# Analysis of Tub4p, a Yeast γ-Tubulin-like Protein: Implications for Microtubule-organizing Center Function

**Laura G. Marschall,* Robert L. Jeng,* Jon Mulholland,‡ and Tim Stearns***

*Department of Biological Sciences, Stanford University, and ‡Department of Genetics, Stanford University Medical School, Stanford, California 94305-5020

***Abstract.*** γ-Tubulin is a conserved component of microtubule-organizing centers and is thought to be involved in microtubule nucleation. A recently discovered *Saccharomyces cerevisiae* gene (*TUB4*) encodes a tubulin that is related to, but divergent from, γ-tubulins. *TUB4* is essential for cell viability, and epitope-tagged Tub4 protein (Tub4p) is localized to the spindle pole body (Sobel, S.G., and M. Snyder. 1995. *J. Cell Biol.* 131:1775–1788). We have characterized the expression of *TUB4*, the association of Tub4p with the spindle pole body, and its role in microtubule organization. Tub4p is a minor protein in the cell, and expression of *TUB4* is regulated in a cell cycle–dependent manner. Wild-type Tub4p is localized to the spindle pole body, and a Tub4p–green fluorescent protein fusion is able to associate with a preexisting spindle pole body, suggesting that there is dynamic exchange between cytoplasmic and spindle pole body forms of Tub4p. Perturbation of Tub4p function, either by conditional mutation or by depletion of the protein, results in spindle as well as spindle pole body defects, but does not eliminate the ability of microtubules to regrow from, or remain attached to, the spindle pole body. The spindle pole bodies in *tub4* mutant cells duplicate but do not separate, resulting in a monopolar spindle. EM revealed that one spindle pole body of the duplicated pair appears to be defective for the nucleation of microtubules. These results offer insight into the role of γ-tubulin in microtubule-organizing center function.

THE number, orientation, and arrangement of microtubules is controlled in most cells by a microtubule-organizing center (MTOC)[1]. In fungi the MTOC is the spindle pole body (SPB), a laminar structure embedded in the nuclear envelope. In animal cells the main MTOC is the centrosome, a nucleus-associated organelle that consists of centrioles and pericentriolar material. The SPB and centrosome are morphologically different but functionally equivalent; they both nucleate the polymerization of microtubules, anchor those microtubules, and duplicate once per cell cycle.

The molecular mechanisms responsible for these functions are not understood, but γ-tubulin is likely to be a key component. γ-Tubulin is related to the α- and β-tubulins that make up the microtubule polymer. It was discovered in the fungus *Aspergillus nidulans* as a suppressor of a mutation in β-tubulin (Oakley and Oakley, 1989). Through the use of homology-based approaches, γ-tubulin genes have now been cloned from animals (Stearns et al., 1991; Zheng et al., 1991), plants (Fuchs et al., 1993; Liu et al., 1994), and other fungi (Stearns et al., 1991; Horio et al., 1991; Luo and Perlin, 1993). These diverse sequences show that γ-tubulin is as evolutionarily conserved as α- and β-tubulin. γ-Tubulin is localized to the MTOC and is not a component of the microtubule polymer, making it unique among the tubulins. It is essential for viability in fungi (Oakley et al., 1990; Stearns et al., 1991; Horio et al., 1991) and is required for centrosome function in vivo (Joshi et al., 1992) and in vitro (Felix et al., 1994; Stearns and Kirschner, 1994). These properties of γ-tubulin, along with the sequence homology between γ-tubulin and α- and β-tubulin, suggest a model in which γ-tubulin plays an essential role at the MTOC, possibly nucleating microtubule growth by direct interaction with α- and/or β-tubulin. Recent work has shown that γ-tubulin is present in the cytoplasm of animal cells as part of a large complex (Stearns and Kirschner, 1994). This complex is a ring-shaped structure and is capable of nucleating microtubules itself (Zheng et al., 1995). The relationship between the cytoplasmic and centrosomal forms of γ-tubulin is not clear, but Moritz et al. (1995) have found that centrosomes contain a similar γ-tubulin–containing ring structure at the base of nucleated microtubules.

Address all correspondence to Tim Stearns, Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020. Tel.: (415) 725-6934. Fax: (415) 725-8309. e-mail: stearns@leland.stanford.edu.

1. *Abbreviations used in this paper*: DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; HA, hemagglutinin; MTOC, microtubule-organizing center; SPB, spindle pole body; YEP, yeast extract peptone medium; ts, temperature-sensitive.

Until recently, an outstanding exception to the universality of γ-tubulin has been the yeast *Saccharomyces cerevisiae*, in which homology-based methods had failed to isolate a γ-tubulin homolog (Stearns et al., 1991; unpublished result). This was unusual because other aspects of the *S. cerevisiae* microtubule cytoskeleton are similar to those in animal cells. For example, the *S. cerevisiae* α- and β-tubulins are ~70% identical to their animal counterparts (Neff et al., 1983; Schatz et al., 1986), which is the same level of homology found in other animal–fungal tubulin comparisons. In addition, the microtubule motor proteins kinesin (Hoyt, 1994) and cytoplasmic dynein (Eshel et al., 1993; Li et al., 1993) are present in forms similar to those in animal cells. In fact, the SPB of *S. cerevisiae* is one of the best-understood MTOCs. The morphology of the organelle in the cell cycle has been studied extensively (Byers and Goetsch, 1975), and several SPB proteins have been identified. These proteins include Kar1p (Vallen et al., 1992), Cdc31p (Spang et al., 1993), and calmodulin (Geiser et al., 1993), which were identified genetically, and the 42-, 90-, and 110-kD SPB components (Rout and Kilmartin, 1991), which were identified biochemically. Recently, Sobel and Snyder (1995) reported the initial characterization of *TUB4*, a gene identified in the *S. cerevisiae* genome sequencing project that encodes a protein that is related to γ-tubulin, but considerably more divergent than other γ-tubulins. This gene is essential for cell viability, and epitope-tagged *TUB4* protein (Tub4p) localizes to the SPB. Nuclear division and microtubule organization are impaired in cells depleted of Tub4p.

Here we report a comprehensive characterization of *TUB4* and the γ-tubulin–like protein that it encodes. Tub4p is a minor protein in the cell and is an intrinsic component of the SPB. Its association with the SPB is dynamic; Tub4p is able to associate with the SPB at multiple stages of the cell cycle. Strains bearing conditional-lethal *tub4* mutations or depleted of Tub4p have specific spindle and SPB defects. Our data support the hypothesis that Tub4p is the γ-tubulin homolog in *S. cerevisiae* and indicate that Tub4p is required for establishment of nucleation capacity at the SPB, but not required for subsequent microtubule growth and attachment.

## Materials and Methods

### Yeast Strains and Media

The yeast strains used in this study are listed in Table I. Yeast extract/peptone (YEP), synthetic dextrose (SD), and sporulation media were as described (Sherman et al., 1983). Strains requiring expression of a plasmid-borne *GAL–TUB4* construct were grown on either YEP + 2% galactose or YEP + 2% galactose/1% glucose. Yeast molecular genetic methods were as described (Stearns et al., 1990). Nocodazole (Janssen Life Sciences Products, Piscataway, NJ) was added to a final concentration of 20 μg/ml. α-Factor (Sigma Chemical Co., St. Louis, MO) was added to media to a final concentration of 5 μg/ml. For the analysis of Tub4p distribution in zygotes, cells were mated by mixing two cultures and concentrating approximately $1 \times 10^7$ cells on a sterile filter. The filter was placed on a YEP/dextrose (YEPD) plate and incubated at 30°C.

### TUB4 Plasmid Constructs

To subclone the *TUB4* coding region and flanking sequences, five PCR primers were produced: Scg.1: GCGGAGCTCATGTTGAACAAATCACCG; Scg.2: GCCGTCGACTTCATGTTAGTCACCATG; Scg.3: GCCAAGCTTATCTATGGGTGGAGAAA; Scg.4: GCCTCTAGACTGAACAAGGAAGGCATC; Scg.5: GCCTCTAGATACTTATTTATGATCAC. Cosmid 8167 DNA was used as the template for all PCRs. PCR was performed using Vent polymerase (New England Biolabs, Beverly, MA).

Vectors were as follows: pTS210, YCp50 (Rose et al., 1987) containing the *GAL1/GAL10* promoter (Johnston and Davis, 1984) and the yeast *ACT1* transcriptional terminator; pTS395, GFP (Prasher et al., 1992) in pTS210; pTS515, three repeats of the hemagglutinin (HA) epitope (Green et al., 1987) in pTS210.

*TUB4* plasmids were as follows: pTS593, *TUB4* (Scg.3+Scg.4) in pTS210; pTS594, *TUB4* (Scg.3+Scg.5) in pTS515; pTS595, *TUB4* (Scg.3+Scg.5) in pTS395 (pTS592 is identical, except that it has a single point mutation in *TUB4*, resulting in the change A334T); pTS597, *TUB4* (Scg.1 + Scg.2) in pSK$^+$ (Stratagene, La Jolla, CA); pTS598, *tub4::HIS3* (1.3-kb EcoRI fragment of pTS597 replaced with *HIS3* gene); pTS606, *TUB4* from pTS593 in pART3.

*TUB4* was sequenced by the dideoxy chain termination method using Sequenase (United States Biochemical Corp., Cleveland, OH) and primers spaced every 200 bp on the *TUB4* sequence. The wild-type sequence was determined from a clone originating from cosmid 8167. The sequence of the *TUB4* mutant in pTS592 was sequenced directly from that plasmid.

### RNA Blotting

Cell cycle stage–specific RNA was prepared by isolating cytoplasmic RNA from yeast cells at various times after release from α-factor arrest (Foreman, P., and R. Davis, unpublished data). 20 μg of cytoplasmic RNA per lane was fractionated on 0.9% agarose/2 M formaldehyde gels, transferred to a Hybond nylon membrane, and hybridized to a $^{32}$P-labeled DNA fragment containing the *TUB4* coding region. The blot was stripped of *TUB4* probe and hybridized to a $^{32}$P-labeled DNA fragment containing the *ACT1* coding region as a control for amount of RNA per gel lane. Densitometry was used to quantify the *TUB4* and *ACT1* signals in each lane.

### Production of Tub4p Antibodies

Bacterial TrpE–Tub4p fusions were created by inserting the *TUB4* coding sequence into pATH11 (Dieckmann and Tzagoloff, 1985). Polyacrylamide gel slices containing the TrpE–Tub4 fusion protein were used to immunize rabbits (BAbCO, Inc., Richmond, CA). Anti-Tub4p antibodies were purified from the rabbit serum by affinity purification against nitrocellulose membrane (Harlow and Lane, 1988) containing a $His_6$-tagged Tub4 protein created by inserting the *TUB4* coding sequence into pET-15b (Novagen, Madison, WI). Both bacterially expressed proteins were purified as insoluble inclusion bodies (Harlow and Lane, 1988).

### Protein Blotting

Protein samples for Western analysis were prepared by disrupting yeast cells with glass beads as described (Kaiser et al., 1987). Anti-Tub4p primary antibody was Tub4-1-4 serum diluted 1:10,000. The primary antibody was detected with HRP-conjugated secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA), followed by enhanced chemiluminescence (Dupont–New England Nuclear, Wilmington, DE).

Protein samples for analysis of soluble Tub4p were made by grinding cells that had been rapidly frozen in liquid nitrogen. Cells were frozen in an equal volume of XB (100 mM KCl, 10 mM Pipes, pH 7.7, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM ATP, 1 mM GTP). Extract was prepared from the cell powder by adding an equal volume of XB and thawing on ice. The extract was separated into soluble and insoluble fractions by centrifugation at 25,000 *g* for 15 min.

### Isolation and Analysis of tub4 Mutants

The generation and characterization of the *tub4* mutants will be described in detail elsewhere. Briefly, the *TUB4* coding sequence was mutagenized by PCR, and a plasmid-shuffle scheme (Sikorski and Boeke, 1991) was used to identify temperature-sensitive (ts) and cold-sensitive (cs) mutants. 36 ts and 5 cs mutants were identified. The mutant *tub4* alleles were recovered from the plasmids and integrated into the genome, such that they replaced the wild-type *TUB4* gene, and were backcrossed once to a wild-type strain. The backcrossed versions of the *tub4-32* and *tub4-34* alleles were used in these experiments.

For Tub4p depletion experiments, TSY509 cells were grown in YEP + 2% galactose + 1% glucose to early log phase. Cells were pelleted,

*Table I. Yeast Strains*

| Name | Genotype | Source |
|---|---|---|
| *S. cerevisiae* strains | | |
| TPS507 | MATa/α *ADE2/ade2 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 ura3-52/ura3-52* | This lab |
| TSY322 | MATa *his3-Δ200 leu2-3,112 lys2-801 ura3-52* (pTS210) | This lab |
| TSY496 | MATα *his3-Δ200 leu2-3,112 lys2-801 tub4::HIS3 ura 3-52* (pTS594) | This study |
| TSY497 | MATa *his3-Δ200 leu2-3,112 lys2-801 tub4::HIS3 ura3-52* (pTS594) | This study |
| TSY498 | MATα *his3-Δ200 leu2-3,112 lys2-801 tub4-32 ura3-52* | This study |
| TSY502 | MATa *his3-Δ200 leu2-3,112 lys2-801 tub4-34 ura3-52* | This study |
| TSY506 | MATa/α *ADE2/ade2 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 ura3-52/ura3-52 TUB4/tub4::HIS3* | This study |
| TSY508 | MATα *his3-Δ200 leu2-3,112 lys2-801 tub4::HIS3 ura3-52* (pTS592) | This study |
| TSY509 | MATa *his3-Δ200 leu2-3,112 lys2-801 tub4::HIS3 ura3-52* (pTS593) | This study |
| TSY514 | MATa *his3-Δ200 leu2-3,112 lys2-801 ura3-52* (pTS592) | This study |
| TSY515 | MATa *his3-Δ200 leu2-3,112 lys2-801 ura3-52* (pTS593) | This study |
| TSY523 | MATa/α *ADE2/ade2 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 ura3-52/ura3-52 TUB4/tub4::HIS3* (pTS607) | This study |
| DBY1710 | MATα *his3-Δ200 ura3-52 kar1-1* | D. Botstein |
| DBY4974 | MATa *his3-Δ200 leu2-3,112 lys2-801 ura3-52* | D. Botstein |
| *S. Pombe* strains | | |
| TSY522 | $h^+/h^-$ *ade6-210/ade6-216 leu1-32 ura4-D18/ura4-D18 tug1+/tug1::ura4+* (pTS606) | This study |

washed once, resuspended in YEPD, and incubated at 30°C. Aliquots were removed from the culture at the times indicated in the individual experiments.

## Fluorescence Microscopy

Immunofluorescence was performed as described (Pringle et al., 1989) with the following modifications. To visualize microtubules, cells were fixed by adding formaldehyde to a final concentration of 3.7% and incubating at room temperature or 30°C for between 1 and 2 h, followed by methanol/acetone treatment. Primary antibody YOL1/34 (Kilmartin et al., 1982) was detected with Texas red–conjugated donkey anti–rat antibodies (Jackson Immunoresearch Laboratories, Inc.). To visualize Tub4p, affinity-purified Tub4-1-4 antibodies were incubated with cells treated as above and detected with fluorescein-conjugated goat anti–rabbit secondary antibodies (Jackson Immunoresearch Laboratories, Inc.). Double labeling of microtubules and Tub4p was carried out by simultaneous incubation with YOL1/34 and Tub4-1-4 antibodies. To visualize the 90-kD SPB component, cells were fixed with 3.7% formaldehyde for only 10 min. Double labeling of Tub4p and the 90-kD SPB component was carried out by simultaneous incubation with Tub4-1-4 and anti-90 mAbs (Rout and Kilmartin, 1990). DNA was visualized by incubation in 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 1 min.

Green fluorescent protein (GFP) fluorescence was visualized either in living cells or in cells that had been fixed with formaldehyde as above. A fluorescein filter set (Hi-Q FITC; Chroma Technology Corp., Brattleboro, VT) was used on a microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY) equipped with an HBO100 mercury lamp and ×100/1.3 objective lens.

The intensity of Tub4p fluorescence at the SPB was measured essentially as described for kinetochores (Nicklas et al., 1995) except that a cooled charge-coupled device camera (Princeton Research Instruments, Inc., Princeton, NJ) was used to acquire the data on a microscope (Axiovert; Carl Zeiss, Inc.) equipped with a bottom port for the camera and a ×100/1.4 objective lens. Cultures of *TUB4* and *tub4-34* cells were grown at 24°C and shifted to 37°C for 3 h. The cells were stained for Tub4p and microtubules as described above. The brightness measurement for the mutant cells is likely to be an overestimate, as only cells in which the SPB could be unambiguously identified were used in the analysis. The position of microtubules was used as a guide to the location of the SPB in mutant cells incubated at the restrictive temperature.

## Electron Microscopy

*TUB4* and *tub4-34* cells were grown at 24°C and shifted to 37°C for 3 h. The cells were rapidly washed once by filtration, and then fixed, embedded, and sectioned as described (Byers and Goetsch, 1975), except that 0.04 M phosphate buffer, pH 6.7, was used instead of cacodylate buffer. In serial sections, the average section thickness was 70 nm.

# Results

## An *S. cerevisiae* Gene with Homology to γ-Tubulin

The *S. cerevisiae* genome project identified an open reading frame on chromosome XII that is most similar to γ-tubulins in the protein database (GenBank accession No. U14913). No known mutations map to the *TUB4* locus (edition XII of the *S. cerevisiae* genetic map). The DNA sequence determined by the genome project was confirmed to be correct by sequencing the *TUB4* open reading frame. The predicted protein product of *TUB4* (Tub4p) is 473 amino acids long and is 41% identical to *Schizosaccharomyces pombe* γ-tubulin, *tug1*$^+$, and between 35% and 40% identical to other γ-tubulins. Tub4p is no more than 29% identical to any α- or β-tubulin. Thus, Tub4p is closest in sequence to γ-tubulin, but is considerably more divergent than the known γ-tubulins, which are all at least 65% identical to each other. This is in contrast to the *S. cerevisiae* α- and β-tubulin proteins, which are no more divergent from their vertebrate homologs than those of other fungi.

A construct that replaces all of the *TUB4* coding sequence, except for 155 bp at the 3′ end, with the yeast *HIS3* gene (*tub4::HIS3*), was used to transform a wild-type diploid strain to histidine prototrophy. In all complete tetrads from this strain, only two spores were viable ($n$ = 40), and all viable spores were His$^-$, confirming that *TUB4* is essential for viability (Sobel and Snyder, 1995). The *tub4::HIS3* deletion mutation can be complemented by either a 2.4-kb fragment of the original *TUB4*-containing cosmid (pTS477) or the *TUB4* coding sequence placed under control of the *GAL1* promoter (pTS593).

To assess functional homology between *TUB4* and γ-tubulin genes from other organisms, two complementation tests were performed. The human γ-tubulin gene (Zheng et al., 1991) was tested for complementation of the *tub4::HIS3* mutation, and *TUB4* was tested for complementation of an *S. pombe* γ-tubulin null mutation (*tug1::ura4*$^+$; Stearns et al., 1991). Western analysis confirmed that the

heterologous proteins were expressed in both cases, yet in neither case was complementation observed (data not shown).

The *TUB4* gene has an exact match to the MluI cell cycle box consensus sequence ACGCGTNA (McIntosh et al., 1991) 127 bp upstream of the ATG. The MluI box is a regulatory site found upstream of many genes that are induced at the G1/S boundary, including *SPC110*, which encodes an SPB component (Kilmartin et al., 1993). The steady state level of *TUB4* transcript was determined by Northern blotting with RNA isolated from yeast cells collected at various times after release from α-factor arrest at G1 of the cell cycle. The *TUB4* mRNA undergoes a cell cycle–dependent oscillation in level typical of that of other MluI box–containing genes (Fig. 1).

### *Tub4p Characterization*

Polyclonal antibodies made against a *TrpE–TUB4* fusion protein recognize wild-type Tub4p as a 55-kD protein in crude yeast extracts (Fig. 2, lane *a*). The anti-Tub4p antibodies also recognize a derivative of Tub4p that has three copies of the HA epitope added to the COOH terminus (Tub4-HA3) as a higher molecular weight form (Fig. 2, lane *b*). This Tub4–HA3 protein is able to fully complement the *tub4::HIS3* mutation. The amount of Tub4p in yeast cells was estimated by comparison to known quantities of bacterially produced Tub4p and was found to be 0.003% of total protein, or ~$10^4$ molecules per cell. α- and β-Tubulin have been estimated to be ~0.05% of total soluble protein in yeast (Kilmartin, 1981). Tub4p is overexpressed ~300-fold when placed under control of the *GAL1* promoter and fully induced (Fig. 2, lanes *d* and *e*). Although this overexpression does not cause any obvious phenotypes (see below), we routinely reduced the level of expression of *TUB4* constructs from the *GAL1* promoter to within a few-fold of that of the endogenous promoter by growing the cells with a mixture of 2% galactose/1% glucose as the carbon source (Fig. 2, lane *b*).

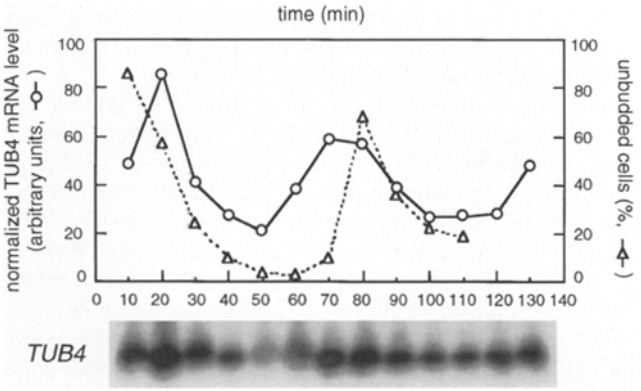


*Figure 1.* Northern blot of RNA from cells synchronized with α-factor and released for the times indicated. The blot was probed with $^{32}$P-labeled *TUB4* DNA, and subsequently probed with $^{32}$P-labeled *ACT1* DNA as a control for equal loading of the RNA samples. *TUB4* mRNA level normalized to *ACT1* level in arbitrary units is plotted on the left axis above the Northern blot (–○–). The percentage of unbudded cells in the culture at the time of RNA extraction is plotted on the right axis (–△–).

About half of the total γ-tubulin in somatic animal cells is in the centrosome; the other half is present as a large cytoplasmic particle, the γ-some, with a sedimentation coefficient of ~25S (Stearns and Kirschner, 1994). To determine whether there is a soluble pool of Tub4p, extract was prepared from a wild-type diploid strain and separated into soluble and insoluble fractions by centrifugation. More than half of the total Tub4p was in the soluble fraction (not shown).

### *Tub4p Is a Component of the Spindle Pole Body*

Anti-Tub4p antibodies were used to localize Tub4p by immunofluorescence microscopy. The anti-Tub4p antibodies stained a bright dot at the vertex of microtubules in interphase cells and at the ends of the spindle in mitotic cells (Fig. 3 *A*), consistent with localization to the SPB. Tub4p staining also colocalized with that of the 90-kD SPB component (see Fig. 6 *g*; Rout and Kilmartin, 1990). The quantity and quality of the Tub4p staining at the SPB did not appear to change over the course of the cell cycle. These results are consistent with those obtained with epitope-tagged Tub4p (Sobel and Snyder, 1995; Marschall, L.G., and T. Stearns, unpublished results).

A fusion between *TUB4* and the coding sequence for GFP was constructed to allow visualization of Tub4p in living cells. The full-length GFP gene was fused to the COOH terminus of the full-length *TUB4* gene and expressed in yeast under control of the *GAL1* promoter, modulated as described above. The fusion protein was produced in yeast at approximately endogenous levels (Fig. 2, lane *c*) and was localized to the SPB in both living and fixed cells (Fig. 3 *C*). The wild-type *TUB4–GFP* con-

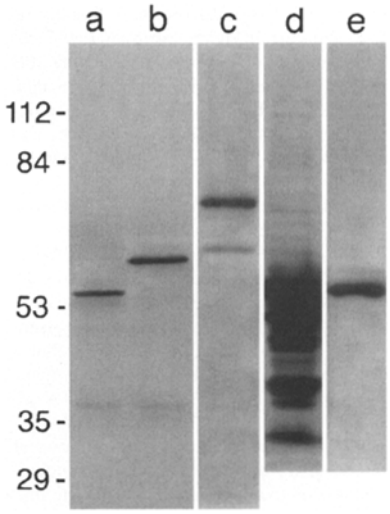


*Figure 2.* Western blots of crude yeast protein extracts probed with anti-Tub4p antibody. The relevant genotypes of the strains used are: (*a*) *TUB4*, (*b*) *tub4::HIS3* (*GAL–TUB4–HA3*), (*c*) *tub4::HIS3* (*GAL-TUB4-GFP*), (*d* and *e*) *TUB4* (*GAL–TUB4*). Equal amounts of protein were loaded in all lanes except *e*, in which 1/100 of the amount was loaded. The cultures used for lanes *b* and *c* were grown with a mixture of 2% galactose/1% glucose as carbon source. The culture used for lanes *d* and *e* were grown with 2% galactose as carbon source. Molecular weight markers (kD) are indicated.

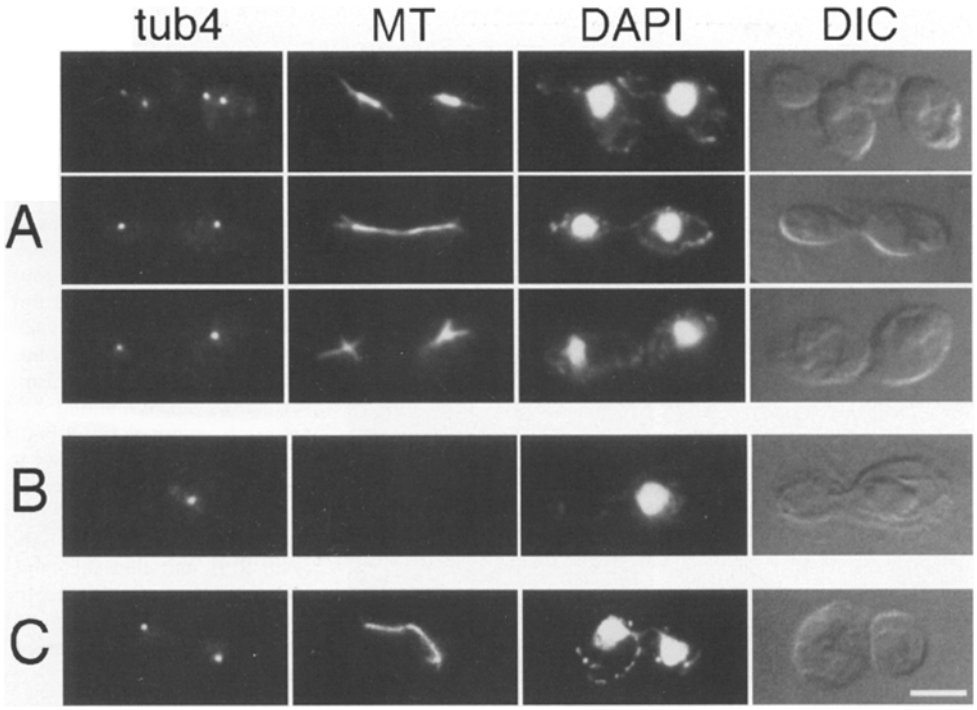


*Figure 3.* Localization of Tub4p. *A* and *B* are a wild-type diploid strain stained with anti-Tub4p antibody, anti–α-tubulin antibody (*MT*), and the DNA stain DAPI. A differential interference contrast (*DIC*) image is also shown. Cells for the experiment shown in *B* were treated with 20 μg/ml nocodazole for 2 h to depolymerize microtubules, and only faint cytoplasmic staining is visible with the anti–α-tubulin antibody. *C* is a wild-type diploid strain with a *GAL–TUB4–GFP* plasmid. The cells for this experiment were grown with a mixture of 2% galactose/1% glucose as carbon source, fixed, and stained with anti–α-tubulin antibody and DAPI. In *C* the SPB fluorescence seen is due to the Tub4–GFP fusion protein. Bar, 5 μm.

struct was not able to complement the *tub4::HIS3* null mutation, but a spontaneous complementing mutant was fortuitously identified among the original constructs. This mutant was sequenced and found to have a single change from wild-type, resulting in a mutation of alanine 334 to threonine. The localization of the complementing and noncomplementing versions of the Tub4–GFP fusion protein was identical; the complementing mutant was used for subsequent experiments.

To determine whether Tub4p requires microtubules to maintain its association with the SPB, cells were treated with nocodazole, an antimicrotubule drug that causes the depolymerization of microtubules, and examined by immunofluorescence. It should be noted that although nocodazole and other benzimidazole drugs depolymerize most of the microtubules in yeast cells, it is possible that a small amount of polymer that is undetectable by immunofluorescence remains. Tub4p localization to the SPB was identical in the presence or absence of nocodazole (Fig. 3 *B*). This experiment was repeated in living cells with Tub4–GFP with the same result: localization of Tub4–GFP to the SPB was independent of the presence of detectable microtubules.

### *Association of Tub4p with the SPB In Vivo*

To test whether newly synthesized Tub4p is able to associate with an existing SPB, a strain bearing a *GAL–TUB4–GFP* plasmid was arrested with α-factor, and then *TUB4–GFP* was induced in the continued presence of α-factor. Cells arrested at the α-factor block have only one SPB (Byers and Goetsch, 1975), so any fluorescence at the SPB would be the result of binding of new Tub4–GFP to that SPB. After 4.5 h of induction, Tub4–GFP was present at the SPB (data not shown). A similar time course of incorporation was observed with mitotically growing cells.

To examine the kinetics of the association of Tub4p with the SPB, an experiment was designed in which an SPB lacking Tub4–GFP is placed in a cytoplasm with excess Tub4–GFP. A strain carrying a plasmid expressing Tub4–GFP was mated to a strain lacking the plasmid. When yeast cells mate, the two haploid nuclei fuse at the SPBs soon after cytoplasmic fusion (Byers and Goetsch, 1975). Because this nuclear fusion would complicate analysis of Tub4–GFP distribution, the strain not expressing Tub4–GFP carried a *kar1-1* mutation, which prevents fusion of the nuclei (Conde and Fink, 1976), thus allowing the presence or absence of Tub4–GFP on the SPBs of either nucleus to be assessed. In live mating cells observed by time-lapse fluorescence microscopy, the mixing of cytoplasms occurred within seconds of fusion of the cells, as assayed by the distribution of the cytoplasmic and nuclear Tub4–GFP. In contrast, binding of Tub4–GFP to the SPB occurred slowly. The time that has elapsed after fusion of two cells can be estimated by budding cycle stage of the resulting zygote. In unbudded zygotes, only one nucleus had a fluorescent SPB (Fig. 4 *A*); in zygotes with a small bud, usually one nucleus had a fully labeled SPB; and the other varied from completely unlabeled, to faintly labeled (Fig. 4 *B*), to fully labeled. In zygotes with a large bud, the SPB had usually duplicated and both SPBs of both nuclei were labeled (Fig. 4 *C*). In control experiments to determine the time represented by these classes of cell morphology, bud emergence occurred ~30 min after cells had visibly fused cytoplasms. Thus, Tub4p is exchangeable at the SPB, but the rate of exchange is relatively slow.

### *Phenotypes of Conditional Mutations in TUB4*

The function of *TUB4* was tested in three ways: construction of temperature-conditional mutants, depletion of Tub4p from cells by repressing expression of the gene, and

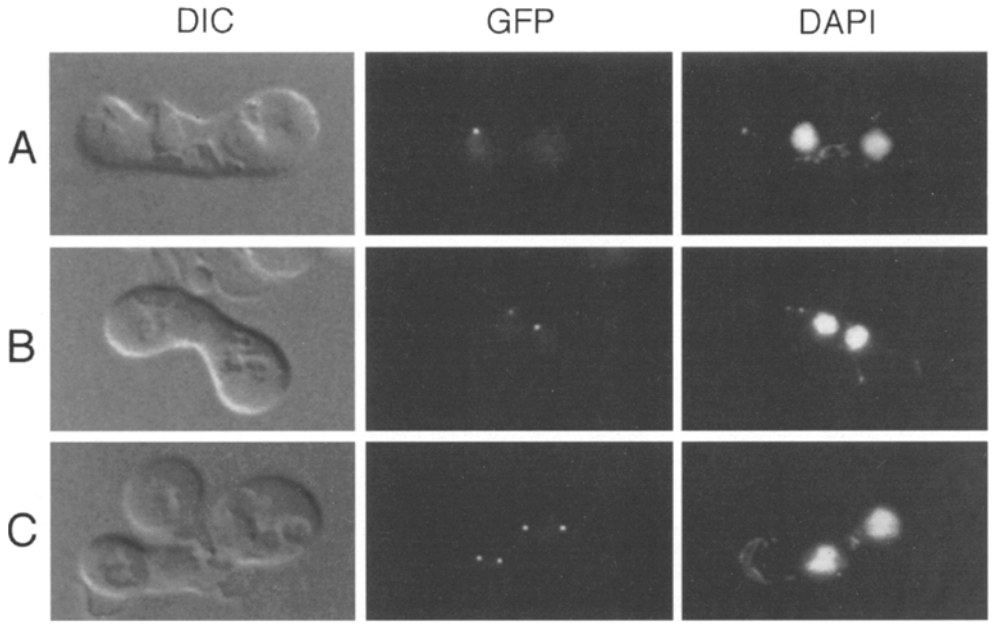


*Figure 4.* Distribution of Tub4–GFP to a SPB in a *kar1* cross. Strain TSY514 *TUB4 (GAL–TUB4–GFP)* was crossed to strain DBY1710 *TUB4 kar1-1* by a quantitative filter mating. The cells were allowed to mate for 3 h, and then fixed and stained with the DNA stain DAPI. The *kar1-1* mutation prevents fusion of nuclei in mating cells; thus, each zygote has two separate nuclei in a common cytoplasm. *A* shows an unbudded zygote, *B* shows a small-budded zygote, and *C* shows a large-budded zygote. Note in *A* that the Tub4–GFP fluorescence is present on only one SPB, but that the faint nuclear fluorescence due to the nuclear localization of excess Tub4–GFP is present in both nuclei.

overexpression of Tub4p. Temperature-conditional mutations were isolated in *TUB4* by PCR-mediated random mutagenesis. A total of 36 ts and 5 cs mutants were isolated. Two recessive ts mutants were chosen for the phenotypic analysis presented here. Asynchronous cultures of *TUB4*, *tub4-32*, and *tub4-34* strains were shifted from the permissive temperature (24°C) to the restrictive temperature (37°C) for 2 h, and the state of the microtubule cytoskeleton was assayed by immunofluorescence (Fig. 5). In both of the *tub4* mutants, there was a slight enrichment for large-budded cells compared to wild-type, but not a cell cycle arrest typical of mutations in the α- and β-tubulin genes of yeast (Stearns and Botstein, 1988; Huffaker et al., 1988; Schatz et al., 1988). There were very few normal mitotic spindles in the *tub4* mutant cells (5% in *tub4-34*); instead, most cells had a monopolar spindle (86% in *tub4-34*). A small percentage of cells had a short bipolar spindle (9% in *tub4-34*). In addition, many of the cells had very long cytoplasmic microtubules, longer than those observed in wild-type cells at any stage of the cell cycle.

To determine the primary defect of the *tub4* ts mutants, *TUB4* and *tub4-34* cells were arrested with α-factor at 24°C, released from the α-factor block at 37°C, and assayed at times after the release. The cells were stained with antibodies against α-tubulin and Tub4p. At the α-factor arrest, both *TUB4* and *tub4-34* cells had normal microtubules and Tub4p staining at the SPB (Fig. 6, *a* and *b*). At 2 h after release, immunofluorescence of the *TUB4* cells showed many cells with mitotic spindles (Fig. 6 *c*). In contrast, at 2 h the *tub4-34* strain had very few normal mitotic spindles and many monopolar spindles, confirmed by the presence of Tub4p at the junction of the cytoplasmic and nuclear microtubules, and not at the other end of the nuclear microtubules (Fig. 6 *d*). Only after 3 h did the cytoplasmic microtubules become more exaggerated in the *tub4-34* strain, often coursing along the entire inner circumference of the cells (Fig. 6 *f*). Thus, failure to make a bipolar spindle is the primary defect in the *tub4* mutants, and the long cytoplasmic microtubules are a secondary defect.

We noted that Tub4p staining in the *tub4-34* strain diminished after longer times at the restrictive temperature. At 2 h the Tub4p staining in *tub4-34* was reduced relative to wild type (Fig. 6, *c* and *d*), and at 3 h there was little or no Tub4p staining in most *tub4-34* cells, whereas the wild type was normal (Fig. 6, *e* and *f*). Similar results were ob-

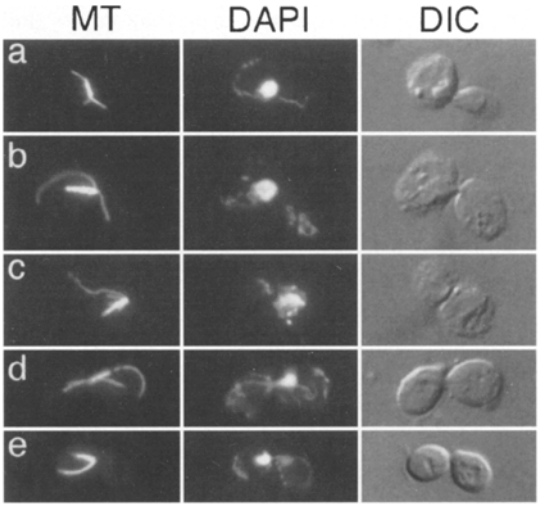


*Figure 5.* Phenotype of temperature-sensitive *tub4* mutants and of Tub4p depletion. *TUB4* (*a*), *tub4-32* (*b*), and *tub4-34* (*c*) strains were grown at 24°C, and then shifted to 37°C for 2 h. For depletion, a *tub4::HIS3* (*GAL–TUB4*) strain was grown in 2% galactose/1% glucose medium and shifted to glucose medium for 6 h (*d*) and 10 h (*e*). The cells were stained with anti–α-tubulin antibodies (*MT*) and DAPI.

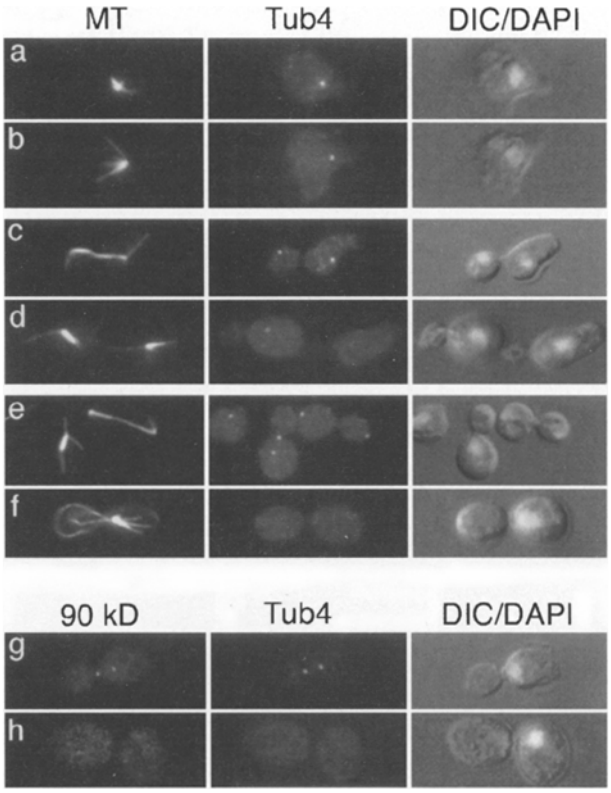


*Figure 6.* Phenotype of *tub4-34* mutant after release from α-factor at restrictive temperature. *TUB4* (*a*, *c*, *e*, and *g*) and *tub4-34* (*b*, *d*, *f*, and *h*) cells were arrested with α-factor at 24°C, and then released from the arrest at 37°C for 0 h (*a* and *b*), 2 h (*c* and *d*), and 3 h (*e–h*). Fixed cells were stained with anti–α-tubulin (*MT*) and anti-Tub4p antibodies and DAPI (*a–f*), or anti–90-kD and anti-Tub4p antibodies (*g* and *h*). The cells in *a–f* were fixed with formaldehyde for 2 h for optimal microtubule staining. The cells in *g* and *h* were fixed with formaldehyde for 10 min for optimal anti–90-kD protein staining. Anti-Tub4p antibody worked well for immunofluorescence under both conditions.

tained in *tub4-32* mutants (see Fig. 8). This effect was quantitated by using a cooled charge-coupled device camera to measure the amount of fluorescence at the SPB in cells stained for Tub4p. The mean SPB fluorescence intensity in *tub4-34* mutant cells grown for 3 h at the restrictive temperature ($n$ = 35) was 9.7% of that in cells of the same strain at the permissive temperature ($n$ = 39). Thus, >90% of the Tub4p at the spindle pole body is lost during the high temperature incubation. In protein blotting experiments, the total amount of Tub4p remained the same in the permissive and restrictive cultures (data not shown), indicating that the loss from the SPB must be due to redistribution of the mutant protein.

To determine whether the loss of Tub4p from the SPB had an effect on other components of the SPB, we examined the localization of the 90-kD SPB component (Rout and Kilmartin, 1990) in double labeling experiments with Tub4p. Tub4p and the 90-kD protein colocalized in wild-type cells that had been incubated at 37°C for 3 h (Fig. 6 *g*), but were absent or greatly reduced in level at the SPB in *tub4-34* cells under the same conditions (Fig. 6 *h*). The amount of anti–90-kD staining directly correlated with that of Tub4p; *tub4-34* cells that retained some Tub4p staining at the SPB also had anti–90-kD staining.

Tub4p function was also assessed by depletion of the protein from cells. A strain in which the only copy of *TUB4* is under control of the *GAL1* promoter (TSY509) was shifted from galactose-containing medium to glucose medium to repress expression of the *TUB4* gene; aliquots were removed from the culture at 2-h intervals and assayed for cell viability and microtubule morphology. The culture continued log phase growth for ~6 h after the shift, and then viability began to decrease. As viability decreased, a change in microtubule morphology became evident; greater than 90% of large-budded cells contained either a short bipolar spindle (62%) (Fig. 5 *d*), a monopolar spindle (19%), or an aberrant spindle that could not be definitively classified (19%). In addition, these cells had unusually long and often more numerous cytoplasmic microtubules. This phenotype is in sharp contrast to the elongated spindle and short cytoplasmic microtubules found in most wild-type large-budded cells. At longer times after the shift, the proportion of large-budded cells with monopolar spindles increased; 10 h after a shift to glucose, 44% of large-budded cells had a monopolar spindle (Fig. 5 *e*), 33% had short spindles, and 23% had a spindle that could not be definitively classified.

In contrast to Tub4p depletion, Tub4p overexpression was not detrimental to yeast cells. Full induction of a *GAL–TUB4* construct resulted in 300-fold overexpression of Tub4p (Fig. 2, lanes *d* and *e*). Strains bearing the *GAL–TUB4* construct grew slightly more slowly on inducing medium, but there were no obvious morphological defects. Immunofluorescence microscopy showed that there were also no obvious microtubule defects in overexpressing cells (Fig. 7). It was not possible to discern an effect on the amount of SPB-localized Tub4p in the overexpressing cells because of the extremely bright cytoplasmic and nuclear staining in those cells.

### *Microtubule Regrowth from the SPB in tub4 Mutants*

To examine the effect of perturbing Tub4p function on microtubule nucleation by the SPB, microtubules were depolymerized in wild-type and *tub4* mutant cells, and then allowed to repolymerize. *TUB4* and *tub4-32* cells were shifted to 37°C for 3 h, and then treated with nocodazole at 4°C for 4 h. Nocodazole treatment at 4°C resulted in more complete microtubule depolymerization than that observed at normal growth temperatures (Jacobs et al., 1988). The nocodazole was rapidly washed out with medium prewarmed to 37°C, and cells were fixed at 0 and 10 min. In both *TUB4* and *tub4-32* cells, microtubules were depolymerized at 0 min (Fig. 8, *a–c*) and extensively polymerized from the SPB by 10 min (Fig. 8, *b* and *d*). In the majority of cells, there was no visible α-tubulin staining at the SPB after the nocodazole treatment. In the rest, there was a dot of staining that is likely due to tubulin remaining at the SPB (Jacobs et al., 1988). Normal microtubule regrowth was apparent even in cells that clearly lacked Tub4p staining at the SPB after the 3-h incubation at the restrictive temperature. It seemed possible that even though Tub4p was no longer localized to the SPB in most cells, Tub4p function might be restored in some way dur-

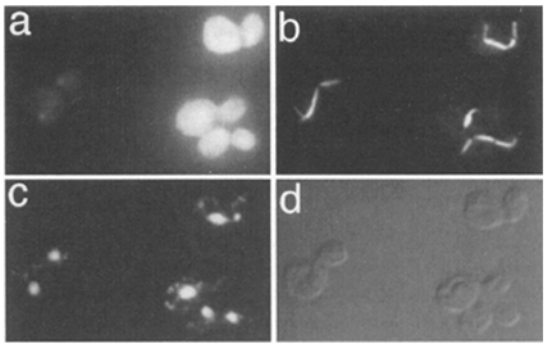


*Figure 7.* Microtubules in cells grossly overexpressing Tub4p. TSY515 (*GAL–TUB4*) was grown under conditions of full induction, fixed, and stained with anti–α-tubulin (*b*) and anti-Tub4p (*a*) antibodies and DAPI (*c*). A differential interference contrast image is also shown (*d*). Most cells in the culture exhibit very bright Tub4p staining. Occasionally, cells appeared to have wild-type levels of Tub4p staining (the left-most cells in the panels); these cells have presumably lost the *GAL–TUB4* plasmid. There were no abnormalities observed in the microtubule cytoskeleton of the overexpressing cells.

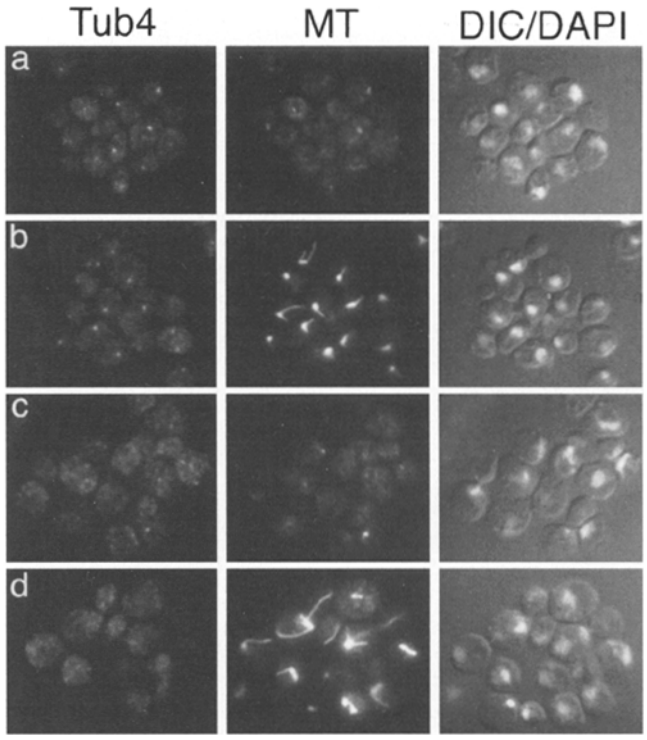


*Figure 8.* Regrowth of microtubules in wild-type and *tub4-32* mutant cells. *TUB4* (*a* and *b*) and *tub4-32* (*c* and *d*) strains were grown at 24°C, shifted to 37°C for 3 h, and then treated with 20 μg/ml nocodazole for 4 h at 4°C. The cells were rapidly washed with medium prewarmed to 37°C and incubated at 37°C for 0 min (*a* and *c*) and 10 min (*b* and *d*). The 0-min time point was fixed immediately after washing. The cells were stained with anti–α-tubulin (*MT*) and anti-Tub4p antibodies and DAPI.

ing the 4°C nocodazole incubation, allowing microtubule nucleation. To control for this, the experiment was repeated with the variation that cells were incubated at 37°C for 1 h before washing out the nocodazole; the regrowth results were the same (not shown).

### *SPB Duplication in a tub4 Mutant*

Thin-section EM was used to determine the state of the SPB in *TUB4* and *tub4* mutant cells. Cultures of *TUB4* and *tub4-34* cells were shifted to 37°C for 3 h and examined. Normal SPB profiles and mitotic spindles were observed in sections of *TUB4* cells (not shown). In *tub4-34* cells, serial sectioning was used to determine whether there were one or two SPBs and whether the SPBs had microtubules associated with them. Among cells where the entire nuclear envelope could be accounted for, most had a duplicated SPB (15 of 18 cells). Fig. 9 *A* shows serial sections of a cell in which one SPB is normal and the other has no associated microtubules. Fig. 9 *B* shows serial sections of a cell in which one SPB has a normal complement of intranuclear microtubules and the other SPB has only two associated microtubules. In five of the 15 cells in which the SPB had duplicated, one of the two SPBs had two or fewer associated microtubules. In both examples shown, the SPBs have a bridge between them. The duplicated SPBs appeared to differ only in their association with microtubules and are normal in their ultrastructure and attachment to the nuclear envelope.

## *Discussion*

### *Is TUB4 the S. cerevisiae γ-Tubulin?*

Tub4p has many of the properties of γ-tubulin that have been described in other organisms: *TUB4* is essential for viability, Tub4p is localized to the SPB in a microtubule-independent manner, and mutations in *TUB4* affect the function of the SPB. However, the divergence of Tub4p from the known γ-tubulins is unusual. We have been unable to demonstrate functional complementation between either human γ-tubulin and Tub4p, or *S. pombe* γ-tubulin and Tub4p. This is not surprising given the sequence divergence and the fact that mammalian α- and β-tubulin genes are not interchangeable with their *S. cerevisiae* cognates (Bond et al., 1986; T. Stearns, unpublished results). It should be noted that the human γ-tubulin gene is able to complement a mutation in the *S. pombe tug1*$^+$ γ-tubulin gene (Horio and Oakley, 1994) with which it shares ~70% amino acid sequence identity.

Despite the sequence divergence, the weight of evidence presented here and by Sobel and Snyder (1995) suggests that *TUB4* is the *S. cerevisiae* γ-tubulin. The possibility remains that *TUB4* encodes a new tubulin that has not been described in any other organism, one that might be the partner of γ-tubulin in the SPB, and therefore have similar properties. We consider this unlikely given the advanced state of the yeast genome project (>90% completed) and the number of tubulin sequences, including γ-tubulins, that have been derived from genome projects in other model organisms. In no case have more than three different tubulin types been found in any organism. This issue will only be answered definitively by the completion of the *S. cerevisiae* genome sequence.

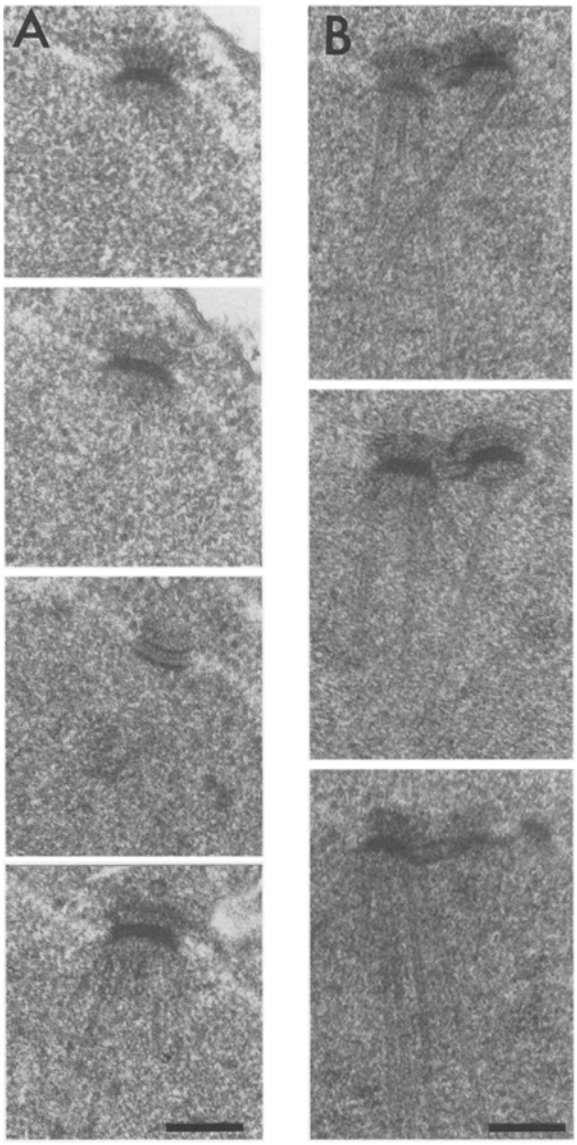


*Figure 9.* EM of *tub4* mutant cells. A *tub4-34* haploid strain was grown at 24°C, and then shifted to 37°C for 3 h. Serial sections of two cells with duplicated SPBs are shown. (*A*) The two SPBs in this cell are one above the other. From the top, the first two sections show that the first SPB has no microtubules. The third section shows the bridge between the two SPBs, and the fourth section shows the second SPB with attached microtubules. Bar, 170 nm; distance from top section to bottom section is ~650 nm. Of seven consecutive sections examined, 4 are shown: numbers 1, 2, 4, and 7. (*B*) The two SPBs are side by side, and the one on the right has only two microtubules. Bar, 150 nm; distance from top section to bottom section is ~350 nm for all sections shown.

### *Tub4p at the Spindle Pole Body*

Both Tub4p and a Tub4–GFP fusion protein are localized to the SPB. As is the case with γ-tubulin in animal cells (Stearns et al., 1991), this localization to the MTOC is not dependent on the presence of detectable microtubule polymer, indicating that Tub4p must bind to at least one other SPB component. This experiment has the caveat that the portion of yeast microtubules associated with the SPB is often recalcitrant to depolymerization. Although there was no detectable microtubule immunofluorescence in most of the cells that we examined, it is possible that there was polymeric tubulin present below the limit of detection. The association of Tub4p with the SPB was examined in more detail using the Tub4–GFP fusion protein. Induction of Tub4–GFP in α-factor–arrested cells resulted in its association with the existing SPB, indicating that Tub4p can associate with an SPB that is already assembled. This is in contrast to results obtained with Kar1p, in which a Kar1–β-galactosidase fusion protein was found to associate only with a new SPB after induction (Vallen et al., 1992). This suggests that Kar1p is incorporated during assembly of the SPB and does not exchange with soluble Kar1p. It also suggests that the SPB might consist of different classes of proteins, some more closely associated with the core of the structure, some less tightly bound. This is similar to results from experiments on the in vitro assembly of centrosomes; γ-tubulin appeared to be peripherally associated with a core of other proteins associated with the centrioles (Stearns and Kirschner, 1994; Felix et al., 1994). Although Tub4–GFP is able to associate with a preexisting SPB, the rate of this association is relatively slow, taking ~30 min after introduction of an SPB lacking Tub4–GFP into a cytoplasm containing Tub4–GFP.

In *tub4* ts mutants, most of the Tub4p was lost from the SPB after 3 h at the restrictive temperature. This likely reflects an inability of the mutant protein to associate or remain associated with the SPB, as the level of the protein determined by Western blotting did not change. Interestingly, loss of Tub4p from SPB was coincident with loss of the 90-kD SPB protein, a component of the inner and outer plaques (Rout and Kilmartin, 1991). It remains to be seen whether the dependency of 90-kD protein localization on Tub4p function reflects a specific interaction or a nonspecific disruption of the structure of the SPB in the *tub4* mutants.

Dramatic overexpression (300-fold) of Tub4p had little phenotypic effect; the microtubules in overexpressing cells appeared to be normal in number and in their association with the spindle body, and there was no major effect on the progression of the cell cycle. Overexpression of γ-tubulin, albeit to lower levels than in our experiments, also has no effect in *Aspergillus* (Oakley et al., 1990). This is intriguing in light of the results of Shu and Joshi (1995) who show that ~100-fold overexpression of γ-tubulin in certain mammalian cell types results in disruption of the endogenous microtubule cytoskeleton and the formation of polymers of γ-tubulin. This difference could be explained by a quantitative difference in the proteins associated with soluble γ-tubulin; if these proteins are involved in keeping γ-tubulin inactive until it is bound to the MTOC, then they might be limiting in certain cultured mammalian cells, resulting in activation of the overexpressed protein in the cytoplasm.

### *Tub4p Function: Implications for γ-Tubulin and the Microtubule-organizing Center*

The main function of MTOCs such as the SPB or the centrosome is to nucleate microtubule assembly from soluble subunits and to anchor one end of those microtubules. It is not known how MTOCs promote assembly of microtu-

bules, but a simple model is that γ-tubulin at the MTOC forms a template for the formation of microtubules from α- and β-tubulin, and that the association of γ-tubulin with the MTOC anchors the end of the nucleated microtubule (Oakley et al., 1990; Stearns et al., 1991; Oakley, 1992). Recent results on the characterization of γ-tubulin in animal cells lend support to this model (Zheng et al., 1995; Moritz et al., 1995). The 25S γ-tubulin–containing complex (Stearns and Kirschner, 1994) is a ring-shaped complex made up of γ-tubulin and several other proteins, which is capable of nucleating microtubule polymerization (Zheng et al., 1995). Sophisticated EM analysis of centrosomes shows that they have within their pericentriolar material similar γ-tubulin–containing ring-shaped structures that seem to be at the base of nucleated microtubules (Moritz et al., 1995).

We assayed the in vivo function of Tub4p by analysis of temperature-sensitive *tub4* mutants at the restrictive condition and by depletion of Tub4p. The most obvious phenotype is that at the restrictive condition, most of the mutant cells have a monopolar spindle. Examination of these cells by serial section EM revealed that although the mutants have a monopolar spindle at the restrictive temperature, they have two unseparated SPBs, and in some cases, it can be seen that one of the SPBs has no microtubules associated with it. The simplest hypothesis is that an SPB formed before inactivation of Tub4p retains its associated microtubules, whereas an SPB formed after inactivation is unable to nucleate microtubules.

There are three important points to be made from this result: (*a*) Tub4p appears to be required to make a spindle pole body that is capable of microtubule nucleation; (*b*) both SPBs in the *tub4* mutant cells have a normal morphology, differing only in their association with microtubules; and (*c*) the presumptive preexisting SPB in the *tub4* mutant cells appears to be normal in its interaction with microtubules. The first point is consistent with the notion that γ-tubulin is intimately associated with the nucleating material of MTOCs. If a SPB is made under conditions in which γ-tubulin is not functional, then that SPB might be expected to be unable to nucleate microtubules. The second point is interesting in light of the phenotypes of mutations in other SPB components. In contrast to the morphologically normal SPBs in a *tub4* mutant, mutations in other SPB components result in either a failure to duplicate the structure, as for calmodulin (Sun et al., 1992), *KAR1* (Rose and Fink, 1987), *MPS1* (Winey et al., 1991), and *CDC31* (Byers, 1981) mutants, or in the formation of a new SPB that is not properly integrated into the nuclear envelope, as in *NDC1* (Winey et al., 1993) and *MPS2* (Winey et al., 1991) mutants. These results suggest that Tub4p is either required to nucleate microtubules directly or required for the assembly of a nucleating structure at the SPB. By this interpretation, Tub4p would be a peripheral component, added to the outside of the core SPB structure. A similar situation has been demonstrated with γ-tubulin in animal cells, where γ-tubulin is required to make a nucleating centrosome from nonnucleating basal bodies in extracts of frog eggs (Stearns and Kirschner, 1994; Felix et al., 1994).

The third point above is the most surprising. Since at least one of the SPBs in a *tub4* mutant has attached microtubules, there are two possible conclusions. One is that Tub4p is required to make an SPB that can nucleate microtubules, but it is not required to maintain the microtubules once nucleated. Alternatively, the mutants that we have isolated may be defective in synthesis of Tub4p, which affects the construction of a new SPB but does not affect an already constructed SPB. We favor the first alternative because there is a demonstrable defect in the function of the mutant proteins; they lose the ability to associate with the SPB at the restrictive temperature. After 3 h at the restrictive temperature, most of the *tub4* mutant cells examined had little or no Tub4p at the SPB, yet there were still microtubules attached to the SPB, and microtubules were able to grow back after depolymerization. The amount of Tub4p remaining at the SPB was <10% of the normal amount; however, the total amount of the protein in the cell did not change. Therefore the observed function of the SPB in the mutants takes place with <10% of the normal amount of Tub4p.

If Tub4p is the *S. cerevisiae* γ-tubulin, then the result that the SPB still has associated microtubules in mutants contradicts some of the predictions of the γ-tubulin template model. The only genetic experiments done with γ-tubulin before this work have examined cells where loss of the γ-tubulin gene occurred a relatively long time before observation, leaving the potential for secondary defects to be expressed. In *Aspergillus*, no or few microtubules were observed in germlings of a *mipA* deletion mutant (Oakley et al., 1990), and in *S. pombe*, few microtubules were observed in cells that were presumed to have lost the plasmid-borne copy of $tug1^+$ (Horio et al., 1991). In both cases, the cells were examined long after the loss event; therefore, the lack of microtubules could be a terminal, rather than the primary, effect of the loss of γ-tubulin. How can our results be reconciled with the existing information about the function of γ-tubulin? One possibility is that Tub4p is required to assemble a microtubule-nucleating site at the SPB, which once assembled no longer requires Tub4p function. Thus, an SPB assembled in the presence of functional Tub4p would no longer require Tub4p function to nucleate and anchor microtubules, whereas an SPB assembled in the absence of functional Tub4p would be unable to nucleate. This could be a characteristic that is peculiar to the yeast SPB, as there are several aspects of this MTOC that differ from many other MTOCs. For example, the yeast SPB has both cytoplasmic and nuclear microtubules throughout the cell cycle (Byers and Goetsch, 1975); there is little of the remodeling found in animal cells, e.g., at the interphase/mitosis transition. Also, microtubules in *S. cerevisiae* are closed at the SPB end (Byers et al., 1978). The nature of the structure at the ends of the microtubules is not known, but it is possible that once a microtubule is nucleated by the SPB, that microtubule never fully depolymerizes. The remaining seed would allow microtubule elongation without true nucleation. Alternatively, Tub4p may be required for nucleation and anchoring, but there may be an excess at the SPB, and the small residual amount left in the mutants is sufficient to carry out these functions.

As a last consideration of the *tub4* mutant phenotype, we suggest that the long cytoplasmic microtubules observed after extended periods at the restrictive condition

do not reflect a specific defect. This phenotype only appears at later times after a shift to the restrictive condition, and other mutations that affect SPB duplication in different ways display the same phenotype (e.g., *NDC1*, Thomas and Botstein, 1986; Winey et al., 1993; and *KAR1*, Rose and Fink, 1987). When cells have only one functional SPB, there are probably fewer microtubule-nucleating sites than in a normal cell. If the tubulin concentration is the same as in normal cells, this might result in fewer and longer microtubules.

We thank Mark Johnston for bringing the *TUB4* sequence to our attention, David Botstein for generous assistance with the EM, James Sabry for invaluable assistance with the fluorescence quantitation, Pam Foreman for kindly providing the RNA used in the *TUB4* Northern blot analysis, David Friedman and Trisha Davis for providing antibodies, Joachim Li for providing plasmids, and Steve Murphy for comments on the manuscript.

This work was supported by grants to T. Stearns from the National Institutes of Health (GM52022), the American Cancer Society, and the Searle Scholars Program. L.G. Marschall was supported by an American Cancer Society postdoctoral fellowship.

Received for publication 1 February 1996 and in revised form 3 April 1996.

*Note added in proof.* The complete sequence of the *S. cerevisiae* genome reveals that *TUB1*, *TUB2*, *TUB3*, and *TUB4* are the only tubulin genes in yeast, and thus that Tub4p is the yeast γ-tubulin.

### *References*

Bond, J.F., J.L. Fridovich-Keil, L. Pillus, R.C. Mulligan, and F. Solomon. 1986. A chicken-yeast chimeric β-tubulin protein is incorporated into mouse microtubules in vivo. *Cell.* 44:461–468.

Byers, B. 1981. Cytology of the yeast life cycle. *In* The Molecular Biology of the Yeast *Saccharomyces*: Life Cycle and Inheritance. J.N. Strathern, E.W. Jones, and J.R. Broach, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 59–96.

Byers, B., and L. Goetsch. 1975. Behavior of spindles and spindle plaques in the cell cycle and conjugation of *Saccharomyces cerevisiae*. *J. Bacteriol.* 124:511–523.

Byers, B., K. Shriver, and L. Goetsch. 1978. The role of spindle pole bodies and modified microtubule ends in the initiation of microtubule assembly in *Saccharomyces cerevisiae*. *J. Cell Sci.* 30:331–352.

Conde, J., and G.R. Fink. 1976. A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. *Proc. Natl. Acad. Sci. USA.* 73:3651–3655.

Dieckmann, C.L., and A. Tzagoloff. 1985. Assembly of the mitochondrial membrane system. *J. Biol. Chem.* 260:1513–1520.

Eshel, D., L.A. Urrestarazu, S. Vissers, J.C. Jauniaux, J.C. Van Vliet-Reedijk, R.J. Planta, and I.R. Gibbons. 1993. Cytoplasmic dynein is required for normal nuclear segregation in yeast. *Proc. Natl. Acad. Sci. USA.* 90:11172–11176.

Felix, M.-A., C. Antony, M. Wright, and B. Maro. 1994. Centrosome assembly in vitro: role of γ-tubulin recruitment in Xenopus sperm aster formation. *J. Cell Biol.* 124:19–31.

Fuchs, U., B. Moepps, H.P. Maucher, and H. Schraudolf. 1993. Isolation, characterization and sequence of a cDNA encoding gamma-tubulin protein from the fern *Anemia phyllitidis L. Sw. Plant Mol. Biol.* 23:595–603.

Geiser, J.R., H.A. Sundberg, B.H. Chang, E.G.D. Muller, and T.N. Davis. 1993. The essential mitotic target of calmodulin is the 110-kilodalton component of the spindle pole body in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 13: 7913–7924.

Green, N., H. Alexander, A. Olson, S. Alexander, T.M. Shinnick, J.G. Sutcliffe, and R.A. Lerner. 1987. Immunogenic structure of the influenza virus hemagglutinin. *Cell.* 28:477–487.

Harlow, E., and D. Lane. 1988. Antibodies : A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 726 pp.

Horio, T., and B.R. Oakley. 1994. Human γ-tubulin functions in fission yeast. *J. Cell Biol.* 126:1465–1473.

Horio, T., S. Uzawa, M.K. Jung, B.R. Oakley, K. Tanaka, and M. Yanagida. 1991. The fission yeast gamma-tubulin is essential for mitosis and is localized at microtubule organizing centers. *J. Cell Sci.* 99:693–700.

Hoyt, M.A. 1994. Cellular roles of kinesin and related proteins. *Curr. Opin. Cell Biol.* 6:63–68.

Huffaker, T.C., J.H. Thomas, and D. Botstein. 1988. Diverse effects of β-tubulin mutations on microtubule formation and function. *J. Cell Biol.* 106:1997–2010.

Jacobs, C.W., A.E.M. Adams, P.J. Staniszlo, and J.R. Pringle. 1988. Functions of microtubules in the *Saccharomyces cerevisiae* cell cycle. *J. Cell Biol.* 107: 1409–1426.

Johnston, M., and R.W. Davis. 1984. Sequences that regulate the divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 4: 1440–1448.

Joshi, H.C., J.P. Monica, L. McNamara, and D.W. Cleveland. 1992. Gamma-tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. *Nature (Lond.).* 356:80–83.

Kaiser, C.A., D. Preuss, P. Grisafi, and D. Botstein. 1987. Many random sequences functionally replace the secretion signal sequence of yeast invertase. *Science (Wash. DC).* 235:312–317.

Kilmartin, J. 1981. Purification of yeast tubulin by self-assembly *in vitro*. *Biochemistry.* 20:3629–3633.

Kilmartin, J., B. Wright, and C. Milstein. 1982. Rat monoclonal anti-tubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93: 576–582.

Kilmartin, J.V., S.L. Dyos, D. Kershaw, and J.T. Finch. 1993. A spacer protein in the *Saccharomyces cerevisiae* spindle pole body whose transcript is cell cycle-regulated. *J. Cell Biol.* 123:1175–1184.

Li, Y.-Y., E. Yeh, T. Hays, and K. Bloom. 1993. Disruption of mitotic spindle orientation in a yeast dynein mutant. *Proc. Natl. Acad. Sci. USA.* 90:10096–10100.

Liu, B., H.C. Joshi, T.J. Wilson, C.D. Silflow, B.A. Palevitz, and D.P. Snustad. 1994. Gamma-tubulin in *Arabidopsis*: gene sequence, immunoblot, and immunofluorescence studies. *Plant Cell.* 6:303–314.

Luo, H., and M.H. Perlin. 1993. The gamma-tubulin-encoding gene from the basidiomycete fungus, *Ustilago violacea*, has a long 5′-untranslated region. *Gene (Amst.).* 137:187–194.

McIntosh, E.M., T. Atkinson, R.K. Storms, and M. Smith. 1991. Characterization of a short, *cis*-acting DNA sequence which conveys cell-cycle stage-dependent transcription in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 11: 329–337.

Moritz, M., M.B. Braunfeld, J.W. Sedat, B. Alberts, and D. Agard. 1995. Microtubule nucleation by γ-tubulin-containing rings in the centrosome. *Nature (Lond.).* 378:638–640.

Neff, N., J.H. Thomas, P. Grisafi, and D. Botstein. 1983. Isolation of the β-tubulin gene from yeast and demonstration of its essential function *in vivo*. *Cell.* 33:211–219.

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.

Oakley, B.R. 1992. Gamma-tubulin: the microtuble organizer? *Trends Cell Biol.* 2:1–5.

Oakley, B.R., C.E. Oakley, Y. Yoon, and M.K. Jung. 1990. Gamma-tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Cell.* 61:1289–1301.

Oakley, C.E., and B.R. Oakley. 1989. Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mipA* gene of *Aspergillus nidulans*. *Nature.* 338:662–664.

Prasher, D.C., V.K. Eckenrode, W.W. Ward, F.G. Prendergast, and M. Cormier. 1992. Primary structure of the *Aequoria victoria* green-fluorescent protein. *Gene (Amst.).* 111:229–233.

Pringle, J.R., R.A. Preston, A.E.M. Adams, T. Stearns, D.G. Drubin, B.K. Haarer, and E.W. Jones. 1989. Fluorescence microscopy methods for yeast. *Methods Cell Biol.* 31:357–435.

Rose, M.D., and G.R. Fink. 1987. KAR1, a gene required for function of both intranuclear and extranuclear microtubules in yeast. *Cell.* 48:1047–1060.

Rose, M.D., P. Novick, J.H. Thomas, D. Botstein, and G.R. Fink. 1987. A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene (Amst.).* 60:237–243.

Rout, M.P., and J.V. Kilmartin. 1990. Components of the yeast spindle and spindle pole body. *J. Cell Biol.* 111:1913-1927.

Rout, M.P., and J.V. Kilmartin. 1991. Yeast spindle pole body components. *Cold Spring Harbor Symp. Quant. Biol.* 56:687–703.

Schatz, P.J., L. Pillus, P. Grisafi, F. Solomon, and D. Botstein. 1986. Two functional α-tubulin genes of the yeast *Saccharomyces cerevisiae* encode divergent proteins. *Mol. Cell. Biol.* 6:3711–3721.

Schatz, P.J., F. Solomon, and D. Botstein. 1988. Isolation and characterization of conditional-lethal mutations in the *TUB1* α-tubulin gene of the yeast *Saccharomyces cerevisiae*. *Genetics.* 120:681–695.

Sherman, F., G.R. Fink, and J.B. Hicks. 1983. Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Shu, H.-B., and H.C. Joshi. 1995. γ-Tubulin can both nucleate microtubule assembly and self-assemble into novel tubular structures in mammalian cells. *J. Cell Biol.* 130:1137–1147.

Sikorski, R.S., and J.D. Boeke. 1991. In vitro mutagenesis and plasmid shuffling: from cloned gene to mutant yeast. *Methods Enzymol.* 194:302–342.

Sobel, S.G., and M. Snyder. 1995. A highly divergent γ-tubulin gene is essential for cell growth and proper microtubule organization in *Saccharomyces cerevisiae*. *J. Cell Biol.* 131:1775–1788.

Spang, A., I. Courtney, U. Fackler, M. Matzner, and E. Schiebel. 1993. The calcium-binding protein cell division cycle 31 of *Saccharomyces cerevisiae* is a component of the half bridge of the spindle pole body. *J. Cell Biol.* 123:405–416.

Stearns, T., and D. Botstein. 1988. Unlinked noncomplementation: isolation of new conditional-lethal mutations in each of the tubulin genes of *Saccharomyces cerevisiae*. *Genetics.* 119:249–260.

Stearns, T., L. Evans, and M. Kirschner. 1991. γ-Tubulin is a highly conserved component of the centrosome. *Cell.* 65:825–836.

Stearns, T., and M. Kirschner. 1994. In vitro reconstitution of centrosome assembly and function: the central role of γ-tubulin. *Cell.* 76:623–638.

Stearns, T., H. Ma, and D. Botstein. 1990. Manipulating the yeast genome using plasmid vectors. *Methods Enzymol.* 185:280–296.

Sun, G.-H., A. Hirata, Y. Ohya, and Y. Anraku. 1992. Mutations in yeast calmodulin cause defects in spindle pole body functions and nuclear integrity. *J. Cell Biol.* 119:1625–1639.

Thomas, J.H., and D. Botstein. 1986. A gene required for the separation of chromosomes on the spindle apparatus in yeast. *Cell.* 44:65–76.

Vallen, E.A., T.Y. Scherson, T. Roberts, K. van Zee, and M.D. Rose. 1992. Asymmetric mitotic segregation of the yeast spindle pole body. *Cell.* 69:505–515.

Winey, M., L. Goetsch, P. Baum, and B. Byers. 1991. *MPS1* and *MPS2*: novel yeast genes defining distinct steps of spindle pole duplication. *J. Cell Biol.* 114:745–754.

Winey, M., M.A. Hoyt, C. Chan, L. Goetsch, D. Botstein, and B. Byers. 1993. NDC1: a nuclear periphery component required for yeast spindle pole body duplication. *J. Cell Biol.* 122:743–751.

Zheng, Y., M.K. Jung, and B.R. Oakley. 1991. Gamma-tubulin is present in *Drosophila melanogaster* and *Homo sapiens* and is associated with the centrosome. *Cell.* 65:817–823.

Zheng, Y., M.L. Wong, B. Alberts, and T. Mitchison. 1995. Nucleation of microtuble assembly by a γ-tubulin-containing ring complex. *Nature (Lond.).* 378:578–583.