The spindle pole body component Spc97p interacts with the γ -tubulin of *Saccharomyces cerevisiae* and functions in microtubule organization and spindle pole body duplication

Michael Knop, Gislene Pereira, Silke Geissler, Katrin Grein and Elmar Schiebel¹

Max-Planck Institut für Biochemie, Am Klopferspitz 18a, 82152 Martinsried, Germany

¹Corresponding author

Previously, we have shown that the γ -tubulin Tub4p and the spindle pole body component Spc98p are involved in microtubule organization by the yeast microtubule organizing centre, the spindle pole body (SPB). In this paper we report the identification of *SPC97* encoding an essential SPB component that is in association with the SPB substructures that organize the cytoplasmic and nuclear microtubules. Evidence is provided for a physical and functional interaction between Tub4p, Spc98p and Spc97p: first, temperature-sensitive *spc97(ts)* mutants are suppressed by high gene dosage of *SPC98* or *TUB4*. Second, Spc97p interacts with Spc98p and Tub4p in the two-hybrid system. Finally, immunoprecipitation and fractionation studies revealed complexes containing Tub4p, Spc98p and Spc97p. Further support for a direct interaction of Tub4p, Spc98p and Spc97p comes from the toxicity of strong *SPC97* overexpression which is suppressed by co-overexpression of *TUB4* or *SPC98*. Analysis of temperature-sensitive *spc97(ts)* alleles revealed multiple spindle defects. While *spc97-14* cells are either impaired in SPB separation or mitotic spindle formation, *spc97-20* cells show an additional defect in SPB duplication. We discuss a model in which the Tub4p–Spc98p–Spc97p complex is part of the microtubule attachment site at the SPB.

Keywords: microtubule organization/ γ -tubulin/Tub4p/Spc97p/Spc98p/spindle pole body

Introduction

The generic term microtubule organizing centre (MTOC) has been coined by Picket-Heaps (1969) to collectively define the microtubule nucleation activity of the morphologically distinct centrosomes, basal bodies, spindle pole bodies (SPBs) and nucleus-associated bodies. How these structurally diverse MTOCs fulfil their common microtubule organizing function is becoming clearer. A genetic approach identified a new class of tubulin, named γ -tubulin, as a suppressor of a temperature-sensitive β -tubulin mutation in the fungus *Aspergillus nidulans* (Weil *et al.*, 1986; Oakley *et al.*, 1990). Since then γ -tubulins have been identified in many different organisms, including human, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Xenopus laevis* and plant cells (Horio *et al.*, 1991; Stearns

et al., 1991; Zheng *et al.*, 1991; Liu *et al.*, 1994; Lopez *et al.*, 1995). These γ -tubulins reveal sequence identities of ~65–70% among themselves, while the identities to the α - and β -tubulin families are in the range 30% (Burns, 1995). Subcellular localization studies identified γ -tubulin as a universal component of MTOCs (Oakley *et al.*, 1990; Horio *et al.*, 1991; Stearns *et al.*, 1991; Zheng *et al.*, 1991). In mammalian centrosomes, γ -tubulin is located in the pericentriolar material, the centrosomal substructure that nucleates microtubule assembly (Stearns *et al.*, 1991). Additional γ -tubulin is concentrated on the surface and in the lumen of isolated centrioles (Moudjou *et al.*, 1996). Experimental evidence suggests that γ -tubulin at the MTOC functions in microtubule organization. Depletion of the essential γ -tubulin in *A.nidulans* (Oakley *et al.*, 1990), *S.pombe* (Horio *et al.*, 1991) and *Drosophila* (Sunkel *et al.*, 1995) caused microtubule defects. Furthermore, microtubule nucleation was disturbed after microinjection of anti- γ -tubulin antibodies into mammalian cells (Joshi *et al.*, 1992) and a γ -tubulin complex obtained from mitotic *Xenopus* egg extracts nucleated microtubules *in vitro* (Zheng *et al.*, 1995).

Recent studies suggest that γ -tubulin functions together with other proteins. *Drosophila* γ -tubulin is part of a complex containing at least two centrosomal microtubule-associated proteins called CP190 and CP60 (Raff *et al.*, 1993; Kellogg *et al.*, 1995; Whitfield *et al.*, 1995). γ -tubulin is also present in a 25S complex in the cytoplasm of mitotic frog extracts (Zheng *et al.*, 1995) and vertebrate somatic cells (Stearns and Kirschner, 1994; Moudjou *et al.*, 1996). Purification of this complex from *X.laevis* identified at least seven proteins, including α -, β - and γ -tubulin, and proteins with apparent molecular weights of 75, 109, 133 and 195 kDa. This γ -tubulin complex is seen as an open ring structure by electron microscopy and acts as an active microtubule-nucleating unit capping the minus end of microtubules *in vitro* (Zheng *et al.*, 1995).

The *Saccharomyces cerevisiae* genome sequencing project identified *TUB4*, encoding γ -tubulin in this organism. Tub4p has been localized by indirect immunofluorescence to the SPB (Sobel and Snyder, 1995). Subsequent studies identified Tub4p as a component of SPB substructures, the outer and the inner plaques (Spang *et al.*, 1996a; Figure 10B), that organize the cytoplasmic and nuclear microtubules respectively. Cells carrying a temperature-sensitive *tub4(ts)* allele showed, depending on the mutant allele, a defect either in microtubule nucleation of the newly formed SPB (Marschall *et al.*, 1996) or in the assembly of a mitotic spindle (Spang *et al.*, 1996a) suggesting at least two functions of Tub4p in microtubule organization.

SPC98 was found as a dosage-dependent suppressor of the conditional lethal phenotype of *tub4-1* cells (Geissler *et al.*, 1996). It codes for the 90 kDa SPB protein, described

previously by Rout and Kilmartin (1990). Genetic and biochemical evidence suggest that Tub4p and Spc98p interact physically and that both proteins function together in microtubule organization by the SPB (Geissler *et al.*, 1996). In agreement with this concerted action is the association of Spc98p with the same SPB substructures as Tub4p (Rout and Kilmartin, 1990; Figure 10B).

Here we report the identification of *SPC97* encoding a further SPB component involved in microtubule organization. We obtained evidence that Tub4p, Spc98p and Spc97p interact physically with each other and that the three proteins are present in the same complexes. Analysis of temperature-sensitive *spc97(ts)* mutants revealed multiple spindle and SPB defects. While *spc97-20* cells were mainly defective in SPB duplication, most of the *spc97-14* cells duplicated the SPB, but were impaired either in SPB separation or in mitotic spindle formation. The relevance of these findings is discussed together with a model in which Tub4p, Spc98p and Spc97p are components of a complex involved in microtubule organization by the SPB.

Results

SPC97 is a dosage-dependent suppressor of spc98-2

We have reported previously that Tub4p and Spc98p are part of a complex involved in microtubule organization by the yeast SPB (Geissler *et al.*, 1996). To identify further components of this complex, we screened for dosage-dependent suppressors of the temperature-dependent growth defect of *spc98-2* cells. The plasmids isolated from suppressed *spc98-2* cells contained *SPC98*, *TUB4* or a gene of unknown function (ORF YHR172w on Chromosome VIII; Johnston *et al.*, 1994) which we named *SPC97* (Spindle Pole body Component with a calculated molecular mass of 97 kDa). Subcloning experiments revealed that *SPC97* was responsible for suppression of *spc98-2* cells (Figure 1A). *SPC97* encodes a protein of 823 amino acids with no homology to Spc98p or to any protein in the database.

SPC97 encodes an essential SPB component

To test whether *SPC97* is essential for cell viability, we analyzed tetrads of the heterozygous *SPC97* deletion strain YMK1-2 (*SPC97::Δspc97::HIS3*). Only two out of the four spores from each tetrad were able to form colonies. These were all His⁻, indicating that the disruption of *SPC97* is lethal for cells (data not shown). This result was confirmed by a plasmid shuffle experiment. Strain YMK10 (*Δspc97::HIS3* pMK8) has a disruption of *SPC97* and is kept alive by *SPC97* on the *URA3*-based plasmid pMK8. YMK10 transformed with the control vector pRS414 was unable to grow on plates containing 5-fluoroorotic acid (5-FOA), which selects against the *URA3* plasmid (Figure 1B, sector pRS414). However, strain YMK10 was able to grow on 5-FOA when a second *SPC97* allele was present, encoded by the *TRP1*-based plasmid pMK26-16 (Figure 1B, sector pRS414-*SPC97*). Summarizing, the spore analysis and the results of the plasmid shuffle experiment are consistent with an essential function of *SPC97* for the viability of yeast cells.

We tested whether Spc97p is a component of the SPB.

Fig. 1. *SPC97* is a dosage-dependent suppressor of *spc98-2* and its gene product is associated with the SPB. (A) A 2 μm-based plasmid (pMK1-10.1) containing a fragment of DNA from chromosome VIII was able to suppress the temperature-dependent growth defect of *spc98-2* cells. The cloned DNA fragment carried three open reading frames (ORF). Subcloning of DNA fragments and suppression analysis revealed that ORF YHR172w was responsible for suppression. ORF YHR172w was designated *SPC97*. For chromosomal gene disruption, the indicated part of YHR172w was replaced by the *HIS3* gene. (B) *SPC97* is essential for growth. Cells of YMK10 (*Δspc97::HIS3* pMK8) containing the *TRP1*-based plasmids pRS414-*SPC97* (pMK26-16), pRS414 or pRS414-*SPC97-3HA* (pMK29-4) were streaked on 5-FOA plates which select against the *URA3*-based plasmid pRS316-*SPC97* (pMK8). Failure of YMK10 (pRS414) cells to grow on 5-FOA indicated that *SPC97* is essential. (C) *SPC97* encodes an SPB component. Spc97p-3HA and Kar1p of YMK22 (*Δspc97::HIS3* pRS414-*SPC97-3HA*) were detected by indirect immunofluorescence. DNA was stained with DAPI. Bar: 5.0 μm.

The subcellular localization of the functional epitope-tagged Spc97p-3HA protein (Figure 1B, sector pRS414-*SPC97-3HA*) was investigated by indirect immunofluorescence with monoclonal anti-HA antibody. Spc97p-3HA was detected as one or two dots per cell near the nuclear periphery (Figure 1C), a staining pattern which is typical for a SPB component (Rout and Kilmartin, 1990; Geissler *et al.*, 1996). The Spc97p-3HA signal colocalized with the signal obtained with antibodies against the SPB protein Kar1p (Rose and Fink, 1987; Spang *et al.*, 1995), confirming that Spc97p is a SPB component. This co-staining was observed in all the inspected cells ($n > 200$) of a non-synchronized culture. Furthermore, Spc97p-3HA localiz-

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Strain background	Number of inspected SPBs	Per cent of total				
		Staining of SPB substructures				No staining
		OP	IP	OP and IP	others	
<i>SPC97</i>	50	0	0	0	0	100
<i>SPC97-3HA</i>	50	28	16	8	0	48

Fig. 2. Spc97p is associated with the outer and inner plaque structures of the SPB. (A–D) SPB with anti-Spc97p-3HA staining, showing silver enhanced gold particles at the outer and inner plaques. The relatively small number of signals associated with the SPBs is explained by the fact that only small gold particles directly exposed on the surface of the thin sections were visualised by the silver enhancement. (E) Distribution of gold particles at the outer and inner plaques of *SPC97* and *SPC97-3HA* cells. Abbreviations: CP, central plaque; IP, inner plaque; M, microtubules; OP, outer plaque. Bar in (A): 100 nm. (B) to (D) have the same magnification as (A).

ation at the SPB was not influenced by the depolymerization of microtubules using nocodazole (data not shown). Taken together, these results indicate that Spc97p is a structural component of the SPB and that its localization is independent of the cell cycle.

Spc97p is associated with the inner and outer plaques of the SPB

SPBs are structured organelles and the function of some substructures have been deduced by electron microscopy. For example the outer and inner plaques of the SPB are the attachment sites for the cytoplasmic and nuclear microtubules respectively (Byers, 1981b). To study the localization of Spc97p at the SPB, permeabilized *SPC97* and *SPC97-3HA* cells were incubated with mouse anti-HA antibodies followed by secondary anti-mouse antibodies coupled to ultra small gold (0.6 nm). Gold particles directly localized on the surface of embedded and thin sectioned cells were then enhanced by silver staining. While about half of *SPC97-3HA* SPBs (52%) were associated with one to three gold particles (Figure 2A–D), none of the *SPC97* SPBs were stained (Figure 2E), indicating specific labelling. Interestingly, all gold particles of *SPC97-3HA* SPBs were associated with the outer or inner

plaques, but not with other SPB substructures (Figure 2). This result is consistent with an association of Spc97p with the SPB substructures, the outer and inner plaques, that organize the cytoplasmic and nuclear microtubules respectively.

Genetic evidence for an interaction of *TUB4*, *SPC98* and *SPC97*

To seek genetic evidence for an interaction of *TUB4*, *SPC98* and *SPC97*, we constructed temperature-sensitive *spc97(ts)* alleles by *in vitro* mutagenesis. Approximately 20 different *ts* alleles of *SPC97* were obtained and then integrated into the native *SPC97* locus. When the suppression ability of high dosage of *TUB4* and *SPC98* was investigated, it became clear that all of the *spc97(ts)* alleles were suppressed by *SPC98*. However, dependent on the *spc97(ts)* allele, the suppression ability of *TUB4* was either equal (Figure 3A, *spc97-14*) or weaker compared with *SPC98* (Figure 3A, *spc97-20*). We further found that *spc98-4* (Figure 3B) and *tub4-1* (Figure 3C) were rescued by *SPC98* or *TUB4* respectively, but not by *SPC97*. In contrast, *spc98-2* was suppressed strongly by *SPC97*, but only very weakly by *TUB4* (Figure 3B). Taken together, the allele-specific, dosage-dependent suppression phenotypes indicate that *TUB4*, *SPC98* and *SPC97* function together.

Further evidence for a genetic interaction of *TUB4*, *SPC98* and *SPC97* was obtained in a screen for mutations that are lethal in combination with the *tub4-1* allele (synthetic lethal screen). This screen resulted in seven complementation groups, one carrying mutations in *SPC98* and another in *SPC97* (data not shown). The other complementation groups will be described elsewhere (S.Geissler, manuscript in preparation).

Overexpression of *SPC97* is lethal and lethality is suppressed by co-overexpression of *TUB4* or *SPC98*

We observed that overexpression of *SPC98* is toxic for yeast cells and this lethal effect was suppressed by co-overexpression of *TUB4* (Geissler *et al.*, 1996). Such a phenotype is characteristic for proteins which are part of complexes requiring coordinated participation of multiple elements (Floor, 1970). Co-overexpression of an interacting protein may restore viability, possibly by balancing the stoichiometry of the overproduced component (Katz *et al.*, 1990; Magdolen *et al.*, 1993; Archer *et al.*, 1995; Geissler *et al.*, 1996). Out of these considerations, we investigated whether overexpression of *SPC97* is toxic for cells and whether this toxicity is suppressed by co-overexpression of *TUB4* or *SPC98*.

SPC97 was cloned behind the strong *Gall* promoter (*Gall-SPC97*). This strong overproduction was toxic for cells, indicated by the growth defect of *Gall-SPC97* cells on galactose plates (Figure 4A, row 2; galactose). We tested whether co-overexpression of *TUB4* or *SPC98* rescued the lethal phenotype of *Gall-SPC97*. Cells expressing *Gall-SPC97* and *Gall-SPC98*, or *Gall-SPC97* and *Gall-TUB4* grew on galactose plates as did cells containing only control vectors (Figure 4A, compare rows 1 and 6 with row 4). Controls established that these rescuing effects were specific. *Gall-SPC97* cells were not rescued by *Gall-Xgam* (Figure 4A, row 5) expressing

Fig. 3. Suppression of *spc97-14*, *spc97-20*, *spc98-2*, *spc98-4* and *tub4-1* by high gene dosage of *TUB4*, *SPC98* or *SPC97*. (A) *spc97-14*, *spc97-20* (B) *spc98-2* and *spc98-4*, and (C) *tub4-1* cells were transformed with the *URA3*-based, 2 μ m plasmids pMK1-3.2 (2 μ -*TUB4*), pMK1-4.1 (2 μ -*SPC98*), pMK1-10.1 (2 μ -*SPC97*), or pRS426 (control). Growth of the transformants was tested at the indicated temperatures on SC plates lacking uracil. Sequence analysis of the *spc97* and *spc98* alleles revealed multiple point mutations which were different in the two *spc98* and two *spc97* alleles respectively.

the γ -tubulin from *X.laevis* which is not functional in yeast (Spang *et al.*, 1996a). Furthermore, all cells grew equally well when the *Gall* promoter was repressed by glucose (Figure 4A, glucose). When triple co-overexpression of *Gall-TUB4*, *Gall-SPC98* and *Gall-SPC97* was investigated, it was found that this was again toxic for

Fig. 4. Overexpression of *SPC97* is toxic and co-overexpression of *TUB4* or *SPC98* rescues *SPC97* toxicity. (A) Strain YPH500 was transformed simultaneously with two different 2 μ m plasmids containing the indicated genes as *Gall* fusions. Growth was assayed as described in Materials and Methods. Plasmids: *SPC97* indicates plasmid pMK52, *SPC98* indicates plasmid pGP1-1, *Xgam* indicates plasmid pSM316, *TUB4* indicates plasmid pSM209-6; for controls plasmids p424-*Gall*, p425-*Gall* and pYES2 were used. The somewhat larger colony sizes on glucose compared with galactose plates is explained by faster growth of strain YPH500 with glucose as carbon source. (B) Overproduced Spc97p-3HA accumulates mainly in the cytoplasm of yeast cells. Cells carrying pRS424-*Gall-SPC97-3HA* (pMK81) were grown in SC medium lacking tryptophan and containing raffinose as carbon source at 30°C to a density of 5×10^6 cells/ml. Galactose (2%) was added to induce the *Gall* promoter. Nine hours after induction, cells were fixed and prepared for immunofluorescence with anti-HA (12CA5) antibodies. DNA was stained with DAPI. The fixed cells were also inspected by phase contrast microscopy (Phase). The arrowheads in (B, DNA) indicate cells with high levels of Spc97p-3HA which arrested with a large bud and the nucleus stuck in the bud neck. The inset shows a 2-fold enlargement of a large budded cell. Bar: 10 μ m.

cells, while a *Gall-Xgam*, *Gall-SPC98* and *Gall-SPC97* combination was not toxic (data not shown).

Overproduction of Spc98p induced a cell-cycle arrest of the cells. Thereby Spc98p was significantly accumulated in the nucleus; this finding led to the discovery of a nuclear localization sequence (NLS) in Spc98p (Geissler *et al.*, 1996). To test this for Spc97p, cell morphology and subcellular localization of Spc97p-3HA was determined after overproduction. In contrast to Spc98p, overproduced Spc97p-3HA was distributed in the cytoplasm of yeast cells (Figure 4B). The nucleus, whose position is indicated by the DAPI staining region, was much more weakly stained (Figure 4B, enlargement), making a NLS in Spc97p unlikely. Furthermore, all cells with high levels of Spc97p arrested in the cell cycle with a large bud and an undivided nucleus stuck in the bud neck, a phenotype similar to what was found for overproduced Spc98p. We noticed that Spc97p was not strongly overproduced in all cells which may be the result of plasmid loss (Knop *et al.*,

Fig. 5. Spc97p interacts with Tub4p and Spc98p in the two-hybrid system. Strain SGY37 (*lexA-op-lacZ*) carrying the indicated *LexA* and *GAL4* gene fusions was incubated on X-gal plates for 3 days at 30°C. Blue colony patches result from interaction of the plasmid encoded proteins. Plasmids are listed in Table III. (A) Spc97p interacts with Tub4p and Spc98p. (B) Tub4p, Spc98p and Spc97p do not interact with themselves in the two-hybrid system. (C) Spc97p and Tub4p interact with different domains of Spc98p. Truncated Spc98p derivatives (numbers indicate amino acids; plasmids were pSG29, pSG30 (Geissler *et al.*, 1996) and pSG31, see Table III) were tested for their interaction with LexA–Spc97p or LexA–Tub4p.

1996). In summary, our results are consistent with the notion that *TUB4*, *SPC98* and *SPC97* act together and that a defined stoichiometry of the three proteins is required for correct function.

***Spc97p* interacts with *Spc98p* and *Tub4p* in the two-hybrid system**

The multiple genetic interactions raised the possibility that Tub4p, Spc98p and Spc97p physically interact. This is an important point, since γ -tubulin in other organisms is present in a large complex and this complex seems to be the functional unit involved in microtubule nucleation (Zheng *et al.*, 1995). To investigate whether Spc97p interacts with Tub4p and Spc98p, we used the two-hybrid system initially described by Fields and Song (1989). *TUB4*, *SPC98* and *SPC97* were expressed as fusions with the DNA-binding protein LexA or the acidic activation domain of the transcription factor Gal4p. In case of an interaction of LexA and Gal4p fusion proteins, a DNA–protein–protein complex is formed that activates transcription of the reporter construct *LexA-op-LacZ*. *LacZ* encodes β -galactosidase and its activity converts the substrate X-gal into a blue indigo dye resulting in blue colonies.

The *SPC98*, *TUB4*, *Xgam* and *SPC97* constructs combined with vector controls did not result in any detectable β -galactosidase activity (Figure 5A, lanes 5–9), and in agreement with a previous report, LexA–Tub4p together with Gal4p–Spc98p gave blue colonies (lane 1), indicating interaction (Geissler *et al.*, 1996). Most interestingly, Spc97p interacted with Tub4p (lane 2) and with Spc98p (lane 4). These signals were specific, since no staining was obtained when Gal4p–Spc97p was combined with the

γ -tubulin from *X.laevis* (lane 3) or full-length Spc110p (data not shown). *SPC110* encodes an SPB component whose N-terminus is located close to the inner plaque (Kilmartin *et al.*, 1993; Spang *et al.*, 1996b; Sundberg *et al.*, 1996).

We also investigated whether Tub4p, Spc98p and Spc97p interact with themselves in the two-hybrid assay. However, no interaction was observed in any of the three cases (Figure 5B). We established by immunoblotting that the hybrid proteins were expressed in yeast (data not shown). Furthermore, we established that the two-hybrid interactions of Spc97p with Spc98p, Spc97p with Tub4p and Spc98p with Tub4p were independent of which protein was fused to LexA and of which to Gal4p (Figure 5A, lanes 1, 2 and 4; Figure 8A, lane 4 and data not shown).

We were interested whether the binding of Tub4p and Spc97p to Spc98p was mediated by overlapping domains in Spc98p. Deletion constructs of Spc98p were tested in combination with Tub4p and Spc97p. A functional Spc98p-truncation derivative lacking the 146 N-terminal amino acids (Gal4p– Δ Spc98p^{147–846}) still interacted with Tub4p (Figure 5C, lane 4) as well as with Spc97p (lane 1). In contrast, while the non-functional Gal4p– Δ Spc98p^{147–551} bound to Spc97p (lane 2), no signal was observed when it was combined with LexA–Tub4p (lane 5), suggesting that different domains in Spc98p are important for the interaction with Tub4p and Spc97p. Finally, a Spc98p derivative containing amino acids 1–324 (Δ Spc98p^{1–324}) did not show interaction with either Tub4p or Spc97p (lanes 3 and 6). We also investigated whether the N-terminal or C-terminal subdomains of Tub4p were sufficient for Spc97p binding. LexA constructs containing the 306 N-terminal amino acids of Tub4p (LexA–Tub4p^{1–306}) or the 305 C-terminal amino acids (LexA–Tub4p^{168–473}) did not mediate binding to either Spc98p (Geissler *et al.*, 1996) or Spc97p (data not shown). In summary, we conclude that Tub4p, Spc98p and Spc97p interact mutually, but not with themselves in the two-hybrid system. Furthermore, while the central domain of Spc98p is sufficient for the interaction with Spc97p, Spc98p binding to Tub4p requires the C-terminal domain as well.

***Tub4p*, *Spc98p* and *Spc97p* are part of the same complex(es)**

The results of the two-hybrid experiment suggest that Tub4p, Spc98p and Spc97p interact physically. To investigate whether these proteins are part of the same complexes, we performed immunoprecipitation experiments. As expected, precipitation of Spc97p-3HA with anti-HA antibody allowed the detection of Spc97p-3HA in the precipitate (Figure 6A, lane 2 and 6D, lane 3). This signal was specific, since no Spc97p-3HA was observed in a strain expressing *SPC97* (Figure 6A, lane 1). Spc98p (Figure 6B, lane 2) and Tub4p (Figure 6C, lane 2) co-precipitated with Spc97p-3HA, revealing that the three proteins are contained in the same complexes. Confirming this result, anti-Spc98p antibodies precipitate not only Spc98p (Figure 6B, lanes 3 and 4), but also Spc97p-3HA (Figure 6A, lane 4) and Tub4p (data not shown; Geissler *et al.*, 1996). Complex formation between Tub4p, Spc98p and Spc97p was further established by the co-precipitation of Spc98p (data not shown) and Spc97p-3HA (Figure 6D, lane 1)

Fig. 6. Co-immunoprecipitation of Tub4p, Spc98p and Spc97p. (A–C) Extracts of *SPC97* and *SPC97-3HA* cells were incubated with anti-HA (lanes 1 and 2), anti-Spc98p (lanes 3 and 4) or without antibodies (lanes 5 and 6) followed by an incubation with protein G–Sepharose. The precipitated proteins were analysed by immunoblotting using anti-HA (A), anti-Spc98p (B) or anti-Tub4p antibodies (C). (D) Extracts of *SPC97-3HA* (lanes 1–4) or *SPC97* (lanes 5–7) cells were incubated with anti-Tub4p (lanes 1 and 5), anti-Spc110p (lanes 2 and 6), anti-HA antibodies (lanes 3 and 7) or without antibody (lane 4). Protein G–Sepharose was added and the immunoprecipitates were tested with anti-HA antibody for the presence of Spc97p-3HA. Positions of molecular weight standards are indicated on the left of the panels.

using anti-Tub4p antibodies. However, anti-Spc110p antibodies (Spang *et al.*, 1995) did not co-precipitate Tub4p, Spc98p (data not shown) or Spc97p-3HA (Figure 6D, lane 2). Controls showed that the immunoprecipitation and the antibody detection were specific (Figures 6A, lanes 3, 5 and 6; 6B, lanes 1, 5 and 6; 6C, lane 1; 6D, lanes 4–7).

Fig. 7. Spc97p co-sediments with Tub4p and Spc98p on sucrose gradients. (A) Extracts of *SPC97-3HA* cells were loaded onto a linear gradient from 5 to 20% sucrose. The gradient was centrifuged for 12 h at 40 000 r.p.m. Fractions were collected and analysed for the presence of Tub4p, Spc98p and Spc97p-3HA by immunoblotting. (B) Fractions four to eight of (A) were subjected to immunoprecipitation using goat anti-Spc98p antibodies. The precipitate was analysed with anti-Spc98p, anti-HA and anti-Tub4p antibodies for the presence of Spc98p, Spc97p-3HA and Tub4p. (C) Extracts from cells expressing *TUB4-3HA* (lanes 1 and 4), *TUB4* and *TUB4-3HA* (lanes 2 and 5), or *TUB4* (lanes 3 and 6) were subjected to immunoprecipitation with anti-HA (lanes 1–3) or anti-Tub4p antibodies (lanes 4–6) chemically cross-linked to protein G–Sepharose. The precipitated proteins were analysed by immunoblotting with anti-Tub4p, anti-HA or anti-Spc98p antibodies as indicated. The asterisk (lane 2) indicates Tub4p which co-precipitated with Tub4p-3HA.

Taken together, these results suggest that Tub4p, Spc98p and Spc97p are present in the same complexes.

Identification of a major 6S complex containing Tub4p, Spc98p and Spc97p

We separated yeast lysates on a linear sucrose gradient and gradient fractions were analysed by immunoblotting for Tub4p, Spc98p and Spc97p-3HA (Figure 7A). All

three proteins peaked in fraction six, while the marker protein β -amylase with a sedimentation coefficient of 5S (molecular weight of 200 kDa) was present in fractions five and six. As reported by Geissler *et al.* (1996), Spc98p was detected as a doublet on polyacrylamide gels due to phosphorylations in this protein (G.Pereira, manuscript in preparation). Noteworthy, minor amounts of Tub4p, Spc98p and Spc97p-3HA were detected up to fraction 14 after prolonged development of the immunoblots (data not shown). This finding is consistent with the notion that Tub4p, Spc98p and Spc97p are also contained in complexes with larger S values. Fraction 15, corresponding to the sediment, contained Tub4p, Spc98p and Spc97p which may reflect the presence of SPBs. Furthermore, Tub4p in fractions three and four could either be the monomeric or dimeric protein, or Tub4p in association with another protein.

We addressed the question whether co-sedimentation of Tub4p, Spc98p and Spc97p-3HA is due to complex formation involving these proteins. Spc98p of sucrose gradient fractions four to eight was precipitated with polyclonal anti-Spc98p antibodies. The precipitate was then tested for the presence of Spc98p, Spc97p-3HA and Tub4p by immunoblotting (Figure 7B). Tub4p and Spc97p-3HA of fractions six and seven co-precipitated with Spc98p, suggesting either a trimeric Tub4p-Spc98p-Spc97p complex or dimeric Spc98p-Tub4p and Spc98p-Spc97p complexes. Tub4p and Spc97p-3HA of fraction five co-precipitated poorly with Spc98p, showing that some Spc98p is not in association with Tub4p or Spc97p.

We tested whether Tub4p complexes exist that contained more than one molecule of Tub4p. Extracts of cells expressing *TUB4-3HA* (Figure 7C, lanes 1 and 4), *TUB4-3HA* and *TUB4* (lanes 2 and 5), or *TUB4* (lanes 3 and 6) were subjected to immunoprecipitation using anti-HA or anti-Tub4p antibodies. Tub4p, Tub4p-3HA and Spc98p were then detected in the precipitate by immunoblotting. The strain backgrounds were confirmed when Tub4p and Tub4p-3HA were visualized in the anti-HA (lanes 1–3) and anti-Tub4p (lanes 4–6) precipitates. In accordance with complexes containing Tub4p and Tub4p-3HA, the anti-HA antibody precipitated Tub4p-3HA and Tub4p (lane 2, asterisk). We excluded that the Tub4p signal resulted from degradation of Tub4p-3HA by cellular proteases (lanes 1 and 4) or from the elution of cross-linked anti-HA antibody from the protein G resin (lane 3). Furthermore, immunodetection of Spc98p in the precipitate confirmed that the precipitated Tub4p and Tub4p-3HA were in complexes with Spc98p (lanes 1, 2 and 4–6). Summarizing, our results indicate that the most prominent Tub4p, Spc98p and Spc97p containing complex has a sedimentation coefficient of ~6S and that at least a subset of Tub4p-containing complexes contain more than one molecule of Tub4p.

***spc97* cells are defective in mitotic spindle formation, SPB duplication and SPB separation**

The phenotype of conditional-lethal mutants provides information about the function of the wild-type gene. We investigated the microtubule and SPB phenotype of *spc97(ts)* cells incubated at the non-permissive temperature. We chose the mutant alleles *spc97-14* and *spc97-20* for further analysis, since they were suppressed differently

by high dosage of *TUB4* and *SPC98* (Figure 3A). The ability of Spc97-14p and Spc97-20p to interact with Tub4p and Spc98p was investigated in the two-hybrid system. While Spc97-14p interacted with Spc98p with wild-type efficiency (Figure 8A, compare lanes 1 and 2), it was defective in its binding to Tub4p (lane 5). In contrast, Spc97-20p interacted only very weakly with Spc98p (lane 3), and not with Tub4p (lane 6). Therefore, it might be possible that *spc97-14* and *spc97-20* cells are defective in different functional steps, raising the possibility that the two *spc97(ts)* alleles show intragenic complementation. However, the temperature-sensitive growth defect of the diploid *spc97-14/spc97-20* strain showed that this was not the case (data not shown).

Phenotypes of *spc97-14* and *spc97-20* cells were determined with or without synchronization of cells by the yeast pheromone α -factor prior to incubation at 37°C. Since both experimental approaches yielded identical results, we only describe the phenotypes of synchronized *spc97(ts)* cells. *spc97-14* and *spc97-20* cells were released from their cell-cycle block by removing α -factor and shifting to the non-permissive temperature. The DNA content and morphology of mutant cells were analyzed every hour. Initially, *spc97-14* and *spc97-20* cells replicated their DNA and formed buds normally. After 2 h, however, 95% of the *spc97-14* and *spc97-20* cells arrested uniformly in the first cell cycle after the temperature shift with a large bud and replicated DNA, while wild-type cells exited mitosis and started another cell cycle (Figure 8B).

Spindle morphologies and localization of the nucleus of *SPC97* wild-type cells and the two *spc97(ts)* mutants were investigated after 3 h at 37°C by immunofluorescence using anti-tubulin antibodies and DAPI staining after formaldehyde fixation. *SPC97* cells with a large bud showed two DAPI staining regions separated by an anaphase spindle (Figure 8C). In contrast, *spc97-14* or *spc97-20* cells contained mostly one DAPI staining region which was located in the bud neck (90 and 71% respectively) or in one cell body (8 and 29% respectively). Large-budded *spc97-14* cells (27%) contained what looked like a short spindle which is a sign that the SPBs were duplicated and separated in these cells (Table I and Figure 8C). The remaining cells exhibited one focus of microtubule organization sitting in the bud neck (63%) or in one of the two cell bodies (8%) (Table I). This phenotype is known as monopolar spindle and is characteristic of yeast mutants with either a defect in SPB duplication or in SPB separation (Byers, 1981a; Rose and Fink, 1987). These cells show only one SPB signal when the SPB is visualized by indirect immunofluorescence. To characterize this defect further, we stained the SPB with anti-Kar1p (Spang *et al.*, 1995) and anti-Spc98p antibodies. This time we fixed the cells with methanol/acetone which preserves SPB antigens (Rout and Kilmartin, 1990), but not very well the structure of the spindle. Only one SPB signal was detected in 60% of large-budded *spc97-14* cells with both antibodies (Figure 8D and Table II). The remaining cells (40%) contained two SPB signals. The somewhat larger percentage of *spc97-14* cells with two SPB signals (40%) compared with cells with an apparent short bipolar spindle (27%) suggests that 13% of *spc97-14* cells have duplicated and separated their SPBs, but contained a monopolar looking spindle. Whether *spc97-14* cells with

one SPB signal have only one SPB or two SPBs sitting close together was investigated by electron microscopy. Thin serial sections through seven whole nuclei of *spc97-14* cells with a large bud indicated that the SPB was duplicated in six cells, while only one SPB was detected in one cell. The duplicated SPBs were of normal appearance. These were either not separated, sitting side by side in

the nuclear envelope (three cells; Figure 9B) or a short spindle was observed organized by two SPBs (three cells; Figure 9C1). In contrast to wild-type spindles (Figure 9A), spindles of *spc97-14* cells showed bundles of microtubules starting from one SPB but bypassing the other (Figure 9C2; small arrowheads).

The majority of *spc97-20* cells (>95%) had elongated cytoplasmic microtubules which were more pronounced compared with *spc97-14* cells (Figure 8C). *spc97-20* cells (86%) exhibited what looked like a monopolar spindle (Table I). This was confirmed by anti-Kar1p and anti-Spc98p staining, which detected only one SPB signal in 89% of cells (Figure 8D and Table II) and two signals in the rest (11%). In a few cells (2.3%) we detected a signal against the half bridge component Kar1p but none against the outer and inner plaque component Spc98p, raising the possibility that these plaque structures are not present or defective in a low percentage of *spc97-20* cells (Table II). Thin serial sections through 15 whole *spc97-20* nuclei identified only one SPB in the majority of nuclei (13 cells; Figure 9D1–D4 and E1–E5), indicating a severe defect in SPB duplication. Single SPBs were of normal appearance, but frequently associated with only a few (Figure 9D) or disorganized microtubules (Figure 9F). In addition, microtubules were observed with no obvious connection to the SPB (Figure 9F2, marked microtubule). These microtubules may have detached from the single SPB. The morphology of the two *spc97-20* spindles was very similar to those observed in *spc97-14* cells (data not shown). In summary, our analysis of *spc97* mutant alleles revealed defects in SPB duplication, SPB separation and mitotic spindle formation.

Discussion

The essential SPB components Tub4p, Spc98p and Spc97p interact with each other and are part of the same complex(es)

The discovery of γ -tubulin as a universal component of MTOCs (Oakley *et al.*, 1990; Stearns *et al.*, 1991) suggests that the microtubule nucleation activity of these morphologically distinct structures shares a molecular basis. This picture was complicated by the finding that γ -tubulin is associated with additional proteins whose properties and functions are largely unknown (Raff *et al.*, 1993; Stearns and Kirschner, 1994; Zheng *et al.*, 1995; Geissler *et al.*,

Fig. 8. *spc97* cells arrest with a large bud, replicated DNA and are defective in mitotic spindle formation. (A) Spc97-14p and Spc97-20p were tested for their interaction with Tub4p and Spc98p in the two-hybrid system. Experimental set up was as described in the legend to Figure 5. (B) *SPC97* (YPH499), *spc97-14* (YMK51.14) and *spc97-20* (YMK51.20) cells were arrested in the cell cycle in G₁ with a 1N DNA content by α -factor ($t = 0$ h). Cells were released from their cell-cycle block by removing α -factor and shifting to 37°C. Samples were taken every hour and analyzed for their DNA content by flow cytometry. (C) α -factor synchronized *SPC97*, *spc97-14* and *spc97-20* cells were released from their cell-cycle block and incubated for 3 h at 37°C. Fixed cells were analyzed by immunofluorescence using anti-tubulin antibody. DNA was stained with DAPI. Cells were also inspected by phase contrast microscopy. (D) Cells were prepared as described in (C) with the exception that acetone/methanol was used for fixation which preserves SPB antigens (Rout and Kilmartin, 1990). SPBs were visualized with anti-Spc98p and anti-Kar1p antibodies. DNA was stained with DAPI. Bar in (C): 5 μ m. (D) is the same magnification as (C).

Table I. Spindle morphology of large-budded *spc97(ts)* cells

Strain	Morphology (per cent occurrence)

YPH499 (<i>SPC97</i>)	83	17	—	—	—	—	—
YMK51.14 (<i>spc97-14</i>)	2	—	20	63	3	7	5
YMK51.20 (<i>spc97-20</i>)	—	—	13	57	—	1	29

Exponentially growing cells were arrested for 3 h at 23°C by the addition of α -factor (10 μ g/ml). Cells were collected, washed twice with prewarmed YPD and released into YPD at 37°C for 3 h. Spindle morphologies of 100–200 large budded cells were determined by indirect immunofluorescence microscopy using anti-tubulin antibody. DNA was stained with DAPI.

1996; Moudjou *et al.*, 1996). Therefore, the identification and functional analysis of proteins in complexes with γ -tubulin is an important task.

In this paper we identified *SPC97* as a dosage-dependent suppressor of conditional lethal *spc98-2* cells. Multiple lines of evidence suggest that the essential SPB component Spc97p interacts with the yeast γ -tubulin Tub4p and Spc98p (Figure 10A). First, the temperature-sensitive phenotype of *spc97(ts)* mutant cells was suppressed by high dosage of *TUB4* or *SPC98* in an allele-specific manner and the growth defect of *spc98* cells was rescued by a high dosage of *SPC97*. Second, overexpression of *SPC97* is toxic for cells and this toxicity is suppressed by co-overexpression of either *TUB4* or *SPC98*. Third, a screen for synthetic lethal mutations in combination with *tub4-1* led to the isolation of *spc97* and *spc98* mutants. Fourth, Tub4p, Spc98p and Spc97p interact mutually in the two-hybrid system. Fifth, Tub4p, Spc98p and Spc97p co-precipitate and band as 6S complexes on a sucrose gradient. Finally, Spc97p was like Tub4p (Spang *et al.*, 1996a) and Spc98p (Rout and Kilmartin, 1990) associated with the outer and inner plaques of the SPB (Figure 10B).

What is the composition of the complexes containing Tub4p, Spc98p and Spc97p? Co-immunoprecipitation of the 6S-Tub4p–Spc97p complexes with anti-Spc98p antibodies is consistent with either dimeric complexes of Spc98p together with Tub4p or Spc97p, or a trimeric complex containing Tub4p, Spc98p and Spc97p. Our analysis does not exclude the presence of additional proteins in the 6S complexes. However, the relatively small S value favours the notion that the complexes are simple in composition containing Tub4p, Spc98p or Spc97p and at the most only a few smaller proteins. We favour a model in which Tub4p, Spc98p and Spc97p mutually interact to form a trimeric complex. Mutual interactions between Tub4p, Spc98p and Spc97p are suggested by the two-hybrid experiments. Each of the three proteins gave a positive signal with the other, but not with themselves (Figure 10A). From these experiments we cannot exclude that the interactions are mediated by a third protein present in yeast cells. However, the finding that different domains of Spc98p are required for the interaction with Tub4p or Spc97p favour the notion that Spc98p has distinct binding sites for Tub4p and Spc97p and therefore interacts physically with both proteins. Bilateral interactions between the three proteins are further indicated by co-overexpression experiments (Figure 10A). Similar toxic overproduction phenotypes have been observed for other components of the yeast cytoskeleton

Table II. Number of SPB signals in large-budded *SPC97*, *spc97-14* and *spc97-20* cells

Strain	Per cent of cells with		
	two Kar1p signals	one Kar1p signal	co-staining of Kar1p and Spc98p
YPH499 (<i>SPC97</i>)	99	1	100
YMK51.14 (<i>spc97-14</i>)	40	60	100
YMK51.20 (<i>spc97-20</i>)	10.8	89.2	97.7 ^a

SPB staining of large-budded *SPC97*, *spc97-14* and *spc97-20* cells 3 h after release from α -factor arrest at 37°C using anti-Kar1p or anti-Spc98p antibodies as SPB specific markers. Both proteins were detected simultaneously in a double labelling experiment using indirect immunofluorescence. Approximately 200 cells were counted. The results were qualitatively confirmed in two independent experiments.

^a2.3% show SPB staining with anti-Kar1p but not with anti-Spc98p antibodies.

such as β -tubulin (*TUB2*) and actin (*ACT1*) (Burke *et al.*, 1989; Magdolen *et al.*, 1993). In these cases, co-overproduction of the interacting proteins α -tubulin (Katz *et al.*, 1990), the β -tubulin binding protein Rbl2p (Archer *et al.*, 1995) or the actin binding protein profilin (Magdolen *et al.*, 1993) neutralized the toxic effects. Therefore, the overexpression phenotypes of *TUB4*, *SPC98* and *SPC97* are most easily explained with a direct interaction of these proteins.

We also detected complexes with higher S values containing Tub4p, Spc98p or Spc97p which may be folding intermediates associated with chaperones such as Tcp1p (Moudjou *et al.*, 1996), multimeric forms of the trimeric Tub4p, Spc98p, Spc97p complex, or any one of the three proteins in association with additional proteins. At least for Tub4p our data suggest that complexes exist which contain more than one molecule of Tub4p.

The question remains as to where the Tub4p–Spc98p–Spc97p complex assembles. In this respect it is interesting to note that the three proteins are located at the inner and outer plaques of the SPB (Figure 10B), meaning on both sides of the nuclear envelope. Since only Spc98p (Geissler *et al.*, 1996) but not Tub4p (S.Geissler, unpublished) and Spc97p (this study) has a detectable nuclear localization sequence (NLS), it is tempting to speculate that the trimeric complex forms in the cytoplasm of yeast cells and is then directed via the NLS of Spc98p into the nucleus. How the ratio of the complex in the cytoplasm and the nucleus is regulated is still an open question. Modifications of a protein in the complex (Moll *et al.*,

Fig. 9. Cells of *spc97-14* are defective in SPB separation or mitotic spindle formation, while the majority of *spc97-20* cells have an SPB duplication defect. *SPC97* (YPH499) (A), *spc97-14* (strain YMK51.14) (B and C) and *spc97-20* cells (YMK51.20) (D–F) were synchronized with α -factor and shifted to 37°C as described in Figure 8C. Cells were then prepared for electron microscopy. Shown are continuous thin serial sections through SPBs. Numbers after the letters indicate serial sections, for example (C2) is the serial section after (C1). (A) Spindle of *SPC97* cell. Arrowheads indicate the position of the two SPBs. (B) Duplicated but unseparated SPBs of *SpC97-14*. (C) Two serial sections through a defective *spc97-14* spindle. (C2) Bundles of microtubules (small arrowheads) pass the SPB on the right in (C1). (D) Thin serial sections through a SPB (large arrowhead) of a *spc97-20* cell with only a few attached microtubules (small arrowheads). A side by side SPB would be expected next to the half bridge shown in (D2). No second SPB was observed in sections below (D1), above (D4) or in other sections through this cell (data not shown). (E) Thin serial sections through a *spc97-20* SPB (marked with large arrowhead). The half bridge is visible in section (E2). (F) Thin serial sections through a *spc97-20* cell. No further SPB was detected in sections below (F1) or above (F3) (data not shown). SPB (large arrowheads) is associated with misdirected microtubules. Some microtubules (small arrow head) are detached from the SPB. Abbreviation: H, half bridge. Asterisks indicate the position of some nuclear pores. Bar in (C1): 140 nm and in (F1): 200 nm. (A) and (B) are the same magnification as (C); (D) and (E) are the same as (F).

1991) or an additional binding protein (Henkel *et al.*, 1992) could regulate this step.

The functions of the *S.cerevisiae* γ -tubulin, Tub4p and the interacting Spc98p have been analysed by studying the phenotype of conditional lethal mutants (Geissler *et al.*,

Whether the structure and function of proteins associated with γ -tubulin are conserved in phylogenetically different organisms is an open question. For the non-tubulin components in the γ -tubulin ring complex of *Xenopus* eggs Mitchison and colleagues discussed a model in which these proteins form a ring-like scaffold on which the 13 γ -tubulin molecules are arranged (Zheng *et al.*, 1995). The γ -tubulin ring directly nucleates microtubules by the interaction of γ -tubulin with tubulin (α -, β -heterodimer). In contrast, Erickson and Stoffler (1996) proposed that γ -tubulin forms a curved protofilament which functions as a stable seed in microtubule nucleation. In the latter model, the accessory proteins stabilize the γ -tubulin protofilament and in addition, may determine its polarity. The molecular mechanism of how microtubules are nucleated by and attached to the yeast SPB may be different compared with other MTOCs by several criteria: first, Tub4p is more divergent from other γ -tubulins, indicated by the moderate 36–40% identity compared with the γ -tubulins from *A.nidulans*, human and *S.pombe*, while these share identities in the range 69–78% (Burns, 1995). Second, the most prominent Tub4p complex seems to be different in size and composition to the γ -tubulin complexes from *Xenopus*, human and *Drosophila* (Raff *et al.*, 1993; Stearns and Kirschner, 1994; Kellogg *et al.*, 1995; Whitfield *et al.*, 1995; Zheng *et al.*, 1995). Finally, microtubule ends that are in contact with the yeast SPB are sealed by a cap-like terminal component connecting the walls of the microtubule cylinder (Byers *et al.*, 1978). The structure of this closed cap appears to be different to the isolated cylindrical γ -tubulin ring complex. Assuming that Tub4p, Spc98p and Spc97p are present in one complex, it is tempting to speculate that the polymerized complex results

in a dome-shaped structure with 13 Tub4p molecules forming the rim of the dome. This Tub4p ring may interact with tubulin and thereby nucleate microtubules.

Materials and methods

Growth media and general methods

Basic yeast methods and growth media were as described (Guthrie and Fink, 1991). *Escherichia coli* strains were transformed by electroporation (Dower *et al.*, 1988). Recombinant DNA methodology was performed as published (Sambrook *et al.*, 1989). DNA sequence of cloned fragments and all PCR products were determined by PCR sequencing, using reagents and machines from Perkin Elmer.

Screening for multicopy suppressor of *spc98-2* and cloning of *SPC97*

Strain ESM278-3 was transformed with a yeast genomic bank constructed in the 2 μ m plasmid pSEY8 (Heitman *et al.*, 1991). Transformants (25 000) were tested for growth at 33°C. DNA of temperature-resistant transformants was extracted (Ausubel *et al.*, 1994) and transformed into *E. coli* DH5 α . Plasmid DNA was isolated and re-transformed into strain ESM278-3 and re-assayed for complementation of the growth phenotype at 33°C. One plasmid (pMK1-10.1) contained the open reading frames (ORFs) YHR170w (partially), YHR171w and YHR172w (*SPC97*). Additionally, plasmids containing *SPC98* (pMK1-4.1) and *TUB4* (pMK1-3.2), which exhibit only a very weak suppression ability of the *spc98-2* allele, were also isolated.

Plasmids

Plasmids are listed in Table III. pMK1-3.2 and pMK1-4.1 contain genomic inserts with *TUB4* and *SPC98* respectively in plasmid pSEY8 and were obtained in the multicopy suppressor screen (see above) together with pMK1-10.1 (*SPC97* on the genomic insert). Subclones of the genomic insert of pMK1-10.1 were constructed as outlined in Figure 1A. For construction of pMK6, *SPC97* was cloned into pBluescript SK⁺ and the internal *Bam*HI–*Sal*I fragments (corresponding to 85% of the ORF) were replaced with a 1326 bp *Bam*HI–*Xho*I fragment containing *HIS3*. For construction of pMK8, pMK9 and pMK26-16, a 3534 bp *Stu*I–*Sal*I (*Sal*I is from the multiple cloning site of pMK1-10.1) containing *SPC97* was cloned into plasmids pRS316, pRS426 and pRS414 previously restricted with *Ecl*136I–*Xho*I. For construction of pMK15, pMK16 and pMK52, a fragment containing *SPC97* was amplified by PCR and cloned into pACTII, pEG202 and p424-*Gall* respectively. The *Not*I site before the stop codon of *SPC97* in plasmid pMK28-2 was introduced by PCR. A fragment of DNA coding for 3-HA epitopes (Spang *et al.*, 1995) was ligated in pMK28-2 restricted with *Not*I to obtain plasmid pMK29-4. For construction of plasmid pMK81, the 3'-end of *SPC97-3HA* from plasmid pMK29-4 was used to replace the corresponding part of *SPC97* in plasmid pMK52. PCR fragments carrying *spc97-14* and *spc97-20* were inserted into pEG202 to give pMK103 and pMK104. PCR fragments carrying *TUB4* and *SPC98* were inserted into pACTII and pEG202 respectively to give pSG45 and pSG56. To construct plasmid pGP1-1, a fragment containing *Gall*–*SPC98* from plasmid pSM289 was cloned into plasmid pRS425.

Constructions of yeast strains

Yeast strains are listed in Table III. Standard yeast techniques were used to manipulate strains (Guthrie and Fink, 1991). To construct strain YMK1-2, cells of strain YPH501 were transformed with plasmid pMK6 previously restricted with *Apal*–*Sac*I to liberate the Δ *spc97::HIS3* disruption cassette. Transformants were selected on His[–] plates. Strain YMK10 is a His⁺ Ura⁺ segregant of strain YMK1-2 previously transformed with plasmid pMK8 (pRS316-*SPC97*). Strains YMK18 and YMK22 were obtained by transforming cells of strain YMK10 with plasmids pMK26-16 (YMK18) or pMK29-4 (YMK22) and selection on SC plates lacking tryptophan, followed by growth on 5-FOA plates. Strains that contain chromosomally integrated copies of the *spc97(ts)* alleles (YMK51.14 and YMK51.20) were obtained as follows: PCR products with *spc97-14* or *spc97-20* (containing the ORF of *spc97-14* or *spc97-20* including flanking sequences) were transformed together with plasmid pRS424 into strain YMK10. Transformants (~5000 colonies/plate) were selected on SC plates lacking tryptophan at 23°C. Colonies were replica plated on 5-FOA plates at 23°C. 5-FOA resistant clones (0.5% of Trp⁺ colonies) were tested for being His[–]. All His[–]

colonies exhibited a temperature sensitive growth phenotype that was complemented by plasmid encoded *SPC97*.

Construction of *spc97(ts)* alleles

SPC97 was mutagenized by PCR (Cadwell and Joyce, 1992). Conditional lethal alleles of *SPC97* were obtained as described (Muhlrad *et al.*, 1992). *spc97-14* and *spc97-20* were obtained in two independent PCRs. Sequence analysis revealed that the two *spc97* alleles carry multiple but from each other distinct point mutations.

Anti-Spc98p antibodies, co-immunoprecipitation, sucrose gradient fractionation and immunoblots

A DNA fragment encoding the 130 N-terminal amino acids of Spc98p was cloned into expression plasmid pQE30 (Quiagen). The histidine-tagged protein was purified according to the manufacturer's recommendation and injected into a goat or a rabbit as recommended by Harlow and Lane (1988). Co-immunoprecipitation: cultures of *SPC97-3HA* (YMK22) or *SPC97* (YMK18) cells were grown in YPD and cells of *TUB4-3HA* (ESM184-1), wild type (YPH500 with pRS316) and *TUB4/TUB4-3HA* (YPH500 with pSM222) were grown in SC medium lacking uracil at 30°C to a density of 2 $\times$ 10⁷ cells/ml. Washed cells were resuspended in lysis buffer (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 10 mM EDTA, 2 mM EGTA, 5% glycerol, 1 mM PMSF, 5 mM benzimidazole, 10 μ M pepstatin A, 0.1 μ M leupeptin, 20 μ g/ml chymostatin and 20 μ g/ml E64) and lysed by vortexing with glass beads on ice until >95% of the cells were lysed. The cell lysate was diluted three times with lysis buffer and adjusted to 1% Triton X-100 and incubated for 30 min on ice. Cell debris were removed by low speed centrifugation (5 min, 2000 g). For immunoprecipitation the following antibodies were used at the indicated dilution: 12CA5 (Babco, 1:20); affinity purified goat anti-Tub4p (1:20; Spang *et al.*, 1996a), goat anti-Spc98p serum (1:200); 12CA5 covalently bound to protein G–Sepharose, affinity purified goat anti-Tub4p covalently bound to protein G–Sepharose. IgG–protein G–Sepharose conjugates were constructed as described (Harlow and Lane, 1988). Cell lysates were incubated for 1 h at 4°C followed by an incubation with protein G Sepharose (Sigma) (only in the cases where the antibody was not yet bound to protein G–Sepharose) for 1 h. The precipitates were washed four times with lysis buffer containing 1% Triton X-100 and thereafter resuspended in UREA sample buffer (Knop *et al.*, 1996) and heated for 10 min at 65°C before subjecting to SDS–PAGE (Laemmli, 1970) and immunoblotting. For sucrose gradient fractionation, 0.5 ml low speed centrifuged cell lysate was layered on top of a linear 5–20% sucrose gradient that was set up in lysis buffer without glycerol. The gradient was centrifuged in an SW41 rotor (Beckmann) for 12 h and 40 000 r.p.m. at 5°C. Fourteen fractions were collected from top to bottom. The pellet was resuspended in lysis buffer (15th fraction). Protease inhibitors were added and the fractions were either precipitated with trichloroacetic acid for analysis by immunoblotting or used without further dilution for immunoprecipitation. The proteins were separated by SDS–PAGE and transferred onto nitrocellulose. The primary antibodies were mouse monoclonal 12CA5, goat anti-Spc98p, rabbit anti-Spc98p or goat anti-Tub4p. As secondary antibodies rabbit anti-mouse, rabbit anti-goat or affinity-purified goat anti-rabbit IgGs coupled to horseradish peroxidase (Jackson Immuno Research Laboratories) were used. The immunoreaction was visualized with an ECL-Kit from Amersham.

Immunofluorescence microscopy and electron microscopy

Immunofluorescence of yeast cells shown in Figures 4B and 8C was performed as described by Knop *et al.* (1996) with 1 h fixation time. All other immunofluorescence experiments were performed as published (Rout and Kilmartin, 1990). The primary antibodies were either a pool of mouse monoclonal anti-98-kDa (Rout and Kilmartin, 1990), rabbit anti-Kar1p (Spang *et al.*, 1995), mouse monoclonal anti- β -tubulin WA3 (Spang *et al.*, 1995) or mouse monoclonal 12CA5 anti-HA antibodies. Secondary antibodies were goat anti-mouse IgG coupled to CY3 or goat anti-rabbit IgG coupled to DTAF (Jackson Immuno Research Laboratories). DNA was stained with DAPI.

Thin-section EM of yeast cells was performed as described (Byers and Goetsch, 1991). Preembedding labelling of yeast cells: cells were fixed by adjusting the culture to 100 mM KPO₄ (pH 6.5) and 3.5% formaldehyde for 10 min at 30°C. Cells were washed twice with SP buffer (40 mM KPO₄ pH 6.5, 1.2 M sorbitol, 1000 g, 5 min). Cells were incubated with glusulase (100 μ l/ml) (DuPont) and Zymolyase 20T (250 μ g/ml) for 1 h at 37°C to obtain spheroplasts. Spheroplasts were washed twice with SP buffer, then resuspended in incubation buffer 1 (PBS with 300 mM NaCl, 1% BSA, 0.1% TX-100, 1 mM PMSF, 10 μ M pepstatin A) with 12CA5 antibodies

Table III. Yeast strains and plasmids

Name	Genotype/construction	Source or reference
Yeast strains		
YPH499	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1</i>	Sikorski and Hieter (1989)
YPH500	<i>MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1</i>	Sikorski and Hieter (1989)
YPH501	<i>MATa/α ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1</i>	Sikorski and Hieter (1989)
YMK1-2	<i>MATa/α ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 SPC97/Δspc97::HIS3</i>	this study
YMK10	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3 pMK8</i>	this study
YMK18	<i>MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3 pMK26-16</i>	this study
YMK22	<i>MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3 pMK29-4</i>	this study
YMK51.14	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc97-14</i>	this study
YMK51.20	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc97-20</i>	this study
YMK51.27	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc97-27</i>	this study
SGY37	<i>Mata ura3-52::URA3-lexA-op-lacZ trp1 his3 leu2</i>	Geissler <i>et al.</i> (1996)
ESM184-1	<i>MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δtub4::HIS3 pSM222</i>	Spang <i>et al.</i> (1996a)
ESM208	<i>MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 tub4-1</i>	Spang <i>et al.</i> (1996a)
ESM260-7	<i>MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc98::HIS3 LEU2-spc98-4-leu2</i>	this study
ESM278-3	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc98-2</i>	this study
Plasmids		
p424- <i>Gall</i>	2 μm, <i>TRP1</i> -based yeast- <i>E.coli</i> shuttle vector carrying the <i>Gall</i> promoter	Mumberg <i>et al.</i> (1995)
p425- <i>Gall</i>	2 μm, <i>LEU2</i> -based yeast- <i>E.coli</i> shuttle vector carrying the <i>Gall</i> promoter	Mumberg <i>et al.</i> (1995)
pACTII	2 μm, <i>LEU2</i> -based vector carrying the <i>GAL4</i> activator domain	Durfee <i>et al.</i> (1993)
pEG202	2 μm, <i>HIS3</i> -based vector carrying the <i>LexA</i> DNA-binding domain	Gyuris <i>et al.</i> (1993)
pGP1-1	<i>SPC98</i> under control of the <i>Gall</i> promoter in plasmid pRS425	this study
pMK1-10.1	<i>SPC97</i> in pSEY8	this study
pMK1-3.2	<i>TUB4</i> in pSEY8	Geissler <i>et al.</i> (1996)
pMK1-4.1	<i>SPC98</i> in pSEY8	this study
pMK6	pBluescript SK ⁺ containing <i>Δspc98::HIS3</i>	this study
pMK8	pRS316 containing <i>SPC97</i>	this study
pMK9	pRS426 containing <i>SPC97</i>	this study
pMK15	pACTII containing <i>SPC97</i>	this study
pMK16	pEG202 containing <i>SPC97</i>	this study
pMK26-16	pRS414 containing <i>SPC97</i>	this study
pMK29-4	pRS414 containing <i>SPC97-3HA</i>	this study
pMK52	p424- <i>Gall</i> containing <i>SPC97</i>	this study
pMK81	p424- <i>Gall</i> containing <i>SPC97-3HA</i>	this study
pMK103	pEG202 containing <i>spc97-14</i>	this study
pMK104	pEG202 containing <i>spc97-20</i>	this study
pRS315	<i>CEN6</i> , <i>LEU2</i> -based yeast- <i>E.coli</i> shuttle vector	Sikorski and Hieter (1989)
pRS316	<i>CEN6</i> , <i>URA3</i> -based yeast- <i>E.coli</i> shuttle vector	Sikorski and Hieter (1989)
pRS425	2 μm, <i>LEU2</i> -based yeast- <i>E.coli</i> shuttle vector	Christianson <i>et al.</i> (1992)
pRS426	2 μm, <i>URA3</i> -based yeast- <i>E.coli</i> shuttle vector	Christianson <i>et al.</i> (1992)
pSEY8	2 μm, <i>URA3</i> -based yeast- <i>E.coli</i> shuttle vector	Heitmann <i>et al.</i> (1988)
pSG31	pACTII containing bases 1–972 of <i>SPC98</i>	this study
pSG45	pACTII containing <i>TUB4</i>	this study
pSG56	pEG202 containing <i>SPC98</i>	this study
pSM209-6	<i>TUB4</i> in pYES2	Spang <i>et al.</i> (1996a)
pSM222	<i>TUB4-3HA</i> in pRS316	Spang <i>et al.</i> (1996a)
pSM313	<i>Gall-TUB4</i> in pRS425	Geissler <i>et al.</i> (1996)
pSM316	γ-tubulin gene from <i>X.laevis</i> in p425- <i>Gall</i> : <i>Gall-Xgam</i>	Geissler <i>et al.</i> (1996)
pYES2	2 μm, <i>URA3</i> -based yeast- <i>E.coli</i> shuttle vector carrying the <i>Gall</i> promoter	Invitrogen

for 2 h at 20°C. Cells were washed three times with PBT and then incubated with goat anti-mouse IgGs coupled to ultra thin gold (BioTrend) for 2 h at 20°C in incubation buffer 2 (as incubation buffer 1 but with 150 mM NaCl). Cells were washed once with PBT and twice with PBS, then fixed with 2% glutaraldehyde in PBS for 10 min at room temperature, followed by an incubation with an aqueous solution of 2% osmium tetroxide and then 1% uranyl acetate for 15 min at 20°C each. Fixed cells were embedded in Spurr resin (Polyscience) as described (Byers and Goetsch, 1991). Sections of embedded cells were layered on Ni grids and treated with silver enhancer according to Danscher (1981). Stained sections were contrasted (lead citrate and uranyl acetate) and inspected for SPB staining by electron microscopy.

Flow cytometry

Cells were prepared for flow cytometry as described (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*, *Spc98p* and *Spc97p*

Gene fusions with the DNA-binding domain of *LexA* were made using plasmid pEG202. Plasmid pACTII was chosen as activation domain vector. Derivatives of pEG202 and pACTII were transformed into the 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.

Co-overexpression and growth assay on plates

Cells of strain YPH500 were transformed with plasmids pMK52 (*Gall-SPC97*), pGP1-1 (*Gall-SPC98*), pSM209 (*Gall-TUB4*) or pSM316 (*Gall-Xgam*) in the combinations described in Figure 4. For controls, plasmids p425-*Gall*, p424-*Gall* and pYES2 (Invitrogen) were used. Precultures of the transformants were grown in selective medium containing 2% raffinose. Serial dilutions of equal amounts of cells were

made up in water and aliquots were spotted either on selective SC plates (either SC medium lacking uracil and tryptophan or lacking leucine and tryptophan) containing 2% glucose/2% raffinose or 2% galactose/2% raffinose as carbon sources. Plates were incubated at 30°C for 3 days.

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