Kaplan *et al.*, 1999 Regulation of *Saccharomyces cerevisiae* kinetochores by the type 1 phosphatase Glc7p. Genes Dev. **13:** 545–555.

Skibbens, R. V., and P. Hieter, 1998 Kinetochores and the checkpoint mechanism that monitors for defects in the chromosome segregation machinery. Annu. Rev. Genet. **32:** 307–337.

Smith, M. M., 1991 Mutations affecting nuclear proteins in yeast. Methods Cell Biol. **35:** 485–523.

Stoler, S., K. C. Keith, K. E. Curnick and M. Fitzgerald-Hayes, 1995 A mutation in *CSE4*, an essential gene encoding a novel chromatin-associated protein in yeast, causes chromosome nondisjunction and cell cycle arrest at mitosis. Genes Dev. **9:** 573–586.

Strunnikov, A. V., J. Kingsbury and D. Koshland, 1995 *CEP3* encodes a centromere protein of *Saccharomyces cerevisiae*. J. Cell Biol. **128:** 749–760.

Tavormina, P. A., and D. J. Burke, 1998 Cell cycle arrest in *cdc20* mutants of *Saccharomyces cerevisiae* is independent of Ndc10p and kinetochore function but requires a subset of spindle checkpoint genes. Genetics **148:** 1701–1713.

Tavormina, P. A., Y. Wang and D. J. Burke, 1997 Differential requirements for DNA replication in the activation of mitotic checkpoints in Saccharomyces cerevisiae. Mol. Cell. Biol. **17:** 3315–3322.

Taylor, S. S., and F. McKeon, 1997 Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. Cell **89:** 727–735.

Wach, A., A. Brachat, R. Pohlmann and P. Philippsen, 1994 New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast **10:** 1793–1808.

Wang, Y., and D. J. Burke, 1995 Checkpoint genes required to delay cell division in response to nocodazole respond to impaired kinetochore function in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:** 6838–6844.

Yu, H. G., M. G. Muszynski and R. Kelly Dawe, 1999 The maize homologue of the cell cycle checkpoint protein MAD2 reveals kinetochore substructure and contrasting mitotic and meiotic localization patterns. J. Cell Biol. **145:** 425–435.

Zeng, X., J. A. Kahana, P. A. Silver, M. K. Morphew, J. R. McIntosh *et al.*, 1999 Slk19p is a centromere protein that functions to stabilize mitotic spindles. J. Cell Biol. **146:** 415–425.

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