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The Fission Yeast Kinetochore Component Spc7 Associates with the EB1 Family Member Mal3 and Is Required for Kinetochore–Spindle Association

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A critical aspect of mitosis is the interaction of the kinetochore with spindle microtubules. Fission yeast Mal3 is a member of the EB1 family of microtubule plus-end binding proteins, which have been implicated in this process. However, the Mal3 interaction partner at the kinetochore had not been identified. Here, we show that the *mal3* mutant phenotype can be suppressed by the presence of extra Spc7, an essential kinetochore protein associated with the central centromere region. Mal3 and Spc7 interact physically as both proteins can be coimmunoprecipitated. Overexpression of a Spc7 variant severely compromises kinetochore–microtubule interaction, indicating that the Spc7 protein plays a role in this process. Spc7 function seems to be conserved because, Spc105, a *Saccharomyces cerevisiae* homolog of Spc7, identified by mass spectrometry as a component of the conserved Ndc80 complex, can rescue *mal3* mutant strains.

INTRODUCTION

Segregation of chromosomes requires the association of spindle microtubules and chromosomes. Attachment of the mitotic spindle fibers occurs at a multicomponent protein complex, the kinetochore, that is assembled on centromeric DNA. This DNA region differs greatly in structure and size among various organisms (reviewed in Pidoux and Allshire, 2000; Cleveland *et al.*, 2003). The budding yeast centromere DNA is the simplest one described and consists of a very well defined 125-base pair region, whereas in higher eucaryotes, centromeric DNA is made up of highly repetitive sequences encompassing up to millions of base pairs. The centromere DNA of the fission yeast *Schizosaccharomyces pombe* lies in between these two extremes: it occupies between 40 and 100 kb on each chromosome and is composed of a central region flanked by inner and outer repetitive sequences. To date, proteins found to be associated with these regions either bind to the central core region or to the outer repeats, thus pointing to the existence of two distinct domains in the fission yeast centromere (reviewed in Pidoux and Allshire, 2000). The heterochromatic outer repeats are required for centromere cohesion (reviewed in Bernard and Allshire, 2002), whereas the central region is needed for the assembly of the kinetochore per se (Saitoh *et al.*, 1997; Goshima *et al.*, 1999; Jin *et al.*, 2002; Pidoux *et al.*, 2003). However, in spite of the different *cis*-acting DNA requirements, a substantial number of kinetochore proteins have been conserved from yeast to humans, among them the four-component Ndc80 complex. This complex is required for kinetochore–microtubule association and spindle checkpoint signaling (He *et al.*, 2001; Janke *et al.*, 2001; Wigge and Kilmartin, 2001; Bharadwaj *et al.*, 2004; McCleland *et al.*, 2004).

The spindle microtubules that attach to kinetochores are highly dynamic structures that alternate between phases of growth and shrinkage (Kirschner and Mitchison, 1986). This dynamic behavior is also observed after microtubules are attached to kinetochores and is coregulated by components of the kinetochore complex and microtubule plus-end associated proteins. The role of kinetochore motors such as CENP-E– and Kin1-related proteins in this process has been amply documented (reviewed in McIntosh *et al.*, 2002; Cleveland *et al.*, 2003; Mimori-Kiyosue and Tsukita, 2003). Microtubule plus-end proteins also have been implicated in the local control of microtubule dynamics and in the attachment of microtubules to kinetochores. Members of the CLIP170 family are associated transiently with prometaphase kinetochores and are required for kinetochore–microtubule attachment (Dujardin *et al.*, 1998; Lin *et al.*, 2001). Recently, the nonmotor microtubule-associated protein (MAP) family CLASP has been identified as CLIP170/CLIP115 interaction partners (Akhmanova *et al.*, 2001; Maiato *et al.*, 2003). CLASP1 has been shown to localize near growing spindle microtubule plus-ends and at the outer corona kinetochore region and is required for microtubule dynamics at the microtubule–kinetochore interface (Akhmanova *et al.*, 2001; Maiato *et al.*, 2003).

Another functionally conserved group of plus-end MAPs is the EB1 family that includes the *S. pombe* Mal3 protein (reviewed in Beinhauer *et al.*, 1997; Tirnauer and Bierer, 2000). Human EB1 was originally identified as an interaction partner of the adenomatous polyposis coli tumor suppressor APC (Su *et al.*, 1995). Members of this family localize along microtubules, but they are preferentially associated with microtubule plus-ends, regulate microtubule dynamics, and

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Table 1. Yeast strains used in this study

Name	Genotype	Source
UFYS135	*h⁺ mal3Δ::his3⁺ ade6-M210 leu1-32 ura4-D18 his3Δ*	U. Fleig
UFYS0203	*h⁻mal3-1 ade6-M210 leu1-32 ura4-D6 Ch¹⁶[ade6-M216]*	U. Fleig
UFY177	*h⁺ mal2-GFP/kan^R ade6-M210 leu1-32 ura4-D18*	U. Fleig
UFY25CX	*h⁺ spi1-25 leu1-32 ade6-M210 ura4-D6 his3Δ[ρ]*	U. Fleig
KG425	*h⁻ ade6-M210 leu1-32 his3Δ ura4-D18*	K. Gould
KG554	*h⁺ ade6-M216 leu1-32 his3Δ ura4-D18*	K. Gould
	h⁻ mal3-pkGFP/ura4⁺ ade6-M210 leu1-32 his3Δ ura4-D18	H. Browning
YUG37	*MATa ura3-52 trp1Δ63 GAL2 LEU2-tTA(leu2::pCM149)*	J. Hegemann
YSH12	*MATa ura3-52 trp1-289 leu2-3112 his3Δ1 bim1:: kan^R*	J. Hegemann
UFY155	*h⁻ spc7-GFP/Kan^R ade6-M210 leu1-32 ura4-D6*	This study
UFY617	*h⁻ spc7-HA/Kan^R ade6-M210 leu1-32 ura4-D6 Ch¹⁶[ade6-M216]*	This study
UFY498	*h⁺ spc7-HA/Kan^R mal2-GFP/Kan^R ade6-M210 leu1-32 ura4-D6*	This study
UFY496	*h⁻ spc7-GFP/KanR mal3Δ::his3⁺ ade6-M210 leu1-32 ura4 his3Δ[ρ]*	
UFY466	*h⁺ spc7-GFP/Kan^R nda3-KM311 ade6 leu1-32 ura4-D6*	This study
UFY637	*h⁺ mal3-pkGFP/ura4⁺ spc7-HA/Kan^R adeM-210 leu1-32 ura4⁻[ρ]*	This study
UFY639	*h⁻ mal3-pkGFP/ura4⁺ spc7-HA/Kan^R cut9-665 ade6-M210 leu1-32*	This study
UFY724	*h⁻ mal2–1 spc7-GFP/Kan^R ade6-M210 ura4⁻[ι] Ch16[ade6-M216]*	
UFY693	*h⁻ spc7-GFP/Kan^R spi1-25 ade6-M210 leu1-32 ura4-D6*	This study
YJO359	*MATa ade2–101 trp1-Δ63 leu2-Δ1 ura3-52 his3-Δ200 lys2-801 SPC105-ProA::His3MX6 sst1::loxP*	This study
UFY699	*MATa ura3 trp1 bim1:: kan^R LEU2-tTA (leu2:: pCM149) SPC105(−50, −1):: tetO-CYC1/Kan^R*	This study
YCJ341	*ade2-101 trp1-Δ63 leu2-Δ1 ura3-52 his3-Δ200 lys2-801 SPC24-ProA::His3MX6 cdc16-1::LYS2*	This study

have an important role in the interaction of the microtubule cytoskeleton with other cellular structures (reviewed in Tirnauer and Bierer, 2000; Schuyler and Pellman, 2001; Gundersen and Bretscher, 2003; Mimori-Kiyosue and Tsukita, 2003). Recently, EB1 and APC have been shown to localize to kinetochores, indicating an involvement in chromosome capture (Fodde *et al.*, 2001; Kaplan *et al.*, 2001). This association of EB1 is restricted to polymerizing microtubules pointing to a role for EB1 in regulating microtubule dynamics at the microtubule–kinetochore interface (Tirnauer *et al.*, 2002a). Fission yeast Mal3 was identified in a screen for components required for genome stability, and although loss of the protein is not lethal it leads to increased chromosome loss and altered microtubule dynamics (Beinhauer *et al.*, 1997). In addition, *mal3* mutant cells showed a significant increase in the number of cells with condensed chromosomes, indicating defects in early aspects of mitosis (Beinhauer *et al.*, 1997). To better understand the role of Mal3 in mitosis, we conducted a screen for extragenic suppressors of the *mal3* mutant phenotype. We identified a total of 10 suppressors that were able to rescue the chromosome loss phenotype of the *mal3* mutant strain. The most frequently isolated extragenic suppressor, the *spc7⁺* gene, codes for an essential kinetochore protein that seems to interact with Mal3 at the microtubule–kinetochore interface.

MATERIALS AND METHODS

Strains and Media

Genotypes of strains are listed in Table 1. For determination of genetic interaction at least three double mutants were tested per cross. *S. pombe* strains were grown in rich or minimal medium (YE5S, or EMM and MM) with appropriate supplements (Moreno *et al.*, 1991). G418 resistance was scored on 100 mg/l G418 (Calbiochem, San Diego, CA). EMM with 5 μg/ml thiamine repressed the *nmt1⁺* promoter. For high-level expression from the *nmt1⁺* promoter cells were grown in thiamine-less liquid EMM for 18–22 h at 30°C. Suppression of thiabendazole (TBZ) hypersensitivity was monitored on selective MM with 5–7.5 μg/ml TBZ. Suppression of minichromosome loss was assayed as described (Beinhauer *et al.*, 1997). *Saccharomyces cerevisiae* strains were grown in rich medium (YPD) or selective medium (SD) with appropriate supplements (Kaiser *et al.*, 1994). Suppression of TBZ hypersensitivity was tested on selective SD with 75 μg/ml TBZ or YPD with 50–100 μg/ml TBZ; resistance to G418 on YPD containing 200 mg/l G418. For repression of the *tetO-CYC1* promoter, cells were grown in nonselective SD containing 100 μg/ml doxycycline.

Identification of spc7⁺ and DNA Methods

Multicopy extragenic suppressors of the *mal3* mutant phenotypes were isolated by transformation of the *mal3-1* strain with a *S. pombe* genomic bank (Barbet *et al.*, 1992). Ura⁺ transformants were replica-plated twice onto MM plates containing 7.5 μg/ml TBZ. Plasmids were isolated from transformants able to grow on TBZ-containing media and tested for the ability to rescue the increased minichromosome loss phenotype of the *mal3-1* strain by visual screening for the suppression of colony sectoring (Niwa *et al.*, 1986; Beinhauer *et al.*, 1997).

The 2.7-kb-long *SPC105* open reading frame (ORF) was expressed from the modified *nmt1* promoter in *S. pombe* plasmid pJR2–41 × U or from the *MET25* promoter in the 2μ–containing *S. cerevisiae* plasmid pRS473MET25. Correct annotation of the SPCC1020.02 ORF was confirmed by amplification of this ORF via polymerase chain reaction (PCR) by using a *S. pombe* cDNA Bank (BD Biosciences Clontech, Palo Alto, CA).

A *spc7* null allele (Δ*spc7*) was generated by replacing the internal 3.06 kb of the 4.09 Kb *spc7⁺* ORF with the *his3⁺* marker in the diploid strain KG425 × KG554. Tetrad analysis of 28 heterozygous Δ*spc7*/*spc7⁺* diploids revealed that only the 2 his⁻ spores/tetrad grew.

Immunoprecipitation

Chromatin immunoprecipitation (ChIP) by using *S. pombe* strains was performed as described previously (Jin *et al.*, 2002). Strains were shifted to 18°C for 2 h before fixation with 3% paraformaldehyde. Strains containing green fluorescent protein (GFP)-tagged Spc7 or Mal2 were used for ChIP. Two microliters of rabbit anti-GFP antibody (Molecular Probes, Eugene, OR) was used in the ChIP, and 25 μl of protein A agarose (Roche Diagnostics, Mannheim, Germany). Multiplex PCR with *Taq*DNA polymerase (Roche Diagnostics) was used for analysis of centromeric chromatin in crude extracts and immunoprecipitates, by using the primer pairs for *cnt* (central core), *otr* (outer repeat), and *fbp1⁺* (euchromatic control) as described previously (Jin *et al.*, 2002) and the *imr* (inner most repeat) pair: 5′-GGATATATGTATTCTTG-CACTC-3′ and 5′-GGCTACCAGCAT TGTTATTCATAACC-3′. PCR reactions contained 1.25 mM $MgCl_2$, 0.25 mM dNTPs, 2 μl ChIP sample, or 2 μl of 1/10 dilution of crude sample, with 50 ng of each primer in a 25-μl reaction.

For coimmunoprecipitation, wild-type strains or a *cut9-665* strain incubated for 4 h at 36°C were washed once with STOP buffer (0.9% NaCl, 1 mM NaN_3, 10 mM EDTA, and 50 mM NaF). Approximately 1×10^9 cells were resuspended in 80 μl of HEPES-lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor; Roche Diagnostics) and lysed using glass beads. Then, 450 μl of HEPES buffer was added, and samples were centrifuged twice for 30 min in a microfuge at 4°C. A 150-μl amount of each sample was incubated on ice for 1 h with 50 μl of anti-hemagglutinin (HA) MicroBeads or anti-GFP MicroBeads (Miltenyi Biotec, Auburn, CA). Immunoprecipitates were isolated using the μMACS epitope-

tagged protein isolation kit according to the manufacturer's instructions (Miltenyi Biotec), except that the immune complexes were washed for maximally 5 min. After elution, the immune complexes were boiled, resolved on SDS-7% polyacrylamide gels, and blotted onto Immobilon-P (Millipore, Billerica, MA). Because Spc7-HA is a 158-kDa protein and Mal3-GFP a 62-kDa protein, blots were cut in half and the top half (>85-kDa proteins) was probed with anti-HA antibody (monoclonal mouse; Roche Diagnostics) and the bottom half with anti-GFP antibody (polyclonal rabbit; Molecular Probes). Immobilized antigens were detected using the ECL Advance Western blotting kit (Amersham Biosciences, Piscataway, NJ).

Affinity Purification and Matrix-assisted Laser Desorption Ionization/Time of Flight (MALDI-TOF) Analysis

We lysed 4000 OD of cells in 15 ml of lysis buffer (50 mM Tris, pH 8.0, 140 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 2 mM dithiothreitol, 10 mM NaF, 100 mM glycerophosphate, and protease inhibitors) with glass beads. The cleared lysate was incubated with 200 μl of IgG-agarose (Sigma-Aldrich, St. Louis, MO) for 4 h at 4°C. Subsequently, the agarose was washed with 20 ml of lysis buffer, 20 ml of wash buffer (10 mM Tris, pH 8.0, 250 mM LiCl, 5 mM $MgCl_2$, 0.5% sodium deoxycholate, and 0.5% NP 40) and 1 ml of 5 mM ammonium acetate (pH 5.0). The sample was eluted with 2 ml of 500 mM ammonium acetate (pH 3.4), dried, and subjected to PAGE plus MALDI-TOF analysis a described previously (Shevchenko *et al.*, 1996).

Microscopy

For *S. pombe* cells, photomicrographs were obtained using a Zeiss Axiovert200 fluorescence microscope coupled to a charge-coupled device camera (Hamamatsu Orca-ER) and Openlab imaging software (Improvision, Coventry, United Kingdom). Immunofluorescence microscopy was done as described previously (Hagan and Hyams, 1988; Bridge *et al.*, 1998). For tubulin staining, primary monoclonal anti-tubulin antibody TAT1 (Woods *et al.*, 1989) followed by fluorescein isothiocyanate (FITC) or Cy3-conjugated secondary sheep anti-mouse antibodies (Sigma-Aldrich) were used. Strains expressing HA- and GFP-fusion proteins were observed in fixed cells by indirect immunofluorescence with mouse anti-HA antibody (Covance, Princeton, NJ) followed by Cy3-conjugated sheep anti-mouse antibodies (Sigma) and rabbit anti-GFP (Molecular Probes) followed by FITC-conjugated goat anti-rabbit antibodies (Sigma-Aldrich). Processed cells were stained with 4,6-diamidino-2-phenylindole (DAPI) before mounting.

RESULTS

Identification of spc7$^+$ as a Suppressor of the mal3 Mutant Phenotype

S. pombe strains carrying a mutant *mal3* allele are hypersensitive to microtubule-destabilizing drugs such as TBZ and show a 400-fold increase in the loss of a nonessential minichromosome (Beinhauer *et al.*, 1997). Furthermore, a Δ*mal3* (mal3 deletion) cell population showed a fourfold increase in the number of undivided condensed chromosomes compared with a wild-type strain (Beinhauer *et al.*, 1997). In addition, the spindle checkpoint that monitors the correct alignment of chromosomes on the spindle seems to be activated in Δ*mal3* cells. Double-mutant strains of Δ*mal3* with a null allele of *mph1*$^+$, which is an evolutionarily conserved component of the spindle checkpoint pathway (He *et al.*, 1998), showed reduced growth compared with the single mutant strains (our unpublished data). To clarify the role of the Mal3 protein in mitosis, we conducted a search for multicopy suppressors of the *mal3* mutant mitotic phenotypes (see *Materials and Methods*). Apart from the wild-type *mal3*$^+$ ORF, we identified a total of 10 genes that were able to suppress the hypersensitivity to TBZ and the increased minichromosome loss of the *mal3-1* strain (Vietmeier-Decker and Fleig, unpublished data). The screen was saturating as most suppressors were isolated several times.

A previously uncharacterized ORF with the systematic name SPCC1020.02 (*S. pombe* genome project at the Sanger Institute) was isolated most frequently, namely, 26 times, in this screen. We named this ORF *spc7*$^+$ (*S. pombe c*entromere). According to the sequence annotation of the *S. pombe* genome, the *spc7*$^+$ ORF is 4095 base pairs in length and codes for a 153.5-kDa protein. We confirmed the annotation of this ORF by identification of a cDNA clone that contained the entire coding sequence (see *Materials and Methods*). The presence of extra *spc7*$^+$ rescued the TBZ hypersensitivity and the increased loss of a nonessential minichromosome of the *mal3-1* strain (Figure 1, A and B, respectively). Extra *spc7*$^+$ also rescued the TBZ hypersensitivity of Δ *mal3* and all other *mal3* mutant strains tested (Figure 1A; our unpublished data), implying that suppression by *spc7*$^+$ is not allele specific and can occur even in the absence of *mal3*$^+$. The original genomic clone that suppressed the *mal3* phenotypes only contained the last 2028 base pairs of the 4095-base pair-long *spc7*$^+$ ORF. This fragment rescued as well as full length *spc7*$^+$ (our unpublished data). The first in frame ATG was predicted to give rise to a Spc7 variant without the N-terminal 820 amino acids. To test whether this prediction was correct, the C-terminal 1635-base pair region of the *spc7*$^+$ ORF was expressed from the modified *nmt1* promoter in *S. pombe* plasmid pJR2–41 × U (Moreno *et al.*, 2000). This Spc7 variant (Spc7-C) was able to fully suppress the *mal3* phenotypes (our unpublished data).

We had tested for genetic interaction between *mal3*$^+$ and other genes encoding microtubule plus-end–associated proteins such as *dis1*$^+$ and *alp14*$^+$/*mtc1*$^+$, which encode members of the TOG/XMAP215 family (Ohkura *et al.*, 1988; Nabeshima *et al.*, 1995; Garcia *et al.*, 2001; Nakaseko *et al.*, 2001). No genetic interaction was observed between Δ*mal3* and the mutant *dis1-288* allele. However, Δ*alp14* Δ*mal3* double mutants were synthetically lethal at all temperature tested, thus indicating that the two proteins have a role in the same essential process. We therefore assayed whether extra *spc7*$^+$ could also rescue the mutant phenotypes of an Δ*alp14* strain (Garcia *et al.*, 2001) but found that *spc7*$^+$ was unable to rescue the temperature sensitivity or the TBZ hypersensitivity of the Δ*alp14* strain (our unpublished data).

Identification of spc7$^+$ *as a Suppressor of SpRan Mutant Phenotype*

We have previously reported the characterization of a partial loss of function mutant of the *S. pombe* Ran GTPase Spi1 that led to aberrant mitosis and severe genome instability (Fleig *et al.*, 2000). Strains carrying this specific mutation, named *spi1-25*, were hypersensitive to TBZ. This mutant phenotype was suppressed partially by the presence of extraMal3 (Fleig *et al.*, 2000). A multicopy suppressor analysis identified several other genes that could rescue the *spi1-25* TBZ hypersensitivity (Karig and Fleig, unpublished data). Among them was the *spc7*$^+$ ORF. As shown in Figure 1C, plasmid-borne expression of *spc7*$^+$ partially suppressed the TBZ hypersensitivity of the *spi1-25* strain. In addition, *spc7*$^+$ also rescued partially the temperature sensitivity of the *spi1-25* strain (our unpublished data).

Spc7 Is an Essential Component of the Fission Yeast Kinetochore

To determine whether *spc7*$^+$ was essential for vegetative growth, one copy of the *spc7*$^+$ ORF was replaced with the *his3*$^+$ marker in a diploid strain (see *Materials and Methods*). Sporulation and subsequent tetrad analysis of this strain revealed that only two of the four spores in a tetrad could grow into a colony, and these were always his$^-$, indicating that *spc7*$^+$ is an essential gene. To determine the subcellular localization of Spc7, a fluorescence-improved version of GFP was fused to the COOH-terminal end of the endogenous *spc7*$^+$ coding region. The strain expressing the Spc7 fusion protein was indistinguishable in phenotype from the isogenic wild-type strain, indicating that the tagged gene was

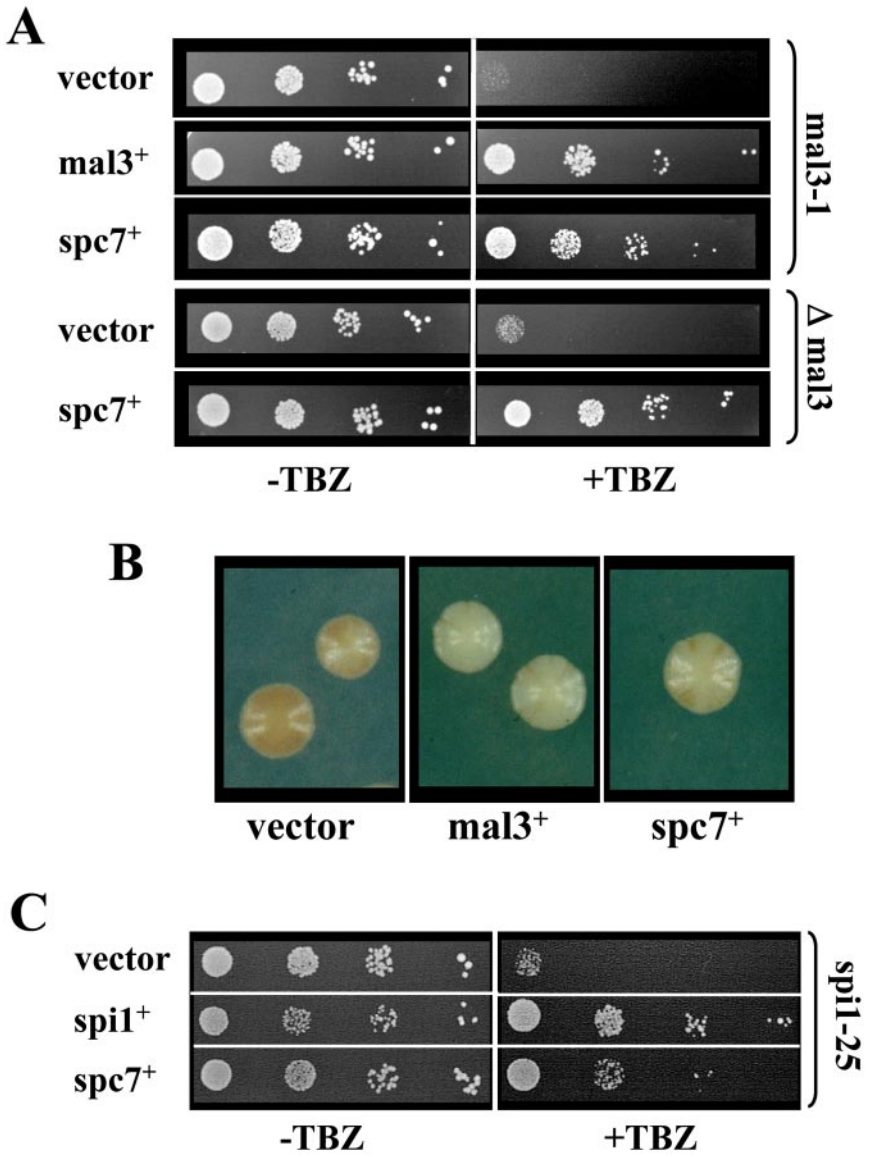


Figure 1. *spc7*$^+$ suppresses the phenotypes of *mal3* and *spi1-25* mutant strains. (A) TBZ hypersensitivity of the *mal3-1* and Δ*mal3* strains is rescued by extra *spc7*$^+$. Left and right, serial dilution patch tests (10^4 to 10^1 cells) of *mal3-1* and Δ*mal3* transformants grown under selective conditions in the absence (−TBZ) or presence (+TBZ) of 7 μg/ml thiabendazole for 5 d at 24°C. Vector control indicates plasmid without insert; mal3$^+$ denotes the presence of wild-type *mal3*$^+$ on a plasmid. (B) Minichromosome loss phenotype of the *mal3-1* strain, indicated by adenine auxotrophy and numerous red sectors in a white colony (vector), is rescued by the presence of *mal3*$^+$ or *spc7*$^+$ on a plasmid. Plates were incubated at 24°C. (C) TBZ hypersensitivity of the *spi1-25* strain is rescued by extra *spc7*$^+$. Panels show serial dilution patch tests of *spi1-25* transformants grown under selective conditions in the absence (−TBZ) or presence (+TBZ) of 7 μg/ml TBZ for 5 d at 24°C. Vector control indicates plasmid without insert, whereas spi1$^+$ denotes the presence of wild-type *spi1*$^+$ on a plasmid.

functional. Immunofluorescence analysis of interphase cells expressing Spc7-GFP revealed a single fluorescent dot near the nuclear periphery, whereas early mitotic cells showed up to six dots associated with the condensed chromatin (Figure 2A, a and b, respectively). Anaphase cells showed two Spc7-GFP dots that cosegregated with the separated chromatin (Figure 2A, c). Cells arrested in mitosis by overexpression of the spindle checkpoint component Mph1 (He *et al.*, 1998) showed up to six fluorescent signals: two signals colocalized with each of the three duplicated chromosomes positioned on a short spindle (Figure 2B). Because this type of localization is characteristic of *S. pombe* kinetochore proteins, we compared the intracellular localization pattern of the Spc7 fusion protein with that of the known kinetochore protein Mal2 (Jin *et al.*, 2002). For this purpose, a strain was used in which the endogenous *spc7*$^+$ ORF had been tagged with the HA epitope and the endogenous *mal2*$^+$ ORF fused to the *GFP* ORF. Colocalization of the Spc7 and Mal2 fusion proteins was observed in interphase and mitotic cells (Figure 2C, e–f) in all cells analyzed (n = 150). Interestingly, in interphase cells 71% of cells had the Mal2 signal closer to the nuclear periphery than the Spc7 signal (n = 76) (Figure 2C, e). Together, these data imply that the Spc7 protein is a component of the fission yeast kinetochore that localizes at centromeres throughout the cell cycle.

We next determined by immunofluorescence analysis whether the subcellular localization of Spc7-GFP was affected in the temperature-sensitive *spi1-25* and *mal2-1* strains, the cold-sensitive *nda3-KM311* (β-tubulin) strain, and the Δ*mal3* strain (Umesono *et al.*, 1983; Fleig *et al.*, 1996, 2000; Beinhauer *et al.*, 1997). For this purpose, the endogenous *spc7*$^+$*-GFP* ORF was crossed into these strains. At the restrictive temperature, Spc7-GFP was localized correctly in the Ran mutant strain *spi1-25* and the *mal2-1* strain, which encodes a defective kinetochore component (Jin *et al.*, 2002). Furthermore, kinetochore localization of Spc7 was not affected in the *nda3-KM311* mutant strain at the nonpermissive temperature, implying that an intact microtubule-cytoskeleton is not required for Spc7 localization (our unpublished data).

As described previously, the Δ*mal3* population showed a fourfold increase in the number of cells with condensed chromosomes, indicating an early mitotic defect. Immuno-

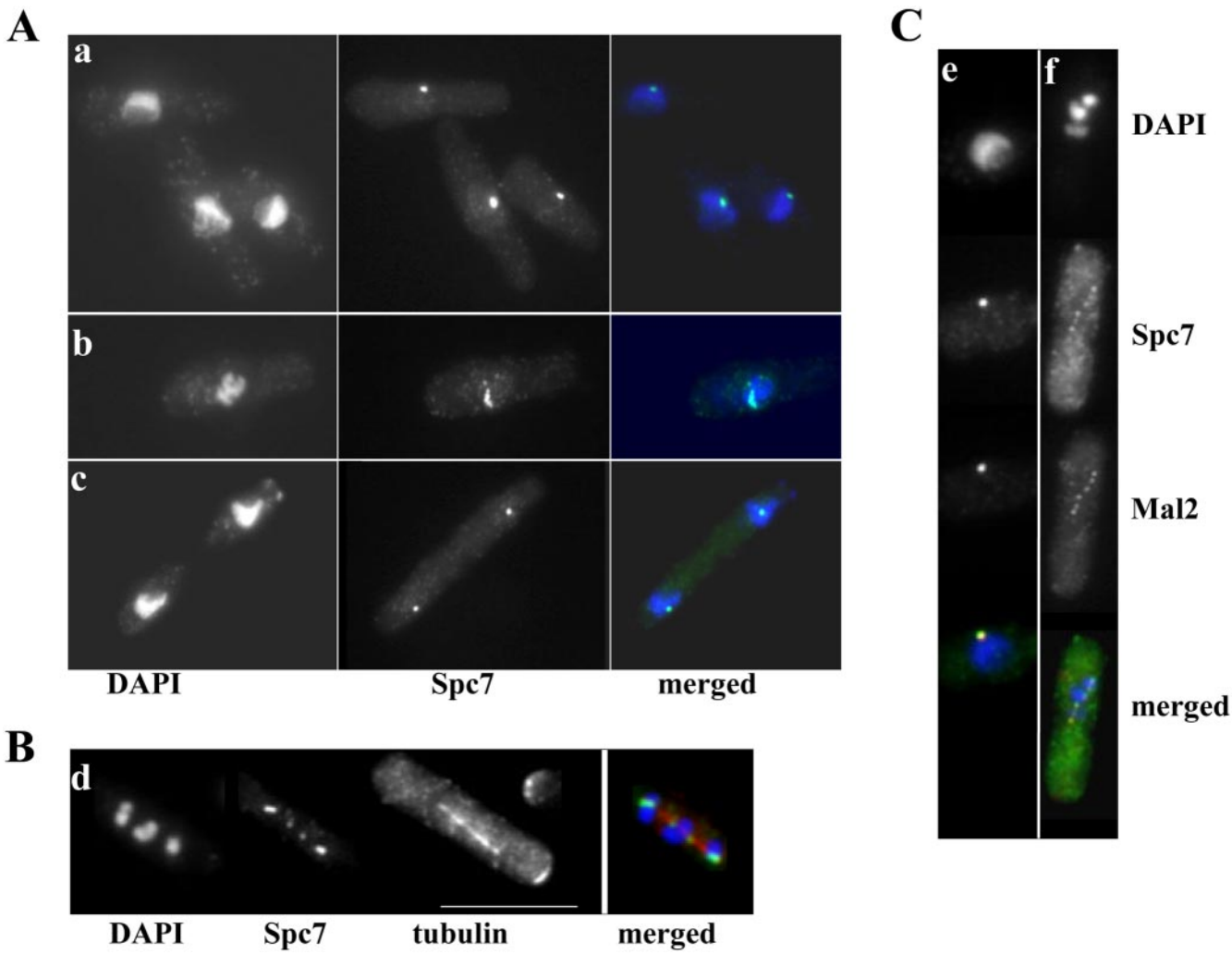
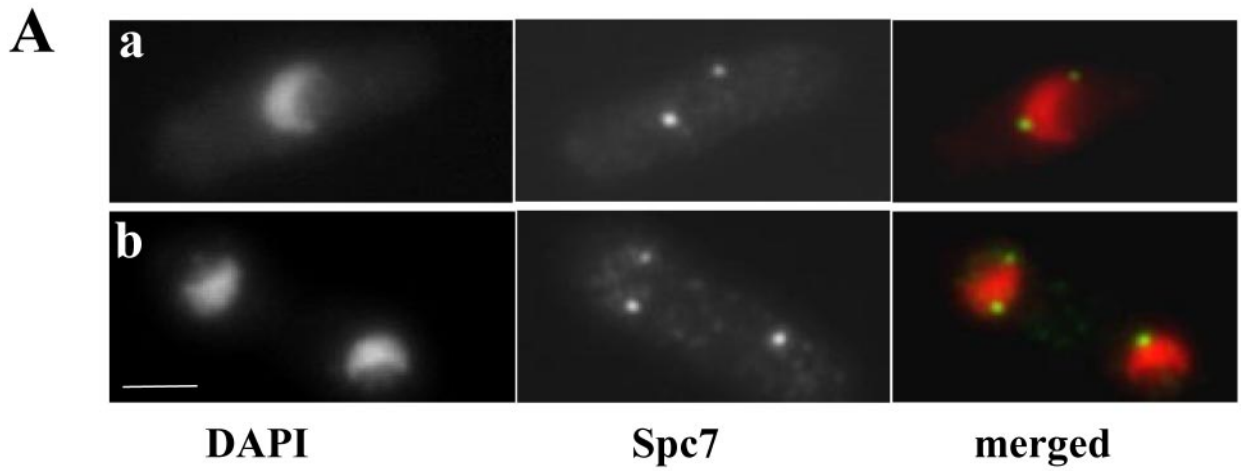


Figure 2. Spc7 localizes to the kinetochore. (A) Localization of the Spc7-GFP protein in wild-type cells in interphase (a) early (b) or late (c) stages of mitosis. Fixed cells were stained with DAPI and anti-GFP antibody. (B) Localization of the Spc7 fusion in a wild-type cell arrested by overexpression of *mph1*$^+$. GFP signals; spindles and condensed chromosomes were simultaneously observed by staining with anti-GFP antibody, anti-tubulin antibody and DAPI. (C) Colocalization of Spc7-HA (green) and Mal2-GFP (red) fusion proteins in interphase cells (e) and cells arrested by overexpression of *mph1*$^+$ (f). Chromosomes, HA- and GFP-signals were simultaneously observed by staining with anti-GFP antibody, anti-HA antibody, and DAPI. Bar, 10 μm.

fluorescence analysis of these fixed cells revealed that the majority (>90%, n = 76) showed six fluorescent Spc7-GFP dots on a metaphase spindle (3 × 2 sister centromeres; our unpublished data). No severe anaphase defects were observed. However clustering of the Spc7 protein at the spindle pole body (SPB) was affected severely in postmitotic Δ*mal3* cells. In wild-type cells, centromeres and the associated kinetochore proteins cluster adjacent to the SPB in interphase cells, and this association with each other and the SPB is only disrupted in M phase (Funabiki *et al.*, 1993; Ekwall *et al.*, 1995; Saitoh *et al.*, 1997). Δ*mal3* G2 interphase cells (7.5%) showed instead of the expected single Spc7-GFP signal, two dots associated with the nuclear periphery (Figure 3A, a). The brighter one of these dots colocalized with the SPB by using the Cut12 SPB marker (our unpublished data; Bridge *et al.*, 1998). Interestingly, >35% of Δ*mal3* postanaphase cells showed two or rarely three Spc7-GFP signals per nucleus (Figure 3A, b and B). These data demonstrate that the Mal3 protein is required for SPB clustering of Spc7, especially in postanaphase cells. However Mal3 is not per se required for clustering of centromeres/kinetochores at the SPB. Microscopic examination of Δ*mal3* cells expressing the GFP-tagged Mal2 kinetochore protein always revealed a single fluorescent dot in G2 cells and two Mal2-GFP dots that colocalized with the separated chromatin in postanaphase cells (our unpublished data; Jin *et al.*, 2002). Because Spc7 clustering does not require an intact microtubule cytoskeleton, it is possible that SPB-associated Mal3 is required for Spc7 clustering. A *Dictyostelium* member of the EB1 family has been shown to be a genuine component of the centrosome (Rehberg and Graf, 2002).

Spc7 Is Associated with the Central Core Region of the Fission Yeast Centromere

The centromeric DNA of *S. pombe* is 40–100 kb in size and consists of a central core (*cnt*) flanked by arrays of inner (*imr*/B) and outer repeats (*otr*/K+L) (Figure 4 shows the centromeric DNA of chromosome I) (reviewed in Clarke, 1998). To identify the centromere region with which the Spc7 protein associates, ChIP was carried out (Partridge *et al.*,



B

defective Spc7 clustering	wild type	Δmal3
interphase	0	7.5
post-anaphase	0	35.8

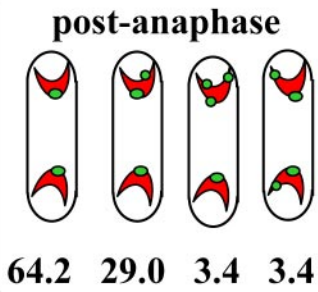


Figure 3. Clustering of Spc7 is defective in a Δ*mal3* strain. (A) Localization of the Spc7-GFP fusion protein in Δ*mal3* cells in G2 (a) and postanaphase (b) cells. Fixed cells pregrown at 24°C were stained with DAPI and anti-GFP antibody. Bar, 5 μm. (B) Shown are the percentages of interphase cells (G2) (n = 120) and postanaphase cells (n = 174) that have more than one Spc7 signal per nucleus. The right-most panel shows the various distributions observed for the Spc7 signal in Δ*mal3* postanaphase cells. Red, chromatin; green, Spc7-GFP signal.

2000). Cells expressing either Mal2-GFP or Spc7-GFP fusion proteins were analyzed in ChIP assays by using anti-GFP antibodies. DNA present in crude extracts or immunoprecipitates by using anti-GFP antibodies were analyzed by multiplex PCR, by using primers to amplify the *cnt*, *imr*, and *otr* regions in the centromere of chromosome I and an unrelated euchromatic control region *fbp*. As previously reported, the Mal2 protein associates with the central core

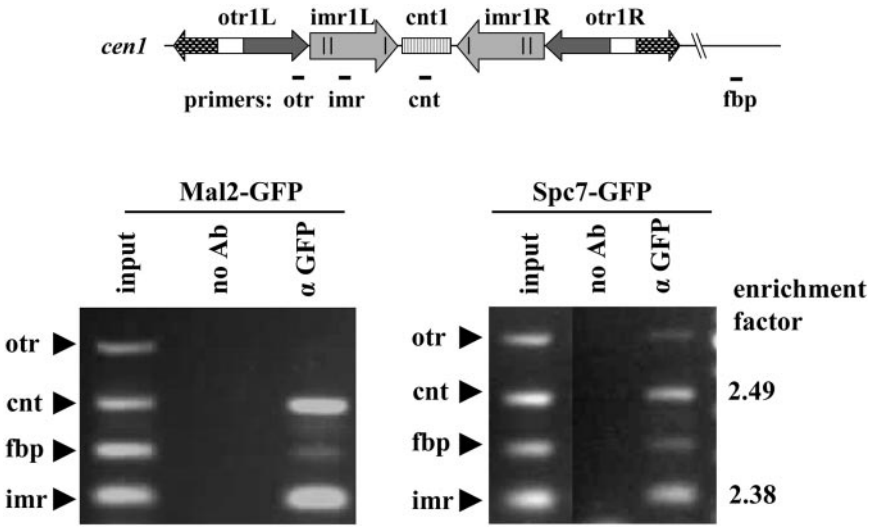


Figure 4. Spc7 is associated with the central domain of *cen1*. Cells expressing Spc7-GFP or Mal2-GFP were fixed and processed for ChIP by using anti-GFP antibodies. Chromatin in immunoprecipitates and crude extracts was analyzed by multiplex PCR, by using primers to amplify regions in *cen1*: *cnt*, *imr*, *otr*, and an euchromatic negative control locus, *fbp*. *cnt* and *imr* sequences are specifically enriched in ChIPs of proteins associated with the central domain such as the Mal2 fusion protein. The *cnt* and *imr* sequences are also enriched in Spc7-GFP ChIPs, indicating association of the Spc7 fusion protein with the central centromere domain.

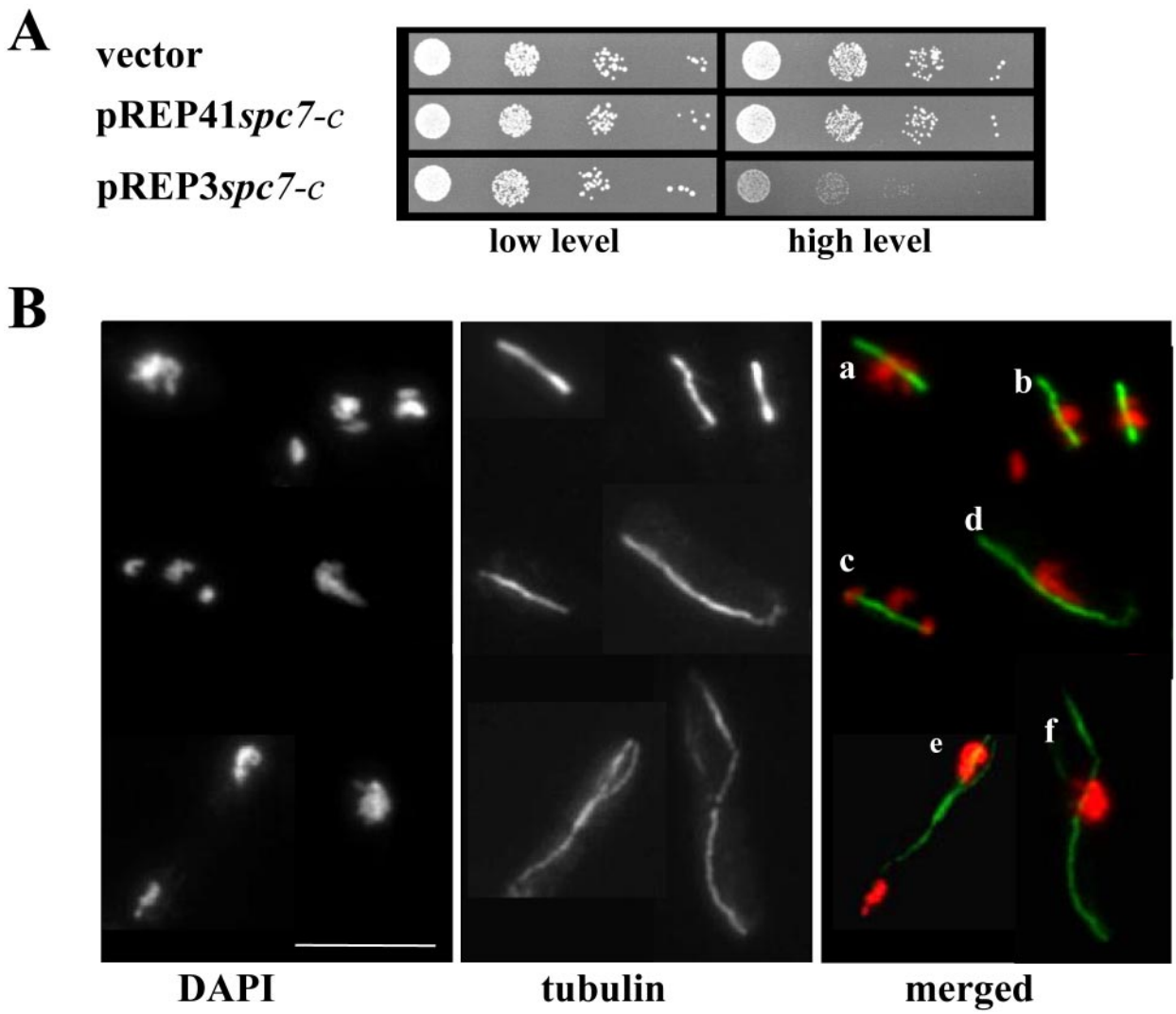


Figure 5. Overexpression of a Spc7 variant in a wild-type strain leads to growth inhibition and severe mitotic defects. (A) Spc7-C was expressed from two different versions of the regulatable *nmt1*$^+$ promoter. pREP3 gives rise to strong and pREP41 to moderate overexpression. Shown are serial dilution patch tests (10^4 to 10^1 cells) of transformants grown under selective conditions for 4 d at 30°C. (B) Photomicrographs of wild-type cells overexpressing *spc7-c* from pREP3. Fixed cells were stained with anti-tubulin antibody and DAPI. Shown is a composite of cells displaying various mitotic defects: nonseparated chromatin (a, d, and f) or unequally/partially divided chromatin on elongating spindles (b, c, and e). Bar, 10 μm.

region as shown by a specific enrichment of the *cnt* and *imr* sequences in the Mal2-GFP ChIP (Jin *et al.*, 2002) (Figure 4). The Spc7 ChIPs also showed an enrichment of the *cnt* and *imr* sequences. These DNA sequences were enriched 2.5- and 2.4-fold, respectively, relative to the *fbp* DNA in Spc7 ChIPs in comparison with the input control. The *otr* sequence was not enriched in Spc7 ChIPs. These data indicate an association of the Spc7 fusion protein with the central domain of the centromere and imply an involvement of this domain in kinetochore–microtubule attachment. Interestingly, the enrichment of the *cnt* and *imr* sequences in Spc7 ChIPs was repeatedly and reproducibly approximately two-fold lower compared with Mal2 ChIPs (Figure 4; our unpublished data).

Spc7 Affects Kinetochore–Microtubule Interactions

We had found that wild-type cells expressing full-length Spc7 from the modified *nmt1* promoter (pREP41; moderate overexpression) had no visible effect on the growth of the cells, nor did overproduction from the wild-type *nmt1*$^+$ promoter (pREP3; strong overexpression) (our unpublished data). Moderate overproduction of the C-terminal part of Spc7 (Spc7-C), which is sufficient to suppress the *mal3* mutant phenotypes, also showed no effect on cell growth (Figure 5A, middle). However, strong overproduction of this Spc7 variant, which is still able to associate with the kinetochore (our unpublished data), led to severe growth inhibition (Figure 5A, bottom). The growth inhibition was not due to a simple displacement of the full-length Spc7 protein by Spc7-C at the kinetochore. The Spc7-GFP fusion protein was properly localized in wild-type cells overexpressing *spc7-c* (our unpublished data). To assay the consequences of Spc7-C overproduction, we looked at immunofluorescence staining of a wild-type strain overexpressing *spc7-c* for 18–22 h. Although interphase cells showed no obvious abnormalities, cells undergoing mitosis were severely affected.

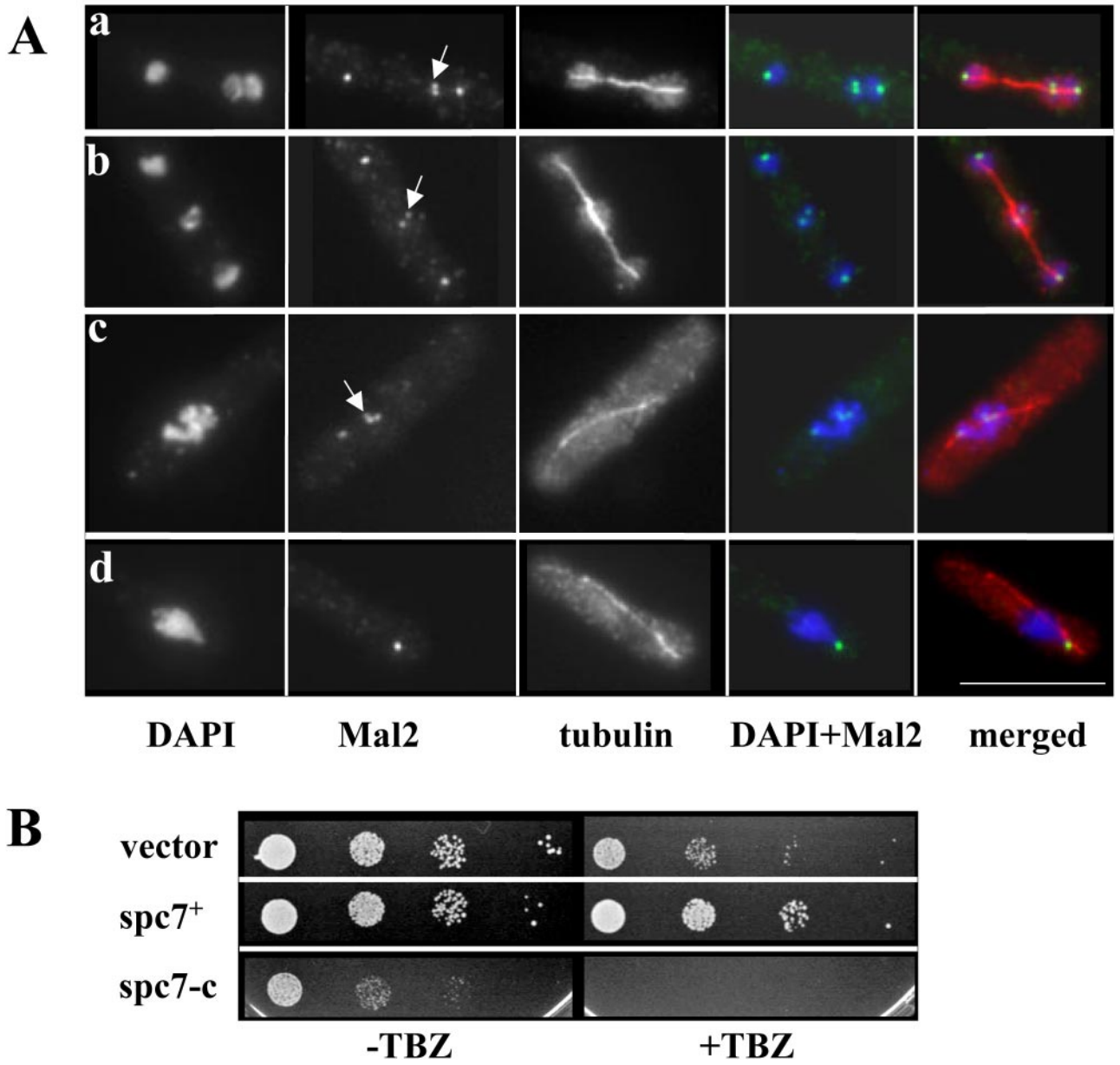


Figure 6. Association between spindle and kinetochore is altered by Spc7-C overproduction. (A) Photomicrographs of *mal2*$^{+}$*-GFP* cells overexpressing *spc7-c*. Fixed cells were stained with DAPI, anti-GFP and anti-tubulin antibody. Shown are elongating spindles without or with unequal segregation of the chromatin. Merged images show chromatin, Mal2-GFP, and the spindle. The arrows indicate kinetochores not attached to the spindle. Bar, 10 μm. (B) Overexpression of Spc7 or Spc7-C from pREP3 alters sensitivity to microtubule-destabilizing drug TBZ. Left and right, serial dilution patch tests (10^4 to 10^1 cells) of wild-type transformants expressing high levels of *spc7*$^{+}$ or *spc7-c* in the absence or presence of TBZ. Vector control indicates plasmid without insert.

After 18 h of induction, 24% of the cells in a population were in mitosis, and this number increased to 40% at later time points (22 h). Sixty-seven percent of mitotic cells showed severe chromosome segregation defects and cells with a "cut" phenotype became more frequent (Figure 5B; our unpublished data). Two predominant defective chromosome resolution phenotypes were observed: 1) no separation of highly condensed chromatin on an elongating spindle (Figure 5B, cells a, d, and f) and 2) unequal segregation of the chromatin (Figure 5B, cells b, c, and e). In the first phenotypic class, chromosomes were not separated on an elongating spindle. Using the Mal2 protein as a kinetochore marker we found 1) that association of Mal2 with the kinetochore was unaffected by Spc7-C overexpression and 2) that not all kinetochores in these cells were associated with the mitotic spindle (Figure 6c, nonattached kinetochore marked by an arrow). In cells where the chromatin had an arrow like appearance, kinetochores were clustered at the "tip" of the arrow (Figure 6d). The second phenotypic class consisted of chromatin that was segregated asymmetrically. Part of the chromosomes were found at one end of a short anaphase spindle (Figure 5B, cell b) or only part of the chromosomal material had been segregated to the two ends of the spindle, whereas the rest remained unseparated at the equatorial

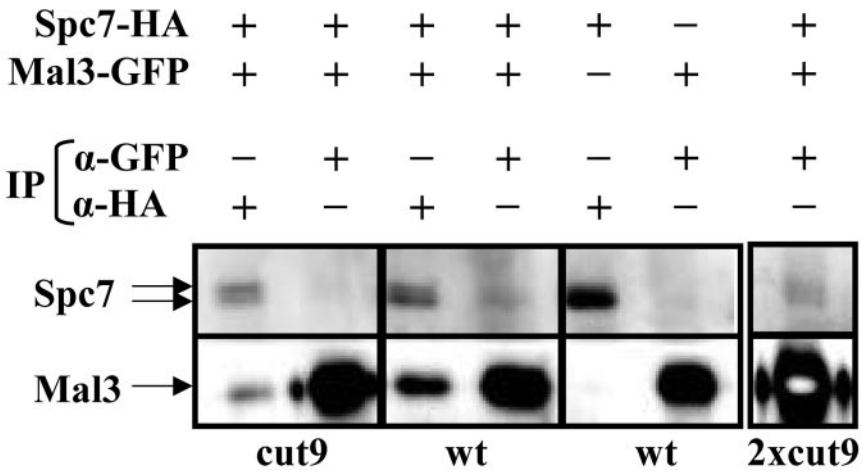


Figure 7. Spc7 and Mal3 coimmunoprecipitate. Protein extracts were prepared from wild-type (wt) and *cut9-665* (cut9) strains expressing Spc7-HA, Mal3-GFP, or both. Lysates were halved: one half was used for immunoprecipitation (IP) with an anti-HA antibody, and the other with an anti-GFP antibody. The immunoprecipitates were resolved by SDS-PAGE, blotted to Immobilon-P, and cut in half at the 85-kDa size marker. The top half was probed with anti-HA antibody, and the bottom half with anti-GFP antibody. Immobilized antigens were detected using the ECL kit. Positions of Spc7 and Mal3 are indicated by arrows. The right most lane (2 × cut9) is identical to lane 2 except that twice the amount of immunoprecipitate was used. This illustrates more clearly the coimmunoprecipitation of Spc7-HA by Mal3-GFP.

plate (Figure 5B, cell c). Rarely, we also observed fully elongated spindles showing asymmetric segregation of the chromosomes (Figure 5B, cell e). Cells belonging to the second phenotypic class often contained kinetochores that did not seem to be attached to spindle microtubules (Figure 6, a and b). We repeated the above-mentioned experiment using a second kinetochore marker, namely, Spc24, and obtained similar results (our unpublished data; Wigge and Kilmartin, 2001).

Interestingly, overexpression of full-length Spc7 or Spc7-C from the wild-type *nmt1*$^+$ promoter in a wild-type strain led to differences in the sensitivity to TBZ. As shown in Figure 6B, overproduction of full-length Spc7 leads to increased resistance to the microtubule-destabilizing drug TBZ, whereas overexpression of the Spc7 variant Spc7-C gave rise to the opposite effect.

Spc7 Coimmunoprecipitates with Mal3

Given the finding that extra *spc7*$^+$ could suppress the mitotic phenotypes of *mal3* mutant strains, we investigated whether the Mal3 and Spc7 proteins interacted. For this purpose, we tested whether Spc7 coimmunoprecipitated Mal3 and vice versa. Immunoprecipitation with anti-GFP or anti-HA antibodies was carried out with protein extracts from strains expressing endogenous Mal3-GFP and/or Spc7-HA. These immunoprecipitates were then analyzed by Western blotting by using anti-GFP and anti-HA antibodies. As shown in Figure 7 immunoprecipitation of Spc7-HA clearly coimmunoprecipitates Mal3-GFP in wild-type cell extracts. We also could coimmunoprecipitate Mal3-GFP with anti-HA antibodies from mitotically arrested *cut9-665* extracts (Figure 7). *cut9*$^+$ codes for a subunit of the anaphase promoting factor (Yamada *et al.*, 1997). Furthermore, immunoprecipitation of Mal3-GFP also coimmunoprecipitated Spc7-HA although the amount of Spc7-HA that was coimmunoprecipitated was low (Figure 7). Western blot analysis showed the existence of two Spc7-HA specific bands. Our preliminary analysis indicates that the slower migrating form is phosphorylated and the predominant form in *cut9-665*–arrested cells (our unpublished data; Figure 7). Both forms could be coimmunoprecipitated by Mal3. Our data thus imply that the Mal3 and Spc7 proteins interact with each other. Interestingly, we were unable to coimmunoprecipitate Spc7 and Mal3 upon prolonged washing steps, indicating that the interaction between Mal3 and Spc7 is not a stable one.

Spc7 Belongs to a Conserved Protein Family That Copurifies with the Conserved Ndc80 Complex

Database searches using PEDANT at MIPS identified nine potential homologues from other fungal organisms. Although the overall sequence identity between these proteins is only ~22%, the amino acid comparison showed a number of conserved sequence motifs of yet unknown function (our unpublished data). Among these homologues is the 105-kDa *S. cerevisiae* Spc105 protein, which shares a number of sequence motifs with the C-terminal part of the 153-kDa Spc7 protein (our unpublished data; Wigge *et al.*, 1998; Nekrasov *et al.*, 2003). To determine whether members of this family have a similar function, we tested the ability of the *S. cerevisiae* Spc105 protein to suppress phenotypes of *mal3* mutant strains. Spc105 is part of the *S. cerevisiae* kinetochore as shown by ChIP and immunofluorescence analysis of a Spc105 fusion protein (our unpublished data; Nekrasov *et al.*, 2003). We identified the Spc105 protein as a component that copurified with the centromere associated, highly conserved Ndc80 complex. Spc24, a known Ndc80 complex component was protein A-tagged and the fusion protein isolated from cell extracts by using affinity chromatography with IgG-Sepharose. The proteins that copurified with protein A-tagged Spc24 were identified by peptide mass fingerprints (MALDI-TOF mass spectrometry). In addition to the previously identified components of the Ndc80 complex, namely, Ndc80, Nuf2, Spc24, and Spc25 (Janke *et al.*, 2001; Wigge and Kilmartin, 2001), we also identified Spc105 in this analysis (Figure 8A). We thus conclude that Spc105 is associated closely with the evolutionarily conserved Ndc80 complex. Next, we protein A-tagged Spc105 and identified the Ydr532c protein as a copurification partner (our unpublished data). The 44-kDa Ydr532c protein has been identified as a component of the SPB and very recently as a new component of the budding yeast kinetochore (Giaever *et al.*, 2002; Huh *et al.*, 2003; Nekrasov *et al.*, 2003).

To test whether Spc105 and Spc7 might have a similar function, we tested whether Spc105 could suppress the TBZ hypersensitivity of *mal3* mutant strains. As shown in Figure 8B, *SPC105* expressed from the regulatable *S. pombe nmt1*$^+$ promoter was able to partially suppress the TBZ hypersensitivity of the *S. pombe mal3-1* and Δ*mal3* strains. In addition, *SPC105* expressed from the *MET25* promoter on a plasmid was able to partially suppress the TBZ hypersensitivity of the *S. cerevisiae* Δ*bim1* strain (Fig-

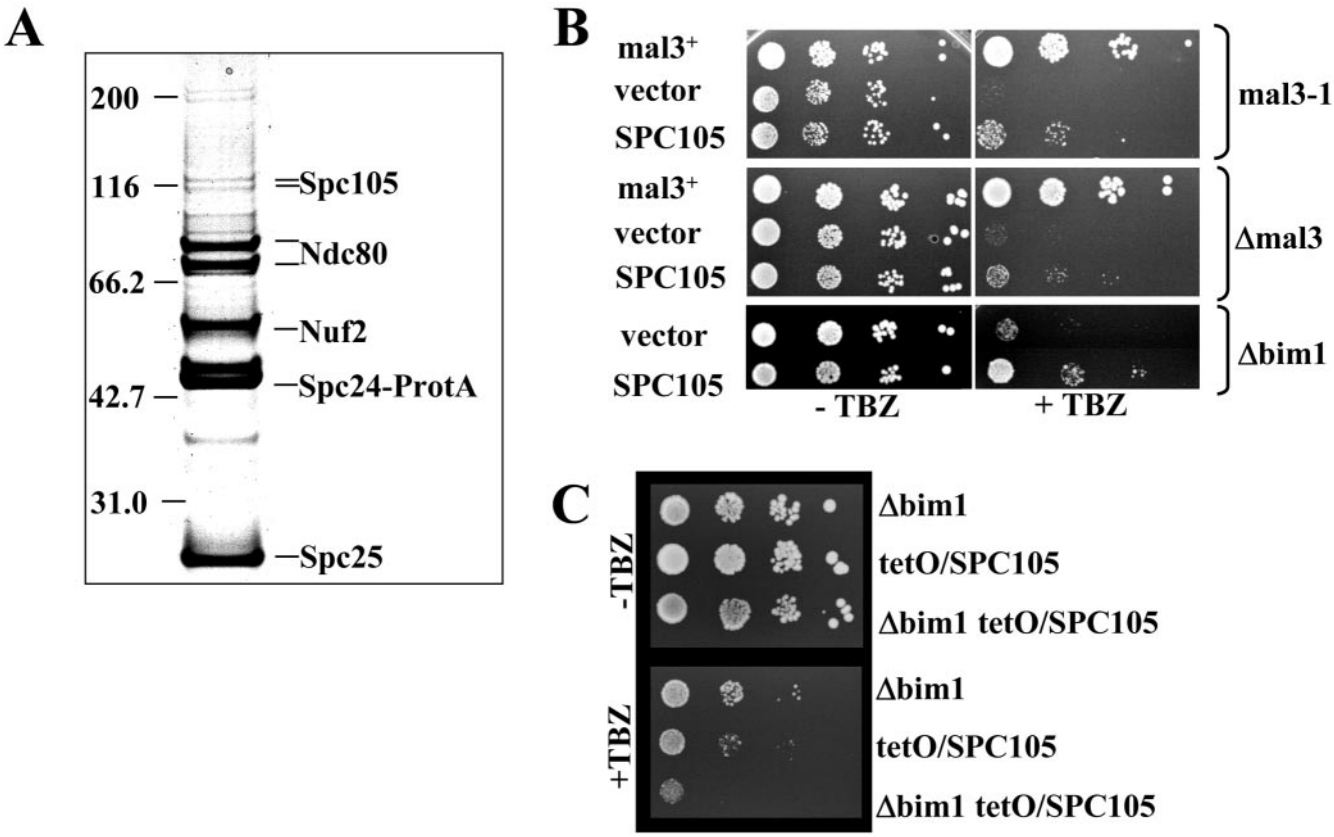


Figure 8. *S. cerevisiae* Spc105, a component of the conserved Ndc80 kinetochore complex, rescues *mal3* mutants. (A) Spc105 copurifies with the Ndc80 complex. Protein A-tagged Spc24 was purified from 4000 OD_{578} of cells by affinity chromatography with IgG-agarose, and the complete preparation was subjected to SDS-PAGE. Copurifying proteins were identified from Coomassie-stained bands by peptide mass finger printing (MALDI-TOF). Bands that are not labeled represent contaminants. (B) TBZ hypersensitivity of the *S. pombe mal3-1* and Δ*mal3* strains and the *S. cerevisiae* Δ*bim1* strain is partially rescued by overexpression of *SPC105*. Panels show serial dilution patch tests (10^4 to 10^1 cells) of *mal3-1*, Δ *mal3*, and Δ *bim1* transformants grown under selective conditions in the absence (−TBZ) or presence (+TBZ) of TBZ. Plates were incubated for 5 d at 24°C (*mal3* strains) or 3 d at 30°C (Δ*bim1* strain). Vector control indicates plasmid without insert. (C) Serial dilution patch tests of Δ*bim1*, *tetO-CYC1/SPC105* single and double mutant strains on YPD with doxycyline with or without 50 μg/ml TBZ. Strains were grown at 30°C for 48 h.

ure 8B). Bim1 is the *S. cerevisiae* member of the EB1 family (Schwartz *et al.*, 1997).

We next tested whether *SPC105* and *BIM1* interacted genetically. For this purpose, we used a strain where *SPC105* was expressed from an ectopic promoter. The endogenous *SPC105* promoter was replaced by the doxycycline regulatable *tetO-CYC1* promoter in a strain with an integrated *tetR*-VP16(tTA) activator allowing regulated expression from the *tetO-CYC1* promoter (Gari *et al.*, 1997). Cells carrying this construct are viable but show a strong increase in the loss of a GFP-marked chromosome fragment and increased sensitivity to TBZ (our unpublished data; Figure 8C). The TBZ-hypersensitivity of the *tetO-CYC/SPC105* strain was increased by the absence of *bim1*$^+$ as a *tetO-CYC/SPC105* Δ *bim1* double mutant strain was more sensitive to TBZ than the single mutant strains (Figure 8C). Furthermore, the double mutant strain showed reduced growth at 36 and at 18°C compared with the single mutant strains (our unpublished data), thus implying that *BIM1* and *SPC105* show genetic interaction.

DISCUSSION

We investigated the role of *S. pombe* EB1 family member in mitosis by screening for suppressors that were able to rescue the *mal3* mutant phenotypes, namely, TBZ hypersensitivity and increased chromosome loss. The most frequently isolated suppressor, *spc7*$^+$, codes for an essential component of the kinetochore, thus demonstrating that Mal3 plays a role at the spindle–kinetochore interface. Members of the EB1 family have been shown to be associated with kinetochores (Juwana *et al.*, 1999; Fodde *et al.*, 2001; Kaplan *et al.*, 2001; Rehberg and Graf, 2002; Tirnauer *et al.*, 2002a), but the interaction partner at the kinetochore has remained unclear. The finding that the *S. cerevisiae* Spc105 protein seems to be a homolog of Spc7 implies that this interaction at the spindle–kinetochore interface has been conserved.

The Spc7 protein is an essential component of the *S. pombe* kinetochore that associates specifically with the central centromere region, implying that this region is required for kinetochore–microtubule association. Although phenotypic analysis of various *S. pombe* kinetochore mutants had suggested that this region is needed for kinetochore–microtubule association (Saitoh *et al.*, 1997; Goshima *et al.*, 1999; Jin *et al.*, 2002; Pidoux *et al.*, 2003), Spc7 is the first central centromere region protein for which a direct involvement at the kinetochore–microtubule interface has been demonstrated. In support of this finding, Dis1, a member of the TOG/XMAP215 family of microtubule-associated proteins, is

associated with this region in mitosis, although the other XMAP215 homologue in *S. pombe*, Alp14/Mtc1, shows a somewhat different localization (Garcia *et al.*, 2001; Nakaseko *et al.*, 2001). Association of Mal3 with a specific centromere region needs yet to be demonstrated. Our attempts to map the centromere region with which Mal3 interacts by using ChIP followed by multiplex PCR were not successful (our unpublished data). However, given the finding that Spc7 and Mal3 could be coimmunoprecipitated, it is feasible that Mal3 is associated with the central centromere region.

Enrichment of the central region DNA sequences in Spc7 ChIPs was repeatedly twofold lower than that of another central region-associated protein, namely, Mal2. This might imply that in an assembled kinetochore Spc7 is physically further away from the centromeric DNA than Mal2. Ultrastructural analysis has shown that *S. pombe* centromeres are multilayered structures (Kniola *et al.*, 2001). In particular, the Ndc80 complex, with which Spc7 is probably associated, was shown to be part of an "anchor structure" close to the SPB and distinct from the localization of the *S. pombe* CENP-A homolog Cnp1 (Kniola *et al.*, 2001). Consistent with this observation, we found that in the majority of interphase cells the Spc7 signal was further away from the nuclear periphery than the Mal2 signal.

Our experimental data indicate that the role of the Spc7 protein at the kinetochore is that of linking the kinetochore to microtubule-plus ends and possibly influencing the dynamics of kinetochore microtubules. First, $spc7^+$ rescued the increased chromosome loss phenotype of a *mal3* mutant strain and can be coimmunoprecipitated with the microtubule plus-end protein Mal3. Second, overexpression of an Spc7 variant (Spc7-C) led to a dominant negative phenotype and kinetochores that were not associated with the mitotic spindle, implying that Spc7 affects kinetochore–microtubule interactions. Third, preliminary evidence indicates that Spc7 might have a role in microtubule dynamics. Extra $spc7^+$ is able to rescue the TBZ-hypersensitivity of specific strains such as *mal3* mutants and the Ran mutant *spi1-25*. Overexpression of full-length Spc7 increases the resistance of cells to the microtubule destabilizing drug TBZ, whereas overproduction of Spc7-C has the opposite effect. Fourth, the *S. cerevisiae* kinetochore protein Spc105 can partially suppress the phenotypes of *S. pombe mal3* mutant strains, suggesting that Spc7 and Spc105 might be functionally homologous. We identified Spc105, as a copurification partner of the Ndc80 complex. The highly conserved Ndc80/HEC1 kinetochore complex is required for the establishment and maintenance of kinetochore–microtubule interactions and plays a role in spindle checkpoint activity (He *et al.*, 2001; Janke *et al.*, 2001; Wigge and Kilmartin, 2001; DeLuca *et al.*, 2002; Bharadwaj *et al.*, 2004; McCleland *et al.*, 2004). Spc105 also has been found recently in affinity purifications of *S. cerevisiae* kinetochore proteins that define the Mtw1p complex (De Wulf *et al.*, 2003; Nekrasov *et al.*, 2003). Furthermore, Spc105 affinity purification has been described to contain components of the Ndc80 complex (Nekrasov *et al.*, 2003). Our finding that Spc105 copurifies with the Ndc80 complex component Spc24 confirms that Spc105 is in proximity to the Ndc80 complex. The fact that Spc105 was not detected in Ndc80 preparations (Nekrasov *et al.*, 2003) might reflect the fact that Spc105 is more closely associated with Spc24 than Ndc80. Alternatively, this might be due to differences in the experimental conditions.

Homologues of the Ndc80 complex also exist in *S. pombe* and have been shown to be part of the kinetochore (Kniola *et al.*, 2001; Wigge and Kilmartin, 2001). It is at present unclear whether Spc7 is closely associated with or part of this complex. However, Spc7 can be coimmunoprecipitated by the *S. pombe* Spc24 protein and vice versa (Kerres and Fleig, unpublished data). Furthermore, Spc24, like Spc7, is associated with the central centromere region (Kerres and Fleig, unpublished data). It is thus feasible to propose that Spc7 is associated with the *S. pombe* Ndc80 complex.

The identification of Spc7/Spc105, as a suppressor of the *mal3* mutant phenotype implies that the Ndc80 complex or proteins in proximity to this complex might possibly play a more direct role in association with microtubule plus-ends than previously envisaged (reviewed in Cheeseman *et al.*, 2002). Interestingly, components of the Ndc80 complex as well as Spc105 but not inner kinetochore proteins such as Ndc10 were identified in enriched SPB preparations (Wigge *et al.*, 1998).

Our experimental data suggest that one of the factors required for kinetochore–microtubule association is an interaction between the kinetochore protein Spc7 and the microtubule plus-end–associated protein Mal3. The Ran GTPase seems to play a role in this specific association as extra Spc7 can rescue a *spi1* mutation, which results in a decrease in the amount of active Ran in cells that are competent for nucleocytoplasmic transport (Fleig *et al.*, 2000; Salus *et al.*, 2002). Recently, Ran has been implicated in kinetochore function as the GTPase activating proteins Ran GAP1 and RanBP2 are associated with metaphase kinetochores and *Caenorhabditis elegans* Ran was localized to kinetochores, where it seemed to play a role in the association of kinetochore microtubules to chromosomes (Bamba *et al.*, 2002; Joseph *et al.*, 2002). Furthermore, in *Xenopus* egg extracts the spindle checkpoint and the kinetochore association of spindle checkpoint proteins is directly regulated by Ran-GTP levels (Arnaoutov and Dasso, 2003).

What is the function of Mal3 at the kinetochore–microtubule interface? Our coimmunoprecipitation analysis suggests that the interaction between Spc7 and Mal3 is not a stable one, thus making it unlikely that the main role of Mal3 is that of continuous microtubule–kinetochore attachment. In support of this is the finding that the kinetochore localization of EB1 was restricted to a subset of early mitotic kinetochores that were associated with polymerizing microtubule plus-ends (Tirnauer *et al.*, 2002a). It is thus more feasible that Mal3 has a role in regulating microtubule dynamics at the kinetochore. The effect of EB1 family members on microtubule dynamics has been amply documented (Beinhauer *et al.*, 1997; Tirnauer *et al.*, 1999; Nakamura *et al.*, 2001; Busch and Brunner, 2004). Absence of EB1 family members leads to reduced microtubule length, whereas overexpression of EB1 bundles microtubules (Bu and Su, 2001; Nakamura *et al.*, 2001; Rogers *et al.*, 2002; Tirnauer *et al.*, 2002b; Ligon *et al.*, 2003). Furthermore, Mal3 affects microtubule dynamics by initiating microtubule growth and inhibiting catastrophe events in interphase microtubule arrays (Beinhauer *et al.*, 1997; Busch and Brunner, 2004). Because overexpression of Spc7 makes wild-type cells more resistant to microtubule-destabilizing drugs, we would like to suggest that Mal3 and Spc7 are part of the complex protein machinery that modulates the dynamic behavior of microtubules at the kinetochore. In this context, it is interesting to note that the ability of EB1 to promote microtubule polymerization was dependent on the presence of the C-terminal part of APC, suggesting that EB1 function is modulated by other proteins (Nakamura *et al.*, 2001). Whether Spc7 has a similar effect on the function of Mal3 or whether it acts in this process but independently of Mal3 remains to be determined.

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