γ-Tubulin–like Tub4p of *Saccharomyces cerevisiae* Is Associated with the Spindle Pole Body Substructures That Organize Microtubules and Is Required for Mitotic Spindle Formation

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Abstract. Tub4p is a novel tubulin in *Saccharomyces cerevisiae* that most closely resembles γ-tubulin. We report in this manuscript that the essential Tub4p is associated with the inner and outer plaques of the yeast microtubule organizing center, the spindle pole body (SPB). These SPB substructures are involved in the attachment of the nuclear and cytoplasmic microtubules, respectively (Byers, B., and L. Goetsch. 1975. *J. Bacteriol.* 124:511–523). Study of a temperature sensitive *tub4-1* allele revealed that *TUB4* has essential functions in microtubule organization. Remarkably, SPB duplication and separation are not impaired in *tub4-1* cells incubated at the nonpermissive temperature. However, SPBs from such cells contain less or misdirected nuclear microtubules. Further analysis revealed that *tub4-1* cells are able to assemble a short bipolar spindle, suggesting that the defect in microtubule organization occurs after spindle formation. A role of Tub4p in microtubule organization is further suggested by an increase in chromosome loss in *tub4-1* cells. In addition, cell cycle arrest and survival of *tub4-1* cells is dependent on the mitotic checkpoint control gene *BUB2* (Hoyt, M.A., L. Totis, B.T. Roberts. 1991. *Cell.* 66:507–517), one of the cell’s monitors of spindle integrity.

The number, direction, and polarity of microtubules are organized by organelles called microtubule-organizing centers (MTOC). In *Saccharomyces cerevisiae*, microtubule organizing functions are provided by the spindle pole body (SPB) (see Fig. 8 A). The SPB is a cylindrical multilaminated structure that is embedded in the nuclear envelope. SPB substructures are detectable by EM (Byers, 1981a,b; Byers and Goetsch, 1975). The central plaque serves to anchor the SPB in the nuclear envelope. The inner and outer plaques nucleate the nuclear and cytoplasmic microtubules, respectively. An additional substructure of the SPB, the half bridge, is an extension of the central plaque along the cytoplasmic margin of the nuclear envelope. The half bridge has important functions in SPB duplication.

The SPB, in common with centrosomes of higher eukaryotes, shows cell cycle–dependent behavior (Byers and Goetsch, 1975). In G1 of the cell cycle, the single SPB in each yeast cell is duplicated. The duplicated SPBs undergo separation to form the poles of the spindle. Motor proteins and microtubules are required for SPB separation and spindle formation (Jacobs et al., 1988; Saunders and Hoyt, 1992). In mitosis, nuclear microtubules organized by the inner plaque of the SPB have essential functions in chromosome segregation (Jacobs et al., 1988). Cytoplasmic microtubules are required for the migration of the nucleus into the bud, but they are not essential for spindle elongation in anaphase B (Sullivan and Huffaker, 1992). Interestingly, a surveillance system involving the *BUB* (Hoyt et al., 1991) and *MAD* genes (Li and Murray, 1991) halts the cell cycle in mitosis in response to microtubule perturbation. After nuclear division and cytokinesis, each yeast cell retains exactly one SPB.

MTOCs from phylogenetically different organisms are heterogeneous in structure. Despite these structural differences, MTOCs contain related proteins that may perform similar functions. Phylogenetically conserved components of MTOCs are centrin (Baum et al., 1986; Errabolu et al., 1994; Lee and Huang, 1993; Salisbury et al., 1984; Spang et al., 1993) and γ-tubulin (Horio et al., 1991; Oakley et al., 1990; Stearns et al., 1991; Zheng et al., 1991). γ-Tubulin is, besides α- and β-tubulin, the third member of the tubulin superfamily. It is assumed that γ-tubulin is a key component of MTOCs involved in microtubule nucleation (Joshi et al., 1992; Stearns and Kirschner, 1994). This conclusion is consistent with the inhibition of microtubule nucleation after disruption of the essential γ-tubulin genes in *Aspergillus nidulans* (Oakley et al., 1990), *Schizosaccharomyces pombe* (Horio et al., 1991), and *Drosophila* (Sunkel et al., 1995), and by the failure of mammalian cells to assemble mitotic spindles after microinjection of anti-γ-tubulin antibodies (Joshi et al., 1992). Furthermore, γ-tubulin binds in...
vitro with high affinity to the minus end of microtubules (Li and Joshi, 1995) that are proximal to the MTOC (McIntosh and Euteneuer, 1984). A highly purified γ-tubulin complex from Xenopus consists of at least seven different proteins and seems to have an open ring structure. This complex caps the minus end of microtubules in vitro (Zheng et al., 1995) and in situ (Mortiz et al., 1995).

Besides α- and β-tubulin, a novel tubulin, Tub4p, that most resembles γ-tubulin has been identified by the yeast genome sequencing project in S. cerevisiae. Due to the relatively low identity to any of the three tubulin subfamilies, it has been suggested that Tub4p represents a new class of mitotic checkpoint control gene (Sobel and Snyder, 1995) that is involved in the organization of the nuclear envelope. Sobel and Snyder (1995) reported that an epitope-tagged Tub4p resides at the SPB. Partial depletion of Tub4p caused a migration failure in the first cell cycle after shifting the cells to the nonpermissive temperature. SPB duplication, SPB separation, and spindle formation were normal in tub4-1 cells. However, no mitotic spindles were observed. In addition, defects in microtubule organization of tub4-1 cells are indicated by an increase in chromosome loss and by the dependence of cell cycle arrest and survival on the mitotic checkpoint control gene BUB2, which is involved in monitoring spindle integrity.

Materials and Methods

Yeast Strains, Media, and Yeast Transformation

Yeast strains used in this study are summarized in Table I. Yeast cells were grown in yeast extract, peptone, and dextrose growth medium (YPD). Synthetic complete medium (SC) was prepared as described by Schiestl and Gietz (1989). Yeast strains were transformed as described by Schiestl and Gietz (1991) with glucose, raffinose, or galactose as carbon sources. Yeast strains were transformed as described by Schiestl and Gietz (1989). The entire coding region of tub4-1 was amplified by PCR with primers TUB4-1 (5'-GCCGGTACCATGGTTGGAAATTTATGACGTC-3') and TUB4-2. Primer TUB4-3 introduces a KpnI restriction site upstream of the start codon of TUB4. The PCR product was restricted with KpnI and NsiI and cloned into the KpnI and NsiI sites of the yeast expression vector pYES2 (Invitrogen, San Diego, CA), which was restricted with KpnI and NsiI. The resulting plasmid pSM209 carries a GALI-TUB4 fusion.

Expression of γ-tubulin from Xenopus in Yeast. The alcohol dehydrogenase (ADH) promoter/terminator from pH6005 (kindly provided by H. Doonley, Genzentrum, Munich, Germany) was cloned into plasmid pBlue SK (Stratagene, La Jolla, CA) to give pBlue-ADH-γ-tubulin from Xenopus laevis on plasmid pTS235 (kindly provided by T. Stearns, Stanford University, CA) (Stearns et al., 1991) was amplified by PCR with primers JIC-III (5'-CAACGCTGATGCTATTAATTTATATTTATATTTATACG-3') and JIC-IV (5'-AGATTGTAATCTACGACGCAGAGATTATTTTGAC-3'). The PCR product was treated with the Sure clone kit (Pharmacia) and then cloned into the HindIII restriction site of pBlue-ADH that had been converted to blunt end with Klenow polymerase (pSs2). The ADH-γ-tubulin fusion on a 3,500-bp Xhol fragment was cloned into LEU2-based vector pRS315 (Sikorski and Hieter, 1989). The resulting plasmid was named pSM8.

Cloning of TUB4 by PCR

The entire coding region of TUB4 was amplified by PCR with primers TUB4-1 (5'-CAACCTCTAGATGTCAGCGCAAATATTGTC-3') and TUB4-2 (5'-CCAATGTGACTGTTGCTGCGTCTC-3') using chromosomal DNA from strain S288C as template (these sequence data are available from EMBL/GenBank/DDBJ under accession number YSCB816). Primers TUB4-1 and TUB4-2 carry an XbaI and NsiI restriction site, respectively. The 2,200-bp PCR product was restricted with XbaI and NsiI and ligated into pRS315 (Sikorski and Hieter, 1989), previously restricted with XbaI and PstI to give plasmid pSM204. TUB4 on pRS316 was named pSM223.

Plasmid Constructions

Construction of a GST-Δtub4 Gene Fusion. The 600-bp EcoRI/SalI fragment of pSM204 carrying the 3' end of TUB4 was cloned into the EcoRI/XhoI sites of the glutathione-S-transferase (GST) expression vector pGEX-5X-1 (Pharmacia, Uppsala, Sweden). The resulting plasmid was named pSM220.

Construction of Epitope-tagged TUB4-ΔHA Gene Fusions. An NolI restriction site was introduced by PCR between the coding region of TUB4 and the TAA stop codon using primers TUB4-4 (5'-TCTATTGCAGG-GCCTTCAATTATTTATGCACGTCG-3') and TUB4-5 (5'-TATGAACTGCGGACATTGATGCCCTTCGCTTTACG-3'). The resulting plasmid was named pSM217. The 114-bp NolI fragment of plasmid pGTEP-I (kindly provided by B. Futter, Cold Spring Harbor, NY) with three repeats coding for the hemagglutinin epitope (YPYDVPDYA) was inserted into the NolI restriction site of pSM217. The orientation and number of inserts were confirmed by sequencing. Plasmid pSM218 carries a single insertion of the 114-bp NolI-fragment (TUB4-ΔHA gene fusion).

TUB4 under the Control of the GALI Promoter. The coding region of TUB4 was amplified by PCR with primers TUB4-3 (5'-CGGGTACCATGGTTGGAAATTTATGACGTC-3') and TUB4-4. Primer TUB4-3 introduces a KpnI restriction site upstream of the start codon of TUB4. The PCR product was restricted with KpnI and NsiI and cloned into the KpnI and NsiI sites of vector pQ30 (Qiagen, Inc., Chatsworth, CA) to give plasmid pSM205. Plasmid pSM205 was restricted with KpnI and SalI. The SalI restriction site is located in the polylinker region of pQ30 next to the PstI site. The KpnI/Sall fragment of pSM205 with the coding region of TUB4 was cloned into the yeast expression vector pYES2 (Invitrogen, San Diego, CA), which was restricted with KpnI and XhoI. The resulting plasmid pSM209 carries a GALI-TUB4 fusion.

Expression of γ-tubulin from Xenopus in Yeast. The alcohol dehydrogenase (ADH) promoter/terminator from pH6005 (kindly provided by H. Doonley, Genzentrum, Munich, Germany) was cloned into plasmid pBlue SK (Stratagene, La Jolla, CA) to give pBlue-ADH-γ-tubulin from Xenopus laevis on plasmid pTS235 (kindly provided by T. Stearns, Stanford University, CA) (Stearns et al., 1991) was amplified by PCR with primers JIC-III (5'-CAACGCTGATGCTATTAATTTATATTTATATTTATACG-3') and JIC-IV (5'-AGATTGTAATCTACGACGCAGAGATTATTTTGAC-3'). The PCR product was treated with the Sure clone kit (Pharmacia) and then cloned into the HindIII restriction site of pBlue-ADH that had been converted to blunt end with Klenow polymerase (pSs2). The ADH-γ-tubulin fusion on a 3,500-bp Xhol fragment was cloned into LEU2-based vector pRS315 (Sikorski and Hieter, 1989). The resulting plasmid was named pSM8.

Construction of a Δtub4-HIS3 Disruption Cassette. TUB4 of pSM204 was restricted with EcoRI and PstI, disrupting the coding region of TUB4. HIS3 on a Smal/NsiI fragment was inserted into TUB4 (pSM206). The Δtub4-HIS3 on an SacI/Sall fragment from pSM208 was ligated into vector pBlue SK that was restricted with SacI and SalI (pSM219).

Construction of a bub2::HIS3 Disruption Cassette. BUB2 was cloned by PCR using chromosomal DNA from strain S288C (gift of R. Mortimer, University of California, Berkeley). The PCR product was subcloned into pUC18 using the Sure clone kit from Pharmacia (pSM51). HIS3 on an SmaI fragment was ligated with pSM51 previously restricted with EcoRV (pSM63). The EcoRV site is located just downstream of the BUB2 start codon.

Construction of tub4-1(ts)

TUB4 was mutated by PCR according to Cadwell and Joyce (1992). The PCR product was restricted with XbaI and NsiI, and then cloned into the XbaI and NsiI sites of plasmid pRS315 (Sikorski and Hieter, 1989). A pool of plasmids were transformed into strain ESM183, and selection was made on SC plates lacking uracil and leucine. Cells, which had lost pSM223, were selected by growth on 5-fluoroorotic acid (5-FOA) plates at 37°C. The Ura colonies were tested for growth at 23° and 37°C. DNA from strains, which could not grow at 37°C, was isolated and transformed into Escherichia coli Sure. Plasmid-DNA was then again transformed into ESM183. Transformants were selected on 5-FOA plates at 37°C, and then tested for temperature sensitivity on YPD plates. The nucleotide sequence of tub4-1(ts) alleles was analyzed.
**Table I. Yeast Strains**

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| S288C  | MATα mal gal2 | R. Mortimer (University of California, Berkeley) |
| BJ5626 | MATα ura3-32/ara3-32 trp1/Trp1 leu2-3,112/Leu2 his3Δ200/His3Δ200 pep4::HIS3/pep4::HIS3 prb1Δ1.6R/prb1Δ1.6R can1/can1 | B. Jones (Carnegie-Mellon University, Pittsburgh, PA) |
| YPH499 | MATα ura3-32 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 | Sikorski and Hieter (1989) |
| YPH500 | MATα ura3-32 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 | Sikorski and Hieter (1989) |
| YPH501 | MATα ura3-32/ara3-32 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/Trp1 his3Δ200/His3Δ200 leu2Δ1/Leu2 can1/Can1 | Sikorski and Hieter (1989) |
| ESM176 | MATα ura3-32 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 pYES2 | this study |
| ESM177 | MATα ura3-32 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 pSM209* | this study |
| ESM178 | MATα ura3-32/ara3-32 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/Trp1 his3Δ200/His3Δ200 leu2Δ1/Leu2 can1/Can1 TUB4+/Δub4-:HIS3 | this study |
| ESM183 | MATα ura3-32 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δub4-::HIS3 pSM223* | this study |
| ESM184 | MATα ura3-32 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δub4-::HIS3 pSM222* | this study |
| ESM204 | MATα ura3-32 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 ΔTUB4+::pSM244* | this study |
| ESM208 | MATα ura3-32 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 tub4-1 | this study |
| ESM210 | MATα ura3-32/ara3-32 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/Trp1 his3Δ200/His3Δ200 leu2Δ1/Leu2 can1/Can1 TUB4+/Δub4-1 | this study |
| ESM215 | MATα ura3-32 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 tub4-1::HIS3 | this study |
| ESM218 | MATα ura3-32 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 tub4-1 | this study |
| YRN212 | MATα ura3-32 lys2-801 ade2-101 trp1Δ1 cysH2* [CFI(CEN6) TRP1 SUP11 CYH2] | J. Hegemann (University of Giessen, Germany) |
| YAS3 | MATα ura3-32 lys2-801 ade2-101 trp1Δ1 cysH2* [CFI(CEN6) TRP1 SUP11 CYH2] TUB::pSM244* | this study |
| YAS4 | MATα ura3-32 lys2-801 ade2-101 trp1Δ1 cysH2* [CFI(CEN6) TRP1 SUP11 CYH2] tub4-1 | this study |
| YAS5 | MATα ura3-32 lys2-801 ade2-101 trp1Δ1 cysH2* [CFI(CEN6) TRP1 SUP11 CYH2] Δtub1::URA3 | this study |
| YAS7 | MATα ura3-32 lys2-801 ade2-101 trp1Δ1 cysH2* [CFI(CEN6) TRP1 SUP11 CYH2] Δtub1::URA3 | this study |
| YAS9 | MATα ura3-32 ade2-101 trp1Δ63 leu2Δ1 cdc15 Δtub1::URA3 | this study |

*psm223 is a pRS316 (Sikorski and Hieter, 1989) derivative carrying TUB4.
*psm222 is a pRS316 derivative carrying TUB4-HA.
*pSM209 is a pYES2 derivative containing GAL-TUB4.
*pSM244 is a pRS306 derivative carrying tub4-1.

**Construction of Yeast Strains**

ESM178. The diploid strain YPH501 (Sikorski and Hieter, 1989) was transformed with the TUB4 disruption cassette of pSM219 restricted with SalI and SacI, and selection was made on SC plates lacking histidine. The construction of ESM178 was confirmed by Southern analysis as described by Spang et al. (1993).

ESM183. ESM178 was transformed with plasmid pSM223 selection being made on SC plates lacking uracil (ESM181-3). Plasmid pSM223 carries TUB4 on the pRS316-based vector pRS316 (Sikorski and Hieter, 1989). ESM181-3 was sporulated and tetrads were dissected. Colonies which were His⁺ Ura⁺ were named ESM183. ESM183 carries the Δub4-::HIS3 disruption and plasmid pSM223.

ESM184. ESM178 was transformed with plasmid pSM222. Transformants were selected on SC plates lacking uracil (ESM181). Plasmid pSM222 carries the TUB4-HA of pSM244 on pRS316. Spores of ESM181 were obtained (ESM184), which were His⁺ Ura⁺, indicating that TUB4-HA is functional.

ESM208, YAS3, and YAS4. tub4-1 was cloned into integration vector pRS306 (Sikorski and Hieter, 1989). We gave plasmid pSM244, which was linearized with the restriction enzyme Hpal. Plasmid pSM244 was then integrated into its chromosomal location by homologous recombination, creating a duplication containing the wild-type copy and the mutant copy flanking the plasmid sequences. Strains YPH500 and YRN212 (gift of J. Hegemann, University of Giessen, Germany) were transformed with the linearized plasmid pSM244, with selection being made on SC plates lacking uracil. The transformants were named ESM204 and YAS3, respectively. Integration of plasmid pSM244 was confirmed by Southern analysis. Cells, which spontaneously excised plasmid pRS306 together with TUB4, were selected for on 5-FOA plates. Temperature-sensitive colonies derived from ESM204 and YAS3 were named ESM208 and YAS4, respectively.

ESM210, ESM218, YAS5, YAS7, and YAS9. ESM208 was crossed with strain YPH499. The diploid strain ESM210 was sporulated. Two colonies of each tetrad revealed a temperature-sensitive growth defect. One MATα tub4-1 colony was named ESM218. SSI7 of ESM218, YPH499, and cdc15 was disrupted with the Δtub1::URA3 disruption cassette of plasmid pG-
were 12CA5 (Hiss Diagnostics GmbH, Freiburg, Germany), anti-90-kD (kindly provided by J. Kilmartin, Medical Research Council, Cambridge, UK) (Rout and Kilmartin, 1990), anti-Karlp (Spang et al., 1995), or affinity-purified anti-Tub4p antibodies. Secondary antibodies in immunofluorescence were rabbit anti-mouse IgG conjugated with FITC and goat anti-rabbit IgG conjugated with CY3.

Immunoelectron microscopy of isolated SPBs was performed as follows: SPBs were embedded into LR White as described by Spang et al. (1993). Sections of enriched SPBs were incubated with mouse monoclonal 12CA5 or anti-β-tubulin (WA3; kindly provided by U. Euteneuer-Schiwa, University of Munich, Germany) antibodies followed by an incubation with rabbit anti-mouse IgGs. Finally, the sections were incubated with protein A bound to gold. Sections of SPBs were also incubated with rabbit anti-Tub4p antibodies. In this case, the second incubation was performed with protein A bound to gold. For double labeling experiments, sections of enriched SPBs were first labeled with mouse monoclonal 12CA5 antibody. After washing, sections were incubated with rabbit anti-mouse IgGs, followed by an incubation with protein A bound to gold. The preparation was fixed with glutaraldehyde for 10 min. The second antibody was either anti-β-tubulin (WA3) or the anti-90-kD antibody. After washing, sections were incubated with goat anti-mouse IgGs coupled to 5 nm gold. No 5-nm signal was observed when the anti-90-kD or anti-tubulin antibodies were omitted from the incubation, indicating that all 12CA5 binding sites were blocked by the rabbit anti-mouse IgGs. Alternatively, the rat monoclonal anti-α-tubulin antibody YOL1/34 (Sera-lab) was used as the second antibody in the double labeling experiment followed by an incubation with rabbit anti-rat IgGs coupled to gold particles. No signal was obtained with secondary antibodies only. Thin-section EM of yeast cells was performed as described by Byers and Goetsch (1991).

Cell Lysates and Immunoblots

Yeast cell lysates were prepared as described by Ausubel et al. (1994). The protein content of samples was measured by the method of Bradford (1976). Proteins were separated by SDS-PAGE (Laemmli, 1970). Immunoblotting was performed as described by Harlow and Lane (1988). Secondary antibodies were goat anti-rabbit or rabbit anti-mouse IgG conjugated with HRP (Bio Rad Laboratories, Hercules, CA). Detection was performed as described by Byers and Goetsch (1991).

Yeast extracts (80 µg) of YPH500 (TUB4; lane 1), ESM192 (ADH-Xgam; lane 2), and an extract from X. laevis (lane 3) were analyzed by immunoblotting with peptide antibodies specific to X. laevis γ-tubulin. (B) X. laevis γ-tubulin does not complement for Tub4p. Growth of strain ESM183 (Δ tub4::HIS3) and TUB4 on the URA3-based plasmid pRS316 carrying plasmids pSM204 (TUB4 on the LEU2-based pRS315; sector 1), the Xgam expression plasmid pSM33 (ADH-Xgam on pRS315; sector 2), or pRS315 (sector 3) on 5-FOA plates at 37°C. Failure of ESM183 pRS315-ADH-Xgam to grow on 5-FOA plates (sector 2) indicates that Xgam does not provide Tub4p functions in S. cerevisiae.

Figure 1. The γ-tubulin from X. laevis does not complement for TUB4. (A) Expression of γ-tubulin from X. laevis in S. cerevisiae. Yeast extracts (80 µg) of YPH500 (TUB4; lane 1), ESM192 (ADH-Xgam; lane 2), and an extract from X. laevis eggs (lane 3) were analyzed by immunoblotting with peptide antibodies specific to X. laevis γ-tubulin.
Tub4p were used for the localization studies. The specificity of these antibodies toward Tub4p was investigated by immunoblotting. Tub4p was not detected in the total yeast extract (Fig. 2 A, lane 1); however, in a fraction enriched for nuclei, a band at ~55 kD reacted with the antibodies (lane 2). A protein with the same migration behavior was stained in a total yeast extract after expression of TUB4 from the strong GAL1 promoter (lane 3). These results suggest that our anti-Tub4p antibodies are specific towards Tub4p. Remarkably, although expression of TUB4 from the GAL1 promoter results in an at least 100-fold increase in Tub4p levels (Fig. 2 A), viability, cell morphology, and microtubule structure were not affected even after a 24-h incubation under inducing conditions (data not shown).

To test whether the affinity-purified anti-Tub4p antibodies stain the SPB, the localization of Tub4p in yeast cells was investigated by indirect immunofluorescence microscopy. Tub4p was localized as one or two dots on the nuclear periphery of the cells (Fig. 2 B). The number of Tub4p signals per cell and their location are in agreement with an association of Tub4p with the SPB. That the Tub4p signal was associated with the SPB was confirmed by a double labeling experiment using antibodies against the 90-kD SPB protein (Rout and Kilmartin, 1990) as a marker for SPBs (data not shown). Unfortunately, the affinity-purified antibodies lost their activity quite rapidly. To circumvent this problem, a functional gene fusion of TUB4 with a sequence coding for three repeats of the hemagglutinin (HA) epitope was constructed. Tub4p-HA was expressed in yeast and was associated with the SPB in indirect immunofluorescence experiments (data not shown).

At least eight substructures of the SPB are detectable by EM (see Fig. 8 A). Most importantly, the outer and inner plaques organize the cytoplasmic and nuclear microtubules, respectively (Byers, 1981a; Byers and Goetsch, 1975). An SPB component involved in microtubule organization should, therefore, localize to the outer and inner plaques of the SPB. The localization of Tub4p with the SPB was determined by immunoelectron microscopy using enriched SPBs and affinity-purified anti-Tub4p antibodies. The ultrathin sections showed numerous SPBs of which ~200 were inspected more closely. The substructures of the SPB—outer, central, and inner plaques—were clearly detectable (Fig. 3, A–D). In addition, the embedded SPBs contained nuclear microtubules attached to the inner plaque, while the cytoplasmic microtubules were lost during the purification of the SPBs (Rout and Kilmartin, 1990). Two to seven gold particles were associated with ~70% of the sectioned SPBs. These gold particles were localized with the inner (up to four gold particles; Fig. 3, A–C; note B is an enlargement of A) and outer (up to three; Fig. 3, B and D) plaques of the SPB. To confirm this result, thin sections of isolated SPBs from TUB4-HA and TUB4 cells were incubated with 12CA5 antibodies that are directed against the HA epitope. While none of the TUB4 SPBs were labeled, 30% of the SPBs from TUB4-HA cells had two to three gold particles associated with the inner plaque (Fig. 3, E and F) and to a lower extent with the outer plaque. Labeling of only 30% of the SPBs may be explained by the fixation sensitivity of the HA-antigen (Spang et al., 1995). In the experiments using the anti-Tub4p and anti-HA antibodies, SPB staining was not observed when sections of SPBs were incubated with the secondary antibody, only indicating that the labeling was dependent on the primary antibodies. Taken together, our results suggest that Tub4p is associated with the sites of microtubule attachment, the inner and the outer plaques of the SPB.

**Spindle Elongation Is Defective in tub4-1 Cells**

To study the function of Tub4p at the SPB, a conditional lethal mutant of TUB4 was constructed. Sequence analysis of the recessive tub4-1 allele revealed that the mutated Tub4p* carried a Phe243Ser substitution. tub4-1 was integrated in its chromosomal location (ESM208). Some mutants affecting microtubule function in *S. cerevisiae* display supersensitivity to the anti-microtubule drug benomyl (Stearns et al., 1990). Cells of tub4-1, however, showed no increased sensitivity towards benomyl (data not shown).

To determine whether the tub4-1 cells exhibited a uniform arrest phenotype, we shifted an asynchronous culture to the restrictive temperature. More than 80% of the tub4-1 cells completed S phase (Fig. 4 A) and arrested in the cell cycle with a single large bud after 3 h at 37°C (data not shown). The number of cells with 2N DNA content, as well as cells with a large bud, declined to 70% after 6 h at 37°C, suggesting that some cells could overcome cell cycle arrest. The nuclear DNA and microtubule organization was visualized by 4',6-diamidino-2-phenylindole (DAPI) staining and indirect immunofluorescence, respectively. A TUB4 strain showed characteristic microtubule structures representing all stages of the cell cycle (Fig. 4 C, Table II).
Figure 3. Tub4p is a component of the inner and outer plaques of the SPB. Sections of enriched SPBs of TUB4 (A–D) or TUB4-HA cells (E and F) were incubated with affinity-purified anti-Tub4p (A–D) or anti-HA (12CA5) antibodies followed by protein A–gold (A–D) or rabbit anti–mouse IgGs followed by protein A–gold (E and F). (A–D) SPBs with Tub4p staining (10 nm gold particles) at the inner and outer plaques. B is an enlargement of A. Four gold particles are associated with the inner plaque (A) and three (B) at the outer plaque of the SPB. (C) SPB with three gold particles at the inner plaque. (D) SPB with two Tub4p signals at the outer plaque. (E and F) Tub4p-HA staining at the inner plaque (three gold particles). C, central plaque; I, inner plaque; M, microtubules; O, outer plaque. Bars: (A) 160 nm; (E) 80 nm. B–D are double the size of A; F is the same magnification as E.

In particular, wild-type large budded cells contained an anaphase spindle with DAPI-staining regions in both the mother and bud cell bodies. While tub4-1 cells grown at the permissive temperature were similar to wild-type cells (data not shown), tub4-1 cells incubated at the nonpermissive temperature displayed four classes of abnormal spindles. Large-budded cells with a very short spindle, a metaphase-like bipolar spindle, or a monopolar spindle, and unbudded cells with a short bipolar spindle were observed (Fig. 4 B). The distribution of these spindle structures is given in Table II. Interestingly, in ~90% of the large budded tub4-1 cells, the cytoplasmic microtubules responsible for nuclear migration appeared elongated. We investigated whether nuclear migration occurs in tub4-1 cells. A mutation that interferes only with nuclear but not with cytoplasmic microtubule function causes cells to arrest with an undivided nucleus located in the bud neck. In comparison, 85% of tub2-401 cells that lack all microtubules have a defect in nuclear migration (Huffaker et al., 1988). In ~50% of tub4-1 cells, the nucleus was located in the mother cell body, indicating a partial defect in nuclear migration.

The formation of a short spindle in the tub4-1 mutant suggested that the SPB was duplicated and separated. To determine if this was indeed the case, we stained cells with antibodies against the 90-kD SPB component. In ~90%, two SPBs were detected in cells (n = 200) with a large bud confirming that SPBs were duplicated and separated in most tub4-1 cells (data not shown).

About 20% of tub4-1 cells arrested in the cell cycle without a bud. We tested whether these cells were in a specific cell cycle stage at the time the culture was shifted to the nonpermissive temperature. As for an unsynchronized culture, 76% of α-factor–synchronized tub4-1 cells arrested with a large bud in the first cell cycle (data not shown). Microtubule staining of these cells was very similar to that of an unsynchronized tub4-1 population. In 90% of tub4-1 cells (n = 200) with a large bud, two SPBs were detected using the 90-kD SPB antigen as marker (Fig. 4 D). Again, 24% of the arrested tub4-1 cells were unbudded, half of which contained either a short spindle or no nucleus (Table II). These unbudded cells rose most likely from large-budded tub4-1 cells that failed to arrest in the cell cycle.

tub4-1 Cells Are Defective in Mitotic Spindle Formation

To understand the defect of tub4-1 cells, the spindle morphology was investigated by EM. SPBs of wild-type (Fig. 5 A) and tub4-1 cells incubated at the nonpermissive temperature (Fig. 5 B) appeared to have identical morphology. SPBs were embedded in the nuclear envelope, and nuclear microtubules were in association with the SPB. At the restrictive temperature, however, nuclear microtubules of tub4-1 cells were severely disorganized. Sections through seven tub4-1 cells showing two SPBs, of which two are shown in Fig. 5, C and D, were inspected more closely. No obvious defect in SPB structure was apparent. SPBs were embedded into the nuclear envelope via the central plaque. The half bridge and the outer plaque appeared normal. Since the inner plaque is hardly visible in sections...
(YPH500) were incubated and prepared for immunofluorescence as described in B. (D) tub4-1 cells (strain YAS5; tub4-1Δssl1::URA3) were synchronized by α-factor. Cells were released from the cell cycle block and incubated at 37°C for 2 h. Cells were fixed with methanol and acetone, and SPBs were detected by indirect immunofluorescence with anti-90-kD antibodies. DNA was stained with DAPI. Bar, 5.0 μm. C and D are the same magnification as B.
through whole cells, it is difficult to judge whether this SPB substructure is defective in tub4-1 cells. SPBs were separated, however, located on one side of the nucleus and not connected by a parallel array of microtubules. Instead, the few microtubules spread out and passed the other SPB (Fig. 5 C). The distance between the two SPBs was variable. SPBs were close together (Fig. 5 D) or separated by 0.5–1 μm. The electronmicrographs are in agreement with the spindle phenotypes of tub4-1 cells (Fig. 4 B) observed by indirect immunofluorescence. The defective spindle in Fig. 5 C corresponds with the short microtubule bundles seen in large-budded tub4-1 cells. The two SPBs close to each other (Fig. 5 D) are consistent with the monopolar spindle seen in some tub4-1 cells. Two classes of microtubule defects were observed when sections through SPBs (n = 20) were inspected. Attached nuclear microtubules were either severely disorganized, pointing in another direction than the axis of the SPB (n = 15; Fig. 5, E and F), or SPBs of tub4-1 cells had no or only a few detectable microtubules (n = 5; Fig. 5, G and H).

Since microtubules are required for SPB separation (Jacobs et al., 1988), the defect in spindle formation in tub4-1 cells may be explained by nonfunctional nuclear microtubules. Alternatively, a spindle may form that then collapses at a later stage in the cell cycle. Such a spindle collapse was observed after eliminating the function of the kinesin-related proteins Kip1p and Cin8p. In the kip1cin8 double mutant, preanneaphase bipolar spindles rapidly collapsed, with previously separated poles being drawn together (Saunders and Hoyt, 1992).

These possibilities were tested using HU-blocked tub4-1 cells. HU prevents DNA replication and arrests cells with a short spindle at the end of S or in G2 phase of the cell cycle (Hartwell, 1976). If the defect in microtubule organization of tub4-1 cells occurred after spindle formation, the HU-blocked cells should have a normal spindle. tub4-1 cells were synchronized in G1 of the cell cycle by the addition of α-factor. Cells were then released from cell cycle arrest at 37°C with or without HU. As controls, wild-type and cdc15 cells were also synchronized by α-factor, and then shifted to 37°C. cdc15 cells arrest in the cell cycle with elongated spindles at the end of mitosis (Schweitzer and Philippson, 1991). Analysis of DNA content by flow cytometry confirmed that DNA replication was inhibited by HU (data not shown). Microtubule organization was analyzed by EM. No obvious defect in spindle formation was observed in HU-blocked tub4-1 cells (n = 5; Fig. 6 A). The two separated SPBs were connected by an ordered, parallel array of microtubules. We noticed that in some cases (n = 3), the spindle was located more toward one side of the nucleus. However, such spindles were also observed in tub4-1 cells incubated at the permissive temperature (data not shown). In contrast, tub4-1 cells not incubated with HU replicated the DNA and revealed defective spindles. In sectioned cells where two SPBs (n = 3) were observed, no ordered array of microtubules characteristic of a short spindle was detectable (Fig. 6 B). As for an unsynchronized culture, the two SPBs were located on the same side of the nucleus. The spindle in wild-type or cdc15 cells was of normal appearance (data not shown). Single SPBs of TUB4, cdc15, and tub4-1 cells were inspected more closely. All SPBs from cdc15 and TUB4 cells and most of the SPBs from tub4-1 cells blocked by HU were associated with a parallel array of microtubules (Table III). In contrast, SPBs of tub4-1 cells had in a single section only few or misdirected microtubules similar to the phenotypes observed for an unsynchronized tub4-1 culture (Fig. 5, E–H). In summary, our results are consistent with the formation of a short spindle in tub4-1 cells that then collapses at later stages in the cell cycle.

### Chromosome Loss Is Increased in tub4-1 Cells

An expected cytotypic consequence of mitotic spindle malfunction is a decrease in the fidelity of chromosome transmission (Hoyt et al., 1990). This was investigated using the indicator strain YRN212 carrying SUP11 on a supernumerary chromosome. SUP11 suppresses the ade2-101 phenotype of YRN212. Therefore, loss of the SUP11-containing chromosome causes a phenotypic change in colony color from white to red. tub4-1 was integrated into strain YRN212, creating a duplication containing the wild-type copy and the mutant copy flanking plasmid sequences (YAS3). Cells of YAS3 that spontaneously excised TUB4 were selected for on 5-FOA plates (YAS4). Chromosome loss of strains YRN212, YAS3, and YAS4 was determined at different temperatures (Table IV). Even at the permissive temperature, chromosome loss of tub4-1 cells was increased at least twofold compared to TUB4 cells. At 30° and 33°C, 50% and 74% of tub4-1 cells had defects in chromosome transmission, while the control strains were not affected (Table IV).
Figure 5. Spindle and SPB morphology of *tub4-1* cells. TUB4 (A) and *tub4-1* cells (B–H) were grown at 23°C. Cells were either incubated at 23°C (B) or shifted to 37°C for 3 h (C–H). Sections of cells with two SPBs (C and D) or one SPB (A, B, and E–H) are shown. Asterisks indicate the position of some nuclear pores. The large arrows in C–F and H point towards SPBs. C, central plaque; E, nuclear envelope; H, half bridge; N, nucleus; O, outer plaque; M, microtubules. Bars: (A) 100 nm; (C) 200 nm. B is the same magnification as A. D–H are the same magnification as C.
Figure 6. tub4-1 cells form a short spindle. tub4-1 cells (YAS5; tub4-1 Asstl::URA3) were synchronized with α-factor. Cells were released in YPD medium with (A) or without (B) HU at 37°C. Cells were incubated for 2 h at 37°C, and then prepared for EM. (A) Shown is a tub4-1 cell arrested in the cell cycle with HU. The two SPBs are connected by a short spindle. (B) Synchronized tub4-1 cells without HU treatment. The separated SPBs were not connected by a parallel array of microtubules. Asterisks indicate the position of some nuclear pores. The large arrows point toward SPBs. E, nuclear envelope; N, nucleus; M, microtubules. Bars: (A) 200 nm; (B) 150 nm.

Cell Cycle Arrest and Survival of tub4-1 Cells Is Dependent on the Checkpoint Control Gene BUB2

A defect in microtubule function activates mitotic checkpoint control, causing BUB and MAD gene-dependent cell cycle arrest (Hoyt et al., 1991; Li and Murray, 1991). We tested whether the cell cycle arrest of tub4-1 cells is dependent on mitotic checkpoint control. The checkpoint control gene BUB2 (Hoyt et al., 1991) of ESM208 (tub4-1) was disrupted (ESM215; tub4-1 bub2). While both strains formed colonies at 30°C, only tub4-1 cells grew at 33°C (data not shown). Furthermore, inactivation of BUB2 decreased the viability of tub4-1 cells shifted to 37°C (Fig. 7 A). More than 60% of the tub4-1 cells survived an incubation period of 4.5 h at 37°C, indicating that cell cycle arrest of tub4-1 cells is in part reversible. In contrast, only 10% of tub4-1 bub2 cells were viable after 4.5 h at 37°C (Fig. 7 A).

While tub4-1 cells arrested in the cell cycle with a single large bud, the tub4-1 bub2 double mutant continued budding (Fig. 7 B). Cells with two buds accumulated in the tub4-1 bub2 culture. Taken together, these results clearly demonstrate a role of BUB2 in the cell cycle arrest of tub4-1 cells.

Discussion

TUB4 of S. cerevisiae encodes a novel tubulin that most resembles the γ-tubulin family. However, Tub4p is with 41% identity only moderately homologous to human γ-tubulin, while the γ-tubulins from X. laevis, D. melanogaster, S. pombe, and A. nidulans are 98, 78, 71, and 68% identical to human γ-tubulin, respectively. Tub4p is even less similar to α (31% identity to Tublp and Tub3p) and β-tubulin (27%, Tub2p) of S. cerevisiae. Based on the analysis of the Tub4p sequence, it has been suggested that Tub4p represents the first member of a new tubulin superfamily (Burns, 1995).

To understand the function of Tub4p in S. cerevisiae, we studied the localization of Tub4p with substructures of the SPB. In addition, the phenotype of the conditional lethal tub4-1 allele was analyzed. tub4-1 cells arrested in the first cell cycle after shifting the cells to the nonpermissive temperature, assuring that the observed defects are a direct consequence of Tub4p malfunction. Our analysis suggests
that TUB4 has important functions in microtubule organization. First, the essential Tub4p is associated with the SPB (Fig. 2 B). Closer inspection showed that Tub4p is a component of the inner and the outer plaques of the SPB (Fig. 3)—exactly the sites of the SPB that organize the nuclear and cytoplasmic microtubules, respectively (Fig. 8 A, Byers and Goetsch, 1975). Second, 50% of tub4-1 cells are defective in nuclear migration (Table II), which is most likely caused by nonfunctional astral microtubules (Sullivan and Huffaker, 1992). Third, tub4-1 cells fail to form a mitotic spindle (Figs. 4-6). Fourth, cell cycle arrest and survival of tub4-1 cells is dependent on the mitotic checkpoint control gene BUB2 (Fig. 7). Since BUB2 is required for proper cell cycle arrest in mitosis in response to the loss of microtubule function (Hoyt et al., 1991), BUB2-dependent cell cycle arrest and survival of tub4-1 cells suggests a defect in microtubule organization. Finally, tub4-1 cells lose a supernumerary chromosome at elevated rates during mitotic growth (Table IV). Some mutants with decreased fidelity in chromosome transmission are defective in microtubule structure (Hoyt et al., 1990, 1992). Examples are mutations in the two genes coding for α-tubulin in S. cerevisiae, and in CIN8 and KIP1 that encode polypep-

dides related to the heavy chain of the motor protein kinesin (Hoyt et al., 1992).

While our manuscript was in preparation, Sobel and Snyder (1995) published a study on Tub4p function based on the partial depletion of Tub4p. They reported multiple defects in spindle formation and a defect in nuclear migration. These deficiencies were observed 17-20 h (or 8.1±0.6 generations) after repression of GAL1-TUB4 by glucose. Since Tub4p levels were not controlled in these experiments, it is unclear whether the described phenotypes are a direct or indirect consequence of Tub4p depletion. Using a similar GAL1-TUB4 depletion strain, we found by indirect immunofluorescence drastically different Tub4p levels in individual cells after growth for 24 h in the repressing glucose medium (E. Schiebel, unpublished results). This may explain the multiple phenotypes of GAL1-TUB4 cells seen after repression of the GAL1 promoter.

What is the role of Tub4p in microtubule organization?
The localization of Tub4p with the SPB substructures that are in contact with microtubule ends (Fig. 3) suggests that Tub4p may be involved in microtubule nucleation and/or attachment of microtubules to the SPB. A model for Tub4p's functions has to consider the phenotype of tub4-1 cells. tub4-1 cells incubated at the nonpermissive temperature duplicate and separate their SPBs. Furthermore, formation of a short bipolar spindle appears normal as suggested by the HU experiment (Fig. 6; Table III). Since mitotic spindles were not observed in tub4-1 cells, it is most likely that the defects in microtubule organization occur after the formation of a short bipolar spindle (Fig. 8 B). It is important to emphasize that only a subset of Tub4p's functions may be affected in tub4-1 cells. Therefore, we cannot exclude that tub4 alleles defective in other TUB4 functions may reveal different phenotypes.

A number of yeast mutants with defects in mitotic spindle formation have been described. For example, in cells carrying the cold-sensitive tub2-405 allele, the short bipolar spindle collapsed at the nonpermissive temperature with both SPBs on opposite sides of the nucleus (Pasqualone and Huffaker, 1994). A similar phenotype was observed when cells containing short spindles were treated with nocodazole. The spindles collapsed, but the SPBs remained on opposite sides of the nucleus (Jacobs et al., 1988). In contrast, inactivation of the kinesin-related motor proteins Cin8p and Kip1p caused rapid collapse of preanaphase spindles, with previously separated SPBs being drawn together (Saunders and Hoyt, 1992). This phenotype was explained by counteracting forces produced by kinesin-related proteins. Since the SPBs were on the same side of the nucleus also in tub4-1 cells, it is likely that the defective microtubule structure disturbs the counteracting forces, such that the separated SPBs are drawn together. Assuming that the attachment of nuclear microtubules to the SPB is weakened in tub4-1 cells, the detachment of some microtubules could be caused by mitosis-specific pulling forces that are transmitted to the SPB via microtubules. Mitosis-specific forces that may act on the MTOC pulling forces that are transmitted to the SPB via microtubules could be caused by mitosis-specific microtubule ends. Mitosis-specific forces has to consider the phenotype of tub4-1 cells incubated at the nonpermissive temperature. Mitosis-specific forces has to consider the phenotype of tub4-1 cells incubated at the nonpermissive temperature. Mitosis-specific forces has to consider the phenotype of tub4-1 cells incubated at the nonpermissive temperature.
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