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

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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). 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.
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 morphologies 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 Karlp (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 epo1- 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, 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 (YPE), 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-Arrest (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 x 10⁷ cells on a sterile filter. The filter was placed on a YEP/dextrose (YPED) 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: GCGGAGCTCAGGTGGAACATCACC; Scg.2: GCCCTCTAGCTGTGTTAAGCAGGCGC; Scg.3: GCCCAAGCTTATCTAGGGTGAGAA; Scg.4: GCCCTTAGAC-TGAAACAGGAAAGGCTAC; Scg.5: GCCCTCTAGACTTATT-TGACCCAG. 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 pSDK1 (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 dye-deoxy 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 32P-labeled DNA fragment containing the TUB4 coding region. The blot was stripped of TUB4 probe and hybridized to a 32P-labeled DNA fragment containing the ACT1 coding region as a control for amount of RNA per gel lane. Denaturation 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 pATH1 (Dieckmann and Tzagoloff, 1985). Polycrylamide 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 His6-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 MgCl₂, 0.1 mM CaCl₂, 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 region was mutagenized by PCR, and a plasmid-shuffle scheme (Sikorski and Boeke, 1992) 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 early log phase. Cells were pelleted,
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 tetrad isolates 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 the 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 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).

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; Marshall, 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 1.](image)

**Figure 1.** Northern blot of RNA from cells synchronized with α-factor and released for the times indicated. The blot was probed with 32P-labeled TUB4 DNA, and subsequently probed with 32P-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 (– – – ).

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![Figure 2.](image)

**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 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 karl-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 cytoplasmains 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 cytoplasmains. 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
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 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-i 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-i 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.

**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.
Tub4p and the 90-kD protein colocalized in 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 d), 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. Tub4p 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 Tub4p 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.

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 microtubu-
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 gernlings of a mip1 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., NDCl, Thomas and Botstein, 1986; Winie et al., 1993; and KARI 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 lower after a shift to the restrictive condition, the microtubules are longer microtubules.

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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 that Tub4p is the yeast β-tubulin.

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