The EMBO Journal vol.15 no.15 pp.3899–3911, 1996

The spindle pole body component Spc98p interacts with the γ-tubulin-like Tub4p of *Saccharomyces cerevisiae* at the sites of microtubule attachment

Silke Geissler, Gislene Pereira, Anne Spang, Michael Knop, Sylvie Souès[1], John Kilmartin[1] and Elmar Schiebel[2]

Max-Planck Institut für Biochemie, Am Klopferspitz 18a, Genzentrum, 82152 Martinsried, Germany and [1]Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, UK

[2]Corresponding author

S.Geissler, G.Peirera and A.Spang contributed equally to this work

Tub4p is a novel tubulin found in *Saccharomyces cerevisiae*. It most resembles γ-tubulin and, like it, is localized to the yeast microtubule organizing centre, the spindle pole body (SPB). In this paper we report the identification of *SPC98* as a dosage-dependent suppressor of the conditional lethal *tub4-1* allele. *SPC98* encodes an SPB component of 98 kDa which is identical to the previously described 90 kDa SPB protein. Strong overexpression of *SPC98* is toxic, causing cells to arrest with a large bud, defective microtubule structures, undivided nucleus and replicated DNA. The toxicity of *SPC98* overexpression was relieved by co-overexpression of *TUB4*. Further evidence for an interaction between Tub4p and Spc98p came from the synthetic toxicity of *tub4-1* and *spc98-1* alleles, the dosage-dependent suppression of *spc98-4* by *TUB4*, the binding of Tub4p to Spc98p in the two-hybrid system and the co-immunoprecipitation of Tub4p and Spc98p. In addition, Spc98-1p is defective in its interaction with Tub4p in the two-hybrid system. We suggest a model in which Tub4p and Spc98p form a complex involved in microtubule organization by the SPB.

Keywords: γ-tubulin/microtubule organization/*SPC98*/spindle pole body/*TUB4*

Introduction

Microtubules are hollow cylinders formed by the self-assembly of tubulin, a heterodimer composed of α- and β-tubulin. They are intrinsically polar polymers such that one end, the so-called plus end, assembles tubulin at a greater rate than the minus end (Bergen and Borisy, 1980; Horio and Hotani, 1986). While microtubules form spontaneously *in vitro* by the self-assembly of tubulin subunits, assembly *in vivo* is organized by organelles named microtubule organizing centres (MTOC). MTOCs reduce the critical concentration required for the assembly process. In addition, they determine number, direction and polarity of microtubules. The polarity is such that the minus end of the microtubule contacts the MTOC (McIntosh and Euteneuer, 1984).

The mechanisms by which MTOCs regulate assembly and establish polarity of microtubules is beginning to become clearer. A recently discovered new member of the tubulin superfamily of proteins, named γ-tubulin (Oakley and Oakley, 1989), is probably involved in these processes. γ-Tubulin was first found as a suppressor of a heat-sensitive β-tubulin mutation in the fungus *Aspergillus nidulans* (Weil *et al.*, 1986). Since then γ-tubulin has been identified in many different organisms, including human, *Drosophila melanogaster*, *Xenopus laevis* and *Schizosaccharomyces pombe* (Horio *et al.*, 1991; Stearns *et al.*, 1991; Zheng *et al.*, 1991). Subcellular localization studies identified γ-tubulin as a component of MTOCs (Oakley *et al.*, 1990; Horio *et al.*, 1991; Stearns *et al.*, 1991; Zheng *et al.*, 1991). In human centrosomes γ-tubulin is located in the pericentriolar material, the centrosomal substructure that nucleates microtubule assembly (Stearns *et al.*, 1991). A function of γ-tubulin in microtubule organization is suggested by the inhibition of microtubule nucleation after disruption of the essential γ-tubulin gene in *A.nidulans* (Oakley *et al.*, 1990), *S.pombe* (Horio and Oakley, 1994) and *Drosophila* (Sunkel *et al.*, 1995). In addition, mammalian cells fail to assemble mitotic spindles after microinjection of anti-γ-tubulin antibody (Joshi *et al.*, 1992). γ-Tubulin is present in a 25S complex in the cytoplasm of frog eggs and vertebrate somatic cells (Stearns and Kirschner, 1994). Purification of this γ-tubulin-containing complex from *X.laevis* identified seven different proteins, including α- and β-tubulin. These proteins form an open ring structure that acts as an active microtubule-nucleating unit capping the minus ends of microtubules *in vitro* (Zheng *et al.*, 1995).

Microtubules in the budding yeast *Saccharomyces cerevisiae* are essential components of the mitotic and meiotic spindles and are important for nuclear movement during cell division and mating. *Saccharomyces cerevisiae* has an essential β-tubulin gene (*TUB2*; Neff *et al.*, 1983) and two α-tubulin genes, *TUB1* and *TUB3* (Schatz *et al.*, 1986). Despite extensive searching, no γ-tubulin has been identified in *S.cerevisiae*. However, a novel tubulin, Tub4p, which most resembles the γ-tubulin family, was found through the yeast genome sequencing project. Tub4p is associated with the yeast MTOC, the spindle pole body (SPB; Sobel and Snyder, 1995; Spang *et al.*, 1996). Interestingly, Tub4p is located at the inner and outer plaques of the SPB close to the ends of the nuclear and cytoplasmic microtubules (Spang *et al.*, 1996; Figure 11), suggesting a role in microtubule organization or attachment to the SPB. This was confirmed from an analysis of the phenotype of the conditional lethal *tub4-1* allele. Under restrictive growth conditions *tub4-1* cells were defective in microtubule organization with only a few or misdirected nuclear microtubules associated with the inner plaque (Spang *et al.*, 1996).

To understand how microtubules are anchored to the yeast SPB, we screened for dosage-dependent suppressors of the

lethal phenotype of the *tub4-1* allele. We identified *SPC98*, which codes for the 90 kDa SPB component previously described by Rout and Kilmartin (1990) and which also localizes to the inner and outer plaques, using a monoclonal antibody approach. A function for Spc98p in microtubule organization is suggested by the defective microtubule structures seen after strong overexpression of *SPC98* and after incubation of *spc98-1* cells under restrictive growth conditions. In addition, we have demonstrated that Spc98p and Tub4p directly interact, suggesting that both SPB components form a complex which is involved in the attachment of microtubules to the yeast SPB.

Results

A screen for proteins involved in Tub4p function

We screened for dosage-dependent suppressors of the temperature-sensitive phenotype of *tub4-1* cells. Strain ESM208 (*tub4-1*) was transformed with a genomic library cloned into the 2 μm-based vector YEp13, which is maintained at 10–40 copies/cell (Guthrie and Fink, 1991). Out of 20 000 transformants 15 were able to grow at the non-permissive temperature. Ten of the plasmids isolated from the suppressed *tub4-1* cells contained *TUB4*, based on restriction analysis and PCRs with *TUB4*-specific primers. The remaining five plasmids carried inserts unrelated to *TUB4*. Their restriction patterns were similar, suggesting that these plasmids contained overlapping genomic fragments. Subcloning of DNA fragments and suppression analysis showed that a 5.0 kb *Sal*I fragment contained in plasmid pSM271 was responsible for the dosage-dependent suppression of *tub4-1* (Figure 1A and B), but this fragment did not rescue a deletion of *TUB4* (data not shown). The 5.0 kb *Sal*I fragment contained one open reading frame, which we named *SPC98* (*s*pindle *p*ole body *c*omponent with a calculated molecular mass of *98* kDa, GenBank accession no. EMBL Z46843). *SPC98* was responsible for suppression of *tub4-1*, as shown by deletion analysis (Figure 1A). In addition, a point mutation changing the potential start codon (ATG) of *SPC98* to ACG coding for Thr impaired the suppression of *tub4-1* (data not shown). Subsequent analysis revealed that *SPC98* on the centromere-based plasmid pRS316 present at 1–4 copies/cell also suppressed the temperature-sensitive growth defect of *tub4-1* cells (data not shown). *SPC98* is identical to ORF N1222 described by the yeast genome sequencing project (Mallet *et al.*, 1995). It encodes a protein of 846 amino acids with no homology to any protein in the database.

SPC98 is essential for cell viability

To test whether *SPC98* is essential for cell viability, a deletion experiment was performed. A derivate of the diploid yeast strain YPH501 (Sikorski and Hieter, 1989) was isolated in which one of the two wild-type alleles of *SPC98* was disrupted by the *HIS3* gene (SGY26, Figure 1A). Analysis of 40 tetrads of strain SGY26 revealed that only two of the spores from each tetrad formed colonies. All of these colonies were His$^-$, indicating that they carried the wild-type *SPC98* allele. Spores which had failed to form viable colonies were examined under the microscope. Microcolonies consisting of two to four large-budded cells were observed (data not shown).

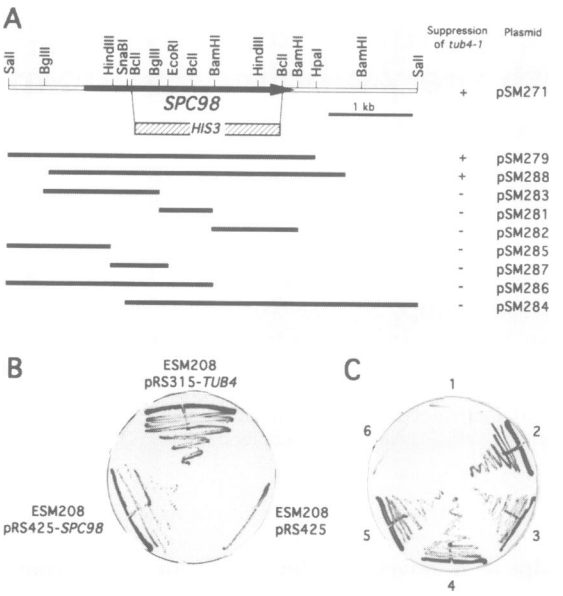


Fig. 1. *SPC98* is a dosage-dependent suppressor of *tub4-1* and is essential for viability. (**A**) Plasmid p2-2-1 of a genomic YEp13 library suppressed the temperature-sensitive phenotype of *tub4-1* cells. The 5.0 kb *Sal*I fragment of p2-2-1 was subcloned into the 2 μm *LEU2*-based vector pRS425 (pSM271). Plasmid pSM271 and subclones of pSM271 in pRS425 were tested for complementation of *tub4-1* as described in (B). For gene disruption experiments, 70% of the coding region of *SPC98* was deleted. The *HIS3* gene on a *Bam*HI fragment was inserted into *Bcl*I restriction sites of *SPC98*. (**B**) Suppression of *tub4-1* by *SPC98*. ESM208 (*tub4-1*) was transformed with plasmids pSM204 (pRS315-*TUB4*, *TUB4* on the centromere *LEU2*-based vector pRS315), pRS425 or pSM271 (pRS425-*SPC98*, *SPC98* on the 2 μm *LEU2*-based plasmid pRS425). Transformants were incubated for 3 days at 37°C. (**C**) *SPC98* is essential for growth. Cells were streaked on 5-FOA plates which select against *URA3* plasmids (pRS316). The growth behaviour of control strains [sectors 5 (*ura3*, growth) and 6 (*URA3*, no growth)] indicated that selection on the 5-FOA plates was effective. ESM243 (Δ*spc98*::*HIS3* pRS316-*SPC98*) with control vector pRS315 (sector 1), ESM243 with pRS315-*SPC98* (pSM297, sector 2), the diploid strain SGY26 (Δ*spc98*::*HIS3*/*SPC98*) carrying plasmid pRS316-*SPC98* (sector 3), ESM243 with pRS315-*MYC-SPC98* (pSM339, sector 4), YPH499 (*ura3*, sector 5) and ESM184 (Δ*tub4*::*HIS3* pRS316-*TUB4*-HA, sector 6) were tested for growth on 5-FOA plates at 23°C.

A plasmid shuffle experiment established that *SPC98* is essential for growth. We constructed ESM243 [Δ*spc98*::*HIS3* pSM296], which has a disruption of *SPC98* and is maintained by *SPC98* on a *URA3*-based plasmid (pSM296). ESM243 did not grow on 5-fluoroorotic acid (5-FOA) plates, which select against the *URA3* plasmid, showing that *SPC98* is essential for growth (Figure 1C, sector 1). In contrast, 5-FOA-resistant colonies were obtained when ESM243 contained an additional *SPC98* on the *LEU2*-based plasmid pSM297 (sector 2) or when a chromosomal copy of *SPC98* was present (*SPC98*/Δ*spc98*::*HIS3* pSM296, sector 3). Taken together, the results of the spore viability and the plasmid shuffle experiments suggest that *SPC98* is essential for the viability of yeast cells.

SPC98 codes for the 90 kDa SPB component

To understand why *SPC98* suppressed the lethal phenotype of *tub4-1* at the non-permissive temperture, we investigated the subcellular localization of epitope-tagged MYC–Spc98p by indirect immunofluorescence with monoclonal

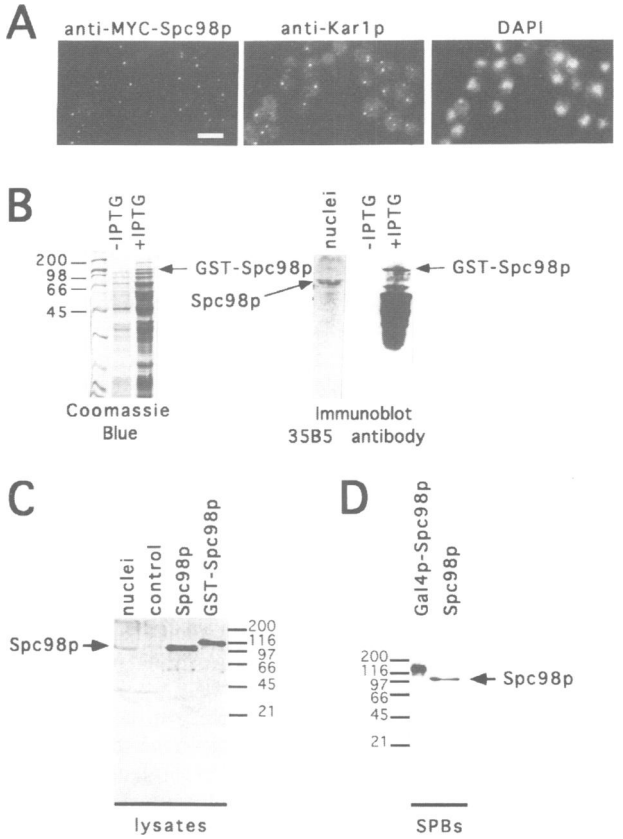


Fig. 2. *SPC98* codes for the 90 kDa SPB protein. (**A**) MYC–Spc98p is associated with the SPB. MYC–Spc98p and Karlp of ESM281 (pRS315-*MYC-SPC98*, pSM339) were detected by indirect immunofluorescence. DNA was stained with DAPI. (**B**) GST–Spc98p was expressed in *E.coli* by the addition of IPTG. *Escherichia coli* extracts were separated by SDS–PAGE. Proteins were then analysed by Coomassie Blue staining or immunoblotting using the 35B5 monoclonal antibody. The signals below GST–Spc98p (immunoblot, +IPTG) are most likely degradation products detected by the 35B5 antibody. No protein was stained by 35B5 in *E.coli* extracts without induction of GST–Spc98p (Figure 2B, immunoblot, –IPTG). Controls with purified GST, GST–Karlp or GST–Spc110p proteins (Spang *et al.*, 1995) established that the 35B5 antibody did not cross-react with GST (data not shown). (**C**) Yeast nuclei (20 μg) and cells lysates of YPH499 (20 μg) carrying control plasmid pYES2 (control) or plasmids with *SPC98* or *GST–SPC98* under control of the *GAL1* promoter were analysed after 6 h growth in galactose/raffinose medium by immunoblotting with 35B5 antibody. (**D**) SPBs of cells with Gal4p–Spc98p or Spc98p were prepared and then analysed by immunoblotting with 35B5 antibody. Bar in (A) = 5 μm.

anti-MYC-tag antibody. *MYC–SPC98* is functional, since it can replace the wild-type gene (Figure 1C, sector 4). In these cells the MYC–Spc98p signal was detected as 1 or 2 dots/cell at the nuclear periphery (Figure 2A) which were coincident in 95% of the cases examined with the signal obtained by antibodies directed against the SPB component Karlp (Spang *et al.*, 1995). No immunofluorescence signal of the anti-MYC antibody was observed in a control strain lacking MYC–Spc98p (data not shown). These results suggest that Spc98p is a SPB component.

Rout and Kilmartin (1990) identified a SPB protein with an apparent molecular weight of 90 kDa which is associated with the same SPB substructures as Tub4p (Rout and Kilmartin, 1990; Spang *et al.*, 1996). This raised the possibility that *SPC98* might encode the 90 kDa SPB component. Two independent approaches indicated that this was indeed the case. First, the monoclonal anti-90 kDa antibody 35B5 (Rout and Kilmartin, 1990) reacted with immunoblots of recombinant GST–Spc98p fusion protein (Figure 2B, +IPTG). Second, a fragment encoding the 5′-end of the open reading frame of *SPC98* was isolated from screening a λgt11 cDNA expression bank with the pooled anti-90 kDa monoclonal antibodies.

Next, we tested whether the 35B5 antibody detects Spc98p or GST–Spc98p in yeast. As described before, the 35B5 antibody stained the 90 kDa SPB component in isolated yeast nuclei (Figure 2C, nuclei; Rout and Kilmartin, 1990). However, no signal was obtained in total yeast lysates probed with 35B5 (control). This changed when *SPC98* was expressed from the strong *GAL1* promoter (Figure 2C, Spc98p). Now a protein with a similar electrophoretic mobility to the 90 kDa SPB component was detected by the antibody (compare nuclei with Spc98p). In addition, GST–Spc98p was stained by 35B5 in yeast cells expressing *GST–SPC98*. We noticed that the mobility of GST–Spc98p was somewhat faster than expected from its molecular weight.

To establish that *SPC98* encodes for the 90 kDa SPB component and not for a protein cross-reacting with 35B5, we substituted the functional, plasmid-encoded *GAL4–SPC98* (SGY34) gene fusion for the chromosomal *SPC98*. SPBs from this and wild-type strains were analysed by immunoblotting with anti-90 kDa antibody. In the case of cross-reactivity, both the 90 kDa SPB protein and Gal4p–Spc98p will be detected by the 35B5 antibody in SPBs of *GAL4–SPC98* cells. However, only a band corresponding in size to Gal4p–Spc98p was stained in SPBs from the *GAL4–SPC98* strain (Figure 2D). Taken together with the SPB staining given by the MYC-tagged Spc98p, we conclude that *SPC98* codes for the 90 kDa SPB component.

Strong overexpression of SPC98 is toxic and causes a defect in microtubule structure

Spc98p levels of *GAL1–SPC98* cells increased at least 100-fold after induction of the *GAL1* promoter by galactose (Figure 2C). This caused a severe growth defect, with individual cells unable to form colonies on galactose plates (Figure 3A, *GAL1–SPC98*, galactose). Some growth was observed at the highest cell densities (100 000 and 10 000 cells), which was expected, since *GAL1–SPC98* cells did not immediately die on galactose plates, allowing at least one duplication. The toxic effects of Spc98p overproduction became irreversible, since after 8 h induction only 15% of the cells could be rescued in the repressing glucose medium (Figure 3B, *GAL1–SPC98*) and further induction caused even greater losses in viability.

The phenotype of *GAL1–SPC98* cells was examined after 6–8 h induction. Cells accumulated with large buds (>85%) and with a predominately 2N DNA content (Figure 3C, *GAL1–SPC98*). The DNA content of control cells was normal in galactose (Figure 3C, control). When uninduced cells with large buds were examined by immunofluorescent staining with anti-tubulin nearly all contained an elongated anaphase spindle which had separated two DAPI staining regions of the nucleus (Figure 3D, glucose, and Table I). However, such anaphase spindles were rarely found in large-budded induced cells (Figure 3D, galactose and Table I). Instead, three types of spindle morphologies were detected (Table I): cells with a short

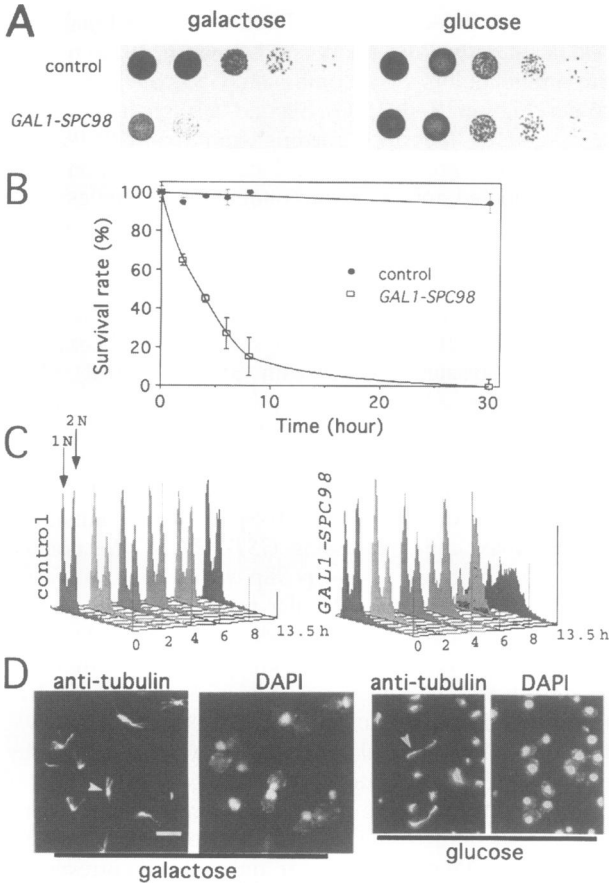


Fig. 3. Overexpression of *SPC98* is lethal and affects microtubule organization. (**A**) Overexpression of *SPC98* is lethal for wild-type cells. Cells of GPY7-1 (control, pYES2) and GPY7-2 (*GAL1–SPC98*) pregrown in raffinose medium were washed. Equal volumes containing 100 000, 10 000, 1000, 100 and 10 cells were then plated on SC plates lacking uracil with galactose/raffinose or glucose/raffinose as carbon source. Plates were incubated at 30°C for 3 days. (**B**) Cells containing the control plasmid pYES2 (control) or *GAL1–SPC98* were grown in raffinose medium to a density of 5×10^7 cells/ml. Cells were then diluted to 2×10^6 cells/ml with galactose/raffinose medium. After dilution or after 2, 4, 6, 8 or 30 h at 30°C 200–500 washed cells were plated onto glucose plates. The number of colonies was determined after 3 days at 30°C. The average of three independent experiments is shown. (**C**) Overexpression of Spc98p causes cell cycle arrest. Cells were pregrown as described in (B). After growth in galactose/raffinose medium, the DNA content was determined by flow cytometry. (**D**) Overexpression of *SPC98* causes a defect in microtubule organization. Cells of GPY7-2 (*GAL1–SPC98*) were grown in galactose/raffinose or glucose/raffinose medium for 6 h at 30°C as described in (B) and (C). Microtubules were detected by indicrect immunofluorescence using anti-tubulin antibodies. DNA was stained with DAPI. The arrowheads in (D) mark the spindle of cells with large buds. Bar in (D) = 5.0 μm.

metaphase-like spindle oriented along the mother bud axis (71%), cells with a misoriented short metaphase-like spindle (17%) and cells with a monopolar spindle (14%). These spindle morphologies were rarely seen in uninduced large-budded cells or in cells of the control strain after growth in galactose medium (Table I). More than 95% of the induced, large-budded *GAL1–SPC98* cells had elongated cytoplasmic microtubules (Figure 3D, galactose). SPB duplication and separation were not defective in most cells after overexpression of *SPC98*, since in >85% of the large-budded cells two SPB signals were obtained with the anti-Kar1p antibody (data not shown), which was used as a marker for SPBs (Spang *et al.*, 1995).

Spc98p contains a nuclear localization sequence

When the *GAL1* promoter was repressed by glucose in *GAL1–SPC98* cells Spc98p was associated with the SPB, as in wild-type cells (Figure 4A). Under inducing conditions, however, increased amounts of Spc98p were detected, which co-localized with the DAPI staining regions, suggesting that most of the protein was transported into the nucleus (Figure 4B). In some cells, dot-like staining regions were observed at the nuclear periphery (Figure 4B, arrow), which either correspond to SPBs or represent aggregated Spc98p.

Since some of the overproduced Spc98p accumulated in the nucleus, we analysed the amino acid sequence of Spc98p for potential nuclear localization sequences (NLS). Amino acids 599–614 of Spc98p (Figure 4C) might correspond to a bipartite NLS; two positively charged clusters are separated by 10 amino acids (reviewed by Dingwall and Laskey, 1991). To confirm that Spc98p contains a functional NLS, a gene fusion of *lacZ* and a short fragment of *SPC98* coding for amino acids 550–631 was constructed, under control of the *GAL1* promoter. When the subcellular distribution of this chimeric protein was investigated under inducing growth conditions, most of the hybrid protein was found in the nucleus (Figure 4E). This contrasted with the distribution of β-galactosidase, which gave a uniform staining pattern of the cells (Figure 4D). No β-galactosidase signal was observed in the immunofluorescence experiment when expression of β-galactosidase or NLS–β-galactosidase was not induced by galactose (data not shown), establishing that the antibodies were specific towards β-galactosidase. In summary, our results indicate that amino acids 550–631 of Spc98p contain a functional NLS which directs β-galactosidase to the nucleus.

Co-overexpression of TUB4 rescues the lethal phenotype of SPC98 overexpression

In common with Spc98p, overproduction of other cytoskeletal elements was toxic for yeast cells (Rose and Fink,

Table I. Spindle morphology of large-budded cells after overexpression of *SPC98*

Strain	Morphology (per cent occurrence)					
GPY7-1 (pYES2, glucose)	92 ± 4	5 ± 1	0	0	1 ± 1	2 ± 1
GPY7-1 (pYES2, galactose)	94 ± 3	5 ± 2	0	0	0	1
GPY7-2 (*GAL1-SPC98*, glucose)	91 ± 2	6 ± 2	0	0	2 ± 2	1
GPY7-2 (*GAL1-SPC98*, galactose)	3 ± 1	0	37 ± 5	34 ± 4	14 ± 2	17 ± 3

Strains GPY7-1 and GPY7-2 were grown in SC medium lacking uracil and with raffinose as carbon source. The exponentially growing cultures (1×10^7 cells/ml) were diluted 1:10 into SC medium containing glucose/raffinose or galactose/raffinose and then incubated for 6 h at 30°C. Spindle morphologies of 100–200 large-budded cells were determined by indirect immunofluorescence microscopy using anti-tubulin antibody. The average of three independent experiments is shown.

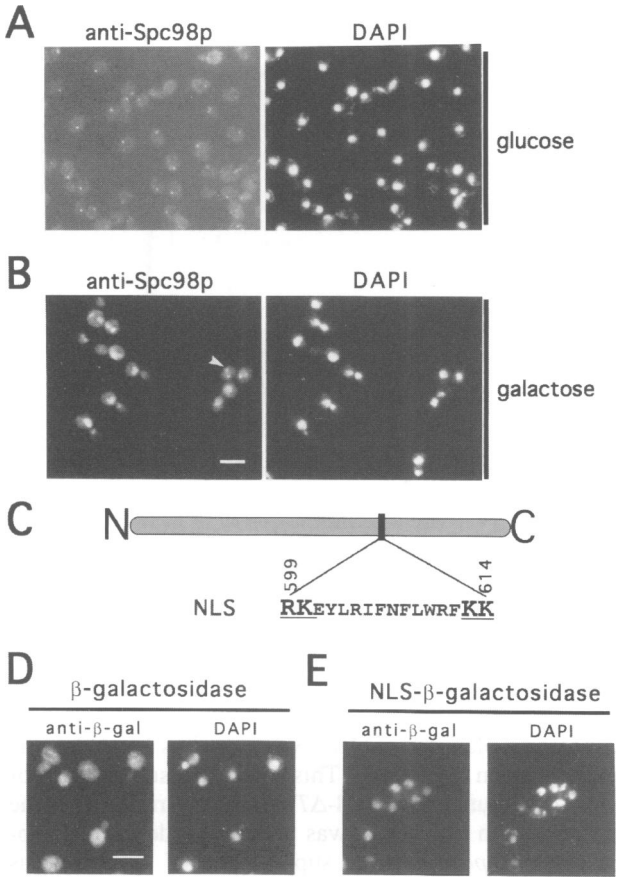


Fig. 4. Spc98p contains a NLS. Overexpressed Spc98p accumulates in the nucleus. GPY7-2 (*GAL1–SPC98*) cells were grown as described in the legend to Figure 3 in glucose/raffinose (**A**) or galactose/raffinose medium (**B**). Spc98p was detected by indirect immunofluorescence with anti-90 kDa antibodies (Rout and Kilmartin, 1990). DNA was stained with DAPI. (**C**) Bipartite NLS of Spc98p. (**D**) β-Galactosidase has a uniform distribution in yeast cells after overproduction. (**E**) β-Galactosidase fused to the NLS of Spc98p was transported into the nucleus. Expression of *lacZ* and *NLS–lacZ* was induced by the addition of galactose to cultures pregrown in raffinose medium. β-Galactosidase was detected by indirect immunofluorescence using monoclonal anti-β-galactosidase antibodies. DNA was stained with DAPI. Bars in (B) and (D) = 5 μm. (A) and (E) are the same magnification as (B).

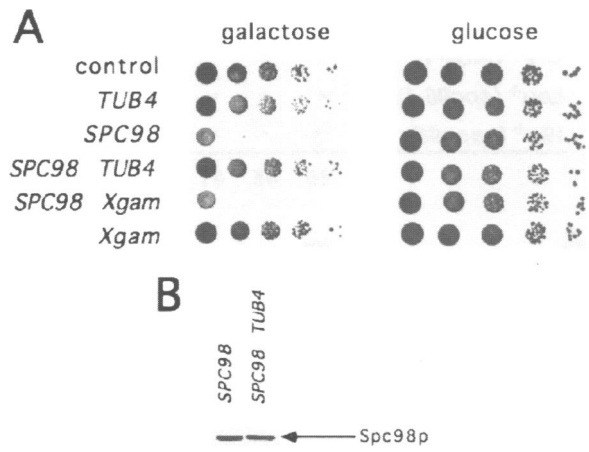


Fig. 5. Co-overexpression of *TUB4* rescues the lethality of *SPC98* overexpression. (**A**) YPH499 with plasmids pYES2 and p425-Gal1 (control), pYES2 and pSM313 (*TUB4*), pSM289 and p425-Gal1 (*SPC98*), pSM289 and pSM313 (*SPC98 TUB4*), pSM289 and pSM316 (*SPC98 Xgam*) or pYES2 and pRS316 (*Xgam*) were incubated on SC plates lacking uracil and leucine with galactose/raffinose or glucose/raffinose as carbon source for 3 days at 30°C. Serial dilutions of these cells were prepared as described in the legend to Figure 3A. (**B**) YPH499 with plasmids pSM289 (*GAL-SPC98*) and p425-Gal1 or pSM289 and pSM313 (*GAL1-TUB4*) were grown in galactose/raffinose medium for 6 h. Cell lysates (40 μg) were tested for Spc98p by immunoblotting with rabbit anti-Spc98p antibodies.

1987; Burke *et al.*, 1989; Magdolen *et al.*, 1993). In some cases, toxicity can be reduced by co-overproduction of an interacting protein (Katz *et al.*, 1990; Magdolen *et al.*, 1993; Archer *et al.*, 1995). We tested whether co-overexpression of *TUB4* reduced the toxic effects of high Spc98p levels. All strains grew equally well when the *GAL1* promoter was repressed by glucose (Figure 5A, glucose) and, while cells carrying the control vectors pYES2 and p425-Gal1 (Figure 5A, galactose, control), *GAL1–TUB4* or *GAL1–Xgam* (γ-tubulin gene from *X.laevis*) grew on galactose plates, overexpression of *SPC98* was toxic for all of these cells (Figure 5A, galactose). This toxicity was suppressed by co-expression of *GAL1–TUB4* (Figure 5A, galactose, compare *SPC98* with *SPC98 TUB4*). This was specific to Tub4p, since overexpression of *Xgam* together with *SPC98* did not suppress the lethality of *SPC98* (*SPC98 Xgam*). Immunoblot analysis confirmed that Tub4p and Xgam were at least 100-fold overproduced in these experiments (data not shown; Spang *et al.*, 1996) and that the rescuing effect of Tub4p overproduction was not simply a result of lower Spc98p levels (Figure 5B).

Genetic interaction between TUB4 and SPC98

To further establish genetic interaction between *SPC98* and *TUB4*, we investigated whether temperature-sensitive alleles of *TUB4* and *SPC98* show synthetic lethality. For this purpose we generated temperature-sensitive alleles of *SPC98* by *in vitro* mutagenesis. These were then integrated into the *LEU2* locus while the natural *SPC98* was disrupted by *HIS3*. The recessive *spc98-1(ts)* was used for the following experiments. In addition, the *tub4-1* locus of strain ESM208 (Spang *et al.*, 1996) was marked with *URA3* by transformation with a *URA3* integrating plasmid containing *tub4-1*, to give strain ESM261. This strain has a duplication of *tub4-1* and a reduced temperature-sensitive phenotype (data not shown).

The strains containing the duplicated *tub4-1* and *spc98-1* were mated (to give ESM277) and sporulated. The auxotrophic markers of spores from 100 tetrads were determined (Figure 6A). This strategy allowed us to follow the segregation of the *tub4-1*, Δ*spc98* and *spc98-1* mutations, since these are tightly linked to the *URA3*, *HIS3* and *LEU2* genes respectively. As expected, the Leu^- His^+ combination was never obtained (marked II in Figure 6A), since these cells do not contain a *SPC98* allele. Most interestingly, no colonies with the marker combination Ura^+ Leu^+ His^+ (*tub4-1 spc98-1 Δspc98::HIS3*) were detected after 4 days at 23°C (Figure 6A, column I), while the other marker combinations were obtained with the expected frequencies (Figure 6A).

After 6 days at 23°C 14 small Ura^+ Leu^+ His^+ colonies (out of the 100 tetrads analysed) appeared on plates of

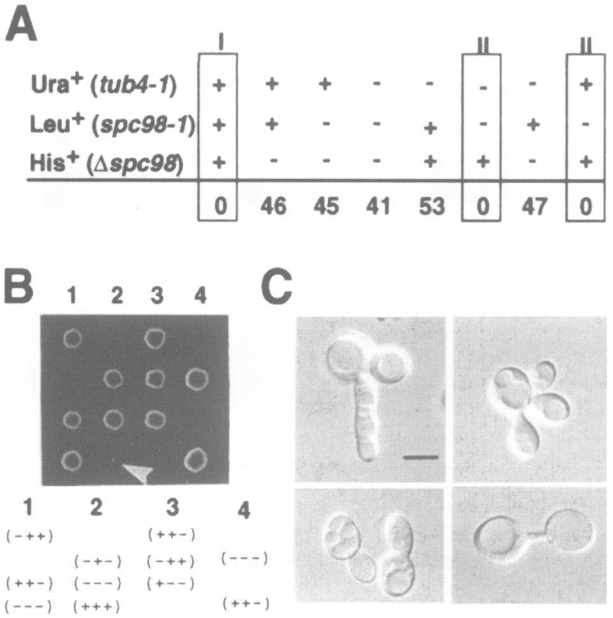


Fig. 6. Genetic interaction of *TUB4* and *SPC98*. (**A**) The auxotrophies of 100 tetrads of ESM277 were analysed after 4 days at 23°C. *tub4-1*, *spc98-1* and Δ*spc98* were linked to *URA3*, *LEU2* and *HIS3* respectively. Note that since *SPC98* is an essential gene, no Leu$^-$ His$^+$ spores (marked as area II) were obtained, since these contain a *SPC98* disruption without a rescuing *spc98-1* allele. (**B**) Small Ura$^+$ Leu$^+$ and His$^+$ colonies (marked with an arrowhead) appeared after 6 days at 23°C. Four tetrads of ESM277 are shown. The auxotrophic requirements are indicated below. (–++) colonies were Ura$^-$ Leu$^+$ His$^+$. (**C**) Morphology of Ura$^+$ Leu$^+$ and His$^+$ cells of (B) grown at 23°C. Cells were analysed by Normarski optics. Bar in (C) = 2.5 μm.

ESM277 (Figure 6B, fourth spore of tetrad 2). A high percentage of large-budded (60%) or misformed cells (30%) were observed in *tub4-1 spc98-1* double mutants grown at 23°C (Figure 6C).

As controls a strain containing a *TUB4* locus marked by *URA3* (ESM294) was crossed with *spc98-1* or a strain carrying Δ*spc98*::*HIS3* with *SPC98* integrated at the *LEU2* locus (ESM283) was crossed with the duplicated *tub4-1* strain (ESM261). In tetrads of these diploid strains Ura$^+$ Leu$^+$ His$^+$ colonies were detected as frequently as colonies with other marker combinations, with the exception of Leu$^-$ His$^+$ cells (Δ*spc98*::*HIS3*). Ura$^+$ Leu$^+$ His$^+$ cells did not show morphological defects at 23°C (data not shown). These data show that the combination of the *tub4-1* and *spc98-1* alleles severely affects viability and growth rate of yeast cells.

TUB4 is a multi-copy suppressor of spc98-4

Strains carrying the conditional lethal *spc98-1–spc98-5* alleles were transformed with a multi-copy genomic library in vector pSEY8 (Heitmann *et al.*, 1988). Plasmids were obtained that suppressed the temperature-sensitive growth defect of *spc98* cells. The inserts of these plasmids were analysed by sequencing. One set of plasmids that suppressed *spc98-4* (Figure 7, pSEY8-*TUB4*) but not the other *spc98(ts)* alleles carried chromosomal DNA corresponding to chromosome XII. Further analysis revealed that these plasmids contained the *TUB4* gene, raising the possibility that *TUB4* may be the dosage-dependent suppressor of *spc98-4*. We tested for this by removing a 926 bp *Cla*I fragment from one of the

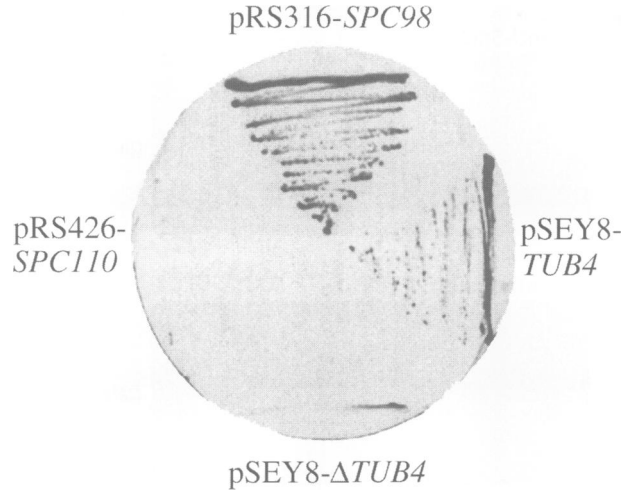


Fig. 7. *TUB4* is a dosage-dependent suppressor of *spc98-4*. *spc98-4* cells (ESM266-4) carrying *SPC98* on the centromere *URA3*-based plasmid pRS316 (pRS316-*SPC98*, pSM296), *TUB4* on the 2 μm *URA3*-based vector pSEY8 (pSEY8-*TUB4*, pMK1-3.2), pSEY8 with partially deleted *TUB4* (pSEY8-Δ*TUB4*, pSM355) and *SPC110* on the 2 μm *URA3*-based vector pRS426 (pRS426-*SPC110*, pSM356). The transformants were incubated for 3 days at 37°C on SC plates lacking uracil.

suppressing plasmids (pMK1-3.2), which is located in the coding region of *TUB4*. This impaired suppression of *spc98-4* (Figure 7, pSEY8-Δ*TUB4*), confirming that the *TUB4* gene in pMK1-3.2 was the dosage-dependent suppressor of *spc98-4*. The suppression of *spc98-4* was specific to *TUB4*, since *SPC110* (encoding another SPB component; Rout and Kilmartin, 1990) on the 2 μm vector pRS426 did not suppress the growth defect of *spc98-4* at 37°C (Figure 7, pRS426-*SPC110*).

Tub4p and Spc98p interact in the two-hybrid system

The multiple genetic interactions between *SPC98* and *TUB4* suggest a physical interaction of the gene products. This possibility was investigated using the two-hybrid system initially described by Fields and Song (1989). *TUB4* and *SPC98* were expressed as fusions with DNA coding for the DNA binding protein LexA and the acidic activation domain of Gal4p respectively. Plasmids carrying these constructs were transformed in a yeast strain that contains a *lexA* operator–*lacZ* reporter construct. Interaction of LexA–Tub4p with Gal4p–Spc98p should result in the formation of a transcription factor activating the transcription of the reporter gene *lacZ* coding for β-galactosidase.

No β-galactosidase activity was detected in cells expressing *LexA* together with *GAL4–SPC98* (Figure 8, spot 3) or *LexA–TUB4* and *GAL4* (spot 2) respectively. However, when *LexA–TUB4* and *GAL4–SPC98* (spot 1) were co-expressed strong β-galactosidase acitivity, indicated by the strong blue colony colour, was observed. A number of controls established that this increase in β-galactosidase activity was dependent on *TUB4* and *SPC98*. No β-galactosidase activity was detected when *LexA–TUB4* was combined with *GAL4–ΔSPC110* (spot 10; Kilmartin *et al.*, 1993) or when *GAL4–SPC98* was expressed with the LexA fusions of the γ-tubulins from *S.pombe* (spot 13, gtb1$^+$) or *X.laevis* (spot 14; Stearns

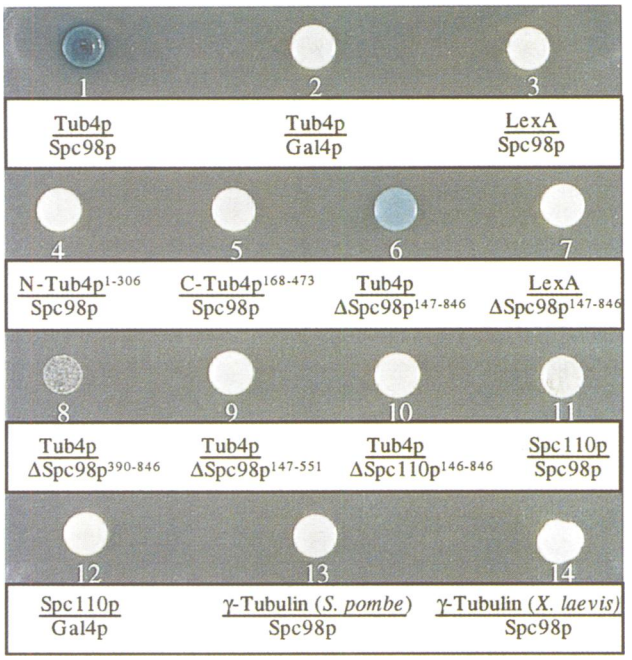


Fig. 8. Tub4p interacts with Spc98p in the two-hybrid system. Strain SGY37 (*lexA–op–lacZ*) carrying the indicated *LexA* (top) and *GAL4* constructs (bottom) was incubated on X-Gal plates for 3 days at 30°C. Blue colour indicates β-galactosidase activity.

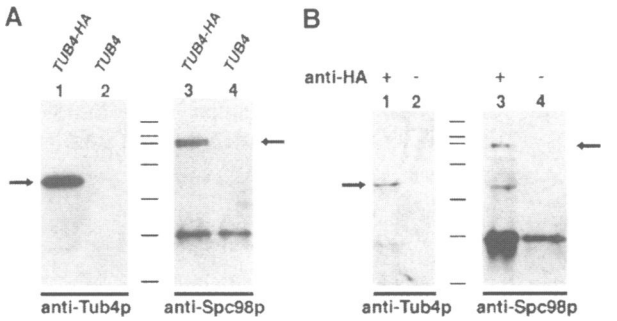


Fig. 9. Co-immunoprecipitation of Tub4p and Spc98p. (**A**) *TUB4–HA* (ESM184, lanes 1 and 3) and *TUB4* cells (YPH500, lanes 2 and 4) were lysed and then incubated with the anti-HA antibody 12CA5 followed by incubation with protein G–Sepharose. The protein G–Sepharose was sedimented by centrifugation and extensively washed to remove non-specifically bound proteins. The precipitate was analysed by immunoblotting with affinity-purified anti-Tub4p (lanes 1 and 2) or anti-Spc98p (lanes 3 and 4) antibodies. The arrows indicate the positions of Tub4p–HA (lane 1) or Spc98p (lane 3). The anti-Spc98p antibodies cross-reacted with a protein of ~31 kDa. Molecular weight standards: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa). (**B**) HA–Spc98p of strain ESM279 was precipitated with anti-HA antibodies (lanes 1 and 3) and protein G–Sepharose. As a control for the specificity of the immunoprecipitation, the anti-HA antibodies were omitted from the precipitation (lanes 2 and 4). The precipitated proteins were tested for the presence of Tub4p and Spc98p by immunoblotting using affinity-purified antibodies. The arrows indicate the positions of Tub4p (lane 1) and HA–Spc98p (lane 3). Standards are the same as in (A).

et al., 1991). Δ*SPC110* codes for the coiled coil domain of the SPB component Spc110p (Kilmartin *et al.*, 1993).

We tested whether subdomains of Spc98p or Tub4p are sufficient for interaction. No positive signal was obtained when *GAL4–SPC98* was co-expressed with *LexA* fused to *N-TUB4* (Figure 8, spot 4) or *C-TUB4* (spot 5) coding for the N- or C-terminal domains of Tub4p respectively. In contrast, Δspc98p$^{147–846}$, carrying a deletion of the 145 N-terminal amino acids of Spc98p combined with Tub4p (spot 6), resulted in a positive signal which was weaker compared with that of Spc98p (spot 1). Further deletions of N- (ΔSpc98p$^{390–846}$, spot 8) or C-terminal (ΔSpc98p$^{147–551}$, spot 9) amino acids of Spc98p impaired interaction with Tub4p. We noticed that LexA–ΔSpc98p$^{390–846}$ protein (spot 8) was somehow toxic to the cells, causing a reduction in growth. Immunoblots with appropiate antibodies showed that expression of most of these proteins was at normal or higher levels (see Materials and methods). In summary, Tub4p clearly interacts with Spc98p in the two-hybrid system. While neither the N- nor C-terminal portions of Tub4p were sufficient for the interaction with Spc98p, the C-terminal 699 amino acids of Spc98p still mediated Tub4p interaction.

Co-immunoprecipitation of Tub4p and Spc98p

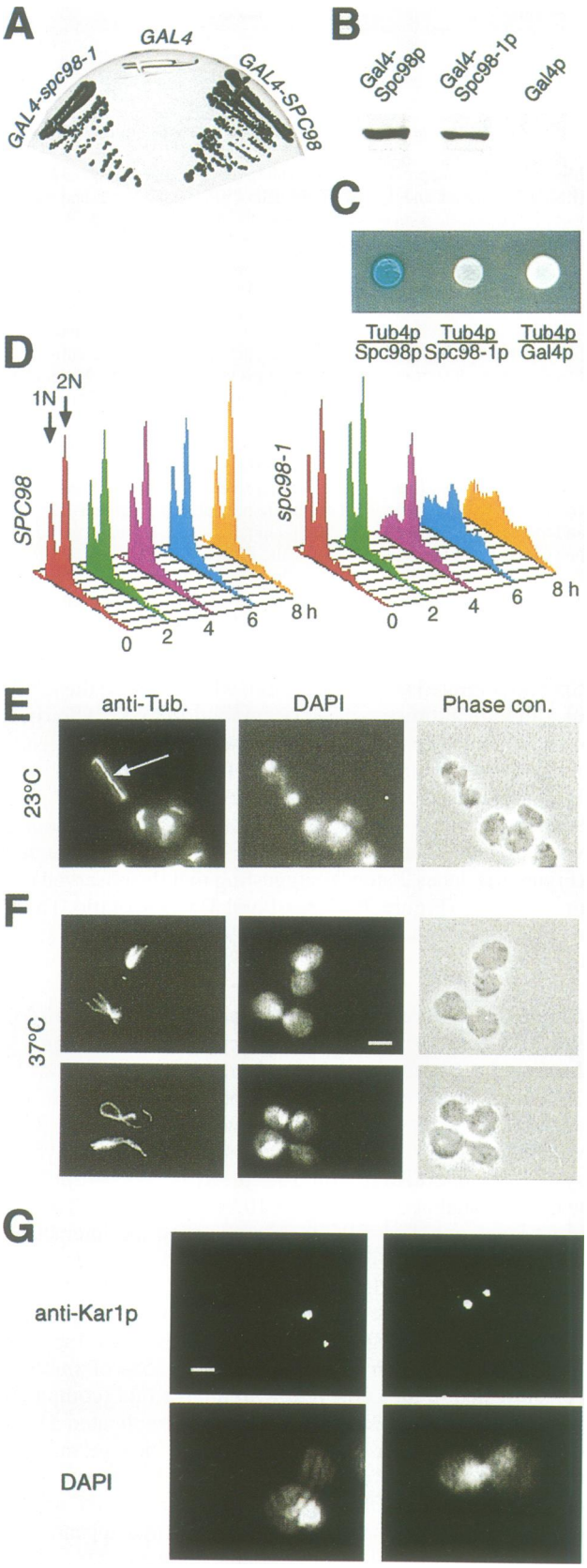
We investigated by immunoprecipitation whether Tub4p and Spc98p are components of one complex. Extracts of *TUB4–HA* or *TUB4* cells were incubated with monoclonal anti-HA antibodies. The precipitated material was then analysed by immunoblotting for the presence of Spc98p and Tub4p. As expected, Tub4p–HA but not Tub4p was precipitated by the anti-HA antibodies (Figure 9A, compare lanes 1 and 2). Most interestingly, Spc98p co-precipitated with Tub4p–HA (lane 3), but was not precipitated from extracts of *TUB4* cells (lane 4). We noticed that the precipitated Spc98p migrated as a double band, which may be the result of a modification or a partial degradation of Spc98p. In a complementary experiment an extract of *HA–SPC98* cells was incubated with or without anti-HA antibodies. The anti-HA antibodies precipitated HA–Spc98p and Tub4p (Figure 9B, lanes 1 and 3). However, when the anti-HA antibodies were omitted, Tub4p and HA–Spc98p were not detected (Figure 9B, lanes 2 and 4), suggesting that the observed co-precipitation (Figure 9B, lanes 1 and 3) was specific. These results confirm that Tub4p and Spc98p belong to the same complex.

Spc98-1p is defective in its interaction with Tub4p; spc98-1 cells arrest in the cell cycle with a large bud and a defect in spindle formation

To understand the function of Spc98p at the SPB, we analysed the temperature-sensitive *spc98-1* allele. *spc98-1* was fused with *GAL4* and then tested in the two-hybrid system for interaction with Tub4p. While *GAL4–spc98-1* was functional at 23°C (Figure 10A) and expressed to the same level as *GAL4–SPC98* (Figure 10B), the interaction of Spc98-1p with Tub4p was impaired (Figure 10C), suggesting that the defect in *spc98-1* cells results from a weaker interaction of Spc98p with Tub4p.

spc98-1 was integrated in its chromosomal location (ESM278). After 4 h at 37°C more than 85% of *spc98-1* cells arrested in the cell cycle with a large bud (compared with 15% at 23°C; data not shown) and replicated DNA (Figure 10D). After 6 and 8 h at the non-permissive temperature *spc98-1* cells with >2N DNA contents were observed (Figure 10D). *spc98-1* cells incubated at the permissive temperature had spindles of normal appearance. Particularly, cells with large buds showed two DAPI

staining regions separated by an anaphase spindle (Figure 10E, arrow). Under restrictive conditions, however, no such spindles were observed in large-budded *spc98-1* cells (Figure 10F). These cells contained short bundles of nuclear microtubules which were either located in one cell body (48%, n = 100) or in the neck of large-budded cells (52%). Corresponding with the localization of the nuclear microtubules, the single DAPI staining region was either located in only one cell body (48%) or in between the mother cell and the bud (52%). Most of the cells (>95%) had elongated cytoplasmic microtubules. Staining of the SPB using anti-Karlp antibodies (Spang *et al.*, 1995) revealed two phenotypes (Figure 10G). Large-budded *spc98-1* cells showed two SPB signals (n = 50) either in one cell body (45%; first cell in Figure 10G) or one signal in the mother cell and the other one in the bud (55%; second cell in Figure 10G). In addition, the spindle orientation was impaired in ~20% of the cells, with the Karlp signal in one cell body. In summary, *spc98-1* cells arrest in the cell cycle with replicated DNA, duplicated and separated SPBs and a defective spindle.



Discussion

SPC98 encodes the 90 kDa SPB component

Tub4p of *S.cerevisiae* is a highly divergent γ-tubulin which has essential functions in microtubule organization. This is shown by the inability of *tub4-1* cells to form a mitotic spindle (Spang *et al.*, 1996). How Tub4p fulfils its function in microtubule organization is not understood. Tub4p may directly interact with microtubule ends, thereby anchoring microtubules to the SPB. In agreement with such a model, Tub4p is associated with the SPB substructures that organize the nuclear and cytoplasmic microtubules (Spang *et al.*, 1996).

The identification of proteins interacting with Tub4p would allow a more detailed understanding of the role of Tub4p in microtubule organization. We identified *SPC98* as a dosage-dependent suppressor of *tub4-1*. Four lines of evidence suggest that *SPC98* encodes the 90 kDa SPB component previously described by Rout and Kilmartin (1990), who identified it by raising monoclonal antibodies against enriched SPBs. First, a functional epitope-tagged MYC–Spc98p localized to the SPB in indirect immunofluorescence experiments, as does the 90 kDa SPB component

Fig. 10. Analysis of *spc98-1*. (**A**) Spc98-1p is defective in its interaction with Tub4p in the two-hybrid system. *GAL4–spc98-1* and *GAL4–SPC98* are functional at 23°C, as shown by a plasmid shuffle experiment (see Figure 1C) using strain ESM243 (Δ*spc98*::*HIS3* pRS316-*SPC98*). (**B**) *GAL4–SPC98* and *GAL4–spc98-1* are expressed to similar levels. Extracts (80 μg) of *GAL4–SPC98*, *GAL4–spc98-1* and *GAL4* cells were analysed by immunoblotting using anti-HA antibodies which are directed against the HA epitope in the Gal4p portion of the hybrid proteins. (**C**) Spc98-1p is defective in its interaction with Tub4p. Strain SGY37 (*lexA–op–lacZ*) carrying the indicated *LexA* (top) and *GAL4* constructs (bottom) was incubated on X-Gal plates for 3 days at 30°C. Blue colour indicates β-galactosidase activity. (**D**) *spc98-1* cells arrest in the cell cycle with replicated DNA. *SPC98* (YPH500) or *spc98-1* cells (ESM278) were grown at 23°C and then shifted to 37°C for 2, 4, 6 or 8 h. The DNA content of the cells was analysed by flow cytometry. (**E** and **F**) *spc98-1* cells are defective in microtubule organization. Microtubules of *spc98-1* cells incubated at 23 (E) or 37°C (F) for 4 h were detected by indirect immunofluorescence using anti-tubulin antibodies. DNA was stained with DAPI. Cells were visualized by phase contrast microscopy. The arrow in (E) points towards a cell with an anaphase spindle. (**G**) SPB staining of *spc98-1* cells. *spc98-1* cells incubated at 37°C for 4 h were fixed with methanol and acetone (Rout and Kilmartin, 1990) and finally stained with affinity-purified anti-Karlp antibodies (Spang *et al.*, 1995). DNA was stained with DAPI. Bar in (F) = 2.5 and in (G) = 1.5 μm. (E) is the same magnification as (F).

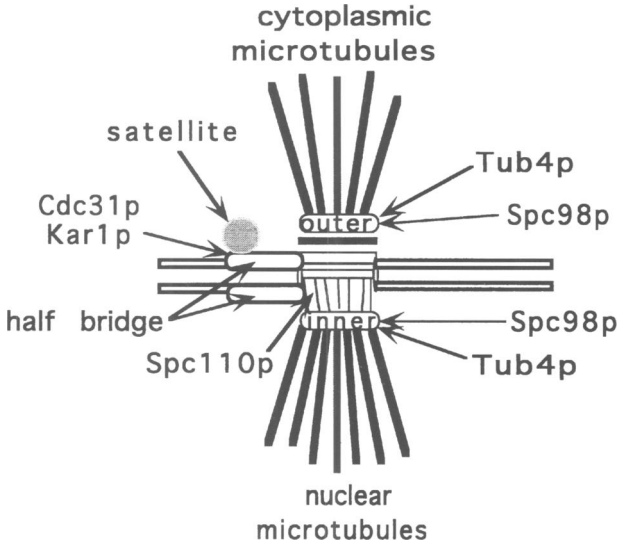


Fig. 11. Localization of Tub4p and Spc98p relative to known SPB components.

(Rout and Kilmartin, 1990). Second, anti-90 kDa monoclonal antibody 35B5 reacted with Spc98p expressed in *E.coli* or yeast and a fragment of *SPC98* was isolated by screening of expression libraries with anti-90 kDa antibodies. Third, overexpression of *SPC98* caused the accumulation of Spc98p in yeast extracts which had the same electrophoretic mobility as the 90 kDa SPB protein of nuclei detected by the 35B5 antibody. Finally, when *SPC98* was substituted by a functional *GAL4–SPC98* gene fusion, the electrophoretic mobility of the 90 kDa SPB component in isolated SPBs was reduced to that of Gal4p–Spc98p.

Nuclear localization signal of Spc98p

We noticed that overexpressed Spc98p accumulated in the nucleus. Further analysis identified a functional NLS between amino acids 550 and 631 of Spc98p which directed β-galactosidase into the yeast nucleus. This Spc98p fragment contains a bipartite NLS (amino acids 599–614) with two clusters of positively charged amino acids separated by a spacer of 10 amino acids (Dingwall and Laskey, 1991). No other potential NLS was identified on Spc98p. Interestingly, Spc98p is associated with the outer and inner plaques of the SPB, which are located on the cytoplasmic and nuclear sides of the nuclear envelope (Figure 11; Byers and Goetsch, 1975; Rout and Kilmartin, 1990). Since the yeast nuclear envelope stays intact during the entire cell cycle, the functional NLS of Spc98p raises the question of how Spc98p is incorporated into SPB substructures in both the nucleus and the cytoplasm.

SPB duplication is initiated in early G_1 phase of the cell cycle with the formation of the satellite on the cytoplasmic side of the nuclear envelope (Figure 11; Byers and Goetsch, 1975). The SPB is then fully duplicated after Start of the cell cycle. How the SPB duplicates and inserts into the nuclear envelope is not understood, especially since no intermediates have been observed in wild-type cells by electron microscopy (Byers, 1981). In *MPS2* (Winey *et al.*, 1991) and *NDC1* (Winey *et al.*, 1993) mutants SPB duplication is defective, so that cells arrest with an incomplete SPB lacking an inner plaque, which remains on the cytoplasmic side of the nuclear envelope. From these observations it was concluded that a precursor of the SPB is formed in the cytoplasm and then inserted into the nuclear envelope (Winey *et al.*, 1991). The nuclear localization of Spc98p suggests that this SPB component is incorporated into the inner plaque after insertion of the SPB precursor into the nuclear envelope.

The question remains as to how Spc98p is assembled into the outer plaque of the SPB, which is located on the cytoplasmic side of the nuclear envelope. One possibility is that a fraction of Spc98p is retained in the cytoplasm by an interacting protein. This Spc98p would then be assembled in the outer plaque of the SPB. Nuclear uptake control by such a specialized accessory protein has been demonstrated for IκB, which binds to the transcription factor NF-κB and thereby prevents nuclear import (Henkel *et al.*, 1992). In addition, the nucleocytoplasmic distribution of Spc98p could be regulated by modification, e.g. phosphorylation. One prominent example of the role of phosphorylation in controlling nuclear import is the transcription factor Swi5p, which is transported into the nucleus in the G_1 phase of the cell cycle. Phosphorylation of three Ser residues proximal to the bipartite NLS by Cdc28p kinase prevents import into the nucleus during the S, G_2 and M phases of the cell cycle (Moll *et al.*, 1991). Alternatively, the NLS of Spc98p may be inefficient, ensuring that a portion of Spc98p remains in the cytoplasm.

Interaction of Tub4p with Spc98p

Strongly elevated Spc98p levels are toxic to yeast cells. Cells arrested with a large bud and replicated DNA, but with only one DAPI staining region and a defective spindle structure. Other components of the yeast cytoskeleton cause toxicity after overexpression. Overexpression of the SPB component Kar1p (Rose and Fink, 1987; Spang *et al.*, 1995) prevents SPB duplication. Elevated β-tubulin levels (*TUB2*) arrest cells in the G_2 phase of the cell cycle. These cells are devoid of microtubules and accumulate β-tubulin in a novel structure (Burke *et al.*, 1989). Further, overexpression of actin is toxic to yeast cells (Magdolen *et al.*, 1993). All these proteins are part of complex structures which require coordinated participation of multiple elements. The order of assembly in such complexes is determined by the affinity and concentration of the subunits. This is suggested from phage studies, where the assembly of protein complexes was sensitive to the relative dosage of the components and required precise stoichiometries (Floor, 1970). Co-overexpression of an interacting protein may therefore restore the stoichiometry required for complex formation. Examples are Rbl2p and α-tubulin, which rescue β-tubulin overexpression lethality (Katz *et al.*, 1990; Archer *et al.*, 1995). Rbl2p is a yeast protein that binds β-tubulin (Archer *et al.*, 1995). Similarly, co-overexpression of the actin binding protein profilin neutralized the toxic effect of elevated actin levels (Magdolen *et al.*, 1993). By analogy with these examples, the rescuing effect of Tub4p overexpression may be explained by the binding of Tub4p to Spc98p.

Additional evidence for the concerted action of Spc98p and Tub4p comes from the multiple genetic interactions of the corresponding genes. *SPC98* was identified as a dosage-dependent suppressor of *tub4-1*, while *TUB4*

suppressed *spc98-4* when present in multiple copies. In addition, *SPC98* and *TUB4* show synthetic toxicity. To explain these observations, we propose a model in which Spc98p and Tub4p first form a complex that then functions in microtubule attachment. Spc98p–Tub4p complex formation would be unstable in *tub4* or *spc98* cells. Overexpression of the wild-type partner would shift the equilibrium towards the Spc98p–Tub4p complex, thereby suppressing the temperature-sensitive phenotype. In agreement with such a model, we found that an additional copy of *tub4-1* reduced the temperature-sensitive phenotype of *tub4-1* cells. Synthetic toxicity of *tub4* and *spc98* is explained by the strongly reduced interaction of the mutated Spc98p* and Tub4p* proteins, causing lethality even at the permissive temperature. The toxicity of overexpressed *SPC98* may be explained by the competition of free Spc98p with potential Spc98p–Tub4p complex binding sites at the SPB.

Such a model predicts that Spc98p and Tub4p physically interact and are therefore in close proximity at the SPB. Physical interaction of Spc98p and Tub4p is indicated by the two-hybrid experiments, in which a positive signal was only obtained when *TUB4* was combined with *SPC98*. In agreement with an interaction of Spc98p and Tub4p, these proteins localized to the same sections of the inner or outer plaques of the SPB, as suggested by double labelling immunoelectron microscopy experiments (Rout and Kilmartin, 1990; Spang *et al.*, 1996). In addition, co-immunoprecipitation suggests that Tub4p and Spc98p are part of one complex. In view of the large size of the SPB plaque structures, it seems likely that Spc98p and Tub4p may be part of an even larger complex containing multiple subunits.

Interestingly, the phenotype of *spc98-1* cells may be the result of a weaker interaction between Tub4p and Spc98p. This is suggested by the two-hybrid experiments, in which the *GAL4–spc98-1 LexA–TUB4* cells gave a drastically reduced signal compared with *GAL4–SPC98 LexA–TUB4* cells. *spc98-1* cells duplicated and separated their SPBs, but did not form a mitotic spindle under restrictive conditions, suggesting that Spc98p has an essential function in mitotic spindle formation. Similar phenotypes were observed in *tub4-1* cells (Spang *et al.*, 1996), raising the possibility that *spc98-1* and *tub4-1* cells are defective in the same step in spindle formation. More detailed studies, using additional *spc98(ts)* alleles, are required to understand the role of Spc98p in microtubule organization.

Microtubule ends of *S.cerevisiae* which are in contact with the SPB are sealed by a cap-like terminal component connecting the walls of the microtubule cylinder (Byers *et al.*, 1978). Considering the function of Tub4p and Spc98p in microtubule organization and their location in the SPB, Tub4p and Spc98p may be involved in the formation of this microtubule cap structure. A microtubule cap with a somewhat different structure formed by a γ-tubulin-containing complex at the minus ends of microtubules was recently identified by *in vitro* (Zheng *et al.*, 1995) and *in situ* (Moritz *et al.*, 1995) studies. It will be interesting to see whether Spc98p is homologous to one of the proteins that form the γ-tubulin ring complex (Zheng *et al.*, 1995). Taking the association of Tub4p with the SPB and its function in microtubule organization into account, Tub4p may be a highly divergent γ-tubulin adapted to the specialized microtubule attachment structures in *S.cerevisiae*.

Materials and methods

Strains, media and general methods

Yeast strains are listed in Table II. Basic yeast methods and growth media were as described by Guthrie and Fink (1991). *Escherichia coli* strains were transformed by electroporation (Dower *et al.*, 1988). Recombinant DNA methodology was performed as described in Sambrook *et al.* (1989). DNA sequences of cloned fragments and all PCR products were determined by the dideoxy chain termination method (Sanger *et al.*, 1977).

Cloning of SPC98 and the screen for multi-copy suppressors of spc98

Strain ESM208 (*tub4-1*) was transformed with a yeast genomic bank constructed in the 2 μm plasmid YEp13 (Lagosky *et al.*, 1987). Transformants (20 000) were tested for growth at 37°C. DNA of temperature-resistant ESM208 transformants was extracted (Ausubel *et al.*, 1994) and transformed in *E.coli* DH5α. Multi-copy suppressors of ESM266 were identified as described for ESM208, however, a yeast genomic bank constructed in the *URA3*-based 2 μm plasmid pSEY8 was used (Heitmann *et al.*, 1988).

Cloning of SPC98 by screening λgt11 expression banks with pooled anti-90 kDa monoclonal antibodies

A total of 3.4×10^7 plaques were screened with the pooled anti-90 kDa monoclonal antibodies using three independent genomic DNA expression banks and one cDNA expression bank. One positive was obtained from the cDNA bank (Clontech YL1008b) which contained the 5′-half of the open reading frame of *SPC98* up to the *Eco*RI site at 1081. All four anti-90 kDa monoclonal antibodies (Rout and Kilmartin, 1990) reacted with this insert.

Plasmids

Plasmids are listed in Table II. Plasmid p2-2-1 was one of the suppressor clones in YEp13. A 5.0 kb *Sal*I fragment of p2-2-1 was cloned in pRS425 (Christianson *et al.*, 1992; pSM271). Subclones of the 5.0 kb *Sal*I fragment of pSM271 were constructed as outlined in Figure 1A. For construction of pSM288, *SPC98* was amplified by PCR and then ligated into pRS425. The 3.7 kb *Sac*I fragment of pSM288 carrying *SPC98* was cloned into the *Sac*I sites of pRS315 (Sikorski and Hieter, 1989; pSM297) and pRS316 (Sikorski and Hieter, 1989; pSM296) respectively. For *SPC98* overexpression, a fragment containing *SPC98* was amplified by PCR and cloned into the yeast expression vector pYES2 (Invitrogen, pSM289). A *GST–SPC98* gene fusion for *GAL1*-controlled expression in yeast was constructed in the yeast expression vector pEG(KT) (Mitchell *et al.*, 1993; pGP2). A *GST–SPC98* gene fusion for expression in *E.coli* was obtained using expression vectors pGEX-5X-1 from Pharmacia (pSM290). The ATG start codon of *SPC98* was mutated to ACG by directed PCR mutagenesis. The PCR product was cloned into pRS315 to give pSM332. For construction of epitope-tagged *MYC–SPC98* or *HA–SPC98* gene fusions, a *Not*I restriction site was introduced by PCR between the ATG initiation codon and the coding region of *SPC98* (pSM331). A small DNA fragment coding for the MYC or three HA epitopes was ligated into the *Not*I restriction site of pSM331 (pSM339 or pSM338). To obtain a Δ*spc98*::*HIS3* disruption cassette, pSM271 was restricted with *Sal*I and then partially restricted with *Hpa*I. The 3.6 kb *Sal*I–*Hpa*I fragment carrying *SPC98* was cloned into the *Xho*I–*Sma*I restriction sites of pBlueskript SK (pSM276). *HIS3* on a 1.7 kb *Bam*HI fragment was inserted into the *Bcl*I sites (pSM294). The 3.6 kb *Bst*XI–*Kpn*I fragment of pSM276 was cloned into pRS425 (pSM279). The 244 bp *Bam*HI–*Xba*I restriction fragment of pSM271 coding for the NLS of Spc98p was ligated into vector YCpIF5 (Foreman and Davis, 1994; pSM303). The *Kpn*I–*Xba*I fragment of pSM303 carrying *GAL1–HA–*Δ*spc98* was then cloned into YEp357 (Myers *et al.*, 1986; pSM305). The 550 bp *Eco*RI–*Hin*dIII fragment of pSM204 was cloned into pRS306 (Sikorski and Hieter, 1989; pSM351). *GAL1–TUB4* of pSM209 (Spang *et al.*, 1996) was ligated into pRS425 (pSM313). The γ-tubulin gene from *X.laevis* (Stearns *et al.*, 1991) was cloned into pRS425-Gal1 (Mumberg *et al.*, 1995; pSM316). Plasmids for the two-hybrid system were constructed using vectors pEG202 (Gyuris *et al.*, 1993) and pACTII (Durfee *et al.*, 1993). PCR fragments carrying *SPC110* (Kilmartin *et al.*, 1993), *TUB4* (Spang *et al.*, 1996) and the γ-tubulin

Table II. Yeast strains and plasmids

Name	Genotype/construct	Source or reference
Yeast strains		
ESM184	MATα *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δtub4::HIS3* pSM222	Spang *et al.* (1996)
ESM208	MATα *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 tub4-1*	Spang *et al.* (1996)
ESM243	MATa *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc98::HIS3* pSM296	this study
ESM261	MATα *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 tub4-1*::pSM244	this study
ESM266-1	MATa *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*::pSM321 *Δspc98::HIS3*	this study
ESM266-4	MATa *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*::pSM324 *Δspc98::HIS3*	this study
ESM277	MATa/α *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1*::pSM321 *TUB4/tub4-1*::pSM244 *SPC98/Δspc98::HIS3*	this study
ESM278	MATα *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc98-1*	this study
ESM279	MATa *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc98::HIS3* pSM338	this study
ESM281	MATa *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc98::HIS3* pSM339	this study
ESM283	MATa *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*::pSM342 *Δspc98::HIS3*	this study
ESM294	MATα *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 TUB4*::pSM351	this study
GPY7-1	YPH499 pYES2	this study
GPY7-2	YPH499 pSM289 (*GAL1-SPC98*)	this study
SGY26	MATa/α *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 SPC98/Δspc98::HIS3*	this study
SGY34	MATa *ura3-52 lys2-801 ade2-101 trp1*D*63 his3*D*200 leu2*D*1* D*spc98::HIS3* pSG26 (*GAL4-SPC98*)	this study
SGY37	MATa *ura3-52::URA3-lexA-op-lacZ trp1 his3 leu2*	this study
W276	MATα *ura3-52::URA3-lexA-op-lacZ trp1 his3 leu2::lexA-op-LEU2*	W.Seufert
YPH499	MATa *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski and Hieter (1989)
YPH500	MATα *ura3-52 lys2-801 ade2-101 trp1*D*63 his3*D*200 leu2*D*1*	Sikorski and Hieter (1989)
YPH501	MATa/α *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1*	Sikorski and Hieter (1989)
Plasmids		
pACTII	2 μm, *LEU2*-based vector carrying the *GAL4* activator domain	Durfee *et al.* (1993)
pEG(KT)	2 μm vector for the expression of GST gene fusions under control of the *GAL1* promoter	Mitchell *et al.* (1993)
pGP2	3.4 kb fragment of pSG25 in pEG(KT): *GAL1–GST–SPC98*	this study
pMK1-3.2	chromosomal fragment carrying *TUB4* in pSEY8	this study
pRY161	2 μm, *LEU2*-based, *GAL1–lacZ*	CSH, Yeast Course
pSEY8	2 μm, *URA3*-based yeast–*E.coli* shuttle vector	Heitmann *et al.* (1988)
pSG26	*SPC98* in pACTII	this study
pSM204	*TUB4* in pRS315	Spang *et al.* (1996)
pSM222	*TUB4–HA* in pRS316	Spang *et al.* (1996)
pSM244	*tub4-1* in pRS306	Spang *et al.* (1996)
pSM271	5.0 *Sal*I fragment of p2-2-1 carrying *SPC98* in pRS425	this study
pSM289	*SPC98* in pYES2: *GAL1–SPC98*	this study
pSM296	*SPC98* in pRS316	this study
pSM297	*SPC98* in pRS315	this study
pSM313	*GAL1–TUB4* in pRS425	this study
pSM316	γ-tubulin gene from *X.laevis* in p425-Gal1: *GAL1–Xgam*	this study
pSM321	*spc98-1* in pRS305	this study
pSM324	*spc98-4* in pRS305	this study
pSM338	*HA-SPC98* in pRS315	this study
pSM339	*MYC-SPC98* in pRS315	this study
pSM342	*SPC98* in pRS305	this study
pSM351	550 bp *Eco*RI–*Hin*dIII fragment of pSM204 (3′-end of *TUB4*) in pRS306	this study
pSM355	derivative of pMK1-3.2 carrying a 926 bp *Cla*I deletion in *TUB4*	this study
pSM356	*SPC110* in pRS426	this study
pRS305	*LEU2*-based integration vector	Sikorski and Hieter (1989)
pRS306	*URA3*-based integration vector	Sikorski and Hieter (1989)
pRS315	*CEN6*, *LEU2*-based yeast–*E.coli* shuttle vector	Sikorski and Hieter (1989)
pRS316	*CEN6*, *URA3*-based yeast–*E.coli* shuttle vector	Sikorski and Hieter (1989)
pRS425	2 μm, *LEU2*-based yeast–*E.coli* shuttle vector	Christianson *et al.* (1992)
pRS426	2 μm, *URA3*-based yeast–*E.coli* shuttle vector	Christianson *et al.* (1992)
pYES2	2 μm, *URA3*-based yeast–*E.coli* shuttle vector carrying the *GAL1* promoter	Invitrogen
p425-Gal1	2 μm, *LEU2*-based yeast–*E.coli* shuttle vector carrying the *GAL1* promoter	Mumberg *et al.* (1995)

genes from *Xgam* and *S.pombe* (Stearns *et al.*, 1991) were cloned into pEG202 to give plasmids pSG14, pSG21, pSG35 and pSG36 respectively. Fragments of *TUB4* coding for amino acids 1–306 or 168–473 were ligated into pEG202 to give pSG33 and pSG34 respectively. PCR fragments carrying *SPC98*, *spc98-1* or *SPC110* (Mirzayan *et al.*, 1992; coding for amino acids 146–846) were inserted into pACTII to give pSG26, pSG38 or pSG28 respectively. Subclones of *SPC98* coding for amino acids 147–846, 147–551 and 390–846 were inserted in pACTII to give pSG29, pSG30 and pSG32 respectively.

Construction of yeast strains

SGY26. YPH501 (Sikorski and Hieter, 1989) was transformed with the *Δspc98::HIS3* cassette of plasmid pSM294.

ESM243. SGY26 was transformed with the *URA3*-based pSM296. After sporulation, a colony which was His^+ Ura^+ was named ESM243.

ESM279 and ESM281. ESM243 was transformed with plasmids pSM338 (pRS315-*HA-SPC98*) or pSM339 (pRS315-*MYC-SPC98*). Cells which spontaneously lost plasmid pSM296 were selected for on 5-FOA plates.

ESM261. ESM208 (*tub4-1*; Spang *et al.*, 1996) was transformed with the integration plasmid pSM244 previously restricted with *Bgl*II.

ESM278. YPH500 was transformed with plasmid pSM326 (*spc98-1* on pRS306) previously restricted with *Nru*I, selection being made on SC plates lacking uracil. Transformants were incubated at 23°C on 5-FOA-containing plates. 5-FOA^R clones which were temperature sensitive for growth were transformed with *SPC98* on plasmid pRS316. *SPC98* on pRS316 restored growth at 37°C, indicating that *spc98-1* is recessive.

ESM294. YPH500 was transformed with pSM351 restricted with *Bcl*I.

ESM266-1, ESM266-4 and ESM283. spc98-1, spc98-4 and *SPC98* were cloned into integration vector pRS305 to give plasmids pSM321, pSM324 and pSM342 respectively. These plasmids were restricted with *Bst*EII. The linearized plasmids were then integrated into the *LEU2* locus of strain ESM243. 5-FOA-resistant colonies were named ESM266-1 (*leu2Δ1*::pSM321), ESM266-4 (*leu2Δ1*::pSM324) and ESM283 (*leu2Δ1*::pSM342) respectively.

ESM288, ESM277 and ESM296. Strains ESM261 and ESM283, ESM261 and ESM266-1 or ESM294 and ESM266-1 were crossed to give ESM288, ESM277 and ESM296 respectively.

SGY37. Strain W276 was crossed to YPH499. A spore which was Ura$^+$ Trp$^-$ Leu$^-$ and Ade$^+$ was named SGY37.

SGY34. This was obtained by sporolation of SGY26 (*SPC98/Δspc98::HIS3*) transformed with pSG26 (*GAL4–SPC98*). Spores which were His$^+$ Leu$^+$ were named SGY34.

Construction of spc98 (ts) alleles

SPC98 was mutagenized by PCR according to Cadwell and Joyce (1992). Conditional lethal alleles of *SPC98* were obtained as described by Muhlrad *et al.* (1992).

Purification of GST–Spc98p fusion protein and polyclonal anti-Spc98p antibodies

The GST–Spc98p fusion protein was expressed and purified by affinity purification using glutathione–Sepharose from Pharmacia. Antibodies against the purified protein were raised in rabbits as described by Harlow and Lane (1988). The anti-Spc98p antibodies were purified as described by Spang *et al.* (1995).

Cell extracts, isolation of nuclei, isolation of SPBs and overexpression of SPC98

Cell extracts were prepared as described by Ausubel *et al.* (1994). Nuclei and SPBs were isolated from strains YPH499 and SGY34 as described by Rout and Kilmartin (1990). Strains GPY7-1 (pYES2) and GPY7-2 (*GAL1–SPC98*) were grown in SC medium lacking uracil with raffinose as a carbon source. Galactose was added to half of the cultures at a density of 5×10^6 cells/ml. Glucose was added to the other half.

Immunofluorescence microscopy and immunoblots

Immunofluorescence of yeast cells was performed as described by Spang *et al.* (1995) or Rout and Kilmartin (1990). The primary antibodies were either a pool of mouse monoclonal anti-90 kDa (Rout and Kilmartin, 1990), rabbit anti-Kar1p (Spang *et al.*, 1995), mouse monoclonal anti-β-tubulin WA3 (Spang *et al.*, 1995), mouse monoclonal anti-MYC or mouse monoclonal anti-β-galactosidase (Boehringer Mannheim) antibodies. Secondary antibodies were goat anti-mouse IgG coupled to CY3 or goat anti-rabbit IgG coupled to FITC or CY3 from Jackson ImmunoResearch Laboratories. DNA was stained with DAPI.

Protein concentrations of samples were determined by the method of Bradford (1976). The proteins were separated by SDS–PAGE (Laemmli, 1970) and transferred onto nitrocellulose (Spang *et al.*, 1993). The primary antibodies were mouse monoclonal 35B5 (Rout and Kilmartin, 1990), rabbit anti-Spc98p, rabbit anti-Spc110p (Spang *et al.*, 1995) or mouse 12CA5 (Babco) antibodies. As secondary antibodies rabbit anti-mouse or goat anti-rabbit IgGs coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories) were used. The immunoreaction was visualized with an ECL kit from Amersham.

Flow cytometry

Cells were prepared for flow cytometry as described by Hutter and Eipel (1979). The DNA content of 20 000 cells was determined using a flow cytometer (Facscalibur; Becton-Dickinson).

Two-hybrid assay for the interaction of Tub4p and Spc98p

Gene fusions with the DNA binding domain of *LexA* were made using plasmid pEG202 (Gyuris *et al.*, 1993). Plasmid pACTII (Durfee *et al.*, 1993) was chosen as the activation domain vector. Derivatives of pEG202 and pACTII were transformed into strain SGY37 bearing the *lexA* operator–*lacZ* reporter construct. For the assay of β-galactosidase activity colonies were grown at 30°C on X-Gal plates lacking histidine and leucine (Gyuris *et al.*, 1993). Raffinose (2%) and galactose (2%) were used as carbon sources. We established that LexA–Tub4p and LexA-C–Tub4p were present in about equal amounts using antibodies directed against the C-terminal portion of Tub4p (Spang *et al.*, 1996). Further, protein levels of the inactive Gal4p–ΔSpc98p$^{147-551}$ and Gal4p–ΔSpc98p$^{390-846}$ constructs were even higher compared with the active Gal4p–Spc98p and Gal4p–ΔSpc98p$^{147-846}$. This was tested with 12CA5 antibody, which is directed against the HA tags of the Gal4p fusion proteins (Durfee *et al.*, 1993). *GAL4–ΔSPC110* and *LexA–SPC110* were expressed in yeast cells, as shown by anti-Spc110p antibodies (Spang *et al.*, 1995; data not shown).

Immunoprecipitation experiments

Cultures (400 ml) of *TUB4–HA* (ESM184), *TUB4* (YPH499) or *HA–SPC98* (ESM279) cells were grown in YPD medium at 30°C to a density of 2×10^7 cells/ml. The cells were harvested by centrifugation and washed with water. Cells were resuspended in lysis buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 10 mM EDTA, 1% TX-100, 5% glycerol, 1 mM benzamidine, 5 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 10 mM pepstatin) and strongly vortexed with glass beads at 4°C until 80% of the cells were lysed. Cell lysates were incubated with 12CA5 antibodies (Hiss GmbH) for 1 h at 4°C, followed by an incubation with protein G–Sepharose (Pharmacia) for 30 min at 4°C. Protein G–Sepharose was washed with lysis buffer, lysis buffer containing 1 M NaCl and lysis buffer. The precipitated material was heated with sample buffer and then analysed by immunoblotting using affinity-purified rabbit anti-Tub4p or rabbit anti-Spc98p antibodies.

Acknowledgements

We thank Drs Nasmyth and Seufert for the chromosomal gene banks in vectors YEp13 and pSEY8 respectively. Plasmids pACTII, YCpIF5, pEG202, pEG(KT), YEp357 and p425-Gal1 were gifts from Drs Elledge, Davis, Brent, Deschenes, Lusty and Funk respectively. Strain W276 was obtained from Dr Seufert. Katrin Grein is acknowledged for excellent technical assistance. Anti-MYC antibodies were a gift from Dr Lehner. This work was carried out with support from the BMFT. G.P. is the recipient of a DAAD scholarship and S.S. was supported by an EU HCM Research Network.

References

Archer,J.E., Vega,L.R. and Solomon,F. (1995) Rb12p, a yeast protein that binds to β-tubulin and participates in microtubule function *in vivo*. *Cell*, **82**, 425–434.

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1994) *Current Protocols in Molecular Biology*. John Wiley and Sons, New York, NY.

Bergen,L.G. and Borisy,G.G. (1980) Head-to-tail polymerization of microtubules *in vitro*. *J. Cell Biol.*, **84**, 141–150.

Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.*, **72**, 248–254.

Burke,D., Gasdaska,P. and Hartwell,L. (1989) Dominant effects of tubulin overexpression in *Saccaromyces cerevisiae*. *Mol. Cell. Biol.*, **3**, 1049–1059.

Byers,B. (1981) In Wettstein,D., Stenderup,A., Kielland-Brandt,M. and Friis,J. (eds), *Multiple Roles of the Spindle Pole Bodies in the Life Cycle of Saccharomyces cerevisiae*. Alfred Benzon Symposia, Munksgaard, Copenhagen, Vol. 16, pp. 119–133.

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

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

Cadwell,R.C. and Joyce,G.F. (1992) *Randomization of Genes by PCR Mutagenesis*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Christianson,T.W., Sikorski,R.S., Dante,M., Shero,J.H. and Hieter,P. (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene*, **110**, 119–122.

Dingwall,C. and Laskey,R.A. (1991) Nuclear targeting sequences—a consensus? *Trends Biochem. Sci.*, **16**, 478–481.
Dower,W.J., Miller,J.F. and Ragsdale,C.W. (1988) High efficiency transformation of *E.coli* by high voltage electroporation. *Nucleic Acids Res.*, **16**, 127–145.
Durfee,T., Becherer,K., Chen,P.-L., Yeh,S.-H., Yang,Y., Kilburn,A.E., Lee,W.-H. and Elledge,S.J. (1993) The retinoblastoma protein associates with the protein phosphotase type 1 catalytic subunit. *Genes Dev.*, **7**, 555–569.
Fields,S. and Song,O. (1989) A novel genetic system to detect protein–protein interactions. *Nature*, **340**, 245–236.
Floor,E. (1970) Interaction of morphogenic genes of bacteriophage T4. *J. Mol. Biol.*, **47**, 293–306.
Foreman,P.K. and Davis,R.W. (1994) Cloning vectors for the synthesis of epitope-tagged, truncated and chimeric proteins in *Saccharomyces cerevisiae*. *Gene*, **144**, 63–68.
Guthrie,C. and Fink,G.R. (1991) *Guide to Yeast Genetics and Molecular Biology*. Academic Press, New York, NY.
Gyuris,J., Golemis,E., Chertkov,H. and Brent,R. (1993) Cdi1, a human G1 and S phase protein phosphatase that assiciates with Cdk2. *Cell*, **75**, 791–803.
Harlow,E. and Lane,D. (1988) *Antibodies*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Heitmann,J., Movva,N.R., Hiestand,P.C. and Hall,M.N. (1988) FK 506-binding protein proline rotamase is a target for the immunosupressive agent FK 506 in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **88**, 1948–1952.
Henkel,T., Zabel,U., Zee,K.v., Müller,J.M., Fanning,E. and Baeuerle,P.A. (1992) Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-κB subunit. *Cell*, **68**, 1121–1133.
Horio,T. and Hotani,H. (1986) Visualization of the dynamic instability of individual microtubules by dark-field microscopy. *Nature*, **321**, 605–607.
Horio,T. and Oakley,B.R. (1994) Human γ-tubulin functions in fission yeast. *J. Cell Biol.*, **126**, 1465–1473.
Horio,T., Uzawa,S., Jung,M.K., Oakley,B.R., Tanaka,K. and Yanagida,M. (1991) The fission yeast γ-tubulin is essential for mitosis and is localized at microtubule organizing centers. *J. Cell Sci.*, **99**, 693–700.
Hutter,K.J. and Eipel,H.E. (1979) Microbial determination by flow cytometry. *J. Gen. Microbiol.*, **113**, 369–375.
Joshi,H.C., Palacios,M.J., McNamara,L. and Cleveland,D.W. (1992) γ-Tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. *Nature*, **356**, 80–83.
Katz,W., Weinstein,B. and Solomon,F. (1990) Regulation of tubulin levels and microtubule assembly in *Saccharomyces cerevisiae*: consequences of altered tubulin gene copy number. *Mol. Cell. Biol.*, **10**, 5286–5294.
Kilmartin,J.V., Dyos,S.L., Kershaw,D. and Finch,J.T. (1993) A spacer protein in the *Saccharomyces cerevisiae* spindle pole body whose transcription is cell-cycle regulated. *J. Cell Biol.*, **123**, 1175–1184.
Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
Lagosky,P.A., Taylor,G.R. and Haynes,R.H. (1987) Molecular characterization of the *Saccharomyces cerevisiae* dihydrofolate reductase gene (*DFR1*). *Nucleic Acids Res.*, **15**, 10355–10371.
Magdolen,V., Drubin,D.G., Mages,G. and Bandlow,W. (1993) High levels of profilin supress the lethality caused by overproduction of actin in yeast cells. *FEBS Lett.*, **316**, 41–47.
Mallet,L., Bussereau,F. and Jacquet,M. (1995) A 43.5 kb segment of yeast chromosome XIV, which contains *MFA2*, *MEP2*, *CAP/SRV2*, *NAM9*, *FKB1/FPR1/RBP1*, *MOM22* and *CPT1*, predicts an adenosine deaminase gene and 14 new open reading frames. *Yeast*, **11**, 1195–1209.
McIntosh,J.R. and Euteneuer,U. (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.
Mirzayan,C., Copeland,C.S. and Snyder,M. (1992) The *NUF1* gene encodes an essential coiled-coil related protein that is a potential component of the yeast nucleoskeleton. *J. Cell Biol.*, **116**, 1319–1332.
Mitchell,D.A., Marshall,T.K. and Deschenes,R.J. (1993) Vectors for the inducible overexpression of glutathione S-transferase fusion proteins in yeast. *Yeast*, **9**, 715–723.
Moll,T., Tebb,G., Surana,U., Robitsch,H. and Nasmyth,K. (1991) The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the *S. cerevisiae* transcription factor SWI5. *Cell*, **66**, 743–758.
Moritz,M., Braunfeld,M.B., Sedat,J.W., Alberts,B. and Agard,D.A. (1995) Microtubule nucleation by γ-tubulin-containing rings in the centrosome. *Nature*, **378**, 638–640.
Muhlrad,D., Hunter,R. and Parker,R. (1992) A rapid method for localized mutagenesis of yeast genes. *Yeast*, **8**, 79–82.
Mumberg,D., Müller,R. and Funk,M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene*, **156**, 119–122.
Myers,A.M., Tzagoloff,A., Kinney,D.M. and Lusty,C.J. (1986) Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusion. *Gene*, **45**, 299–310.
Neff,N.F., Thomas,J.H., Grisafi,P. and Botstein,D. (1983) Isolation of the β-tubulin gene from yeast and demonstration of its essential function *in vivo*. *Cell*, **33**, 211–219.
Oakley,B.R., Oakley,E., Yoon,Y. and Jung,M.K. (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,C.E. and Oakley,B.R. (1989) Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mipA* gene of *Aspergillus nidulans*. *Nature*, **338**, 662–664.
Rose,M.D. and Fink,G.R. (1987) *KAR1*, a gene required for function of both intranuclear and extranuclear microtubules in yeast. *Cell*, **48**, 1047–1060.
Rout,M.P. and Kilmartin,J.V. (1990) Components of the yeast spindle and spindle pole body. *J. Cell Biol.*, **111**, 1913–1927.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.
Schatz,P.J., Pillus,L., Grisafi,P., Solomon,F. and Botstein,D. (1986) Two functional α-tubulin genes of the yeast *Saccharomyces cerevisiae* encode divergent proteins. *Mol. Cell. Biol.*, **6**, 3711–3721.
Sikorski,R.S. and Hieter,P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19–27.
Sobel,S.G. and Snyder,M. (1995) A highly divergent γ-tubulin gene is essential for cell growth and proper microtubule organization in *Saccharomyces cerevisiae*. *J. Cell Biol.*, **131**, 1775–1788.
Spang,A., Courtney,I., Fackler,U., Matzner,M. and Schiebel,E. (1993) The calcium-binding protein cell division cycle 31 of *Saccharomyces cerevisiae* is a component of the half bridge of the spindle pole body. *J. Cell Biol.*, **123**, 405–416.
Spang,A., Courtney,I., Grein,K., Matzner,M. and Schiebel,E. (1995) The Cdc31p-binding protein Kar1p is a component of the half-bridge of the yeast spindle pole body. *J. Cell Biol.*, **128**, 863–877.
Spang,A., Geissler,S., Grein,K. and Schiebel,E. (1996) The γ-tubulin like Tub4p of *Saccharomyces cerevisiae* is associated with the spindle pole body substructures that organize microtubules and is required for mitotic spindle formation. *J. Cell. Biol.*, in press.
Stearns,T. and Kirschner,M. (1994) *In vitro* reconstitution of centrosome assembly and function: the central role of γ-tubulin. *Cell*, **76**, 623–637.
Stearns,T., Evans,L. and Kirschner,M. (1991) γ-Tubulin is a highly conserved component of the centrosome. *Cell*, **65**, 825–836.
Sunkel,C.E., Gomes,R., Sampaio,P., Perdigao,J. and Gonzáles,C. (1995) γ-Tubulin is required for the structure and function of the microtubule organizing centre in *Drosophila* neuroblasts. *EMBO J.*, **14**, 28–36.
Weil,C.F., Oakley,C.E. and Oakley,B.R. (1986) Isolation of *mip* (microtubule-interacting protein) mutations of *Aspergillus nidulans*. *Mol. Cell. Biol.*, **6**, 2963–2968.
Winey,M., Goetsch,L., Baum,P. and Byers,B. (1991) *MPS1* and *MPS2*: novel yeast genes defining distinct steps of spindle pole body duplication. *J. Cell Biol.*, **114**, 745–754.
Winey,M., Hoyt,M.A., Chan,C., Goetsch,L., Botstein,D. and Byers,B. (1993) NDC1: a nuclear periphery component required for yeast spindle pole body duplication. *J. Cell Biol.*, **122**, 743–751.
Zheng,Y., Jung,K. and Oakley,B.R. (1991) γ-Tubulin is present in *Drosophila melanogaster* and *Homo sapiens* and is associated with the centrosome. *Cell*, **65**, 817–823.
Zheng,Y., Wong,M.L., Alberts,B. and Mitchison,T. (1995) Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. *Nature*, **378**, 578–583.

Received on February 20, 1996; revised on April 22, 1996

Note added in proof

The numbering in Figure 4C is incorrect, the indicated NLS, corresponds to amino acids 580–595.