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An α-Tubulin Mutant Demonstrates Distinguishable Functions Among the Spindle Assembly Checkpoint Genes in *Saccharomyces cerevisiae*

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ABSTRACT

Cells expressing a mutant allele of α-tubulin, *tub1-729*, are cold sensitive and arrest as large-budded cells with microtubule defects. The cold sensitivity of *tub1-729* is suppressed by extra copies of a subset of the mitotic checkpoint genes *BUB1*, *BUB3*, and *MPS1*, but not *MAD1*, *MAD2*, and *MAD3*. This suppression by checkpoint genes does not depend upon their role in the *MAD2*-dependent spindle assembly checkpoint. In addition, *BUB1* requires an intact kinase domain as well as Bub3p to suppress *tub1-729*. The data suggest that *tub1-729* cells are defective in microtubule-kinetochore attachments and that the products of specific checkpoint genes can act either directly or indirectly to affect these attachments.

DURING cell division, DNA is replicated and then partitioned equally between two newly formed cells. Incorrect partitioning of DNA during mitosis or meiosis causes aneuploidy, which can lead to deleterious consequences such as cancer and birth defects. Microtubules are essential for proper DNA segregation: They position the nucleus for cell division, form the mitotic spindle, and contribute to force generation during anaphase (reviewed in Mitchison 1988; Koshland 1994). At each of these steps microtubules interact with other proteins or protein complexes that regulate, assist, or monitor microtubule function. For example, motors bind to microtubules in mitosis and help generate the force required to separate sister chromatids during anaphase. To understand the process of cell division, it is necessary to understand microtubules and the proteins that interact with them.

To learn more about the various roles of microtubules in the cell, we have taken a genetic approach using the budding yeast, *Saccharomyces cerevisiae*. In budding yeast, there are two functionally interchangeable α-tubulin genes, *TUB1* and *TUB3* (Schatz *et al.* 1986, 1988), and one β-tubulin gene, *TUB2* (Thomas *et al.* 1985). A genetic screen for conditional alleles of the major α-tubulin gene, *TUB1*, isolated 35 cold-sensitive mutants (Schatz *et al.* 1988). These mutants fall into three classes: Class 1 mutants have a reduced number of microtubules; class 2 mutants have extra microtubules; and class 3 mutants have abnormal microtubules. The diversity of the phenotypes observed in this mutant collection suggests that these α-tubulin alleles may be deficient in distinct aspects of microtubule function. Analyses of these mutants can lead to an understanding of microtubule processes as well as to the identification of other proteins that interact with tubulin.

When shifted to the nonpermissive temperature (15°), cells expressing *tub1-729*, a class 1 allele, as its sole source of α-tubulin, accumulate as large-budded cells with either a short spindle or a single dot of microtubule staining (Schatz *et al.* 1988; Guénette *et al.* 1995). A screen for low-copy suppressors of the cold sensitivity of *tub1-729* identified only one gene, *BUB3* (*b*udding *u*ninhibited by *b*enomyl) eight times (Guénette *et al.* 1995). *BUB3* encodes a protein involved in the spindle assembly checkpoint that binds to and is phosphorylated by Bub1p (Hoyt *et al.* 1991; Roberts *et al.* 1994). Direct testing revealed that *BUB1* also suppresses the cold sensitivity of *tub1-729* cells, but another spindle assembly checkpoint gene, *MAD2* (*m*itotic *a*rrest *d*eficient), does not (Guénette *et al.* 1995). Bub1p and Bub3p, together with Mps1p (*m*ono *p*olar *s*pindle), Mad1p, Mad2p, and Mad3p, are part of the spindle assembly checkpoint. These proteins monitor microtubule-kinetochore attachments and prevent cells from entering anaphase until all of the chromosomes are attached to spindle microtubules. Mutations in these checkpoint genes—either deletion of *BUB1* and *-3* and *MAD1*, *-2*, and *-3* or a point mutation in the essential *MPS1*—eliminate the spindle assembly checkpoint, allowing cells to enter anaphase when their mitotic spindle is disrupted either by the microtubule depolymerizing drug, benomyl (Hoyt *et al.* 1991; Li and Murray 1991) or by mutations that destabilize the spindle (Pangilinan and Spencer 1996). The higher eukaryotic homologs of these six mitotic checkpoint genes localize to unattached kinetochores during pro-metaphase and all except Mps1p are released from the kinetochore after microtubules bind (Chen *et al.* 1996, 1998; Li and Benezra 1996; Taylor and McKeon 1997; Tay-

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lor *et al.* 1998; Martinez-Exposito *et al.* 1999; Abrieu *et al.* 2001). The kinetochore localization of these proteins supports the idea that they monitor microtubule-kinetochore attachment and help to convey a signal to halt cell-cycle progression through downstream factors.

In this article, we show that genetic interactions with *tub1-729* distinguish among the mitotic checkpoint genes. The results demonstrate that, like *BUB1* and *BUB3* (Guénette *et al.* 1995), *MPS1* suppresses the cold sensitivity of *tub1-729*. However, *BUB2* and *MAD1*, *2*, and *3* do not suppress. Strikingly, the suppression by *BUB1*, *BUB3*, or *MPS1* is independent of Mad2p and therefore independent of the Mad2p-dependent mitotic checkpoint pathway. Additional genetic analyses indicate that *BUB1* and *BUB3* require one another to suppress the cold sensitivity of *tub1-729*, while *MPS1* requires neither *BUB1* nor *BUB3*. Further experiments suggest that the mutant tub1-729p α-tubulin is defective in its interactions with the kinetochore, as assayed by arrest phenotype, frequency of chromosome loss, and genetic interactions with mutations in genes encoding kinetochore proteins. The data suggest that Bub3p contributes to stabilizing microtubule-kinetochore attachment and that it is this activity that suppresses the phenotypes of *tub1-729*.

MATERIALS AND METHODS

Strains and media: All yeast strains used in this study are derivatives of FSY182, -183, or -185 (Weinstein and Solomon 1990) except where noted (Table 1). We used standard methods for yeast manipulations (Guthrie and Fink 1991; Solomon *et al.* 1992).

Plasmid construction: To construct a *LYS2*-marked plasmid expressing *BUB3* under the control of its own promoter, the *BUB3* open reading frame was amplified by PCR and ligated into either the pRS317 vector (Sikorski and Hieter 1989) or the YEp426 vector (Ma *et al.* 1987). The upstream primer used to amplify *BUB3* (5′-ATAGCGGCCGCGTGACAACCA AGC-3′) contains a *Not*I site (underlined). The downstream primer (5′-TCTGTCTTCTTGCGTATAGG-3′) contains a *Sal*I site (underlined). These two primers generate a PCR product containing the *BUB3* gene as well as ~250 bp of upstream and downstream sequence (~1500 bp). The PCR product was digested with *Not*I and *Sal*I and ligated into a *Not*I/*Sal*I-digested pRS317 or YEp426 vector to make pKC52 and pKC74. Both of these vectors rescue the benomyl supersensitivity of *bub3*Δ cells.

To construct a *LYS2*-marked plasmid expressing either *tub1-729* or *TUB1* under the control of its own promoter, the *TUB1* coding region was amplified by PCR from pRB629 or pRB539, respectively. The upstream *TUB1* primer (5′-GCGCGCCG GCGGCCGCTGCTCACACCAAGCATCA-3′) contains a *Not*I site (underlined). The downstream *TUB1* primer (5′-CCGCC GGCCCTGCAGCAATACGATGAGACTCAT-3′) contains a *Pst*I site (underlined). These two primers amplified a region including the α-tubulin open reading frame as well as 500 bp of upstream sequence and 260 bp of downstream sequence (~2200 bp). The PCR products were digested with *Not*I and *Pst*I and ligated into *Not*I/*Pst*I-digested pRS317 (Sikorski and Hieter 1989) to generate pKC43 (*tub1-729*) and pKC44 (*TUB1*).

Gene disruptions: To delete the *MAD2* open reading frame with *KAN*^{R}, we digested the *mad2::*KAN^R knockout plasmid (pKS198-MAD2; a gift of K. Simons and P. Sorger) with *Nde*I and *Sac*II and transformed this fragment into KCY1955 to generate SKY294. Proper integration was verified using PCR. *Mad2::KAN*^{R} is benomyl supersensitive and this sensitivity can be rescued by pRC4.

BUB3 was disrupted using the *LEU2* disruption construct, pTR27 (a gift from M. A. Hoyt). pTR27 was digested with *StuI* and *SacI* and transformed into the wild-type diploid FSY185 to generate KCY576. Proper integration of the *LEU2* gene at the *BUB3* locus was verified by PCR. KCY576 was sporulated to generate *bub3::LEU2* haploids sensitive to the microtubule depolymerizing drug, benomyl. This benomyl supersensitivity can be rescued by a low-copy *BUB3* plasmid.

To delete the *TUB3* open reading frame with *KAN*^{R}, we used the PCR-based knock-out strategy described by Wach *et al.* (1997). Using primers containing both 40 bp of *TUB3*-flanking DNA and 20 bp of DNA found in the pFA6a-*KAN*MX6 plasmid (a gift from P. Phillipsen), we amplified a fragment containing the *KAN*^{R} gene surrounded by *TUB3* flanking DNA. We purified this fragment and transformed it into KCY2011 to generate KCY2024. Proper integration was verified using PCR.

Cell morphology and nuclear position: To assay the budding phenotypes of *TUB3/p-tub1-729*, *mad2*Δ cells, we transformed these cells with *MAD2*- or *BUB3*-expressing plasmids (KCY-2072, KCY2070, KCY2077, and KCY2080) and grew these cells to mid-log phase (1×10^7 cells/ml) at the permissive temperature (30°). After shifting them to restrictive temperature (13°), we counted the cells at varying intervals and determined the number of large-budded cells (Figure 2C). For the purpose of this assay, large-budded cells were defined as cells with buds greater than half the size of their mothers' buds. To assay the budding phenotypes and nuclear positioning in *tub1-729* (with or without excess *BUB3*), *tub1-724*, and wild-type cells, the cells were grown for 20 hr at the permissive (30°) or restrictive (15°) temperatures. Nuclear positioning and bud size were analyzed as described in Schatz *et al.* (1988) and Table 2 and Figure 4. The identity of each of the samples in both assays was hidden during these analyses.

Chromosome loss assays: To construct a strain that would enable us to test the frequency of chromosome loss in *tub1-729* cells, we crossed KCY2024 to YPH278 (a gift from P. Hieter) to generate KCY2045. We sporulated and dissected this diploid to generate KCY2098, which expresses *tub1-729* as the sole source of α-tubulin and contains the *ade2-101* mutation and a mini-chromosome expressing *SUP11*. The colonies produced by these cells are white as long as the mini-chromosome expressing the ochre suppressor *SUP11* is maintained. To measure the frequency of chromosome loss in *tub1-729* cells, we used the method described by Christianson *et al.* (1992). We grew cells at the permissive temperature in media that selects for the maintenance of the mini-chromosome and then plated these cells to YEPD media. We determined the viable number of cells in each culture (N_1) and the percentage of cells that contained the mini-chromosome (P_2). We diluted these cells 20-fold and grew the cells at a semipermissive temperature (20°) for 36 hr in medium that does not select for mini-chromosome maintenance. We plated these cells to YPD at the permissive temperature and determined the number of viable cells per milliliter (N_2) and the number of cells that still contained the mini-chromosome (*i.e.*, number of white colonies, P_2). The frequency of mini-chromosome loss (m) is described by $m = 1 - e^{\ln(P_2/P_1)/g}$, where g represents the number of cell divisions at the semipermissive temperature and is calculated by $g = \ln(N_2/N_1)/\ln 2$. Over 1200 colonies per strain were analyzed to produce the data shown in Table 3.

Synthetic lethality of *tub1-729* with kinetochore mutants: To

test whether cells expressing *tub1-729* as the sole source of α-tubulin are viable in combination with kinetochore mutations, we crossed KCY2103 (*tub1::HIS3*, *tub3::KAN*^{R}, p*tub1-729-LEU2-CEN*, p*TUB1-URA3-CEN*) to four different kinetochore mutants: *ndc10-1* (JK418); *ndc80-1*; *nuf2-61* (PSY455); and *dam1-1* (MJYM14-pb 889). The *ndc10-1* and *ndc80-1* alleles are in the W303 yeast background; both of these strains were backcrossed at least three times into the S288C yeast background before being crossed to KCY2103. We isolated *tub1-729* kinetochore double mutants from these crosses and struck them to selective media or selective media containing 5-fluoroorotic acid (5-FOA) to select for the loss of the wild-type α-tubulin plasmid expressing *URA3*. After 5 days of growth at 30°, the plates were photographed (Figure 5).

RESULTS

A subset of the mitotic checkpoint genes suppresses the cold sensitivity of *tub1-729*: Cells expressing the mutant *tub1-729* as the sole source of α-tubulin are cold sensitive and arrest with no microtubules (Schatz *et al.* 1988; Guénette *et al.* 1995). These phenotypes are suppressed partially by extra copies of either of two mitotic checkpoint genes, *BUB1* or *BUB3* (Guénette *et al.* 1995). The interaction is specific; other α-tubulin mutants with the same arrest phenotype are not suppressed by these genes. Extra copies of a third checkpoint gene, *MPS1*, suppress the cold sensitivity of *tub1-729* as efficiently as *BUB1* or *BUB3* (Figure 1A). However, the other known mitotic checkpoint genes—*BUB2*, *MAD1*, *MAD2* (Guénette *et al.* 1995), or *MAD3*—do not suppress the cold sensitivity of *tub1-729* (Figure 1A).

We quantitated the *BUB3* suppression by measuring plating efficiency of mutant and suppressed cells plated at permissive (30°) and restrictive (18°) temperatures. *BUB3* on a *CEN*-plasmid increases the colony formation at 18° by more than three orders of magnitude (0.0001–0.3%; see Figure 1A). Higher levels of *BUB3*, present on a high-copy 2μ plasmid, increase plating efficiency to 19.4%. Similar results were obtained for *BUB1*. However, higher levels of *MPS1* overexpression can arrest the cell cycle (Hardwick *et al.* 1996), and we have found that 2μ plasmids expressing either *MAD1* or *MAD3* have a detrimental effect on *tub1-729* cells (data not shown). Therefore, although high-copy plasmids expressing either *BUB1* or *BUB3* are more efficient suppressors of the phenotypes of *tub1-729*, we focused on the effects of a lower level of expression derived from *CEN* plasmids in order to compare the effects of *BUB1* and *BUB3* with those of other mitotic checkpoint genes.

The kinase activity of *BUB1* is required to suppress the cold sensitivity of *tub1-729*: Bub1p is a serine/threonine kinase whose enzymatic activity is required for the spindle assembly checkpoint (Roberts *et al.* 1994). A mutant allele of *BUB1*, *bub1K733R*, which contains a single amino acid substitution in the conserved kinase domain, does not rescue the benomyl sensitivity of cells deleted for *BUB1* (Roberts *et al.* 1994). Although the kinase activity of Bub1K733Rp is lost, the mutant protein still binds to Bub3p (Roberts *et al.* 1994). To determine if the kinase activity of Bub1p is required for the suppression of *tub1-729*, we transformed *tub1-729* cells with low-copy plasmids expressing either wild-type *BUB1* or *bub1K733R*. The overexpression of mutant *BUB1*, *bub1K-733R*, does not suppress the cold sensitivity of *tub1-729* (Figure 1B). This result suggests that the kinase activity of *BUB1* is required for the suppression of *tub1-729*.

***MPS1*, *BUB1*, and *BUB3* can suppress the cold sensitivity of *tub1-729* in the absence of *MAD2*:** *BUB1*, *BUB3*, and *MPS1* are each components of the mitotic checkpoint pathway that prevents cells from entering anaphase until all of the chromosomes are attached to the microtubules of the mitotic spindle. We tested whether the suppression of *tub1-729* by *MPS1*, *BUB1*, or *BUB3* depends upon that checkpoint function by making a strain (SKY294) that expresses *tub1-729* and in which the mitotic checkpoint is disrupted by the deletion of *MAD2* (Li and Murray 1991). However, all of the known components of the mitotic checkpoint pathway (*BUB1*, *BUB3*, *MAD1*, *MAD2*, and *MAD3*) are essential in *tub1-729* cells in which tub1-729p is the sole source of α-tubulin (*tub1*Δ, *tub3*Δ, and p*tub1-729*; data not shown). Therefore, we generated KCY2010 cells, which express *tub1-729* as well as a chromosomal copy of the minor α-tubulin gene, *TUB3*. The KCY2010 cells are still cold sensitive, but at 13° instead of 18° (Figure 2A). Their cold sensitivity is also suppressed by the same subset of mitotic checkpoint genes (Figure 2A). We used *TUB3/p-tub1-729* cells to test whether *BUB1*, *BUB3*, and *MPS1* could rescue the phenotypes of *tub1-729* cells that were deleted for *MAD2*.

*TUB3/p-tub1-729, mad2*Δ cells (KCY2042) are viable at 30°. However, they are more cold sensitive than *TUB3/p-tub1-729* cells since the checkpoint pathway is disrupted, and they fail to grow at 13°–15° (Figure 2B). Cold sensitivity is rescued by extragenomic copies of *BUB1*, *BUB3*, and *MPS1* (Figure 2B), despite the abrogation of the mitotic checkpoint. Extra copies of *BUB2* or *MAD3* did not effect the cold sensitivity of *TUB3/p-tub1-729* cells. We have observed an occasional slight suppression by *MAD1*, but this result is not consistent.

It is possible that overexpression of specific checkpoint genes could suppress *tub1-729* cold sensitivity by strengthening the mitotic checkpoint. To test that possibility, we monitored the progression of *TUB3/p-tub1-729, mad2*Δ cells through the cell cycle after shift to the restrictive temperature (13°). We conducted this experiment at 13° rather than 15° (Figure 2B) because we observed a tighter cell cycle arrest at the lower temperature. Our first observation was that KCY2042 cells (*TUB3/p-tub1-729, mad2*Δ) failed to arrest as large-budded cells when shifted to the restrictive temperature (Figure 2C, triangles). In contrast, KCY2042 cells in which the mitotic checkpoint has been restored by transformation with a *CEN* plasmid bearing *MAD2* accumulate significantly at the large-bud stage after one to two cell cycles (Figure 2C, diamonds; Table 2). This suggests

TABLE 1

Strains and plasmids

Strain/plasmid	Genotype	Reference
Strains		
FSY157	*MAT*α *ura3-52 leu2-3, 112 his3*Δ*200 lys2-801 tub1*Δ*::HIS3 tub3*Δ*::TRP1* plus pRB634	Schatz *et al.* (1988)
FSY160	*MAT*α *ura3-52 leu2-3, 112 his3*Δ*200 lys2-801 tub1*Δ*::HIS3 tub3*Δ*::TRP1* plus pRB629	Schatz *et al.* (1988)
FSY182	*MAT*α *ura3-52 leu2-3, 112 his3*Δ*200 lys2-801 tub1*Δ*::HIS3 tub3*Δ*::TRP1* plus pRB539	Schatz *et al.* (1988)
FSY185	*a*/α *ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3*Δ*200/his3*Δ*200 lys2-801/lys2-801 ade2/ADE2*	Weinstein and Solomon (1990)
JK418	MAT**a** *leu2-3, 112 lys2 ura3 trp1 his3 ndc10-1* (W303)	Goh and Kilmartin (1993)
KCY576	*a*/α *ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3*Δ*200/his3*Δ*200 lys2-801/lys2-801 ade2/ADE2 BUB3/bub3::LEU2*	This study
KCY927	FSY160 plus pRC4	This study
KCY929	FSY160 plus pKH502	This study
KCY931	FSY160 plus pKH130	This study
KCY933	FSY160 plus pKF44	This study
KCY935	FSY160 plus pMA1183	This study
KCY936	FSY160 plus pMA1145	This study
KCY1087	*MAT***a** *ura3-2 leu2-3,112 lys2-801 his3*Δ*200 tub1::hisg tub3::hisg* plus pKC43	This study
KCY1168	KCY1087 plus pRS315	This study
KCY1170	KCY1087 plus pTR168	This study
KCY1295	KCY1087 plus pTR170	This study
KCY1876	FSY160 plus pCT3	This study
KCY1955	FSY160 plus pDK7	This study
KCY1956	FSY160 plus pE58	This study
KCY2010	*MAT*α *tub1::HIS3 TUB3 trp1-1 lys2-801 leu2-3,112 ura3-52 his3*Δ*200* plus pRB629	This study
KCY2011	*MAT***a** *tub1::HIS3 TUB3 trp1-1 lys2-801 leu2-3,112 ura3-52 his3*Δ*200* plus pRB629	This study
KCY2013	KCY2010 plus pCT3	This study
KCY2014	KCY2010 plus pDK7	This study
KCY2015	KCY2010 plus pE58	This study
KCY2016	KCY2010 plus pKF44	This study
KCY2017	KCY2010 plus pMA1183	This study
KCY2018	KCY2010 plus pMA1145	This study
KCY2019	KCY2010 plus pKH130	This study
KCY2020	KCY2010 plus pRC4	This study
KCY2021	KCY2010 plus pKH502	This study
KCY2024	*MAT***a** *tub1::HIS3 tub3::KAN*^{R} *trp1-1 lys2-801 leu2-3,112 ura3-52 his3*Δ*200* plus pRB629	This study
KCY2042	*MAT***a** *ura3-52 leu2-3,112 lys2-801 his3*Δ*200 trp1-1 tub1::HIS3 TUB3 mad2::KAN*^{R} plus pRB629	This study
KCY2048	FSY160 plus pC2	This study
KCY2070	KCY2042 plus pCT3	This study
KCY2071	KCY2042 plus pKH130	This study
KCY2072	KCY2042 plus pRC4	This study
KCY2073	KCY2042 plus pKH502	This study
KCY2074	KCY2042 plus pE58	This study
KCY2075	KCY2042 plus pKF44	This study
KCY2076	KCY2042 plus pMA1138	This study
KCY2077	KCY2042 plus pMA1145	This study
KCY2080	KCY2042 plus pC2	This study
KCY2085	*MAT*α *ura3-52 leu2-3,112 his3*Δ*200 lys2-801 trp1-1 tub1::HIS3, tub3::KAN*^{R} plus pRB629	This study
KCY2098	*MAT*α *ade2-101 ura3-52 leu2-3,112 his3*Δ*200 lys2-801 tub1::HIS3 tub3::KAN*^{R} CFIII (CEN3.L.Y YPH278) *URA3 SUP11* plus pRB629	This study

(*continued*)

TABLE 1

(Continued)

Strain/plasmid	Genotype	Reference
KCY2103	KCY2085 plus pDK7	This study
KCY2155	KCY2098 plus pRS317	This study
KCY2156	KCY2098 plus pKC52	This study
KCY2157	KCY2098 plus pKC74	This study
KCY2171	*MAT*α *ura3-52 his3*Δ*200 leu2-3,112 trp1-1 tub1::HIS3 tub3::KAN^R ndc10-1* plus pDK7 and pRB629	This study
KCY2216	*MAT*α *ura3-52 his3*Δ*200 leu2-3,112 trp1-1 tub1::HIS3 tub3::KAN^R dam1-1* plus pDK7 and pRB629	This study
KCY2247	*MAT***a** *ura3-52 his3*Δ*200 leu2-3,112 trp1-1 tub1::HIS3 tub3::KAN^R ndc80-1* plus pDK7 and pRB629	This study
KCY2202	*MAT*α *ura3-52 his3*Δ*200 leu2-3,112 trp1-1 lys2-801 tub1::HIS3 bub3::LEU2* plus pKC43	This study
KCY2258	*MAT***a** *ura3-52 his3*Δ*200 leu2-3,112 trp1-1 tub1::HIS3 tub3::KAN^R nuf2-61* plus pDK7 and pRB629	This study
KCY2262	*MAT***a** *ura3-52 his3*Δ*200 leu2-3,112 trp1-1 lys2-801 tub1::HIS3 bub1::KAN^R* plus pRB629	This study
KCY2350	KCY2202 plus pCT3	This study
KCY2352	KCY2202 plus pE58	This study
KCY2354	KCY2202 plus pKIF44	This study
KCY2356	KCY2202 plus pMA1183	This study
KCY2358	KCY2202 plus pMA1145	This study
KCY2360	KCY2202 plus pKH130	This study
KCY2362	KCY2202 plus pRC4	This study
KCY2364	KCY2202 plus pKH502	This study
KCY2366	KCY2262 plus pCT3	This study
KCY2368	KCY2262 plus pE58	This study
KCY2370	KCY2262 plus pKF44	This study
KCY2372	KCY2262 plus pMA1183	This study
KCY2374	KCY2262 plus pMA1145	This study
KCY2376	KCY2262 plus pKH130	This study
KCY2378	KCY2262 plus pRC4	This study
KCY2380	KCY2262 plus pKH502	This study
KCY2397	KCY2098 plus pKC44	This study
MJYM14-9b (889)	*MAT***a** *his3*Δ*200 leu2-3,112 ura3-52 trp1*Δ*1 dam1-1*	Jones *et al.* (1999)
ndc80-1	*MAT***a** *ura3-52 his3*Δ*200 leu2-3,112 trp1-1 lys2-801 ndc80-1* (W303)	Wigge *et al.* (1998)
PSY455	*MAT***a** *leu2 ura3 trp1 nuf2-61*	Osborne *et al.* (1994)
SKY294	*MAT***a** *tub1::HIS3 tub3::TRP1 mad2::KAN^R* plus pDK7 and pRB629	This study
YPH278	*MAT*α *ade2-101 leu2-1 ura3-52 his3-200 lys2-801* CFIII (CEN3.L.Y YPH278) *URA3 SUP11*	Spencer *et al.* (1990)
Plasmids		
pAS53	*tub3::hisg-URA3-hisg*	Abruzzi *et al.* (2002)
pC2	*BUB3-URA3-2*μ	Guénette *et al.*(1995)
pCT3	*URA3-CEN*	Gift of C. Thompson and R. Young
pDK7	*TUB1-URA3-CEN*	Kirkpatrick and Solomon (1994)
pE58	*MPS1-URA3-CEN*	Schutz and Winey (1998)
pFA6-kanMX6	*KAN^R* for constructing PCR knockouts	Wach *et al.* (1997)
pKC43	*tub1-729-LYS2-CEN*	This study
pKC44	*TUB1-LYS2-CEN*	This study
pKC52	*BUB3-LYS2-CEN*	This study
pKC74	*BUB3-LYS2-2*μ	This study
pKF44	*BUB1-URA3-CEN*	Farr and Hoyt (1998)
pKH130	*MAD1-URA3-CEN*	Hardwick and Murray (1995)

(*continued*)

TABLE 1

(Continued)

Strain/plasmid	Genotype	Reference
pKH502	*MAD3-URA3-CEN*	Hardwick *et al.* (2000)
pKS198-d*MAD2*	*mad2::KAN*^{R} deletion plasmid	Gift from K. Simons and P. Sorger
pLV61	*tub1::hisg-URA3-hisg*	Fleming *et al.* (2000)
pTR27	*bub3::LEU2*	Hoyt *et al.* (1991)
pTR168	*BUB1-LEU2-CEN*	Roberts *et al.* (1994)
pTR170	*bub1K733R-LEU2-CEN*	Roberts *et al.* (1994)
pMA1183	*BUB2-URA3-CEN*	Gift from A. Hoyt
pMA1145	*BUB3-URA3-CEN*	Roberts *et al.* (1994)
pRB624	*tub1-724-LEU2-CEN*	Schatz *et al.* (1988)
pRB539	*TUB1-LEU2-CEN*	Schatz *et al.* (1988)
pRB629	*tub1-729-LEU2-CEN*	Schatz *et al.* (1988)
pRC4	*MAD2-URA3-CEN*	Chen *et al.* (1999)
pRS315	*LEU2-CEN*	Sikorski and Hieter (1989)
pRS317	*LYS2-CEN*	Sikorski and Hieter (1989)
YEp426	*LYS2-2μ*	Ma *et al.* (1987)

that *tub1-729* cells accumulate as large-budded cells due to the *MAD2*-dependent spindle assembly checkpoint. Although a plasmid expressing *MAD2* can restore the mitotic checkpoint in KCY2042 cells, plasmids expressing *BUB3* did not. KCY2042 cells containing extra copies of *BUB3* on either a low-copy *CEN* plasmid (Figure 2C, squares) or a high-copy 2μ plasmid (Figure 2C, circles) continue to grow and do not accumulate as large-budded cells. We conclude that *BUB3* does not suppress the phenotypes of *tub1-729* by restoring the mitotic checkpoint and that the suppression by *BUB3* requires a function independent of its role in the mitotic checkpoint.

Genetic requirements for *BUB1*, *BUB3*, and *MPS1* suppression of *tub1-729* cold sensitivity: To learn more about how *BUB1*, *BUB3*, and *MPS1* suppress the cold sensitivity of *tub1-729*, we examined the suppressing activity of these genes in the absence of *BUB1* or *BUB3*. We constructed *TUB3/p-tub1-729, bub1*Δ cells (KCY2262) and *TUB3/p-tub1-729*, *bub3*Δ cells (KCY2202) and transformed them with a panel of mitotic checkpoint genes (Figure 3, A and B). As expected, restoration of wild-

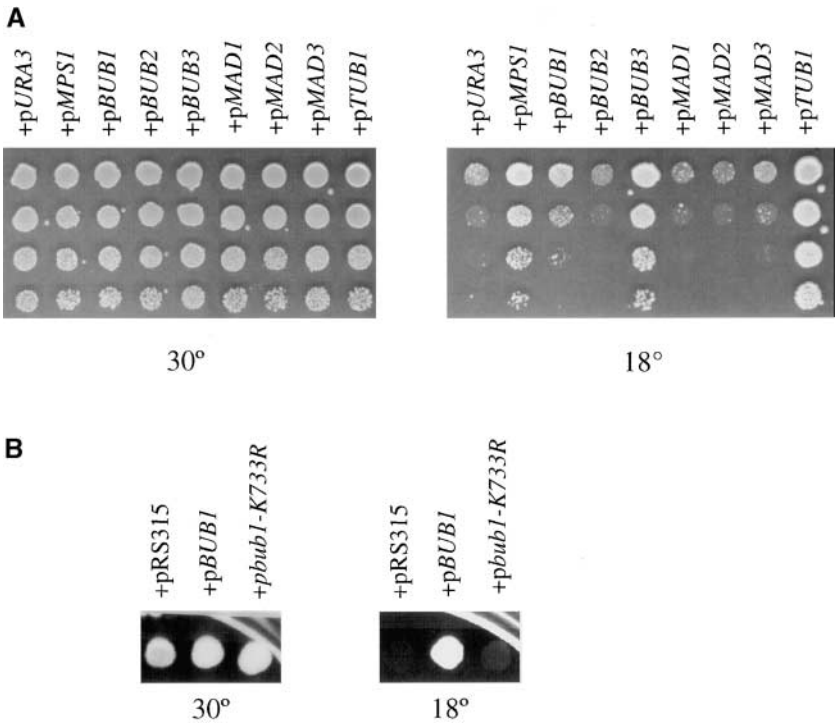

Figure 1.—A subset of the mitotic checkpoint genes suppresses the cold sensitivity of *tub1-729*. (A) *tub1-729* cells (FSY160; *tub1*Δ, *tub3*Δ, p*tub1-729*) were transformed with plasmids expressing the mitotic checkpoint genes (see above), an empty plasmid (negative control), or a plasmid expressing wild-type α-tubulin (positive control). Serial dilutions of these cells were plated to the permissive (30°) and restrictive (18°) temperatures. *MPS1*, *BUB1*, and *BUB3* suppress the cold sensitivity of *tub1-729*; however, *BUB2*, *MAD1*, *MAD2*, and *MAD3* do not. (B) The kinase activity of Bub1p is required for the suppression of the cold sensitivity of *tub1-729*. *Tub1-729* cells were transformed with a plasmid expressing *BUB1*, a plasmid expressing a kinase-defective allele of *BUB1* (*bub1K733R*), or a control plasmid (pRS315). These cells were grown at the permissive (30°) or nonpermissive (18°) temperature. In contrast to wild-type *BUB1*, the kinase-defective allele of *BUB1* does not suppress the cold sensitivity of *tub1-729*.

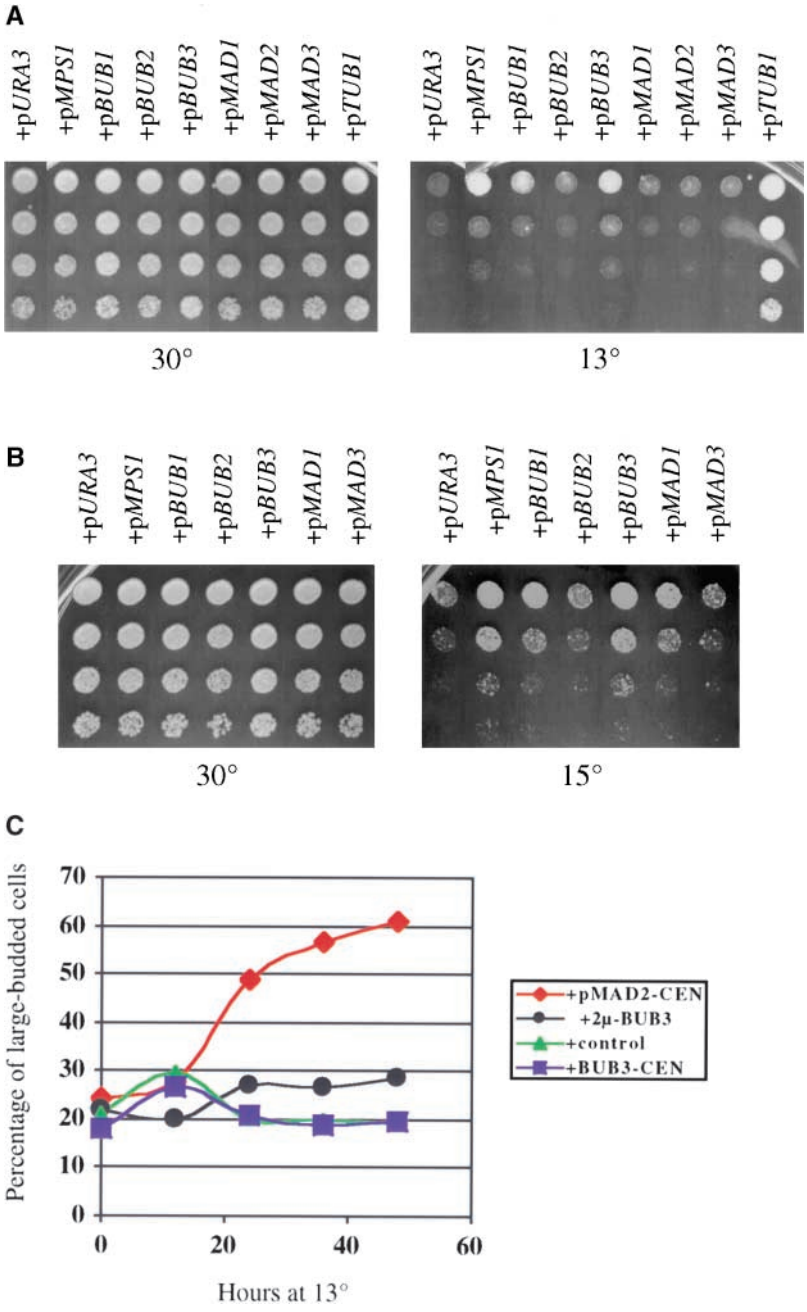

FIGURE 2.—*MPS1*, *BUB1*, and *BUB3* suppress the cold sensitivity of *tub1-729* when the *MAD2*-dependent checkpoint is eliminated. (A) *TUB3/p-tub1-729* cells (KCY2010; *tub1*Δ, *TUB3*, p*tub1-729*) were transformed with the same constructs as shown in Figure 1A and serial dilutions of these cells were plated to the permissive (30°) and restrictive (13°) temperatures. Although these cells are less temperature sensitive due to the expression of the minor α-tubulin gene, *TUB3*, they are suppressed by the same subset of the mitotic checkpoint genes as shown in Figure 1A. (B) *TUB3/p-tub1-729* cells deleted for *MAD2* (KCY2042; *tub1*Δ, *TUB3*, *mad2*Δ, p*tub1-729*) were transformed with a control plasmid or plasmids expressing mitotic checkpoint genes (see above) and serial dilutions of these cells were plated to the permissive (30°) and restrictive (15°) temperatures. *MPS1*, *BUB1*, and *BUB3* can suppress the cold sensitivity of *TUB3/p-tub1-729* cells even in the absence of *MAD2*. (C) *TUB3/p-tub1-729, mad2*Δ cells transformed with a control plasmid, a *MAD2* plasmid, or high- or low-copy plasmids expressing *BUB3* were shifted to the restrictive temperature (13°) and the number of large-budded cells was monitored over time. *TUB3/p-tub1-729, mad2*Δ cells do not accumulate as large-budded cells at the restrictive temperature (triangles). However, if normal *MAD2* expression is restored by transforming these cells with a *MAD2* plasmid, these cells accumulate as large-budded cells after 24 hr at 13° (diamonds). Although the overexpression of *BUB3* rescues the cold sensitivity of *TUB3/p-tub1-729, mad2*Δ cells (see above), it does not restore the spindle assembly checkpoint in these cells; they do not accumulate as large-budded cells at the restrictive temperature (circles and squares). The identity of each of these samples was hidden from the experimenter for the duration of the experiment.

type *BUB1* rescues the cold sensitivity of *TUB3/p-tub1-729, bub1*Δ cells, and restoration of wild-type *BUB3* rescues the cold sensitivity of *TUB3/p-tub1-729, bub3*Δ *cells*. *BUB1* and *BUB3* require one another to suppress the cold sensitivity of *tub1-729*; *BUB1* cannot rescue *TUB3/p-tub1-729, bub3*Δ cells and *BUB3* cannot rescue *TUB3/p-tub1-729, bub1*Δ cells. An extra genomic copy of *MPS1* can suppress the cold sensitivity of *tub1-729* cells in the absence of either *BUB3* or *BUB1*. As previously observed, we saw no suppression by *BUB2*, *MAD1*, *MAD2*, or *MAD3*. This experiment suggests that Bub1p and Bub3p function together to suppress the cold sensitivity of *tub1-729* and that Mps1p functions independently.

***Tub1-729* cells accumulate as large-budded cells with their nucleus adjacent to or straddling the mother-bud neck:** To investigate the nature of the suppression of *tub1-729* by a subset of the mitotic checkpoint genes, we focused on the effects of *BUB3* and conducted a more careful examination of the morphology and nuclear positioning of *tub1-729* cells at the permissive (30°) and restrictive temperatures (15°). At the permissive temperature, *tub1-729* cells resemble wild-type cells; they have a wild-type distribution of large- and small-budded cells, and the position of the nuclei in the large-budded cells is typical for cells progressing through mitosis (Table 2, A and B). When *tub1-729* cells are shifted to the

TABLE 2

Budding morphology and nuclear position of *tub1-729* cells at permissive and nonpermissive temperatures

	A		B			
						Other
Wild type, 30°	64%	36%	7%	6%	23%	0%
Wild type, 15°	57%	43%	5%	14%	23%	1%
tub1-729, 30°	59%	41%	7%	6%	26%	2%
tub1-729, 15°	26%	74%	27%	27%	19%	1%
tub1-724, 30°	63%	37%	9%	5%	21%	2%
tub1-724, 15°	16%	84%	69%	8%	7%	0%

(A) The budding morphology of wild-type, *tub1-729*, and *tub1-724* cells was analyzed at permissive (30°) and nonpermissive temperatures (15°). The budding morphology was divided into two classes: cells with no bud or a small bud and cells with a large bud. The percentage of cells in each of these categories is shown. (B) We further characterized the phenotypes of the large-budded cells by examining the position of their nucleus. The percentage of the large-budded cells with their nuclei randomly positioned in the mother cell, near or straddling the bud neck, or separated in the mother and daughter cell is presented above. All assays of cell morphology and nuclear positioning were done by blind screening.

restrictive temperature (15°) for 20 hr, the percentage of cells that are large-budded cells almost doubles (74 *vs.* 41%). DNA staining of *tub1-729* cells grown at the restrictive temperature revealed that the nuclei of most of the large-budded cells are either in the mother cell or adjacent to or spanning the mother-bud neck (Table 2B). This suggests that at the restrictive temperature, *tub1-729* cells accumulate prior to the onset of anaphase. We compared this arrest phenotype to that of another class 1 α-tubulin mutant, *tub1-724*. That mutant also arrests as large-budded cells at the restrictive temperature, but almost all of the large-budded cells accumulate with their nucleus in a random position in the mother cell. The contrast between the nuclear positioning phenotypes of *tub1-729* and *tub1-724* cells suggests that the nuclear positioning phenotypes observed for *tub1-729* cells are not a simple consequence of loss of microtubules at restrictive temperature.

***Tub1-729* cells overexpressing Bub3p no longer accumulate as large-budded cells with their nuclei positioned at the mother-bud neck:** Since a high-copy plasmid expressing *BUB3* rescues the cold sensitivity of *tub1-729*, we examined the budding morphology and nuclear positioning in *tub1-729* cells containing either a control plasmid or a 2μ plasmid expressing *BUB3*. *Tub1-729* cells overexpressing *BUB3* still delay in mitosis as large-budded cells (76 *vs.* 74%); however, there is a dramatic decrease in the number of cells that accumulate as large-budded cells with their nuclei near or spanning the mother-bud neck (Figure 4) and an increase in the number of large-budded cells that accumulate with their nuclei in a random position in the mother cell (27–60%) (data not shown). The overexpression of *BUB3* causes a shift in the point at which cells accumulate in the cell cycle; *tub1-729* cells overexpressing *BUB3* accumulate as large-budded cells with their nuclei almost exclusively in a random position in the mother cell. The overexpression of *BUB3* causes *tub1-729* cells to more closely resemble other class 1 α-tubulin mutants that have general microtubule defects such as *tub1-724* (see Table 2).

***Tub1-729* cells exhibit a higher frequency of chromosome loss that is reduced by *BUB3* overexpression:** That *tub1-729* cells accumulate at the metaphase-to-anaphase transition at the restrictive temperature suggested that these cells might have defects in proper chromosome segregation. To test this hypothesis, we followed mini-chromosome loss (Spencer *et al.* 1990). We constructed a yeast strain (KCY2098) that expresses *tub1-729* as the sole source of α-tubulin, the *ade2-101* gene, and the ochre suppressor, *SUP11*, from a mini-chromosome. We transformed this strain with *CEN*-plasmid expressing *BUB3*; a 2μ plasmid expressing *BUB3*; and, as controls, plasmids expressing wild-type α-tubulin (*TUB1*) or with no insert. We monitored the number of viable cells that contain the mini-chromosome both before and after 36 hr of growth at the semipermissive temperature (20°) in media that do not select for the mini-chromosome. Using the methods described by Christianson and colleagues we determined the frequency of chromosome loss (Christianson *et al.* 1992; see materials and methods). The results (Table 3) demonstrate that the higher frequency of chromosome loss in *tub1-729* cells relative to wild-type cells (3.8×10^{-2} *vs.* 0.2×10^{-2}) is suppressed by increasing levels of *BUB3*.

Mutations in the kinetochore components *DAM1* and *NUF2* are lethal in combination with *tub1-729*: As shown above, the arrest phenotype of *tub1-729* suggests that the mutation affects the ability of cells to progress from metaphase to anaphase. This defect is suppressed by overexpression of three mitotic checkpoint proteins whose mammalian homologs localize to the kinetochore (Chen *et al.* 1996, 1998; Li and Benezra 1996; Taylor and McKeon 1997; Taylor *et al.* 1998; Martinez-Exposito *et al.* 1999; Abrieu *et al.* 2001). Therefore, we tested whether *tub1-729* cells are affected by mutations in kinetochore components. We generated *tub1-729* cells that contain a *URA3-CEN* plasmid expressing wild-type α-tubulin (KCY2103) and each of four different temperature-sensitive kinetochore mutants (*ndc10-1*, *ndc80-1*, *nuf2-61*, or *dam1-1*; see materials and methods). Two of these double mutants—*tub1-729, dam1-1* and *tub1-729, nuf2-61*—are not viable in the absence of the plasmid expressing wild-type α-tubulin (Figure 5).

DISCUSSION

Accurate segregation of chromosomes between mother and daughter cells requires that microtubules attach to

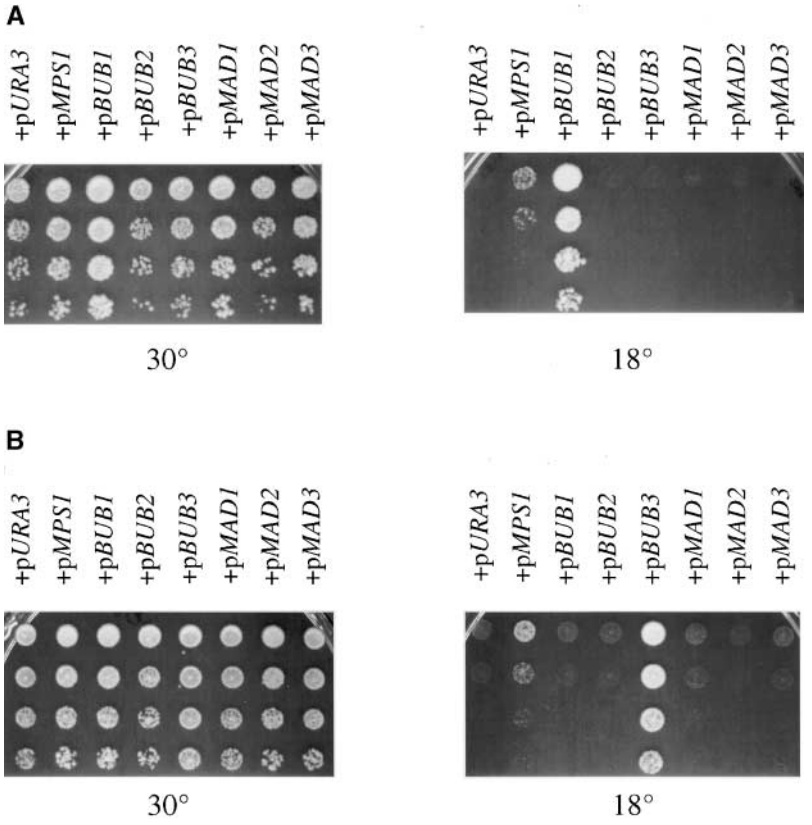

FIGURE 3.—*BUB1* and *BUB3* require one another to suppress the cold sensitivity of *tub1-729*; however, suppression by *MPS1* requires neither *BUB1* nor *BUB3*. *TUB3/p-tub1-729* cells deleted for (A) *BUB1* (*bub1*Δ, *tub1*Δ, *TUB3*, p*tub1-729*) or (B) *BUB3* (*bub3*Δ, *tub1*Δ, *TUB3*, p*tub1-729*) were transformed with the plasmids shown above and serial dilutions of these cells were plated to the permissive (30°) and restrictive (18°) temperatures. As expected, a plasmid copy of *BUB1* suppresses the temperature sensitivity of *TUB3/p-tub1-729* cells deleted for *BUB1*, and a plasmid copy of *BUB3* suppresses the temperature sensitivity of *TUB3/p-tub1-729* cells deleted for *BUB3*. A genomic plasmid expressing *MPS1* rescues the temperature sensitivity of *tub1-729* in the absence of either *BUB3* or *BUB1*. *BUB1* and *BUB3* require one another to suppress the cold sensitivity of *tub1-729*.

the kinetochores of all of the chromosomes prior to the initiation of anaphase. The spindle assembly checkpoint arrests cells and prevents entry into anaphase until all the microtubule-kinetochore attachments are made. In *S. cerevisiae* six genes are essential for the spindle assembly checkpoint: *BUB1* and *BUB3* (HOYT *et al.* 1991); *MAD1*, *2*, and *3* (LI and MURRAY 1991); and *MPS1* (WEISS and WINEY 1996). In higher eukaryotes, the products of the highly conserved homologs of these genes (CHEN *et al.* 1996, 1998; LI and BENEZRA 1996; TAYLOR and MCKEON 1997; CAHILL *et al.* 1998; JIN *et al.* 1998; TAYLOR *et al.* 1998) all localize to unattached kinetochores, and all except Mps1p disappear from kinetochores after microtubules have attached (CHEN *et al.* 1996, 1998; LI and BENEZRA 1996; TAYLOR and MCKEON 1997; TAYLOR *et al.* 1998; MARTINEZ-EXPOSITO *et al.* 1999; ABRIEU *et al.* 2001). When kinetochores are not attached to microtubules, the proteins involved in the checkpoint bind to and inhibit the ubiquitin ligase specificity factor, Cdc20p, which is a component of the anaphase-promoting complex (APC; reviewed in AMON 1999; BURKE 2000). The APC degrades Pds1p, an inhibitor of sister chromatid separation. When Cdc20p function is inhibited, Pds1p levels remain high and cells remain in metaphase.

The experiments presented here focus on the interactions between a subset of the mitotic checkpoint genes and a mutant allele of α-tubulin, *tub1-729*. The data suggest that *MPS1*, *BUB1*, and *BUB3* have a role at the kinetochore that is distinct from their function in the mitotic checkpoint pathway. We propose that *tub1-729* microtubules are deficient in kinetochore binding and that excess *MPS1*, *BUB1*, and *BUB3* suppress the phenotypes of *tub1-729* by affecting this attachment.

A subset of the mitotic checkpoint genes rescue the cold sensitivity of *tub1-729*: *BUB1*, *BUB3*, and *MPS1* are partial suppressors of the defects of *tub1-729*. Modest overexpression of each rescues the cold sensitivity of *tub1-729* cells, but not the sensitivity to the microtubule-destabilizing drug benomyl (GUÉNETTE *et al.* 1995; data not shown). We conclude that *tub1-729p* has multiple defects and that *BUB1*, *BUB3*, or *MPS1* suppresses a subset of them.

The ability of *BUB1*, *BUB3*, and *MPS1* to suppress *tub1-729* is specific. Extra copies of four other mitotic checkpoint genes—*BUB2*, *MAD1*, *MAD2*, or *MAD3*—have no effect on *tub1-729*. Conversely, *BUB1*, *BUB3*, and *MPS1* are not general suppressors of microtubule defects; they do not suppress all other cold-sensitive α-tubulin mutants (such as *tub1-724*; see Table 2; GUÉNETTE *et al.* 1995; data not shown) nor do they suppress mutants that effect the levels of tubulin polypeptides such as a deletion of the minor α-tubulin gene, *TUB3* (data not shown). These results suggest that excess *MPS1*, *BUB1*, and *BUB3* suppress a specific microtubule defect found in *tub1-729*.

A potential structural role for kinase components of the mitotic checkpoint: *BUB1* and *MPS1* encode serine/threonine kinases. The kinase activities of these two proteins are required for the spindle assembly check-

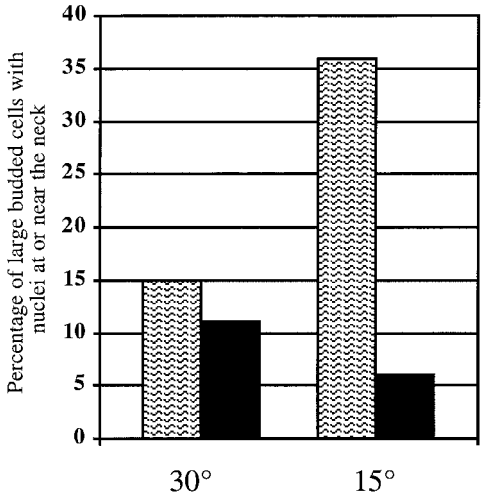

FIGURE 4.—*BUB3* overexpression reduces the number of large-budded *tub1-729* cells that accumulate at the restrictive temperature with their nuclei positioned near the mother-bud neck. When *tub1-729* cells are shifted to the restrictive temperature they accumulate as large-budded cells and ~35% of these cells have their nuclei positioned adjacent to or spanning the mother-bud neck. The overexpression of *BUB3* reduces the percentage of *tub1-729* cells that accumulate as large-budded cells with their nuclei positioned at or near the mother-bud neck to ~7%. ▨, *tub1-729*; ■, *tub1-729* + p*BUB3*-2μ.

point. Mutations in the kinase domain of either *BUB1* (*bub1K733R*) or *MPS1* (*mps1-1*) disrupt the spindle assembly checkpoint (ROBERTS *et al.* 1994; HARDWICK *et al.* 1996; WEISS and WINEY 1996). Similarly, *BUB1*'s kinase domain is required for the suppression of *tub1-729* (Figure 1B). *MPS1* requires both *BUB1* and *BUB3* to activate the spindle assembly checkpoint when overexpressed. This suggests that *MPS1* and *BUB1* have partially overlapping functions at the beginning of a signaling pathway (HARDWICK *et al.* 1996; FARR and HOYT 1998). Interestingly, *MPS1* requires neither *BUB1* nor *BUB3* to suppress

TABLE 3

Chromosome loss in *tub1-729* cells overexpressing *BUB3*

All strains are *tub1*Δ, *tub3*Δ, *SUP11-CEN-URA3*, *ade2-101* plus the following plasmids	Frequency of mini-chromosome loss ($\times 10^{-2}$)
p*tub1-729-LEU2-CEN* p*LYS2-CEN*	3.8
p*tub1-729-LEU2-CEN* p*BUB3-LYS2-CEN*	1.9
p*tub1-729-LEU2-CEN* p*BUB3-LYS2-2μ*	0.7
p*TUB1-LEU2-CEN* p*LYS2-CEN*	0.2

Tub1-729 cells containing the *ade2-101* mutations and a mini-chromosome expressing *SUP11* were transformed with an empty vector control, a high- or low-copy plasmid expressing *BUB3*, or a plasmid expressing wild-type *TUB1*. The frequency of mini-chromosome loss was determined using the method described by CHRISTIANSON *et al.* (1992; see MATERIALS AND METHODS). *Tub1-729* cells have a higher rate of chromosome loss than wild-type cells. This enhanced rate of chromosome loss is suppressed by increasing levels of *BUB3*.

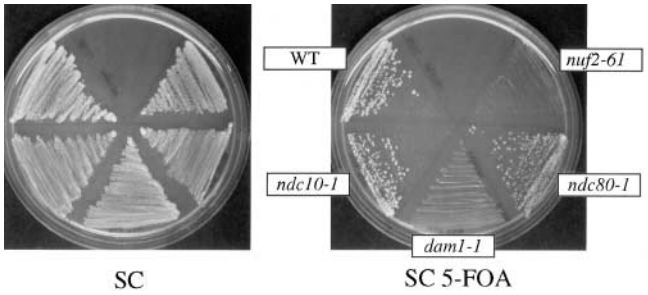

FIGURE 5.—*Tub1-729* cells require *NUF2* and *DAM1* for viability at the semipermissive temperature. A wild-type control and four different kinetochore component/*p-tub1-729* double mutants (*ndc10-1/tub1-729*, *ndc80-1/tub1-729*, *nuf2-61/tub1-729*, and *dam1-1/tub1-729*) expressing wild-type α-tubulin from a *URA3*-marked plasmid were streaked to either SC (synthetic complete) media or SC media containing 5-FOA. 5-FOA selects for the loss of the *URA3* maker. *Tub1-729/nuf2-61* and *tub1-729/dam1-1* double mutants are inviable in the absence of the wild-type α-tubulin plasmid at 25°.

the cold sensitivity of *tub1-729* (Figure 3). This observation suggests that *BUB1* and *MPS1* do not suppress the cold sensitivity of *tub1-729* by performing partially overlapping functions at the top of a signaling cascade.

The suppression of *tub1-729* may depend upon a structural role for the *BUB1* and *MPS1* gene products. That possibility is supported by several findings. First, the suppression depends upon overexpression of either gene (Figure 1A) and the ability of these genes to suppress *tub1-729* is additive (data not shown). Second, excess Bub3p—which forms a complex with Bub1p (ROBERTS *et al.* 1994; BRADY and HARDWICK 2000) and is phosphorylated by it—also suppresses *tub1-729*. In fact, co-overexpression of *BUB1* and *BUB3* from low-copy plasmids suppresses the cold sensitivity of *tub1-729* better than either one alone (data not shown). Third, Bub1p and Bub3p require one another to suppress the cold sensitivity of *tub1-729* (Figure 3). The Bub1p-Bub3p complex forms independently of any of the other spindle assembly checkpoint genes and persists throughout the cell cycle. In animal cells, Bub3p may regulate the kinase activity of Bub1p and may help Bub1p localize to unattached kinetochores (ROBERTS *et al.* 1994; TAYLOR *et al.* 1998). These results suggest that the *BUB1/BUB3* complex could be important for the suppression of *tub1-729*.

***MPS1*, *BUB1*, and *BUB3* suppression of *tub1-729* does not act through the *MAD2*-dependent checkpoint pathway:** Our results demonstrate that *MPS1*, *BUB1*, and *BUB3* do not require an intact mitotic checkpoint pathway to suppress the phenotypes of *tub1-729*. Recent work on the mammalian spindle assembly checkpoint suggests that *BUBR1*—a protein bearing homology to both *BUB1* and *MAD3* (TAYLOR *et al.* 1998)—and *BUB3* can bind to *CDC20* in a *MAD2*-independent manner and inhibit the APC, thereby preventing the onset of anaphase (TANG *et al.* 2001). This finding raises the possibil-

ity that overexpression of *MPS1*, *BUB1*, or *BUB3* could act by similarly restoring the checkpoint in cells deleted for *MAD2*. We performed two experiments to address this concern. First, we showed that the overexpression of *BUB3* does not rescue the benomyl supersensitivity of *mad2*Δ cells (data not shown), indicating that they were unable to arrest their cell cycle when microtubules were depolymerized. Second, we shifted KCY2042 (*tub1*Δ, *TUB3*, *ptub1-729*, *mad2*Δ) cells overexpressing *BUB3* to the restrictive temperature (13°). Unlike *MAD2* cells, the KCY2042 cells overexpressing *BUB3* did not arrest as large-budded cells (Figure 2C). These two experiments illustrate that excess *BUB3* does not restore the spindle assembly checkpoint in *mad2*Δ cells. They offer further evidence that *BUB3* suppresses the phenotypes of *tub1-729* using a novel mechanism that is independent of its known role in the mitotic checkpoint.

***Tub1-729* cells exhibit phenotypes similar to kinetochore mutants:** The mutation in *tub1-729p* changes alanine 422 to valine (Guénette *et al.* 1995). Residue 422 is in C-terminal helix 12, which protrudes from the surface of the microtubule (Nogales *et al.* 1998, 1999) and may mediate interactions of microtubules with protein ligands (Paschal *et al.* 1989). This model is compatible with several results above, suggesting that *tub1-729p* affects microtubule-kinetochore attachment. The arrest phenotype of *tub1-729* cells—large budded, with their nuclei predominantly in the mother or adjacent to or spanning the mother-bud neck (Guénette *et al.* 1995; Table 2)—is similar to that of kinetochore mutants, such as *ctf19* (Hyland *et al.* 1999), *ctf13-30*, *ndc10-42* (Doheny *et al.* 1993), *cep3-1* and *-2* (Strunnikov *et al.* 1995), and *skp1-4* (Connelly and Hieter 1996). That the *tub1-729* arrest phenotype is a consequence of defective microtubule-kinetochore attachment is supported by the finding that the mutant cells at permissive temperature show a rate of plasmid loss higher than that of wild-type cells, an effect substantially rescued by excess *BUB3*.

Finally, we find that *tub1-729* cells require *DAM1* and *NUF2* function. Nuf2p and Dam1p are both part of protein complexes that are present at the kinetochore. Available data suggest that they may be involved in facilitating microtubule-kinetochore interactions. Nuf2p is part of a complex containing Ndc80p, Spc24p, and Spc25p. This complex is present at the centromere and is localized to a subset of short spindle microtubules (Janke *et al.* 2001; Wigge and Kilmartin 2001). *DAM1* was originally found in a screen for genes that are synthetically lethal with *mps1-1* (Jones *et al.* 1999). Dam1p forms a complex with Duo1p and Dad1p, and this complex localizes to mitotic spindles (Jones *et al.* 1999; Cheeseman *et al.* 2001; Enquist-Newman *et al.* 2001) and to kinetochores (Cheeseman *et al.* 2001). Interestingly, *dad1-1* interacts with the same subset of spindle assembly checkpoint genes—*MPS1*, *BUB1*, and *BUB3*—but not with *MAD1*, *-2*, and *-3*. Perhaps the Dam1p, Duo1p, and Dad1p complex and tub1-729p act in a similar way and consequently interact genetically with a subset of the mitotic checkpoint genes including *MPS1*, *BUB1*, and *BUB3*. Although it is possible that *tub1-729* cells require *NUF2* and *DAM1* for some microtubule function that is independent of the kinetochore, the observation that *tub1-729* interacts with a specific set of kinetochore mutants provides additional evidence that *tub1-729* may be deficient in microtubule-kinetochore interactions.

The data presented above are consistent with a model in which *MPS1*, *BUB1*, and *BUB3*, in addition to their participation in the spindle assembly checkpoint, play a second and distinct role stabilizing microtubule-kinetochore interactions. We propose that this role accounts for their ability to suppress the cold sensitivity of *tub1-729*. In this capacity, it is possible that Bub1p, Bub3p, and Mps1p could act as the "sensor" in the spindle assembly pathway. Bub1p, Bub3p, and Mps1p may localize to the kinetochore and mediate microtubule kinetochore attachment. Once microtubules attach to the kinetochore, Bub1p, Bub3p, and Mps1p could signal to downstream factors that would consequently modulate cell-cycle progression.

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