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A Conserved Interaction between Moe1 and Mal3 Is Important for Proper Spindle Formation in *Schizosaccharomyces pombe*

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Moe1 is a conserved fission yeast protein that negatively affects microtubule stability/assembly. We conducted a two-hybrid screen to search for Moe1-binding proteins and isolated Mal3, a homologue of human EB1. We show that Moe1 and Mal3 expressed in bacteria form a complex and that Moe1 and Mal3 expressed in fission yeast cosediment with microtubules. Deletion of either *moe1* or *mal3* does not result in lethality; however, deletion of both *moe1* and *mal3* leads to cell death in the cold. The resulting cells appear to die of chromosome missegregation, which correlates with the presence of abnormal spindles. We investigated the cause for the formation of monopolar spindles and found that only one of the two spindle pole bodies (SPBs) contains γ-tubulin, although both SPBs appear to be equal in size and properly inserted in the nuclear membrane. Moreover, the *moe1 mal3* double null mutant in the cold contains abnormally short and abundant interphase microtubule bundles. These data suggest that Moe1 and Mal3 play a role in maintaining proper microtubule dynamics/integrity and distribution of γ-tubulin to the SPBs during mitosis. Finally, we show that human Moe1 and EB1 can each rescue the phenotype of the *moe1 mal3* double null mutant and form a complex, suggesting that these proteins are part of a well-conserved mechanism for regulating spindle functioning.

INTRODUCTION

Microtubules are polymers of α- and β-tubulins that are highly conserved in eukaryotic cells (Hyman and Karsenti, 1996). Microtubules play critical roles in establishing the spatial distribution of molecules and organelles and in chromosome segregation, all of which require microtubules to undergo remodeling in a cell cycle-dependent manner. The mechanisms that drive microtubule remodeling are not entirely clear. One area of intense study centers around the observation that microtubules assembled in vitro are intrinsically "dynamic" in that they seem to constantly undergo cycles of polymerization and depolymerization. The temporal and spatial regulation of these cycles must be well-coordinated with progression of the cell cycle.

Like many higher eukaryotes, the fission yeast *Schizosaccharomyces pombe* has a complex and dynamic microtubule cytoskeleton. Immediately before mitosis, *S. pombe* microtubules undergo a dramatic reorganization from an interphase configuration to a prophase-like stage (Nabeshima *et al.*, 1998; reviewed by Hagan, 1998). During this reorganization, the interphase microtubules depolymerize while a spindle is nucleated within the nucleus by the spindle pole bodies (SPBs). At the end of mitosis, the spindle depolymerizes and the interphase microtubules reemerge from microtubule-organizing centers (MTOCs) located near the newly formed septum. The microtubule cytoskeleton is also important for cell polarity, namely, in maintaining a bipolar cell extension and an elongated cell morphology. The establishment of polarity is thought to depend on microtubules, which lay parallel to the cell body, and thus appear to provide a physical link to allow the cell ends to coordinate their extensions (Mata and Nurse, 1997).

We have recently characterized a highly conserved protein, Moe1 (for Microtubule overextended), that appears to play a role in promoting microtubule disassembly/instability (Chen *et al.*, 1999). Null mutants in *moe1* (*moe1*Δ) accumulate abnormally long and abundant microtubule bundles that are resistant to microtubule-destabilizing agents such as thiabendazole (TBZ). Despite the fact that *moe1*Δ cells exhibit numerous microtubule abnormalities, these cells remain viable. Interestingly, combining a *moe1*Δ with a loss-of-function mutation in the Ras1-Cdc42 G-protein signaling pathway (Chang *et al.*, 1994) creates a synthetic lethality (Chen *et al.*, 1999): the double mutants are impaired in proper spindle

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Abbreviations used: GFP, green fluorescence protein; GST, glutathione *S*-transferase; LBD, LexA DNA-binding domain; MTOC, microtubule-organizing centers; PCR, polymerase chain reaction; SPB, spindle pole bodies; TBZ, thiabendazole.

formation and chromosome segregation. Our data support a hypothesis that proper spindle formation requires the cycling of microtubule polymerization and depolymerization, and *moe1*Δ can impede this process by keeping tubulins in the polymerized state.

The mechanism by which Moe1 affects microtubule functioning is not clear. Curiously, we have been unable to detect any physical association between Moe1 and microtubules in the cell. Moe1 is largely cytosolic and concentrates near the nuclear periphery, and can accumulate in the nucleus when it is overexpressed (Chen *et al.*, 1999; Yen, and Chang, in press). Thus, Moe1 may influence microtubule functioning, at least in part, by acting through microtubule-binding proteins. To search for such proteins, we carried out a yeast two-hybrid screen using Moe1 as bait and isolated Mal3 (Beinhauer *et al.*, 1997), which is a member of a conserved family of microtubule-binding proteins that include human EB1 (Su *et al.*, 1995) and budding yeast Bim1 (Schwartz *et al.*, 1997). Cells lacking *mal3* are hypersensitive to TBZ and contain abnormally short and thin microtubules (Beinhauer *et al.*, 1997). Thus, it seems that Mal3 plays a key role in maintaining tubulins in the polymerized state. Despite the fact that microtubules are abnormal in *mal3*Δ cells, no obvious spindle defects have been reported. Intriguingly, overexpression of *mal3,* however, induces numerous spindle abnormalities (Beinhauer *et al.*, 1997). This suggests that Mal3 may interact stoichiometrically with components of a large protein complex that is necessary for proper spindle functioning. The identities of these Mal3-binding proteins are still unknown, however. Moreover, human EB1 may play a role in tumorigenesis because of its binding to a tumor suppressor APC (adenomatous polyposis coli, Su *et al.*, 1995). The mechanisms by which EB1 (or APC) affects tumor development remain unresolved. The identification of additional conserved Mal3-binding proteins would undoubtedly shed light on this issue.

Here we present evidence that proper spindle formation and chromosome segregation require a cooperation between Moe1 and Mal3. We present evidence showing that Moe1 and Mal3 play a role in spindle formation by maintaining proper microtubule dynamics and distribution of γ-tubulin to the SPBs. Finally, we show that EB1 and human Moe1 can rescue the phenotypes of the *moe1*Δ *mal3*Δ double mutant and physically interact, indicating that the interaction between Moe1 and Mal3 is highly conserved evolutionarily. It is possible that EB1 and Moe1 may participate in tumorigenesis by affecting spindle functioning, which leads to genome instability.

MATERIALS AND METHODS

Yeast Strains and Microbial Manipulation

Our generic wild-type strain is SP870 (*h*^{90}, *leu1-32*, *ade6-M210*, *ura4-D18*; Chang *et al.*, 1994). *S. pombe* cells were grown in either rich medium (YEAU, yeast extract medium supplemented with 75 mg/L uracil and adenine) or synthetic minimal medium with appropriate auxotrophic supplements (Alfa *et al.*, 1993). To examine phenotypes of cold-sensitive mutants, cells were pregrown at 30°C to early log phase (2–5 × 10^6 cells/ml) and then shifted to 20°C for up to 72 h. To estimate the duration of anaphase at 20°C, cells were synchronized by hydroxyurea (Chen *et al.*, 1999) at 30°C; after the cells were washed, they were resuspended in fresh medium and incubated at 20°C. To express proteins under the control of the thiamine-repressible *nmt1* promoter, freshly transformed cells were grown at 30°C for 16 h in the absence of thiamine to derepress the expression of proteins. For all experiments in which cells were spread on plates, it was necessary to plate twice as many *moe1*Δ cells to compensate for the low plating efficiency.

Plasmid Constructions

To express a triple HA1-tagged Moe1 under the control of the *nmt1* promoter, a *Bgl*II *moe1* fragment was created by polymerase chain reaction (PCR) and cloned into the *Bam*HI site in pSLF173 (Forsburg and Sherman, 1997) to generate pHA-MOE1. The same *Bgl*II fragment of *moe1* was cloned into the *Bam*HI site of pVJL11 (Chang *et al.*, 1994) to allow for the expression of a LexA DNA-binding domain (LBD)-Moe1 fusion protein for the yeast two-hybrid system. To express LBD-Moe1ΔC fusion protein, an *Nco*I/*Sal*I fragment was excised from pLBD-MOE1, removing the *moe1*-coding sequence for amino acid residues 314–567. The remaining vector was self-ligated, and a stop codon was added at the *Xba*I site by an amber linker (New England Biolabs, Beverly, MA) to create pLBD-MOE1ΔC. A 2.2-kb DNA fragment containing the *mal3* gene along with its flanking regions was amplified by PCR and cloned into the *Eco*RV site of pBluescript II S/K− (Stratagene, La Jolla, CA) to create pBSMAL3. A 0.9-kb *Eco47*III/*Bsg*I fragment in pBSMAL3 was replaced by the budding yeast *ADE2* to create pMAL3A. To express a GAD-MAL3ΔN fusion protein, an *Eco*RI/*Sma*I fragment was subcloned from pREP3-Mal3 (Beinhauer *et al.*, 1997) to pGADGH (Chang *et al.*, 1994) to create pGAD-MAL3ΔN. To express GAD-MAL3ΔC, a *Bam*HI/*Eco*RI fragment from pGAD-MAL3 (see below) was subcloned into pGADGH to create pGAD-MAL3ΔC. To express a glutathione *S*-transferase (GST)-Mal3 fusion protein in *Escherichia coli*, a *Bam*HI/*Kpn*I fragment from pGAD-MAL3, capable of encoding amino acid residues 5–308 of Mal3, was cloned into pRB259 (Chang *et al.*, 1994) to create pGST-MAL3. The *EB1* gene was amplified from the cDNAs of human erythroleukemia K562 cells (CLONTECH, Palo Alto, CA) by PCR to contain unique *Bam*HI sites for cloning. To express the *EB1* gene under the control of the *mal3* promoter, we first amplified a 0.7 kb DNA fragment containing mostly the 5′-flanking sequence of *mal3* (nucleotide position −703 to 4) by PCR to contain *Bam*HI and *Sac*I. This fragment was cloned into pSP2 (Cottarel *et al.*, 1993) to create pSP2MAL3P. The amplified *Bam*HI fragment of *EB1* was blunt ended and subcloned into the *Sac*I site in pSP2MAL3P to generate pMAL3P-EB1. The *Bam*HI fragment of *EB1* was cloned into pVJL11 to create pLBD-EB1. To express LBD-HSMOE1, the *Bam*HI fragment encoding the human Moe1 was excised from pHSMOE1 (Chen *et al.*, 1999) and cloned into pGADGH to create pGAD-HSMOE1. The coding sequence for green fluorescence protein (GFP) was excised from pALG (Li *et al.*, 2000) and cloned into pTrcHisC (CLONTECH) at the *Pst*I/*Kpn*I sites to create pHT-GFP.

Yeast Two-Hybrid Screen and β-Galactosidase Assay

The yeast two-hybrid screen was carried out using reporter strain L40 (Vojtek *et al.*, 1993), which carries the reporter gene cassettes *lexA-HIS3* and *lexA-lacZ*. The bait was pLBD-MOE1. The cDNA library used contains *S. pombe* cDNAs cloned into the *Eco*RI and *Xho*I sites in pGADGH (Chang *et al.*, 1994). Approximately 6 million cDNA clones were screened. One hundred fifty-four clones rendered cells both His^+ and $lacZ^+$, of which 84 were judged to interact specifically with Moe1 because they did not interact with the control, Lamin (Vojtek *et al.*, 1993). The *mal3* cDNA clone, named pGAD-MAL3, was isolated once, and it contains the coding sequence for amino acids 5–308 plus ~900 bp of 3′-flanking sequence. We note that *mal3* has an *Eco*RI site in its coding region, so it is highly probable that most of the *mal3* cDNAs were truncated during the library construction and, hence, were not isolated more frequently. We were unable to detect any two-hybrid interaction be-

tween Mal3 and other known components in the Ras1 morphogenic pathway, i.e., Ras1, Scd1, Scd2, Cdc42sp, and Shk1 (Chang *et al.*, 1994; Marcus *et al.*, 1995). Mal3 is the only microtubule-binding protein isolated from this screen, and we will describe the characterization of other Moe1-binding proteins elsewhere (Yen and Chang, in press). The β-galactosidase activity was determined by either a filter color assay using 5-bromo-4-chloro-3-indolyl -β-D-galactoside or a quantitative assay using *o*-nitrophenyl-β-D-galactoside (Hoffman and Winston, 1990).

Antibody Preparation

The Moe1 antigen was purified from *E. coli* (strain BL21[DE3]pLysS), which was transformed with pHT-MOE1 (Chen *et al.*, 1999) to express full-length Moe1 tagged with polyhistidine (His-Moe1). For the first boost, ≈250 μg of HT-Moe1 premixed with an equal volume of Freund's complete adjuvant were injected subcutaneously into rabbits. For subsequent monthly boosts, ≈125 μg of HT-Moe1 premixed with an equal volume of Freund's incomplete adjuvant were administrated. We found that this antibody with a 1:1000 dilution recognized a single band of 62 kDa (the predicted molecular mass of Moe1) in wild-type *S. pombe* cell extract but not in *moe1*Δ cells (Figure 3A).

Strain Constructions

To generate *mal3*Δ cells, strain SP870 was transformed with a *Bam*HI/*Apa*I fragment released from pMAL3A, and cells prototrophic for adenine were isolated. Proper gene deletion was confirmed by PCR. All examined cells have the same phenotype; one of them was chosen for detailed study and named MAL3A. As reported by Beinhauer *et al.* (1997), our *mal3*Δ cells also displayed numerous phenotypes that are common among mutants defective in microtubule functioning: hypersensitivity to TBZ (see below), aberrant cell morphologies (T-shaped and bent), and an off-center nucleus (data not shown). Unlike the reported *mal3*Δ strain, however, our *mal3*Δ cells did not display any appreciable growth defect at 20°C (the optimal growth temperature for yeast is 30°C; Figure 4A). We speculate that this difference is most likely due to differences in the genetic backgrounds between the two strains. A diploid strain heterozygous for *moe1*Δ and *mal3*Δ was generated by crossing strain MOE1U (*moe1::ura4*) with MAL3A (*mal3::ADE2*). Its tetrads were dissected to obtain a *moe1*Δ *mal3*Δ strain (ME1UML3A). Strain ME1UML3A was transformed with a linearized pVINMT81 (a derivative of pVINCE; Marcus *et al.*, 1995) and seeded on plates containing 5-fluoro-orotic acid to select cells that had lost *ura4*. One of these was named ME1NML3A. We created strain ECP16 by crossing strain 318 (West *et al.*, 1998), containing a Cut11 tagged with GFP (*cut11-gfp-ura4*), and ME1UML3A and selected for *moe1*Δ *mal3*Δ *cut11-gfp* cells after tetrad dissection.

Microtubule Cosedimentation Assay

Purified bovine tubulins (25 μg, purity > 99%, Cytoskeleton Inc., Denver, CO) were dissolved in 80 μl GMC buffer (80 mM 1,4-piperazinediethanesulfonic acid (PIPES), pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 30% glycerol with a cocktail of protease inhibitors [Sigma, St. Louis, MO], which includes 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and pepstatin A, and 2 μg/ml aprotinin) and incubated at 37°C for 30 min to allow for microtubule polymerization. Polymerized microtubules were diluted to 0, 3, 10, or 30 μM in GMC immediately before mixing with the cell lysate. pREP3-MAL3 (Beinhauer *et al.*, 1997), pHA-MOE1, and pAAUGST (Gilbreth *et al.*, 1998) were used to express Mal3, Moe1, and GST, respectively, in *moe1*Δ *mal3*Δ cells (ME1NML3A). Yeast lysates were prepared from cells grown at 30°C to log phase; the lysis buffer was the DB buffer (Hirata *et al.*, 1998). The lysates were cleared by centrifugation at 20,000 × *g* for 5 min at 4°C, and the supernatants were diluted to 8 mg/ml and stored at −80°C. Lysates were thawed in the presence of 1 mM GTP and dithiothreitol and centrifuged at 20,000 × *g* for 30 min at 4°C before use. Soluble lysates of 10 μl were mixed with 10 μl cushion buffer (80 mM 1,4-piperazinediethanesulfonic acid (PIPES), pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 60% glycerol, and a cocktail of protease inhibitors) and 20 μl preassembled bovine microtubules for 10 min at room temperature. The entire sample was then laid on top of 100 μl cushion buffer and centrifuged at 20,000 × *g* for 30 min. Aliquots of supernatant and pellet were analyzed by Western blotting.

Protein-Binding Assays

GST, GST-Mal3, polyhistidine (His_6), His-Moe1, and His-GFP were expressed in *E. coli* using pRP259, pGST-MAL3, pTrcHisB (CLONTECH), pHT-Moe1 (Chen *et al.*, 1999), and pHT-GFP, respectively. For the glutathione bead pull-down assay, GST and GST-Mal3 (6 and 3 μg, respectively) were collected by the beads and then mixed with crude lysate containing 3 μg each His-GFP or His-Moe1. The RIPA buffer (Harlow and Lane, 1988) was added to the mixture in a 4:1 ratio, and the resulting sample was incubated at 4°C for 4 h with rotation. Afterward, the beads were washed four to five times with RIPA and twice with PBS before being analyzed by immunoblots. For the Far-Western assay, 3 μg His_6 and His-Moe1 were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and then incubated with 1 μg GST or GST-Mal3 for 1 h at 4°C. The bound GST-tagged proteins were revealed by immunoblots.

Western Blot Analysis

Moe1 and Mal3 were detected by rabbit polyclonal antibodies specific for Moe1 (1:1000 dilution) and Mal3 (1:500 dilution; Beinhauer *et al.*, 1997), respectively. The presence of microtubules made of bovine tubulins was detected by an anti-α-tubulin monoclonal antibody (1:200; Sigma, T-4026). The detections of GST- and polyhistidine-tagged proteins were as described by Chen *et al.* (1999). All antibodies were diluted in Tris-buffered saline with 3% bovine serum albumin and 0.5% Tween-20.

Cell Survival Test

Various strains were pregrown at 30°C in YEAU to early log phase. Equal numbers of cells were spread on YEAU plates and incubated at 20°C. Over time, these plates were returned to 30°C, and the number of colonies that emerged was counted after 3 d.

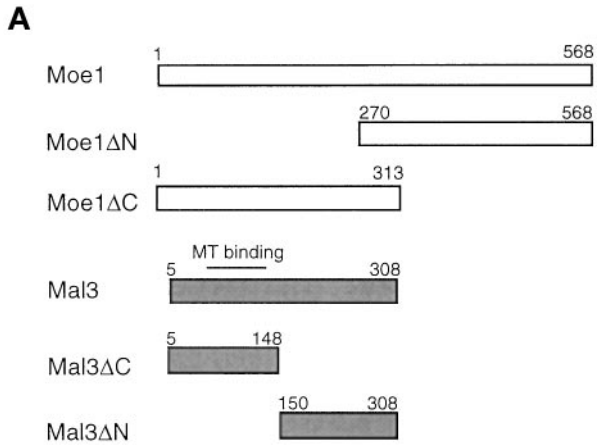
Fluorescence Microscopy

To visualize microtubules, cells were fixed with 0.05% glutaraldehyde and 3% paraformaldehyde in the PEM buffer (Alfa *et al.*, 1993) and then incubated with an anti-α-tubulin monoclonal antibody, TAT1 (1:5; Woods *et al.*, 1989). To visualize Sad1 and microtubules and γ-tubulin and microtubules simultaneously, cells were fixed with 4% paraformaldehyde for 1 h. The primary rabbit antibodies used to stain Sad1 (Hagan and Yanagida, 1995) and γ-tubulin (Sigma, T3559) were both diluted 1:100. Secondary antibodies were from Sigma: anti-mouse IgG conjugated with fluorescein isothiocyanate (1:50) and anti-rabbit IgG conjugated with tetramethylrhodamine B isothiocyanate (1:40). To view F-actin, cells were fixed with 4% paraformaldehyde for 30 min and stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR; 20 units/ml, 1 h). To view DNA, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml). The number of microtubules in the cell was measured by adjusting the focal plane up and down while counting, and a total of 30 cells were examined.

RESULTS

Physical Interaction between Mal3 and Moe1

To search for *S. pombe* components that form complexes with Moe1, we carried out a yeast two-hybrid screen using full-



B

GAD \ LBD	Moe1	Moe1ΔN	Moe1ΔC
Mal3	++	-	+
Mal3ΔN	++	-	+
Mal3ΔC	-	-	-

Figure 1. Two-hybrid interactions between full-length and truncated proteins. (A) A schematic representation of various truncated proteins tested by the two-hybrid system. A region essential for microtubule (MT) binding (amino acid residues 67–121), deduced from previously published results (Juwana *et al.*, 1999), is marked by a line above Mal3. (B) A summary of the two-hybrid interactions between various combinations of proteins. The binding between full-length Moe1 and Mal3 produced 12 units of β-galactosidase activity. The plasmids expressing GAD-Mal3, GAD-Mal3ΔN, GAD-Mal3ΔC, LBD-Moe1, LBD-Moe1ΔN, and LBD-Moe1ΔC were pGADMAL3, pGAD-MAL3ΔN, pGAD-MAL3ΔC, pLBDMOE1, pLBD-MOE1ΔN (Chen *et al.*, 1999), and pLBD-MOE1ΔC, respectively.

length Moe1 as bait (see MATERIALS AND METHODS) and isolated a cDNA clone encoding a nearly full-length Mal3 (amino acid residues 5–308; Figure 1). We performed two biochemical assays using recombinant proteins purified from *E. coli* to determine whether Moe1 and Mal3 bind directly in vitro. As shown in Figure 2A, GST-tagged Mal3 (GST-Mal3), but not GST alone, bound specifically to polyhistidine-tagged Moe1 (His-Moe1) but not to the His-GFP control. His-Moe1 immobilized on the membrane also bound specifically to GST-Mal3 in a Far-Western (filter overlay) assay (Figure 2B). These data indicate that Moe1 and Mal3 bind directly in vitro. Next, the two-hybrid assay was used to map the binding sites between Moe1 and Mal3 (Figure 1). Our data demonstrate that the N terminus of Moe1 (amino acid residues 1–313; Figure 1) was necessary to bind the C terminus of Mal3 (residues 150–308), a region distinct from the conserved putative microtubule-binding site.

Moe1, Mal3, and Microtubules Form a Complex

We carried out a microtubule cosedimentation assay to determine whether Moe1 and Mal3 can associate with microtubules. Yeast lysates, prepared from *moe1*Δ *mal3*Δ cells containing various overexpressed proteins, were mixed with microtubules that were preassembled in vitro from bovine tubulins. As shown in Figure 3, Mal3, but not the GST control, cosedimented with microtubules, which is consistent with the fact that Mal3 can associate with microtubules in yeast cells (Beinhauer *et al.*, 1997). Human Mal3 homologues (EB1 and RP1) have also been shown to bind microtubules in vitro (Berrueta *et al.*, 1998; Juwana *et al.*, 1999). In addition, Moe1 alone did not associate with microtubules but it did so efficiently when Mal3 was added in the lysates (Figure 3). These results suggest that Mal3, Moe1, and microtubules can form a complex.

Synthetic Interaction Induced by moe1Δ *and* mal3Δ

To investigate whether the physical interaction between Moe1 and Mal3 is essential for viability, we analyzed the phenotype of a *moe1*Δ *mal3*Δ strain (see MATERIALS AND METHODS). As shown in Figure 4A, the growth of *moe1*Δ *mal3*Δ cells was markedly reduced at 20°C (Figure 4A). Using a cell survival assay, in which cells were preincubated at 20°C for various times before being tested at 32°C for colony formation, we further determined that *moe1*Δ *mal3*Δ cells lost viability readily at 20°C (Figure 4B). Based on these results, we conclude that Moe1 and Mal3 are essential for viability in the cold.

Because both Moe1 and Mal3 have been suggested to play a role in proper chromosome segregation, we asked whether *moe1*Δ *mal3*Δ cells die of chromosome missegregation at 20°C. As shown in Figure 4, C and D, cells containing grossly missegregated chromosomes accumulated steadily over time at 20°C. This suggests that chromosome missegregation is one of the major causes of cell death in the *moe1*Δ *mal3*Δ strain at 20°C.

During the course of examining interphase cells, we found that a large number of *moe1*Δ *mal3*Δ cells, 37%, seemed to grow monopolarly with F-actin accumulated in only one end of the cell (Figure 4E). This type of cell was quite rare in *moe1*Δ, *mal3*Δ or wild-type cells (≈4.3%). It seems that Moe1 and Mal3 are also required for maintaining proper cell polarity.

Loss-of-Function in moe1 *and* mal3 *Affects Spindle Formation*

Because Moe1 and Mal3 have been shown to affect spindle functioning, we asked whether abnormal spindle functioning causes chromosome missegregation in *moe1*Δ *mal3*Δ cells. Various strains were pregrown at 30°C to early log phase, shifted to 20°C, and then examined microscopically after two generations. We found that the *moe1*Δ *mal3*Δ strain growing at 20°C contained considerably more cells in "prophase" (5.6%, as judged by the presence of unseparated condensed chromosomes and absence of the spindle). In comparison, the percentages of prophase cells in wild-type, *mal3*Δ, and *moe1*Δ cells were < 0.5, < 0.5, and 1.9%, respectively. These data illustrate that *moe1*Δ *mal3*Δ cells exhibit a substantial mitotic delay at 20°C.

We then investigated whether the mitotic delay was caused by abnormal spindle formation. The percentage of

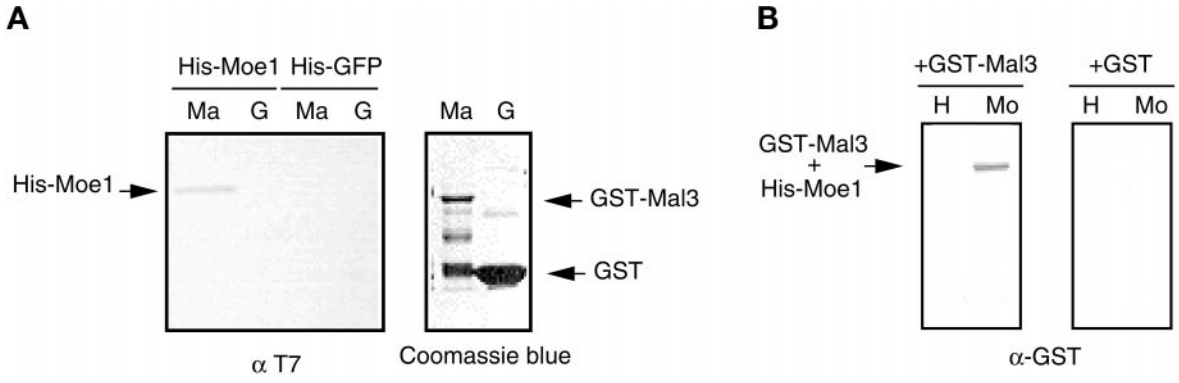


Figure 2. In vitro binding between Moe1 and Mal3. (A) GST control and GST-Mal3 (G and Ma, on top of each gel) bound to the glutathione beads were mixed with crude bacterial lysates containing either the His-GFP control or His-Moe1. The His-tagged proteins bound to the beads were analyzed by Western blots, shown on the left, using an antibody against a T7 epitope that is present in all His-tagged proteins. The presence of GST proteins on the glutathione beads was determined by Coomassie blue staining. (B) Polyhistidine control and His-tagged Moe1 (H and Mo, top) were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and then incubated with either purified GST or GST-Mal3, indicated on top. The presence of the GST tag was detected by Western blots.

*moe1*Δ *mal3*Δ cells containing a detectable spindle at 20°C was only slightly lower than normal (7 versus ~10% in wild-type or either single mutant); however, these cells contained disproportionally large numbers of V-, star-, or fan-shaped spindles, anomalies that were rarely detectable at 30°C (Figure 5, A and B). We have evidence (below) that these spindles are monopolar. Note that the chromosomes in these cells were unseparated. Furthermore, *moe1*Δ *mal3*Δ cells in anaphase frequently contained a spindle that appeared too long and curved within the cell (Figures 5A, f and g, and 4B). In some cells, the excessively long spindle seemed to push one of the nuclei backward such that the same daughter cell ended up with two nuclei (Figure 5A, g). For the remaining spindles with a normal appearance, almost none of them seem to function properly in chromosome segregation. Approximately 95% of *moe1*Δ *mal3*Δ cells reaching midanaphase with a spindle of 6–7 μm contained unseparated chromosomes (Figure 5C). In contrast, the chromosomes in more than 80% of wild-type, *moe1*Δ, and *mal3*Δ cells in a similar stage of anaphase were separated.

The presence of an excessively long spindle in the double mutant may reflect abnormal spindle nucleation/morphogenesis. Alternatively, the defect may reside in the nuclear membrane such that the spindle is not seated properly and can break free. To investigate the latter possibility, we generated a *moe1*Δ *mal3*Δ strain that contains a nuclear membrane protein, Cut11, fused at the C terminus with GFP (Cut11-GFP). Our data show that the nuclear membrane was intact and wrapped around the spindle (Figure 5, h). We asked further whether the long spindle is a result of a prolonged anaphase, during which the spindle continues to elongate, although the nuclei have already reached the cell ends (Hagan *et al.*, 1990). We thereby estimated the duration of anaphase by measuring the time it took for the bulk of a

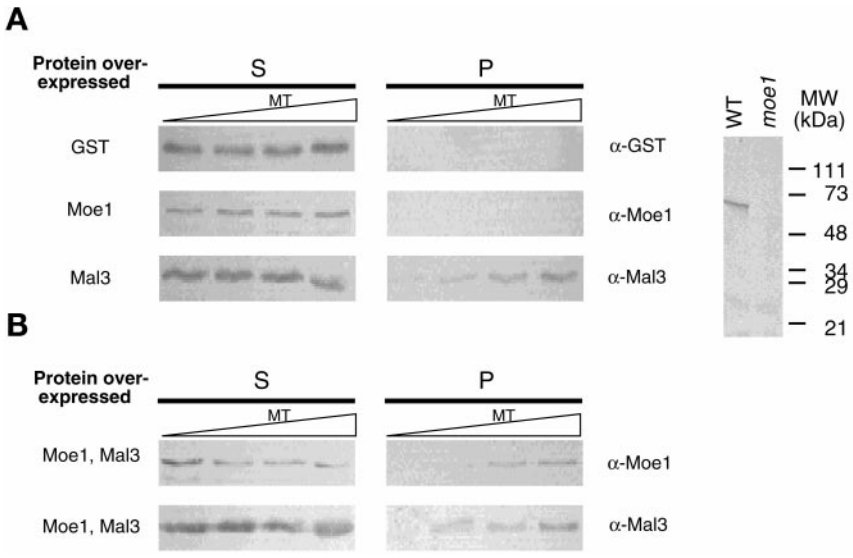


Figure 3. Microtubules, Moe1, and Mal3 form a complex in yeast lysate. Microtubules (MT) of various concentrations (from left to right, 0, 3, 10, and 30 μM) were mixed with yeast lysates prepared from *moe1*Δ *mal3*Δ cells (strain ME1NML3A) overexpressing various proteins, as indicated on the left. The mixtures were centrifuged through a cushion buffer and both the supernatant (S) and pellet (P) were analyzed by immunoblots with antibodies indicated on the right. The anti-Moe1 antibody recognizes a single band of 62 kDa in wild-type (WT), but not *moe1*Δ, cell lysates (A, right). The plasmids used to express Mal3, Moe1, and GST were pREP3-MAL3, pHA-MOE1, and pAAUGST.

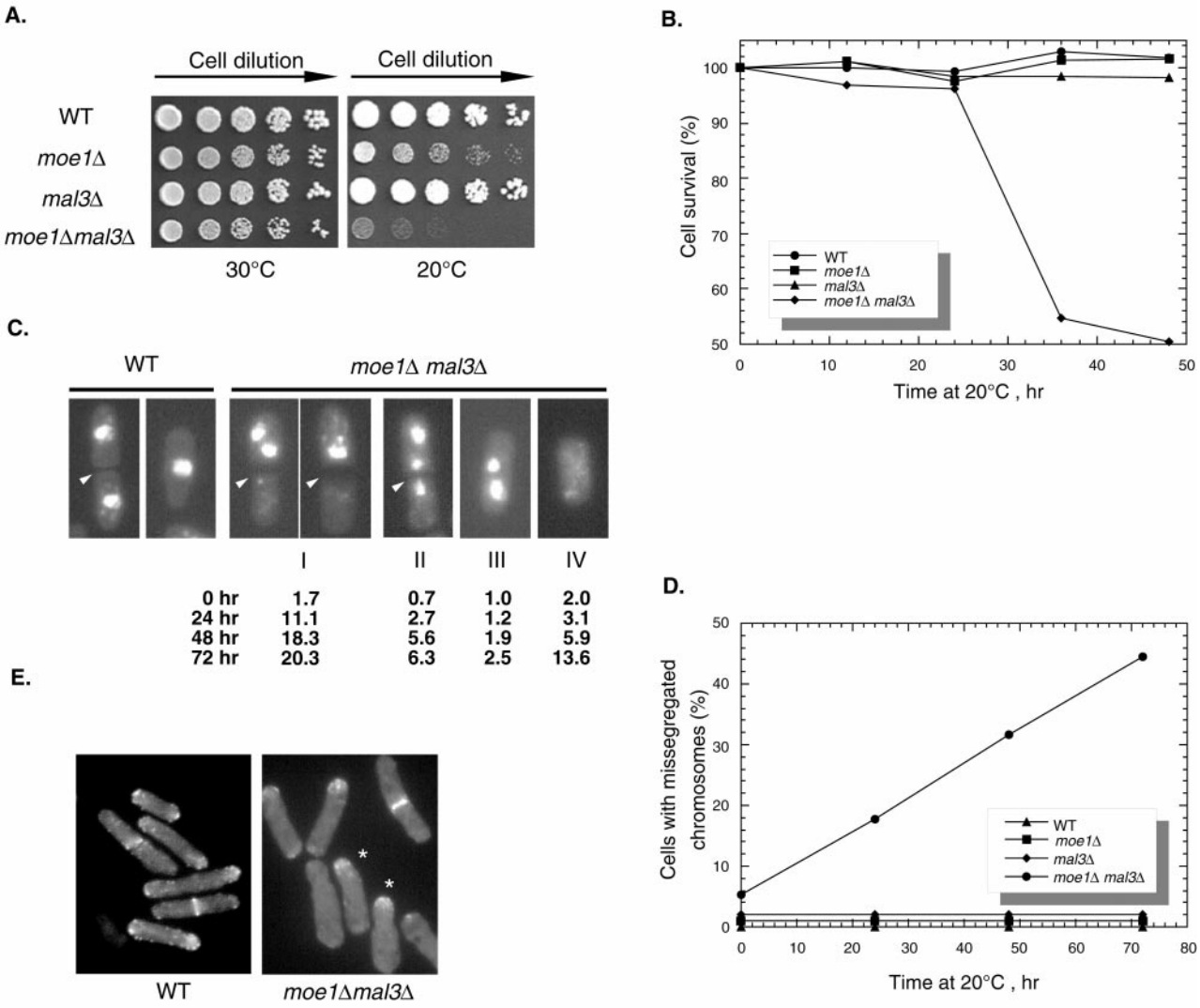
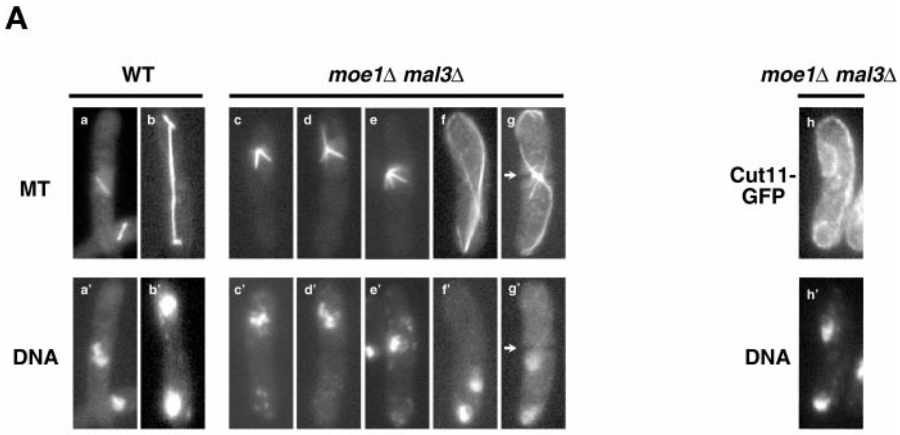


Figure 4. Phenotypes of *mal3*Δ *moe1*Δ cells. (A) Serial dilutions of cells (1:5) were spotted on YEAU plates. These plates were incubated at either 30°C for 3 d or at 20°C for 6 d before being photographed. (B) Cells (relevant genotypes are shown in the inset) were spread on plates (YEAU) and preincubated at 20°C for the indicated times and then transferred to 30°C. The colonies that emerged were counted. (C) *moe1*Δ *mal3*Δ cells pregrown at 30°C to log phase were shifted to 20°C for the indicated times, and the presence of missegregated chromosomes (groups I–IV) was revealed by 4′,6-diamidino-2-phenylindole (DAPI) staining. The percentages of these aberrant cells are indicated at the bottom of each panel. A wild-type cell that just completed separation (left) and one that was in interphase (right) are shown as controls. Arrowheads, positions of septa. (D) The percentages of three groups of cells with missegregated chromosomes, as shown in C (groups I–IV), are combined and plotted against time after being shifted to 20°C. (E) F-actin was revealed by rhodamine-conjugated phalloidin. *, *moe1*Δ *mal3*Δ cells that grew monopolarly with F-actin detected at only one end of the cell. Strains used were SP870 (WT, wild-type) and ME1UML3A (*moe1*Δ *mal3*Δ). In C and D more than 400 cells were examined at each time point.

synchronized culture (see MATERIALS AND METHODS) to pass from anaphase (as judged by the presence of binuclear cells) to cytokinesis (as judged by the presence of septated cells). Our data suggest that both *moe1*Δ and *moe1*Δ *mal3*Δ cells spent essentially the same amount of time in anaphase (≈1 h at 20°C), but *moe1*Δ *mal3*Δ cells had twice as many cells with abnormally long spindles as did *moe1*Δ cells (Figure 5B). Thus, it seems unlikely that the long spindle observed in *moe1*Δ *mal3*Δ cells is caused simply by a prolonged anaphase.

Abnormal Microtubule Functioning in moe1Δ mal3Δ *Cells*

Proper microtubule functioning is undoubtedly important for spindle formation. Because both *moe1*Δ and *mal3*Δ cells have been shown to display numerous abnormalities in microtubules, we investigated whether microtubules are abnormal in *moe1*Δ *mal3*Δ cells. As reported previously, *mal3*Δ cells are hypersensitive, whereas *moe1*Δ cells are resistant, to TBZ (Beinhauer *et al.*, 1997; Chen *et al.*, 1999; see also Figure



B

Relavant Genotype	Growth Temperature	Distribution of various spindles, %		
WT	30°C	100	<0.3	<0.3
	20°C	100	<0.3	<0.3
*moe1*Δ	30°C	89.6	1.4	9.0
	20°C	79.4	2.6	17.0
*mal3*Δ	30°C	100	<0.3	<0.3
	20°C	100	<0.3	<0.3
*moe1*Δ *mal3*Δ	30°C	81.2	1.2	16.4
	20°C	44.3	16.9	39.5

C

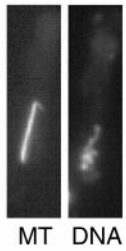


Figure 5. Abnormal spindles in *moe1*Δ *mal3*Δ cells. Cells pregrown at 30°C to log phase were transferred to 20°C and examined after 24 h. (A) The spindles were examined by immunostaining (MT, a–g), and Cut11-GFP was visualized directly (h). An arrowhead indicates the position of a septum. (B) The relative abundance of various forms of spindles, observed in A, among all the cells that contain a spindle are tabulated. (C) An example of a *moe1*Δ *mal3*Δ cell in anaphase (spindle length of 7 μm) containing unseparated chromosomes. The strains used were SP870 (wild-type, WT), ME1UML3A (*moe1*Δ *mal3*Δ), MOE1U (*moe1*Δ), MAL3A (*mal3*Δ), and ECP16 (*moe1*Δ *mal3*Δ *cut11::gfp*).

6A). Furthermore, microtubules in both strains display abnormal morphologies at 20°C: they are abnormally short and thin in *mal3*Δ cells but are abnormally long and abundant in *moe1*Δ cells (Figure 6B). Interestingly, we found that *moe1*Δ *mal3*Δ cells were as hypersensitive to TBZ as were *mal3*Δ cells, which to some degree correlates with the observation that their microtubules were as short as those in *mal3*Δ cells (Figure 6). Additionally, these short microtubule bundles were as abundant as those in *moe1*Δ cells (on average four to six bundles per cell as opposed to two to three, as seen in

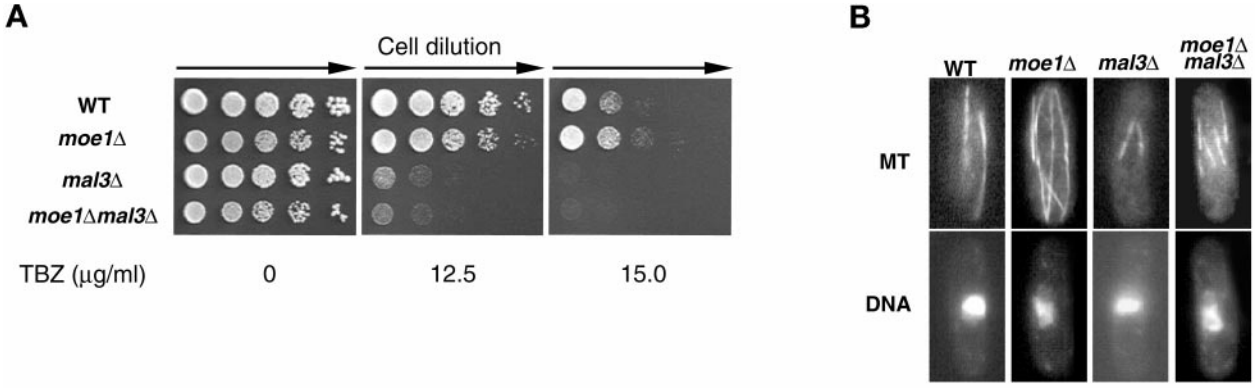


Figure 6. TBZ sensitivity and abnormal microtubules in various strains. (A) Serial dilutions of cells (1:5) were spotted on YEAU plates containing TBZ. The plates were incubated at 30°C for 3 d. (B) See legend to Figure 5 for the growth conditions of cells. Microtubules (MT) were revealed by immunostaining. Microtubule numbers in the cell (see text) were counted by adjusting the focal plane up and down. Strains used were SP870 (WT, wild-type), MOE1U (*moe1*Δ), MAL3A (*mal3*Δ), and ME1UML3A (*moe1*Δ *mal3*Δ).

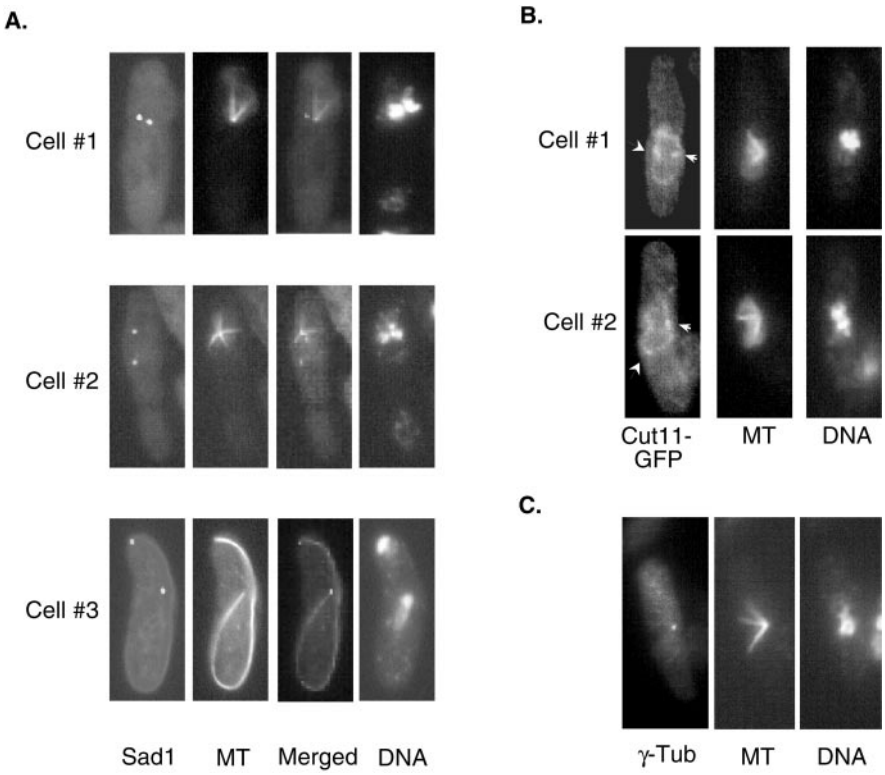


Figure 7. Asymmetric spindle nucleation from the SPBs in *moe1*Δ *mal3*Δ cells. See legend to Figure 5 for the growth conditions of cells. (A) *moe1*Δ *mal3*Δ cells (strain ME1UML3A) were double-stained with antibodies to view Sad1 (Sad1) and the spindle (MT), and two imagines were merged by Photoshop (Adobe Systems, Inc). (B) Strain ECP16 (*moe1*Δ *mal3*Δ *cut11::gfp*) was double-stained with antibodies to GFP and α-tubulin to visualize Cut11-GFP and spindle (MT) simultaneously. SPBs are marked by both arrowheads and arrows; the former also mark the inactive SPBs. Note that the lower SPB in cell 2 is not on the same focal plane as the upper one; therefore, it appears smaller. (C) ME1UML3A were doubled-stained to reveal γ-tubulin (γ-Tub) and microtubules (MT). A total of 25 cells with monopolar spindles were examined, one of which is shown.

wild-type cells; Figure 6B). The Mal3 homologue in budding yeast, Bim1, has been shown to play a role in modulating microtubule dynamics (Tirnauer *et al*., 1999). Because the microtubule abnormalities in the *mal3* and *bim1* mutants are very similar, it is possible that Mal3 also affects microtubule dynamics in *S. pombe*. Therefore, we believe that the presence of these abnormally abundant and short microtubules in *moe1*Δ *mal3*Δ cells is indicative of a global alteration in microtubule dynamics or integrity or both, which may affect spindle formation.

Asymmetric Spindle Nucleation from the SPBs in moe1Δ mal3Δ *Cells*

The SPB is the fungal MTOC responsible for spindle formation. During interphase, the SPB duplicates, and the resulting SPBs are later inserted into the nuclear membrane during prophase (Ding *et al*., 1997). We investigated whether any obvious abnormalities in the SPB can be detected to account for the formation of abnormal spindles in *moe1*Δ *mal3*Δ cells. SPBs were visualized either by immunostaining with an antibody against Sad1, an SPB constituent (Hagan and Yanagida, 1995), or by tracking Cut11-GFP, which also localizes to SPBs (West *et al*., 1998). In the majority of *moe1*Δ *mal3*Δ cells in M phase (~90%), Sad1 appeared as two dots of equal size that closely associated with the DNA mass (Figure 7A). We reached the same conclusion by examining Cut11-GFP (Figure 7B); moreover, it is obvious that these two dots of Cut11-GFP were always inserted in the nuclear membrane (Figure 7B). Therefore, it is unlikely that the integrity and duplication of the SPBs are dramatically altered in *moe1*Δ *mal3*Δ cells.

As described above, *moe1*Δ *mal3*Δ cells contained a large number of cells with a V-, fan-, or star-shaped spindle. We double-stained these cells to view the spindle and the SPB at the same time and found that most of these abnormal spindles (≈85%) were nucleated from only one of the SPBs (Figure 7, A and B). This result indicates that these abnormal spindles are monopolar. In contrast, despite their excessive length, the abnormally long S-shaped spindles are bipolar, as evidenced by the presence of two SPBs of equal size attached to the ends of the spindle (Figure 7A).

γ-Tubulin is a conserved and ubiquitous component in MTOCs and critical for proper spindle formation by presumably acting as a microtubule-nucleating site (Horio *et al*., 1991). Thus, we investigated whether γ-tubulin is improperly localized to the SPBs in those *moe1*Δ *mal3*Δ cells containing a monopolar spindle. Our data showed that in almost all of these abnormal cells (≥95%) there was only a single γ-tubulin dot, with which the spindle is associated (Figure 7C). This suggests that the majority of *moe1*Δ *mal3*Δ cells with a monopolar spindle contain only one SPB that is functional with detectable γ-tubulin. There was no obvious abnormality in γ-tubulin localization in interphase cells or in cells with bipolar spindles (data not shown). Hence, deletion of both *moe1* and *mal3* do not seem to cause a global reduction in γ-tubulin protein levels but appear to affect proper localization of γ-tubulin to SPBs during mitosis.

Genetic and Physical Interaction between EB1 and HsMoe1

Moe1 and Mal3 both have homologues in humans, HsMoe1 and EB1, which efficiently suppress the phenotypes of *moe1*Δ

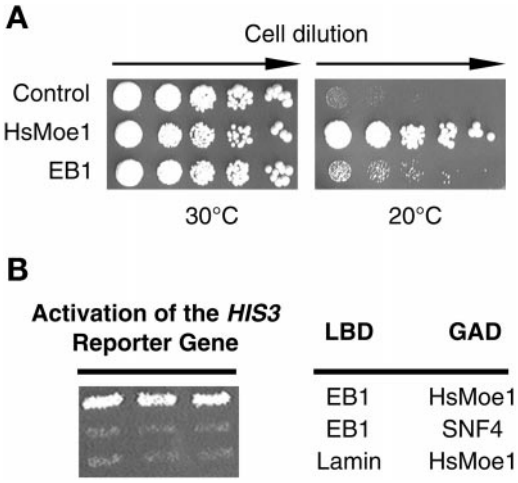


Figure 8. Genetic and physical interaction between human Moe1 and EB1 in yeast. (A) Human Moe1 and EB1 were expressed in *moe1*Δ *mal3*Δ cells (strain ME1NML3A). These cells were spotted on YEAU plates after a series of 1:5 dilutions. The plates were incubated at either 30°C for 3 d or 20°C for 6 d. Plasmids tested were pARTCM (Empty vector control; Chang *et al.*, 1994), pHSMOE1 (expressing human Moe1 under the control of the *adh1* promoter), and pMAL3P-EB1 (expressing EB1 under the control of a *mal3* promoter). (B) The two-hybrid reporter cells carrying the indicated hybrid proteins were replica-plated on medium lacking histidine. Shown here are cell patches that were able to grow in the absence of histidine, which indicates activation of the *HIS3* reporter gene. The plasmids tested were pGAD-HSMOE1, pLBD-EB1, pLBD-Lamin (Vojtek *et al.*, 1993), and pGAD-SNF4 (Fields and Song, 1989).

and *mal3*Δ cells (Beinhauer *et al.*, 1997; Chen *et al.*, 1999), respectively. Can HsMoe1 and EB1 also interact? To address this issue, we overexpressed HsMoe1 or EB1 to see whether they rescue the phenotype of *moe1*Δ *mal3*Δ cells. As shown in Figure 8A, both HsMoe1 and EB1 were able to rescue the cold-dependent lethality of *moe1*Δ *mal3*Δ cells, which correlates with the observations that these transformed cells had far more cells with a normal spindle and thus far fewer with missegregated chromosomes (data not shown). Furthermore, we found that HsMoe1 and EB1, like their yeast counterparts, also physically interacted, as determined by the yeast two-hybrid system (Figure 8B). Therefore, these data strongly indicate that the genetic and physical interactions between Moe1 and Mal3 have been conserved extensively during evolution, and we propose that, in humans, HsMoe1 and EB1 also participate in proper spindle functioning and chromosome segregation.

DISCUSSION

In this study, we demonstrate that proper spindle functioning in *S. pombe* requires a cooperation between Moe1 and Mal3. Loss of function of both *moe1* and *mal3* results in the formation of abnormal spindles, which apparently leads to chromosome missegregation and cell death in the cold. We show that the interphase microtubule bundles in *moe1*Δ *mal3*Δ cells are abnormally short and abundant, which is interpreted as an indication of global alterations in microtubule dynamics and/or integrity. We reveal at least one of the causes for the formation of monopolar spindles: γ-tubulin localization to only one of the SPBs. Based on these results, we propose that Moe1 and Mal3 are necessary for proper microtubule dynamics/integrity and proper association between γ-tubulin and the SPBs.

Is the regulation of γ-tubulin and SPBs association by Moe1 and Mal3 conserved in higher eukaryotes? SPBs in *S. pombe* are functionally analogous to the centrosome in animal cells. Both SPBs and centrosomes also contain numerous conserved components, of which γ-tubulin is central for microtubule nucleation (reviewed by Schiebel, 2000). The association between γ-tubulin and centrosomes requires dynein and the dynactin complex (Quintyne *et al.*, 1999; Young *et al.*, 2000). Dynein is a minus-end motor protein whose ability to transport cargo (such as γ-tubulin) is stimulated through a direct binding to dynactin. Interestingly, EB1 has been found to coprecipitate with several components of the dynactin complex and with a dynein intermediate chain (Berrueta *et al.*, 1999). Thus, it is possible that in mammalian cells EB1 can play a role in the transport of γ-tubulin to the centrosome through its binding to dynactin. The mechanism by which γ-tubulin is transported to SPBs in *S. pombe* is poorly understood. It is enticing to speculate that dynein or dynactin or both may play a role in this process, and they can be further influenced by both Moe1 and Mal3. There is one caveat with this model, however. In a recent study of what appears to be the sole dynein heavy chain in *S. pombe* (encoded by *dhc1*), Yamamoto and colleagues (1999) have shown that the primary function of Dhc1 is to control nuclear movement during meiosis. No obvious defects in the microtubule cytoskeleton can be detected in the mitotic cell cycle.

Our data show that in those *moe1*Δ *mal3*Δ cells that contain a monopolar spindle, only one of the SPBs contains γ-tubulin. This raises an intriguing possibility that the two SPBs in the same *S. pombe* cell are in fact different from one another. Indeed, there is evidence that the two SPBs in wild-type cells are different biochemically. For example, the Cdc7 protein kinase has been shown to preferentially associate with one of the SPBs in a cell cycle-dependent manner (Sohrmann *et al.*, 1998; Cerutti and Simanis, 1999). Furthermore, we note that one of the centrioles in the centrosome must recruit additional proteins to reach a state of competency necessary for proper microtubule nucleation (reviewed by Andersen, 1999). We wonder whether one of the SPBs must also recruit additional proteins, such as γ-tubulin, to function properly in *S. pombe*, after SPB duplication.

How do Moe1 and Mal3 interact to affect microtubule functioning? We observed that some of the microtubule phenotypes induced by *moe1*Δ, namely, excessive length and stability, are no longer present in *moe1*Δ *mal3*Δ cells, which supports a hypothesis that Moe1 influences microtubule length and stability by acting through Mal3. We note that Moe1 also plays a role in modulating the number of microtubules. This role does not seem to involve Mal3 because *moe1*Δ *mal3*Δ cells have the same abnormal number of microtubules as do *moe1*Δ, but not *mal3*Δ, cells. Alternatively, Moe1 and Mal3 may affect microtubule functioning independently, and the microtubule morphology in *moe1*Δ *mal3*Δ cells is a combined effect caused by both *moe1*Δ and *mal3*Δ. It is puzzling that *moe1*Δ *mal3*Δ cells contain interphase

microtubules that are shorter (and more abundant) but spindles that are longer than normal. We have two interpretations. A long spindle that is resistant to depolymerization may block the formation of interphase microtubules in the next cell cycle, which results in the formation of short microtubules. Alternatively, long microtubules are generated in both interphase and mitosis, but most of them are unstable in interphase and thus appear shorter.

We believe that Moe1 interacts with Mal3 and/or microtubules in a transient and highly regulated manner. Although Moe1 can bind Mal3 when both are expressed in *E. coli*, we have been unable to coprecipitate Moe1 and Mal3 from fission yeast lysates (Chen and Chang, unpublished results). We speculate that Moe1 and Mal3 are capable of binding directly, but the accessibility of their binding domains is tightly regulated in *S. pombe*.

Mal3 homologues are present in budding yeast, fission yeast, and humans. Moe1 is present in fission yeast and humans (and other higher eukaryotes such as *Drosophila, Caenorhabditis,* and *Arabidopsis*; Chen *et al*., 1999) but absent from budding yeast. We believe that this intriguing distribution of the Moe1-Mal3 protein complexes among various species underscores the specific need for an organism to regulate its microtubule dynamics and/or the spindle functioning. For example, unlike most eukaryotic systems including fission yeast, budding yeast does not have a characteristic prophase (Kilmartin and Adams, 1984; O'Toole *et al*., 1999). The spindle nucleation in budding yeast does not coincide with entry into M phase; in fact, it has a spindle throughout most of its interphase. In contrast, spindle formation in fission yeast must be synchronized with the onset of mitosis. It is possible that fission yeast requires molecules such as Moe1 that are not present in budding yeast to fine-tune these events.

EB1 was first isolated based on its ability to bind APC in the C terminus (Su *et al*., 1995), a region that is frequently truncated in colon cancer. More important, it has been reported that cell lines derived from colorectal tumors display a high degree of aneuploidy (Lengauer *et al*., 1997), a phenotype similar to the chromosome missegregation seen in *moe1*Δ *mal3*Δ cells. Because our data indicate that EB1 and HsMoe1 can genetically and physically interact in yeast, it is highly probable that they can interact in humans to influence spindle functioning and genome stability.

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