A Genetic Analysis of Interactions with Spc110p Reveals Distinct Functions of Spc97p and Spc98p, Components of the Yeast γ-Tubulin Complex

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The spindle pole body (SPB) in *Saccharomyces cerevisiae* functions as the microtubule-organizing center. Spc110p is an essential structural component of the SPB and spans between the central and inner plaques of this multilamellar organelle. The amino terminus of Spc110p faces the inner plaque, the substructure from which spindle microtubules radiate. We have undertaken a synthetic lethal screen to identify mutations that enhance the phenotype of the temperature-sensitive *spc110–221* allele, which encodes mutations in the amino terminus. The screen identified mutations in *SPC97* and *SPC98*, two genes encoding components of the Tub4p complex in yeast. The *spc98–63* allele is synthetic lethal only with *spc110* alleles that encode mutations in the N terminus of Spc110p. In contrast, the *spc97* alleles are synthetic lethal with *spc110* alleles that encode mutations in either the N terminus or the C terminus. Using the two-hybrid assay, we show that the interactions of Spc110p with Spc97p and Spc98p are not equivalent. The N terminus of Spc110p displays a robust interaction with Spc98p in two different two-hybrid assays, while the interaction between Spc97p and Spc110p is not detectable in one strain and gives a weak signal in the other. Extra copies of *SPC98* enhance the interaction between Spc97p and Spc110p, while extra copies of *SPC97* interfere with the interaction between Spc98p and Spc110p. By testing the interactions between mutant proteins, we show that the lethal phenotype in *spc98–63 spc110–221* cells is caused by the failure of Spc98–63p to interact with Spc110–221p. In contrast, the lethal phenotype in *spc97–62 spc110–221* cells can be attributed to a decreased interaction between Spc97–62p and Spc98p. Together, these studies provide evidence that Spc110p directly links the Tub4p complex to the SPB. Moreover, an interaction between Spc98p and the amino-terminal region of Spc110p is a critical component of the linkage, whereas the interaction between Spc97p and Spc110p is dependent on Spc98p.

INTRODUCTION

The centrosome organizes microtubules both spatially and temporally within a cell. Although at the ultrastructural level centrosomes may differ across a spectrum of organisms, the essential function of microtubule nucleation remains the same. Accordingly, conserved components are being found in diverse species. A prominent centrosomal component that is highly conserved is γ-tubulin (Oakley and Oakley, 1989; Oakley *et al.*, 1990; Zheng *et al.*, 1991; Raff *et al.*, 1993; Horio and Oakley, 1994). γ-Tubulin localizes to centrosomes and is essential for microtubule nucleation (Horio *et al.*, 1991; Stearns *et al.*, 1991; Zheng *et al.*, 1991). In addition to being at the centrosome, γ-tubulin is present in large cytoplasmic complexes (Stearns and Kirschner, 1994; Moudjou *et al.*, 1996). A

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soluble 25S complex from *Xenopus* extracts called the γ-tubulin–containing ring complex (γ-TuRC) is a helical ring structure capable of nucleating microtubules in vitro and capping the minus ends of stabilized microtubules (Moritz *et al.*, 1995; Zheng *et al.*, 1995). One current model postulates that γ-tubulin and associated proteins form a scaffold upon which the α/β tubulin heterodimers assemble into a 13-protofilament microtubule (Oakley *et al.*, 1990; Zheng *et al.*, 1995). How γ-tubulin is anchored at the centrosome remains unknown.

In *Saccharomyces cerevisiae*, the spindle pole body (SPB) serves as the organism's microtubule-organizing center (Byers, 1981a, b). The multilayered structure includes an outer plaque radiating cytoplasmic microtubules, a central plaque embedded in the nuclear envelope, and an inner plaque radiating nuclear microtubules (Rout and Kilmartin, 1990; Bullitt *et al.*, 1997). Spc110p is an essential structural component that spans between the central and inner plaques. A central coiled-coil domain (residues 155–798) is flanked by N-terminal and C-terminal regions. The C-terminal region of Spc110p has a calmodulin-binding site and is localized to the central plaque (Sundberg *et al.*, 1996), while its N terminus faces the inner plaque (Spang *et al.*, 1996b). The coiled-coil region is largely dispensable because a strain carrying an *SPC110* allele with three quarters of the central α-helical domain deleted still has a nearly normal growth rate (Kilmartin *et al.*, 1993).

Spc110p is regulated throughout the cell cycle. Transcription of *SPC110* is under the control of an *Mlu*I cell cycle box and is induced in late G_1 at the time that the SPB duplicates (Kilmartin *et al.*, 1993). Later in the cell cycle as the spindle forms, Spc110p is phosphorylated. Phosphorylation is removed at the time of anaphase (Friedman *et al.*, 1996).

A mutational analysis of Spc110p revealed three regions with distinct functions (Sundberg and Davis, 1997). Region I consists of residues 1–163, N-terminal to the coiled coil. Region II includes amino acid residues 772–836, and region III is the calmodulin-binding site between amino acid residues 897–917. Temperature-sensitive mutations in region I of *SPC110* lead to a mitotic arrest dependent on the *MAD1* checkpoint. The spindles appear morphologically normal when analyzed either in electron micrographs of thin sections or by immunofluorescence. Region II in the C terminus of Spc110p is required for stable attachment of Spc110p to the central plaque. Mutations here do not interfere with the initial assembly of Spc110p onto the SPB, but Spc110p separates from the SPB during mitosis. Finally, temperature-sensitive mutations in the calmodulin-binding site cause early misassembly of spindle pole components and broken spindles (Kilmartin and Goh, 1996; Stirling *et al.*, 1996; Sundberg *et al.*, 1996).

To learn more about the function of the N terminus, we performed a synthetic lethal screen for mutations that would enhance the subtle phenotype of the region I mutant *spc110–221*. The screen identified both *SPC97* and *SPC98*, two genes that encode spindle pole components with many similar properties. Spc97p and Spc98p are similar in size and share short stretches of sequence similarity. Together with the yeast homologue of γ-tubulin, Tub4p, they form a 6S soluble complex (Sobel and Snyder, 1995; Geissler *et al.*, 1996; Marschall *et al.*, 1996; Spang *et al.*, 1996a; Knop *et al.*, 1997). Two-hybrid assays suggest that each one can bind Tub4p. Since the 6S complex contains one molecule each of Spc97p and Spc98p and at least two molecules of Tub4p, one possible arrangement is that Spc97p and Spc98p each bind one molecule of Tub4p (Geissler *et al.*, 1996; Knop *et al.*, 1997). All three proteins are found at the inner plaque of the SPB in the nucleus and at the outer plaque of the SPB in the cytoplasm. Finally, recent results suggest that the N terminus of Spc110p interacts equally well with Spc98p and Spc97p (Knop and Schiebel, 1997). Despite these similarities, Spc97p and Spc98p must have unique functions because each is essential for cell viability. By performing a detailed genetic analysis and two-hybrid analyses in two different systems, we demonstrate that Spc97p and Spc98p can be distinguished on the basis of their interactions with Spc110p. Our results argue that Spc98p is the component of the Tub4p complex primarily responsible for interaction with Spc110p.

MATERIALS AND METHODS

Strains and Media

Strains used in this study are listed in Table 1. Strains TNY2–2B and TNY2–4B were obtained by dissection of the diploid created by mating HSY14 (Sundberg and Davis, 1997) with GZY7–27C (Zhu, 1996). Strains TNY135 and TNY136 contain *spc110–250*, a null allele encoding a truncated version of Spc110p of 79.4 kDa. Strains TNY160–165 are the original strains isolated in the synthetic lethal screen. Strain TNY161 was backcrossed five times to to give strain TNY137. Strain TNY165 was backcrossed two and three times to give strains TNY75–16D and TNY76–1C, respectively. Strain TNY76–1C was mated to strain TNY75–16D to create the diploid DCY101. Strains TNY163 and TNY162 were backcrossed once to give strains TNY113–10A and TNY114–39C, respectively. Strain TNY150, which has *URA3* integrated at the *SPC98* locus, was constructed by transformation of strain TNY2–2B with plasmid pTN32. Strain TNY150 was mated to strain TNY76–1C to create the diploid TNY117.

YPD, SD-uracil low adenine, and YPD low adenine (Geiser *et al.*, 1991; Muller, 1996) were described previously. SD-lys is SD medium supplemented with 1× amino acid mix without lysine, 50 μg/ml adenine, and 25 μg/ml uracil. The complete 100× amino acid mix was described previously (Geiser *et al.*, 1991).

Plasmids

Plasmids used in this study are listed in Table 2. Plasmid pTN9 was created by cloning the *Sac*II–*Cla*I fragment containing *SPC97*

Table 1. Yeast strains

Strain	Genotype	Source
DCY101	*spc98-63/spc98-63 spc110-221/spc110-221 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 lys2Δ::HIS3/lys2Δ::HIS3 leu2-3,112/leu2-3,112 ura3-1/ura3-1 pHS26*	This study
EMY84-3D	*MAT**a** ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 trp1-1*	E. Muller
EMY84-6C	*MATα ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 trp1-1*	E. Muller
TNY2-2B	*spc110-221 MAT**a** ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 trp1-1 ura3-1 pHS26*	This study
TNY2-4B	*spc110-221 MATα ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 trp1-1 ura3-1 pHS26*	This study
TNY31	*SPC97/spc97-114 spc110-220/spc110-221 MAT**a**MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/trp1-1 ura3-1/ura3-1 pHS26*	This study
TNY47	*spc97-114/SPC97 spc110-221/spc110-224 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/trp1-1 URA3/ura3-1 pHS26*	This study
TNY49	*spc97-114/SPC97 spc110-221/spc110-225 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/trp1-1 URA3/ura3-1 pHS26*	This study
TNY51	*spc97-114/SPC97 spc110-221/spc110-222 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/trp1-1 URA3/ura3-1 pHS26*	This study
TNY64-5C	*spc97-114 SPC110 MATα ade2-1oc ade3Δ his3-11,15 leu2-3,112 lys2Δ::HIS3 trp1-1 pHS26*	This study
TNY75-16D	*spc98-63 spc110-221 MATα ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 ura3-1 pHS26*	This study
TNY76-1C	*spc98-63 spc110-221 MAT**a** ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 ura3-1 pHS26*	This study
TNY82	*spc97-62/SPC97 spc110-221/spc110-220 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 TRP1/trp1-1 ura3-1/ura3-1 pHS26*	This study
TNY83	*SPC97/spc97-62 spc110-222/spc110-221 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/TRP1 ura3-1/ura3-1 pHS26*	This study
TNY84	*SPC97/spc97-62 spc110-224/spc110-221 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/TRP1 ura3-1/ura3-1 pHS26*	This study
TNY86	*SPC97/spc97-62 spc110-226/spc110-221 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 LYS2/lys2Δ::HIS3 trp1-1/TRP1 ura3-1/ura3-1 pHS26*	This study
TNY88	*SPC97/spc97-113 spc110-220/spc110-221 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/TRP1 ura3-1/ura3-1 pHS26*	This study
TNY89	*SPC97/spc97-113 spc110-222/spc110-221 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/TRP1 ura3-1/ura3-1 pHS26*	This study
TNY91	*SPC97/spc97-113 spc110-225/spc110-221 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/TRP1 ura3-1/ura3-1 pHS26*	This study
TNY92	*SPC97/spc97-113 spc110-226/spc110-221 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 LYS2/lys2Δ::HIS3 trp1-1/TRP1 ura3-1/ura3-1 pHS26*	This study
TNY106	*spc98-63/SPC98 spc110-221/spc110-220 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/trp1-1 ura3-1/ura3-1 pHS26*	This study
TNY107	*spc98-63/SPC98 spc110-221/spc110-222 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/trp1-1 ura3-1/ura3-1 pHS26*	This study
TNY108	*spc98-63/SPC98 spc110-221/spc110-224 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/trp1-1 ura3-1/ura3-1 pHS26*	This study
TNY109	*spc98-63/SPC98 spc110-221/spc110-225 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/lys2Δ::HIS3 trp1-1/trp1-1 ura3-1/ura3-1 pHS26*	This study
TNY110	*spc98-63/SPC98 spc110-221/spc110-226 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/LYS2 trp1-1/trp1-1 ura3-1/ura3-1 pHS26*	This study
TNY113-10A	*spc97-113 spc110-221 MAT**a** ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 ura3-1, pHS26*	This study
TNY114-39C	*spc97-62 spc110-221 MATα ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 trp1-1 ura3-1 pHS26*	This study
TNY117	*spc110-221/spc110-221 spc98-63/SPC98::URA3::SPC98 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 pHS26*	This study
TNY127	*spc97-114/SPC97 spc110-221/spc110-226 MAT**a**/MATα ade2-1oc/ade2-1oc ade3Δ/ade3Δ his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 lys2Δ::HIS3/LYS2 trp1-1/trp1-1 ura3-1/ura3-1 pHS26*	This study
TNY135	*spc110-250 MAT**a** ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 trp1-1 ura3-1 pHS26*	This study
TNY136	*spc110-250 MAT**a** ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 ura3-1 pHS26*	This study
TNY137	*spc97-114 spc110-221 MAT**a** ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 ura3-1 pHS26*	This study
TNY150	*spc110-221 SPC98::URA3::SPC98 MAT**a** ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 trp1-1 ura3-1*	This study
TNY160	*nsl2-118 spc110-221 MATα ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 ura3-1 pHS26*	This study
TNY161	*spc97-114 spc110-221 MATα ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 ura3-1 pHS26*	This study
TNY162	*spc97-62 spc110-221 MATα ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 ura3-1 pHS26*	This study
TNY163	*spc97-113 spc110-221 MATα ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 ura3-1 pHS26*	This study
TNY164	*nsl2-85 spc110-221 MAT**a** ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 ura3-1 pHS26*	This study
TNY165	*spc98-63 spc110-221 MATα ade2-1oc ade3Δ his3-11,15 lys2Δ::HIS3 leu2-3,112 ura3-1 pHS26*	This study
PJ69-4A	*MAT**a** trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	(James *et al.*, 1996)
Y190	*MAT**a** trp1-901 leu2-3,112 ura3-52 his3-200, ade2-101 gal4Δ gal80Δ URA3::GAL1-lacZ LYS2::GAL1-HIS3*	(Harper *et al.*, 1993)

Table 2. Plasmids

Plasmid	Parent plasmid	Relevant markers[a]	Source or reference
pACTII		*GAL4*-activator domain HA epitope *LEU2* 2μ	(Durfee *et al.*, 1993)
pAS2		*GAL4*-DNA-binding domain HA epitope *TRP1* 2μ	(Harper, *et al.*, 1993)
pBluescriptII SK+		*bla*, f1 origin	Stratagene
pDC1	pTN9	*spc97-114*	This study
pDC2	pTN9	*spc97-62*	This study
pDC3	pTN9	*spc97-113*	This study
pDC4	PCR Blunt	*spc98-63*	This study
pDC5	PCR Blunt	*spc98-63*	This study
pDC7	pHS90	*spc98-63* (aa 1-846 containing S754F)	This study
pDC9	pHS89	*spc98-63*	This study
pDV1A	pACTII	*SPC110* (aa 1-150)	This study
pDV4	pACTII	*SPC97* (aa 1-823)	This study
pDV6	pACTII	*SPC97* (aa 1-548)	This study
pDV8	pAS2	*SPC98* (aa 1-846)	This study
pDV9	pTHS37	*SPC98* (aa 1-361)	This study
pDV13	pAS2	*TUB4* (aa 1-473)	This study
pDV16	pACTII	*TUB4* (aa 1-473)	This study
pDV17	pACTII	*SPC110* (aa 1-183)	This study
pDV18	pAS2	*SPC97* (aa 1-823)	This study
pDV20	pACTII	*spc110-221* (aa 1-183; see Sundberg and Davis, 1997 for description of mutations)	This study
pDV21	pAS2	*spc97-62* (aa 1-823 containing L22F)	This study
pDV22	pAS2	*spc97-114* (aa 1-823 containing E56K)	This study
pDV23	pAS2	*spc98-63* (aa 1-846 containing S754F)	This study
pGEMT		Bacterial vector for cloning PCR products	Promega
pLAMIN	pGBT-CYH	*GAL4*-DNA-binding domain-lamin fusion *TRP1 CEN*	D. Lockshon
pHS26	pTD29	*SPC110 ADE3 LYS2* 2μ origin	(Sundberg *et al.*, 1996)
pHS88	pGF29	*SPC98 URA3* 2μ origin	(Sundberg and Davis, 1997)
pHS89	pRS316	*SPC98 URA3 CEN6*	(Sundberg and Davis, 1997)
pHS90	pGEMT	PCR fragment containing *SPC98* (1-846) flanked by *Sma*I (at Start) and *Xho*I	H. Sundberg
pHS91	pGF29	*TUB4 URA3* 2μ origin	(Sundberg and Davis, 1997)
pHS92	pGF29	*SPC97 URA3* 2μ origin	(Sundberg and Davis, 1997)
pJG109	YCp50	*SPC110 URA3 CEN*	(Geiser, 1993)
pMM66	pRS316	*URA3* lacks the *Nco*I site *CEN6*	M. Moser
pRD54	pRS316	*GAL1-HA*	Gift of R. Deshaies
pRS306		*URA3* f1 origin	(Sikorski and Hieter, 1989)
pRS316		*URA3 CEN6 ARSH4* f1 origin	(Sikorski and Hieter, 1989)
pTD103	pRS306	*spc98-63 URA3*	This study
pTD104	pRS306	*SPC98 URA3*	This study
pTD105	pRS306	*Bgl*II–*Sac*II fragment of *spc98-63* inserted at *Bam*HI and *Sac*II *URA3*	This study
pTD106	pRS306	*Bgl*II–*Sac*II fragment of *SPC98* inserted at *Xho*I and *Sac*II sites *URA3*	This study
pTHS36	pACTII	*SPC98* (aa 149-846) inserted at *Nco*I and *Xho*I	H. Sundberg
pTHS37	pACTII	*SPC98* (aa 1-846) inserted at *Sma*I and *Xho*I	H. Sundberg
pTN1	YCp50	*SPC97 URA3 CEN*	This study
pTN2	pTN1	4.8-kb *Sac*I deletion from pTN1 *URA3 CEN*	This study
pTN3	pRS316	*Bgl*II–*Mun*I fragment containing *SPC97 URA3 CEN6*	This study
pTN4	pTN3	1.3-kb *Bam*HI deletion from *SPC97 URA3 CEN6*	This study
pTN5	pBluescriptII SK+	*Cla*I–*Not*I fragment containing *SPC97*	This study
pTN6	pTN5	*SPC97*[b]	This study
pTN9	pMM66	*SPC97*[b] *URA3 CEN6*	This study
pTN26	pRD54	*GAL1-HA-SPC97*[b] *URA3 CEN6*	This study
pTN31	pGF27	*SPC97*[b] *TRP1* 2μ origin	This study
pTN32	pRS306	*SPC98 URA3*	This study

[a] Unless stated otherwise, all markers from the parent plasmid are present in the new construct.
[b] An *Nco*I site was introduced at the initial ATG of *SPC97* and an internal *Nco*I site was abolished by a silent mutation.

from pTN6 into pMM66, which is pRS316 without the *Nco*I site in *URA3*.

Most two-hybrid constructs were created by cloning PCR products. For construction of pDV1A and pDV17, the template was plasmid pHS31 (Friedman *et al.*, 1996). For construction of pDV20, the template was plasmid pHS42, which contains the *spc110–221* allele (Sundberg and Davis, 1997). When necessary, primers were tagged with restriction enzyme sites to facilitate cloning in frame with *GAL4* in pACTII.

Plasmid pDV23 was made in several steps. First, a PCR fragment encoding the C-terminal region of Spc98–63p, which includes S754F, was cloned into pHS90 to replace the corresponding fragment of wt *SPC98* and generate pDC7. Then, the fragment containing the entire coding sequence of *spc98–63* from pDC7 was inserted into pAS2.

For plasmid pDV21, a PCR fragment containing the first 630 base pairs (bp) of *spc97–62* was generated from the gap-repaired plasmid pDC2. The PCR fragment was then used to replace the corresponding fragment of *SPC97* in plasmid pDV18. pDV22 was similarly created, except the template for PCR was the gap-repaired plasmid pDC1. All constructs made by inserting PCR fragments were sequenced.

Synthetic Lethal Screen

Strains carrying the *spc110–221* allele are unable to grow at 37°C but form normal-sized colonies at 30°C. A plasmid shuffle technique was used to screen for mutations that enhance the phenotype conferred by *spc110–221* by preventing growth at 30°C (Muller, 1996). These synthetic lethal mutations make *spc110–221* cells growing at 30°C dependent on plasmid pHS26 carrying wt *SPC110*. Plasmid pHS26 contains an additional gene, the *ADE3* gene, which allows formation of a red pigment. Therefore, cells that require plasmid pHS26 for viability form solid red colonies. Cells harboring other mutations that are not synthetic lethal with *spc110–221* can lose plasmid pHS26 and give rise to colonies that sector white. Therefore, the primary screen was for cells that form solid red colonies at 30°C. The details of the screen are given below.

TNY2–2B (pHS26) and TNY2–4B (pHS26) were mutagenized to 3–15% survival with 1% ethyl methanesulfonate, and cells were plated on YPD low-adenine plates. Solid red colonies were streaked twice on YPD low-adenine to confirm the phenotype. The secondary screen distinguished the mutants that had integrated the plasmid into a chromosome from the mutants that still had the plasmid as a separate entity. Each candidate was crossed with a haploid wt strain (either EMY84–3D or EMY84–6C). In the diploid, the synthetic lethal interaction between *nsl* and *spc110–221* is abolished by the presence of wt *NSL* and *SPC110* genes. Therefore, if the plasmid has not integrated, the diploid cannot lose the plasmid and the colonies remain solid red. Only mutants that were able to sector white when mated to a wt haploid were taken through the tertiary screen.

The tertiary screen identified cells that retained the pHS26 plasmid because the mutation rendered them dependent upon *ADE3*, *LYS2*, or some other region of the plasmid. Another plasmid carrying *SPC110* and *URA3* (pJG109) was introduced into the solid red candidates. Only strains that were able to sector white when provided with another source of Spc110p were considered further. Of the 75 reproducibly solid red colonies isolated, 59 were eliminated through the secondary and tertiary screens. The 16 synthetic lethal candidates that passed both secondary and tertiary screens were placed in complementation groups.

Complementation Analysis

Both mating types of all synthetic lethal candidates were made. All possible pairwise matings were then done between the mutants. Complementation between two synthetic lethal mutants was assessed by determining whether the resulting diploids could lose pHS26 and sector white. One expected complementation group consisted of mutants having additional mutations in *spc110–221*. The intragenic lethal mutations were identified by mating each of the synthetic lethal strains to a strain having a nonfunctional truncated *spc110* allele (either TNY135 or TNY136). If the resulting diploid was not able to lose pHS26, the mutant was placed in the *SPC110* complementation group.

Cloning of NSL1

Approximately 10 genome equivalents of a centromere-based (YCp50) yeast genomic library (Rose *et al.*, 1987) were transformed into the *nsl1–114 spc110–221* mutant. The transformed colonies were plated on SD-ura low ade at 30°C for 3–4 d, after which sectoring colonies were picked. The sectoring colonies were restreaked on SD-ura low ade until pure white colonies were obtained. The complementing library plasmid was rescued from a pure white colony as described (Hoffman and Winston, 1987) and transformed into XL1-blue bacteria. The library plasmids recovered from the bacteria were digested with *Hin*dIII to ascertain whether the insert contained *SPC110*. For library plasmids that did not contain *SPC110*, the ends of the library insert were sequenced with primers specific to YCp50 and then analyzed using the *Saccharomyces* Genome Database.

Mapping of Mutations in SPC97 and SPC98

The *spc97* mutations were mapped by gap repair (Orr-Weaver and Szostak, 1983) combined with a plasmid-shuffle technique (Davis, 1990). Plasmids pDC1, pDC2, and pDC3 were recovered from TNY137 (*spc97–114*), TNY162 (*spc97–62*), and TNY163 (*spc97–113*), respectively. Each was sequenced using the DNA Sequencing Kit (Perkin Elmer-Cetus Applied Biosystems, Norwalk, CT). The *spc97–62* allele contained a C>T change at bp 64 (as measured from the initial ATG), leading to the mutation L22F. The *spc97–113* allele had a G>A change at bp 512, which gives the mutation R171K. The *spc97–114* allele contained a G>A change at bp 166, causing the mutation E56K.

The mutation in *spc98–63* responsible for synthetic lethality was identified by sequencing. The final 370 bp of *spc98–63* were recovered from two different colonies of TNY76–1C by PCR using Vent polymerase (New England Biolabs, New Haven, CN). The two fragments were cloned into PCR-Blunt plasmids (Invitrogen, San Diego, CA) yielding pDC4 and pDC5. Sequencing of each plasmid revealed a single C>T point mutation at bp 2261 in *spc98–63*, which results in the mutation S754F. Proof that this mutation conferred synthetic lethality was obtained by testing the ability of the C2261T *spc98* to confer synthetic lethality at three different gene dosages. A *CEN* plasmid was created by replacing the last 359 bp of wt *SPC98* on pHS89 with the same region of pDC5 carrying the single mutation C2261T to make plasmid pDC9. The low-copy number vector pDC9 suppressed the synthetic lethality of the *spc98–63 spc110–221* mutant. We then tested whether the C2261T mutation was sufficient to confer synthetic lethality when integrated at *URA3*. The integrating plasmids pTD103 and pTD104 carrying *spc98* with C2261T and wt *SPC98*, respectively, were cut with *Stu*I to allow integration at *ura3–1* in strain TNY76–1C carrying *spc98–63* and *spc110–221*. We found that even two copies of the C2261T allele were sufficient to suppress the synthetic lethality. We next integrated a fragment of *spc98* carrying the C2261T mutation at the *SPC98* locus. Plasmids pTD105 and pTD106 carrying only the 3′-end of C2261T *spc98* or *SPC98*, respectively, were cut with *Afl*II and transformed into strain TNY2–2B carrying *spc110–221* and wt *SPC98*. This results in the replacement of *SPC98* by one complete copy of the gene and one copy lacking the promoter and the 5′-coding region of the gene, thus yielding only one expressed full-length copy. If the single C2261T mutation is sufficient to confer synthetic lethality, then some of the colonies transformed with the fragment carrying the single mutation C2261T (pTD105) should display the synthetic lethal phenotype, i.e., be solid red at 30°C. We observed that 45% of the colonies transformed with pTD105 were solid red and 0.3% of the colonies transformed with the wt fragment (pTD106) were solid red. Therefore, the C2261T mutation in *spc98–63* is sufficient to confer synthetic lethality in a haploid *spc110–221* strain when it is the only expressed copy of the gene.

Test for Allele Specificity

Six different temperature-sensitive alleles of *SPC110* were tested for their interaction with the *spc97* and *spc98* alleles. We obtained the double-mutant strains by crossing each synthetic lethal strain (having either *spc97* or *spc98* and *spc110–221*) with a strain carrying

another *spc110* allele. We concluded that a particular *spc97* or *spc98* allele exhibited lethality with a *spc110* allele if >90% of the tetrads had two viable spores and two inviable spores (or spores that formed solid red colonies) at 30°C. At least 15 tetrads were analyzed for each diploid.

Two-Hybrid Assay

To analyze two-hybrid interactions, we used the *ADE2* reporter in the PJ69–4A strain and the *lacZ* reporter in the Y190 strain. Yeast transformations were done using the high-efficiency lithium acetate method of Gietz and Schiestl (1995). A Gal4p–DNA-binding domain (Gal4DB) fusion plasmid and a Gal4p-activation domain (Gal4AD) fusion plasmid were cotransformed into PJ69–4A, and equal aliquots of transformants were plated onto SD-trp,-leu (−TL), and SD-trp,-leu,-ade (−TLA) plates. Plates were incubated at 30°C, and growth of colonies was scored between day 3 and day 6. Colonies plated on −TL plates were typically fully grown after 3 d, while colonies plated on −TLA plates took between 3 and 6 d to be fully grown. The growth rate of colonies on −TLA plates, as compared with that of colonies on −TL plates, was used as a measure of the strength of the signal generated by a given combination of Gal4AD and Gal4DB plasmids. Once cells were fully grown, the plating efficiency on −TLA plates, as compared with the plating efficiency on −TL plates, was used as another measure of the strength of the signal generated by the two-hybrid interaction. (See footnotes to Table 5 for specific designation of different growth rates.) We have observed that scoring expression of the reporter gene in a population of transformants is superior to analyzing the activity of the reporter in a single transformant, e.g., by measuring β-galactosidase in a liquid culture. Our previous two-hybrid analyses (Geiser *et al.*, 1993) have indicated that the β-galactosidase activity can vary substantially between transformants, perhaps due to variable gene expression. In addition, preparing cultures for measuring enzyme activity can impose a selection on growth that changes the level of gene expression. By examining the growth of a population of initial transformants, this selection is precluded. Finally, our methods of analysis yield results that are consistent from transformation to transformation. All transformations were performed at least in duplicate. Each Gal4p-fusion construct was checked for its ability to activate transcription of the reporter genes nonspecifically. pACTII was used as a negative control to be cotransformed with a Gal4DB-based construct, while pLamin was cotransformed with a Gal4AD-based construct.

Gal4DB and Gal4AD plasmids were also transformed into Y190. We used Y190 even though PJ69–4A also contains a *lacZ* reporter because the promoter driving *lacZ* in PJ69–4A is activated by the cell permeabilization required to measure β-galactosidase by colony lifts (James *et al.*, 1996). Y190 transformants were grown on −TL plates at 30°C for 3 d, and then *lacZ* activity was measured by the colony lift filter assay using X-gal as the substrate (Bartel and Fields, 1995). In some cases, the filter assays were repeated using 5-d-old transformants.

Expression of the fusion proteins was analyzed in an immunoblot of whole-cell extracts (Friedman *et al.*, 1996) using anti-HA antibodies (monoclonal antibody 12CA5 from Boehringer Mannheim (Indianapolis, IN) or polyclonal antibody from Santa Cruz Biotechnology, Santa Cruz, CA). All fusion proteins to be compared were included on a single blot. At least two transformants were analyzed for each construct. For the comparison of the mutant Spc97p and the mutant Spc98p proteins with their wt counterparts, the levels of expression were normalized to major proteins in yeast cells as detected by staining the blot with Ponceau S.

Table 3. Complementation groups from the synthetic lethal screen with *spc110-221*

		Phenotype of double mutant[a]			
Group	Alleles	21°C	25°C	30°C	Gene
I	nsl1-62	S	S	R	NSL1 = SPC97
	nsl1-113	S	S	R	
	nsl1-114	R	R	R	
II	nsl2-85	R	R	R	NSL2
	nsl2-118	R	R	R	
III	nsl3-63	R	R	R	NSL3 = SPC98

[a]The double mutant refers to an *nsl spc110-221* mutant. These results were observed for the original isolates and were confirmed through at least one backcross. The phenotype is based on the red/white colony sectoring assay. S, Colonies sector; the double mutant does not display lethality at the indicated temperature. R, Colonies are solid red; the double mutant displays lethality at the indicated temperature.

RESULTS

A Synthetic Lethal Screen with spc110–221 Gives Three Complementation Groups

We performed a synthetic lethal screen with the *spc110–221* allele using the red/white colony-sectoring assay combined with the plasmid-shuffle technique described in MATERIALS AND METHODS. The *spc110–221* allele encodes mutations in the N-terminal region, region I. Strains carrying *spc110–221* grow well at temperatures below 34°C. Out of 2×10^5 colonies screened for each mating type, we isolated 16 strains that contain mutations that lower the permissive temperature of the *spc110–221* mutant to 30°C or below. Ten of these strains have additional mutations in the *spc110–221* gene. The remaining six strains have mutations that define three complementation groups designated *NSL1*, *NSL2*, and *NSL3* for N-terminal synthetic lethal (Table 3).

NSL1 Is SPC97

When a yeast genomic library was transformed into a member of group I (*nsl1–114*), a library insert containing a portion of chromosome VIII complemented the synthetic lethality of *nsl1–114* and *spc110–221*. Six potential ORFs flanked by *CDC23* and *ENO2* are contained in the insert. Subcloning narrowed the complementing region to a 3-kilobase (kb) segment containing YHR172w. This gene was subsequently identified as *SPC97* (Knop *et al.*, 1997). A plasmid containing a deletion of a 1.3 kb *Bam*HI fragment from *SPC97* does not complement the *nsl1* mutation. Therefore, *SPC97* is necessary to complement the synthetic lethal interaction of *spc110–221* and *nsl1–114*. This 3-kb segment also complements the other alleles of *NSL1* but not the alleles of *NSL2* or *NSL3*.

Gap repair indicated that the mutations in the *spc97* alleles clustered in the N-terminal part of the protein (see MATERIALS AND METHODS). The *spc97–114* mutation resulted in an amino acid change from glutamate to lysine at position 56 of the protein. This change of a negatively charged amino acid to a positively charged amino acid confers the most severe phenotype of the *spc97* alleles such that the *spc110–221 spc97–114* double mutant is not viable at any temperature (Table 3). Leucine 22 is changed to phenylalanine in the *spc97–62* allele. In the *spc97–113* allele, arginine 171 is changed to a lysine. These conservative replacements have a milder effect and only decrease the permissive temperature of the *spc110–221* to 30°C (Table 3). None of these alleles confer a phenotype in a strain carrying a single copy of wt *SPC110* on the chromosome.

NSL3 Is SPC98

Because we found that *spc97* exhibited synthetic lethality with *spc110–221*, we reasoned that mutations in *SPC98* or *TUB4*, which encode other components in the Tub4p complex, may have been obtained in the screen. We transformed low copy number (*CEN*) plasmids containing either *SPC97*, *SPC98*, or *TUB4* into the *nsl2 spc110–221* mutants and the *nsl3–63 spc110–221* mutant. The *nsl2 spc110–221* mutants were not suppressed by *SPC97*, *SPC98*, or *TUB4* on low-copy number plasmids. Therefore, *NSL2* does not encode an identified component of the Tub4p complex. In contrast, *SPC98* on a low-copy number plasmid fully suppressed the synthetic lethality conferred by the *nsl3–63* allele. To determine whether *nsl3–63* is linked to *SPC98*, we first constructed a strain containing *spc110–221* and a copy of *SPC98* marked with the *URA3* gene (see MATERIALS AND METHODS). This strain was then mated to the *nsl3–63 spc110–221* strain, and the resulting diploid was dissected. In the 37 tetrads in which *URA3* segregated 2:2, all synthetic lethal spores were uracil auxotrophs, indicating that *NSL3* is tightly linked to *SPC98*.

As final proof that *nsl3–63* is an allele of *SPC98*, we demonstrated that the single mutation S754F in Spc98–63p is necessary and sufficient for synthetic lethality with *spc110–221*. The C2261T mutation in *spc98–63* was fortuitously identified by sequencing the final 370 bases of *spc98–63*. To determine whether this mutation was sufficient to confer synthetic lethality, we tested three different dosages of *spc98* carrying the C2261T mutation: on a *CEN* plasmid, as a second integrated copy, and finally as a single copy integrated in place of wt *SPC98*. In a diploid strain (DCY101) homozygous for both *spc110–221* and *spc98–63*, synthetic lethality is observed in the presence of the low-copy number plasmid carrying C2261T *spc98* (pDC9). In a haploid *spc110–221 spc98–63* strain, even one
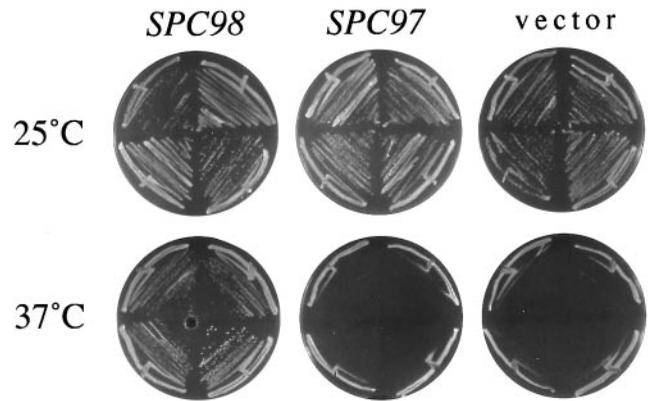
Figure 1. Suppression of the temperature-sensitive *spc110–221* allele by *SPC98*. A haploid *spc110–221* mutant (TNY2–2B) is suppressed by *SPC98* on a *CEN* plasmid (pHS89) but not by *SPC97* on a multicopy plasmid (pTN31). The vector plasmid was pRS316.

extra copy of C2261T *spc98* is sufficient to suppress the synthetic lethality. However, a single integrated copy of *spc98* carrying the C2261T mutation conferred synthetic lethality to a *spc110–221* strain as described in MATERIALS AND METHODS.

Further Genetic Interactions of SPC110 with SPC97 and SPC98

SPC98 is a dosage-dependent suppressor of the temperature sensitivity of a diploid *spc110–221* mutant, but not of the temperature sensitivity conferred by any of the other *spc110* alleles (Sundberg and Davis, 1997). *SPC97* and *TUB4* are not dosage-dependent suppressors of any of our *spc110* alleles in diploids. Here we show that *SPC98* on a centromere plasmid is sufficient for suppression of the temperature sensitivity in a haploid *spc110–221* mutant (Figure 1). In contrast, a multicopy plasmid containing *SPC97* does not suppress the phenotype of a haploid *spc110–221* strain. These results suggest that the *spc110–221* allele confers a specific defect in interaction with Spc98p.

Defects at the N and C termini of Spc110p affect distinct functions (Sundberg and Davis, 1997). We tested whether the effects of the *spc97* and *spc98* mutations were specific to alleles of *spc110* encoding N-terminal mutations in Spc110p. The *spc98–63* allele is specific and exhibits synthetic lethality with only N-terminal *spc110* alleles and not with any of the C-terminal *spc110* alleles. In contrast, the *spc97* alleles are lethal with both N-terminal and C-terminal alleles of *spc110* (Table 4). Even the mild *spc97* alleles, *97–62* and *97–113*, affect both N-terminal and C-terminal *spc110* alleles.

Interactions between Spc98p and Spc110p

The diverse and allele-specific genetic interactions observed between *SPC98* and *SPC110* suggest that the

Table 4. Synthetic lethality between temperature-sensitive *spc110* alleles and *spc97* and *spc98* alleles

Location of mutations[a]		Allele	spc97-62	spc97-113	spc97-114	spc98-63
N terminus	Region I	spc110-221	SL[b]	SL	SL	SL
	Region I	spc110-222	SL	SL	SL	SL
C terminus	Region II	spc110-226	NO	NO	SL	NO
	Region III	spc110-220	NO	NO	SL	NO
	Regions II + III	spc110-225	NO	SL	SL	NO
	Truncation	spc110-224	SL	NO	SL	NO

[a]Mutations in the *spc110* alleles fall into three regions: region I is residues 1-163 N-terminal to the coiled-coil domain. Region II is aa 772-836. Region III is aa 897-917 and is the calmodulin-binding site.
[b]SL, Synthetic lethality is detected between the two alleles at 30°C. NO, Double mutants are able to sector at 30°C. Genetic analysis is described in MATERIALS AND METHODS. The diploid strains that were sporulated to obtain these results are listed in Table 1.

two proteins interact. We sought evidence for physical interactions between Spc110p and Spc98p using a two-hybrid assay in which a positive interaction confers adenine prototrophy by allowing expression of the *ADE2* gene. Three Gal4p-Spc110p-fusion constructs containing residues localized to the N-terminal region of Spc110p were created. Spc110p$^{1-183}$ includes all of region I and 33 amino acid residues predicted to be part of the coiled-coil domain. Spc110p$^{1-150}$ includes the majority of region I and none of the predicted coiled-coil sequence. To examine the effects of mutations in Spc110p on interactions, we also created the Spc110–221p$^{1-183}$ construct. These three fusion constructs of Spc110p are stably expressed, and their levels of expression are similar as analyzed by Western blotting (Figure 2A). A construct encoding full-length Spc110p fused to the Gal4p-activation domain was not used because it is completely inactive in the two-hybrid assay and fails to interact even with calmodulin (Geiser *et al.*, 1993).

All three constructs that contain N-terminal sequences of Spc110p showed an interaction with Spc98p as measured by expression of the *ADE2* reporter gene (Table 5A). Spc110p$^{1-183}$ gives the strongest signal, whereas the shorter construct Spc110p$^{1-150}$ gives a weaker signal, suggesting that the extra residues in Spc110$^{1-183}$ are important for binding to Spc98p. Mutations in the *spc110–221* allele substantially reduced the interactions between Spc110–221p and Spc98p (Table 5B), indicating that the binding site for Spc98p is disrupted in Spc110–221p. The S754F mutation in Spc98–63p abolishes the interaction with Spc110–221p$^{1-183}$ consistent with the lethal phenotype of the *spc110–221 spc98–63* mutant. Finally, the S754F mutation has no detectable effect on interaction with wt Spc110p$^{1-183}$ in the two-hybrid assay (Table 5B).

Interactions between Spc110p and Spc97p

In contrast to Spc98p, Spc97p failed to interact with any of the Spc110p constructs as measured by expres-

sion of the *ADE2* reporter gene in strain PJ69–4A. However, using a different two-hybrid strain (Y190) and monitoring expression of a different reporter
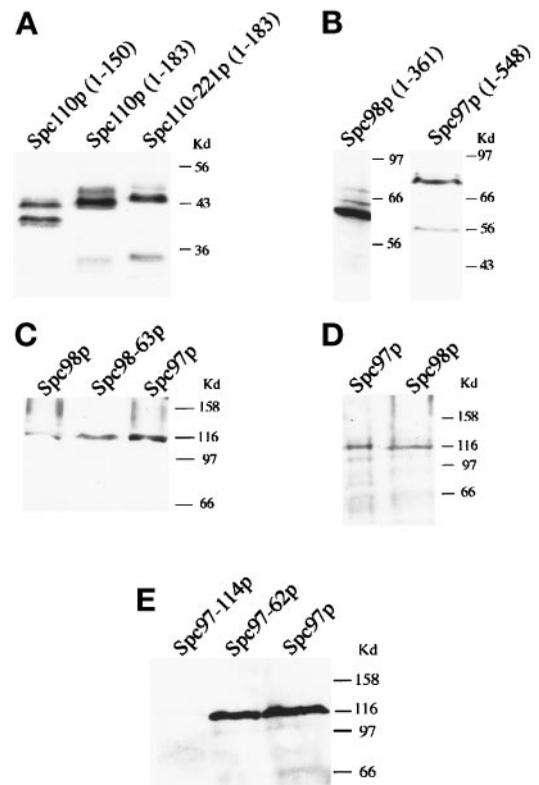
Figure 2. Immunoblots of Gal4p-fusion proteins in the two-hybrid strains. Panels A, B, C, and E are analyzed in strain PJ69–4A; panel D is analyzed in strain Y190. Each panel indicates a different blot. Whole-cell extracts obtained from an approximately equal amount of cells growing in midlog phase are loaded in each lane. panels A, B, and E are probed with rabbit anti-HA antibodies; panels C and D are probed with mouse anti-HA antibodies. In panel A, all Gal4p-Spc110p constructs migrate as doublets with molecular weights ranging from 40 to 44 kDa; in panel B, Gal4p-Spc98p$^{1-361}$ migrates as a 64-kDa protein, and Gal4p-Spc97p$^{1-548}$ migrates as an 84-kDa protein.

Table 5. Two-hybrid interactions between Spc110p and components of the Tub4p complex

Gal4p-DNA binding domain fused to	Gal4p-activation domain fused to	Growth on [-trp,-leu,-ade] plates[a]	Colony color[b]
A. wt proteins			
Spc98p	Spc110p (aa 1-183)	++++	B[c]
Spc97p	Spc110p (aa 1-183)	−	B[d]
Tub4p	Spc110p (aa 1-183)	−/+	LB (≤1%)
Spc98p	Spc110p (aa 1-150)	+++	B
Spc97p	Spc110p (aa 1-150)	−	W (3d); B (5d)[e]
B. Mutant proteins			
Spc98p	Spc110-221p (aa 1-183)	++	W
Spc97p	Spc110-221p (aa 1-183)	nd	W
Spc98-63p	Spc110p (aa 1-183)	++++	B
Spc98-63p	Spc110p (aa 1-150)	++	nd
Spc98-63p	Spc110-221p (aa 1-183)	−	nd
Spc97-62p	Spc110p (aa 1-183)	−	W
Spc97-114p	Spc110p (aa 1-183)	−	W
C. Controls			
lamin[f]	Spc110p (aa 1-183)	−	W
lamin	Spc110p (aa 1-150)	−	W
lamin	Spc110-221p (aa 1-183)	−	W

[a]Growth of cells on SD-trp, -leu, -ade (−TLA) plates is scored between day 3 and when colonies are fully developed by day 5 or 6. When colonies are fully developed, the plating efficiency on −TLA plates is compared to the plating efficiency on SD-trp, -leu plates (−TL). ++++, plating efficiency on −TLA is ≥90% of −TL; colonies are fully developed on day 3; +++, ≥90% plating of −TL, colonies are small on day 3 and fully developed by day 5; ++, 50-90% plating of −TL; colonies are barely visible on day 3, fully developed by day 5; +, 50% plating of −TL; colonies are barely visible on day 3, fully developed by day 6; +/−, 25% plating of −TL; colonies are not visible on day 3, fully developed by day 6; −/+, no growth on day 3; colonies are barely visible on day 5, but do not get bigger thereafter; −, no growth. Results are tabulated from at least two independent transformations.

[b]Colony-lift filter assays using X-gal are performed to measure β-galactosidase activity. (%) indicates the % of the total number of colonies transferred onto nitrocellulose filters that display color. A (≤5%) is considered background rather than a true interaction that occurs when 50-95% of transferred colonies display color after a 30° incubation overnight. Generally, "blue" colonies are blue within the first 1–3 h after incubation, but the final scoring reported here is for an overnight incubation to include weak interactions. B, blue; LB, light blue; W, white; nd, not determined. Observations are made from at least two independent transformations.

[c]Blue colonies develop within 1 h of incubation at 30°C.

[d]Blue colonies develop after 2 h incubation at 30°C.

[e]Assays were performed using 3- or 5-d-old transformants.

[f]Lamin instead of the vector pAS2 was used as the negative control because pAS2 by itself activates transcription of the reporter genes.

(*lacZ*) allowed detection of a weak interaction between Spc97p and Spc110p$^{1-183}$ (Table 5). We looked for conditions that would allow detection of the interaction between Spc97p and Spc110p in the PJ69−4A strain background. We found that increasing the level of wt Spc98p sufficiently enhanced the interaction between Gal4AD-Spc110p$^{1-183}$ and Gal4DB-Spc97p to al-

low adenine prototrophy in strain PJ69−4A (Table 6A and Figure 3). The specificity of this three-way interaction is indicated by the fact that overexpressing *TUB4* did not enhance the interaction between Spc110p and Spc97p (Table 6A), and overexpressing *SPC97* did not enhance the interaction between Spc110−221p and Spc98p (Table 6B). Thus, Spc98p promotes the interaction between Spc110p and Spc97p fusion proteins in strain PJ69−4A. By Western blotting, the level of expression of Gal4-Spc97p and Gal4-Spc98p is similar in strain PJ69−4A and strain Y190 (Figure 2, C and D). Thus, differences in expression of the fusion proteins in the two strains are not the cause of the discrepancy.

We next examined the interactions between mutant Spc97p and Spc110p fusion proteins. No interaction was detected between Spc97p and Spc110−221p$^{1-183}$ as measured either by expression of *ADE2* in the presence of extra copies of *SPC98* (Table 6A) or by expression of β-galactosidase in strain Y190 (Table 5B). Since the interaction between Spc97p and Spc110−221p$^{1-183}$ was not detectable, we could not test whether the synthetic lethal mutation in Spc97−62p had any further effects on interaction with Spc110−221p$^{1-183}$. However, the mutation in the Spc97−62p fusion protein reduced interactions with the wt Spc110p fusion protein in the absence (Tables 5B) or presence of extra Spc98p (Table 6A and Figure 3). By Western blotting, Gal4-Spc97−62p is expressed at a similar level as wt Gal4-Spc97p (Figure 2E), indicating that decreased interactions are not due to a decreased amount of fusion protein. No interaction is detectable between Spc97−114p and Spc110p, but the low level of the Spc97−114p fusion protein may be the cause (Figure 2E).

Interactions between Spc110p and Tub4p

Although *TUB4* was not identified in our synthetic lethal screen, we tested for interactions between Tub4p and Spc110p because it forms a complex with Spc97p and Spc98p. When fused to the Gal4pDB, Tub4p gives a background signal in both two-hybrid systems (−/+). Interaction with *Spc110p*$^{1-183}$ was not detectable above this background (Table 5). Extra copies of *SPC98* promoted the interaction between Spc110p and Tub4p to a level that was detectable above background (Table 6A). Note that extra copies of *SPC97* have no effect on this interaction.

Genetic Evidence That Interaction between Spc97p and Spc110p Is Dependent on Spc98p

Our genetic and two-hybrid results led to a two-part hypothesis. First, Spc98p is primarily responsible for the interaction between the Tub4p complex and Spc110p. Second, the interaction between Spc97p and Spc110p is dependent on Spc98p. We performed additional analyses to test this hypothesis. Consistent
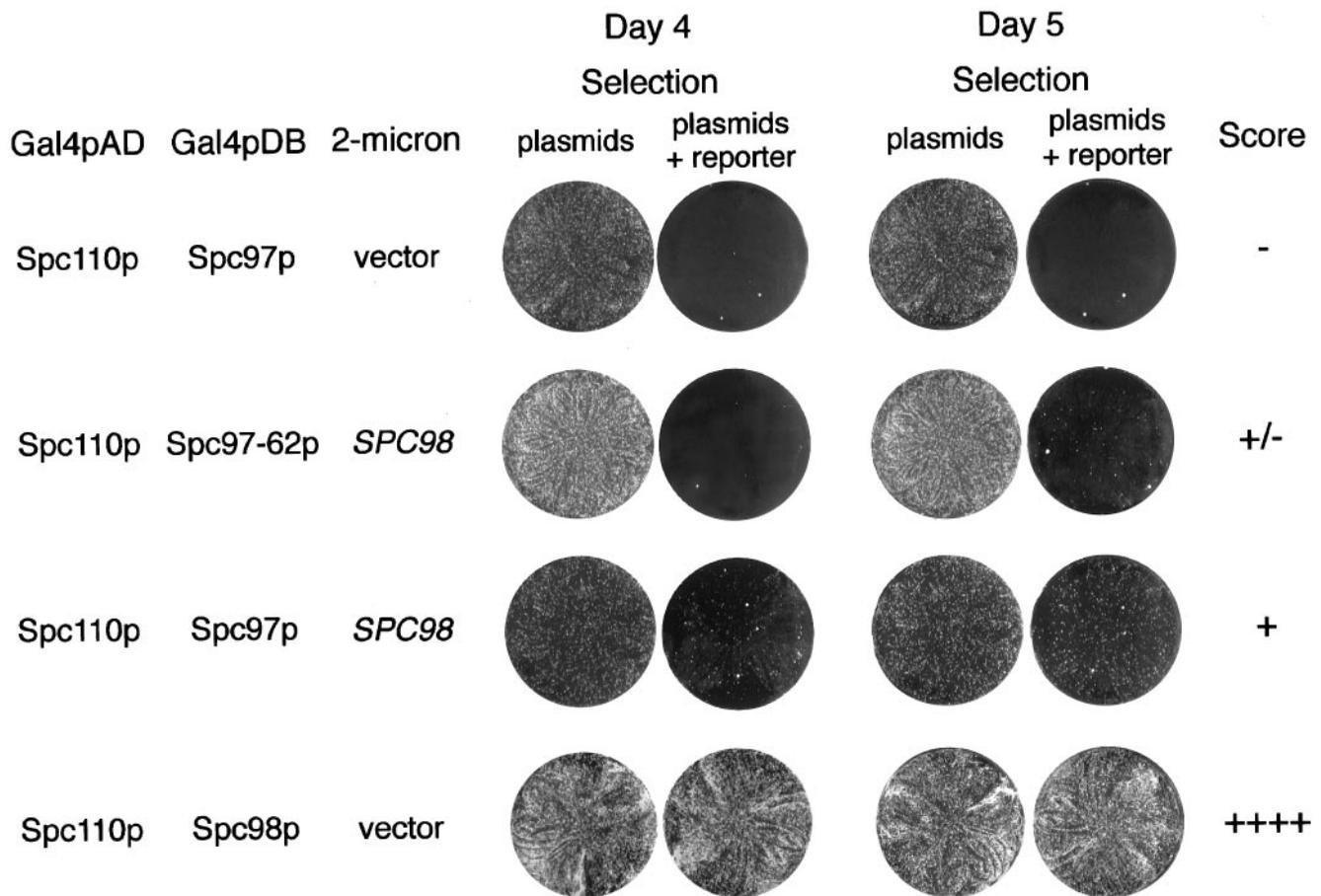
Figure 3. Examples of three-way interactions in the two-hybrid system as scored by expression of the *ADE2* gene. Each row shows a combination of two Gal4p-fusion proteins plus a third protein produced from the 2μ plasmid. The plasmids are given in Table 2. The two plates on the left indicate growth after 4 d, and the two on the right show growth after 5 d. The medium for selection of the plasmids is SD-trp,-leu,-ura. Interaction between proteins confers adenine prototrophy. The medium for selection of the plasmids and the reporter is SD-trp,-leu,-ura,-ade. The scoring designation for each combination is given in the last column.

with the hypothesis, *SPC98* is a dosage-dependent suppressor of the synthetic lethality between *spc97* and *spc110*, but *SPC97* is not a suppressor of the synthetic lethality between *spc98* and *spc110* (Table 7). The ability of *SPC98* to suppress correlates with the severity of the *spc97* allele. The most severe allele, *spc97–114*, is only suppressed at 25°C, whereas the milder alleles, *spc97–62* and *spc97–113*, are suppressed at 30°C.

Interactions among Components of the Tub4p Complex

Our hypothesis predicts that the synthetic lethality between *spc97* and *spc110* is caused indirectly by a reduced interaction between the Spc97p mutant proteins and Spc98p. To test this prediction, we characterized the interactions among the compo-

nents of the Tub4p complex using a two-hybrid assay.

We first analyzed the interactions among the wt proteins. wt Spc97p, Spc98p, and Tub4p all interact with each other in pairwise combinations (Table 8A) as shown previously (Knop *et al.*, 1997). However, our results differ from the previous results in two ways. First, we found that residues 1–361 of Spc98p are sufficient for interaction between Spc98p and Tub4p, and additional sequences C-terminal of residue 551 in Spc98p are not required (Table 8A). Second, interaction between two molecules of Tub4p is readily detected in our two-hybrid system.

Our interest lay in characterizing the effects of the synthetic lethal mutations isolated in this study on interactions among the components of the Tub4p complex. In agreement with our prediction, the L22F mutation in Spc97–62p reduced interaction

Table 6. Three-way interactions in the two-hybrid assay between Spc110p and Spc97p, Tub4p, or Spc98p

Gal4p–DNA-binding domain fused to	Gal4p-activation domain fused to	Protein overexpressed[a]	Growth on [-trp,-leu,-ura,-ade] plates[b]
A. Interactions with Spc97 or Tub4p			
Spc97p	Spc110p (aa 1-183)	Vector	−
Spc97p	Spc110p (aa 1-183)	Spc98p	+
Spc97p	Spc110p (aa 1-183)	Tub4p	−
Tub4p	Spc110p (aa 1-183)	Vector	−/+
Tub4p	Spc110p (aa 1-183)	Spc98p	+/−
Tub4p	Spc110p (aa 1-183)	Spc97	−/+
Spc97-62p	Spc110p (aa 1-183)	Spc98p	+/−
Spc97-114p	Spc110p (aa 1-183)	Spc98p	−
Spc97p	Spc110-221p (aa 1-183)	Spc98p	−
B. Interactions with Spc98p			
Spc98p	Spc110p (aa 1-183)	Vector	++++
Spc98p	Spc110p (aa 1-183)	Spc97p	++++
Spc98p	Spc110p (aa 1-150)	Vector	+++
Spc98p	Spc110p (aa 1-150)	Spc97p	++
Spc98p	Spc110-221p (aa 1-183)	Vector	++
Spc98p	Spc110-221p (aa 1-183)	Spc97p	+/−
Spc98-63p	Spc110p (aa 1-183)	Vector	++++
Spc98-63p	Spc110p (aa 1-183)	Spc97p	++++
Spc98-63p	Spc110p (aa 1-150)	Vector	++
Spc98-63p	Spc110p (aa 1-150)	Spc97p	++
Spc98-63p	Spc110-221p (aa 1-183)	Vector	−
Spc98-63p	Spc110-221p (aa 1-183)	Spc97p	−

[a] Overexpression plasmids are 2μ based and contain *URA3* as the selectable marker. The indicated gene products are expressed from their own promoters in these plasmids. Plasmids are given in Table 2.
[b] Comparisons of growth rate are made between SD-trp,-leu,-ura plates *vs.* SD-trp,-leu,-ura,-ade plates in order to select for the third cotransformed plasmid. See footnotes under Table 5 for explanations of the scoring technique.

with all of the Spc98p fusion proteins (Table 8). The E56K mutation in Spc97–114p abolished these interactions. The effects of the S754F mutation on the interaction of Spc98–63p with Spc97p is complex. The mutation actually enhanced interaction with the N-terminal (1–548) sequences of Spc97p but reduced the interaction with full-length Spc97p (Table 8B). Finally, the mutations in Spc97–62p, Spc97–114p, and Spc98–63p did not affect their interactions with Tub4p (Table 8B).

Table 7. Dosage-dependent suppression of synthetic lethality at 30°C[a]

	Synthetic lethal mutant							
	spc110-221 spc97-114		spc110-221 spc97-113		spc110-221 spc97-62		spc110-221 spc98-63	
Plasmid	25°C	30°C	25°C[b]	30°C	25°C	30°C	25°C	30°C
2 μ SPC97	+++	+++	NA[b]	+++	NA	+++	−	−
2 μ SPC98	+	−	NA	++	NA	+	+++	+++
2 μ TUB4	−	−	NA	−	NA	−	++	+

[a] Each synthetic lethal strain was transformed with the indicated plasmid, and transformants were restreaked on selective plates at 25°C and 30°C. The plasmids were pHS92 (2μ *SPC97*), pHS88 (2μ *SPC98*), and pHS91 (2μ *TUB4*). The strains were (in order from left to right): TNY137, TNY163, TNY162, and TNY76-1C. Sectoring was assessed after 8 d. The ability of a solid red mutant to sector white at the indicated temperature indicates whether its synthetic lethality is suppressed. +++, the mutant is suppressed; the colonies are almost completely white. ++, the mutant is suppressed; the colonies have some red sectors. +, the mutant is suppressed; the colonies have larger red sectors. −, the mutant is not suppressed; the colonies are as solid red as colonies with empty vector.
[b] Not applicable; these alleles do not confer a synthetic lethal phenotype at 25°C. See Table 3.

Table 8. Two-hybrid interactions among components of the Tub4p complex

Gal4p-DNA binding domain fused to	Gal4p-activation domain fused to	Growth on [-trp,-leu,-ade] plates [a]
A. wt proteins		
Spc97p	Spc98p	++++
Spc97p	Spc98p (aa 148-846)	+++
Spc97p	Spc98p (aa 1-361)	+
Spc97p	Tub4p	++++
Spc97p	Spc97p	−
Spc98p	Spc97p	++++
Spc98p	Spc97p (aa 1-548)[b]	++
Spc98p	Tub4p	++++
Spc98p	Spc98p	−
Tub4p	Spc98p	++
Tub4p	Spc98p (aa 148-846)	+++
Tub4p	Spc98p (aa 1-361)	+++
Tub4p	Spc97p	++++
Tub4p	Spc97p (aa 1-548)	+++
Tub4p	Tub4p	+++
B. Mutant proteins		
Spc97-62p	Spc98p	+++
Spc97-62p	Spc98p (aa 148-846)	++
Spc97-62p	Spc98p (aa 1-361)	−
Spc97-62p	Tub4p	++++
Spc97-114p	Spc98p	+/−
Spc97-114p	Spc98p (aa 148-846)	−
Spc97-114p	Spc98p (aa 1-361)	−
Spc97-114p	Tub4p	++++
Spc98-63p	Spc97p	++
Spc98-63p	Spc97p (aa 1-548)	++++
Spc98-63p	Tub4p	++++

[a] See Table 5 for explanations of this scoring technique.

[b] Numbers in parentheses indicate amino acid residues of the tested protein that are fused to the Gal4p domain. If no numbers are indicated, the full-length protein is included.

Table 9. *GAL* overexpression of *SPC97* in *spc110* diploid strains

Strain[a]	Permissive temperature	
	GAL1 vector[b]	*GAL1-SPC97*
spc110-220/spc110-220	32°C	30°C
spc110-221/spc110-221	34°C	21°C
spc110-224/spc110-224	30°C	Dead
spc110-225/spc110-225	30°C	25°C
spc110-226/spc110-226	32°C	21°C
SPC110/SPC110	37°C	37°C[c]

[a] The strains used were (in order of appearance) HSY20, HSY21, HSY46, HSY48, HSY83 (Sundberg and Davis, 1997) and JGY46 (Geiser *et al.*, 1991).

[b] The *GAL1* vector is pRD54 and the *GAL1-SPC97* plasmid is pTN26.

[c] The colonies are healthy but smaller than ones that just have the *GAL* vector.

Evidence That Spc97p Sequesters Spc98p from Spc110p

One distinction between *SPC97* and *SPC98* is that *SPC98* is a dosage-dependent suppressor of *spc110−221* but *SPC97* is not (see Figure 1). We explored whether this lack of suppression was merely due to insufficient *SPC97* being expressed from the high-copy number vector by testing whether even higher expression levels could suppress the temperature sensitivity conferred by any of the *spc110* alleles. Even overexpression of *SPC97* using a *GAL*-inducible promoter did not suppress the temperature sensitivity of the *spc110* mutants. Instead, the *GAL* overexpression of *SPC97* from a *CEN* plasmid lowered the permissive temperature of all the *spc110* mutants but had little effect on a wt strain (Table 9).

To further explore the toxic effects of overexpression of *SPC97*, we analyzed the effects of extra copies of *SPC97* on the interaction between Spc98p and Spc110p by the two-hybrid assay. While extra copies of *SPC97* had no effect on the interaction between Spc98p and

Spc110p$^{1-183}$, it reduced the interaction of Spc98p with either Spc110p$^{1-150}$ or Spc110−221p$^{1-183}$ (Table 6B). Since the mutation in Spc98−63p reduces its ability to interact with full- length Spc97p (Table 8B), extra copies of *SPC97* had no effect on the interaction of the mutant Spc98−63p with Spc110p$^{1-150}$ or with Spc110−221p$^{1-183}$ (Table 6B).

Overexpression of SPC110 Is Toxic to the spc97 Mutants

The toxic effect of overexpression was not confined to *SPC97*. Overexpression of *SPC110* confers a temperature-sensitive growth defect to two of the *spc97* mutant strains, *spc97–114* and *spc97–62* (Figure 4). The toxic effect is strongest with the *spc97–114* mutant, which is killed at 37°C by overexpression of *SPC110* and is independent of the *spc110−221* allele. The *spc97* alleles, by themselves, do not have a phenotype at 37°C, and a strain containing only the *spc110−221* allele grows well with extra copies of *SPC110* at 37°C (Figure 4). Furthermore, overexpression of *SPC110* does not cause any growth defects in the *spc98−63 spc110−221* mutant. Thus, overexpression of *SPC110* causes temperature sensitivity only in the presence of the *spc97* mutations.

DISCUSSION

Previous analyses of *SPC110* suggest a role for Spc110p in anchoring the inner plaque to the central plaque at the SPB (Kilmartin and Goh, 1996; Sundberg *et al.*, 1996; Sundberg and Davis, 1997; Knop and Schiebel, 1997). A screen for mutations that enhance the temperature-sensitive phenotype conferred by an *spc110* allele encoding mutations in the N terminus of Spc110p identified *SPC97* and *SPC98*. Spc97p, Spc98p, and Tub4p form a soluble 6S complex in yeast cells

Figure 4. Overexpression of Spc110p is toxic to *spc97* mutants. Each strain contains *SPC110* on a multicopy plasmid (pHS26). The relevant genotype is given beside each row. The strains were (in order from top to bottom): TNY2–4B, TNY64–5C, TNY137, TNY114–39C, TNY113–10A, and TNY76–1C. Serial dilutions containing 1×10^5, 1×10^4, 1×10^3, 1×10^2, and 10 cells were plated for each strain on YPD and incubated at 30°C and 37°C for 52 h.
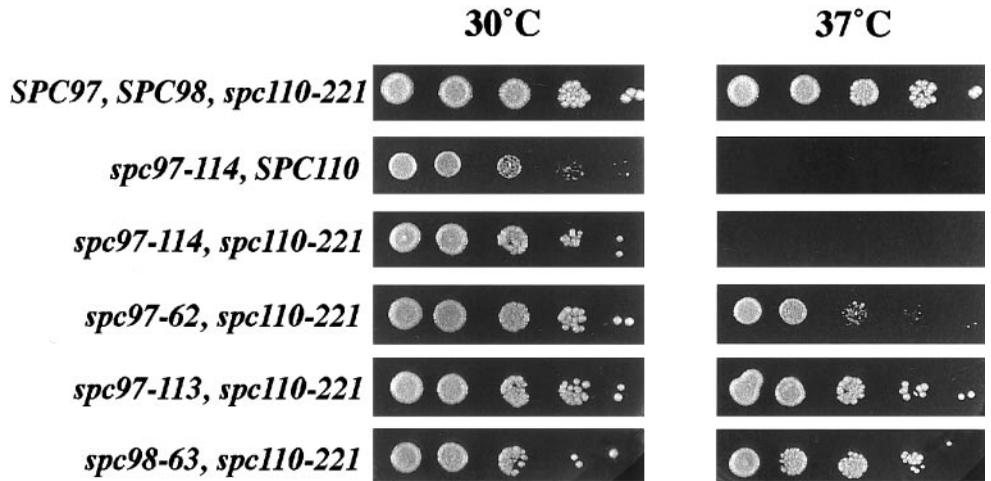
and are found at the inner and outer plaques of the SPB (Rout and Kilmartin, 1990; Spang *et al.*, 1996a; Knop *et al.*, 1997). All three proteins are required for proper spindle formation, but it remains to be shown how the Tub4p complex assembles into the inner and outer plaques of the SPB (Geissler *et al.*, 1996; Marschall *et al.*, 1996; Spang *et al.*, 1996a; Knop *et al.*, 1997). Although they share many properties, Spc97p and Spc98p are not similar in their interactions with Spc110p. The genetic and two-hybrid results together strongly argue that Spc98p is primarily responsible for attaching the Tub4p complex to the N terminus of Spc110p (Figure 5). The evidence is summarized in Table 10.

Interaction between the N terminus of Spc110p and Spc98p generated a robust signal in two different two-hybrid systems. Interaction of Spc110p with Spc97p and Tub4p did not. Overexpression of *SPC98* enhanced the interaction between Spc110p and Spc97p and between Spc110p and Tub4p. Thus, Spc98p mediates or stabilizes the interactions between Spc110p and the other two components of the Tub4p complex.

At the nonpermissive temperature, the *spc110–221* allele causes cells to arrest at the *MAD1* checkpoint with spindles that appear morphologically normal (Sundberg and Davis, 1997). *SPC98* is the only gene in the Tub4p complex that can suppress the temperature-sensitive growth of *spc110–221* mutants ([Sundberg and Davis, 1997] and further characterized herein). Thus, the interaction between Spc98p and Spc110p is specifically impaired in these cells.

Mutations in either *SPC98* or *SPC97* worsen the phenotype conferred by *spc110–221*, but they do so through different mechanisms. The *spc98–63* allele
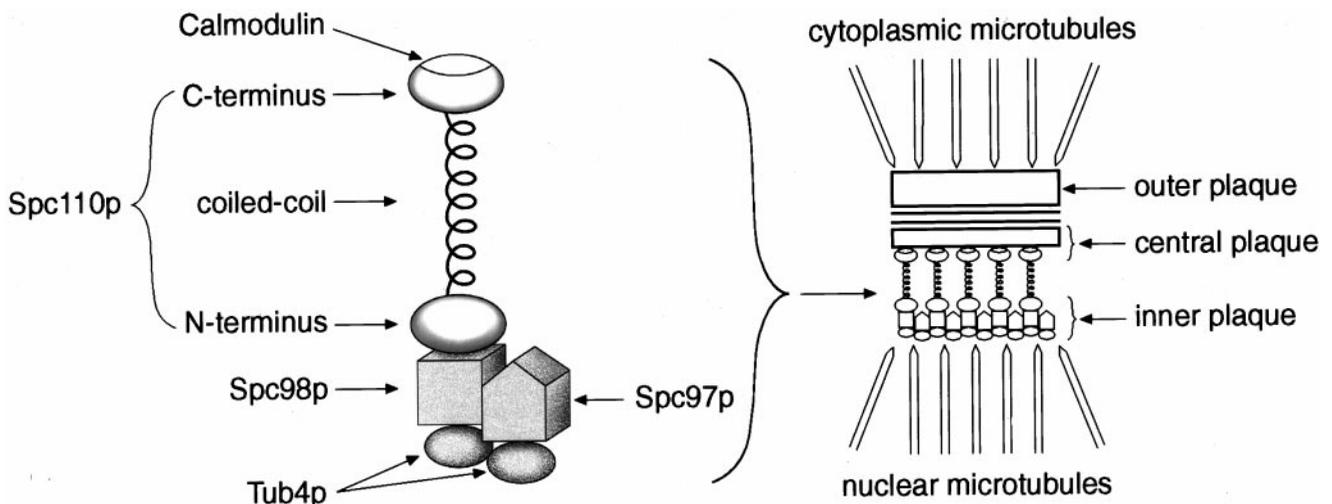
Figure 5. Model of the linkage of the Tub4p complex to Spc110p at the SPB.

Table 10. Characteristics of Spc97p and Spc98p

Properties	Spc97p	Spc98p
Similarities		
Binds to Tub4p	Yes[a]	Yes[b]
Present in Tub4p-complex	Yes[a]	Yes[a]
Primary sequence similarity	Yes	Yes
Mutants show synthetic lethality with *spc110-221* (contains mutations in the N-terminal region I)	Yes	Yes
Differences		
Overexpression suppresses *spc110-221*	No	Yes
Interaction with wt Spc110p in two-hybrid assays	Weak	Robust
Overexpression influences interactions with Spc110p in two-hybrid assay	Weakens Spc98p-Spc110p interaction	Enhances Spc97p-Spc110p interaction
Mutants show synthetic lethality with *spc110* alleles encoding mutations in C-terminal regions	Yes	No
Interaction with wt Spc110p reduced by synthetic lethal mutations	Yes	No
Overexpression toxic to *spc110* mutants	Yes	No
Overexpression of *SPC110* is toxic to mutants	Yes	No

[a] Knop *et al.* (1997).
[b] Geissler *et al.* (1996).

further compromises the impaired interaction between Spc98p and Spc110–221p. As measured in a two-hybrid assay, the S754F mutation encoded by *spc98–63* completely abolishes the interaction of Spc98–63p with Spc110–221p. The specificity of this effect was shown in two ways. First, the S754F mutation has no effect on the interaction of Spc98–63p with wt Spc110p. Second, *spc98–63* enhances the phenotypes conferred only by alleles of *spc110* encoding N-terminal mutations and not by alleles encoding C-terminal mutations even though the latter group of alleles confers more severe phenotypes. This type of allele specificity is strong genetic evidence for a direct interaction between Spc98p and Spc110p.

In contrast to the *spc98* allele, the *spc97* alleles indirectly affect interaction with Spc110–221p by decreasing interaction with Spc98p. The evidence for this indirect effect is twofold. First, overexpression of *SPC110* is toxic to *spc97–114 spc110–221* and *spc97–62 spc110–221* mutants at 37°C. This toxic effect is the opposite of that expected if the *spc97* alleles directly decreased the binding of mutant Spc97p to Spc110–221p (also see further discussion below). Second, as expected for an effect mediated through a third protein (in this case Spc98p), the effects of the *spc97* alleles are not specific to the *spc110–221* allele. The lack of specificity is shown in three ways. The *spc97* alleles decrease the permissive temperature of the C-terminal Spc110p mutants, which have a normal N-terminal region. Moreover, in addition to decreasing interaction with Spc110–221p, the L22F mutation decreases the interaction of Spc97–62p with wt Spc110p as measured in the two-hybrid assay. Finally, the reduction in binding to Spc110p occurs because the mutation in Spc97–62p reduces its binding to Spc98p.

The distinction between Spc98p and Spc97p is further demonstrated by the effects of overexpressing each of the genes. Overexpression of *SPC98* enhances the interaction between Spc97p and Spc110p as measured by the two-hybrid assay. In vivo, overexpression of *SPC98* suppresses the synthetic lethality of all *spc97 spc110–221* mutants. In contrast, overexpression of *SPC97* interferes with the ability of wt Spc98p to interact with Spc110p[1–150] or Spc110–221p and lowers the permissive temperatures of *spc110* mutants. Because the decreased interaction between Spc98–63p and Spc97p prevents the toxic effect of overproduced Spc97p, we believe the toxicity is due to the sequestration of Spc98p by Spc97p.

The diverse dosage-dependent effects described here suggest that the stoichiometry of Spc97p and Spc98p is delicately balanced. Spc98p is responsible for targeting the Tub4p complex to the nucleus and is the only protein in the complex that contains a nuclear localization signal (Geissler *et al.*, 1996; Pereira *et al.*, 1998). Moreover, overexpressing *SPC98* causes the accumulation of Spc98p in the nucleus, whereas overexpressing *SPC97* leads to the accumulation of Spc97p in the cytoplasm (Knop *et al.*, 1997; Knop and Schiebel, 1997). It is tempting to speculate that the appropriate ratio of Tub4p complex in the cytoplasm and in the nucleoplasm must exist to ensure a balance in the nucleation of nuclear and cytoplasmic microtubules.

The stoichiometry of Spc110p to the components of the Tub4p complex is also balanced. High expression of *SPC97* from a *GAL1* promoter is toxic to all *spc110* mutants. The overproduction of wild-type Spc97p may sequester Spc98p in the cytoplasm and limit the amount of Spc98p that can incorporate into the inner plaque. This deficit of Spc98p in the nucleus is exac-

erbated in the *spc110* mutants by the weakened attachment of the inner plaque to the SPB, whether by disruption of the C terminus or N terminus of Spc110p (Sundberg and Davis, 1997). Conversely, a high level of *SPC110* is toxic to *spc97* mutants at 37°C. Overproducing Spc110p may sequester Spc98p in the nucleus, resulting in a deficit of Spc98p in the cytoplasm. This deficit is worsened by mutations in Spc97p that impair binding to Spc98p and therefore interfere with the ability of Spc97p to keep Spc98p in the cytoplasm.

Recently, Tassin *et al*. (1997) reported the identification of human and *Xenopus* proteins, of 100 and 116 kDa, respectively, that cross-react with monoclonal antibodies raised against yeast Spc110p. The vertebrate Spc110p-related proteins localize to centrosomes and are phosphorylated during mitosis. Interestingly, a portion of the Spc110p-related protein identified in *Xenopus* extract copurifies with a γ-TuRC–like complex of 25S. Furthermore, antibodies against Spc110p inhibit microtubule nucleation on isolated centrosomes. We and others find no evidence for a soluble complex containing Spc110p and the proteins of the Tub4p complex in yeast (Vinh, unpublished results; Knop and Schiebel, 1997). However, the evidence presented here and that reported previously (Knop and Schiebel, 1997) demonstrate that Spc110p interacts with the Tub4p complex in yeast. The association of Spc110p with the microtubule nucleation machinery has been detected cytologically. Mutant cells containing Spc110p defective in binding calmodulin exhibit an aberrant intranuclear structure that emanates microtubules and contains Spc110p, Tub4p, and Spc98p (Sundberg *et al.*, 1996; Sundberg and Davis, 1997). Strong overexpression of wt Spc110p results in the formation of a polymeric structure that anchors microtubules (Kilmartin and Goh, 1996). Therefore, Spc110p is physically associated with structures that polymerize microtubules in both the SPB and the centrosome, two organelles that are structurally diverse. These observations suggest that the underlying mechanism of microtubule nucleation is conserved between yeast and higher eukaryotes.

ACKNOWLEDGMENTS

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