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Saccharomyces cerevisiae BUB2 Prevents Mitotic Exit in Response to Both Spindle and Kinetochore Damage

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ABSTRACT

The spindle assembly checkpoint-mediated mitotic arrest depends on proteins that signal the presence of one or more unattached kinetochores and prevents the onset of anaphase in the presence of kinetochore or spindle damage. In the presence of either damage, *bub2* cells initiate a preanaphase delay but do not maintain it. Inappropriate sister chromatid separation in nocodazole-treated *bub2* cells is prevented when mitotic exit is blocked using a conditional *tem1*[c] mutant, indicating that the preanaphase failure in *bub2* cells is a consequence of events downstream of *TEM1* in the mitotic exit pathway. Using a conditional *bub2*[tsd] mutant, we demonstrate that the continuous presence of Bub2 protein is required for maintaining spindle damage-induced arrest. *BUB2* is not required to maintain a DNA damage checkpoint arrest, revealing a specificity for spindle assembly checkpoint function. In a yeast two-hybrid assay and *in vitro*, Bub2 protein interacts with the septin protein Cdc3, which is essential for cytokinesis. These data support the view that the spindle assembly checkpoint encompasses regulation of distinct mitotic steps, including a *MAD2*-directed block to anaphase initiation and a *BUB2*-directed block to *TEM1*-dependent exit.

THE cell division cycle in most eukaryotes is tightly regulated by checkpoints that monitor and ensure appropriate passage past critical steps essential for successful cell division. The spindle assembly checkpoint serves to arrest cells at prometaphase until each sister chromatid pair has achieved bipolar attachment to the spindle (Rieder and Salmon 1998; Skibbens and Hieter 1998).

Chromatid attachment to the spindle can be affected by impaired spindle microtubules or kinetochores, and the spindle assembly checkpoint is experimentally induced using antimicrotubule drugs such as nocodazole or conditional alleles that interfere with kinetochore protein function. A single unattached kinetochore appears to emit an inhibitory signal through the spindle assembly checkpoint pathway that prevents the onset of anaphase and subsequent mitotic events (Spencer and Hieter 1992; Nicklas *et al.* 1995; Rieder *et al.* 1995). In *Saccharomyces cerevisiae*, the *MAD1-3* (Li and Murray 1991) and the *BUB1-3* (Hoyt *et al.* 1991) genes were first identified through genetic screens for the inability to delay or arrest in the presence of microtubule-damaging drugs. When kinetochore structure is compromised *S. cerevisiae* cells arrest prior to anaphase despite having intact microtubules, and this arrest is dependent on a subset set of genes that are involved in the response to spindle damage induced by antimicrotubule drugs (Wang and Burke 1995; Pangilinan and Spencer 1996). Mad1, Mad2, Bub1, and Bub3 proteins have been shown to localize to kinetochores in experimental systems that permit immunocytochemical localization of chromosomal structures (reviewed by Skibbens and Hieter 1998). The kinetochore localization and associated biochemical properties of this group of proteins have suggested a model wherein an unattached kinetochore emits a signal that prevents Cdc20 protein from directing the anaphase promoting complex (APC)-mediated degradation of the anaphase inhibitor Pds1 protein and, consequently, metaphase-to-anaphase transition (Cohen-Fix *et al.* 1996; Visintin *et al.* 1997; Elledge 1998; Lim *et al.* 1998; Schott and Hoyt 1998). This aspect of the spindle assembly checkpoint is highly conserved, supported by observations of functional interactions between *MAD2* and *CDC20* in widely divergent organisms (Fang *et al.* 1998; Hwang *et al.* 1998; Kallio *et al.* 1998; Kim *et al.* 1998).

In contrast, the role of the Bub2 protein has only recently begun to emerge. Like the other *BUB* and *MAD* genes, it is not required for cell viability in budding yeast and its absence causes a hypersensitivity to microtubule-depolymerizing drugs (Hoyt *et al.* 1991). Unlike the other *MAD* and *BUB* genes, the *bub2*Δ mutant exhibits a partial preanaphase arrest (Pangilinan and Spencer 1996). Several recent studies have implicated *BUB2* in the control of a mitotic regulatory step independent of *MAD2* in the presence of spindle damage (Alexandru *et al.* 1999; Fesquet *et al.* 1999; Fraschini *et al.* 1999; Li 1999). The late regulatory step is proposed to be exit from mitosis as defined by a decrease in M-phase-specific cyclin-dependent kinase activity, controlled by an elabo-

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rate "mitotic exit network" (Jasperson *et al.* 1998). The execution of mitotic exit in *S. cerevisiae* depends on regulated GTPase (*TEM1*), protein kinase (*CDC15*, *CDC5*, *DBF2*, *DBF20*), and phosphatase (*CDC14*) activities that ultimately control an *HCT1*-associated form of the anaphase promoting complex (Peters 1999). APC^{HCT1}-directed polyubiquitination of CLB2 leads to its destruction and establishment of G1 cyclin-associated forms of cyclin-dependent kinase (Morgan 1999).

A role for *S. cerevisiae BUB2* in mitotic exit is supported by evidence from characterization of the *Schizosaccharomyces pombe* homolog *cdc16*$^{+}$, which has been shown to regulate both Clb2 protein degradation and cytokinesis (Fankhauser *et al.* 1993). The *S. cerevisiae BUB2* gene complements the temperature-sensitive allele *cdc16-116* in *S. pombe* (Fankhauser *et al.* 1993). The possibility that *BUB2* serves to prevent premature exit from mitosis when anaphase is inhibited has served as a working hypothesis.

This study uses synchronized budding yeast strains to show that *bub2*Δ mutants are unable to maintain a mitotic arrest when kinetochore damage is induced. This is significant to our current understanding of the role of *BUB2*, as it has been suggested that *BUB2* is not involved in the cellular response to kinetochore damage but rather senses another (as yet undefined) aspect of spindle structure or function (Wang and Burke 1995; Tavormina and Burke 1998; Li 1999). We show that the inappropriate sister chromatid separation in the presence of spindle damage in *bub2*Δ mutants is dependent on the *TEM1*-mediated signaling of mitotic exit. In addition, we have constructed a conditional *BUB2* allele (BUB2^{tsd}) and used it to show that the continuous presence of Bub2 protein is required for spindle assembly checkpoint-mediated arrest to be maintained. *BUB2* is not required for the maintenance of the DNA damage checkpoint-mediated mitotic arrest induced in *cdc13-1* mutants, precluding a global role for *BUB2* in maintaining mitotic arrest in response to diverse triggers. In a yeast two-hybrid screen with Bub2 protein as bait, the septin *CDC3* was identified. Recombinant Bub2 and Cdc3 proteins also associate *in vitro*. Cdc3 belongs to the highly conserved septin family of proteins essential for cytokinesis (Kim *et al.* 1991; Field and Kellog 1999). These results suggest the possibility that the interaction between Cdc3 and Bub2 proteins links the spindle assembly checkpoint to a septin-mediated role in cytokinesis or mitotic exit.

MATERIALS AND METHODS

Plasmids and strains: The bub2^{tsd} allele, a conditional allele with a permisive temperature of 25° and restrictive temperature of 37°, was constructed in plasmid pRaj154 (*BUB2* promoter-*UBIQUITIN-DHFR*^{ts}-*BUB2*-pRS304) by combining the following fragments in a four-way ligation: (i) a 600-bp segment from immediately upstream of the *BUB2* open reading frame generated by PCR using oligonucleotides OLF208 (5′-gactacgagctcgctgttgacgggggctcta-3′) and OLF209 (5′-cggaattc gcaaaagttaacaag-3′) and cut with *Sac*I and *Sal*I; (ii) an *Eco*RI/*Hin*dIII fragment from pJW8 (Dohmen *et al.* 1994) carrying the ubiquitin-DHFR^{ts} fusion moiety; (iii) a 474-bp NH_2-terminal segment of the *BUB2* open reading frame generated by PCR using oligonucleotides OLF210 (5′-cccaagcttatgacctcaatt gaa gat-3′) and OLF211 (5′- acgcgtcgacagtgaaaagttga tacg-3′) and cut with *Hin*dIII and *Sal*I; and (iv) *Sac*I- and *Sal*I-treated pRS304 (Sikorski and Hieter 1989) whose unique ApaI site had been destroyed. Plasmid pWS103 (Shou *et al.* 1999), carrying a conditional degron allele of *TEM1* (designated as *tem1*^{c} in the integrating vector pRS304; Sikorski and Hieter 1989), was a gift from R. Deshaies. It encodes a *UBIQUITIN-LacI-Tem1* fusion gene driven by the galactose responsive GAL1 promoter.

For yeast two-hybrid analysis, the GAL4 DNA-binding domain was fused with a full-length *BUB2* open reading frame in plasmid pRaj72, constructed by ligation of a 937-bp PCR amplification product from genomic DNA (using oligonucleotides OLF161 (5′-atccgtcgacccatggagatgacctcaattgaagatctgata-3′) and OLF162 (5′-catgccatgggcgtcgacttacggtatatatatgtct gggt-3′) into *Nco*I-*Sal*I-cut pAS2 (gift of S. Elledge) as a *Nco*I-*Sal*I fragment. Yeast two-hybrid host strains and control plasmids were obtained from S. Elledge (Bai and Elledge 1997) and from CLONTECH (Palo Alto, CA). The GST-CDC3 fusion encoded in plasmid pRaj134 was constructed by PCR amplification of the *CDC3* open reading frame from YRaj127 using oligonucleotides OLF229 (5′-cgcgccatggaggaattcatgagtttaaag gaggaacacgtg-3′) and OLF230 (5′-ggcgtcgacctaacgtaaaaatcc cttcttc-3′) cloned into *Eco*RI/*Sal*I-digested pGEX4T-1 (Pharmacia, Piscataway, NJ).

Yeast transformation and genetic manipulation were performed according to published methods (Guthrie and Fink 1991). Experimental and control strain sets within any single experiment were isogenic derivatives related by DNA-mediated transformation or backcrossing. Yeast strains and genotypes are shown in Table 1. Strain YRaj132, carrying the *BUB2*^{tsd} allele, was generated by transforming YRaj127 with ApaI-linearized pRaj154, followed by selection for *TRP1*. This strain expresses the *BUB2*^{tsd} from a natural *BUB2* promoter and also carries, in tandem, a nonfunctional truncated *BUB2* gene ending at amino acid 158. Strains YRaj144 (*cdc13-1 bub2*Δ) and YRaj140 (*cdc13-1*) were generated by transforming *Xho*I-linearized pVL451 (gift of V. Lundblad) into YRaj130 and YRaj127, respectively, under selection for *URA3*. 5-Fluoroorotic acid-resistant derivatives were subsequently screened for temperature sensitivity at 37°. The introduction of the *bub2*Δ*::LEU2* deletion has been described previously (Pangilinan and Spencer 1996). In the case of YFS1214 and YFS1215 used for the green fluorescent protein (GFP) sister chromatid labeling experiments, the *bub2*Δ*::URA3* disruption plasmid pTR24 (Hoyt *et al.* 1991) was used for one-step gene replacement in strains AFS173 and AFS387 (Straight *et al.* 1996; gifts from Straight lab) to introduce *bub2*Δ*::URA3* into wild-type and *mad2-1* backgrounds, respectively. The *mad2*Δ*::HIS3* allele was obtained from M. Mayer and P. Hieter (unpublished results). Conditional Tem1 protein expression was achieved in strains YRaj164, YRaj166, and YRaj169 by transforming strains YFS1214, YFS1215, and AFS387, respectively, with *Eco*RI-linearized pWS103. The *TEM1* conditional degron allele, *tem1*^{c}, disrupted the resident *TEM1* gene. *Tem1*^{c}-carrying haploids were inviable in glucose media and arrested with separated nuclei.

Analysis of new bud formation, mitotic arrest, and viability: Standard cell culture procedures were followed (Guthrie and Fink 1991). For α factor synchronization experiments, log phase cells were incubated with 50 μg/ml α factor (Sigma, St. Louis) for 2 hr at 25° followed by washing and release into appropriate medium at the stated temperatures. For visualiza-

TABLE 1

Yeast strain genotypes

Name	Genotype	Source or reference
AFS173	*MAT***a** *ura3-1 ade2-1 trp1-1 leu2-3,112::lacO-LEU2 his3-11,15::GFP-LacI-HIS3*	Straight *et al.* (1996)
AFS387	*MAT***a** *ura3-1 ade2-1 trp1-1 mad2-1 leu2-3,112::lacO-LEU2 his3-11,15::GFP-LacI-HIS3*	Straight *et al.* (1996)
YRaj121	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ1 leu2Δ1 bub2Δ::LEU2*	This study
YRaj127	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ1 leu2Δ1*	This study
YRaj132	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ1 leu2Δ1* $bub2^{tsd}$*::TRP1*	This study
YRaj140	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ1 leu2Δ1 cdc13-1*	This study
YRaj144	*MAT***a** *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 cdc13-1 bub2Δ::LEU2*	This study
YRaj158	*MAT***a** *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 bub2Δ::LEU2 mad2Δ::H153*	This study
YRaj164	*MAT***a** *ura3-1 ade2-1 trp1-1 bub2Δ::URA3* $tem1^c$*::TRP1 leu2-3,112:: lacO-LEU2 his3-11,15::GFP-LacI-HIS3*	This study
YRaj169	*MAT***a** *ura3-1 mad2-1 ade2-1 trp1-1* $tem1^c$*::TRP1 leu2-3,112::lacO-LEU2 his3-11,15::GFP-LacI-HIS3*	This study
YFS449	*MAT***a** *ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ1 leu2Δ1 ctf13-30 mad2-1* + CFIII(CEN3.L.YPH278) *URA3 SUP11*	Pangilinan and Spencer (1996)
YFS451	*MAT***a** *ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ1 leu2Δ1 mad2-1* + CFIII(CEN3.L.YPH278) *URA3 SUP11*	Pangilinan and Spencer (1996)
YFS822	*MAT***a** *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 bub2Δ::LEU2* + CFIII(D8B.d.YPH281) *URA3 SUP11 LEU2*	This study
YFS884	*MAT***a** *ura3-52 lys2-801 ade2-101 leu2Δ1 ctf13-30* + CFIII(D8B.d.YPH281) *URA3 SUP11 LEU2*	This study
YFS824	*MAT***a** *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1* + CFIII(D8B.d.YPH281) *URA3 SUP11 LEU2*	This study
YFS961	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ1 leu2Δ1 ctf13-30 bub2Δ::LEU2* + CFIII(D8B.d.YPH281) *URA3 SUP11 LEU2*	This study
YFS1214	*MAT***a** *ura3-1 ade2-1 trp1-1 bub2Δ::URA3 leu2-3,112::lacO-LEU2 his3-11,15::GFP-LacI-HIS3*	This study
YFS1215	*MAT***a** *ura3-1 ade2-1 trp1-1 mad2-1 bub2Δ::URA3 leu2-3,112::lacO-LEU2 his3-11,15::GFP-LacI-HIS3*	This study
YFS1280	*MAT***a** *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 mad2Δ::HIS3*	This study

tion of GFP fluorescence, cells were fixed in 4% paraformaldehyde. In experiments using nocodazole, drug concentration was at 15 μg/ml. Cells were prepared for microscopy by fixation in 3.7% formaldehyde in SK (20 mm sorbitol, 50 mm phosphate buffer pH 7.5) and staining with 4′,6-diamidino-2-phenylindole (DAPI) at a final concentration of 300 ng/ml. Very large budded, uninucleate cells, indicating a preanaphase delay or arrest morphology, were defined as mother cells containing a single nuclear DNA mass and with a single daughter bud. New bud formation was observed as the presence of a second bud (a total of three connected cell bodies) containing a single nuclear DNA mass.

Cell viability was assayed using microcolony formation as follows. After nonpermissive temperature or nocodazole treatment, cell aliquots were returned either to permissive temperature or nocodazole-free medium, respectively, and plated at a density of ∼500 cells/cm^2 on YPD. Inviable cells were scored as those that failed to produce at least 50 cell bodies/microcolony after incubation for 20 hr, at which time wild-type microcolonies comprised >100 cell bodies. In most cases inviable microcolonies contained <10 cell bodies.

Yeast two-hybrid screening: The plasmid pRaj72 was transformed into the yeast two-hybrid strain CG1945 (CLONTECH). The yeast cDNA library (gift of S. Elledge) was transformed into pRaj72 (GalBD-BUB2 fusion) carrying CG1945, and transformants were directly selected on plates lacking tryptophan, leucine, and histidine and containing 10 mm 3-aminotriazole (3-AT; Sigma). Approximately 10,000 independent transformants were screened, yielding seven colonies with robust and reproducible growth on selective media by 4–7 days. These colonies were tested for β-galactosidase activity using the filter assay (Bai and Elledge 1997). The interacting plasmid from one of three colonies showing β-galactosidase activity was recovered and retransformed along with pRaj72 (GalBD-Bub2) into strain YJ69-2a (James *et al.* 1996), which carries an *ADE2* as well as *HIS3* reporter gene. These transformants were selected on medium lacking tryptophan and leucine (for establishment of the plasmids). Subsequent plating on medium also lacking histidine and adenine and containing 20 mm 3-AT confirmed the strength and plasmid dependence of the two-hybrid interaction. Sequence analysis of the insert in the interacting library plasmid revealed the presence of coding sequence from *CDC3* (amino acids 315 to 521) cloned in frame C-terminal to the pACT2-GAL4 activation domain (GalAD-Cdc3). The GalBD-Cdc16 fusion expressing the $cdc16^+$ of *S. pombe* was a gift from C. Albright (Furge *et al.* 1998). The GalBD-p53 and GalAD-SVT plasmids expressing fusions of p53 and SV40 T antigen, respectively, were from CLONTECH.

***In vitro* protein interaction assays:** GST-CDC3 fusion and GST proteins were expressed in *Escherichia coli* DH5-alpha (Life Technologies) from plasmid pRaj134 and pGEX4T-1 (Pharmacia), respectively, and purified according to manufacturer's instructions (Pharmacia). Cell lysis and binding reactions were

performed in ice-cold phosphate-buffered saline pH 7.4, 1% Triton X-100. ^{35}S-labeled BUB2 protein was synthesized *in vitro* using a coupled transcription, translation rabbit reticulo-lysate system (Promega, Madison, WI). Reactions were carried out according to manufacturer's instructions. Equal volumes of plain glutathione beads or beads carrying GST or GST-CDC3 were incubated with 35 μl out of a total of 150 μl of *in vitro*-translated extract in a total volume of 100 μl on ice for 1 hr. The bead samples were then pelleted, and unbound material was removed in four successive washes with ice-cold binding buffer. After the final wash, proteins were eluted in 50 μl binding buffer containing 10 mm glutathione and 10 μl of 5× SDS polyacrylamide gel electrophoresis sample buffer was added. The samples were boiled and analyzed on duplicate 10% SDS polyacrylamide gels (25 μl volume/lane). One gel was processed for autoradiography and the other for silver staining.

RESULTS

Kinetochore-induced preanaphase arrest requires MAD2 for establishment and BUB2 for maintenance: *CTF13* (Doheny *et al.* 1993) encodes a component of *CBF3*, an essential kinetochore protein complex (Russel *et al.* 1999). The temperature-sensitive allele *ctf13-30* induces a preanaphase delay, which is morphologically visible as an accumulation of very large budded uninucleate cells. This class of delaying cells occurs at a frequency of ~1–2% in wild-type cultures, compared to 80% in *ctf13-30* cultures at the nonpermissive temperature (Spencer and Hieter 1992; Doheny *et al.* 1993). The preanaphase arrest in *ctf13-30* cells depends on the spindle assembly checkpoint (Wang and Burke 1995; Pangilinan and Spencer 1996) and requires *MAD1*, *MAD2*, *BUB1*, and *BUB3* gene functions. Although it has been suggested that *BUB2* is dispensable for the checkpoint arrest induced by kinetochore damage (Wang and Burke 1995), we have observed that the *ctf13-30*-induced delay is reduced in *ctf13-30 bub2*Δ populations (Pangilinan and Spencer 1996). This reduction might reflect a smaller proportion of cells entering a delay or, alternatively, the presence of a shorter delay.

Examination of synchronous cultures supports the latter interpretation. *ctf13-30* kinetochore damage was induced at a semipermissive temperature (34°) sufficient to induce a preanaphase delay without a significant loss of viability, allowing observation of the delay dynamics as well as the associated impact on viability. *ctf13-30* strains lacking MAD2 or BUB2 were compared to isogenic relatives with an intact checkpoint. α-Factor-arrested cultures were released (at t = 0) into pheromone-free medium at 34° and followed for two cell cycles. At each time point, preanaphase delay was measured as the frequency of very large budded uninucleate cells, and viability was measured as the frequency of microcolony-forming units plated at permissive temperature (25°). As expected, strains without a *ctf13-30* defect never produce delaying cells and suffer no loss of viability on shift to 34° (Figure 1, A and B). In cells with an intact checkpoint, *ctf13-30* produces a sustained delay that does not completely subside but accumulates in

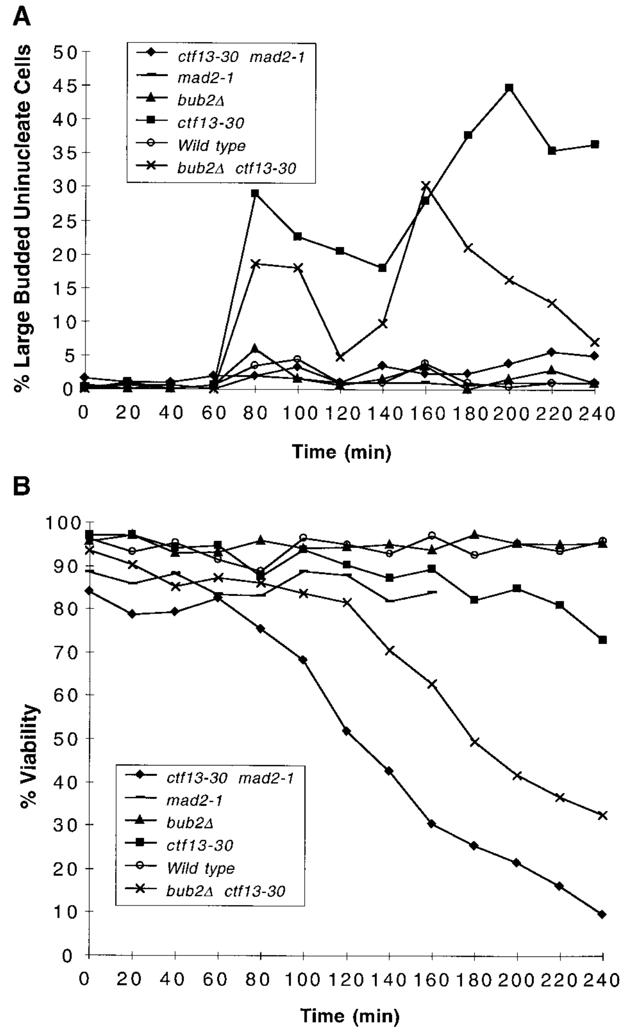

Figure 1.—Damaged kinetochore-induced mitotic arrest in wild-type and *bub2*Δ cells. (A) Preanaphase delay in synchronous *ctf13-30* cultures at 34°. Indicated strains were arrested in α-factor at 25° and released from arrest into pheromone-free medium at 34° (t = 0 min). Aliquots taken at 20-min intervals were fixed in formaldehyde, stained with DAPI, and scored for the presence of anaphase delay. The frequency (%) of large budded uninucleate cells is shown for each strain. Morphological parameters were as previously defined (Spencer and Hieter 1992), where scored cells are those with bud diameter >75% that of the mother sphere and a single DAPI-staining chromosomal mass at the mother-bud junction. This criterion identifies cells that are very rarely observed in wild-type cell cycles in the absence of mitotic checkpoint induction. (B) Loss of viability in synchronous *ctf13-30* cultures at 34°. Aliquots of samples removed for assay in (A) were diluted, sonicated, and plated on YPD for 24 hr at 25° and scored for microcolony formation. Strains were wild type (YFS824), *mad2-1* (YFP451), *mad2-1 ctf13-30* (YFS449), *bub2*Δ (YFS822), *bub2*Δ *ctf13-30* (YFS961), and *ctf13-30* (YFS884). Nearly identical time course results were obtained in two independent experiments, and representative results from a single experiment are shown.

the next cell cycle (Figure 1A). In contrast, *mad2-1 ctf13-30* cells are completely unable to delay and this is accompanied by a steady loss of viability beginning when cells with an intact checkpoint normally start to delay (compare to *ctf13-30* at 60–80 min; Figure 1, A and B). Interestingly, the *bub2*Δ *ctf13-30* strain initially produces delaying cells to approximately the same level as *ctf13-30* cells with an intact checkpoint, but the frequency of delayed cells declines sharply at 120 min (Figure 1A). The population remains synchronous and repeats this pattern through a second cell cycle. The decline at 120 min is accompanied by onset of loss of viability, which appears later in *bub2*Δ *ctf13-30* than in *mad2-1 ctf13-30* mutants (Figure 1, A and B). This suggests that *BUB2* has a role in delay maintenance while *MAD2* is important for delay establishment, and both genes are important for the viability of cells containing kinetochore damage.

MAD2 and BUB2 control distinct steps within mitosis, and both control sister chromatid separation: Exposure of cells to microtubule-destabilizing drugs like nocodazole also induces the spindle assembly checkpoint. At high concentration (15 μg/ml), nocodazole completely blocks formation of a mitotic spindle, and cells arrest in a prometaphase state. Failure of the spindle assembly checkpoint causes these cells to exit mitosis without segregating chromosomes to the spindle poles; they are unable due to the absence of a spindle. In such cells, the appearance of a new cycle can be very simply assayed as new (second) bud formation (Hoyt *et al.* 1991). Although the reason for retention of the first daughter cell is not well understood, the appearance of a second bud serves as an important marker of inappropriate cell cycle progress in spindle assembly checkpoint mutants.

Temporal analysis of the appearance of a new bud in *bub2*Δ, *mad2*Δ, and *mad2*Δ *bub2*Δ strains underscored the difference in the roles of the two genes in cells exposed to nocodazole. The onset of new bud formation in *mad2*Δ is ~30 min earlier than in *bub2*Δ cells (Figure 2A). The lag in new bud formation seen in *bub2*Δ mutants (relative to *mad2*Δ mutants) is consistent with a partial function of the checkpoint. New bud formation in the *bub2*Δ *mad2*Δ double mutant preceded that in *mad2*Δ by 30 min (Figure 2A).

Our results differ somewhat from others recently reported (Fesquet *et al.* 1999; Li 1999) in which the kinetics of new bud formation in *bub2*Δ and *mad2*Δ cell populations is inverted or indistinguishable, respectively. Appearance of a new bud as an indicator of a new cell cycle measures an event located far downstream of the control point of interest at the metaphase/anaphase transition. Perhaps differences in laboratory strain genetic background create different rate-limiting steps in mitotic progression. Alternatively, closely spaced time points may be required to reproducibly discern differences between mutants. In agreement with Li (1999) and Fesquet *et al.* (1999), the double mutant preceded the single mutants, providing strong evidence

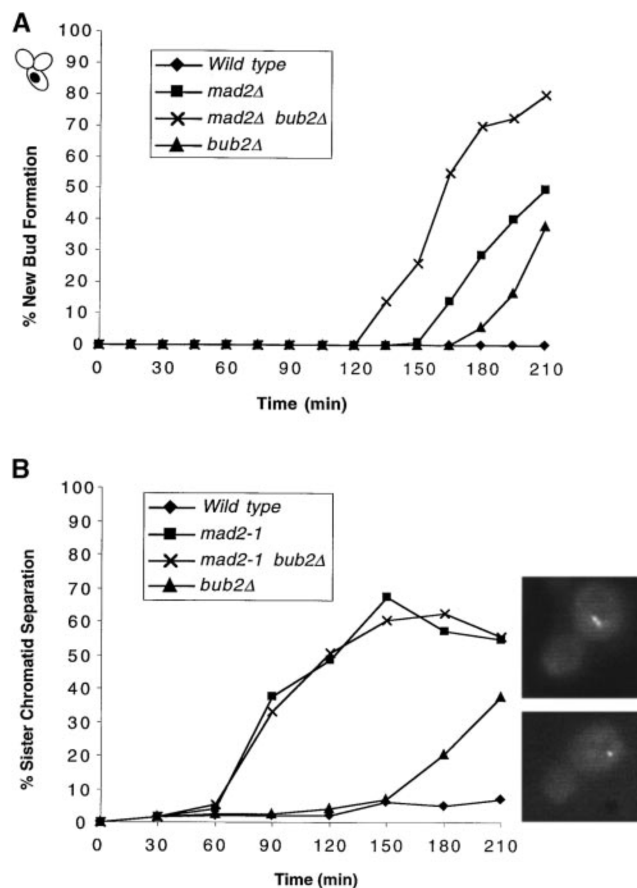

Figure 2.—Kinetics of new bud formation and sister chromatid separation in nocodazole. (A) A time course analysis of arrest failure as exhibited by the kinetics of second bud formation was performed. α-Factor-synchronized cells of the indicated strains were released into YPD at 25° containing 15 μg/ml nocodazole at $t = 0$ min. Aliquots were removed at indicated time points, sonicated, and fixed in formaldehyde for DAPI staining and microscopy. New (second) bud formation during nocodazole arrest was detected as the appearance of three or more associated cell bodies despite the persistence of a single DAPI-stained nucleus. Similar data were obtained in three independent experiments, and representative results are shown. Strains were wild type (YRaj127), *mad2*Δ (YFS1280), *bub2*Δ (YRaj121), and *mad2*Δ *bub2*Δ (YRaj158). (B) Cell cultures of the indicated strains in logarithmic phase were synchronized in G1 with α-Factor (see materials and methods). To enhance GFP-based visualization of sister chromatids, the expression of the Lac repressor-GFP fusion was induced during the last 30 min of α-factor arrest by transferring cells to synthetic complete dextrose medium lacking histidine and containing 10 mm 3-AT (Sigma) according to Straight *et al.* (1996). Synchronized cells were then released into pheromone-free YPD containing 15 μg/ml nocodazole at 25°, and aliquots were removed at indicated time points, sonicated, fixed in 4% paraformaldehyde, and visualized for GFP fluorescence. Similar data were obtained in two independent experiments, and representative results are shown. Strains were wild type (AFS173), *mad2-1* (AFS387), *bub2*Δ (YFS1214), and *mad2-1 bub2*Δ (YFS1215).

for the hypothesis that *MAD2* and *BUB2* control distinct steps in mitotic progress with additive timing. The lack of epistasis for bud formation contrasts with analysis

of sister chromatid separation in the double mutant (below).

A direct comparison of *MAD2* and *BUB2* function in the control of the metaphase/anaphase transition is provided by the visualization of GFP-marked sister chromatids (Straight *et al.* 1996). The kinetics of sister chromatid separation in *mad2*Δ, *bub2*Δ, and *mad2*Δ *bub2*Δ double mutants was assayed in synchronous populations. α-Factor-arrested cells were released into medium containing nocodazole at 15 μg/μl and aliquots removed every 30 min for analysis. Sister chromatid separation occurred with identical kinetics in *mad2*Δ and *mad2*Δ *bub2*Δ double mutants (Figure 2B), indicating that for this phenotype *MAD2* gene function is epistatic to *BUB2.* Sister chromatid separation occurred 90 min later in *bub2*Δ cells (Figure 2B), indicating that *BUB2* plays a role in preventing sister chromatid separation at prometaphase arrest. Wild-type cells maintain arrest throughout the duration of this experiment. Our observations confirm and extend results presented by Fraschini *et al.* (1999).

This analysis is consistent with the working hypothesis that *bub2*Δ cells establish but do not maintain a prometaphase arrest in the presence of nocodazole. Interestingly, this assay directly measures an effect on the morphological transition known to be controlled by the *MAD2*-dependent checkpoint step and indicates that sister chromatid separation accompanies the mitotic exit allowed in cells that lack *BUB2.*

The sister chromatid separation phenotype of *bub2*Δ cells requires the mitotic exit network: The sister chromatid separation seen in *bub2*Δ cells exposed to nocodazole may be due either to activation of the normal *CDC20*-directed mechanism for Pds1 protein degradation or to an indirect effect of premature mitotic exit. Anaphase normally accompanies a *CDC20*-mediated degradation of Pds1 protein. However, it has been demonstrated that mitotic exit, when induced in cells lacking *CDC20* function, by overexpression of Hct1 protein leads to degradation of Pds1 protein (Visintin *et al.* 1997). We therefore tested the effect of *bub2*Δ on sister chromatid separation in cells exposed to nocodazole, when the mitotic exit pathway is blocked.

Tem1 protein is a GTPase essential for the activation of the phosphatase *CDC14* and the mitotic exit pathway (Shou *et al.* 1999). A conditional Tem1 protein expression system has been developed (Shou *et al.* 1999) based on the "N-terminal degron" concept (Dohmen *et al.* 1994), wherein a destabilizing residue at the N terminus of a protein greatly reduces its half-life. Despite the very short half-life, *TEM1* function is provided by overexpression from a galactose responsive promoter. When the promoter is shut off on incubation in glucose, Tem1 protein levels are depleted rapidly and *tem1* phenotype is evident within a single cell cycle (Shou *et al.* 1999). The *tem1* degron allele (*tem1^c*) was introduced into the strains used for analysis of sister chromatid separation

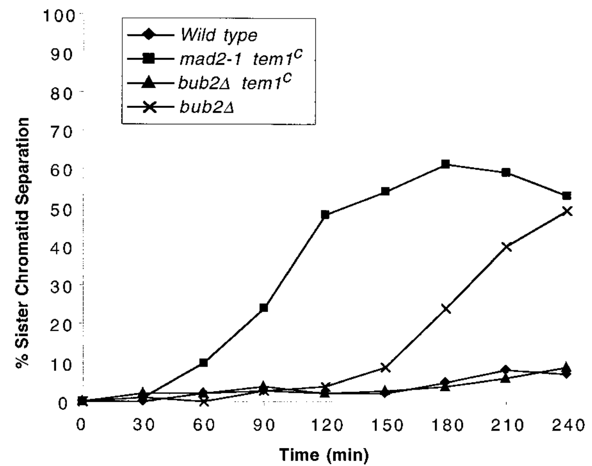

Figure 3.—Effect of *TEM1* on sister chromatid separation in nocodazole-treated *bub2*Δ cells. Asynchronous cultures grown in YPG (2% galactose, 2% raffinose) were arrested in α-factor and then released into pheromone-free YPD (2% dextrose) containing 15 μg/ml nocodazole at 25°. Aliquots were removed at the indicated time points, sonicated, and fixed in 4% paraformaldehyde for visualization of GFP fluorescence. Similar sister chromatid separation kinetics were observed in two independent experiments, and representative results are shown. Strains were wild type (AFS173), *mad2-1 tem1^c* (YRaj169), *bub2*Δ *tem1^c* (YRaj164), and *bub2*Δ (YFS1214).

in the previous section. Single cycle shutoff was achieved as judged by appearance of a typical *tem1* mutant phenotype (cells arrested prior to cytokinesis after completed nuclear division; data not shown). The strains compared in Figure 3 were propagated in galactose-containing, glucose-free medium (*TEM1* function present) and brought to a G1 arrest using α-factor. Following arrest, cells were released into galactose-free, glucose-containing medium to extinguish Tem1 protein function in cells that have the conditional allele (*tem1^c*) and aliquots were removed at various time points for analysis. Whereas *mad2-1 tem1* cells showed separated sister chromatids, *bub2*Δ *tem1* cells maintained sister chromatid cohesion for the duration of the experiment (Figure 3). Thus, the mitotic exit pathway functions downstream of *TEM1* are responsible for the sister chromatid separation in *bub2*Δ mutants but are not required for sister chromatid separation in *mad2*Δ mutants.

***BUB2* is not required for maintenance of DNA damage-induced arrest:** The arrests induced by spindle damage or DNA damage are mediated by stabilization of Pds1p (Yamamoto *et al.* 1996; Cohen-Fix and Koshland 1997; Gardner *et al.* 1999) in response to distinct pathways. For example, Hardwick *et al.* (1999) have used the *cdc13-1* allele to demonstrate that the DNA damage checkpoint-mediated arrest does not require the spindle assembly checkpoint genes *MAD1*, *MAD2*, and *MAD3.* However, the emerging role of Bub2 protein in preventing premature mitotic exit is one that could be recruited in response to either type of damage. We

therefore asked whether *BUB2* is required for the maintenance of a DNA damage-induced mitotic arrest.

Cells with the temperature-sensitive *cdc13-1* mutation accumulate single-stranded DNA under nonpermissive conditions and exhibit a DNA damage checkpoint-dependent arrest (Garvik *et al.* 1995; Polotnianka *et al.* 1998; Gardner *et al.* 1999), mediated through the stabilization of Pds1p (Lim and Surana 1996; Cohen-Fix and Koshland 1997). To study the role of *BUB2*, logarithmically growing *cdc13-1* and *cdc13-1 bub2*Δ double mutant strains were synchronized in α-factor and then released under restrictive (37°) or permissive (25°) conditions for *cdc13-1*. Under restrictive conditions, arrest was evidenced by accumulation of large budded cells with undivided nuclei (Figure 4A). The *cdc13-1 bub2*Δ double mutant behaved just like the *cdc13-1* single mutant. Thus, while both the DNA damage- and spindle damage-induced checkpoints act through stabilization of Pds1p to prevent anaphase, only the spindle damage-induced checkpoint breaks down prematurely in the absence of Bub2 protein.

Interestingly, the DNA damage arrest phenotype is epistatic to the spindle damage arrest phenotype. This was tested in cells subjected to both *cdc13-1*-induced DNA damage as well as nocodazole-induced spindle damage in the presence and absence of *BUB2* function. Comparison of arrest competence was measured by scoring the frequency of cells exhibiting new bud formation. *cdc13-1* and *cdc13-1 bub2*Δ strains were released from α-factor arrest at restrictive (37°) or permissive (25°) temperature into rich medium containing 15 μg/ml nocodazole. The *cdc13-1*-induced arrest was maintained in the *bub2*Δ background in the presence of nocodazole. The *bub2*Δ strain showed new bud formation at both 25° and 37° as expected while the *cdc13-1 bub2*Δ double mutant showed new bud formation only at 25° and not at 37° (Figure 4B).

Taken together, these data confirm that the arrest mechanisms employed in response to DNA and spindle damage are distinct and that the *BUB2*-controlled negative regulation of mitotic exit is not essential for the development of a robust DNA damage-mediated arrest.

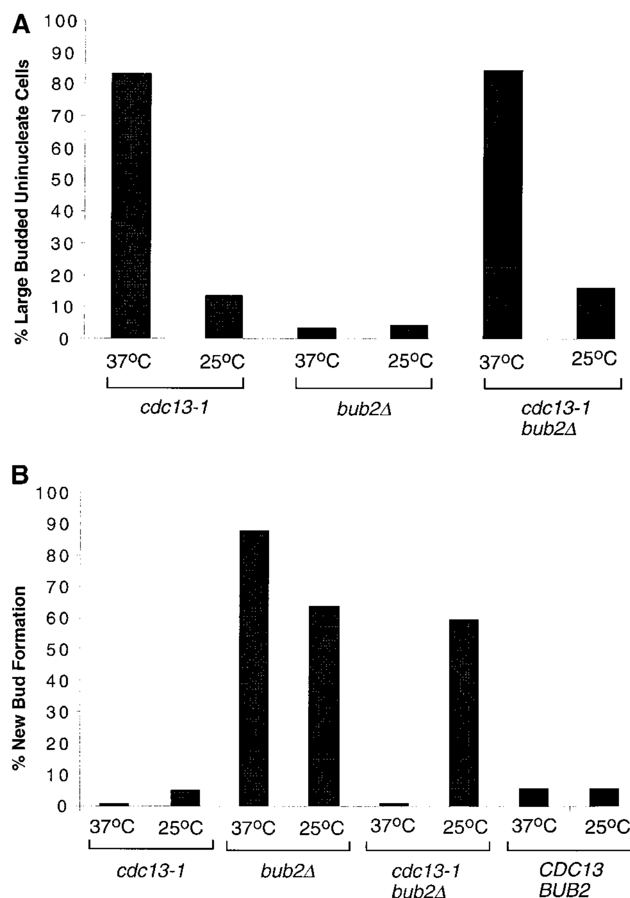

Figure 4.—Effect of *bub2*Δ on *cdc13-1*-induced arrest. (A) Exposure to 37°. Indicated strains were synchronized in α-factor and released into pheromone-free YPD at either 25° or 37°. After 4 hr, samples were sonicated, fixed in formaldehyde, stained with DAPI, and scored for the presence of large budded uninucleate cells as defined in Figure 1. (B) Exposure to both 37° and nocodazole. Each strain was arrested in α-factor and released into pheromone-free YPD containing 15 μg/ml nocodazole at 25° or 37°. After 4 hr, samples were sonicated, fixed in formaldehyde, stained with DAPI, and scored for the presence of arrest failure as indicated by new bud formation. New (second) bud formation during nocodazole arrest was detected as the appearance of three or more associated cell bodies despite the persistence of a single DAPI-stained nucleus. Stains were wild type (YRaj127), *cdc13-1* (YRaj140), *bub2*Δ (YRaj121), and *cdc13-1 bub2*Δ (YRaj144). Similar results were obtained in three independent experiments, and representative data are shown.

Continuous function of Bub2 protein is required for maintenance of spindle assembly checkpoint-mediated arrest: To test whether Bub2 protein is required continuously once the spindle assembly checkpoint-mediated arrest has been established, we constructed a conditional bub2 allele (*BUB2^{tsd}*) using a "temperature-sensitive degron" (Dohmen *et al.* 1994). This was constructed by fusing a temperature-sensitive derivative of the dihydrofolate reductase (DHFR) protein, whose stability is controlled by the N-end rule pathway of protein degradation (Dohmen *et al.* 1994) to the N terminus of Bub2 protein. This resulting fusion protein was expected to be stable at 25° and to be degraded rapidly at 37°. The construct replaced the wild-type *BUB2* gene by integration into the chromosomal *BUB2* locus (see materials and methods) such that expression was driven by the *BUB2* promoter. The Bub2^{tsd} fusion protein provides *BUB2* function at 25° as evidenced by sustained arrest in the presence of nocodazole (Figure 5, left). However at 37° there is a clear loss of Bub2 protein function as cells fail to arrest in the presence of nocodazole (Figure 5, center). To test whether Bub2 protein function is required continuously once the arrest is established, cells that were exposed to nocodazole at 25° for 4 hr (achieving 85% population arrest) were then shifted to 37°. These exhibited a breakdown of the arrest as

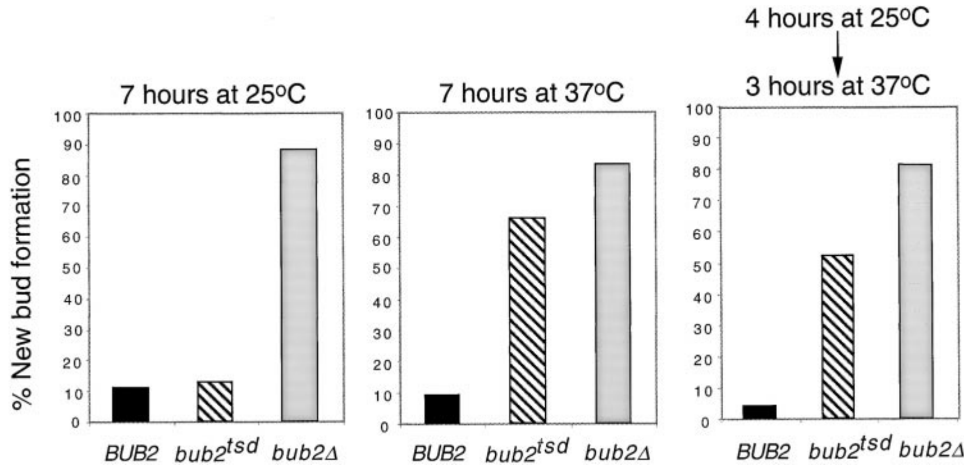

Figure 5.—Conditional expression of *BUB2*. Log phase populations grown in YPD at 25° were transferred to YPD containing 15 μg/ml nocodazole at the indicated temperatures and cultured for the indicated time periods. Samples were sonicated, fixed in formaldehyde, stained with DAPI, and scored for the presence of arrest failure as indicated by new (second) bud formation. Strains were *BUB2* (YRaj127), $bub2^{tsd}$ (YRaj132), and *bub2*Δ (YRaj121). Similar results were obtained in two independent experiments, and representative data are shown.

evidenced by the appearance of new (second) bud formation (Figure 5, right). Thus the Bub2 protein is required continuously for maintenance of the spindle assembly checkpoint-mediated block even when its function is manipulated well after establishment of prometaphase arrest.

Bub2 protein exhibits a physical interaction with the septin protein Cdc3: To better understand the role of *BUB2* through its interactions with other genes, a two-hybrid screen (Bai and Elledge 1997) using a yeast cDNA library has been initiated. One interacting plasmid obtained (Figure 6) has been analyzed in depth. The library insert encodes an in-frame GAL4 fusion containing the C-terminal 206 amino acids of the *CDC3* gene product. *CDC3* encodes a member of the septin family of proteins that play important roles in cytokinesis and cell morphology (Field and Kellog 1999). An *in vitro* test was performed to further explore the two-hybrid association between Cdc3 and Bub2 proteins. A bacterially expressed GST-CDC3 fusion protein was used in an affinity trap for ^{35}S-methionine-labeled Bub2 protein obtained after coupled *in vitro* transcription/translation. Bub2 protein was specifically retained on beads carrying GST-CDC3 protein but not on beads carrying GST protein alone (Figure 6B). The *in vitro* affinity between Bub2 and Cdc3 proteins indicates that the yeast two-hybrid interaction may represent a direct contact between these proteins *in vivo*.

cdc3-1 cells appear to have an intact spindle assembly checkpoint and show no synthetic lethality in *bub2*Δ or *BUB2* overexpression backgrounds (not shown). *BUB2* has a well-conserved homologue in the distantly related fungus *S. pombe* (Fankhauser *et al.* 1993). *S. cerevisiae* Bub2 and *S. pombe* $cdc16^{+}$ proteins exhibit 39% identity and 58% similarity (data not shown) in BLASTP alignment using default parameters (Tatusova and Madden 1999). $Cdc16^{+}$ stabilizes $p34^{CDC2}$ kinase activity and has been shown to negatively regulate the timing of cytokinesis in fission yeast, as well as placement and number of the septa formed on mitotic exit (Minet *et al.* 1979; Fankhauser *et al.* 1993). Furthermore, *S. cerevisiae BUB2* complements the temperature-sensitive allele *cdc16-116* in *S. pombe*, although it is unable to complement a $cdc16^{-}$ null mutation (Fankhauser *et al.* 1993). We therefore asked whether the Cdc16 protein of *S. pombe* interacts with Cdc3 protein of *S. cerevisiae* in a two-hybrid assay and found that it does (Figure 6A). The conservation of this interaction, together with known late mitotic functions for each of these proteins, suggests that the association indicated may have significant biological consequences.

DISCUSSION

The spindle assembly checkpoint controls the timing of the metaphase-to-anaphase transition, inhibiting sister chromatid separation until all chromatid pairs have achieved bipolar spindle attachment. However, mitotic progression is also controlled at a later point prior to the execution of events leading to exit and cytokinesis (Jasperson *et al.* 1998). Emerging evidence suggests the existence of an aspect of the spindle assembly checkpoint that regulates a late mitotic control step and depends on the *BUB2* protein (Alexandru *et al.* 1999; Fesquet *et al.* 1999; Fraschini *et al.* 1999; Li 1999; this work). These two distinct aspects of the spindle assembly checkpoint are controlled by *MAD2*- and *BUB2*-dependent pathways and are both required for full prometaphase arrest in response to spindle or kinetochore damage.

A key issue in the description of these two activities of the spindle assembly checkpoint is whether or not they represent a branched response following a common initiation signal. The *MAD2*-dependent pathway is clearly induced by an unattached or defective kinetochore (Wang and Burke 1995; Pangilinan and Spencer 1996). It has been generally suggested that *BUB2* may not have a role to play when the spindle assembly checkpoint responds to defective kinetochores but rather responds to a distinct spindle damage structure

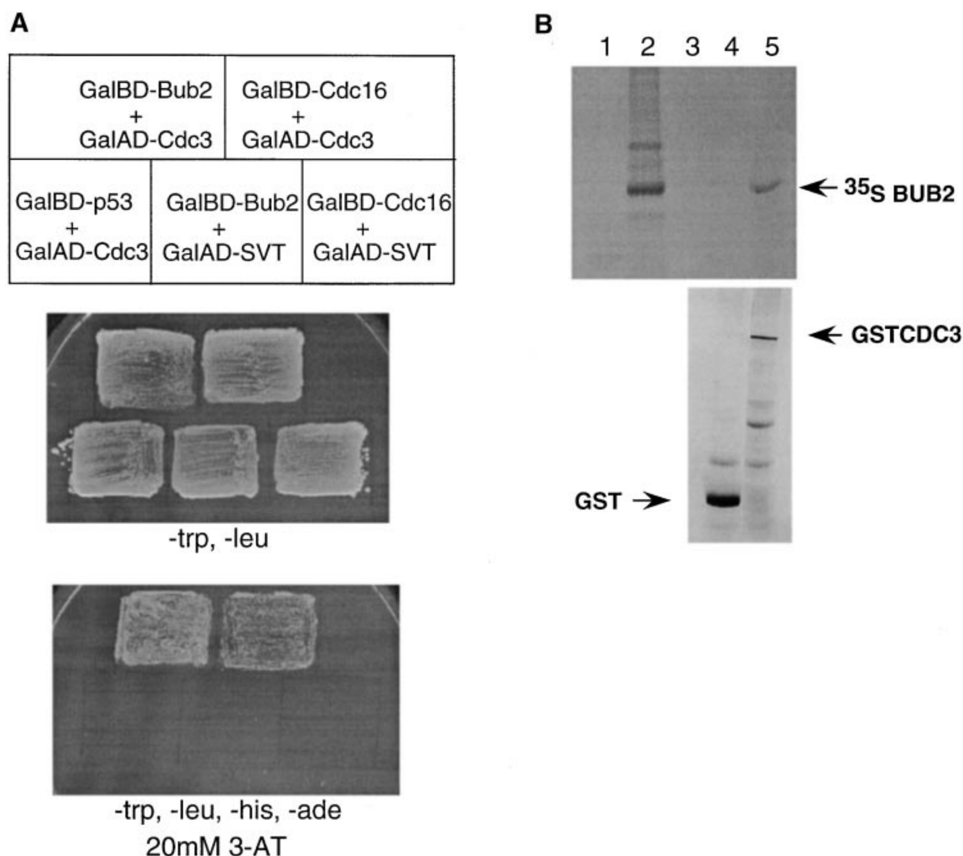

Figure 6.—Bub2 and Cdc3 proteins interact in the yeast two-hybrid system and *in vitro*. (A) Yeast reporter strain J69-2A was cotransformed with GAL4-binding domain (BD) or activation domain (AD) fusions as indicated (top). Transformants were patched onto synthetic complete medium lacking tryptophan and leucine (middle) or synthetic complete medium lacking tryptophan, leucine, adenine, and histidine that also contained 20 mm 3-AT (bottom). GalAD-SVT and GalBD-p53 contain a GAL4-activation domain/SV40 large T antigen fusion gene and GAL4/DNA-binding domain/human p53 fusion gene, respectively. These well-characterized constructs (CLONTECH) serve as negative controls not expected to exhibit interaction with *CDC3*, *BUB2*, or *CDC16* fusions. (B) Interaction between Bub2 and Cdc3 proteins *in vitro*. (Top) An autoradiogram of ^{35}S-labeled *in vitro*-translated Bub2 protein and its binding properties. Lane 1, ^{35}S-labeled *in vitro* transcription/translation product without supplied plasmid template (negative control); lane 2, ^{35}S-labeled *in vitro transcription/*translation product from *BUB2* template (^{35}S-Bub2 protein); lane 3, eluate from glutathione beads incubated with ^{35}S-Bub2 protein; lane 4, eluate from GST-bound glutathione beads incubated with ^{35}S-Bub2 protein; lane 5, eluate from GST-CDC3-bound beads incubated with ^{35}S-Bub2 protein. (Bottom) Relevant lanes (4 and 5) from a duplicate but silver-stained gel. GST and GST-CDC3 proteins are as indicated. Molecular sizes were confirmed using prestained marker from Bio-Rad (not shown).

(Wang and Burke 1995; Li 1999). However, the presence of a single chromosome containing a centromere DNA mutation (one that generates a mitotic delay in checkpoint proficient cells) induces the occasional production of cells with extra buds in cycling *bub2*Δ strains (Pangilinan and Spencer 1996). This phenotype, albeit occurring at a low frequency, represents inappropriate mitotic exit despite the presence of a functional spindle. In agreement with this result, a synchronous *bub2*Δ *ctf13-30* double-mutant population exhibits a transient accumulation of delayed cells indicating that kinetochore damage-induced arrest is established but is not maintained. Thus a kinetochore damage-induced checkpoint arrest requires Bub2 protein for its full function in two independent assays. We suggest therefore that the *MAD2*- and *BUB2*-dependent pathways are both triggered by a signal from an unattached kinetochore.

Analysis of *mad2 bub2* double mutants indicates that these two genes act in additive functions within mitosis, in general agreement with previously published work (Alexandru *et al.* 1999; Fesquet *et al.* 1999; Fraschini *et al.* 1999; Li *et al.* 1999). This conclusion is established by the observation of additive effects of mutant alleles on a postmitotic phenotype and the appearance of new (second) bud formation in a nocodazole block. Interestingly, the timing of inappropriate sister chromatid separation exhibits epistasis in the *mad2 bub2* double mutant, with the effect of the *mad2* mutant rendering the *bub2* defect irrelevant. This result is consistent with the hypothesis that the *MAD2*-dependent prevention of sister chromatid separation in the presence of spindle damage requires a later *BUB2*-dependent step for maintenance.

The sister chromatid separation seen in nocodazole-exposed *bub2*Δ cells could be explained either as an indirect consequence of onset of mitotic exit and late mitotic APC activity or as a direct consequence of inability to hold off anaphase. This latter possibility would be supported by the observation that under normal conditions the degradation of Pds1p is essential for subsequent degradation of Clb2p and for mitotic exit (Cohen-Fix and Koshland 1999; Tinker-Kulberg and Morgan 1999). On the other hand, the ability of late mitotic APC activity to nonspecifically degrade Pds1 protein, when the latter has been stabilized through lack of Cdc20 protein function, has been demonstrated by Visintin *et al.* (1997). Under conditions of spindle

damage in *mad2 bub2* mutants, degradation kinetics of Pds1 and Clb2 proteins appear indistinguishable (Fraschini *et al.* 1999). Moreover, when the kinetics of sister chromatid separation are followed, there is no difference between *mad2* and *mad2 bub2* mutants, suggesting that *BUB2* does not influence a rate-limiting step in sister chromatid separation. Under conditions of spindle damage, we asked whether the sister chromatid separation in *bub2* mutants is largely a consequence of onset of the mitotic exit pathway. Our results (Figure 3) that *bub2*Δ *tem1* cells, which do not exit mitosis, do maintain sister chromatid cohesion in nocodazole is direct evidence that inappropriate sister chromatid separation seen in *bub2* cells is due to the onset of the exit pathway. This is consistent with the observation that overexpression of Tem1 protein mimics a *bub2* phenotype in nocodazole-treated cells (Alexandru *et al.* 1999). Taken together these results demonstrate a situation wherein the inhibitory effect of Pds1 protein on exit (Cohen-Fix and Koshland 1999; Tinker-Kulberg and Morgan 1999) is apparently prevented in cells lacking *BUB2* function, raising the intriguing possibility that Pds1 protein acts through a *BUB2*-dependent pathway to control exit. This in turn would be consistent with the finding by Tavormina and Burke (1998) that the mitotic arrest in *cdc20* mutants requires *BUB2* function.

In theory, the importance of a regulatory block to mitotic exit when anaphase is delayed would be encountered in other preanaphase blocks. The initial characterization of *bub2* mutants indicated that *BUB2* was not required for maintenance of α-factor or hydroxyurea-induced arrests in G1 or S phase, respectively (Hoyt *et al.* 1991). Interestingly, recent analysis of the roles of components of the DNA damage checkpoint has revealed partially separable controls at metaphase and mitotic exit (Sanchez *et al.* 1999). We determined whether *BUB2* played a role in maintenance of the DNA damage checkpoint, which, like the spindle assembly checkpoint, acts predominantly through the stabilization of Pds1 protein. The *cdc13-1* allele induces the DNA damage checkpoint, at the restrictive temperature of 37° (Lim and Surana 1996; Cohen-Fix and Koshland 1997). The results demonstrate that a *cdc13-1*-induced arrest is maintained in cells lacking Bub2 protein and indicate that the role of the *BUB2* pathway in preventing premature exit when anaphase is blocked is not a global one.

While the mechanism by which *BUB2* maintains a spindle damage-induced arrest is not apparent, the *bub2^{tsd}* allele indicates the presence of an active and continuous role for the Bub2 protein in the maintenance of the mitotic arrest induced by nocodazole. Recent work on the roles of *DBF2* kinase in late mitosis (Fesquet *et al.* 1999) and on *CLB2* and *CLB3* stability throughout mitosis (Alexandru *et al.* 1999; Fraschini *et al.* 1999) suggest that these are regulated directly or indirectly by *BUB2* when spindle damage is present. An analogy can be drawn between *BUB2* and the function of its *S. pombe* homologue *cdc16$^+$* (Fankhauser *et al.* 1993). In *S. pombe*, *cdc16* and *byr4* proteins form a two-component GTPase-activating protein that maintains *spg1* protein in its GDP-bound form until it is appropriate for the GTP-bound form, which facilitates mitotic exit, to prevail (Furge *et al.* 1998). We found that Bub2 protein of *S. cerevisiae* interacts with byr4 protein of *S. pombe* in the yeast two-hybrid system (data not shown), adding another piece of evidence that *BUB2* and *cdc16$^+$* are functional counterparts in *S. cerevisiae* and *S. pombe*, respectively. Li (1999) has shown that *BFA1*, the *S. cerevisiae* homologue of *S. pombe byr4$^+$*, is required for the spindle assembly checkpoint. The *TEM1* gene of *S. cerevisiae* is a homologue of *spg1$^+$* of *S. pombe*, and it has been demonstrated that Tem1 protein-dependent signaling facilitates mitotic exit by activating the Cdc14 phosphatase and, consequently, Clb2p degradation (Shou *et al.* 1999). These results are consistent with the observation that the continuous presence of Bub2 protein and its biochemical activity is required for its role in the maintenance of the spindle assembly checkpoint-mediated arrest.

In a yeast two-hybrid screen for proteins that interact with *BUB2* we isolated the Cdc3 protein, a septin essential for cytokinesis (Kim *et al.* 1991). Bub2 and Cdc3 proteins also interact *in vitro*, suggesting that they are able to make direct contact with each other in the cell. Furthermore, *cdc16$^+$*, the *S. pombe* homologue of *BUB2*, interacts with Cdc3 protein of *S. cerevisiae* in a two-hybrid test.

Although the biological steps affected by a candidate *BUB2/CDC3* interaction have not yet been defined, a rapidly growing body of circumstantial evidence suggests several interesting views. While Cdc3 protein localizes predominantly to the mother-bud neck (Longtine *et al.* 1999), Bub2 protein localizes to the spindle pole bodies (Fraschini *et al.* 1999; Li 1999). Li (1999) has proposed that the localization of Bub2 protein at the spindle pole body may suggest a role in monitoring passage of the spindle pole through the mother-bud neck. If so, such a role would be facilitated by an interaction between a neck-localized Cdc3 protein and Bub2 protein. It is interesting to note that in *S. pombe*, *sid2$^+$* encodes a spindle pole body-associated kinase whose septin-dependent activity peaks during onset of cytokinesis (Sparks *et al.* 1999). *DBF2* is the *S. cerevisiae* homologue of *sid2$^+$*, and its kinase activity is influenced by *BUB2* under conditions of spindle damage (Fesquet *et al.* 1999; Sparks *et al.* 1999). This provides intriguing indirect evidence for a biological significance of the observed yeast two-hybrid interaction between *BUB2* and the septin *CDC3*.

In summary, we demonstrate that *BUB2* acts in a pathway that is required for maintenance of the spindle assembly checkpoint-mediated arrest and that this pathway is required even when kinetochore damage is the

inducer of arrest. Furthermore, the continuous presence of the Bub2 protein is required for this role and such a requirement is consistent with the proposed role for *BUB2* in regulating GTPase activity of Tem1 protein. The premature breakdown of metaphase in *bub2*Δ cells is a consequence of activation of the *TEM1*-directed mitotic exit pathway and does not precede the *TEM1*-directed step. We have noted that the *BUB2*-controlled step is irrelevant in cells arrested at metaphase by the DNA damage checkpoint, suggesting that Bub2 protein function is not a universal requirement for preventing mitotic exit until anaphase has occurred. Finally, we have uncovered an intriguing physical association of Bub2 protein with Cdc3, a septin that functions in cytokinesis and cell morphogenesis. Experimental analysis of the relationships among essential mitotic events and their control will likely continue to reveal layers in a network of coordinate regulation governing the many ordered processes that comprise the division of one cell into two.

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LITERATURE CITED

Alexandru, G., W. Zacharie, A. Schleiffer and K. Nasmyth, 1999 Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. EMBO J. **18:** 2707–2721.

Bai, C., and S. J. Elledge, 1997 Gene identification using the yeast two-hybrid system. Methods Enzymol. **283:** 141–156.

Cohen-Fix, O., and D. Koshland, 1997 The anaphase inhibitor of *Saccharomyces cerevisiae* Pds1p is a target of the DNA damage checkpoint pathway. Proc. Natl. Acad. Sci. USA **94:** 14361–14366.

Cohen-Fix, O., and D. Koshland, 1999 Pds1p of budding yeast has dual roles: inhibition of anaphase initiation and regulation of mitotic exit. Genes Dev. **13:** 1950–1959.

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

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.

Elledge, S. J., 1998 Mitotic arrest: Mad2 prevents sleepy from waking up the APC [comment]. Science **279:** 999–1000.

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

Fankhauser, C., J. Marks, A. Reymond and V. Simanis, 1993 The S. pombe cdc16 gene is required both for maintenance of p34cdc2 kinase activity and regulation of septum formation: A link between mitosis and cytokinesis? EMBO J. **12:** 2697–2704.

Fesquet, D., P. J. Fitzpatrick, A. L. Johnson, K. M. Kramer, J. H. Toyn *et al.*, 1999 A Bub2p-dependant spindle checkpoint pathway regulates the Dbf2p kinase in budding yeast. EMBO J. **18:** 2424–2434.

Field, C. M., and D. Kellog, 1999 Septins: Cytoskeletal polymers or signalling GTPases? Trends Cell Biol. **10:** 387–394.

Fraschini, R., E. Formenti, G. Lucchini and S. Piatti, 1999 Budding yeast Bub2 is localized at spindle pole bodies and activates the mitotic checkpoint via a different pathway from MAD2. J. Cell Biol. **145:** 979–991.

Furge, K. A., K. Wong, J. Armstrong, M. Balasubramanian and C. F. Albright, 1998 Byr4 and Cdc16 form a two component GTPase activating protein for Spg1 GTPase that controls septation in fission yeast. Curr. Biol. **8:** 947–954.

Gardner, R., C. W. Putnam and T. W. Weinert, 1999 RAD53, DUN1 and PDS1 define two parallel G2/M checkpoint pathways in budding yeast. EMBO J. **18:** 3173–3185.

Garvik, B., M. Carson and L. Hartwell, 1995 Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol. Cell. Biol. **15:** 6128–6138.

Guthrie, C., and G. R. Fink (Editors), 1991. *Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology*, Vol. 194. Academic Press, San Diego, CA.

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.

Hoyt, M. A., L. Totis and B. T. Roberts, 1991 *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. Cell **66:** 507–517.

Hwang, L. H., L. F. Lau, D. L. Smith, C. A. Mistrot, K. G. Hardwick *et al.*, 1998 Budding yeast Cdc20: a target of the spindle checkpoint [see comments]. Science **279:** 1041–1044.

James, P., J. Halladay and E. A. Craig, 1996 Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics **144:** 1425–1436.

Jasperson, S. L., J. F. Charles, R. L. Tinker-Kulberg and D. O. Morgan, 1998 A late mitotic regulatory network controlling cyclin destruction in *Saccharomyces cerevisiae*. Mol. Biol. Cell **9:** 2803–2817.

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.

Kim, H. B., B. K. Haarer and J. R. Pringle, 1991 Cellular morphogenesis in the *Saccharomyces cerevisiae* cell cycle: localization of the CDC3 gene product and the timing of events at the budding site. J. Cell Biol. **112:** 535–544.

Kim, S. H., D. P. Lin, S. Matsumoto, A. Kitazono and T. Matsumoto, 1998 Fission yeast Slp1: an effector of the Mad2-dependent spindle checkpoint [see comments]. Science **279:** 1045–1047.

Li, R., 1999 Bifurcation of the mitotic checkpoint pathway in budding yeast. Proc. Natl. Acad. Sci. USA **96:** 4989–4994.

Li, R., and A. W. Murray, 1991 Feedback control of mitosis in budding yeast. Cell **66:** 519–531. (erratum: Cell **79:** following 388).

Lim, H. H., and U. Surana, 1996 Cdc20, a beta-transducin homologue, links RAD9-mediated G2/M checkpoint control to mitosis in *Saccharomyces cerevisiae.* Mol. Gen. Genet. **253:** 138–148.

Lim, H. H., P. Y. Goh and U. Surana, 1998 Cdc20 is essential for the cyclosome-mediated proteolysis of both Pds1 and Clb2 during M phase in budding yeast. Curr. Biol. **8:** 231–234.

Longtine, M., H. Fares and J. R. Pringle, 1999 Role of the yeast Gin4p protein kinase in septin assembly and the relationship between septin assembly and septin function. J. Cell Biol. **143:** 719–736.

Minet, M., P. Nurse, P. Thuriaux and J. M. Mitchison, 1979 Uncontrolled septation in a cell division cycle mutant of the fission yeast *Schizosaccharomyces pombe.* J. Bacteriol. **137:** 440–446.

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

Nicklas, R. B., S. C. Ward and G. J. Gorbsky, 1995 Kinetochore chemistry is sensitive to tension and may link mitotic forces to a cell cycle checkpoint. J. Cell Biol. **130:** 929–939.

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

Peters, J.-M., 1999 Subunits and substrates of the anaphase-promoting complex. Exp. Cell Res. **248:** 339–349.
Polotnianka, R. M., J. Li and A. J. Lustig, 1998 The yeast Ku heterodimer is essential for protection of the telomere against nucleolytic and recombinational activities. Curr. Biol. **8:** 831–834.
Rieder, C. L., and E. D. Salmon, 1998 Vertebrate cell kinetochore and its roles during mitosis. Trends Cell Biol. **8:** 310–318.
Rieder, C. L., R. W. Cole, A. Khodjakov and G. Sluder, 1995 The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. J. Cell Biol. **130:** 941–948.
Russel, 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.
Sanchez, Y., J. Bachant, H. Wang, F. Hu, D. Liu *et al.*, 1999 Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. Science **286:** 1166–1171.
Schott, E. J., and M. A. Hoyt, 1998 Dominant alleles of *Saccharomyces cerevisiae* CDC20 reveal its role in promoting anaphase. Genetics **148:** 599–610.
Shou, W., J. H. Seol, A. Shevchenko, C. Baskerville, D. Moazed *et al.*, 1999 Exit from mitosis is triggered by Tem-1 dependant release of the protein phosphatase Cdc14 from nucleolar RENT complex. Cell **97:** 233–244.
Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.
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.
Sparks, C. A., M. Morphew and D. McCollum, 1999 Sid2p, a spindle pole body kinase that regulates the onset of cytokinesis. J. Cell Biol. **146:** 777–790.
Spencer, F., and P. Hieter, 1992 Centromere DNA mutations induce a mitotic delay in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA **89:** 8908–8912.
Straight, A. F., A. S. Belmont, C. C. Robinett and A. W. Murray, 1996 GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion. Curr. Biol. **6:** 1599–1608.
Tatusova, T. A., and T. L. Madden, 1999 BLAST 2 Sequences, a new tool for comparing protein and nucleic acid sequences. FEMS Microbiol. Lett. **174:** 247–250.
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 assembly checkpoint genes. Genetics **148:** 1701–1713.
Tinker-Kulberg, R. L., and D. O. Morgan, 1999 Pds1 and Esp1 control both anaphase and mitotic exit in normal cells and after DNA damage. Genes Dev. **13:** 1936–1949.
Visintin, R., S. Prinz and A. Amon, 1997 CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. Science **278:** 460–463.
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.
Yamamoto, A., V. Guacci and D. Koshland, 1996 Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s). J. Cell Biol. **133:** 99–110.

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