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Receptors determine the cellular localization of a γ-tubulin complex and thereby the site of microtubule formation

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The yeast microtubule organizing centre (MTOC), known as the spindle pole body (SPB), organizes the nuclear and cytoplasmic microtubules which are functionally and spatially distinct. Microtubule organization requires the yeast γ-tubulin complex (Tub4p complex) which binds to the nuclear side of the SPB at the N-terminal domain of Spc110p. Here, we describe the identification of the essential SPB component Spc72p whose N-terminal domain interacts with the Tub4p complex on the cytoplasmic side of the SPB. We further report that this Tub4p complex-binding domain of Spc72p is essential and that temperature-sensitive alleles of *SPC72* or overexpression of a binding domain-deleted variant of *SPC72* (Δ*N-SPC72*) impair cytoplasmic microtubule formation. Consequently, polynucleated and anucleated cells accumulated in these cultures. In contrast, overexpression of the entire *SPC72* results in more cytoplasmic microtubules compared with wild-type. Finally, exchange of the Tub4p complex-binding domains of Spc110p and Spc72p established that the Spc110p domain, when attached to ΔN-Spc72p, was functional at the cytoplasmic site of the SPB, while the corresponding domain of Spc72p fused to ΔN-Spc110p led to a dominant-negative effect. These results suggest that different components of MTOCs act as receptors for γ-tubulin complexes and that they are essential for the function of MTOCs.

Keywords: microtubule nucleation/Spc72p/spindle pole body/Tub4p/γ-tubulin

Introduction

Microtubules are part of the cytoskeleton of eukaryotic cells with essential functions in chromosome segregation in mitosis and meiosis, cell polarity, organelle positioning, secretion and cellular movement (reviewed in Huffaker *et al*., 1987; Hyman and Karsenti, 1996). Microtubules are hollow cylinders, and the wall of the cylinder consists of tubulin, a heterodimer of α- and β-tubulin (reviewed by Mandelkow and Mandelkow, 1993). Microtubules form by the self-assembly of tubulin, a process that starts *in vivo* at so-called microtubule organizing centres (MTOCs) (reviewed in Brinkley, 1985; Kellogg *et al*., 1994; Pereira and Schiebel, 1997). The generic term MTOCs groups morphologically distinct structures with a common microtubule organization activity such as centrosomes, spindle pole bodies (SPBs) and basal bodies (Pickett-Heaps, 1969).

The microtubule organization capability of MTOCs frequently is cell-cycle regulated. For example, the number of microtubules at mammalian centrosomes increases 5-fold at the onset of mitosis (Kuriyama and Borisy, 1981). Another example is the SPB from *Schizosaccharomyces pombe* which organizes nuclear microtubules only in mitosis (Hagan and Hyams, 1988; Masuda *et al*., 1992). Furthermore, one cell may have multiple MTOCs which are morphologically, spatially and functionally distinct. In this respect, plant cells are particularly interesting, since a number of different microtubule arrays exist: these include the cortical nuclear-associated microtubules in interphase, the pre-prophase band in G_2 phase, and the mitotic spindle and the phragmoplast microtubules during mitosis (reviewed in Smirnova and Bajer, 1992; Marc, 1997). There is increasing evidence that the nuclear surface of higher plants serves as a MTOC during interphase and during telophase (Stoppin *et al*., 1994). Other MTOCs may exist, including the phragmoplast (Cleary *et al*., 1992). In the fission yeast *S.pombe*, interphase microtubules are organized mainly by a not very well defined MTOC that is localized at the cell equator (Hagan and Hyams, 1988). However, at the onset of mitosis, microtubules are organized from the two SPBs into a typical spindle (Ding *et al*., 1997).

The SPB of *Saccharomyces cerevisiae* offers an example of one MTOC that can organize functionally and spatially distinct classes of microtubules (Byers and Goetsch, 1975). The *S.cerevisiae* SPB is embedded in the nuclear envelope during the entire cell cycle. Substructures named the outer, central and inner plaques have been described by electron microscopy (Byers and Goetsch, 1975; Byers, 1981) (Figure 8). The outer and inner plaques organize the cytoplasmic and nuclear microtubules, respectively. The cytoplasmic microtubules have functions in nuclear positioning and nuclear movement (Palmer *et al*., 1992; Sullivan and Huffaker, 1992), while the nuclear microtubules are involved in spindle formation and chromosome segregation in mitosis and meiosis (Jacobs *et al*., 1988).

A universal component of MTOCs is γ-tubulin which was first discovered in the fungus *Aspergillus nidulans* (Weil *et al*., 1986; Oakley and Oakley, 1989). Since then, the function of γ-tubulin in microtubule formation has been established by antibody microinjection experiments (Joshi *et al*., 1992), genetic studies (Oakley *et al*., 1990; Horio *et al*., 1991; Sobel and Snyder, 1995; Marschall *et al*., 1996; Spang *et al*., 1996a) and biochemical approaches (Li and Joshi, 1995; Zheng *et al*., 1995). Biochemical studies using extracts of frog eggs (Zheng *et al*., 1995), mammalian cells (Stearns and Kirschner, 1994; Moudjou *et al*., 1996), *S.cerevisiae* (Knop and Schiebel, 1997) and *A.nidulans* (Akashi *et al*., 1997) cells revealed that γ-tubulin is part of larger complexes. Purification of such a 25S complex from *Xenopus laevis*

eggs identified α-, β- and γ-tubulin and at least four additional proteins (Zheng *et al*., 1995). The *S.cerevisiae* γ-tubulin, Tub4p, forms a stable complex with two other proteins, Spc98p and Spc97p (Geissler *et al*., 1996; Knop and Schiebel, 1997), and this Tub4p complex is localized at the outer and inner plaques of the SPB (Rout and Kilmartin, 1990; Knop *et al*., 1997) (Figure 8). Conditional lethal mutants in *SPC98* and *SPC97* revealed a function of the encoded proteins in microtubule organization by the SPB (Geissler *et al*., 1996; Knop *et al*., 1997).

γ-Tubulin complexes assemble in the cytoplasm of cells (Stearns and Kirschner, 1994; Moudjou *et al*., 1996; Pereira *et al*., 1998). From *S.cerevisiae* we know that Spc98p and Spc97p of the assembled γ-tubulin complex (Tub4p complex) bind to the N-terminal domain of the SPB component Spc110p (Knop and Schiebel, 1997). Analogously, other γ-tubulin complexes may bind to MTOCs via such γ-tubulin complex-binding proteins (GTBPs). A GTPB may localize to only one MTOC within a cell and thereby contribute to the characteristic microtubule organization properties of an MTOC.

Spc110p is only associated with the inner plaque, while the Tub4p complex is located with the outer and inner plaques. Therefore, a protein other then Spc110p has to function as a GTBP at the outer plaque. The goal of this study was to identify the yeast GTBP at the outer plaque. Using the yeast two-hybrid system, we identified a new, essential SPB component of the outer plaque, named Spc72p, whose N-terminal domain interacts with Spc98p and Spc97p of the Tub4p complex. We further established that Spc72p fulfils similar functions in microtubule organization at the outer plaque as Spc110p at the inner plaque.

Results

Cloning of SPC72 and its interaction with the Tub4p complex in the two-hybrid system

Previously, we have shown that the N-terminal domain of Spc110p interacts with Spc98p and Spc97p, but not with Tub4p (Knop and Schiebel, 1997) in the yeast two-hybrid system (Fields and Song, 1989). However, an interaction with Tub4p was observed after co-overexpression of *SPC98* and *SPC97*. We used these criteria to search for further SPB components that interact with the Tub4p complex. Our screen resulted in a prey plasmid containing ~270 codons of the 5′ end of the open reading frame (ORF) YAL047c. Since further experiments showed that ORF YAL047c encodes a SPB component, we renamed YAL047c as *SPC72*, for SPB component with a molecular weight of 72 kDa. *SPC72* is located on chromosome I and it encodes a protein of 622 amino acids. The analysis of the amino acid sequence revealed that stretches of Spc72p have a high probability of forming coiled-coil structures (Figure 1A), a structural motif that has been found in other SPB components such as Spc42p (Donaldson and Kilmartin, 1996) and Spc110p (Kilmartin *et al*., 1993). Spc72p does not show significant homology to any protein in the database.

Subdomains and the entire coding region of Spc72p were tested for their interactions with Tub4p, Spc98p and Spc97p using the yeast two-hybrid system (Figure 1B). In agreement with our screening criteria, the N-terminal domain of Spc72p ($Spc72p^{1–271}$; the numbers denote amino acids) showed two-hybrid interactions with Spc97p (Figure 1B, lane 4) and Spc98p (lane 5) but not with Tub4p (lane 3). We noticed that the strength of these interactions was dependent on whether $Spc72p^{1–271}$ was fused to Gal4p or lexA (compare lanes 4 and 8, and lanes 5 and 11). However, an indication that the two-hybrid

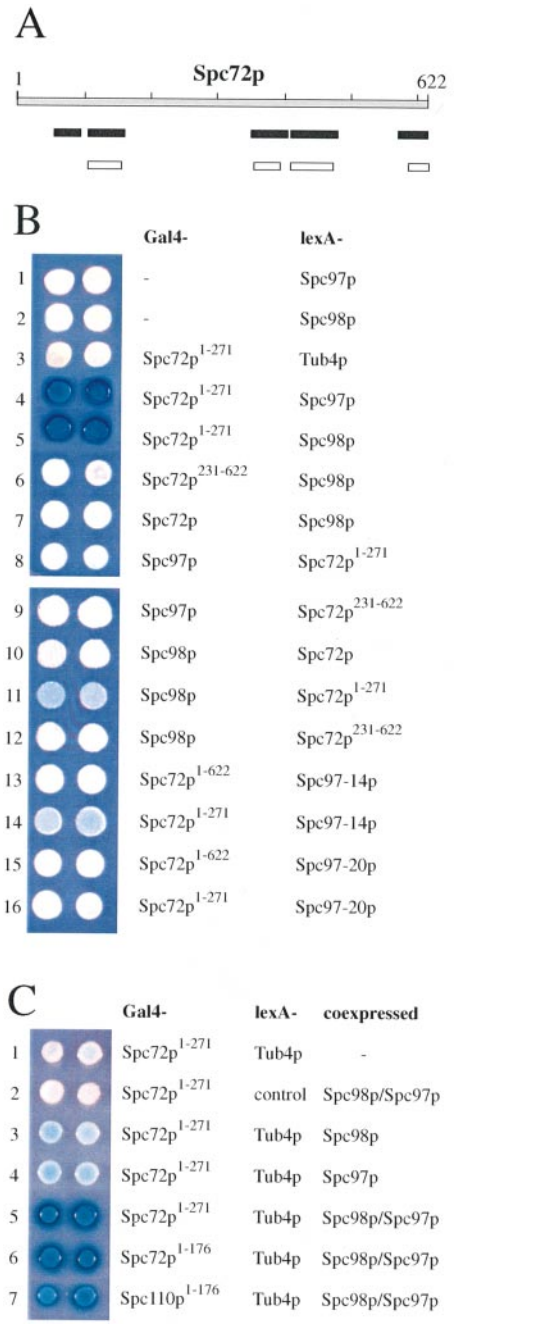


Fig. 1. Spc72p interacts with Spc97p and Spc98p in the two-hybrid system. (**A**) The potential coiled-coil regions of Spc72p (bars below the Spc72p scheme) were predicted using either the Coils software of Lupas *et al*. (1991) (▬) or the Paircoil program of Berger *et al*. (1995) (▭). (**B**) The N-terminal domain of Spc72p interacts with Spc98p and Spc97p in the two-hybrid system. The empty plasmid was used as a control (–). The blue colony colour on X-Gal plates indicates interaction. (**C**) Tub4p interacts with Spc72p after co-overexpression of *SPC98* and *SPC97*. *SPC98* and/or *SPC97* were expressed under the control of the *Gal1* promoter from *CEN-TRP1* plasmids harbouring one or both promoter fusions. The two-hybrid interactions were assayed as described (Knop and Schiebel, 1997).

binding of Spc72p$^{1–271}$ to Spc97p is specific came from the observation that the temperature-sensitive *spc97-20* (lane 16) and *spc97-14* alleles (lane 14) showed no or a strongly reduced interaction with Spc72p$^{1–271}$. In contrast to Spc72p$^{1–271}$, no binding of the C-terminal domain (lanes 6; data not shown) or the entire Spc72p (lane 7; data not shown) to Spc98p or Spc97p was observed.

Similarly to the N-terminal domain of Spc110p (Spc110p$^{1–176}$) (Knop *et al*., 1997; Figure 1C, lane 7), a strong interaction of Spc72p$^{1–271}$ with lexA–Tub4p was only observed when *SPC98* and *SPC97* were co-overexpressed simultaneously (Figure 1C, compare lanes 3 and 4 with 5), suggesting that the N-terminal domain of Spc72p interacted indirectly with lexA–Tub4p via binding to Spc98p and Spc97p in the Tub4p complex. It is noteworthy that the first 176 amino acids of Spc72p (Spc72p$^{1–176}$) were sufficient for this interaction and behaved as Spc72p$^{1–271}$ in all these experiments (lane 6; data not shown). In conclusion, the two-hybrid interactions of the subdomains of Spc72p with components of the Tub4p complex is remarkably reminiscent of Spc110p (Knop and Schiebel, 1997). In addition, the failure of Gal4–Spc72p to interact with Spc98p and Spc97p explains why the entire *SPC72* was not obtained in the initial two-hybrid screen.

Biochemical and genetic interactions of Spc72p with the Tub4p complex

We looked for biochemical evidence for an interaction of the Tub4p complex with Spc72p. A yeast strain harbouring a functional chromosomal gene fusion of *SPC72* with three copies of the haemagglutinin epitope (3HA) was constructed. *SPC72-3HA* cells were lysed under conditions which extracted ~30% of SPC72p-3HA, hardly any of Spc110p and most of Spc98p, Spc97p and Tub4p from the cells (data not shown). Spc72p-3HA was then precipitated with anti-HA (12CA5) antibodies. Besides Spc72p-3HA, also Spc98p, Spc97p and Tub4p (Figure 2A), but not α- and β-tubulin (data not shown), were detected in the immunoprecipitate, indicating a physical interaction between the Tub4p complex and Spc72p. Analysis of the immunoprecipitation supernatants revealed that only a small percentage of the Tub4p complex co-precipitated with Spc72p-3HA, while >90% of the extracted Spc72p-3HA was precipitated. This suggests that only a minor fraction of the Tub4p complex is associated with Spc72p. We established that the co-precipitation of Tub4p, Spc98p and Spc97p with Spc72p-3HA was specific: no Tub4p complex was precipitated by the anti-HA antibodies from an extract of *SPC72* cells (Figure 2A, lanes 1). To confirm that Tub4p, Spc98p, Spc97p and Spc72p interact, we precipitated Spc97p-3HA by anti-HA antibodies. Spc98p, Tub4p and Spc72p were detected in the precipitate and this co-precipitation was not observed from an extract containing Spc97p (Figure 2B).

We then tested whether the Tub4p complex binds to GST–Spc72p$^{1–271}$ purified from *Escherichia coli*, as is the case for GST–Spc110p$^{1–204}$ (Knop and Schiebel, 1997). Yeast extract containing Tub4p complex was incubated with recombinant GST, GST–Spc42p$^{1–214}$ or GST–Spc72p$^{1–271}$ bound to a glutathione resin. Binding of Tub4p, Spc98p and Spc97p to the resins was analysed by immunoblotting, because these species are minor components in total yeast cell lysate. A fraction of the Tub4p complex present in the crude lysate bound to GST–Spc72p$^{1–271}$, but not to GST or GST–Spc42p$^{1–214}$, indicating specific binding (Figure 2C).

Genetic evidence for an interaction of Spc72p with the Tub4p complex came from overexpression studies of *SPC72*. We found that moderate overexpression of *SPC72* from the centromere-based *GalS* promoter caused a slight growth defect (Figure 2D, compare lanes 1 and 2, galactose). This defect was increased further by expressing *SPC72* from the stronger *Gal1* promoter (lane 3, galactose) and was finally lethal when the *Gal1–SPC72* promoter fusion was on a 2 μm multicopy plasmid (lane 4, galactose). In contrast, all cells grew equally on the repressing glucose plates. Interestingly, the toxic effects of *SPC72* overexpression were much weaker in strain ESM387-3 which carries the chromosomal *Gal1–TUB4*, *Gal1–SPC98* and *Gal1–SPC97* derivatives (Figure 2D, lanes 5–8). This result is explained most easily by a binding of Spc72p to the assembled Tub4p complex. Taken together, our biochemical and genetic analyses confirmed that the N-terminal domain of Spc72p interacts with the Tub4p complex.

Spc72p is an essential component of the outer plaque of the SPB

Spc72p may be a cytoplasmic Tub4p complex-binding protein, it could represent an additional subunit of the Tub4p complex, or it may function as a GTBP at the outer plaque. Only in the latter case we would expect to find Spc72p exclusively at the outer plaque of the SPB. To address these possibilities, we investigated the localization of Spc72p by indirect immunofluorescence and immunoelectron microscopy. We used affinity-purified anti-Spc72p$^{1–271}$ antibodies for these experiments which were specific for Spc72p. This is indicated by the fact that predominantly one protein band with ~85 kDa was detected in a cell lysate of *SPC72* cells (Figure 3A, lane 3) and this band was shifted towards a higher molecular weight in an *SPC72-3HA* cell extract (compare lanes 3 and 4). A comparison with the anti-HA antibody (lanes 1 and 2) showed that the anti-Spc72p antibodies (lanes 3 and 4) were more specific. On immunoblots, some Spc72p was shifted to higher molecular weights, and sometimes even multipe bands were resolved. One reason for this behaviour could be phosphorylation of Spc72p. By indirect immunofluorescence, Spc72p was detected as one or two dots at the nuclear periphery of all cells of an unsynchronized culture by the anti-Spc72p antibodies (Figure 3B). Double labelling experiments with anti-tubulin antibodies established that Spc72p is associated with the spindle poles, exactly where the SPBs are situated. An identical cellular distribution was observed with a functional Spc72p–green fluorescent protein (Spc72p–GFP) fusion (data not shown).

The substructural localization of Spc72p at the SPB was investigated by immunoelectron microscopy. Isolated SPBs were incubated with the anti-Spc72p antibodies, followed by secondary antibodies coupled to colloid gold (15 nm). One to six gold particles were associated with the outer plaque region of the SPBs (n = 30; Figure 3C), suggesting that Spc72p is a component of the outer plaque. It is noteworthy that the inner plaque which is recognizable

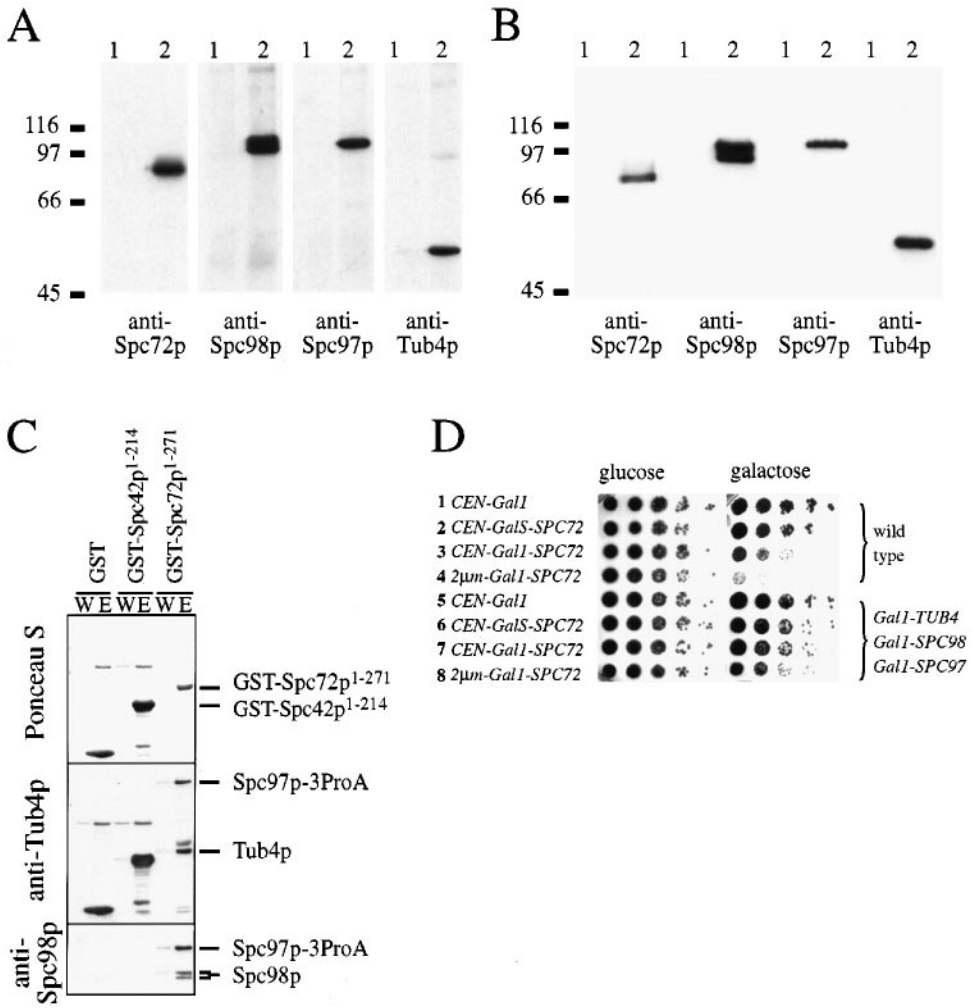


Fig. 2. Spc72p interacts with the Tub4p complex. (**A**) Co-immunoprecipitation of Spc72p-3HA with the γ-tubulin complex. Extracts of wild-type (lanes 1, YPH499) or *SPC72-3HA* (lanes 2, ESM479) cells were incubated with anti-HA (12CA5) antibodies covalently cross-linked to protein A–Sepharose. Spc72p, Spc98p, Spc97p and Tub4p were detected in the immunoprecipitate by immunoblotting as indicated. (**B**) Co-immunoprecipitation of Spc97p-3HA with Spc98p, Tub4p and Spc72p. Extracts of wild-type (lanes 1, YPH499) or *SPC97-3HA* (lanes 2, YMK22; Knop *et al.*, 1997) cells were used. Spc72p, Spc98p, Spc97p and Tub4p were detected in the immunoprecipitate by immunoblotting as indicated. (**C**) *In vitro* binding of the Tub4p complex to the N-terminal domain of Spc72p. Cells of strain YMK47 (*SPC97-3ProA*; Knop and Schiebel, 1997) were lysed with glass beads. The cleared extract was applied to glutathione–Sepharose columns, on which GST (expressed from pGEX-5X-1), GST–Spc42p$^{1–241}$ (pSM363) or GST–Spc72p$^{1–271}$ (pGP68) were immobilized. After washing the columns with buffer, bound proteins were eluted with buffer containing 10 mM glutathione. Tub4p and Spc98p in the wash (W, corresponding to the last 500 µl of the wash fraction) and eluate (E) fractions were detected by immunoblotting. Spc97p-3ProA was detected due to the binding of protein A to IgGs of the primary and secondary antibodies. The antibodies specific for Tub4p were raised against a GST–Tub4p fusion protein and therefore also recognize GST and GST–Spc42p$^{1–241}$ in the immunoblots. The protein of ~70 kDa is most probably Hsp70 from *E.coli* which co-purifies with many fusion proteins. (**D**) Genetic evidence for an interaction of Spc72p with the Tub4p complex. Cells of YPH499 (rows 1–4) and ESM387–3 (rows 5–8) were transformed with p413-*Gal1* (rows 1 and 5), p413-*GalS–SPC72* (rows 2 and 6; pSM573), p413-*Gal1–SPC72* (rows 3 and 7; pSM572) or p423-*Gal1–SPC72* (rows 4 and 8; pSM574). Serial dilutions of transformants grown in raffinose medium were plated out on selective plates containing either raffinose and glucose (glucose) or raffinose and galactose (galactose) as carbon sources.

by the attached microtubules was never stained by the anti-Spc72p antibodies.

To determine whether *SPC72* is an essential gene, the entire coding region of *SPC72* was disrupted in the diploid yeast strain YPH501 using the *kanMX4* gene as disruption marker (ESM418). Analysis of tetrads from ESM418 suggested that *SPC72* is indeed essential (data not shown). The essential function of *SPC72* was confirmed by a plasmid shuffle experiment (Figure 7B). In summary, *SPC72* encodes an essential SPB component that is associated with the outer plaque of the SPB.

Temperature-sensitive SPC72 mutants are defective in spindle elongation, cytoplasmic microtubule organization and nuclear migration

The phenotype of mutants provides information about the function of the wild-type gene. We were interested especially in the phenotype of *spc72* mutants with a defect in the N-terminal Tub4p complex-binding domain. Therefore, we constructed an N-terminal variant of *SPC72* (Δ*N-SPC72*), that carried a deletion of amino acids 2–176. However, Δ*N-SPC72* was unable to keep cells alive in the absence of the wild-type *SPC72* (Figure 7B). To

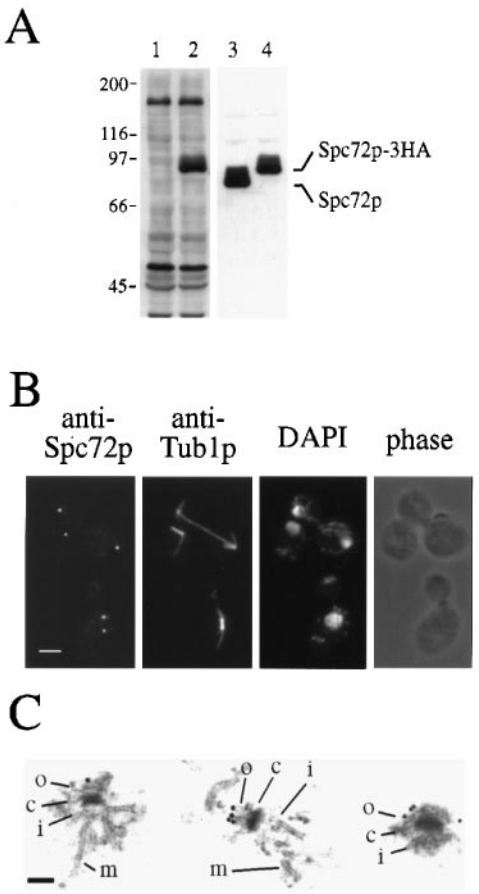


Fig. 3. Spc72p is an essential protein that is associated with the outer plaque of the SPB. (**A**) The anti-Spc72p antibodies are specific. Immunoblots of crude extracts of wild-type *SPC72* (YPH499; lanes 1 and 3) or *SPC72-3HA* cells (ESM479; lanes 2 and 4) were probed with monoclonal anti-HA (lanes 1 and 2) or affinity-purified polyclonal anti-Spc72p (lanes 3 and 4) antibodies. (**B**) Spc72p is an SPB component. Spc72p and tubulin of wild-type cells (YPH499) were stained by indirect immunofluorescence using affinity-purified rabbit anti-Spc72p and mouse monoclonal anti-tubulin (Wa3) antibodies. DNA was stained with DAPI. Bar: 4 μm. (**C**) Spc72p is associated with the outer plaque. Isolated SPBs of strain YPH499 were prepared for immunoelectron microscopy as described in Materials and methods. Spc72p was detected using the affinity-purified rabbit anti-Spc72p antibodies. Secondary antibodies were goat anti-rabbit IgGs conjugated to 15 nm gold particles. Only the outer, but not the inner plaque of the SPB was labelled. The inner plaque is still associated with the nuclear microtubules, while the cytoplasmic microtubules of the outer plaque were lost during the SPB purification (Rout and Kilmartin, 1990). Abbreviations: c, central plaque; i, inner plaque; m, microtubule; o, outer plaque. Bar: 100 nm.

overcome this problem, we constructed and analysed temperature-sensitive *spc72(ts)* mutants with defects in the Tub4p complex-binding region of Spc72p (Figure 4A). Synchronized cultures of *SPC72* and *spc72-7* were shifted from 23 to 37°C. The DNA content, microtubule phenotypes and the distribution of the DNA were determined over time. *SPC72* and *spc72-7* cells replicated their DNA with similar kinetics (Figure 4B). However, while *SPC72* cells continued in the cell cycle, as indicated by the appearance of cells with 1N DNA content after 3 h, a *spc72-7* culture also showed cells with a DNA content >2N after 3 h.

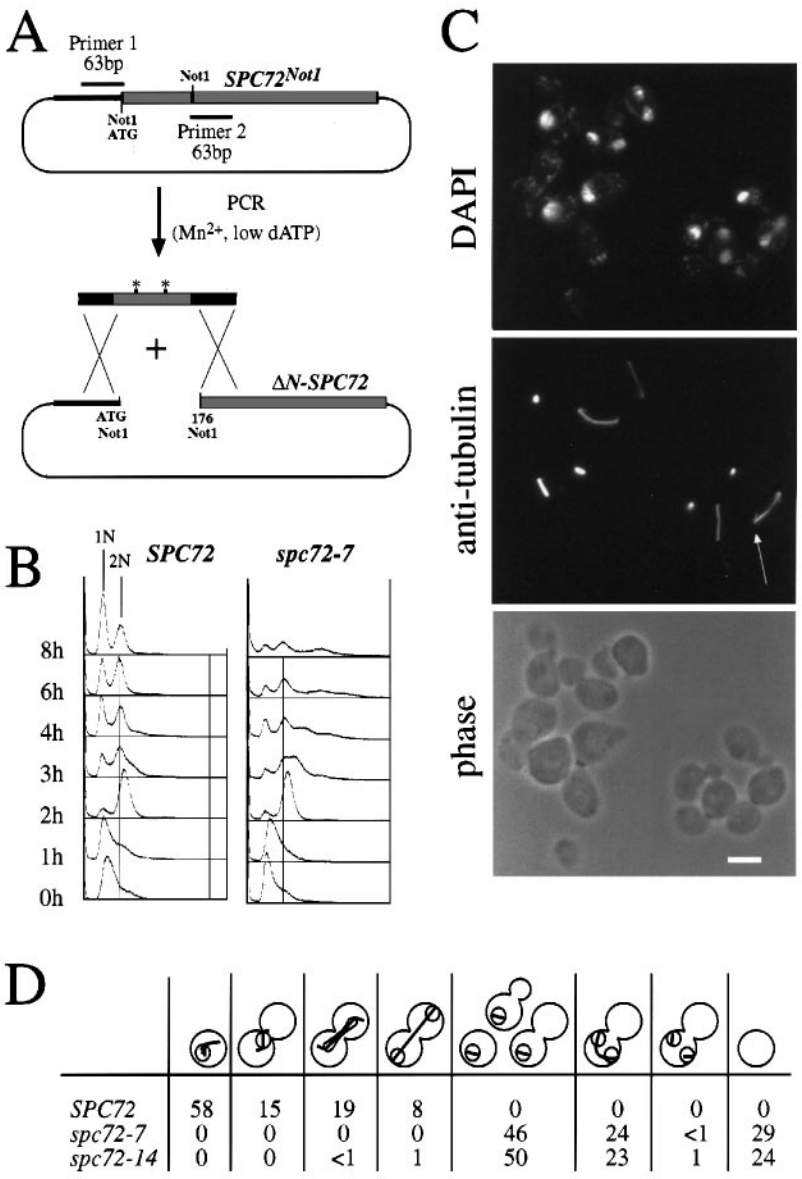
When *spc72-7* cells were analysed using immunofluorescence microscopy, it was clear that most cells had strongly reduced cytoplasmic microtubule arrays (Figure 4C; for comparison, see Figures 3B and 5D, first column). We also noticed cells with an anaphase spindle in one cell body that still exhibited cytoplasmic microtubule remnants associated with the SPBs (Figure 4C, arrow). Figure 4D shows the distribution of the various microtubule phenotypes observed in wild-type, *spc72-7* and *spc72-14* cells 3 h after release fom the α-factor arrest at 37°C. As expected from this cytoplasmic microtubule deficiency, 29% of *spc72-7* cells were anucleated and lacked any microtubules, and about the same percentage of *spc72-7* cells had two separate 4,6′-diamidino-2-phenylindole (DAPI)-staining regions in the schmoo-containing mother cell (Figure 4C and D). These two DAPI regions were still connected by long misaligned nuclear microtubules, indicating that nuclear division was not complete. The residual cells (~46%) had one DAPI-staining region with a short spindle of random orientation. The nucleus was either in the mother cell or in a cell without a bud. Nearly identical results were obtained with *spc72-14* cells which differ from *spc72-7* cells in their temperature sensitivity (data not shown and see Figure 4D). Taken together, our *spc72(ts)* cells show a clear defect in cytoplasmic microtubule functions, as indicated by the nuclear migration and spindle orientation defects. However, they show additional defects in spindle elongation or nuclear division that are not easily explainable.

Overexpression of ΔN-SPC72 is lethal and results in multi-nucleated and anucleated cells, while more cytoplasmic microtubules are observed after SPC72 overexpression

Since the nuclear phenotypes of *spc72-7* and *spc72-14* cells are not fully understood, we looked for additional experiments that could lead to a better understanding of Spc72p's function. We reasoned that overproduction of ΔN-Spc72p should displace Spc72p from the outer plaque. A ΔN-Spc72p-containing outer plaque may then be unable to bind Tub4p complex and thereby would fail to organize cytoplasmic microtubules. On the contrary, overproduced Spc72p may increase the number of Tub4p complex-binding sites at the outer plaque and thereby elevate the number of cytoplasmic microtubules.

Mild overexpression of *ΔN-SPC72* from the galactose-inducible *GalS* promoter was lethal for the cells, as indicated by the failure of *GalS–ΔN-SPC72* cells to grow on galactose plates, while *GalS–SPC72* and *GalS* control cells grew (Figure 5A). Analysis of cell extracts confirmed *GalS*-dependent expression of ΔN-Spc72p and Spc72p (Figure 5B).

To gain an understanding of why overexpression of *ΔN-SPC72* is toxic, we investigated whether overproduced ΔN-Spc72p and Spc72p are capable of binding to the SPB and whether they replace endogeneous Spc72p from the SPB. We established that GFP–ΔN-Spc72p associated with the SPB after mild overproduction (data not shown). In addition, we found that 64% of the nuclei of *SPC72–GFP* cells did not show a Spc72p–GFP signal at the SPB after induction of *GalS–ΔN-SPC72* for 9 h (corresponding to two doubling times of the *GalS* cells), while the rest of the *SPC72–GFP* nuclei showed one SPB signal. In contrast, all nuclei of cells, in which the central plaque of the SPB was marked by GFP fused to Spc42p (Spc42p–



SPC72	58	15	19	8	0	0	0	0
spc72-7	0	0	0	0	46	24	<1	29
spc72-14	0	0	<1	1	50	23	1	24

Fig. 4. *spc72-7* is defective in nuclear migration and nuclear division. (**A**) Strategy for the construction of temperature-sensitive alleles of *SPC72* which carry mutations specifically in the Tub4p complex-binding domain of Spc72p. Codons 1–176 of *SPC72* were mutagenized by PCR. The PCR product was combined with the non-mutagenized 3′ region of *SPC72* by homologous recombination as described (Muhlrad *et al.*, 1992). The regions where recombination occurs reside within the primer of the PCR product and, therefore, were not mutagenized. (**B**) *SPC72* (YMK179-9) and *spc72-7* (YMK179–3) cells were arrested by α-factor in the cell cycle in G_1 with a 1N content (t = 0 h). Cells were released from their cell-cycle block by removing α-factor and they were simultaneously shifted to 37°C. Samples were taken every hour and analysed for their DNA content by flow cytometry. (**C**) *spc72-7* cells fail to organize cytoplasmic microtubules and have a nuclear migration defect. Synchronized *spc72-7* cells of (B) were fixed with formaldehyde after 3 h at 37°C. The fixed cells were analysed by indirect immunofluorescence using anti-tubulin antibodies. DNA was stained with DAPI. Cells were also inspected by phase contrast microscopy. Bar: 6 μm. (**D**) Phenotypes of *spc72-7* cells. The spindle and nuclear migration defects of >200 synchronized *SPC72*, *spc72-7* and *spc72-14* cells incubated for 3 h at 37°C were determined. The distribution is given in percentages.

GFP), revealed one or two SPB signals after induction of *GalS–ΔN-SPC72* (Figure 5C). Most probably, Spc72p–GFP that was already assembled into the outer plaque at the time of the *GalS–ΔN-SPC72* induction was not replaced by ΔN-Spc72p, while ΔN-Spc72p competed with Spc72p for the incorporation into the newly formed SPB. Similarly, the SPB of *GalS–SPC72* cells was stained more strongly by the anti-Spc72p antibodies compared with the *GalS* control (data not shown), and overproduced Spc72p replaced Spc72p–GFP from a newly formed SPB (Figure 5C), suggesting that more Spc72p bound to the outer plaque of *GalS–SPC72* cells than in wild-type.

The positions of the nucleus and microtubule structures in *GalS*, *GalS–SPC72* and *GalS–ΔN-SPC72* cells were analysed after growth in the inducing galactose-containing medium. Most interestingly, many *GalS–ΔN-SPC72* cells were unbudded and contained no, two or four nuclei (Figure 5D). We noticed cells which appear to have only one nucleus. As reported for the *tub2-401* mutant (Sullivan and Huffaker, 1992), we assume that these cells probably have more than one nuclei situated on top of or next to each other where they cannot be resolved by fluorescence microscopy. This is also suggested by the large number of anucleated cells in the *GalS–ΔN-SPC72* culture

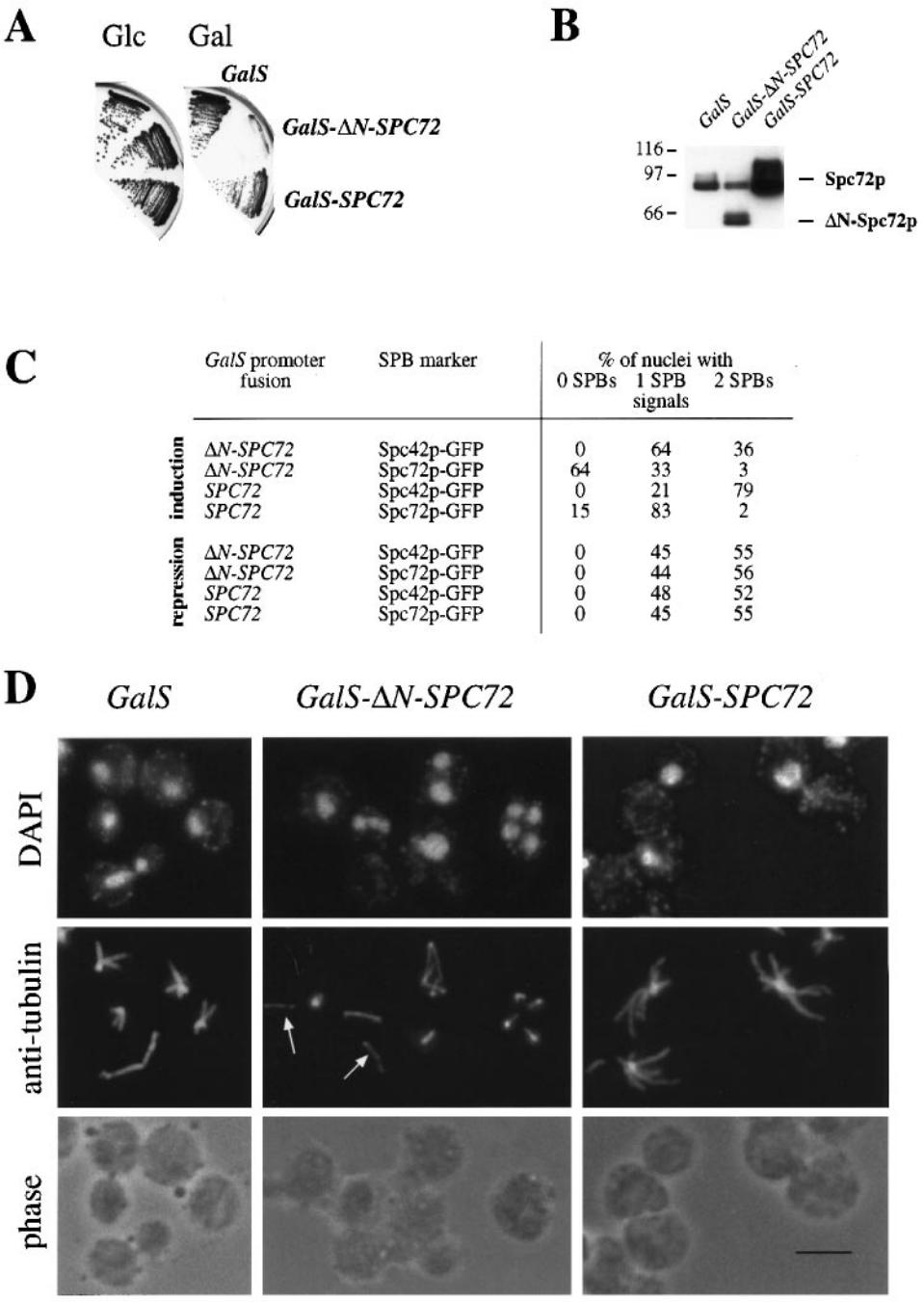


Fig. 5. Overexpression of Δ*N-SPC72* is dominant lethal and results in multi- and anucleated cells, while overexpression of *SPC72* causes an increase in cytoplasmic microtubule staining. (**A**) Overexpression of Δ*N-SPC72* is lethal. Cells of YPH499 containing the control plasmid p415-*GalS*, p415-*GalS*–Δ*N-SPC72* (pMK252) or p415-*GalS*–*SPC72* (pMK253) were grown on selective plates containing either glucose and raffinose (Glc) or galactose and raffinose (Gal) as carbon sources. Note that the *GalS* promoter is repressed by glucose and induced by galactose. (**B**) Expression levels of *GalS*–Δ*N-SPC72* and *GalS*–*SPC72*. YPH499 cells containing plasmids p415-*GalS*, p415-*GalS*–Δ*N-SPC72* or p415-*GalS*–*SPC72*^{NotI} were grown in raffinose/galactose medium for 3 h at 30°C. Expression of the Spc72p derivatives was determined by immunoblotting using anti-Spc72p antibodies. Since YPH499 (*SPC72*) carrying p415-*GalS*–Δ*N-SPC72* also expressed *SPC72*, Spc72p and ΔN-Spc72p were detected in the immunoblot. However, the signals are not comparable, because our anti-Spc72p antibodies are directed against the N-terminal 271 amino acids of Spc72p, of which the first 176 are missing in ΔN-Spc72p. (**C**) Overproduced ΔN-Spc72p and Spc72p replace Spc72p–GFP but not Spc42p–GFP from the SPB. Cells of *SPC42–GFP* (ESM440) or *SPC72–GFP* (ESM504) were transformed with the p415-*GalS*–Δ*N-SPC72* and p415-*GalS*–*SPC72* plasmids. Transformants were grown in selective medium containing either raffinose/glucose or raffinose/galactose as carbon sources for 9 h at 30°C (corresponding to two doubling times of the *GalS* cells). The cells were fixed briefly and the DNA was stained with DAPI. The number of GFP signals associated with ~100 nuclei was determined by fluorescence microscopy. (**D**) Overexpression of Δ*N-SPC72* results in multi- and anucleated cells, while overexpression of *SPC72* gives rise to more cytoplasmic microtubules. Wild-type cells (YPH499) carrying plasmids p415-*GalS*, p415-*GalS*–Δ*N-SPC72* or p415-*GalS*–*SPC72* were grown in glactose medium as described in (C). Microtubules of fixed cells were analysed by indirect immunofluorescence using anti-tubulin antibodies. DNA was stained with DAPI. Cells were also inspected by phase contrast microscopy. Bar: 5 μm.

Table I. Number of nuclei per cell upon Δ*N-SPC72* overexpression

Expression of	Percentage of cells with			
	0	1	2	>2 nuclei
GalS	0	100	0	0
GalS–SPC72	0	100	0	0
GalS–ΔN-SPC72	35	44	19	2

Cells were grown to early logarithmic growth phase on selective medium containing raffinose. The *GalS* promoter was induced for 9 h (corresponding to two doubling times of the *GalS* control strain in this medium). Cells were fixed briefly and the DNA was visualized with DAPI as described in Materials and methods.

(Table I). They resulted from cells that did complete anaphase nuclear division in the mother cell body, giving birth to one anucleated and one binucleated cell. It is noteworthy that multi- and anucleated cells were a specific phenotype of Δ*N-SPC72* overexpression. Such cell types were not found in the *GalS* or *GalS–SPC72* cultures (Table I). Furthermore, hardly any cytoplasmic microtubules were observed in *GalS–*Δ*N-SPC72* cells, while the nuclear microtubules were of normal appearance: depending on the cell-cycle stage of the cell, a monopolar spindle organized by one SPB (cell with four nuclei in Figure 5D), a parallel array of microtubules organized by two SPBs, or even two nuclei with an anaphase B spindle within one cell body were observed. We also noticed anucleated cells with one microtubule and nucleated cells with microtubules that were not connected with the SPB (Figure 5D, arrows), raising the possibility that microtubules detached from the SPB in *GalS–*Δ*N-SPC72* cells or that microtubule formation took place independently of the SPB.

Mild overexpression of *GalS–SPC72* dramatically increased the tubulin signal in the cytoplasm, suggesting that more cytoplasmic microtubules were organized by the outer plaque (Figure 5D). In addition, ~60% of the *GalS–SPC72* cells were large budded, with a single DAPI-staining region in the mother cell body, positioned close to the bud neck (Figure 5D). About 80% of the nuclei contained two SPBs (GFP–Spc42p; Figure 5C) and most probably a short spindle which was masked by the strong cytoplasmic microtubule staining. Taken together, the nuclear phenotype of *SPC72* overexpression is consistent with the notion that these cells pause in mitosis due to a defect in mitotic spindle assembly.

GalS, *GalS–*Δ*N-SPC72* and *GalS–SPC72* cells were inspected by thin section electron microscopy. The size and morphology of five SPBs examined from *GalS–SPC72* (Figure 6B) and *GalS–*Δ*N-SPC72* (Figure 6E) cells were similar to those of the SPB from *GalS* cells (Figure 6A). Since the cytoplasmic microtubules are difficult to detect by electron microscopy in yeast, it was impossible to judge by this experiment whether their number was affected. However, the nuclear microtubules are easily detectable and their appearance in *GalS–*Δ*N-SPC72* (Figure 6C and D) and *GalS–SPC72* cells (Figure 6B) was the same as in the *GalS* control (Figure 6A). In contrast to *GalS* cells (Figure 6A), *GalS–*Δ*N-SPC72* cells assembled the anaphase spindle in one cell body (Figure 6C). Furthermore, thin serial sections through

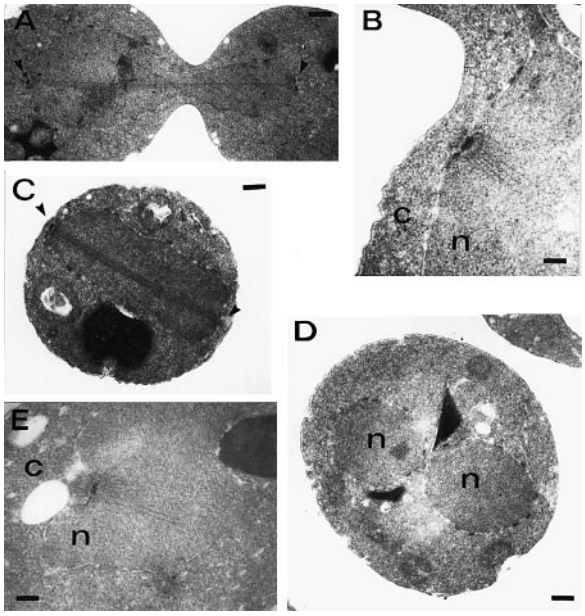


Fig. 6. Thin section electron microscopic analysis of *GalS*, *GalS–*Δ*N-SPC72* and *GalS–SPC72* cells. YPH499 cells containing p415-*GalS*, p415-*GalS–*Δ*N-SPC72* (pMK252) and p415-*GalS–SPC72* (pMK253) were grown in galactose medium as described in Figure 5D. Thin serial sections of embedded cells were inspected by electron microscopy. Note that the contrast of the specimen is relatively weak due to growth in synthetic complete medium without leucine. (**A**) A wild-type spindle of *GalS* cells. (**B**) A section through a *GalS–SPC72* SPB. (**C**) A spindle of a *GalS–*Δ*N-SPC72* cell. (**D**) A *GalS–*Δ*N-SPC72* cell with two separated nuclei. (**E**) An SPB of a *GalS–*Δ*N-SPC72* cell. Arrowheads in (A) and (C) point towards the SPBs. Bars in (A), (C) and (D) are 320 nm and in (B) and (E) 200 nm. Abbreviations: c; cytoplasm; n, nucleoplasm.

several *GalS–*Δ*N-SPC72* cells confirmed that some cells contained two completely separated nuclei (for an example, see Figure 6D). In addition, cells were found that did not contain nuclear membrane structures (data not shown). In conclusion, the phenotypes of Δ*N-SPC72* and *SPC72* overexpression are consistent with a function of the N-terminal domain of Spc72p in cytoplasmic microtubule organization, most likely by recruiting the Tub4p complex to the outer plaque.

The Tub4p complex-binding domain of Spc110p is functional at the outer plaque

A comparison of the amino acid sequences of the Tub4p complex-binding sites of Spc72p and Spc110p did not indicate any homology. This raises the possibility that the two Tub4p complex-binding domains evolved independently and that they may contain features which are important for their specific functions at the inner and outer plaques. To test this possibility, we constructed Δ$N^{2–176}$*-SPC110* (Δ*N-SPC110*), *SPC72*$^{2–176}$*–SPC110*$^{177–944}$ (*N-SPC72–SPC110*) and *SPC110*$^{1–176}$*–SPC72*$^{177–622}$ (*N-SPC110–SPC72*) derivatives (Figure 7A) and tested whether they are expressed and functional.

We noticed that Δ*N-SPC110* does not provide *SPC110* function (Figure 7B) and that its overexpression from the *GalS* promoter resulted in a lethal phenotype (Figure 7D), indicating that the Tub4p complex-binding domain of Spc110p is essential for its function. In contrast to Δ*N-SPC110*, hardly any transformants were obtained with a plasmid carrying *N-SPC72–SPC110* expressed from the

SPC110 promoter, suggesting that *N-SPC72–SPC110* is dominant lethal. Consequently, *GalS–N-SPC72–SPC110* expression was lethal (Figure 7D), such that 95% of the cells were no longer viable 1 h after the induction of the *GalS* promoter (data not shown). Most interestingly, the *N-SPC110–SPC72* gene fusion rescued a *SPC72* deletion (Figure 7B), raising the possibility that the Tub4p-binding domain of Spc110p functions at the outer plaque. To exclude that the rescuing effect of N-Spc110p was due to a simple stabilization of the C-terminal domain of Spc72p, we constructed a *KAR1–SPC72* gene fusion which did not function for *SPC72* (data not shown). Finally, immunoblots established that *N-SPC110–SPC72* (Figure 7C), *GalS–ΔN-SPC110*, *GalS–SPC110* and *GalS–N-SPC72–SPC110* were expressed in yeast (Figure 7E).

We were interested in whether N-Spc110–Spc72p fulfils Spc72p function at the SPB. This is indicated by the fact that N-Spc110–Spc72p produced from the *GalS* promoter replaced endogenous GFP–Spc72p from the newly formed SPB (data not shown). For a more detailed study, *N-SPC110–SPC72* and *SPC110* were integrated into the *leu2* locus of Δ*spc72::kanMX4* cells to exclude artefacts caused by loss or an increased copy number of the plasmid. *N-SPC110–SPC72* cells had a slightly longer doubling time (2.8 h) at 30°C than *SPC72* cells (2.4 h), and a small population of large budded *N-SPC110–SPC72* cells had two separate DAPI-staining regions in the mother cell body (Figure 7F, arrow), while such cells were not observed in the *SPC72* culture (Table II). The cytoplasmic microtubules were often longer in *N-SPC110–SPC72* cells undergoing anaphase as compared with *SPC72* cells. Summarizing, our results demonstrate that the N-terminal domain of Spc110p functions for the corresponding domain of Spc72p, suggesting that the Tub4p-binding domain of Spc110p is functional at the outer plaque.

Discussion

A universal component of MTOCs involved in microtubule organization is γ-tubulin (Horio *et al*., 1991; Liu *et al*., 1993; Spang *et al*., 1996a; Ding *et al*., 1997) and possibly other subunits of γ-tubulin complexes (Rout and Kilmartin, 1990; Spang *et al*., 1996a; Knop *et al*., 1997). γ-Tubulin complexes assemble in the cytoplasm of cells (Stearns and Kirschner, 1994; Moudjou *et al*., 1996; Akashi *et al*., 1997; Pereira *et al*., 1998), followed by their binding to MTOCs. This suggests that MTOCs must have GTBPs which dock the γ-tubulin complex to the scaffold of the MTOC. Using yeast as a model system, we have started to investigate the nature, specificity and regulation of GTBPs.

Spc72p is the GTBP of the outer plaque

We recently identified the SPB component Spc110p (Kilmartin *et al*., 1993; Kilmartin and Goh, 1996) as a GTBP of the inner plaque (Knop and Schiebel, 1997) (Figure 8). Here, we describe the identification of the essential SPB component Spc72p as a GTBP of the outer plaque. This notion is supported by the findings that Spc72p is an outer plaque component whose N-terminal domain interacts with Spc98p and Spc97p, but not with Tub4p, in the yeast two-hybrid system. Further proof for an interaction of Spc72p with the Tub4p complex came from the co-immunoprecipitation of the Tub4p complex with Spc72p-3HA and from the *in vitro* binding of the Tub4p complex to purified GST-Spc72p$^{1–271}$. In the immunoprecipitation experiment, we noticed that the phosphorylated and the unphosphorylated forms of Spc98p were precipitated by Spc72p-3HA with about equal efficiency, which was unexpected since previous experiments indicated that Spc98p at the outer plaque is in its unphosphorylated form (Pereira *et al*., 1998). We assume that phosphorylated Tub4p complex from the inner plaque bound to Spc72p-3HA after its extraction from the SPB. This assumption is in agreement with the finding that both forms of Spc98p bound to GST-Spc72p$^{1–271}$ *in vitro*.

Conditional lethal spc72 mutants showed defects in cytoplasmic microtubule functions

We studied the function of Spc72p in yeast cells using temperature-sensitive alleles of *SPC72*. If Spc72p is the

Fig. 7. The Tub4p-binding domain of Spc110p is functional at the outer plaque. (**A**) Construction of *N-SPC72–SPC110* and *N-SPC110–SPC72* hybrids. *Not*I restriction sites were introduced by recombinant PCR after codon 1 and codon 176 of *SPC72* and *SPC110* [now named *SPC110^{NotI}* (pMK231) and *SPC72^{NotI}* (pMK234)]. Using these *Not*I sites, we constructed Δ*N-SPC72* (pMK232), Δ*N-SPC110* (pMK230), *N-SPC72–SPC110* (pMK233) and *N-SPC110–SPC72* (pMK235). (**B**) The Tub4p complex-binding domain of Spc110p is essential for its function and *N-SPC110–SPC72* rescues a *SPC72* null mutant. Strain ESM335 (Δ*spc110::HIS3* pRS316-*SPC110* 'SPC110 shuffle strain') was transformed with plasmid pRS414 (sector 'control plasmid'), and pRS414 derivatives carrying *SPC110* (pSM187), Δ*N-SPC110* (pMK230) or *SPC110^{NotI}* (pMK231). Transformants were incubated on 5-FOA-containing plates at 30°C. Strain ESM448 (Δ*spc72::kanMX4*, pRS316-*SPC72*, 'SPC72 shuffle strain') transformed with pRS315 is unable to grow on 5-FOA which selects against the *URA3*-based pRS316-*SPC72* plasmid (sector 'control plasmid'), indicating that *SPC72* is an essential gene. *SPC72* (pSM447) or *N-SPC110–SPC72* (pMK235) on a *LEU2*-based plasmid enables ESM448 to grow on 5-FOA, indicating that *N-SPC110–SPC72* provides *SPC72* function. In contrast, Δ*N-SPC72* (pMK232) did not allow growth of ESM448 on 5-FOA, suggesting that the N-terminal domain of Spc72p is essential for its function. All transformants grew equally well on selective plates without 5-FOA (data not shown). (**C**) Expression of *N-SPC110–SPC72*. Extracts from strains YMK174 (*SPC110* and *SPC72*; lanes 1 and 3) and YMK173 (*SPC110* and *N-SPC110–SPC72*; lanes 2 and 4) were probed with anti-Spc110p$^{3–175}$ (lanes 1 and 2) or anti-Spc72p$^{1–271}$ (lanes 3 and 4) antibodies. Spc110p (lane 1) or Spc72p (lane 3) were detected by anti-Spc110p or anti-Spc72p in the wild-type strain, respectively. N-Spc110p–Spc72p is detected by the anti-Spc110p (lane 2) but not by the anti-Spc72p antibody (lane 4). (**D**) Overexpression of Δ*N-SPC110* or *N-SPC72–SPC110* is lethal. Cells of YPH499 were transformed with the control plasmid p415-*GalS* (sector '*GalS*'), or with p415-*GalS* carrying Δ*N-SPC110* (pMK239), *SPC110^{NotI}* (pMK240) or *N-SPC72–SPC110* (pMK243). The transformants were streaked on plates containing raffinose and glucose (data not shown) or raffinose and galactose as carbon source. While all transformants grew equally well on the glucose plates, *GalS–ΔN-SPC110* and *GalS–N-SPC72–SPC110* did not grow on galactose which induces the *GalS* promoter. (**E**) *GalS*-dependent expression of Δ*N-SPC110*, *SPC110^{NotI}* and *N-SPC72–SPC110*. Cells of YPH499 carrying plasmids p415-*GalS*, p415-*GalS–ΔN-SPC110* (pMK239), p415-*GalS–SPC110^{NotI}* (pMK240) or p415-*GalS–N-SPC72-SPC110* (pMK243) were tested by immunoblotting with anti-Spc110p$^{3–175}$, anti-Spc110p$^{293–756}$ and anti-Spc72p$^{1–271}$ for expression of the *GalS* promoter fusions. Note that the chromosomally encoded Spc72p and Spc110p of YPH499 were also detected by the antibodies. (**F**) *N-SPC110–SPC72* cells show a weak nuclear migration failure. *N-SPC110–SPC72* (ESM173) and *SPC72^{NotI}* (YMK174) cells were grown in YPD medium at 30°C to mid-logarithmic phase. The microtubules of fixed cells were stained by indirect immunofluorescence with anti-tubulin antibodies. DNA was stained with DAPI. Cells were also inspected by phase contrast microscopy. The arrow points to a cell with two nuclei in one cell body. Bar: 5 μm.

GTBP at the outer plaque, we expected to find that the cytoplasmic microtubules are defective in *spc72(ts)* cells, resulting in nuclear migration and nuclear positioning defects (Palmer *et al*., 1992; Sullivan and Huffaker, 1992). This was indeed the case: a high proportion of anucleated *spc72(ts)* cells and large budded *spc72(ts)* cells with one or two DAPI-staining regions randomly positioned in the mother cell body were observed. In addition, the mitotic spindle of large budded *spc72(ts)* cells was not aligned along the mother–bud axis as is the case in wild-type cells (Palmer *et al*., 1992). A direct proof of a cytoplasmic microtubule defect in *spc72(ts)* cells is the reduction or complete loss of cytoplasmic microtubule staining in indirect immunofluorescence experiments. However, compared with other mutants which affect cytoplasmic microtubule functions (Sullivan and Huffaker, 1992; Eshel *et al*., 1993; Li *et al*., 1993; Cottingham and Hoyt, 1997), *spc72(ts)* cells did not complete anaphase nuclear division in the mother cell body and therefore failed to give rise to multi-nucleated cells. Instead, ~50% of *spc72(ts)* cells formed a short spindle, or 24% of the cells formed an anaphase spindle randomly positioned in the mother cell, but failed to divide the nucleus. The reason for these nuclear phenotypes of *spc72(ts)* cells is unknown. Possible

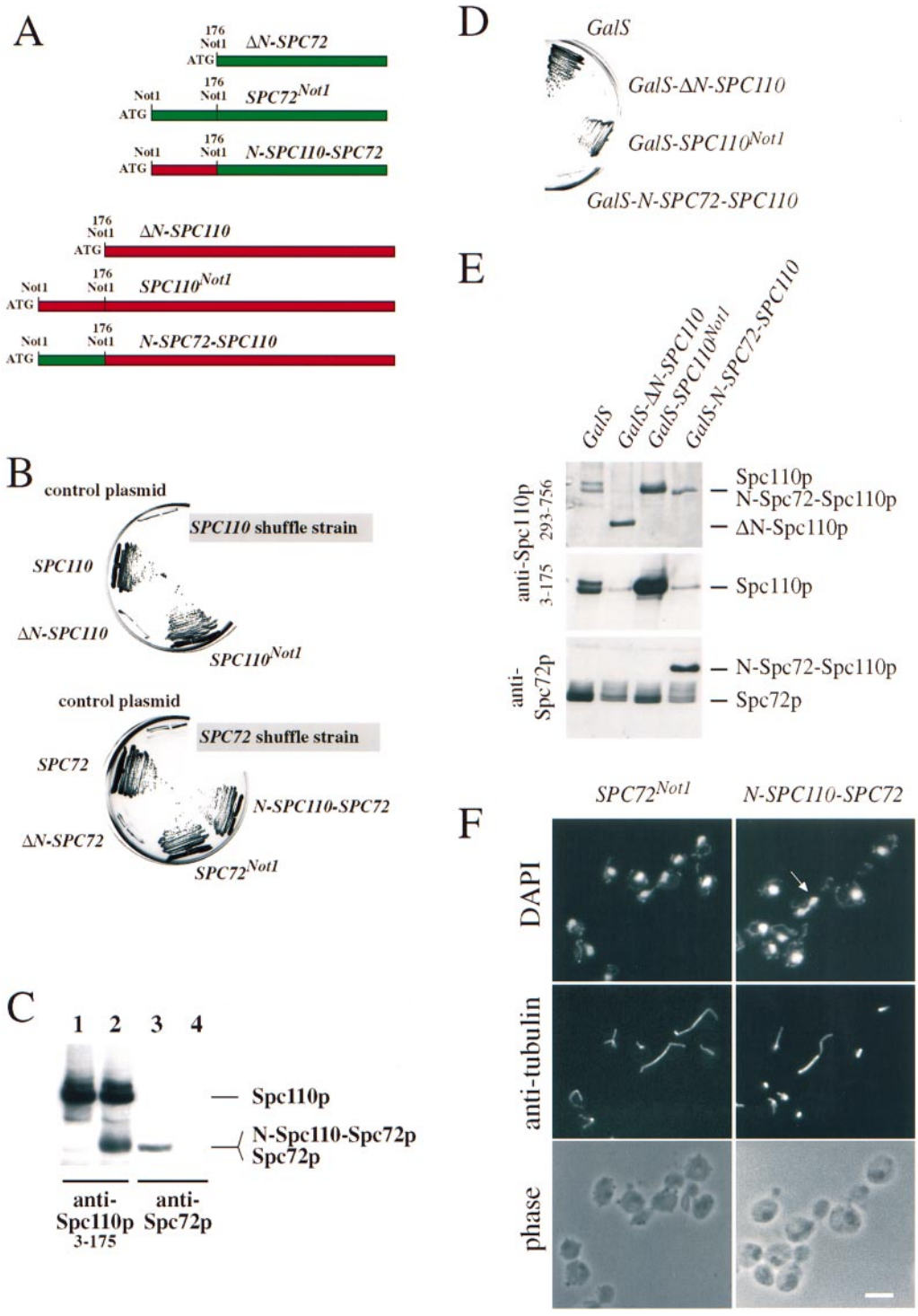


Table II. Phenotypes of cells expressing the *N-SPC110–SPC72* gene fusion

N-SPC110-SPC72	54	10	17	12	5	2
SPC72^{Not1}	52	18	29	0	0	0

Cells were grown to early logarithmic phase and prepared for fluorescence microscopy as shown in Figure 7F. Approximately 200 cells were counted (values in percentages).

explanations are that Spc72p may have additional functions in cell-cycle regulation, nuclear microtubule organization or nuclear division. Alternatively, aggregates of Spc72-7p, which we observed occasionally by indirect immunofluorescence in the cytoplasm of *spc72-7* cells (data not shown), may bind other components of the SPB and thereby influence SPB functions. This would then result in the activation of the mitotic checkpoint (Hoyt *et al*., 1991; Li and Murray, 1991) in addition to the nuclear migration phenotype.

In contrast to *spc72-7* cells, overexpression of Δ*N-SPC72* resulted in polynucleated cells. Hardly any cytoplasmic microtubules were detectable in *GalS–*Δ*N-SPC72* cells, while the nuclear microtubules were of normal appearance and they were probably functional, indicated by the segregation of sister chromatids and the formation of anaphase spindles. Remarkably, the phenotype of *GalS–*Δ*N-SPC72* overexpression is nearly identical to that of *tub2-401* cells which are selectively defective in cytoplasmic microtubule functions (Sullivan and Huffaker, 1992). This similarity supports the notion that Spc72p has a specific function in cytoplasmic microtubule organization. The specific cytoplasmic microtubule defect of Δ*N-SPC72* is explained by the finding that ΔN-Spc72p competes with GFP–Spc72p for the incorporation into the outer plaque of newly formed SPBs. Such SPBs are then unable to recruit Tub4p complex to the outer plaque, which as a consequence fails to organize cytoplasmic microtubules.

Further support for Spc72p's function as a GTBP comes from the observation that the moderate overexpression of *SPC72* gives rise to more Spc72p signal at the SPB, accompanied by an increase in cytoplasmic microtubule staining. The elevated level of Spc72p at the outer plaque may recruit more Tub4p complex to this location of the SPB, with the consequence that more cytoplasmic microtubules are formed. Overexpression of *SPC72* showed toxic effects, but these cells were not defective in cytoplasmic microtubule functions, since the nucleus was positioned in the bud neck. Instead, *GalS–SPC72* cells were delayed in the formation of an anaphase spindle. Based on the observation that overproduced Spc72p did not accumulate inside the nucleus (data not shown), we favour the idea that the cytoplasmic Spc72p binds Tub4p complex and may prevent its nuclear import via Spc98p (Pereira *et al*., 1998). The depletion of Tub4p complex in the nucleus then interferes with nuclear microtubule functions which then leads to the activation of the mitotic spindle checkpoint (Hoyt *et al*., 1991; Li and Murray, 1991). This model is supported by the observation that co-overexpression of *TUB4*, *SPC98* and *SPC97*, which gives rise to large amounts of Tub4p complex (Pereira *et al*., 1998), suppresses the lethal effect of multicopy *Gal1–SPC72* overexpression.

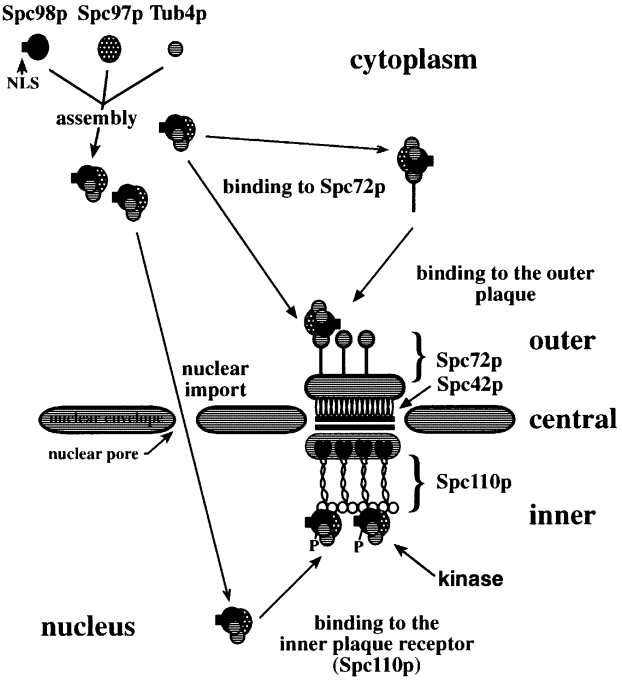


Fig. 8. Spc72p is the GTBP at the outer plaque. Shown is a schematic picture of an SPB (Byers and Goetsch, 1975; Bullitt *et al*., 1997). The localization of Tub4p (Spang *et al*., 1996a), Spc42p (Donaldson and Kilmartin, 1996), Spc72p (this study), Spc97p (Knop *et al*., 1997), Spc98p (Rout and Kilmartin, 1990) and Spc110p (Rout and Kilmartin, 1990; Spang *et al*., 1996b) at the SPB has been determined by immunoelectron microscopy. The outer plaque organizes the cytoplasmic microtubules, while the nuclear microtubules are connected with the inner plaque. The central plaque is embedded in the nuclear envelope (Byers and Goetsch, 1975). The Tub4p complex assembles in the cytoplasm and is imported into the nucleus via an essential nuclear localization sequence in Spc98p (Pereira *et al*., 1998). The Tub4p complex binds to the inner plaque via the binding of Spc98p and Spc97p to the N-terminal domain of Spc110p (Knop and Schiebel, 1997). Spc110p and Spc98p at the inner plaque are phosphorylated in a cell-cycle-dependent manner (Friedmann *et al*., 1996; Stirling and Stark, 1996; Pereira *et al*., 1998). At the outer plaque, the Tub4p complex interacts with the N-terminal domain of Spc72p (this study) either prior to binding of Spc72p to the SPB or to SPB-bound Spc72p.

The Tub4p-binding domain of Spc110p functions at the outer plaque

Sequence analysis of the Tub4p complex-binding domains of Spc110p and Spc72p revealed no homology, raising the possibility that these domains evolved independently and did not arise by gene duplication. Whether GTBPs contribute to the specific microtubule properties of an MTOC is an important question. Therefore, we tested whether hybrid proteins between the N-terminal domain of Spc72p and the C-terminal Spc110p, and *vice versa*, are functional. Our results show that *N-SPC110–SPC72* rescues a *SPC72* null mutant, indicating that *N-SPC110–SPC72* either fulfils *SPC72* function or by-passes its requirement. That N-Spc110p–Spc72p functions directly for Spc72p at the outer plaque is suggested by the association of the hybrid with the SPB upon its overexpression, thereby displacing Spc72p–GFP from the outer

plaque. Does N-Spc110–Spc72p fully substitute for Spc72p? Analysis of a chromosomal integrated *N-SPC110–SPC72* allele identified a weak growth defect and a small percentage of anucleated cells and cells with two DAPI-staining regions connected by a spindle in one cell body. These phenotypes indicate that nuclear migration is delayed in at least some *N-SPC110–SPC72* cells. Our conclusion is supported further by the observation that more *N-SPC110–SPC72* cells of a logarithmically growing culture have a 2N DNA content compared with the *SPC72* control (data not shown). The nature and extent of these defects are comparable with the weak cytoplasmic microtubule defects of *DYN1* (Eshel *et al*., 1993; Li *et al*., 1993) and *KAR9* (Miller and Rose, 1998) deletion mutants incubated at 30°C. However, in contrast to Δ*dyn1* cells, the nuclear migration defect of *N-SPC110–SPC72* cells was not increased by reducing the growth temperature to 14°C (data not shown).

N-SPC72–SPC110 expressed from the *SPC110* promoter on a centromere-based plasmid resulted in a dominant lethal phenotype which was dependent on the *N-SPC72* portion, since the non-functional Δ*N-SPC110* version did not affect cell viability. The only way to analyse *N-SPC72–SPC110* was the expression of the hybrid gene from a regulated promoter, e.g. the *GalS* promoter. Although this promoter is weaker than *Gal1* (Mumberg *et al*., 1995), it still caused overexpression of *N-SPC72–SPC110*. Analysis of *GalS–SPC72–SPC110* cells clearly showed more nuclear microtubules than in wild-type cells. However, these cells also failed to duplicate the SPB (M.Knop, unpublished). Although our observations are consistent with the view that N-Spc72–Spc110p functions as a GTBP at the inner plaque, it remains unclear why even low levels of N-Spc72–Spc110p are lethal for cells. Either *N-SPC72–SPC110* expressed from the *SPC110* promoter also affects SPB duplication as does *GalS–SPC72–SPC110*, or the nuclear microtubules organized by N-Spc72–Spc110p are not functional, resulting in a defective spindle followed by cell death. Taken together, our results are consistent with the notion that the N-terminal domains of Spc110p and Spc72p fulfil an essential role by the organization of the nuclear spindle and the cytoplasmic microtubules respectively, and that these functions are connected to the ability of these domains to bind to the Tub4p complex.

GTBPs represent MTOC-specific components of microtubule attachment sites

The components of the yeast Tub4p complex have been localized to the outer and inner plaques (Rout and Kilmartin, 1990; Spang *et al*., 1996a; Knop *et al*., 1997), suggesting that they represent universal components of the microtubule organization machinery. In contrast, Spc110p and Spc72p are the first side-specific proteins of the SPB involved in microtubule organization. Our data suggest that Spc110p and Spc72p have at least two functionally distinct domains: an N-terminal domain that interacts with the Tub4p complex and a C-terminal domain which binds to at least another SPB component. Cell-cycle-dependent modification of the N-terminal domain of Spc110p and Spc72p could in fact modify the microtubule organization properties of the inner and outer plaques. In this respect, it is interesting that Spc110p is a phosphoprotein (Friedmann *et al*., 1996; Stirling and Stark, 1996) and that Spc72p was resolved by SDS–PAGE into multiple bands (Figure 2B), suggesting that it is modified. The C-terminal domains of Spc110p and Spc72p carry the information as to which side of the SPB the proteins bind. Spc110p has been purified in complex with Spc42p, calmodulin and an SPB component with an apparent mol. wt of 35 kDa (Knop and Schiebel, 1997), indicating a physical interaction between the four SPB components (Figure 8). How Spc72p is bound to the SPB is still an open question.

In conclusion, based on the analysis of the yeast SPB, we favour the idea that each MTOC has a specific set of GTBPs which interact with components of the γ-tubulin complex as well as with proteins of the MTOC. In some cases, GTBPs may already bind to cytoplasmic γ-tubulin complexes, explaining their variation in size and complexity. The binding properties, post-transitional modification and abundance of GTBPs may contribute to the specific microtubule-organizing properties of an MTOC.

Materials and methods

Growth media and general methods

Basic yeast methods and growth media were as described (Sherman, 1991). Yeast strains were grown in yeast extract, peptone, dextrose (YPD) medium containing 100 mg/l adenine. For *Gal1*- or *GalS*-controlled gene expression, yeast strains were grown in synthetic complete (SC) medium containing raffinose (2%) as carbon source. Galactose (2%) or glucose (2%) were added to induce or repress the *Gal1* or *GalS* promoters, respectively. Yeast strains were transformed by the lithium acetate method (Schiestl and Gietz, 1989). The *E.coli* strains were transformed by electroporation (Dower *et al*., 1988). PCR was performed with a mixture of 0.4 U of Vent polymerase (New England Biolabs) and 2 U of *Taq* polymerase (Gibco-BRL) per 100 μl reaction. Recombinant DNA methodology was as described by Sambrook *et al*. (1989).

Two-hybrid screen

A two-hybrid screen was performed using the entire coding sequence of *SPC98* fused to the DNA-binding domain of the *GAL4* gene in the *TRP1*-based plasmid pGBT9 (Fields and Song, 1989). Strain Y190 (Bai and Elledge, 1996) carrying pGBT9-*SPC98* was then transformed with a yeast cDNA library fused to the *GAL4* activation domain. Among the 20 000 Leu^+ Trp^+ transformants, 25 grew on plates containing 50 mM aminotriazole and showed β-galactosidase activity. The prey plasmids were transformed into SGY37 with plasmid pMK16 (pEG202-*SPC97*) or pEG202. For three prey plasmids, expression of β-galactosidase was dependent on plasmid pMK16. These prey plasmids were transformed into strain SGY37 together with pSG21 (pEG202-*TUB4*) and with or without plasmid pMK155 (p414-*Gal1-SPC97 Gal1-SPC98*). One prey plasmid resulted in expression of β-galactosidase when *SPC98* and *SPC97* were co-expressed. Sequence analysis revealed that the positive prey plasmid contained an in-frame fusion of *GAL4* to the first 270 codons of *SPC72* (YAL047c).

Plasmids and yeast strains

Plasmid and yeast strains used during this study are listed in Table III. Where PCR products were used for cloning, the resulting plasmids were either sequenced (pSM447, pSM572, pMK230–257) or PCR products from two independent reactions were cloned (two-hybrid plasmids). In all cases, the independent constructs behaved identically. Chromosomal encoded terminal protein fusions with GFP or 3HA were constructed using a PCR targeting strategy with GFP–KanMX (Wach *et al*., 1997) or 3HA-KanMX (M.Knop, B.Windsor, K.Siegers and C.Schiebel, in preparation) modules.

Construction of temperature-sensitive alleles of SPC72

Codons 1–176 of *SPC72* were mutagenized by PCR (Cadwell and Joyce, 1992) using Primer 1 that is homologous to the 63 bp upstream of the start codon of *SPC72* and Primer 2 that binds to codons 177–198 of

Table III. Yeast strains and plasmids

Name	Genotype/construction	Source or reference
Yeast strains		
ESM335	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc110::HIS3* pCM94	this study
ESM356-1	*MATa ura3-53 leu2Δ1 trp1Δ63 his3Δ200*	this study
ESM357-9	*MATα ura3-53 leu2Δ1 trp1Δ63 his3Δ200*	this study
ESM387-3	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63::Gal1-SPC97-3HA::TRP1 his3Δ200 leu2Δ1::Gal1-TUB4::LEU2 ura3-52::Gal1-SPC98::URA3*	Pereira *et al*. (1998)
ESM418	*MATa/α ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2 SPC72/Δspc72::kanMX4*	this study
ESM440	*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 SPC42-GFP-kanMX4*	this study
ESM448	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc72::kanMX4* pSM447-1	this study
ESM479	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 SPC72-3HA-kanMX4*	this study
ESM504	*MATα ura3-53 leu2Δ1 trp1Δ63 his3Δ200 SPC72-GFP-kanMX4*	this study
FY1679	*MATa/α ura3-52/ura3-52 TRP1/trp1Δ63 LEU2/leu2Δ1 HIS3/his3Δ200*	Eurofan
SGY37	*MATa ura3-52::URA3-lexA-op-LacZ trp1 his3 leu2*	Geissler *et al*. (1996)
Y190	*MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112*	Bai and Elledge (1996)
YMK173	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1::pMK241 Δspc72::kanMX4*	this study
YMK174	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1::pMK242 Δspc72::kanMX4*	this study
YMK175	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1* pMK238	this study
YMK176	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1* pMK252	this study
YMK177	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1* pMK253	this study
YMK178	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1* pMK254	this study
YMK179-3	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1::pspc72-7 Δspc72::kanMX4*	this study
YMK179-6	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1::pspc72-14 Δspc72::kanMX4*	this study
YMK179-9	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1::pSPC72 Δspc72::kanMX4*	this study
YMK22	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3* pMK29	Knop *et al*. (1997)
YMK47	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc97::HIS3* pMK38	Knop and Schiebel (1997)
YPH499	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski and Hieter (1989)
YPH500	*MATα ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	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		
p413-*Gal1*	pRS413 containing the *Gal1* promoter	Mumberg *et al*. (1995)
p413-*GalS*	pRS413 containing the *GalS* promoter	Mumberg *et al*. (1995)
p414-*Gal1*	pRS414 containing the *Gal1* promoter	Mumberg *et al*. (1995)
p415-*GalS*	pRS415 containing the *GalS* promoter	Mumberg *et al*. (1995)
p423-*Gal1*	pRS423 containing the *Gal1* promoter	Mumberg *et al*. (1995)
pACTII	2 μm, *LEU2*-based vector carrying the *GAL4* activator domain	Durfee *et al*. (1993)
pATH11	*E.coli* expression vector containing TrpE	Koerner *et al*. (1991)
pCM94	pRS316 containing *SPC110*	this study
pEG202	2 μm, *HIS3*-based vector carrying the *lexA* DNA-binding domain	Gyuris *et al*. (1993)
pGBT9	2 μm, *TRP1*-based vector carrying the *GAL4* DNA-binding domain	Fields and Song (1989)
pGEX-5X-1	*E.coli* expression vector containing GST under control of the lacZ promoter	Pharmacia
pGP62[a]	pACTII containing $SPC72^{1–622}$	this study
pGP63	pACTII containing $SPC72^{1–271}$	this study
pGP64	pACTII containing $SPC72^{231–622}$	this study
pGP65	pEG202 containing $SPC72^{1–622}$	this study
pGP66	pEG202 containing $SPC72^{1–271}$	this study
pGP67	pEG202 containing $SPC72^{231–622}$	this study
pGP68	pGEX-5X-1 containing $SPC72^{1–271}$	this study
pMK103	pEG202 containing *spc97-14*	Knop *et al*. (1997)
pMK104	pEG202 containing *spc97-20*	Knop *et al*. (1997)
pMK15	pACTII containing *SPC97*	Knop *et al*. (1997)
pMK151	pACTII containing $SPC110^{1–204}$	Knop and Schiebel (1997)
pMK155	p414-*Gal1* containing *SPC97* and *Gal1–SPC98* in reverse orientation	Knop and Schiebel (1997)
pMK16	pEG202 containing *SPC97*	Knop *et al*. (1997)
pMK215	pACTII containing $SPC72^{1–176}$	this study
pMK230	pRS414 containing *pSpc110-ATG-NotI-SPC110*$^{177–944}$ (*ΔN-SPC110*)	this study
pMK231	pRS414 containing *pSpc110-ATG-NotI-SPC110*$^{1–176}$*-NotI-*SPC110$^{177–944}$ (*SPC110*^{NotI})	this study
pMK232	pRS315 containing *pSpc72-ATG-NotI-SPC72*$^{177–622}$ (*ΔN-SPC72*)	this study
pMK233	pRS414 containing *pSpc110-ATG-NotI-SPC72*$^{1–176}$*-NotI-SPC110*$^{177–944}$ (*N-SPC72-SPC110*)	this study
pMK234	pRS315 containing *pSpc72-ATG-NotI-SPC72*$^{1–176}$*-NotI-SPC72*$^{177–622}$ (*SPC72*^{NotI})	this study
pMK235	pRS315 containing *pSpc72-ATG-NotI-SPC110*$^{1–176}$*-NotI-SPC72*$^{177–622}$ (*N-SPC110-SPC72*)	this study
pMK238	p415-*GalS* containing a *Bam*HI-ATG-*Not*I-*Xba*I linker	this study
pMK239	p415-*GalS* containing *ATG-NotI-SPC110*$^{177–944}$ (*GalS–ΔN-SPC110*)	this study
pMK240	p415-*GalS* containing *ATG-NotI-SPC110*$^{1–176}$*-NotI-SPC110*$^{177–944}$ (*GalS–SPC110*^{NotI})	this study
pMK241	pRS305 containing *pSpc72-ATG-NotI-SPC110*$^{1–176}$*-NotI-SPC72*$^{177–622}$ (*N-SPC110–SPC72*)	this study
pMK242	pRS305 containing *pSpc72-ATG-NotI-SPC72*$^{1–176}$*-NotI-SPC72*$^{177–622}$ (*SPC72*^{NotI})	this study
pMK243	p415-*GalS* containing *ATG-NotI-SPC72*$^{1–176}$*-NotI-SPC110*$^{177–944}$ (*GalS–N-SPC72–SPC110*)	this study
pMK252	p415-*Gals* containing *ATG-NotI-SPC72*$^{177–622}$ (*GalS–ΔN-SPC72*)	this study

Table III. Continued

Name	Genotype/construction	Source or reference
pMK253	p415-*GalS* containing *ATG-NotI- SPC72*$^{1–176}$*-NotI-SPC72*$^{177–622}$ (*GalS–SPC72*^{NotI})	this study
pMK254	p415-*GalS* containing *ATG-NotI-SPC110*$^{1–176}$*-NotI-SPC72*$^{177–622}$ (*GalS–N-SPC110–SPC72*)	this study
pMK256	p415-*GalS* containing *ATG-NotI–GFP-NotI-SPC72*$^{176–622}$ (*GalS–GFP–ΔN-SPC72*)	this study
pMK257	p415-*GalS* containing *ATG-NotI–GFP-NotI-SPC110*$^{176–944}$ (*GalS–GFP–ΔN-SPC110*)	this study
pMK29	pRS414 containing *SPC97-3HA*	Knop *et al*. (1997)
pMK38	pRS414 containing *SPC97-3ProA*	Knop and Schiebel (1997)
pMK51	p414-*Gal1* containing *SPC97*	Knop and Schiebel (1997)
pRS305	*LEU2*-based yeast 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)
pRS414	*CEN6*, *TRP1*-based yeast-*E.coli* shuttle vector	Sikorski and Hieter (1989)
pRS423	2 μm, *HIS3*-based yeast-*E.coli* shuttle vector	Christianson *et al. (*1992)
pSG21	pEG202 containing *TUB4*	Geissler *et al.* (1996)
pSG26	pACTII containing *SPC98*	Geissler *et al*. (1996)
pSG46	pACTII containing *TUB4*	Geissler *et al.* (1996)
pSG56	pEG202 containing *SPC98*	Geissler *et al*. (1996)
pSM187	pRS414 containing *SPC110*	this study
pSM363	pGEX-5X-1 containing *SPC42*$^{1–214}$	this study
pSM405	pGBT9 containing *SPC98*	this study
pSM430	pATH11 containing *SPC72*$^{1–425}$	this study
pSM438	p414-*Gal1* containing *SPC98*	Knop and Schiebel (1997)
pSM447	pRS316 containing *SPC72*	this study
pSM464	pRS315 carrying the Δ*spc72::kanMX4* disruption cassette	this study
pSM572	p413-*Gal1* containing *SPC72*	this study
pSM573	p413-*GalS* containing *SPC72*	this study
pSM574	p423-*Gal1* containing *SPC72*	this study
pSPC72	pRS305 containing *SPC72*	this study
p*spc72-14*	pRS305 containing *spc72-14*	this study
p*spc72-7*	pRS305 containing *spc72-7*	this study

[a]*SPC72*$^{1–622}$ indicates that codons 1–622 of *SPC72* have been cloned into plasmid pACTII.

SPC72 as shown in Figure 4A. The mutagenized 5′ region of *SPC72* was combined with the not mutagenized 3′ region of *SPC72* as outlined in Figure 4A. Conditional lethal alleles of *SPC72* were selected as described (Muhlrad *et al*., 1992).

Binding of Tub4p complex to recombinant GST–N-Spc72p

Binding of the Tub4p complex to recombinant GST fusion proteins was performed as described before (Knop and Schiebel, 1997). Plasmids were pGEX-5X-1 (GST), pSM363 (GST–Spc42p$^{1–214}$) and pGP68 (GST–Spc72$^{1–271}$). An extract of cells from strain YMK47 (*SPC97-3ProA*) was used. The samples were analysed by immunoblotting with affinity-purified rabbit anti-Tub4p and rabbit anti-Spc98p antibodies. Spc97-3ProA was always detected on the immunoblot due to the binding of the protein A part to IgGs.

Immunological techniques

Antibodies specific for Spc72p were produced against recombinant TrpE–Spc72p$^{1–425}$ protein. In brief, a *Bam*HI restriction site was introduced by PCR just upstream of the ATG start codon of *SPC72*. Using this *Bam*HI site, a 1279 bp *Bam*HI–*Cla*I fragment of *SPC72* (codons 1–425) was cloned into the *Bam*HI–*Cla*I sites of vector pATH11 (pSM430). The TrpE–Spc72p$^{1–425}$ fusion protein was induced by indoleacrylic acid as described (Koerner *et al*., 1991). The 87 kDa TrpE–Spc72p$^{1–425}$ fusion protein was purified from inclusion bodies. The protein was solubilized in SDS–PAGE buffer and separated by SDS–PAGE (Laemmli, 1970). Proteins were transferred onto a nitrocellulose membrane and stained by Ponceau S. The TrpE–Spc72p$^{1–425}$-containing membrane strip was cut out and solubilized with dimethylsulfoxide (DMSO). Antibodies were raised as described (Harlow and Lane, 1988). For affinity purification of the antibody, CNBr-Sepharose (Pharmacia) with immobilized GST–Spc72p$^{1–271}$ was used.

The polyclonal rabbit anti-Tub4p (Spang *et al*., 1996a), anti-Spc97p (Knop and Schiebel, 1997), anti-Spc98p (Knop *et al*., 1997), anti-Spc110p$^{3–175}$ (Spang *et al*., 1996b) and anti-Spc110p$^{293–756}$ (Stirling *et al*., 1994) have been described before. Rabbit anti-Tub1p and rabbit anti-Tub2p were a kind gift from F.Solomon, and the mouse monoclonal anti-β-tubulin antibody (Wa3) was a gift from U.Euteneuer-Schliwa. The mouse monoclonal anti-HA antibodies (12CA5) were obtained from Hiss Diagnostics, and anti-Myc antibodies (9E10) were purchased from Boehringer Ingelheim. Secondary antibodies used in immunofluorescence, immunoelectron microscopy and immunoblotting were goat anti-mouse and goat anti-rabbit antibodies coupled to Cy2 or Cy3, or goat anti-rabbit antibodies coupled to colloid gold particles or to horseradish peroxidase (all from Jackson Immuno Research Laboratories), respectively.

Immunofluorescence of formaldehyde-fixed yeast cells was performed as described (Knop *et al*., 1996) with 1 h fixation time. Microtubules were stained using the Wa3 antibodies. Spc72p or Spc110p were detected using affinity-purified anti-Spc72p or anti-Spc110p$^{3–175}$ antibodies. DNA was stained with DAPI. For double detection of GFP-labelled proteins and DNA, cells carrying the indicated GFP constructs were fixed with 4% paraformaldehyde/0.1 M KPO_4 pH 6.5 for 5 min. Cells were washed twice with phosphate-buffered saline (PBS) and then incubated with 10 μg/ml DAPI in PBS for 10 min. Cells were harvested and resuspended in PBS containing 1 μg/ml DAPI.

Immunoprecipitation of Spc72-3HA or Spc97-3HA was performed as described (Knop *et al*., 1997; Pereira *et al*., 1998) using TBS-T (20 mM Tris pH 7.5, 135 mM NaCl, 2.5 mM KCl, 1% Triton X-100) as buffer. The anti-HA antibodies were cross-linked to protein A–Sepharose beads (Harlow and Lane, 1988). For immunoblotting, proteins were transferred onto nitrocellulose membranes. The blocked membranes were incubated with the indicated primary antibodies. Secondary antibodies were coupled to peroxidase. The immunoreaction was visualized by an ECL Kit from Amersham.

SPB isolation, electron microscopy and immunoelectron microscopy

SPBs were isolated from strain YPH499 as described previously (Rout and Kilmartin, 1990). Immunoelectron microscopy using anti-Spc72p antibodies was performed as follows. Isolated SPBs were fixed with 4% paraformaldehyde/0.5% glutaraldehyde in Bt-Mg (10 mM BisTris pH 6.5, 0.1 mM $MgCl_2$) for 25 min at room temperature. Fixation reactions were diluted 5-fold with cold Bt-Mg and the SPBs were centrifuged onto round cover slides as described (Mitchison and Kirschner, 1984). After immunodecoration of the SPBs with the appropriate antibodies, the SPBs were post-fixed in 2% glutaraldehyde (10 min), 2% osmium tetroxide (15 min). The samples were dehydrated followed by embedding in Spurr low viscosity resin (Polyscience). Yeast cells were prepared for

thin section electron microscopy following a published protocol (Byers and Goetsch, 1991).

Flow cytometry

Yeast cells were prepared for flow cytometry as described (Hutter and Eipel, 1979). Samples were measured using a FACS-calibur (Becton-Dickson).

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