Human γ-Tubulin Functions in Fission Yeast

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Abstract. γ-Tubulin is a phylogenetically conserved component of microtubule-organizing centers that is essential for viability and microtubule function. To examine the functional conservation of γ-tubulin, we have tested the ability of human γ-tubulin to function in the fission yeast *Schizosaccharomyces pombe*. We have found that expression of a human γ-tubulin cDNA restores viability and a near-normal growth rate to cells of *S. pombe* lacking endogenous γ-tubulin. Immunofluorescence microscopy showed that these cells contained normal mitotic spindles and interphase microtubule arrays, and that human γ-tubulin, like *S. pombe* γ-tubulin, localized to spindle pole bodies, the fungal microtubule-organizing centers. These results demonstrate that human γ-tubulin functions in fission yeast, and they suggest that in spite of the great mor-

phological differences between the microtubule-organizing centers of humans and fission yeasts, γ-tubulin is likely to perform the same tasks in both. They suggest, moreover, that the proteins that interact with γ-tubulin, including, most obviously, microtubule-organizing center proteins, must also be conserved. We have also found that a fivefold overexpression of *S. pombe* γ-tubulin causes no reduction in growth rates or alteration of microtubule organization. We hypothesize that the excess γ-tubulin is maintained in the cytoplasm in a form incapable of nucleating microtubule assembly. Finally, we have found that expression of human γ-tubulin or overexpression of *S. pombe* γ-tubulin causes no significant alteration of resistance to the antimicrotubule agents benomyl, thiabendazole, and nocodazole.

ICROTUBULE organization is controlled in eukaryotic cells by organelles called microtubule-organizing centers (MTOCs)[1] (Pickett-Heaps, 1969). MTOCs exhibit remarkable structural variation among phyla and yet they have similar functions (Pickett-Heaps, 1969; Brinkley, 1985). They nucleate microtubule assembly such that microtubules assemble onto MTOCs, rather than assembling free in the cytoplasm, and thus they help to control the spatial arrangement of microtubules. The number of microtubules associated with MTOCs changes dramatically in a cell cycle–specific fashion in most organisms. MTOCs, thus, appear to be central to the temporal regulation of microtubule assembly. Centrosomes, mammalian MTOCs, establish the number of protofilaments in microtubules (Evans et al., 1985), and they establish the polarity of microtubules with the minus end of microtubules proximal to the MTOC (Euteneuer and McIntosh, 1981; McIntosh and Euteneuer, 1984). While it has not been established that MTOCs of lower organisms carry out the same functions, it is usually assumed that they do.

An important question with respect to microtubule-organizing centers is how, given their structural diversity, they carry out the same or similar functions. The likely answer is that they all contain common elements, presumably proteins, that allow them to control microtubule organization temporally and spatially. One promising candidate for a common protein of MTOCs that may be involved in controlling microtubule organization is γ-tubulin. γ-Tubulin is a recently identified third member of the tubulin superfamily (Oakley and Oakley, 1989), and it is a phylogenetically conserved component of microtubule-organizing centers (Oakley et al., 1990; Zheng et al., 1991; Stearns et al., 1991; Horio et al., 1991; Joshi et al., 1992). It is essential for viability of *Aspergillus nidulans* (Oakley et al., 1990) and *Schizosaccharomyces pombe* (Stearns et al., 1991; Horio et al., 1991), for nuclear division in *A. nidulans* and *S. pombe* (Oakley et al., 1990; Stearns et al., 1991; Horio et al., 1991), and assembly of microtubules from MTOCs in vivo and in vitro (Oakley et al., 1990; Horio et al., 1991; Joshi et al., 1992; Félix et al., 1994; Stearns and Kirschner, 1994). It is postulated to have a central role in nucleating microtubule assembly (Oakley et al., 1990; Oakley, 1992; Joshi et al., 1992).

We reasoned that if γ-tubulin is a common, central component of the microtubule organizing machinery of cells, it might perform the same tasks in all MTOCs and, thus, it might be functionally conserved. To explore this possibility, we have tested the ability of human γ-tubulin to function in

1. *Abbreviations used in this paper*: ADH, alcohol dehydrogenase; DAPI, 4',6-diamidino-2-phenylindole; MTOC, microtubule-organizing center.

the fission yeast *S. pombe*. Human and *S. pombe* γ-tubulins are relatively divergent among γ-tubulins, sharing 71.6% amino acid identity (Zheng et al., 1991; Stearns et al., 1991; Horio et al., 1991). We have also examined the effects of overexpression of *S. pombe* γ-tubulin on growth and microtubule organization, as well as the effects of overexpression of human γ-tubulin and overexpression of *S. pombe* γ-tubulin on resistance to antimicrotubule agents.

Materials and Methods

Strains and Media

An *S. pombe* diploid carrying a wild-type γ-tubulin allele (*gtb1*) and a γ-tubulin gene disrupted by the *S. pombe* Ura4 gene was described previously (Horio et al., 1991), and we have now named this strain NC377. NC377 is homozygous for *leu1-32*. A haploid strain HM123 (*h⁻*, *leu1-32*) was used as a wild type. *ade6* mutants HM143 (*h^90*, ade6-210) and HM144 (*h^90*, ade6-216) were used as control strains for generation time measurements. YPD (1% yeast extract, 2% peptone, 2% dextrose, and 1.7% agar for solid medium) was used as a complete medium. 50–70 μg/ml adenine was added for *ade6* mutant strains. SD agar (0.2% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 2% dextrose, and 2% agar) and EMM (BIO 101, Inc., La Jolla, CA) were used as minimal media with appropriate supplements. For sporulation, sporulation agar medium (described by Gutz et al., 1974) was used. For testing sensitivity to antimicrotubule agents, stock solutions of benomyl (provided by Agricultural Biochemicals Division, Du Pont, Wilmington, DE), thiabendazole (Sigma Chemical Co., St. Louis, MO), and nocodazole (Sigma Chemical Co.) were prepared in DMSO at concentrations of 20, 20, and 5 mg/ml, respectively. Benomyl and thiabendazole were added to complete medium before autoclaving. Nocodazole was added to the medium after autoclaving, immediately before use. Complete medium containing the same amount of DMSO as test cultures was used for control cultures.

Construction of an *S. pombe* Strain Expressing Human γ-Tubulin

An EcoRI fragment containing a full-length human γ-tubulin cDNA (Zheng et al., 1991) was cloned into the polylinker of pBluescriptSK+ (Stratagene, La Jolla, CA) to create plasmid pH3. An EcoRV/SacI fragment from pH3 containing the cDNA was ligated into plasmid pAS248 (Toda et al., 1991), which had been digested with SmaI and SacI. The SmaI and SacI sites are in a small polycloning site immediately downstream of the *S. pombe* alcohol dehydrogenase (ADH) promoter (Russell and Hall, 1983). We named the resulting plasmid pTH5.

NC377 was transformed with pTH5 (Fig. 1). Selection was for complementation of the *leu1-32* mutation of NC377 by the *Saccharomyces cerevisiae* LEU2 gene carried on pTH5. A diploid transformant was sporulated and a Leu⁺, Ura⁺ haploid was selected. The selected haploid strain (AH004) carries a disrupted genomic γ-tubulin gene and pTH5, which expresses human γ-tubulin.

Construction of a Strain Overexpressing *S. pombe* γ-Tubulin

Plasmid pCT134, which is a multicopy vector that contains a full-length *S. pombe* γ-tubulin gene (Horio et al., 1991), was transformed into NC377. A transformant was sporulated, and a Ura⁺, Leu⁺ haploid strain was selected and designated AH001. It carries a disrupted genomic γ-tubulin gene and a functional *S. pombe* γ-tubulin gene carried in multiple copies on pCT134.

Western Blotting and Quantitation of γ-Tubulin on Western Blots

We used γ-tubulin antibodies that have been described previously (Oakley et al., 1990; Zheng et al., 1991). Each *S. pombe* strain was cultured in defined minimal medium, EMM, with appropriate supplements at 32°C. Whole-cell extracts were prepared by crushing the cells with glass beads. Extracts were separated by SDS-PAGE and electroblotted to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA). Antigens were detected by the procedure of Sandhu et al. (1991) except that 5% nonfat dry

milk was used in the blocking buffer rather than 1%. Secondary antibodies were alkaline-phosphatase-labeled goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and they were used at the manufacturer's recommended dilution. To eliminate nonspecific binding, secondary antibodies were preadsorbed with an *A. nidulans* acetone powder or a mixture of *A. nidulans* and *S. pombe* acetone powders prepared according to Harlow and Lane (1988). Signals were detected by chemiluminescence using Lumi-phos 530 (Boehringer Mannheim GmbH, Mannheim, Germany). The signal was recorded on X-OMAT film (Eastman Kodak Co., Rochester, NY).

To determine the relative amounts of γ-tubulin expressed in AH001 and wild-type strains, a series of dilutions of extracts of each strain was made, and Western blots were carried out by the procedure described above. The intensity of the γ-tubulin band in each extract was measured using a gel analysis system (MicroScan 1000; Technology Resources, Inc., Nashville, TN). We found that the signal in the wild-type strain was consistently equal to that of a fivefold more dilute extract of AH001.

RNase Protection Assays

Total RNA was prepared from each of the strains cultured in EMM, with appropriate supplements, at 32°C using a method described by Aves et al. (1985). To detect the *S. pombe* γ-tubulin transcript, a 3.5-kb PvuII-HindIII fragment that contains all but the 27 COOH-terminal basepairs of the *S. pombe* γ-tubulin coding sequence was inserted into pBluescriptSK+ (Stratagene) that had been digested with EcoRV and HindIII. The resulting plasmid was linearized by digesting with XbaI and a 1141-bp antisense RNA probe containing exons 5 and 6 and a portion of exon 4 was synthesized as described by Sambrook et al. (1989) using T3 RNA polymerase. To detect the human γ-tubulin transcript, a plasmid (pH3) containing a full-length human γ-tubulin cDNA (Zheng et al., 1991) was linearized by digesting the ClaI, and a 320-bp antisense RNA probe was synthesized using T3 RNA polymerase. RNase protection assays were carried out following the method described by Melton et al. (1984). Each probe and an RNA preparation of each strain were mixed in 100 μl of hybridization buffer (40 mM Pipes, pH 6.8, 400 mM NaCl, 1 mM EDTA, and 80% formamide). The hybridization mixtures were boiled and then cooled gradually to 44°C and 49°C overnight to detect the *S. pombe* γ-tubulin transcript and the human γ-tubulin transcript, respectively. Mixtures were digested by adding 300 μl of RNase mixture (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 μg RNaseA, and 120 U RNase T1) and incubating for 30 min at 32°C. Digestion was stopped by adding 20 μl of 10% SDS and 0.1 mg of proteinase K and incubating at 37°C for 15 min. The mixtures were extracted once with phenol/chloroform and precipitated with ethanol. Pellets were resuspended in gel loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), boiled for 3 min, and then loaded onto a denaturing acrylamide gel. To detect the *S. pombe* γ-tubulin transcript, samples equivalent to 200 μg total RNA were loaded, and to detect the human γ-tubulin transcript, samples equivalent to 8 μg total RNA were loaded. The gels were dried and the signal was recorded on Kodak X-OMAT film. In some instances, the signal intensity was quantified with a blot analyzer (Betascope 603; Betagen, Waltham, MA).

Immunofluorescence Microscopy

For microtubule staining in unextracted cells, we used the following method based on the procedure described by Hagan and Hyams (1988). *S. pombe* strains were cultured in EMM medium with appropriate supplements at 32°C. To fix the cells, 30% paraformaldehyde in PEM buffer (100 mM Pipes, pH 6.9, 2 mM EGTA, and 1 mM MgCl₂) was added to the cultures to give a final concentration of 3%. 2 min after the addition of formaldehyde, glutaraldehyde was added to give a final concentration of 0.2%. The cultures were fixed for 1 h at 32°C with gentle shaking. The following procedures were then carried out at room temperature unless otherwise specified. Fixed cells were harvested and washed twice, briefly, with PEM. Excess aldehyde was quenched by two incubations, for 10 min each, with 100 mM glycine in PEM. Next, the cells were washed once with PEM and washed for 10 min with PEM containing 1 M sorbitol (PEMS). The cell wall was digested with 0.6 mg/ml lyticase (Sigma Chemical Co.) and 0.2 mg/ml Novozym 234 (Novo Biolabs, Bagsvaerd, Denmark) in PEMS at 37°C for 1 h. After the cell wall digestion, cells were given two brief washes in PEMS followed by a 10-min wash in the same solution. The cells were permeabilized by incubating with 1% Triton X-100 in PEM for 5 min, washing three times with PEM, then incubating in methanol on ice for 8 min. They were then washed twice with PEM, then incubated in PEMBL (PEM containing 1 mg/ml bovine serum albumin [Sigma Chemical Co.] and 100

mM lysine) for ≥30 min. Primary and secondary antibodies were diluted in PEMBL, and incubations for both primary and secondary antibodies were overnight. Each incubation was followed by three washes in PEMBL (a brief wash, a 1-h wash, and a 4-h wash). Microtubules were detected with the anti-α-tubulin antibody TAT1 (Woods et al., 1989). The secondary antibody was Cy3-labeled goat anti-mouse IgG (H+L) (Jackson Immuno-Research Laboratories, Inc.) used at the manufacturer's recommended dilution. To eliminate nonspecific binding, secondary antibodies were preadsorbed with a mixture of *A. nidulans* and *S. pombe* acetone powders (Oakley et al., 1990) prepared according to the method of Harlow and Lane (1988). After antibody treatments, DNA was stained with 0.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS for 5 min, followed by a PBS rinse.

For anti-γ-tubulin staining, permeabilized cells were prepared using a modification of the procedure of Masuda et al. (1990). AH004 cells were cultured at 32°C in EMM containing 50 μg/ml of adenine until a cell density of 2–5 × 10⁶ was reached. The cells were harvested and washed in 100 mM Tris-H₂SO₄ (pH 9.4) once, followed by an incubation in the same buffer containing 10 mM DTT at 32°C for 5 min. The cells were washed twice in EMM containing adenine and 1 M sorbitol, and were then incubated at 32°C in the same medium containing 0.6 mg/ml lyticase for 1–2 h until >80% of the cells were spheroplasted. The cells were washed twice in PBL buffer (100 mM MES, pH 6.5, 5 mM EDTA, 1 mM trolox [Aldrich Chemical Co., Milwaukee, WI], 10% DMSO, and 0.5 mM PMSF) containing 1 M sorbitol. The following procedures were then carried out at room temperature unless otherwise specified. The cells were permeabilized and extracted using two different sets of conditions. Moderate extraction conditions were used to preserve microtubules. The cells were incubated in PBL containing 1 M sorbitol and 1% Triton X-100 on ice for 8 min. They were then washed 2 × 5 min in PBL. For more intensive extraction of the cytoplasm, the cells were incubated in PBL containing 1% Triton X-100 and 150 mM NaCl on ice for 8 min. The cells were then washed 2 × 5 min in the same buffer without Triton X-100.

The cells prepared by each method described above were fixed in PBL buffer containing 2.6% formaldehyde and 0.05% glutaraldehyde for 15 min. They were washed in PBL twice, and then excess aldehyde was quenched by incubating in PBL containing 100 mM glycine for 10 min twice. The cells were stored at 4°C in PBS containing 2 mg/ml BSA. Before the immunofluorescent staining, the cells were incubated in methanol at −20°C for 8 min, then incubated in acetone for 30 s. Dehydrated cells were suspended in PEMBL and incubated for 30 min, antibodies affinity purified against human γ-tubulin were added at a dilution of 1/50 in PEMBL, and they were incubated for 1–2 h. Primary antibodies were removed with a brief wash in PEMBL, followed by 10- and 30-min washes. For double staining, the cells were then incubated with the anti-α-tubulin antibody TAT1 (Woods et al., 1989) for 1–2 h and washed as above. Cy3-labeled goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) and, for double staining, FITC-labeled goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) were used as secondary antibodies. Secondary antibodies were preabsorbed with acetone powder in PBS as described above and were diluted in PEMBL. Cells were incubated with secondary antibodies for 1–2 h and washed as described above. They were then washed in PBS once and stained with DAPI as described above.

Images were recorded on film (T-Max 400; Eastman Kodak Co.) developed to give an ASA of 400.

Results

An S. pombe Strain Expressing only Human γ-Tubulin Is Viable

To determine if human γ-tubulin functions in *S. pombe*, we constructed a plasmid (pTH5) carrying the selectable *S. cerevisiae LEU2* marker and a human γ-tubulin cDNA (Zheng et al., 1991) inserted downstream of the *S. pombe* ADH promoter (Russell and Hall, 1983). The ADH promoter is constitutive and drives expression at moderate levels. We transformed pTH5 into the heterozygous *S. pombe* diploid NC377 (Fig. 1). NC377 is homozygous for the *leu1-32* mutation, and it is heterozygous for the γ-tubulin (*gtb1*) gene. One copy of *gtb1* is wild-type and the other is disrupted by the *ura4⁺* gene. The *ura4⁺* gene interrupting *gtb1* is the
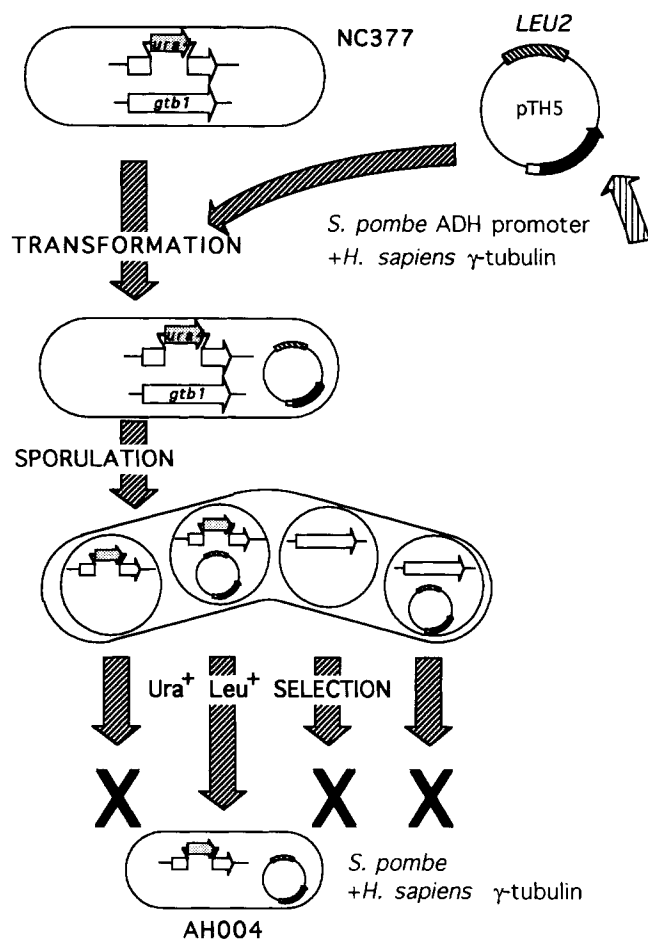

Figure 1. Construction of an *S. pombe* strain expressing human γ-tubulin.

only functional *ura4* gene in NC377. Horio et al. (1991) have previously shown that haploid progeny of NC377 carrying the disruption of *gtb1* are inviable. In addition, plasmid loss experiments indicated that loss of γ-tubulin inhibited microtubule function (Horio et al., 1991). A resulting transformant was sporulated, and a Ura⁺, Leu⁺ strain was selected and designated AH004. In principle, all Ura⁺, Leu⁺ progeny of NC377 should carry the disrupted *S. pombe* γ-tubulin gene and plasmid pTH5 (Fig. 1) and, thus, the only functional γ-tubulin in AH004 should be that encoded by the human γ-tubulin cDNA on pTH5.

We verified in two ways that AH004 was expressing human γ-tubulin, but not *S. pombe* γ-tubulin. First, γ-tubulin was detected by immunoblotting using two different preparations of antibodies. Human γ-tubulin is slightly larger than *S. pombe* γ-tubulin (Zheng et al., 1991; Stearns et al., 1991; Horio et al., 1991). The mass of human γ-tubulin as predicted from the amino acid sequence is 51,203 D, and that of *S. pombe* γ-tubulin is 49,912 D. In our gel system, human γ-tubulin runs with an apparent molecular mass ∼2,000 D greater than that of *S. pombe* γ-tubulin (results not shown). Antibodies raised and affinity purified against *A. nidulans* γ-tubulin (Oakley et al., 1990) detected single protein bands in whole cell extracts of (*a*) a wild-type strain; (*b*) a strain carrying the *S. pombe* γ-tubulin gene on a multicopy plasmid (strain AH001); and (*c*) strain AH004 (Fig. 2). The band de-
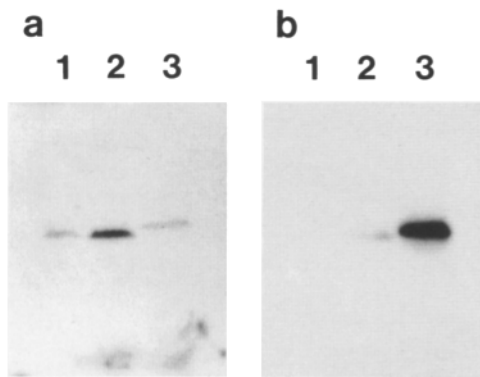
Figure 2. Immunoblot analysis of the expression of γ-tubulin in S. pombe strains. γ-Tubulin was detected in S. pombe whole cell extracts using (a) anti–γ-tubulin antibodies raised and affinity purified against Aspergillus nidulans γ-tubulin (Oakley et al., 1990) and (b) antibodies raised against A. nidulans γ-tubulin and affinity purified against human γ-tubulin (Zheng et al., 1991). Whole-cell extracts (equivalent to 5×10^6 cells) of an S. pombe wild type (HM123, lane 1), the strain overexpressing S. pombe γ-tubulin (AH001, lane 2), and the strain expressing human γ-tubulin (AH004, lane 3) were loaded.

Figure 3. Transcripts of each γ-tubulin detected by RNase protection assays. Transcripts of (a) S. pombe γ-tubulin and (b) human γ-tubulin were detected in total RNA of the wild type (lane 1), AH001 (lane 2), and AH004 (lane 3). The fifth exon, a portion of the fourth exon, and the sixth exon of the S. pombe γ-tubulin are indicated by arrows and numbers in a. Quantitation of these bands revealed that AH001 is expressing about sixfold more γ-tubulin messenger RNA than the wild-type strain.

tected in the AH004 extract was the size of human γ-tubulin and was of slightly higher apparent molecular mass than the S. pombe γ-tubulin detected in the other two strains. No band the size of S. pombe γ-tubulin was detected in AH004. Antibodies affinity purified against human γ-tubulin (Zheng et al., 1991) recognized the same size band as the other antibodies in AH004 extracts but, as expected, gave a more intense signal (Fig. 2 b). S. pombe γ-tubulin was recognized very weakly by these antibodies. These results suggest strongly that only the human γ-tubulin is expressed in AH004 cells.

Second, we verified that only human γ-tubulin mRNA was transcribed in AH004 using RNase protection assays. As shown in Fig. 3 a, the S. pombe genomic γ-tubulin sequence protected fragments of the expected size in the wild-type strain and in AH001, but did not protect any fragment in AH004. The S. pombe γ-tubulin mRNA is, thus, not detectable in AH004. The human γ-tubulin probe protected a band of the expected size in AH004, but did not protect any fragment in AH001 or the wild-type strain. Human γ-tubulin mRNA is, thus, present in AH004, and S. pombe γ-tubulin mRNA is not detectable. This result, coupled with the results of immunoblotting, shows that the γ-tubulin encoded by the human γ-tubulin cDNA is the only γ-tubulin expressed in AH004 cells.

To verify that the human γ-tubulin cDNA on pTH5 was required for viability, we grew AH004 on medium containing leucine for several generations. In the absence of a selection for the LEU2 gene, pTH5 should be lost rapidly if the human γ-tubulin cDNA is not essential for viability. A colony of AH004 was inoculated into complete medium and grown overnight (six to seven generations). The liquid culture was diluted and inoculated onto solid complete medium. 133 colonies were selected at random and tested for their ability to grow on medium lacking leucine. All 133 colonies grew on medium without leucine. This result indicates that pTH5 was retained in all cases in the absence of a selection for the LEU2 gene and, thus, that the human γ-tubulin encoded by
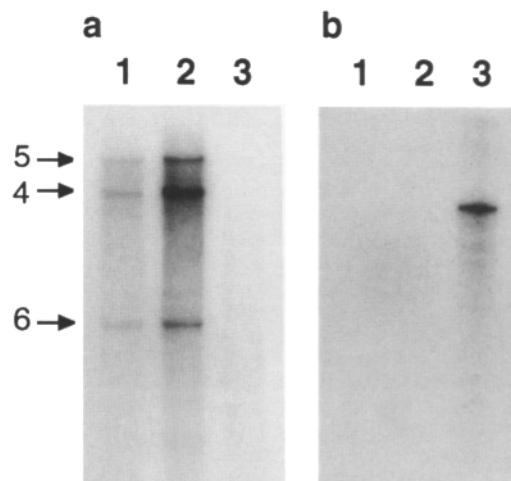
pTH5 is essential for viability in the absence of S. pombe γ-tubulin. As a control, we examined the loss of pTH5 in a strain (AH033) that carried a wild-type S. pombe γ-tubulin gene. Strain AH033 (h^-, leu1-32, ura4-D18) carrying pTH5 was inoculated into medium containing leucine and grown overnight (six to seven generations), diluted and inoculated onto solid medium containing leucine. Of 81 colonies tested, 41 required leucine and had thus lost pTH5. This result indicates that, as expected, pTH5, and the human γ-tubulin gene carried thereon, is not essential for viability in a strain carrying a wild-type S. pombe γ-tubulin gene.

AH004 did not show any obvious defect in growth at any temperature (20–36°C) tested (Fig. 4). To determine if there were small differences in growth rates, the growth rates of AH004 and a strain (HM144) that is wild-type for γ-tubulin and, like AH004, carries the ade6-216 mutation, were measured in liquid culture at 32°C. (Ade6 mutations slightly inhibit growth even in complete medium supplemented with adenine.) The generation time for AH004 was 142 ± 7.4 min, while that of HM144 was 127 ± 6.1 min (mean and standard deviations for three experiments). Human γ-tubulin thus supports a near-normal rate of cell replication in S. pombe.

Human γ-Tubulin Supports Normal Arrays of Microtubules in S. pombe

Since microtubules are absolutely required for cell division, we expected that the microtubules in AH004 would be normal. S. pombe has two types of MTOCs, the spindle pole bodies that nucleate the assembly of mitotic spindles and cytoplasmic MTOCs that nucleate the assembly of cytoplasmic microtubules after mitosis (Hagan and Hyams, 1988). Both types of MTOCs contain γ-tubulin (Horio et al., 1991). Immunofluorescence microscopy of AH004 using the monoclonal anti–α-tubulin antibody TAT1 (Woods et al., 1989)
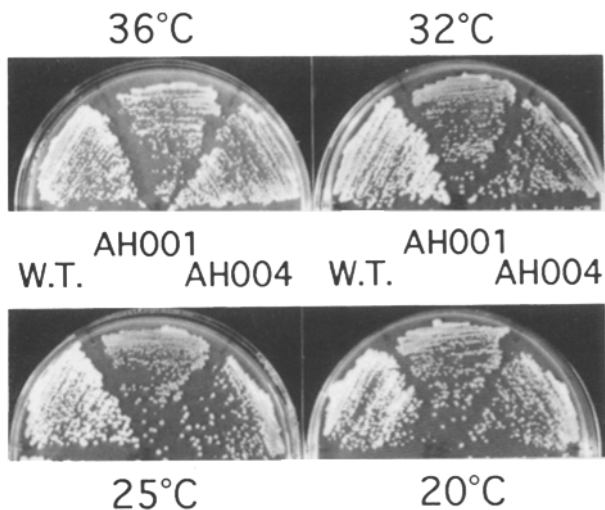
Figure 4. Growth of strains expressing human or *S. pombe* γ-tubu-lins. *(Left to right)* (HM123), an *S. pombe* wild-type strain (*W.T.*), AH001 (which carries the *S. pombe* γ-tubulin gene on a multicopy plasmid), and AH004 (which carries a plasmid expressing human γ-tubulin). Cells were streaked onto complete medium (YPD) and incubated at the temperatures shown. The plates were incubated for 3 d (36°C, 32°C), 5 d (25°C), or 8 d (20°C). Growth was similar for the three strains. AH001 and AH004 appear darker because these strains carry color markers (*ade6* mutations).

revealed spindles and cytoplasmic microtubules indistinguishable from those in the wild type (Fig. 5). The configuration of cytoplasmic microtubules (Fig. 5, *wide arrows*) indicated that functional cytoplasmic MTOCs were present.

A Fivefold Overexpression of S. pombe γ-Tubulin Is Not Harmful

S. pombe γ-tubulin is expressed at higher than normal levels in strain AH001 (Fig. 2). Quantitation of Western blots of serial dilutions of whole-cell extracts of AH001 and a wild-type strain revealed that the level of γ-tubulin is five times higher in AH001 than in the wild-type strain. AH001 was not obviously inhibited for growth, however, at any temperature tested (Fig. 4). In liquid culture at 32°C, the doubling time for AH001 was 141 ± 6.2 min (mean and standard deviations for three experiments). The doubling time for a strain (HM143) that carries a single *S. pombe* γ-tubulin gene and (like AH001) the *ade6-210* mutation was 144 ± 6.7 min. Immunofluorescence microscopy (Fig. 5) revealed that microtubules in AH001 are normal.

Human γ-Tubulin Localizes to the Spindle Pole Body

Since the most obvious intracellular location of γ-tubulin in *S. pombe* is the spindle pole body, and since localization of γ-tubulin to the spindle pole body is assumed to be necessary for its function, we examined strain AH004 to determine if human γ-tubulin was located at the spindle pole body. Initial immunofluorescence localization experiments with AH004 and with AH001, which carries the *S. pombe* γ-tubulin gene on a multicopy vector, revealed a great deal of cytoplasmic fluorescence (results not shown). Brightly staining spots were often seen at locations expected of spindle pole bodies, but the brightness of the cytoplasmic staining precluded

definitive identification. Since γ-tubulin is overexpressed in these strains, we interpreted these results as indicating that the extra γ-tubulin present in these strains remains predominantly in the cytoplasm. The nucleoplasm showed notably less γ-tubulin staining than the cytoplasm (results not shown).

To determine if human γ-tubulin was located at the spindle pole bodies, we used procedures that allowed the majority of the cytoplasmic γ-tubulin to be extracted. We spheroplasted AH004 cells and treated them with a buffer containing 1% Triton X-100 before fixing them. We then visualized γ-tubulin with an antibody affinity purified against human γ-tubulin. Under these conditions, γ-tubulin localized to a spot associated with each nucleus (Fig. 6, *b* and *c*), suggesting that, as expected, human γ-tubulin was located at the spindle pole body. This result was verified by using permeabilization buffer osmotically balanced with sorbitol. Under these conditions, mitotic spindles are preserved and the γ-tubulin was located in spindle pole bodies associated with the mitotic spindle (Fig. 6, *d–i*). Although γ-tubulin staining spots were sometimes visible in the positions expected of cytoplasmic MTOCs, the preservation of cytoplasmic microtubules in postanaphase cells in these preparations was not adequate to allow us to identify cytoplasmic MTOCs with confidence.

Resistance to Antimicrotubule Agents Is Not Significantly Altered in Strains Expressing Human γ-Tubulin or Overexpressing S. pombe γ-Tubulin

Since γ-tubulin is essential for microtubule function, it was worthwhile to determine if overexpression of γ-tubulin or expression of exogenous γ-tubulin would alter the sensitivity of *S. pombe* cells to antimicrotubule agents. We consequently tested the growth rates of HM123, AH001, and AH004 on benomyl, thiabendazole, and nocodazole. In each case, we used a variety of concentrations of antimicrotubule agents up to completely inhibitory concentrations.

As mentioned previously, AH004 grew slightly slower than the other strains in the absence of antimicrotubule agents. It also grew slightly slower in the presence of antimicrotubule agents. Consequently, to compare inhibition of the three strains, we normalized the growth rates of each strain treated with antimicrotubule agent to the growth rate of the same strain in the absence of antimicrotubule agent. We found that inhibition of growth was similar for each of the three strains for each antimicrotubule agent (results not shown). We, thus, find no evidence that overexpression of *S. pombe* γ-tubulin or expression of human γ-tubulin alters the sensitivity of *S. pombe* cells to these antimicrotubule agents.

Discussion

Although *S. pombe* and human γ-tubulins share only 71.6% identity (Zheng et al., 1991; Stearns et al., 1991; Horio et al., 1991), our results demonstrate that human γ-tubulin supports growth and apparently normal arrays of microtubules in *S. pombe* strains lacking endogenous γ-tubulin. Human γ-tubulin probably does not function quite as well in *S. pombe* as the endogenous γ-tubulin, however, because growth rates for the strain expressing only human γ-tubulin are slightly slower than those of a genetically similar strain expressing *S. pombe* γ-tubulin.
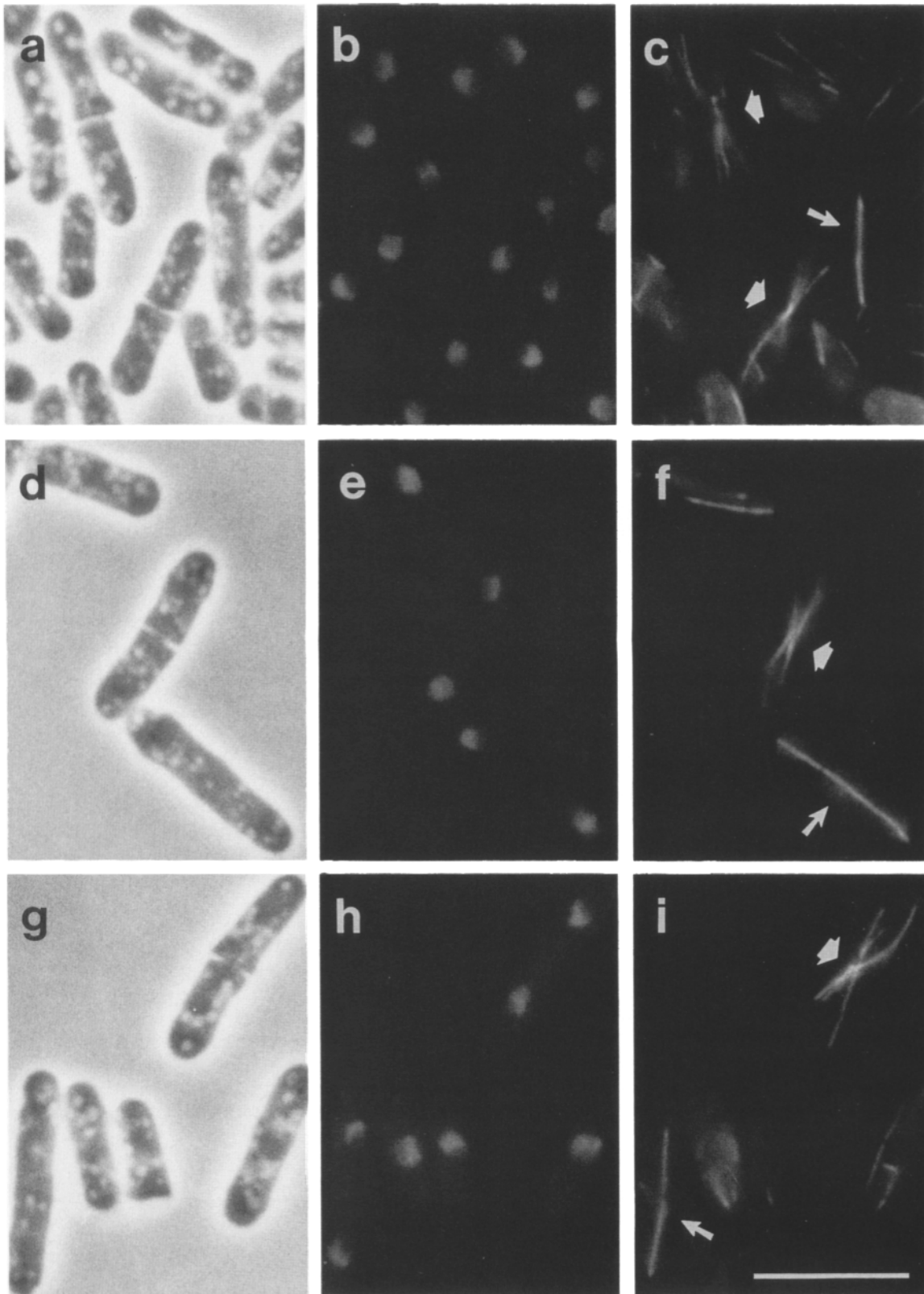
Figure 5. Immunofluorescent staining of microtubules using an anti-α-tubulin antibody. *S. pombe* strains HM123 (*a–c*), AH004 (*d–f*), and AH001 (*g–i*) were fixed and treated for immunofluorescent staining. Micrographs showing phase contrast (*a*, *d*, and *g*), DAPI staining (*b*, *e*, and *h*), and immunofluorescent staining using the monoclonal anti-α-tubulin antibody TAT1 (*c*, *f*, and *i*) of the same field of each strain are shown. Mitotic spindles (*narrow arrows*) and microtubules extending from cytoplasmic MTOCs (*wide arrows*) are indicated in *c*, *f*, and *i*. Bar, 10 μm.
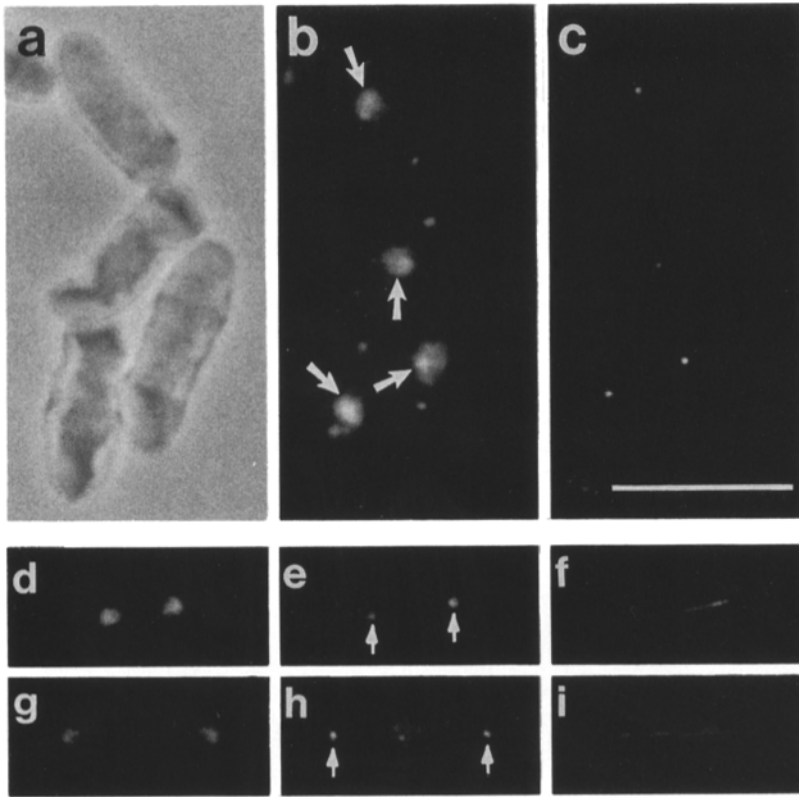
Figure 6. Immunofluorescence microscopy of AH004, an *S. pombe* strain expressing human γ-tubulin. (*a–c*) A field of interphase cells extensively extracted to remove cytoplasmic γ-tubulin (see Materials and Methods). *a* is a phase-contrast image, *b* shows DAPI staining, and *c* shows staining with anti–γ-tubulin antibodies affinity purified against human γ-tubulin. Specificity of these anti–γ-tubulin antibodies is demonstrated in *b* of Fig. 2. Nuclei are shown with arrows in *b* and the other DAPI-stained structures are presumably mitochondria. The nuclei have swollen because of the extraction conditions. *c* shows that there is a γ-tubulin spot associated with each nucleus. *d–f* and *g–i* show two mitotic cells that have been less extensively extracted. *d* and *g* show DAPI staining, *e* and *h* show anti–γ-tubulin staining, and *f* and *i* show spindle staining with an anti–α-tubulin antibody. Spindles are fainter than normal because of extraction, and *g–i* show a slightly later stage with a longer spindle. Although there is some staining of cytoplasmic γ-tubulin, the most obvious location of γ-tubulin is at the spindle pole bodies at the ends of the mitotic spindles (*e* and *h*, arrows). Bar, 10 μm.

It is difficult to compare the levels of human γ-tubulin in AH004 to those of *S. pombe* γ-tubulin in AH001 and wild-type strains because our antibody preparations have different affinities for the different γ-tubulins. It is likely, however, that since the human γ-tubulin cDNA is on a multicopy plasmid and is controlled by the ADH promoter, the level of human γ-tubulin in AH004 is higher than that of endogenous γ-tubulin in wild-type strains. It is possible, consequently, that human γ-tubulin only supports viability in *S. pombe* when it is expressed at high levels. We do not believe this is likely, however, because *S. pombe* is insensitive to endogenous γ-tubulin levels over a fivefold range and, thus, the levels of expression of γ-tubulin seem relatively unimportant.

It is unlikely that γ-tubulin would be functionally conserved from fission yeasts to humans if γ-tubulin did not perform similar functions in the two species. Evidence that γ-tubulin is essential for the formation of normal microtubule arrays in *A. nidulans* (Oakley et al., 1990), *S. pombe* (Horio et al., 1991), and mammalian cells (Joshi et al., 1992) indicates that γ-tubulin has an essential role in organizing microtubules. This view is supported by recent findings that indicate that γ-tubulin and/or associated proteins are essential for nucleation of microtubules from centrosomes in vitro (Félix et al., 1994; Stearns and Kirschner, 1994). γ-Tubulin may, thus, have a universal role in the nucleation of microtubules from MTOCs.

Our finding that human γ-tubulin localizes to the *S. pombe* spindle pole body suggests that proteins that interact with γ-tubulin are probably conserved, at least in the domains that interact with γ-tubulin. It is, thus, likely that some centrosomal proteins have γ-tubulin–binding domains similar to those of spindle pole body proteins. These data suggest that, in spite of their structural diversity, MTOCs may show

significant conservation at the protein level. Information obtained by studying spindle pole bodies in genetically tractable organisms such as *S. pombe* may consequently lead to a better understanding of the essential components of centrosomes. Candidate centrosomal γ-tubulin–binding proteins have already been identified in *Drosophila melanogaster* (Raff et al., 1993), and it would be interesting to determine if they have homologues in *S. pombe*. It is also possible that proteins with conserved γ-tubulin–binding domains are present in the cytoplasmic MTOCs of *S. pombe* and, perhaps, at the regions at the edges of midbodies of mammalian cells that contain γ-tubulin (Julian et al., 1993) and in other MTOCs (Gueth-Hallonet et al., 1993). In plant cells, γ-tubulin distribution is quite different from animal and fungal cells (Liu et at., 1993, 1994), and it would be of interest to determine if plant γ-tubulin would function in animal or fungal cells.

Our results also demonstrate that at least some aspects of human γ-tubulin can be studied in the biochemically and genetically tractable fission yeast. For example, one might be able to look for compounds that inhibit mammalian γ-tubulin activity more rapidly, easily, and less expensively by using a fission yeast strain expressing human γ-tubulin than by using human cells.

The apparently complete lack of toxicity associated with a fivefold overexpression of γ-tubulin is similar to results reported in abstract form for the filamentous fungus *A. nidulans* (Yoon, Y., and B. R. Oakley. 1992. *Mol. Biol. Cell* 3:48a), but is very different from results obtained with α- and β-tubulin. In both *S. cerevisiae* and *S. pombe*, overexpression of β-tubulin is toxic (Burke et al., 1989; Katz et al., 1990; Hiroaka et al., 1984). Substantial overexpression of α-tubulin, moreover, causes moderate toxicity in *S. cere-*

visiae (Weinstein and Solomon, 1990). In higher eukaryotes, the levels of α- and β-tubulins are strongly regulated at the posttranscriptional level (Yen et al., 1988).

Although we have not determined the amount of γ-tubulin at the spindle pole body and in the cytoplasm of overexpressing cells, immunofluorescence microscopy of unextracted cells suggests that much more is present in the cytoplasm than at the spindle pole body. This result indicates that most of the excess γ-tubulin produced remains in the cytoplasm rather than becoming located at the spindle pole body. The likely explanation is that the γ-tubulin–binding capacity of the spindle pole body is limited by the amounts of γ-tubulin–binding proteins present.

In view of the accumulating evidence that γ-tubulin plays an important role in microtubule nucleation (Oakley et al., 1990; Horio et al., 1991; Joshi et al., 1992; Félix et al., 1994; Stearns and Kirschner, 1994), it is, perhaps, surprising that the excess γ-tubulin in the cytoplasm of overexpressing cells has no effect on the microtubule network. One explanation is that the excess γ-tubulin is simply not properly folded. There is evidence that γ-tubulin is associated with a chaperonin complex (Melki et al., 1993). It is possible that the chaperonin complex is present in limiting amounts and that any γ-tubulin synthesized in excess of the capacity of the chaperonin complexes is not folded properly. It is unlikely, however, that a fivefold overexpression of γ-tubulin, which is a relatively low abundance protein, would saturate chaperonin complexes, unless γ-tubulin were folded by a distinct low abundance class of chaperonin complexes. One would expect that an improperly folded protein would be unstable, moreover, and we see no evidence in Western blots of proteolytic cleavage of γ-tubulin in overexpressing strains (Fig. 2).

A second possibility is that, after synthesis, γ-tubulin is normally maintained in an inactive form that becomes active only in the context of the MTOC, and that excess synthesis simply results in more of the inactive form. This touches on a general problem with MTOC proteins. When MTOC proteins are synthesized, they must not nucleate the assembly of microtubules until they become part of MTOCs. If they did, inappropriate assembly of microtubules would occur, and the microtubule cytoskeleton would become disorganized. Activation of the microtubule nucleating ability of MTOC proteins could involve a number of processes including, but not limited to, posttranslational modifications of the MTOC proteins, binding of MTOC proteins together to form active complexes, or release of MTOC proteins from sequestering complexes.

In this regard, it is interesting to note that a candidate γ-tubulin sequestering complex has been found in *Xenopus* eggs and in frog and human cultured cells. In these cells γ-tubulin is found in large (∼25 S) complexes that have been termed γ-somes (Stearns and Kirschner, 1994). Although γ-somes bind to microtubules (Stearns and Kirschner, 1994), they apparently do not nucleate microtubule assembly because microtubules are not nucleated at random positions in the cytoplasm. While Stearns and Kirschner have hypothesized that the failure of γ-somes to nucleate microtubule assembly is simply a matter of concentration, it is tempting to speculate that γ-somes may serve to sequester γ-tubulin in a microtubule-nucleation–inactive form until release during MTOC formation. In this regard, it would be interesting to determine if γ-somes are present in *S. pombe*

and if they are more abundant in strains overexpressing γ-tubulin.

Regulation at the level of assembly of components onto the MTOC could be partially responsible for the observations that the ability of MTOCs to nucleate microtubule assembly is regulated in a cell cycle-specific fashion (Snyder and McIntosh, 1975; Telzer and Rosenbaum, 1979; Buendia et al., 1992). It is clear, however, that the simple presence of γ-tubulin at the MTOC is not sufficient to create a nucleation-active MTOC (Oakley et al., 1990; Horio et al., 1991; Masuda et al., 1992) and, thus, there must be additional levels of regulation.

References

Aves, S. J., B. W. Durkacz, A. Carr, and P. Nurse. 1985. Cloning, sequencing and transcriptional control of the *Schizosaccharomyces pombe cdc10* 'start' gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:457–463.

Brinkley, B. R. 1985. Microtubule organizing centers. *Annu. Rev. Cell Biol.* 1:145–172.

Buendia, B., G. Draetta, and E. Karsenti. 1992. Regulation of the microtubule nucleating activity of centrosomes in *Xenopus* egg extracts: role of cyclin A–associated protein kinase. *J. Cell Biol.* 116:1431–1442.

Burke, D., P. Gasdaska, and L. Hartwell. 1989. Dominant effects of tubulin overexpression. *Mol. Cell. Biol.* 9:1049–1059.

Euteneuer, U., and J. R. McIntosh. 1981. Structural polarity of kinetochore microtubules in PtK₁ cells. *J. Cell Biol.* 89:338–345.

Evans, L., T. Mitchison, and M. Kirschner. 1985. Influence of the centrosome on the structure of nucleated microtubules. *J. Cell Biol.* 100:1185–1191.

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

Gueth-Hallonet, C., A. Antony, J. Aghion, A. Santa-Maria, I. Lajoie-Mazenc, M. Wright, and B. Maro. 1993. γ-tubulin is present in acentriolar MTOCs during early mouse development. *J. Cell Sci.* 105:157–166.

Gutz, H., H. Heslot, U. Leupold, and N. Loprieno. 1974. *Schizosaccharomyces pombe.* In Handbook of Genetics. R. C. King, editor. Plenum Publishing Corporation; New York. pp. 395–446.

Hagan, I. M., and J. S. Hyams. 1988. The use of cell division mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe. J. Cell Sci.* 89:343–357.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. p. 633.

Hiraoka, Y., T. Toda, and M. Yanagida. 1984. The NDA3 gene of fission yeast encodes β-tubulin: a cold-sensitive nda3 mutation reversibly blocks spindle formation and chromosome movement in mitosis. *Cell.* 39:349–358.

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

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

Julian, M., Y. Tollon, I. Lajoie-Mazenc, A. Moisand, H. Mazarguil, A. Puget, and M. Wright. 1993. γ-Tubulin participates in the formation of the midbody during cytokinesis in mammalian cells. *J. Cell Sci.* 105:145–156.

Katz, W., B. Weinstein, and F. Solomon. 1990. Regulation of tubulin levels and microtubule assembly in *Saccharomyces cerevisiae*: consequences of altered tubulin gene copy number. *Mol. Cell Biol.* 10:5286–5294.

Liu, B., J. Marc, H. C. Joshi, and B. A. Palevitz. 1993. A γ-tubulin related protein associated with the microtubule arrays of higher plants in a cell cycle-dependent manner. *J. Cell Sci.* 104:1217–1228.

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

Masuda, H., T. Hirano, M. Yanagida, and W. Z. Cande. 1990. In vitro reactivation of spindle elongation in fission yeast *nuc2* mutant cells. *J. Cell Biol.* 110:417–425.

Masuda, H., M. Sevik, and W. Z. Cande. 1992. In vitro microtubule-nucleating activity of spindle pole bodies in fission yeast *Schizosac-*

charomyces pombe: cell cycle–dependent activation in *Xenopus* cell-free extracts. *J. Cell Biol.* 117:1055–1066.

McIntosh, J. R., and U. Euteneuer. 1984. Tubulin hooks as probes for microtubule polarity: an analysis of the method and an evaluation of data on microtubule polarity in the mitotic spindle. *J. Cell Biol.* 98:525–533.

Melki, R., I. Vainberg, R. L. Chow, and N. J. Cowan. 1993. Chaperonin-mediated folding of vertebrate actin-related protein and γ-tubulin. *J. Cell Biol.* 122:1301–1310.

Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmid containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035–7056.

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

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

Oakley, B. R. 1992. γ-Tubulin: the microtubule organizer? *Trends Cell Biol.* 2:1–5.

Pickett-Heaps, J. D. 1969. The evolution of the mitotic apparatus: an attempt at comparative ultrastructural cytology in dividing plant cells. *Cytobios.* 3:257–280.

Raff, J. W., D. R. Kellogg, and B. M. Alberts. 1993. Drosophila γ-tubulin is part of a complex containing two previously identified centrosomal MAPs. *J. Cell Biol.* 121:823–835.

Russell, P. R., and B. D. Hall. 1983. The primary structure of the alcohol dehydrogenase gene from the fission yeast *Schizosaccharomyces pombe. J. Biol. Chem.* 258:143–149.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandhu, G. S., B. W. Eckloff, and B. C. Kline. 1991. Chemiluminescent substrates increase sensitivity of antigen detection in Western blots. *Biofeedback.* 11:14–16.

Snyder, J. A., and J. R. McIntosh. 1975. Initiation and growth of microtubules from mitotic centers in lysed mammalian cells. *J. Cell Biol.* 67:744–760.

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

Stearns, T., and M. Kirschner. 1994. In vitro reconstitution of centrosome assembly and function: the central role of γ-tubulin. *Cell.* 76:623–637.

Telzer, B. R., and J. L. Rosenbaum. 1979. Cell cycle–dependent, in vitro assembly of microtubules onto the pericentriolar material of HeLa cells. *J. Cell Biol.* 81:484–497.

Toda, T., M. Shimanuki, and M. Yanagida. 1991. Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast *FUS3* and *KSS1* kinases. *Genes & Dev.* 5:60–73.

Weinstein, B., and F. Solomon. 1990. Phenotypic consequences of tubulin overproduction in *Saccharomyces cerevisiae*: differences between alpha-tubulin and beta-tubulin. *Mol. Cell. Biol.* 10:5295–5304.

Woods, A., T. Sherwin, R. Sasse, T. H. MacRae, A. J. Baines, and K. Gull. 1989. Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. *J. Cell Sci.* 93:491–500.

Yen, T. J., P. S. Machlin, and D. W. Cleveland. 1988. Autoregulated instability of β-tubulin mRNAs by recognition of the nascent amino terminus of β-tubulin. *Nature (Lond.).* 334:580–585.

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