A Highly Divergent γ-Tubulin Gene Is Essential for Cell Growth and Proper Microtubule Organization in *Saccharomyces cerevisiae*

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Abstract. A *Saccharomyces cerevisiae* γ-tubulin–related gene, *TUB4*, has been characterized. The predicted amino acid sequence of the Tub4 protein (Tub4p) is 29–38% identical to members of the γ-tubulin family. Indirect immunofluorescence experiments using a strain containing an epitope-tagged Tub4p indicate that Tub4p resides at the spindle pole body throughout the yeast cell cycle. Deletion of the *TUB4* gene indicates that Tub4p is essential for yeast cell growth. Tub4p-depleted cells arrest during nuclear division; most arrested cells contain a large bud, replicated DNA, and a single nucleus. Immunofluorescence and nuclear staining experiments indicate that cells depleted of Tub4p contain defects in the organization of both cytoplasmic and nuclear microtubule arrays; such cells exhibit nuclear migration failure, defects in spindle formation, and/or aberrantly long cytoplasmic microtubule arrays. These data indicate that the *S. cerevisiae* γ-tubulin protein is an important SPB component that organizes both cytoplasmic and nuclear microtubule arrays.

MICROTUBULE networks are involved in a wide variety of cellular processes, including intracellular transport, cell motility, nuclear migration and the segregation of chromosomes during mitosis. The microtubule organizing center (MTOC)[1] nucleates microtubule assembly and is responsible for establishment of the microtubule array (reviewed in Brinkley, 1985; Kalt and Schliwa, 1993). MTOCs are morphologically diverse among different species, but they serve similar functions and duplicate in a similar manner. One important MTOC component found in many different organisms is γ-tubulin (Oakley and Oakley, 1989; reviewed in Oakley, 1992; Joshi, 1993). γ-Tubulin has been shown to be important for microtubule assembly in vivo in several organisms (Oakley et al., 1990; Horio et al., 1991; Joshi et al., 1992; Sunkel et al., 1995). In mammalian cells, microinjection of anti-γ–tubulin antibodies into cells with depolymerized microtubules blocked subsequent microtubule assembly (Joshi et al., 1992). When the γ-tubulin gene was disrupted in *Aspergillus nidulans, Schizosaccharomyces pombe,* and *Drosophila melanogaster,* defects in nuclear migration and division were observed (Oakley et al., 1990; Horio et al., 1991; Sunkel et al., 1995; see Discussion). In organisms where γ-tubulin has been localized, it is found at the

MTOC (Oakley et al., 1990; Horio et al., 1991; Stearns et al., 1991; Zheng et al., 1991), and in some species it is located at other sites as well (Julian et al., 1993; Gard, 1994; Lajoie-Mazenc et al., 1994).

Although γ-tubulin has been found in diverse species including those of plants, animals and fungi, it has not been reported in Baker's yeast, *Saccharomyces cerevisiae,* which has been used extensively for studies in chromosome segregation and microtubule function (Page and Snyder, 1993). *S. cerevisiae* has relatively simple microtubule arrays which are organized by an electron-dense structure called the spindle pole body (SPB) (reviewed in Byers, 1981; Rose et al., 1993; Winey and Byers, 1993; Snyder, 1994). The SPB is a complex, trilaminar disk-shaped structure embedded in the nuclear envelope. It is comprised of a central electron-dense plaque, or layer, which lies in the plane of the nuclear envelope and is interposed between the outer and inner plaques, which face the cytoplasm and nucleus, respectively. A "half bridge" lies adjacent to the central plaque in the plane of the nuclear envelope. The association of the SPB with the envelope is permanent because in fungi the nuclear envelope remains intact throughout the cell cycle. Therefore, in *S. cerevisiae* two distinct microtubule arrays, nuclear and cytoplasmic, are present during the cell cycle. Cytoplasmic arrays are important for nuclear orientation and migration, SPB separation, and perhaps play an ancillary role in bud formation and/or growth (Huffaker et al., 1988; Jacobs et al., 1988; Snyder et al., 1991; Lillie and Brown, 1992; Palmer et al., 1992). Nuclear arrays are important for SPB separation,

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1. *Abbreviations used in this paper.* HA, hemagglutinin; MCB, MluI cell cycle box; MTOC, microtubule organizing center; SPB, spindle body pole.

formation of the spindle apparatus, and mitosis (Huffaker et al., 1988; Jacobs et al., 1988; Hoyt et al., 1992; Roof et al., 1992; Saunders and Hoyt, 1992; Sullivan and Huffaker, 1992). The nuclear and cytoplasmic arrays project from the inner and outer plaques of the SPB, respectively.

SPB duplication and the reconfiguration of microtubule arrays are highly regulated events in the cell cycle (Byers and Goetsch, 1974, 1975; Byers, 1981; Adams and Pringle, 1984; Kilmartin and Adams, 1984). In G1, cytoplasmic arrays emanate from a single SPB (Adams and Pringle, 1984; Kilmartin and Adams, 1984). SPB duplication occurs after the G1/S transition and is thought to occur via the half bridge (Byers and Goetsch, 1974, 1975; Byers, 1981). First, a satellite forms adjacent to this structure on the cytoplasmic face of the nuclear envelope. Next, fully duplicated SPBs separated by a full bridge are observed. During S phase, a short spindle with a prominent nuclear microtubule array is apparent; at mitosis the nuclear microtubules elongate to form a long spindle apparatus which separates the replicated DNA (Byers and Goetsch, 1975; Adams and Pringle, 1984; Kilmartin and Adams, 1984).

Genetic and immunological approaches have been used to identify a number of SPB components. Kar1p and Cdc31p, which localize to the half bridge (Vallen et al., 1992; Spang et al., 1993, 1995), were identified through genetic screens (Hartwell et al., 1973; Conde and Fink, 1976) and are important for SPB duplication (Baum et al., 1986; Rose and Fink, 1987). Strains containing temperature-sensitive mutations in *KAR1* or *CDC31* fail to duplicate SPBs properly (Baum et al., 1986; Rose and Fink, 1987). A number of other SPB components were identified through immunological studies (Rout and Kilmartin, 1990, 1991). A 42-kD protein localizes to the central plaque, and a 90-kD polypeptide, Spc90p, localizes to both the inner and outer plaques where microtubules appear to originate (Rout and Kilmartin, 1990; Rout and Kilmartin, 1991). A 110-kD calmodulin-binding polypeptide, Nuf1p/Spc110p, has been identified that forms a spacer element between the central and inner plaques (Geiser et al., 1993; Kilmartin et al., 1993; Stirling et al., 1994). Several other proteins, including Spc80p and Nuf2p, are associated with the SPB, but may not be integral to it (Rout and Kilmartin, 1990; Osborne et al., 1994). Finally, several mutants that affect SPB duplication have been isolated (Winey et al., 1991). Despite a sig-

nificant number of genetic screens to identify mutants with microtubule-associated defects (Hoyt et al., 1990; Spencer et al., 1990; Stearns et al., 1990; Page and Snyder, 1992) and direct approaches using PCR (Stearns et al., 1991), a γ-tubulin homolog has never been reported in *Saccharomyces cerevisiae*. It has been questioned whether *S. cerevisiae* has such a molecule. The identification of a γ-tubulin–related protein in *S. cerevisiae* would be important as it should facilitate genetic analysis of its function and the identification of interacting proteins.

Recently, a γ-tubulin–related protein, Tub4p, was discovered in the chromosome XII sequencing project (see below). Here we present its first characterization. Tub4p is highly divergent from γ-tubulin homologs found in other species. Its localization to the SPB, and the phenotypes of strains depleted for Tub4p are reported. The results indicate that Tub4p is important for proper organization of both nuclear and cytoplasmic microtubule arrays.

Materials and Methods

Yeast Strains, Media, and Microbiological Techniques

All yeast strains are listed in Table I. Yeast transformations were performed using the one step method (Chen et al., 1992). Genetic manipulations and growth media were as described unless indicated otherwise (Sherman et al., 1986). All plasmids were constructed using standard molecular biological techniques (Sambrook et al., 1989).

Deletion of TUB4

A *tub4::HIS3* allele was generated by polymerase chain reaction (PCR) (Baudin et al., 1993) using primers 5'-TATCGGTTAGTCACATGT-ATAAGAATTCAACATATAACTATCTATGGGTGAGCGCGCCTC GTTCAGAATG - 3' and 5'-TCCTGAACAAGGAAGGCATCATT-ATACTAATTTATGATCACCGTCGGCATACTCTTGGCCTCCTCT AGTA - 3' to amplify the *HIS3* gene from plasmid pRS313 (Sikorski and Hieter, 1989). The resulting DNA fragment contains the entire *HIS3* gene flanked by 50 bp of upstream *TUB4* sequence at the 5' terminus, and 50 bp of downstream *TUB4* sequence at the 3' terminus. This fragment was transformed into diploid yeast strain Y270 (Chen et al., 1992), and three independent transformants (Y1232, Y1233, Y1234) were identified that contained the *tub4::HIS3* allele correctly substituted at the *TUB4* locus as determined by PCR analysis. These were sporulated, and tetrads were dissected and analyzed.

Construction of GAL1::TUB4 Depletion Strains

TUB4 was cloned under the control of the *GAL1* promoter in plasmid

Table I.

Strain	Genotype
Y262	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200*
Y270	*MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200*
Y1232	*MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 tub4::HIS3/TUB4*
Y1233	same as Y1232, independent transformant
Y1234	same as Y1232, independent transformant
Y1235	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 tub4::HIS3 p270*
Y1236	*MATa*, same as Y1235, different segregant
Y1237	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 p269*
Y1238	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 p270*
Y1239	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 TUB4::3XHA,URA3*
Y1240	*MATα same as Y1238, different segregant*
Plasmid	
p269	*GAL10/GAL1 ADE2 URA3 CEN6 ARSH4*
p270	*GAL10/GAL1::TUB4 ADE2 URA3 CEN6 ARSH4*

p269 to generate plasmid p270. p269 is pRS316/*ADE2/GAL1-10*, a vector constructed by Dr. R. Padmanabha (Bristol Meyers, Wallingford, CT) which contains both the *ADE2* gene and the *GAL1-GAL10* promoter fragment cloned into pRS316 (Sikorski and Hieter, 1989). p270 was constructed by ligation of p269 (digested with BamHI, partially filled in with Klenow fragment, dATP and dGTP, and then digested with SacI) with a PCR product containing the *TUB4* gene (digested with XhoI, partially filled in with Klenow fragment, dCTP and dTTP, and then digested with SacI). Two primers, 5'-ATTACTCGAGTTGCTTGTATAAGAATTC-AACATATAACT-3' and 5'-ATTAGAGCTCCTATAATAAAACTA-TTGGGCGGTGG-3', were used to amplify the *TUB4* gene.

To construct yeast strains containing *TUB4* under the control of the *GAL1* promoter, Y1233 (a *tub4::HIS3/TUB4* diploid strain) was transformed with p270. Two independent transformants were sporulated, and tetrads were dissected. Y1235 and Y1236 (*tub4::HIS3* strains containing p270) are two His⁺ Ura⁺ segregants from the independent transformants; these segregants grow on medium containing galactose and do not grow on medium containing glucose. Y1238 and Y1240 are two control TUB4 p270 segregants derived from the same transformants as above.

Analysis of Tub4p-depletion Strains

Cells were depleted of Tub4p as follows: yeast strains were grown in galactose medium under conditions that select for the plasmid (synthetic complete medium lacking uracil). Log-phase cells were diluted to approximately 5×10^4 cells/ml in YPD or YPAD (Sherman et al., 1986) containing 4% glucose. Cell concentrations were monitored using a hemacytometer until at least 4 h after cell division cessation. To confirm that the arrest was not due to depletion of nutrients in the medium, arrested strains were diluted into fresh glucose medium. Log phase growth did not resume indicating that the arrest was not due to lack of nutrients.

For visualization of nuclear DNA, cells were fixed in 3.7% formaldehyde for 60 min. The cells were washed twice with 1.2 M sorbitol, 50 mM potassium phosphate, pH 6.8, and then visualized in 70% glycerol, PBS, 2% *n*-propyl gallate, and 0.25 μg/ml Hoechst 33258, a DNA binding dye, using differential interference contrast microscopy and epifluorescence (Snyder and Davis, 1988). Bud size was quantitated as follows (see Table III): if the bud was less than one half the size of the mother cell, it was counted as a small bud; if the bud was equal to or larger than one half the size of the mother cell, it was counted as a large bud.

Flow-cytometric analysis was performed as described (Hutter and Eipel, 1978). Cells were fixed in 75% ethanol overnight, washed in PBS, treated with RNAse A (Sigma Chemical Co., St. Louis, MO), sonicated, and stained with propidium iodide.

Construction of Epitope-tagged Tub4p Strains

Y1239 was constructed by integration of an epitope-tagged allele of *TUB4* into the chromosomal *TUB4* locus of a wild-type diploid yeast strain (Y270) using the PCR epitope-tagging method (Schneider et al., 1995). This allele was generated using primers 5'-TATGGTTGGCGAGTT-GGAAGAGGACCTGGATGCGACGGTGATCATAAATTAGTAAG-GGAACAAAAGCTGGA-3' and 5'-CTATAATAAAACTATTGG-GCGGTGGTAAAATTCCTGAACAAGGAAGGCATCATTACTATA-GGGCGAATTGGG-3', which contain homology to the *TUB4* locus, to amplify a region of a plasmid that contains a sequence encoding three copies of a hemagglutinin (HA) epitope, as well as the *URA3* gene (kindly provided by B. Schneider and B. Futcher, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY) (Wilson et al., 1984; Schneider et al., 1995) and a second copy of the 3X-HA epitope coding sequence (which is not used in these studies). The resulting DNA fragment contains the last 55 bp of *TUB4* coding sequence upstream of the termination codon, three copies of the HA epitope coding sequence (Schneider et al., 1995), 39 amino acids, a stop codon, the *URA3* gene, another three copies of the HA epitope coding sequences, and 55 bp of *TUB4* downstream noncoding sequence. The fragment was transformed into yeast strain Y270, and haploid segregants containing the HA epitope-tagged allele were recovered. Correct substitution of the epitope-tagged *TUB4* allele, *TUB4::3XHA*, was confirmed in the haploid segregants by both PCR and immunoblot analysis. The final, integrated allele contains the entire *TUB4* coding sequence under the control of its wild-type promoter, followed by three copies of the HA epitope-tag coding sequences, 39 codons, and a stop codon. This allele is marked with the *URA3* gene (146-bp downstream from the stop codon) and segregates 2:2 upon sporulation of *TUB4::3XHA/TUB4* heterozygotes.

Indirect Immunofluorescence

Cells were prepared for indirect immunofluorescence by two different methods. For the Tub4p-3XHA localization experiments, and the tubulin and Spc90p double immunofluorescence experiments, spheroplasts were prepared, fixed with methanol and acetone, and stained, using procedures similar to those described by Rout and Kilmartin (1990). Care was taken to optimize spheroplasting conditions such that cells were permeabilized to antibodies, but that buds remained attached to the mother cells. The primary antibodies used were 12CA5, a mouse anti–HA-epitope monoclonal antibody (BABCO, Richmond, CA), for the Tub4p-3XHA localization experiments, and rabbit anti-β-tubulin antibodies (kindly provided by the F. Solomon laboratory) (Bond et al., 1986) and a mouse anti-Spc90 monoclonal antibody (a kind gift from the J. Kilmartin laboratory, MRC Laboratory of Molecular Biology, Cambridge, England) (Rout and Kilmartin, 1990) for the Tub4p-depletion studies. For tubulin and nucleolar double immunofluorescence experiments, cells were fixed with formaldehyde for 60 min and stained with the rat anti–α-tubulin monoclonal antibody YOL1/34 (Kilmartin et al., 1982) and a mouse anti–Nsr1p monoclonal antibody (Copeland, C., H. Friedman, J. Woolford, and M. Snyder, unpublished results) as described (Pringle et al., 1991). In all experiments, cells were incubated with primary antibodies overnight. For detection of primary antibodies, CY3-conjugated goat anti–mouse antibodies (Jackson Immunoresearch, West Grove, PA) and FITC-conjugated goat anti–rabbit antibodies (Cappel Laboratories, Malvern, PA) were used. Hoechst 33258 was used for visualization of the yeast nuclei and mitochondria (Snyder and Davis, 1988).

For quantitation of the SPB staining results, care was taken to examine cells in all focal planes to ensure that no SPBs were missed.

Results

S. cerevisiae Has a γ-Tubulin–related Gene

A tubulin-related gene, *TUB4*, was identified in the *S. cerevisiae* chromosome XII sequencing project (Accession No. U14913; gene L8167.21; see Fig. 1 for the Tub4p protein sequence). It is located on the long arm of chromosome XII, between *CDC42* and *SEC13*.

Comparison of the predicted 473–amino acid Tub4p sequence with other tubulin sequences in the Genbank database reveals that the *S. cerevisiae* protein is most closely related to members of the γ-tubulin family (Fig. 1 and 2). There are at least 17 full-length γ-tubulin–related sequences in Genbank. Of these, 15 are very similar to one another (Fig. 2) and can be classified according their species taxonomy. Two others, those from *C. elegans* and *E. histolytica*, are highly divergent both from the other 15 and from each other. The Tub4p sequence shares a mean amino acid sequence identity of 35.6% with 16 of the γ-tubulin sequences (range = 32–38%) and is 29% identical to that of *C. elegans*. Tub4p is not significantly closer in identity to the fungal γ-tubulins (those of *S. pombe*, *A. nidulans*, and *U. violacea*, which group together; Fig. 2) than to other γ-tubulin–related proteins. As shown in Fig. 1, many residues that are characteristic of γ-tubulins are also present in Tub4p. In comparison, the Tub4p sequence is 26 and 28% identical to the *S. cerevisiae* Tub1p and Tub3p α-tubulin sequences, respectively, and 27% identical to the Tub2p β-tubulin sequence (see Fig. 2). Similarly, the predicted Tub4p sequence is very divergent from the eight randomly chosen α- and β-tubulins from other organisms that were examined. Tub4p shares mean amino acid sequence identities of 26.9% with the β-tubulins (range = 26–27%) and 27.1% with the α-tubulins (range = 24–29%). Thus, Tub4p is most closely related to members of the γ-tubulin family. We suggest that it is a highly divergent member of this family (see Discussion).
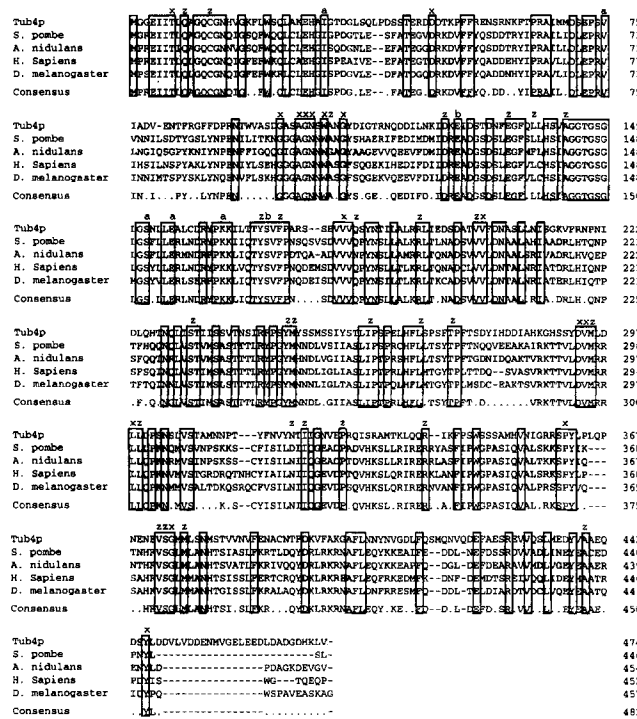
Figure 1. Alignment of the predicted Tub4p protein sequence with several γ-tubulin sequences. Boxes indicate identical residues found in all five family members. Residues common to γ-tubulin family members are indicated by a letter above the Tub4p residue. (x) Tub4p residue found in all γ-tubulin sequences (see list in Fig. 2), but not present in Tub1p, Tub2p, or Tub3p. (z) Tub4p residue found in most (≥14/17) γ-tubulin sequences (see Fig. 2), but not present in Tub1p, Tub2p, or Tub3p. (a) Residue found in Tub4p, Tub1p, and Tub3p and all γ-tubulin sequences, but not present in Tub2p. (b) Residue found in Tub4p, Tub2p, and all γ-tubulin sequences, but not present in Tub1p or Tub3p. Tub1p and Tub3p are the *S. cerevisiae* α-tubulins (Schatz et al., 1986b) and Tub2p is the β-tubulin (Neff et al., 1983).

The carboxy terminal 2–27 amino acids are particularly divergent among various members of the γ-tubulin family, both in sequence and in length (Fig. 1 residues 447–474 for Tub4p; Stearns et al., 1991). Tub4p is also divergent in this segment and has many acidic residues (12/28 terminal amino acids are acidic); this feature is similar to Tub1p, Tub2p, and Tub3p, but unlike most other γ-tubulins.

124-bp upstream of the putative initiator methionine codon of *TUB4* lies an MluI cell cycle box (MCB). MCB elements are found upstream of a number of genes whose expression is elevated after progression through the G1/S transition (Lowndes et al., 1991; Lowndes and Johnston, 1992). Another gene encoding a spindle pole body component, *NUF1/SPC110*, has an upstream MCB element and is cell cycle regulated; its mRNA is highest after the G1/S transition (Kilmartin et al., 1993). Thus, it is possible that the expression of a number of spindle pole body components is cell cycle regulated.

The Tub4 Protein Is Present at the Yeast Spindle Pole Body

γ-Tubulin localizes to the microtubule organizing center in many different organisms (Oakley et al., 1990; Horio et al.,
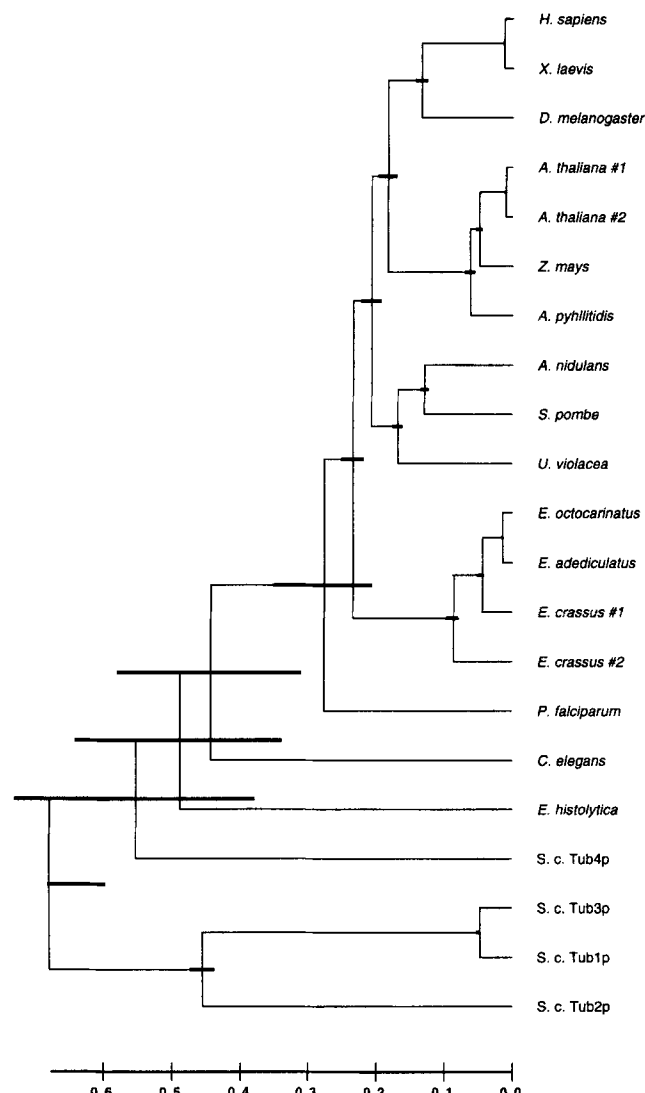

Figure 2. Dendrogram of γ-tubulin–related proteins. *S. cerevisiae* α-tubulin (Tub1p and Tub3p) and β-tubulin (Tub2p) are included. Protein sequences were aligned using the GeneWorks program that uses the unweighted pair group method with arithmetic mean (UPGMA) for determining the calculated relationships (Nei, 1987). The horizontal lines connecting two species names are proportional to the evolutionary distance between their γ-tubulin sequences. The standard error of each branch point is indicated by a thicker line. Where the error bar for a branch point extends horizontally beyond the branch point below or above it, it is not possible to determine which branched first. Thus, Tub4p is not significantly closer to any particular member of the γ-tubulin family, but it is significantly different from Tub1p, Tub2p, and Tub3p. References are as follows: *H. sapiens* (Zheng et al., 1991), *X. laevis* (Stearns et al., 1991), *D. melanogaster* (Zheng et al., 1991), *A. thaliana* #1 and 2 (Liu et al., 1994), *Z. mays* (accession #X78891), *A. phyllitidis* (Fuchs et al., 1993), *A. nidulans* (Oakley and Oakley, 1989), *S. pombe* (Horio et al., 1991, Stearns et al., 1991), *U. violacea* (Luo and Perlin, 1993), *E. octocarinatus* (Liang and Heckmann, 1993), *E. aediculatus* (Accession #X85233), *E. crassus* #1 and 2 (accession #X85234 and X85235, respectively), *P. falciparum* (Maessen et al., 1993), *C. elegans* (Sulston et al., 1992), *E. histolytica* (accession #U20322), *S. cerevisiae* Tub1p and Tub3p (Schatz et al., 1986b), and *S. cerevisiae* Tub2p (Neff et al., 1983).

1991; Stearns et al., 1991; Zheng et al., 1991), and for some organisms it has been found at other sites as well (Julian et al., 1993; Gard, 1994; Lajoie-Mazenc et al., 1994). To determine where Tub4p localizes in budding yeast, a strain was constructed that contains three copies of a short HA peptide coding sequence (i.e. an epitope tag; Wilson et al., 1984) integrated into *TUB4* after the last codon of the open reading frame (Schneider et al., 1995; see Materials and Methods). Haploid cells containing only this *TUB4::3XHA* allele under the control of its natural *TUB4* promoter grew at ~90% the growth rate of wild-type cells, indicating that the Tub4p-3XHA protein complements Tub4p function to near wild-type levels. Proteins prepared from strains containing *TUB4::3XHA* were blotted and probed with a monoclonal antibody that recognizes the HA epitope tag. A reactive band of approximately 59 kD, the expected molecular weight for the Tub4p-3XHA protein, was observed (data not shown). This band was not detected in protein samples prepared from cells that lacked the HA construct.

The subcellular distribution of the epitope-tagged Tub4p was determined by indirect immunofluorescence using spheroplasts fixed with methanol and acetone (Kilmartin et al., 1993; see Materials and Methods). In cells containing a short or medium-sized mitotic spindle, strong Tub4p staining was detected at each pole (Fig. 3, a–d). The staining was concentrated specifically at the polar region and did not colocalize with tubulin, which localizes throughout the spindle. Polar staining was sometimes detected in unbudded cells, but was less intense than that observed in cells with duplicated and separated SPBs. SPB staining was also observed in cells with a long spindle, but these cells were infrequent in the population. SPB staining was not observed in control strains that lacked the HA-tagged protein (Fig. 3, e and f), and was still observed in experiments in which the tubulin antibody was not included (not shown). These latter experiments indicate that the Tub4p signal is not due to crossover signals from nonspecific secondary antibodies or the anti-tubulin antibody. Thus, we conclude that Tub4p is present at the SPB throughout the cell cycle, but is most apparent in cells containing a short or long spindle.

TUB4 Is Essential for Yeast Cell Growth

To determine if the *TUB4* gene is essential, a deletion mutation was constructed in which 98% of the protein coding sequence (codons 3 through 465) of *TUB4* was substituted with the *HIS3* gene (Baudin et al., 1993). This deletion allele was introduced into a *his3Δ/his3Δ* diploid yeast strain and correct replacement at the *TUB4* locus was confirmed by PCR analysis. The heterozygous *tub4Δ/TUB4* strains were sporulated, and tetrads were dissected. Analysis of 48 tetrads from three independent transformants revealed that the majority of tetrads (73%) produced two His⁻ segregants and two segregants which did not form colonies (Table II). Inspection of the cells that did not form colonies revealed that they usually (58 of 60 samples examined) germinated, and most (54/60) divided to form three–five cell bodies (i.e., large buds and/or mother cells). These results suggest that *TUB4* is essential for mitotic growth.

Surprisingly, 8 of 48 tetrads produced one or two His⁺ progeny (Table II). These His⁺ spores are derived from three independent transformants. We hypothesized that

these His⁺ segregants contained an additional wild-type *TUB4* gene, perhaps through chromosome nondisjunction during mitotic or meiotic growth. An analogous situation has been previously noted for the *tub1Δ/TUB1* heterozygous α-tubulin disrupted cells (Schatz et al., 1986a). To determine if the His⁺ survivors contained a wild-type *TUB4* gene, all ten His⁺ segregants were tested for the presence of a wild-type and mutant *TUB4* gene by PCR analysis. All His⁺ segregants contained both alleles. To determine if other markers might also segregate aberrantly in these strains, the mating type of each segregant was analyzed. In 75 His⁻ segregants analyzed, all mated with *MATa* or *MATα* mating tester strains. In contrast, one half of the His⁺ segregants (5/10) did not mate with the tester strains and were capable of sporulation, yielding four-spore tetrads. These results suggest that these segregants gained an additional copy of chromosome III, probably by nuclear missegregation. The other half of the His⁺ segregants were found to be *MATa* or *MATα*, suggesting that the strain had acquired the extra wild-type *TUB4* allele by chromosome missegregation. Dissection of control *TUB4/TUB4* strains from our background did not reveal any nonmating cells; heterozygous strains produced normal segregation of auxotrophic markers. In summary, these data indicate that *tub4Δ* cells cannot survive without a wild-type copy of the *TUB4* gene and that *TUB4* is essential for cell growth. The results further indicate that *tub4/TUB4* heterozygous strains missegregate one or more chromosomes (or their nuclei) during mitotic and/or meiotic growth.

Cells Depleted of Tub4p Arrest at Nuclear Division

To gain further insight into the function of *TUB4* during vegetative growth, strains depleted of Tub4p were analyzed. Two independent *tub4Δ* haploid segregants, each containing a plasmid with the *TUB4* gene placed under the control of the inducible *GAL1* promoter, were constructed (Johnston and Davis, 1984; see Materials and Methods). Identical results were obtained for both strains. Log phase cells were transferred from galactose medium, which induces expression of the *TUB4* gene, to medium containing glucose, which represses expression from the *GAL1* promoter (Johnston and Davis, 1984). The *tub4Δ* cells containing the *GAL1::TUB4* plasmid continued to grow exponentially for 6.8 ± 0.5 generations (~11–14 h) and then arrested after 8.1 ± 0.6 generations (~17–20 h; based on six experiments; Fig. 4). In contrast, control *TUB4* strains that contain either the *GAL1::TUB4* plasmid or the *GAL1* vector continued to grow and divide. We presume the long delay before arrest in the *tub4Δ* strain is due to the presence of excess protein produced from the strong *GAL1* promoter.

Analysis of Tub4p-depleted cells reveals a defect during nuclear division. Of cells that contain nuclei, 78.5 ± 3.5% of Tub4p-depleted cells arrest with large buds (as compared with 36.8 ± 4.5% for wild-type cells). Staining with Hoechst 33258, a DNA binding dye, indicates that 72 ± 3% of these cells contain a single nucleus that resides either in one cell body or in the neck in contrast to 18 ± 2% for wild-type cells (Fig. 5 and Table III). These fractions do not change significantly after incubation for several hours after arrest (*t* = 19 and *t* = 21 h examined; Table
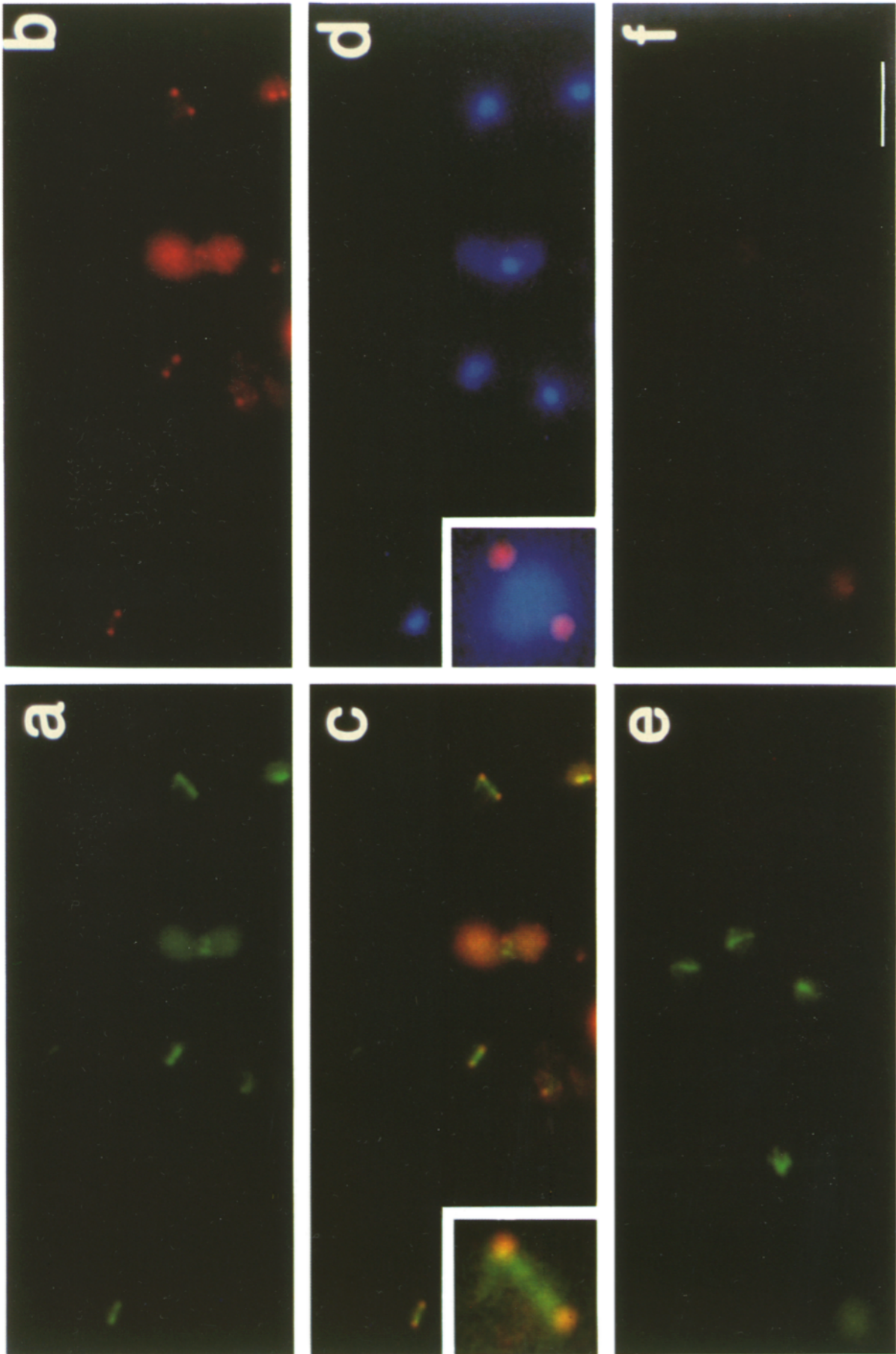
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Table II. Summary of Tetrads Derived from tub4Δ/TUB4 Heterozygotes (Y1232)

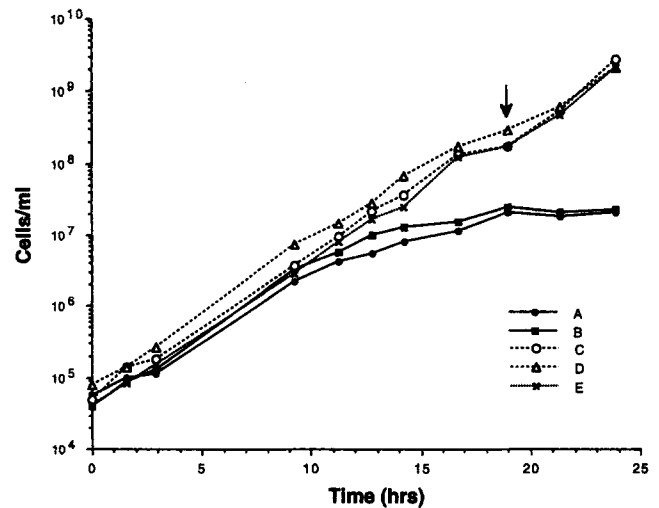| Tetrads	
With Only His⁻ Progeny	
Viable/Dead	Number of Tetrads
4:0	0
3:1	0
2:2	35
1:3	5
0:4	0
Total	40
With His⁺ Progeny	
His⁺/His⁻/Dead	Number of Tetrads
1:2:1	2
1:1:2	4
2:0:2	2
Total	8

Figure 4. Cell division rate of Tub4p-depleted and wild-type cells after the shift to glucose medium. *tub4Δ* cells containing a plasmid with *TUB4* under the control of the *GAL1* promoter (Y1235) and *TUB4* cells containing the vector alone (Y1237) were grown in galactose medium and then shifted to medium containing glucose. Cell number was followed as a function of time. Samples *A* and *B* are two independent *tub4Δ* segregants containing the *GAL1::TUB4* plasmid (Y1235 and Y1236). Samples *C* and *D* are two independent *TUB4* segregants containing the *GAL1::TUB4* plasmid (Y1238 and Y1240) and sample E is a *TUB4* strain containing the *GAL1* vector lacking *TUB4* sequences (Y1237). The arrow indicates the time at which cultures *C*, *D*, and *E* were diluted into fresh medium to maintain log phase growth. This experiment yielded similar results when repeated six times.

III). Both the proportion of large-budded cells that have undergone nuclear migration to the neck and the proportion of cells which have not are each significantly higher in Tub4p-depleted strains than in wild-type cells (see Table III). In fact, Tub4p-depleted strains have a very high fraction of large-budded cells that do not have their nucleus at the neck, whereas these cells are rare in wild-type populations (33–36% vs. 0.7–0.9%; a 36–52-fold enrichment; Table III). Finally, a significant portion of *tub4*-arrested cells do not appear to contain a nucleus. At the first arrest point examined (t = 19), 25.3 ± 0.2% of Tub4p-depleted cells lack a nucleus; this fraction increases to 33.4 ± 0.3% two hours later (t = 21; Table III). In contrast, 0.2% of cells in these strains are anucleate in the population prior to arrest.

To determine whether the cells arrest either prior to, during or after S phase, the DNA content was analyzed in Tub4p-depleted strains. *tub4Δ* cells containing the *GAL1:: TUB4* plasmid were incubated for different periods of time in the presence of glucose. The cells were fixed, stained with propidium iodide, which binds DNA, and analyzed by FACS® and compared with similarly treated wild-type cells. As shown in Fig. 6, log phase cultures of haploid wild-type and mutant strains contain both cells with 1N DNA content and cells with a 2N content. In contrast, most of Tub4p-depleted cells arrest with a 2N content of DNA. Incubation of cells for up to 2 h after arrest yields similar results (data not shown). Thus, *TUB4* function is not required for DNA replication, but is required for nuclear division.

Cells Depleted of Tub4p Have Defects in Their Microtubule Arrays

The microtubule arrays were examined in Tub4p-depleted cells. Wild-type yeast strains generally have short cytoplasmic microtubules throughout the cell cycle (Adams and Pringle, 1984; Kilmartin and Adams, 1984). They duplicate their SPB after the G1/S transition, and when cells have a small- to medium-sized bud, they set up a short spindle with prominent nuclear microtubule arrays. At mitosis, the nuclear spindle elongates to separate the daughter chromosomes.

To determine whether Tub4p-depleted cells exhibit defects in either cytoplasmic or nuclear microtubule arrays, the structure of the microtubule arrays and the localization of a SPB protein, Spc90 (Rout and Kilmartin, 1990), were examined in Tub4p-depleted cells. Both arrested and control cells were spheroplasted, fixed with methanol and acetone, and stained for double indirect immunofluorescence microscopy using anti-tubulin antibodies and an antibody that recognizes the 90-kD SPB protein. Methanol/acetone fixation allows detection of the 90-kD polypeptide. Under appropriate spheroplasting conditions, many large-budded cells are observed in the Tub4p-depleted cells, as described above. The types of budded cells observed for Tub4p-depleted and control strains are shown in Figs. 7 and 8; quantitation of the types of budded cells, as categorized according to their 90 kD and tubulin localization pattern, is presented in Table IV. The results are

Figure 3. Localization of the Tub4p-3XHA fusion protein. Yeast cells containing a *TUB4::3XHA* gene (Y1239) (*a–d*) or a wild-type gene (Y262) (*e* and *f*) were stained for double indirect immunofluorescence microscopy with an anti-tubulin antibody (*a* and *e*) and an anti-HA-epitope antibody (*b* and *f*). Panel *c* shows superimposition of the two signals from panels *a* and *b*. (*d*) Hoechst 33258 DNA stain of the same cells. Insets depict four-fold additional enlargements of a single cell: (*c, Inset*) anti-tubulin and anti-HA staining. (*d, Inset*) anti-HA staining and Hoechst DNA stain. Bar, 5 μm.
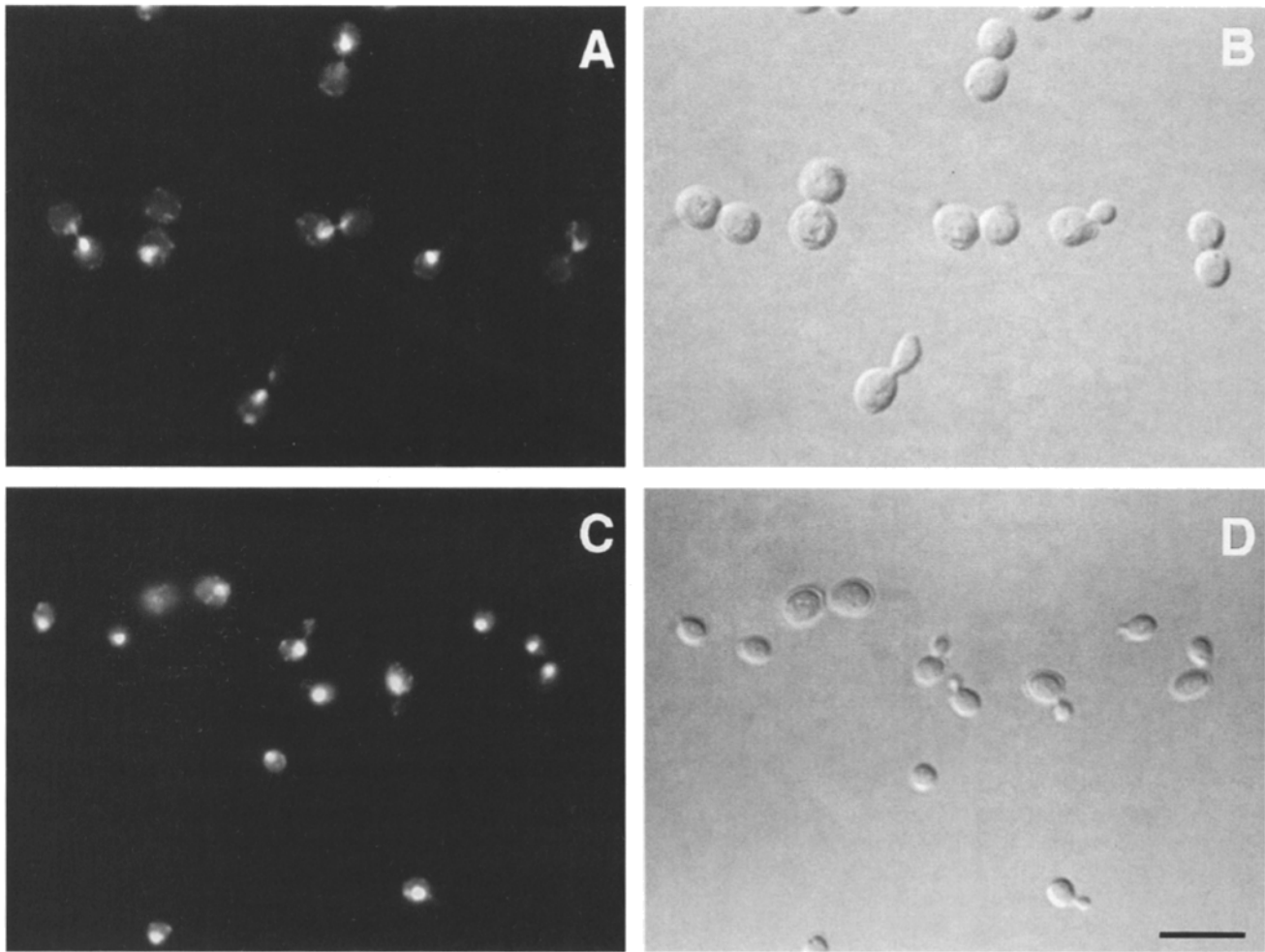
Figure 5. Cells depleted of Tub4p arrest with a large bud and single nucleus. (*A* and *B*) *tub4Δ* cells containing a plasmid with *TUB4* under the control of the *GAL1* promoter (Y1236) were grown for 19 h in the presence of glucose. (*C* and *D*) *TUB4* cells with the vector alone (Y1237) were treated similarly. Cells were stained with the DNA binding dye Hoechst 33258 and photographed using fluorescence microscopy (*A* and *C*) and differential interference contrast microscopy (*B* and *D*). Some cells have shifted their position slightly between photographic exposures. Note that *tub4Δ* cells arrest with a large bud and a single nucleus, which is often at the neck. Bar, 10 μm.

Table III. Percentage of Tub4p-depleted and Wild-type Cells at Different Stages of the Cell Cycle

	Unbudded	Small budded	Large budded			Other	Anucleate*	n
Wild-type (TUB4)								
pGAL::*TUB4*	36.8 ± 4.5	24.6 ± 2.4	0.9 ± 0.2	17.1 ± 1.6	20.5 ± 5.0	0.1 ± 0.1	0.1 ± 0.1	528/271
pGAL::vector	35.5	20.0	0.7	18.7	24.9	0.2	0.0	550
Tub4-depleted (tub4Δ) pGAL-*TUB4*								
9 h (log)	32.1 ± 2.2	20.6 ± 2.5	2.0 ± 0.2	24.4 ± 5.3	21.0 ± 0.8	0.1 ± 0.1	0.2 ± 0	662/532
19 h	11.5 ± 2.6	7.1 ± 0.8	32.5 ± 5.7	39.0 ± 1.4	6.1 ± 0.2	3.8 ± 0.7	25.3 ± 0.2	719/510
21 h	11.2 ± 1.4	6.9 ± 0.3	36.1 ± 3.0	36.1 ± 3.4	7.2 ± 1.4	2.6 ± 0.6	33.4 ± 0.3	592/525

Wild-type *TUB4* strains containing a *GAL1::TUB4* plasmid (Y1238 and Y1240) or GAL1 vector (Y1237) and *tub4Δ* strains containing a *GAL1::TUB4* plasmid (Y1235 and Y1236) were grown in galactose medium and then shifted to medium containing glucose (see legend to Fig. 4 and Materials and Methods). The cells were fixed and stained with Hoechst 33258; the percentage of cells at various stages of the cell cycle was determined. For both strains containing the *TUB4* plasmid, the results of two experiments were quantitated and the average of the results is indicated. For the *TUB4* strain with the *GAL1::TUB4* plasmid, cells were quantitated at two different exponential-growth time points (7.5 and 19 h). For the *tub4* strain with the *GAL1::TUB4* plasmid, two independent *tub4* transformants were analyzed, and the results averaged. For these *tub4 pGAL1::TUB4* cells, at 9 h the cells are in the exponential-growth phase. At 19 and 21 h, the cells have arrested (see Fig. 4).

*The anucleate cells were not categorized with respect to bud size and are not included in the other categories.
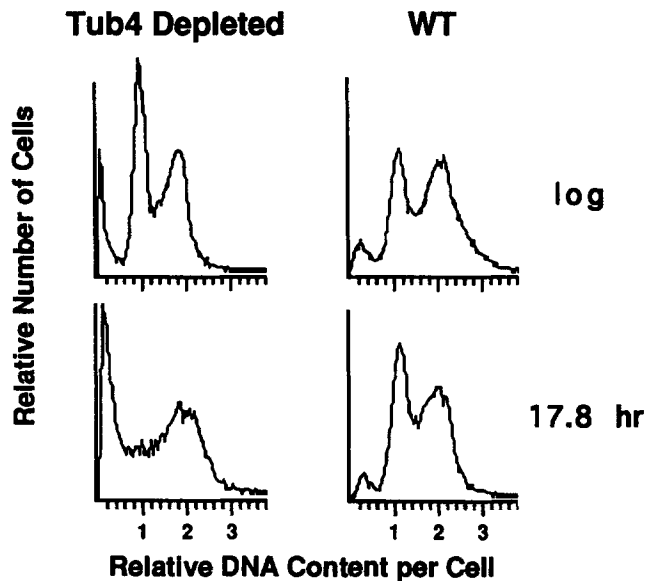
Tub4 Depleted   WT

Figure 6. Tub4p-depleted haploid cells arrest with a 2N content of DNA. (*Left*) *tub4Δ* cells containing *pGAL1::TUB4* (Y1236) were grown in galactose medium and shifted to medium containing glucose. (*Right*) Log-phase *TUB4* cells containing the vector alone (Y1237) were treated similarly. Cells were stained with propidium iodide and subjected to flow-cytometric analysis. The top panels depict the relative data from log phase cells (7 h after incubation in glucose, which is prior to the arrest caused by Tub4p depletion). The bottom panels show the results of cells incubated for 17.8 h in the presence of glucose. Results similar to the bottom panels were found for cells incubated 16.6 h in glucose, an earlier arrest timepoint (data not shown). The ordinates indicate the relative number of cells analyzed. The abscissas indicate the relative DNA content of the cells as determined by propidium iodide staining.

organized according to the two major types of Tub4p-depleted cells: budded cells in which the nucleus has migrated to the neck and those in which it has not.

Wild-type cells in which the nucleus has migrated to the neck, or is positioned between the mother and daughter cells, usually contain two SPBs with a short spindle between them (Fig. 7 *f* and Table IV *A*, col. 2). The remainder of these cells usually have one SPB and short cytoplasmic microtubule arrays (Fig. 7 *g* and Table IV *A*, col. 1). In contrast, in Tub4p-depleted cells that contain their nucleus at the neck, the majority of cells (61%) contain only a single 90-kD staining focus (Fig. 7, *a* and *b* and Table IV *A* col. 1 and 3). However, many cells contain two SPBs, indicating that SPB duplication can occur in these cells (Fig. 7, *c–e*, and Table IV *A*, col. 2 and 4). For cells that have duplicated and separated SPBs, staining of the spindle is often diminished relative to wild-type cells, and for some rare cells no spindle staining is observed (Fig. 7 *e*). These results indicate a defect in nuclear microtubule organization. Surprisingly, most (77%) Tub4p-depleted cells in which the nucleus has migrated to the neck have prominent and extremely long cytoplasmic microtubule arrays (Fig. 7, *a–d*; Table IV *A*, col. 3 and 4) relative to wild-type cells (10%) irrespective of whether they contain a single visible SPB or two SPBs. Some of these microtubules appear to extend around the entire circumference of the bud (Fig. 7 *a*).

The second type of budded cells, those that do not have their nucleus at the neck, subdivides into several major categories. Approximately 70% of the wild-type cells have one SPB with several short cytoplasmic microtubules (Fig. 8 *e*), or, much less frequently, two SPBs and a short spindle. Most of these wild-type cells have either a small- or medium-sized bud, as expected for cells that have not undergone nuclear migration (Adams and Pringle, 1984; Kilmartin and Adams, 1984). Some Tub4p-depleted cells also have a single detectable SPB and microtubule arrays similar to those in wild-type cells (Fig. 8 *a*; Table IV *B*, col. 1), with the important exception that the Tub4p-depleted cells usually have a large bud. Cells in the largest category have prominent spindle microtubules, but have few or no detectable cytoplasmic microtubules (Fig. 8 *d*; Table IV *B*, col. 2). This phenotype is consistent with defects in the nucleation of cytoplasmic microtubules. The second largest class is comprised of cells that have a single detectable SPB with enhanced cytoplasmic arrays (Fig. 8, *b–c*; Table IV *B*, col. 3).

One concern with these experiments is that cells prepared for immunofluorescence using methanol/acetone fixation protocols may not preserve microtubule arrays as well as those fixed by other means (Osborne et al., 1994). Therefore, cells fixed with formaldehyde, which preserves microtubule arrays well (Kilmartin and Adams, 1984; Pringle et al., 1991), were stained with an anti-tubulin antibody and with a control antibody that recognizes a nucleolar protein, Nsr1p (Copeland, C. , H. Friedman, J. Woolford, and M. Snyder, unpublished). The results observed were similar to those found for the methanol/acetone-fixed cells; the same classes of cells described above were observed (data not shown).

In summary, these results indicate that microtubule assembly is abnormal in Tub4p-depleted cells relative to wild-type cells. As described in detail in the discussion, these results are consistent with the view that both nuclear and cytoplasmic arrays are aberrant in Tub4p-depleted cells.

Discussion

Tub4p Is a Highly Divergent γ-Tubulin

This paper describes the characterization of the *S. cerevisiae TUB4* gene. The high proportion of identical residues (mean 35.6%) and the conservation of many γ-tubulin–specific residues indicate that Tub4p is a member of the γ-tubulin family rather than a member of another existing tubulin family or a novel tubulin family. Consistent with this classification, Tub4p localizes to the MTOC as do other γ-tubulins that have been localized (Oakley et al., 1990; Horio et al., 1991; Stearns et al., 1991; Zheng et al., 1991).

Previous attempts to identify a γ-tubulin gene in *S. cerevisiae* using PCR-based strategies were unsuccessful (Stearns et al., 1991). We expect that this is because the *S. cerevisiae* gene is divergent from those of other species. Though it is possible that another, more closely related γ-tubulin will be identified when the remaining 20% of the *S. cerevisiae* genome is sequenced, this would not exclude the classification of Tub4p as a highly divergent γ-tubulin.
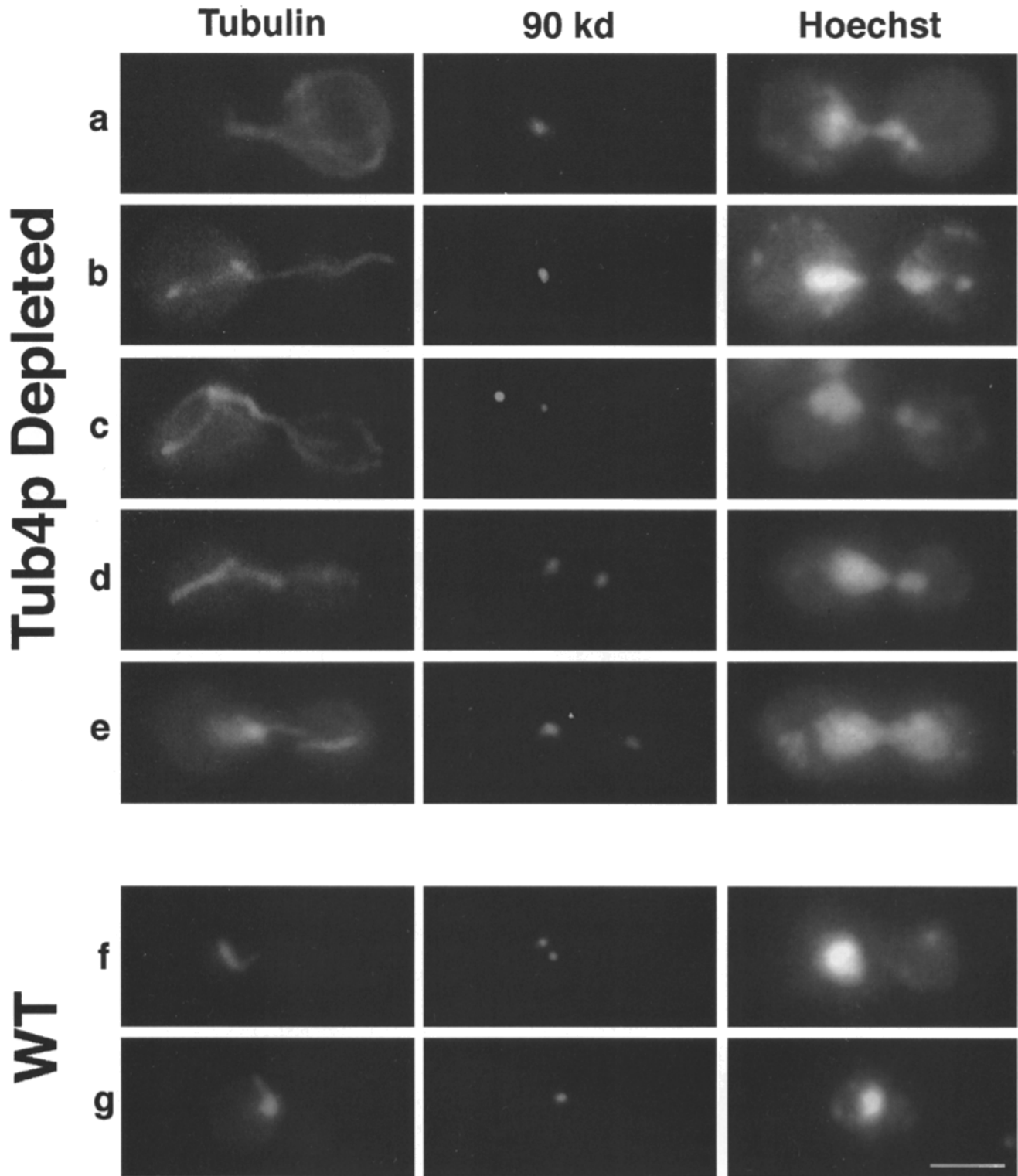
Figure 7. Spindle morphologies of budded Tub4p-depleted cells in which the nucleus has migrated to the bud neck. *tub4Δ* cells containing the p*GAL1::TUB4* plasmid (Y1235) (*a–e*) were grown in medium containing galactose and then incubated in medium containing glucose for 16.6 h. Log phase *TUB4* cells containing the vector alone (Y1237) (*f* and *g*) were treated similarly. Cells were stained for tubulin, 90 kD and Hoechst 33258, as indicated. Examples of the major phenotypes are shown and include: (*a* and *b*) Tub4p-depleted cells with one 90-kD focus and enhanced cytoplasmic microtubules; (*c* and *d*) Tub4p-depleted cells with two 90-kD foci, a spindle, and enhanced cytoplasmic microtubules; (*e*) Tub4p-depleted cell with two 90-kD foci but whose microtubules do not connect to form a spindle; (*f*) wild-type cell with two 90-kD foci and a spindle; (*g*) wild-type cell with one 90-kD focus and short cytoplasmic microtubules. In cells that have two 90-kD staining foci, but no spindle (as in *e* in this figure), the two SPBs are frequently near each other on the side of the nucleus distal to the bud. Bar, 2 μm.

Table IV. Summary of the Microtubule and Spindle Morphologies in Budded Tub4-depleted and Wild-type Cells

	A. Migrated nucleus					B. Unmigrated nucleus				
					n					n
Wild-type	31	59	6	4	118	69	29	0	2	48
Tub4-depleted	14	9	47	30	127	26	37	33	11	70

Budded cells in which the nucleus had migrated (A) and those in which the nucleus had not migrated (B) were scored according to the configurations of their spindles; the percentage of cells in each class are indicated. For cells whose nucleus had migrated, the nucleus is depicted as being between the mother and daughter cell; however, cells in which the nucleus was only positioned at the neck were frequently observed. For cells in which the nucleus had not migrated to the neck, small, medium- and large-budded cells were also scored; for wild-type cells the buds are mostly small or medium size, whereas for Tub4p-depleted cells, the buds are large. Note that cells in the third and fourth categories of both A and B have dramatically enhanced cytoplasmic microtubule arrays.

In both S. cerevisiae and S. pombe, γ-tubulin–related proteins are present at the SPB throughout the yeast cell cycle (Horio et al., 1991). However, in fission yeast the SPB is competent to nucleate microtubule assembly only in mitotic cells (Horio et al., 1991), whereas in S. cerevisiae it probably nucleates microtubule assembly throughout the cell cycle since microtubules always emanate from the SPB (Byers, 1981). Thus, it is likely that in S. pombe regulation of γ-tubulin by the mitotic cdc2 kinase is important for nucleation of assembly (Masuda et al., 1992). For S. cerevisiae, we expect that the level of Tub4p probably is regulated and increased after the G1/S transition when SPB duplication occurs. An MCB element is located upstream of TUB4; MCB elements are present in a number of genes whose RNA levels are elevated after progression through the G1/S transition (Lowndes et al., 1991; Lowndes and Johnston, 1992). In immunofluorescence experiments, Tub4p-3XHA is easily detected at the SPB in cells with short spindles (which should be in S phase or G2), but difficult to detect in unbudded G1 cells. Thus, whereas S. pombe regulates its activity, we speculate that S. cerevisiae regulates the level of protein. Whether Tub4p
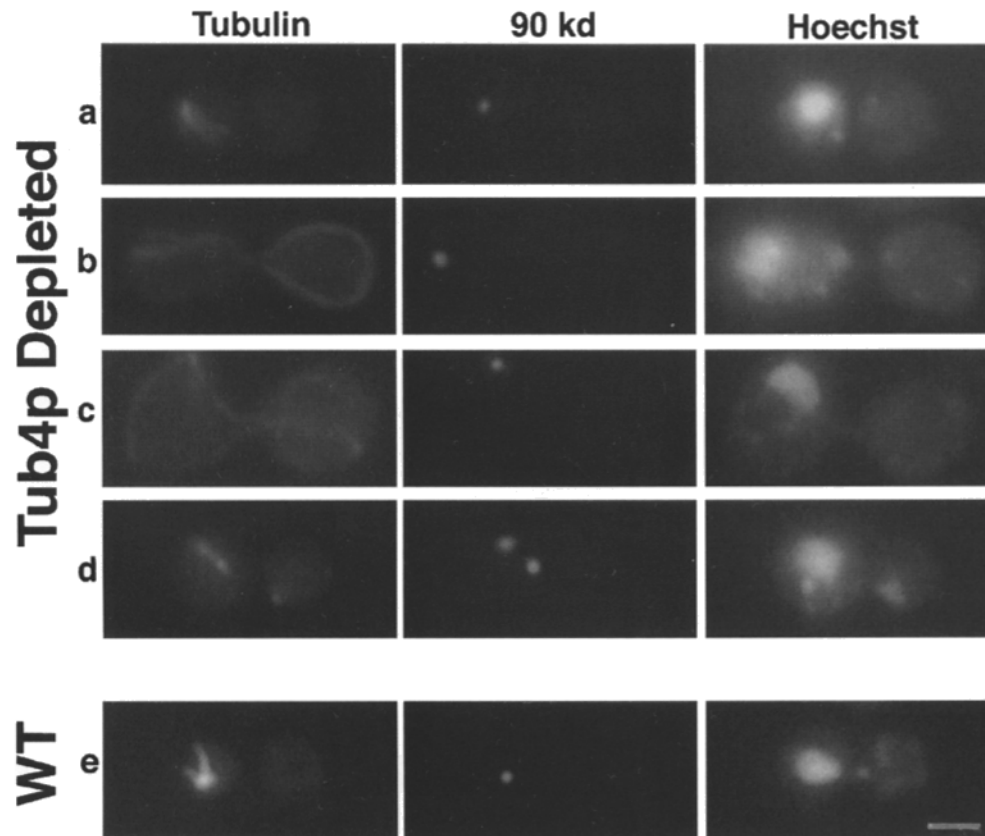

Figure 8. Spindle morphology of budded Tub4p-depleted cells in which the nucleus has not migrated to the bud neck. Staining and methods are as described for Fig. 7. (a) Tub4p-depleted cell with one 90-kD focus and short cytoplasmic microtubules; (b and c) Tub4p-depleted cell with one 90-kD focus and enhanced cytoplasmic microtubules; (d) Tub4p-depleted cell with two 90-kD foci and a spindle, but no cytoplasmic microtubules; (e) wild-type cell with one 90-kD focus and short cytoplasmic microtubules; cells with one 90-kD focus and a large bud are rare in wild-type populations. Bar, 2 μm.

activity is also regulated post-translationally during the cell cycle is not known.

The Role of Tub4p in Nuclear and Cytoplasmic Microtubule Organization

The identification of Tub4p in *S. cerevisiae* has allowed the analysis of large, genetically homogeneous populations of vegetative cells depleted of γ-tubulin; this has not been possible in previous studies of *S. pombe, D. melanogaster,* or *A. nidulans* (Oakley et al., 1990; Horio et al., 1991; Sunkel et al., 1995). In *S. pombe,* only rare cells that have lost the wild-type gene in a mutant background can be analyzed; these are infrequent cells in the population whose genotypes are inferred from their aberrant phenotype; how long they have been arrested and whether there are other mutant cells in the population cannot be deduced. Similarly, the *D. melanogaster* mutants still retain one functional γ-tubulin homolog, and mutants that die after many cell divisions (at late larval stages) are analyzed. Finally, for *A. nidulans* analysis is restricted to germinating populations of cells. The preparation of genetically homogeneous populations of Tub4p-depleted cells allows the study of all cells in a vegetatively growing population, and therefore reduces the chances of missing specific phenotypic classes.

Analysis of Tub4p-depleted cells indicates that Tub4p is important for the organization of both nuclear and cytoplasmic microtubule arrays. There are several lines of evidence which indicate that Tub4p is important for organization of the mitotic spindle. Depletion of Tub4p blocks nuclear division; approximately 72% of Tub4p-depleted cells arrest as large budded cells with one nucleus. Most Tub4p-depleted cells arrest with a 2N content of DNA, a single 90-kD focus of staining, and no apparent spindle (Figs. 7, *a* and *b*, 8, *a–c*; Table IV, *A* and *B*, columns 1 and 3); if such cells contain duplicated SPBs as expected, then a deficiency in nuclear microtubules would result in failure to set up a spindle apparatus. In addition, the nuclear microtubule staining sometimes appears diminished in intensity in Tub4p-depleted cells, and a small fraction of cells with duplicated spindle poles have no detectable nuclear microtubules and/or spindle (e.g. Fig. 7 *e*). It is likely that these poles separate via cytoplasmic microtubules as suggested for *ndc1* cells (Page and Snyder, 1993). Finally, loss of nuclear microtubule function could account for the aberrant chromosome segregation suggested by the tetrad analysis of *tub4Δ/TUB4* heterozygotes.

Tub4p is important for organization of cytoplasmic microtubules. About one third of the Tub4p-depleted cells are large budded but have not undergone nuclear migration; such cells are rare in wild-type (33–36% for Tub4p-depleted, as compared 0.7–0.9% for wild-type; Table III). Many (37%) of these cells in which the nucleus has not migrated to the neck have a short spindle with faint or undetectable cytoplasmic microtubules. A nuclear migration defect is expected for cells that lack cytoplasmic microtubules (Huffaker et al., 1988; Jacobs et al., 1988; Palmer et al., 1992; Sullivan and Huffaker, 1992). In addition to cells that lack cytoplasmic microtubules, most other Tub4p-depleted cells have enhanced cytoplasmic arrays (see next section), indicating that Tub4p is important in the proper organization of cytoplasmic arrays. We speculate that either the prominent cytoplasmic arrays sterically hinder nu-

clear migration, or the cytoplasmic microtubules have not been properly captured and, therefore, nuclear migration fails (Table IV*B*, last two columns).

There are many cells in which nuclear arrays are not detected, yet cytoplasmic arrays are prevalent (Table 4). Similarly, there are cells in which cytoplasmic arrays are not visible, even though nuclear microtubules are present. One possibility is that, upon depletion, Tub4p (and, therefore, microtubules) may be preferentially lost from one plaque or the other. This situation could arise from arrest at different stages of the cell cycle. Alternatively, preferential loss from one plaque or the other could result if Tub4p assembled cooperatively in a given plaque.

In summary, Tub4p is important for organization of both nuclear and cytoplasmic arrays in *S. cerevisiae*. A likely interpretation of these results is that Tub4p resides in both the inner and outer plaques of the SPB, where it nucleates the assembly of the nuclear and cytoplasmic microtubule arrays, respectively. Loss of Tub4p from the inner plaque would result in nuclear microtubule defects, whereas loss of Tub4p from the outer plaque would result in cytoplasmic microtubule defects.

In general, the phenotypes observed in Tub4p-depleted cells have both similarities and differences with those reported for other organisms (Oakley et al., 1990; Horio et al., 1991; Sunkel et al., 1995). In *S. pombe,* defects in nuclear division were observed in a small fraction of the cells (see above), but defects in cytoplasmic arrays were not reported (Horio et al., 1991). For *D. melanogaster,* a probable null mutation in one of the two γ-tubulin genes results in a slight decrease in mitotic cells, and a variety of mitotic defects including increased polyploidy, and abnormal MTOC size and morphology. Our results are most similar to those reported for *A. nidulans* (Oakley et al., 1990). Oakley et al. (1990) found that strains deleted for *mipA,* which encodes γ-tubulin, germinate and have few or no detectable microtubules. These cells exhibit defects in nuclear division and nuclear migration indicating that nuclear and cytoplasmic functions are affected. However, one important difference between the results reported for *S. cerevisiae* and *A. nidulans* is that Tub4p-depleted cells often have enhanced cytoplasmic arrays.

Enhanced Cytoplasmic Arrays in tub4Δ Mutants

The presence of enhanced cytoplasmic arrays in the majority of Tub4p-depleted cells is unexpected for a protein thought to be involved in microtubule assembly. There are several, not mutually exclusive, explanations for this observation. Tub4p-depleted cells, through lack of γ-tubulin, may be unable to nucleate microtubules from one surface or region of the SPB (e.g. the inner or outer plaque); free excess α-tubulin-β-tubulin heterodimers would then be available to polymerize from the remaining microtubules, and consequently the cells form longer microtubule arrays than wild-type cells. The other hypothesis is that Tub4p-depleted cells arrest at a interval in the cell cycle when microtubule polymerization is particularly enhanced. The arrested cells polymerize longer-than-normal microtubules because of failure to traverse this interval. Consistent with the second hypothesis, mutations in other yeast genes that affect SPB function (*CMD1;* calmodulin) or spindle func-

tion (*CDC20*, and *CIK1*) also arrest during nuclear division with enhanced microtubule arrays (Sethi et al., 1991; Page and Snyder, 1992; Sun et al., 1992).

Conclusion

The data presented above provide the first molecular description of a highly divergent γ-tubulin gene of *S. cerevisiae*, the localization of its encoded protein, and the analysis of its mutant phenotypes. Further characterization of Tub4p and the proteins with which it interacts physically and genetically should provide insights into MTOC composition, and the mechanisms of assembly and regulation of a microtubule nucleation center.

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