Ase1p Organizes Antiparallel Microtubule Arrays during Interphase and Mitosis in Fission Yeast^V

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Submitted October 14, 2004; Revised January 10, 2005; Accepted January 21, 2005

Monitoring Editor: Tim Stearns

Proper microtubule organization is essential for cellular processes such as organelle positioning during interphase and spindle formation during mitosis. The fission yeast *Schizosaccharomyces pombe* presents a good model for understanding microtubule organization. We identify fission yeast *ase1p*, a member of the conserved ASE1/PRC1/MAP65 family of microtubule bundling proteins, which functions in organizing the spindle midzone during mitosis. Using fluorescence live cell imaging, we show that *ase1p* localizes to sites of microtubule overlaps associated with microtubule organizing centers at both interphase and mitosis. *ase1Δ* mutants fail to form overlapping antiparallel microtubule bundles, leading to interphase nuclear positioning defects, and premature mitotic spindle collapse. FRAP analysis revealed that interphase *ase1p* at overlapping microtubule minus ends is highly dynamic. In contrast, mitotic *ase1p* at microtubule plus ends at the spindle midzone is more stable. We propose that *ase1p* functions to organize microtubules into overlapping antiparallel bundles both in interphase and mitosis and that *ase1p* may be differentially regulated through the cell cycle.

INTRODUCTION

Proper microtubule organization is essential for diverse cellular functions, ranging from organelle positioning during interphase to bipolar spindle formation and elongation during mitosis (Kirschner and Mitchison, 1986; Hyman and Karsenti, 1998; Reinsch and Gonczy, 1998; Morris, 2003; Pearson and Bloom, 2004). One key determinant of microtubule organization is the microtubule organizing center (MTOC). MTOCs exist in all eukaryotes and share three major properties: nucleation, organization, and attachment of microtubule arrays to the proper cellular organelles. In many cell types, a well-characterized canonical MTOC is the centrosome. Centrosomes function to nucleate microtubules, to organize the microtubules into a radially symmetric structure with the minus ends anchored at the centrosome and the distal plus ends extending toward the cell cortex and to attach the microtubule arrays to the nucleus (Bornens, 2002). However, other highly differentiated cell types such as myotubes, polarized epithelial cells, and neuronal cells display more complex arrangement of MTOCs that organize microtubules into nonradial, linear arrays (Tassin *et al.*, 1985; Butner and Kirschner, 1991; Meads and Schroer, 1995). For examples, myotubes appear to nucleate microtubules everywhere around the nuclear periphery and then arrange them as linear arrays parallel to the long axis of the myotube (Toyama *et al.*, 1982). Polarized epithelial cells have a dominant centrosome as well as noncentrosomal linear arrays of

microtubules that are attached via their minus ends at the apical cell membrane and run parallel to the basal-apical axis of the cell (Tucker *et al.*, 1998; Mogensen *et al.*, 1993). Neuronal cells have long axons with linear bundles of microtubules oriented with their plus ends pointing away from the cell body (Wuerker and Kirkpatrick, 1972; Lewis *et al.*, 1989). Although microtubule nucleation, a process mediated by the γ -tubulin ring complex of proteins (γ -TuRC), and to a lesser extent, centrosome attachment to the nucleus, have been well studied, the mechanism of how linear microtubule arrays are organized is unknown.

The fission yeast has emerged as an excellent model system for studying the microtubule cytoskeleton (Hagan, 1998). Its genome is sequenced (Wood *et al.*, 2002). Its genetics and cytology are relatively simple compared with larger eukaryotes (Forsburg, 2001). Cellular components can be dynamically monitored by imaging living cells expressing fluorescently tagged proteins (Tran *et al.*, 2004), providing a convenient assay for examining the effects of mutation. Evolutionary conservation makes insights gain from fission yeast highly relevant to understanding similar processes in higher eukaryotes. The fission yeast has three classes of MTOCs that are regulated during the cell cycle: the spindle pole body (SPB), the equatorial MTOC (eMTOC), and multiple interphase MTOCs (iMTOCs; Hagan and Petersen, 2000). The SPB, the equivalent of the centrosome, embeds into the nuclear envelope and organizes the mitotic spindle and astral microtubules during mitosis (Hagan and Petersen, 2000). During interphase, the SPB is attached to the nuclear envelope and associates with one of the interphase microtubule bundles (Hagan and Petersen, 2000). The eMTOC appears as a partial ring of γ -TuRC at the start of cytokinesis and contracts with the actin-myosin ring during cell division (Hagan and Petersen, 2000). The eMTOC nucleates a postanaphase array (PAA) of microtubules from the septum site during cytokinesis (Hagan and Petersen,

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-10-0899>) on February 2, 2005.

^V The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

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2000). At the completion of cell division, the eMTOC dots of γ -TuRC break down to form multiple iMTOC dots of γ -TuRC at the cell nuclear periphery (Zimmerman *et al.*, 2004). The function of eMTOC and its associated PAA microtubules are not well understood. Recent experiments suggest that eMTOCs may function to maintain the position of the actin contractile ring at the cell middle (Pardo and Nurse, 2003). Interphase microtubules are organized by multiple iMTOCs as linear bundles that run parallel to the long axis of the cell (Drummond and Cross, 2000; Tran *et al.*, 2001). In general, each iMTOC is associated with the nuclear membrane and organizes microtubules in antiparallel manner, with the dynamic microtubule plus ends extending toward the cell tips and the stable minus ends overlapping at the nuclear periphery (Drummond and Cross, 2000; Tran *et al.*, 2001). This symmetrical and linear organization enables the microtubule plus ends to produce a balance of pushing forces to position the nucleus at the center of the cell (Tran *et al.*, 2001). How the symmetric linear bundles of microtubules are organized is not known.

All three classes of the fission yeast MTOC share the organization of linear arrays of microtubule found in higher eukaryotic cells such as neurons, myotubes, and polarized epithelial cells. In particular, the fission yeast MTOCs may represent a conserved organizational pathway leading to the formation of linear microtubule structures with overlapping antiparallel microtubules. In this study, we identify *ase1p*, a fission yeast homolog of the conserved ASE1/PRC1/MAP65 family of microtubule bundling proteins found at the spindle midzone during mitosis (Chan *et al.*, 1999; Mollinari *et al.*, 2002; Schuyler *et al.*, 2003; Verbrugghe and White, 2004; Verni *et al.*, 2004). Surprisingly, fission yeast *ase1p* has dual functions at interphase and mitosis. *ase1p* functions to stabilize the antiparallel overlapping bundles of minus-ended microtubules associated with the iMTOCs during interphase, the overlapping cytoplasmic astral microtubule minus ends associated with the SPBs, and the plus-ended microtubule spindle midzone during mitosis. As a consequence, mutant cells lacking *ase1p* have severe defects in microtubule organization, leading to nuclear positioning defects during interphase and spindle elongation defects during mitosis. Our studies suggest that the dynamics of *ase1p* appear to be differentially regulated between interphase and mitosis. We propose a model for how *ase1p* organizes linear arrays of microtubules to function in diverse cellular processes.

MATERIALS AND METHODS

Strains and Preparations

Standard *Schizosaccharomyces pombe* genetic and molecular biology techniques and media were used as described in the "Nurse Lab Manual" (http://www.sanger.ac.uk/PostGenomics/S_pombe/docs/nurse_lab_manual.pdf).

We constructed the *ase1p*-GFP and *ase1Δ* strains by the PCR-based, LiAc transformation method previously described (Bahler *et al.*, 1998). Briefly, a Blast search at the Sanger Center *S. pombe* gene server for genes similar to the ASE1/PRC1/MAP65 family of microtubule bundling protein identified the *S. pombe* ORF SPAPB1A10.09. We named this gene *S. pombe ase1+*. GFP tagging of *ase1+* at its C-terminal chromosomal locus was performed using the pFA6a-GFP-kanMX6 plasmid (Bahler *et al.*, 1998) and these primers: Forward, 5'-CTA CCA ACA TTT TTT CTG CTC CAC TCA ACA ATA TTA CAA ATT GTA CAC CGA TGG AGG ATG AAT GGG GAG AAG AAG GCT TTC GGA TCC CCG GGT TAA TTA A-3', and Reverse, 5'-CTT TTA TGA ATT ATC TAT ATG CTG TAT TCA TAT GCA AAA ATA TGT ATA TTT AAA TTT GAT CGA TTA GGT AAA TAA GAA GCG AAT TCG AGC TCG TTT AAA C-3'. Knockout of *ase1+* was performed using these primers: Forward, 5'-AGT TTT TAT ATC TTC CTT TAT ATT CTA TTA ATT GAA TTT CAA ACA TCG TTT TAT TGA GCT CAT TTA CAT CAA CCG GTT CAC GGA TCC CCG GGT TAA TTA A-3', and Reverse, 5'-CTT TTA TGA ATT ATC TAT ATG CTG TAT TCA TAT GCA AAA ATA TGT ATA TTT AAA TTT GAT

CGA TTA GGT AAA TAA GAA GCG AAT TCG AGC TCG TTT AAA C-3'. The resulting PRC products were transformed into haploid strains. G418-resistant transformed cells were screened by PCR for proper integration. The *ase1p*-GFP fusion protein was functional, as judged by proper cell morphology and cytoskeletal organization. The *ase1Δ* strain was viable, but with striking defects in microtubule organization.

Strains used in this study were: PT47 *h⁻ leu1.32 + nmt1::GFP-tub1*; PT65 *h⁺ leu1.32 nup107::nup107-GFP:kanMX6 + nmt1::GFP-tub1*; PT104 *h⁻ leu1.32 + nmt1::GFP-sad1*; PT286 *h⁻ ade6.210 leu1.32 ura4.D18*; PT306 *h⁺ leu1.32 ura4.D18 ase1::ase1-GFP:kanMX6*; PT309 *h⁺ leu1.32 ura4.D18 ase1::kanMX6*; PT311 *h⁺ leu1.32 ura4.D18 ase1::kanMX6 + nmt1::GFP-tub1*; PT335 *h⁺ leu1.32 ase1::ase1-GFP:kanMX6 nup107::nup107p-GFP:kanMX6 + nmt1::GFP-tub1*; PT337 *h⁺ leu1.32 ura4.D18 ase1::kanMX6 + nmt1::GFP-sad1*; PT338 *h⁺ leu1.32 ura4.D18 ase1::ase1-GFP:kanMX6 + nmt1::CFP-tub1*; PT403 *h⁺ leu1.32 cdc25.22^{ts} ase1::ase1-GFP:kanMX6*.

Western Blotting

Cell cycle mutant *cdc25-22* expressing *ase1p*-GFP (PT403) was shifted to the restrictive 36°C for 4 h, arresting cells in G₂. Synchronized cells were then released from the G₂ arrest by shifting back to the permissive 25°C. Total cell extracts were prepared at sequential time points, and Western blot analysis of *ase1p*-GFP was performed as described (Celton-Morizur *et al.*, 2005). Briefly, exponentially growing cells were centrifuged, washed in STOP buffer, and concentrated in 250 μ l phosphate-buffered saline containing 2 mM EDTA, 2 μ g/ml aprotinin, 2 μ g/ml pepstatin, 5 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Acid-washed glass beads, 250 μ l, were added and tubes submitted to 15 min of vortexing at maximum speed on a Vortex-Genie2 (Fischer Scientific, Pittsburgh, PA). After addition of 250 μ l of 2 $\times$ sample buffer (125 mM Tris, pH 6.8, containing 6% SDS, 10% 2-mercaptoethanol, and 20% glycerol), extracts were boiled for 5 min and centrifuged, and supernatants were recovered. Extracts were loaded on 6% SDS-PAGE and blotted on nitrocellulose. GFP was probed with an anti-GFP monoclonal antibody and a peroxidase-coupled anti-mouse and a chemoluminescent revelation kit.

Fluorescence Live Cell Imaging and Fluorescence Recovery after Photobleaching

Fluorescent live cell imaging was performed as previously described (Tran *et al.*, 2004). Briefly, midlogarithmic growing cells were placed in sealed growth chambers containing 2% agarose media. Images were acquired digitally by the MetaMorph-controlled (Universal Imaging, Downingtown, PA) Nikon E600fn microscope (Melville, NY) equipped with a PlanApo 100 $\times$ /1.45 NA TIRF oil immersion objective lens, a Yokogawa spinning-disk confocal scanner (PerkinElmer Life Sciences, Boston, MA), and a deep-cooled Hamamatsu ORCA II-ER CCD camera (Bridgewater, NJ). For single optical-section time lapse, images were collected at 1-s exposure and 5-s intervals. For 3D time lapse, each image stack consisted of 11 optical sections of 0.5- μ m spacing and 20-s intervals between stacks.

Fluorescence recovery after photobleaching (FRAP) was performed on the Zeiss LSM510-Meta confocal microscope (Thornwood, NY) equipped with a PlanApo 63 $\times$ /1.4 NA oil immersion objective lens and a 10 mW 488 nm argon-krypton laser for both GFP imaging (2% laser power) and FRAP (100% laser power). Time-lapse FRAP images were scanned at 2-s intervals.

Perfusion chambers were constructed by placing a coverslip coated with 1 mg/ml poly-lysine on two strips of double-stick tape on top of a glass slide. Cells incubated for 10 min with 10 mg/ml concanavalin A were resuspended in fresh media before flowing through the perfusion chamber, where upon they bind tightly to the surface of the coverslip. Subsequent perfusions of 50 μ g/ml methyl 2-benzimidazolecarbamate (MBC; Sigma, St. Louis, MO) and wash-out were performed by manual pipetting.

Data Analysis

Analysis of microtubule dynamics, nuclear positions, and intensity scans were all performed in MetaMorph as previously described (Tran *et al.*, 2001).

RESULTS

ase1p Belongs to the ASE1/PRC1/MAP65 Family of Microtubule-bundling Proteins

Previously, we have shown that proper nuclear positioning in the fission yeast during interphase requires multiple bundles of interphase microtubules that are organized in an antiparallel manner, with the minus ends overlapping and anchoring to the nuclear surface and the plus ends facing and interacting with the cell tips (Tran *et al.*, 2001). These semistable overlapping microtubule regions are termed iMTOCs (Tran *et al.*, 2001). iMTOCs have been shown to contain *alp4p* and *rsp1p*. *Alp4p* is an essential component of

the γ -TuRC (Vardy and Toda, 2000). Rsp1p is a chaperone protein regulating the formation of iMTOCs during interphase (Zimmerman *et al.*, 2004). In an effort to dissect the molecular structure of the iMTOCs, we searched the *S. pombe* genome for genes encoding orthologues of proteins known to function in microtubule bundling in different organisms. The ASE1/PRC1/MAP65 family has been recently characterized in the budding yeast *Saccharomyces cerevisiae*, mammalian cells, and plant cells, as having a function in bundling overlapping microtubules (Chan *et al.*, 1999; Molinari *et al.*, 2002; Schuyler *et al.*, 2003). The fission yeast genome encodes a single member (SPAPB1A10.09) of this family, which has not been previously characterized. To test whether this gene may function in microtubule organization in the fission yeast, we deleted SPAPB1A10.09 complete ORF to study its function in the fission yeast. We also created the fusion of SPAPB1A10.09 and the green fluorescent protein (GFP) to monitor its localization dynamics. Our results revealed that SPAPB1A10.09 protein localizes to sites of microtubule overlaps and that it functions to bundle microtubules (see below). Thus, we named SPAPB1A10.09 the fission yeast *ase1p* to be consistent with the yeast nomenclature.

ase1Δ cells Are Hypersensitive to the Microtubule-depolymerizing Drug MBC and Show Prolonged Growth Phase and Nonlinear Cell Shape

To study the possible roles of *ase1p* in microtubule organization, we constructed an *ase1Δ* fission yeast strain. *ase1Δ* cells are viable, suggesting that *ase1⁺* is not an essential gene. We compared wild-type fission yeast cells to *ase1Δ* cells. Wild-type cells grew in typical linear manner, reaching lengths of $13.41 \pm 2.12 \mu\text{m}$ ($n = 100$) before undergoing cell division with a medial septum position (Figure 1, A and C). In contrast, *ase1Δ* cells often grew slightly longer than wild-type cells, reaching lengths of $15.40 \pm 2.18 \mu\text{m}$ ($n = 100$) before dividing. Further, *ase1Δ* cells did not grow in a strict linear manner, with some cells showing bent or wavy shape and a slight septum positioning defects (Figure 1, A and C). We next examined the doubling time of wild-type and *ase1Δ* cells. *ase1Δ* cells showed consistent slower doubling time throughout a range of temperature examined (Figure 1B). At 25°C, wild-type cells doubled every $4.07 \pm 0.18 \text{ h}$ ($n = 5$) compared with *ase1Δ* at $5.93 \pm 0.78 \text{ h}$ ($n = 5$). The doubling time decreased with increasing temperature (Figure 1B). We further examined *ase1Δ* cells under different concentrations of MBC, the microtubule depolymerization drug (Tran *et al.*, 2001). With no added MBC, both wild-type and *ase1Δ* colonies grew robustly at 25°C, with *ase1Δ* cells showing slower doubling rate (Figure 1D). At increasing MBC concentrations, *ase1Δ* colonies showed consistent increasing growth retardation. Although 3 $\mu\text{g/ml}$ MBC showed no effect on wild-type colonies, the *ase1Δ* colonies showed severe inhibition of growth (Figure 1D). We conclude that *ase1Δ* cells have defects in the microtubule cytoskeleton.

ase1Δ Cells Have No Overlapping Regions of Interphase Microtubule Organization

We compared interphase microtubule organization of wild-type to *ase1Δ* cells by imaging and analyzing cells expressing GFP-tub1p. Striking defects in microtubule organization are seen in *ase1Δ* cells. Instead of the 3–5 discrete bundles of microtubules aligned parallel to the long axis of the cell found in wild-type cells, *ase1Δ* cells displayed 3–9 discrete microtubule bundles, which were not aligned with the long axis of the cell (Figure 2A). Moreover, although wild-type cells have an average of 4 ± 1 ($n = 100$) microtubule bundles

Figure 1. *ase1Δ* cells grow slower, are longer, and are sensitive to microtubule depolymerization drug. (A) Differential interference contrast (DIC) image of wild-type (PT286) and *ase1Δ* (PT309) cells. *ase1Δ* cells are generally longer, have nonlinear cell shape, and have septum-positioning defects compared with wild-type (yellow bars). Bar, 5 μm . (B) Doubling time of wild-type (PT286) and *ase1Δ* (PT309) cells under a range of temperature. Student's *t* statistics showed that *ase1Δ* cells takes longer to double than wild-type ($p < 0.05$). (C) Histograms of cell lengths at time of mitosis. Wild-type (PT286) cells are $13.41 \pm 2.12 \mu\text{m}$ ($n = 100$), and *ase1Δ* (PT309) cells are $15.40 \pm 2.18 \mu\text{m}$ ($n = 100$) [mean $\pm$ SD; $p < 0.05$]. (D) Carbendazim (MBC) sensitivity assay for wild-type (PT286) and *ase1Δ* (PT309) cells grown on YE5S plates with varying concentrations of MBC.

per cell, *ase1Δ* cells have an average of 6 ± 1 ($n = 100$) microtubule bundles per cell (Figure 2B).

Intensity measurements along the entire length of the microtubule bundles further showed that microtubule bundles in *ase1Δ* cells does not have a medial region of higher fluorescent intensity as observed in microtubule bundles in wild-type cells (Figure 2C). Further, the relative intensity along the length of the microtubules in *ase1Δ* cells appeared equivalent to the intensity of the nonoverlapping regions of microtubule in wild-type cells (Figure 2C), suggesting a total absence of microtubule overlap inside the *ase1Δ* microtubule bundles.

Finally, we compared the ratio of microtubules extending toward opposite cell tips. In wild-type cells, an iMTOC-organized interphase microtubule bundle is bilaterally symmetric, with the minus ends overlapping at the cell center and the distal plus ends extending toward the cell tips. This organization necessarily leads to an equal number of microtubules facing one tip of the cell compared with the opposite cell tip, leading to a ratio of 1. Accordingly, in the wild-type cells, throughout the approximate 1-h duration of observed interphase, this ratio remained relatively constant at 1 (Figure 2, D and E). In contrast, in *ase1Δ* cells, this ratio varied tremendously, ranging from a ratio of 1, to a highly asymmetric ratio of 4-to-1 (Figure 2, D and E).

In conclusion, the approximate doubling of microtubule structures and the lack of overlapping medial regions in *ase1Δ* cells suggest that a wild-type microtubule bundle consists of two half-bundles that are bundled together at the overlapping medial region by *ase1p*. These results also indicate that although *ase1p* may play a role in bundling

Figure 2. *ase1Δ* cells have disorganized interphase microtubules and fail to organize microtubule overlapping regions. (A) Wild-type (PT47) and *ase1Δ* (PT311) cells expressing GFP-tub1p. 3D projection images are rotated 90° for cross-sectional view of cells. Wild-type cells have discrete linear bundles of microtubules with medial regions of overlaps aligned parallel to the cell long axis. *ase1Δ* cells have bundles of microtubules with no apparent medial regions of overlaps and that are not parallel to the cell axis. Bar, 5 μm. (B) Histogram of the number of microtubule bundles per wild-type (green) and *ase1Δ* cells (red; n = 100). (C) Intensity scans of individual microtubule bundles 1, 2, and 3 taken from A. Comparisons of wild-type microtubule intensity (green) to *ase1Δ* microtubule intensity (red). *ase1Δ* cells do not have a medial region of higher fluorescence intensity, consistent with not having medial regions of microtubule overlaps. (D) 3D projection time-lapse images of bilaterally symmetric microtubule organization in wild-type and *ase1Δ* interphase cells. Each 5-min interval produces a complete turnover of microtubule organization. (E) Plot of changes in microtubule symmetry during interphase. Wild-type cells (green) have microtubule bundles that are bilaterally symmetric, leading to a symmetry ratio of 1. *ase1Δ* cells (red) do not have bilaterally symmetric microtubule bundles, leading to wide fluctuations and deviation from the symmetric ratio of 1.

overlapping microtubules, it does not play a role in microtubule nucleation.

ase1p Stabilizes Overlapping Microtubules at the iMTOC Sites, but Is Not Required for Microtubule Nucleation

To understand the function of *ase1p* at sites of microtubule overlaps, we examined the stability of iMTOC-organized microtubules in wild-type and *ase1Δ* cells expressing GFP-tub1p. We imaged microtubules in cells in a perfusion chamber before and after the addition of 50 μg/ml MBC. Before the addition of MBC, wild-type and *ase1Δ* cells both showed long and dynamic bundles of microtubules (Figure 3A; Movie_1_wt_MT_MBC.mov and Movie_1_ase1Δ_MT_MBC.mov). Within 1 min after perfusion of MBC, all microtubules had begun to depolymerize (Figure 3A, 1 min). In wild-type cells, microtubule depolymerization started from the distal microtubule tips and proceeded toward overlapping medial regions where it abruptly stopped, leaving remnants of the stabilized microtubule overlaps (Figure 3A, 5 min). These remnants of microtubule overlaps were located around the nuclear periphery and remained stable during the 5-min incubation in MBC (Figure 3A). Similarly, microtubules in *ase1Δ* cells also quickly depolymerized upon MBC perfusion (Figure 3A). However, microtubule depolymerization proceeded to completion in *ase1Δ* cells, resulting in very few remnants of microtubule overlaps observed at the nuclear periphery region (Figure 3A, 5 min; and Figure 1C, 0 s). Although wild-type cells retained an average of 4 ± 1 (n = 100) microtubule remnants per cell, *ase1Δ* cells

retained an average 1 ± 1 (n = 100) microtubule remnants per cell (Figure 3B).

In wild-type cells, the stable remnants of microtubule-overlapping regions repolymerized microtubule symmetrically from both ends immediately upon the removal of MBC by perfusion with fresh cell media (Figure 3A, 6 min; and Figure 3C). Within 5 min after MBC-washout, the multiple interphase microtubule bundles were reestablished (Figure 3A, 7 min). In contrast, upon MBC-washout, new microtubules were observed to polymerize from sites at the nuclear region and elsewhere in the cytoplasm in *ase1Δ* cells (Figure 3C, yellow arrows). These results suggest that *ase1p* helps to stabilize microtubule structures from MBC-induced depolymerization and may also play a role in restricting sites of microtubule nucleation to the nuclear region. However, *ase1p* does not appear to function in microtubule nucleation.

ase1p Does Not Affect Microtubule Growth and Shrinkage Dynamics

To determine if *ase1p* affects microtubule dynamics, we measured growth and shrinkage rates of individual microtubule bundles in wild-type and *ase1Δ* cells. Because we have shown previously that in wild-type cells, the two ends of interphase microtubule bundles display similar plus-end dynamics (Tran *et al.*, 2001), we therefore considered the stable overlapped medial regions as the iMTOCs, the origin for microtubule polymerization (Figure 4A, 0 s, yellow as-

Figure 3. *ase1p* is required to stabilize and anchor microtubule overlapping regions to the nuclear membrane from MBC-induced microtubule depolymerization. (A) Wild-type (PT47) and *ase1Δ* (PT311) cells expressing GFP-tub1p. A flow chamber was constructed for efficient perfusion of 50 $\mu\text{g}/\text{ml}$ MBC and subsequent washout with fresh media. Before MBC treatment, both wild-type and *ase1Δ* cells have long microtubule bundles (-1 min). Immediately after MBC treatment (0 through 5 min), microtubules initiate catastrophe and depolymerize from the distal cell tips toward the cell center (1 min). After 5 min of MBC treatment, stable remnants of microtubules are still localized to the cell center in wild-type cells (5 min). Similar treatment and duration for *ase1Δ* cells yielded fewer stable microtubule remnants around the cell center. On washout (6 min), microtubule repolymerized from the stable remnants at the cell center in wild-type cells. In *ase1Δ* cells, microtubule repolymerization can be seen in the cytoplasm far from the cell center (6 min). (B) Histogram of the number of stable microtubule remnants remaining per cell after 5 min of MBC treatment ($n = 100$). (C) High temporal resolution of microtubule repolymerization after wash-out of MBC in wild-type and *ase1Δ* cells expressing GFP-tub1p. Immediately after MBC wash-out (0 s) the wild-type cell repolymerized its microtubules from the stable microtubule remnants constricted at the cell nucleus in the middle of the cell. In contrast, microtubule repolymerization in the *ase1Δ* cell occurred at the nuclear region as well as the cell cytoplasm away from the nuclear region (yellow arrows). Bar, 5 μm .

terisk). From this medial region, each half of the interphase microtubule bundles were measured to grow at 1.88 ± 0.78 $\mu\text{m}/\text{min}$ ($n = 20$), and to shrink at 12.35 ± 3.24 $\mu\text{m}/\text{min}$ ($n = 20$; Figure 4, A and B, green arrows; Table 1). During the time when both halves of each bundle exhibited growth, we measured a combined growth rate at 3.65 ± 0.48 $\mu\text{m}/\text{min}$ ($n = 10$), or approximately twofold the rate of each half. Similarly, during the time when both halves of each bundle exhibited shrinkage, we measured a combined shrinkage rate at 27.66 ± 3.37 $\mu\text{m}/\text{min}$ ($n = 10$), or approximately twofold the rate of each half (Figure 4, A and B, green arrows; Table 1). In contrast, because individual bundles of microtubule in *ase1Δ* cells have no stable overlapping regions, we measured end-to-end lengths as a function of time. Interphase microtubule bundles in *ase1Δ* cells exhibited a growth rate of 2.29 ± 0.79 $\mu\text{m}/\text{min}$ ($n = 20$) and a shrinkage rate of 11.30 ± 3.60 $\mu\text{m}/\text{min}$ ($n = 20$; Figure 4, A and B, red and orange arrows; Table 1). Student's *t*-test analysis re-

Figure 4. Microtubule growth and shrinkage dynamics is similar in wild-type and *ase1Δ* cells. (A) Time-lapse inverted-intensity images of wild-type (PT47) and *ase1Δ* (PT311) cells expressing GFP-tub1p. The wild-type cell shows a microtubule bundle organized in a bilaterally symmetric manner with medial stable region SPB and iMTOC at the cell center and dynamic distal microtubule ends facing the cell tips (dark and light green arrows). Each microtubule end grows and shrinks independently, but still remain connected at the medial region (yellow asterisk). The *ase1Δ* cell shows two microtubules (red and orange arrows) growing at different time and different orientation. There is no stable medial region that organizes the two microtubules. Bar, 5 μm . (B) Plot of individual microtubule growth and shrinkage velocities. Data were taken from microtubules presented in A. The various color arrows are matched with A. The double-arrows color lines mark duration where the microtubule growing tips make contact with the cell wall. Numbers represent microtubule velocity of each growth and shrinkage phase in $\mu\text{m}/\text{min}$.

vealed that the growth and shrinkage rates of individual microtubule bundles in *ase1Δ* cells were similar to the growth and shrinkage rates of each halves of the wild-type microtubule bundles (Table 1). Therefore, our results suggest that *ase1p* functions to bundle and stabilize overlapping existing microtubules during interphase, but does not play a role in microtubule plus-end dynamics.

Misorganized Interphase Microtubules in *ase1Δ* Cells Cannot Properly Push the Nucleus to the Cell Center

The position of the fission yeast interphase nucleus has been shown to determine the subsequent position of the contractile ring and septum during cell division (Tran *et al.*, 2000). An essential function of interphase microtubule bundles in the fission yeast is to produce polymerization-driven pushing forces for proper nuclear positioning at the cell middle (Tran *et al.*, 2001). The organization of the interphase microtubule bundles, with antiparallel overlapping medial regions anchored to the nuclear membrane, has been proposed to create a force balance, where each half of a bundle can independently exert pushing forces to bring the nucleus to the cell middle (Tran *et al.*, 2001). We compared nuclear movements in wild-type and *ase1Δ* cells by imaging the cells expressing GFP-sad1p, a marker for the SPB, iMTOCs, and

Table 1. Growth and shrinkage velocities of wild-type vs. *ase1Δ* cells

	Wild-type		<i>ase1Δ</i> (n = 20)	Statistics (wt vs. <i>ase1Δ</i>)	
	Half bundle (n = 20)	Full bundle (n = 10)		Half bundle	Full bundle
Free growth velocity (mm/min) ^a	1.88 ± 0.24	3.65 ± 0.48	2.29 ± 0.79	=	≠
Contacted growth velocity (mm/min) ^b	0.78 ± 0.44	1.58 ± 1.41	2.20 ± 0.99	≠	≠
Shrinkage velocity (mm/min)	12.35 ± 3.24	27.66 ± 3.27	11.39 ± 3.60	=	≠

Values reported as mean ± SD. Student's *t* statistics assuming two-tailed equal variance at *p* < 0.05.

^aFree growth velocity is measured for microtubule growing unhindered in the cytoplasm.

^bContacted growth velocity is measured for microtubule growing while remaining in contact with the cell tip.

the nuclear membrane (Hagan and Yanagida, 1995; Tran *et al.*, 2001). In wild-type cells, the SPB and iMTOCs appeared as several highly fluorescent motile dots around the nuclear membrane. The SPB and iMTOCs were observed to oscillate independently in a direction parallel to the long axis of the cell (Figure 5, A and B, green arrows; Movie_3_wt_sad1GFP.mov). The oscillation is periodic, with each cycle occurring every 4–6 min (Figure 5B, green arrows and lines). In contrast, SPB oscillation in *ase1Δ* cells appeared erratic, with no apparent periodicity (Figure 5, A and B, red arrow and line; Movie_3_ase1Δ_sad1GFP.mov). We observed movement of the SPB in one direction, and instead of switching back immediately and moving in the opposite direction, it abruptly stopped for a sustained length of time (Figure 5, A and B). SPB and iMTOC oscillation in wild-type cells is consistent with an antiparallel microtubule bundle producing a balance of pushing forces to dynamically adjust the nucleus to the cell center. SPB oscillation in *ase1Δ* cells is consistent with the interpretation that the half microtubule bundle nucleated from and anchored to the nuclear membrane can produce pushing forces to move the nucleus in one direction; however, without the overlapping antiparallel opposite half, no reverse movement of the nucleus in the opposite can occur to produce an oscillation.

The failure of *ase1Δ* cells to produce proper nuclear pushing lead to a nuclear positioning and subsequent septum positioning defects, with two unequally sized cells after cell division (Figures 1C and 5C). Using GFP-sad1p to monitor the position of the nucleus, we quantified nuclear positioning defects in wild-type and *ase1Δ* cells. In a random population of cells, nuclear position can be expressed as a ratio of the short-to-long lengths of the position of the nucleus to the respective short and long cell tips. In wild-type cells, the nuclear short-to-long ratio range from 0.8 to 1 (n = 100), with 1 being perfectly centered (Figure 5D). In contrast, *ase1Δ* cells displayed a larger range of nuclear short-to-long ratio from 0.5 to 1 (n = 100), with significantly fewer cells able to place their nuclei at the cell center (Figure 5D). We conclude that failure to form proper antiparallel interphase microtubule bundles will lead to nuclear positioning defects and septum positioning defects.

ase1p Stabilizes the Bipolar Spindle during Mitosis

During mitosis three distinct phases of spindle elongation have been defined according to their distinct elongation velocities (Nabeshima *et al.*, 1998). Detailed structural studies using electron microscopy further revealed that each phase corresponds to distinct total microtubule numbers

Figure 5. Nuclear positioning is defective in *ase1Δ* cells. (A) Time-lapse images of wild-type (PT104) and *ase1Δ* (PT337) cells expressing the SPB, iMTOC, and nuclear membrane marker GFP-sad1p. In the wild-type cell, the SPB (dark green arrow) and one iMTOC (light green arrow) are present in this optical section. They undergo microtubule-dependent oscillation within the cell. In contrast, the *ase1Δ* cell shows only the single SPB (red arrow), which remains relatively static, with no oscillation. Bar, 5 μm. (B) Plot of SPB and iMTOC oscillations in wild-type and *ase1Δ* cells. Data were taken from SPB and iMTOC presented in A. (C) DIC and merged image of wild-type and *ase1Δ* cells expressing GFP-sad1p. The wild-type cells have linear rod-shaped cell morphology, with the nuclei located at the geometrical center of the cells. In contrast, the *ase1Δ* cells appear long, nonlinear, with the nucleus misplaced from the cell center (yellow arrow). Bar, 5 μm. (D) Comparison of nuclei positions in wild-type and *ase1Δ* cells. The septum divides the cell into two halves. Ratio of short-to-long cell halves provides a measure of nuclear and septum deviation from the geometrical center of the cell (n = 100).

Figure 6. *ase1Δ* cells have defective spindle organization and elongation. (A) 3D projection time-lapse images of wild-type (PT65) and *ase1Δ* (PT335) mitotic cells expressing GFP-tub1p. Although the wild-type cell forms discrete bundles of single linear astral microtubules during anaphase B (45 min, yellow arrows), the *ase1Δ* cell forms disorganized, nonlinear astral microtubules that can separate from the SPB (35 and 41 min, red arrows). Bar, 5 μm. (B) High temporal resolution plot of the three phases of spindle elongation. The wild-type cell (green) shows smooth transitions between the different phases. The *ase1Δ* cell (red) shows abrupt and frequent spindle collapse, particularly during phase II, or metaphase and anaphase A. (C) Zoom image of phase II of the spindles in B, showing abrupt and frequent spindle collapse during phase II or metaphase and anaphase A in the *ase1Δ* (red) cell. (D) Kymographs of spindle elongation in wild-type and three *ase1Δ* cells. *ase1Δ* cells have relatively shorter spindles. Bar, 10 μm. (E and F) Intensity scans of wild-type and *ase1Δ* spindles at phase III, or Anaphase B, taken from cells in D. The wild-type cell has a middle region of higher intensity, indicative of the overlapping spindle midzone. In contrast, the *ase1Δ* cell has no middle region of higher intensity, indicative of no spindle midzone. (G) The spindle structures of wild-type and *ase1Δ* cells at phase III. Wild-type cell shows stable spindle structure that has extended to the cell tips. *ase1Δ* cells show abrupt and premature collapse of the spindle, leading to short spindle structures that have not reached the cell tips. Bar, 5 μm. (H) Histogram of the ratio of spindle length-to-cell length at the instant of spindle breakdown in wild-type and *ase1Δ* cells (Table 2).

and antiparallel organization (Ding *et al.*, 1993). The three phases are: I) spindle formation, where the spindle is initially organized and reach an average length of ~3 μm; II) metaphase and anaphase A, where chromosomes are congressed to the metaphase plate then subsequently segregated to opposite SPBs, without accompanying spindle elongation; and III) Anaphase B, where rapid spindle elongation occurs to separate the sister nuclei to opposite cell tips (Nabeshima *et al.*, 1998).

To test if *ase1p* plays a role in the organization of the mitotic spindle, we examined spindle structure and function throughout mitosis by imaging wild-type and *ase1Δ* cells expressing GFP-tub1p. Consistent with previous reports (Nabeshima *et al.*, 1998), wild-type cells formed spindles that displayed distinct three-phase elongation kinetics (Figure 6, A and B, Table 2; Movie_2_wt_MT.mov). Throughout mitosis, the spindle continually elongated. In particular, during phase III the cells showed prominent, discrete elongating spindles with midzone regions of microtubule overlap (Figure 6E). This midzone region is sustained until the spindle has reached the maximum length approximately equaled to the cell length (Figure 6A). In contrast, *ase1Δ* cells displayed spindle defects during phase II and III. During phase II, *ase1Δ* spindles failed to sustain a stabilized length. Instead, the spindle displayed frequent collapse, leading to periods of spindle length shrinkage and growth in its effort to elongate (Figure 6, A and B, Table 2; Movie_2_ase1Δ_MT.mov). During phase III, *ase1Δ* cells failed to form the overlapping microtubule midzone. Intensity measurements of wild-type spindles showed that the midzone region has two times the intensity compared with nonmidzone regions, and that each spindle halves are symmetric and equivalent in intensity (Figure 6, D and E). In contrast, intensity measurements of *ase1Δ* spindles showed that the medial region of double fluorescence intensity corresponding to the spindle midzone was not present, and that the spindle halves were not equivalent (Figure 6, D and E). In addition, *ase1Δ* spindles displayed premature spindle breakdown and failed to segregate the nuclei completely to the opposite cell tips (Figure 6, G and H). Wild-type spindles underwent breakage when they reached lengths of 12.29 ± 1.10 μm ($n = 20$), or $91 \pm 8\%$ ($n = 20$) of the cell lengths (Table 2). In contrast, *ase1Δ* spindles prematurely broke at lengths of 9.21 ± 1.96 μm ($n = 20$), or $60 \pm 19\%$ ($n = 20$) of the cell lengths (Table 2). Infrequently (5%, $n = 60$ spindles), the spindles would exhibit total collapse at phase II or early phase III, leading to two broken halves of a bipolar spindle (Figure 6G). These results suggest that *ase1p* is required for the formation of the spindle midzone, which is crucial for structural integrity of the bipolar spindle.

ase1p Localizes to All Microtubule Organizing Centers throughout the Cell Cycle

To monitor the localization of *ase1p* throughout the cell cycle, we performed two-color 3D imaging of living fission yeast cells expressing *ase1p*-GFP and CFP-tub1p (tubulin). *ase1p*-GFP was found in close association with all three classes of fission yeast MTOCs during the cell cycle. In interphase, each cell has several iMTOC-organized linear bundles of microtubules that are organized along the cell long axis as revealed by CFP-tub1p (Figure 7A). Each iMTOC-organized microtubule bundle, in turn, has a central region with higher fluorescent intensity compared with the rest of the bundle, consistent with the central region being a region of microtubule minus-ends overlap (Tran *et al.*, 2001). *ase1p*-GFP was associated with the region of microtubule

Table 2. Three-phase spindle elongation in wild-type vs. *ase1Δ* cells

	Wild-type (n = 20)	<i>ase1Δ</i> (n = 20)	Statistics (wt vs. <i>ase1Δ</i>)
Phase I (spindle formation)			
Duration (min)	11.42 ± 5.45	13.45 ± 3.29	=
Final spindle length (μm)	2.53 ± 0.47	2.60 ± 0.45	=
Elongation velocity (μm/min)	0.25 ± 0.08	0.20 ± 0.05	≠
Phase II (metaphase and anaphase A)			
Duration (min)	11.90 ± 7.25	17.04 ± 6.72	≠
Final spindle length (μm)	3.23 ± 0.29	2.82 ± 0.44	≠
Elongation velocity (μm/min)	0.09 ± 0.08	0.02 ± 0.02	≠
Phase III (anaphase B)			
Duration (min)	16.39 ± 3.12	14.85 ± 5.00	=
Final spindle length (μm)	12.29 ± 1.10	9.21 ± 1.96	≠
Elongation velocity (μm/min)	0.57 ± 0.11	0.49 ± 0.08	≠
Cell length at mitosis (μm)	13.62 ± 1.06	14.51 ± 0.64	≠
Ratio of final spindle-to-cell length	0.91 ± 0.08	0.60 ± 0.19	≠

Values reported as mean ± SD. Student's *t* statistics assuming two-tailed equal variance at *p* < 0.05

overlap of all interphase microtubule bundles as bars of varying lengths (Figure 7A, white arrows). During mitosis, when microtubules form a bipolar spindle, ase1p-GFP localized to the spindle midzone where plus ends of spindle microtubule overlap (Figure 7A, yellow arrow). During mitosis, ase1p-GFP also localized to the sites of astral microtubule overlaps on the SPB cytoplasmic surface (Figure 7A, red arrows). Finally, during late mitosis, ase1p-GFP localized to the site of the septum, where PAA microtubule bundles are forming (Figure 7A, blue arrow).

To test if there is a one-to-one correspondence of interphase microtubule bundles and ase1p-GFP bars, we independently measured cells expressing GFP-tub1p and cells expressing ase1p-GFP. Wild-type interphase cells displayed a range of 3–5 bundles of microtubules per cell, with an average of 4 ± 1 (*n* = 100) microtubules bundles per cell (Figure 7B). Similarly, wild-type interphase cells displayed a range of 3–6 bars of ase1p-GFP per cell, with an average of 4 ± 1 (*n* = 100) ase1p-GFP bars per cell. We conclude that ase1p localizes to regions of minus-ends microtubule overlaps associated with iMTOCs, SPBs, and eMTOCs throughout the cell cycle, and with region of plus-ends microtubule overlap at the spindle midzone during mitosis. This is consistent with ase1p playing a role in microtubule bundling at these sites, independent of microtubule polarity.

ase1p Has Different Dynamics at Interphase and Mitosis

Our results indicated that ase1p functions at both interphase and mitosis to bundle overlapping microtubules. The transition from interphase to mitosis is accompanied by a complete change in microtubule organization and dynamics. In the fission yeast, interphase microtubules are highly dynamic, exhibiting growth and shrinkage cycles every 4–5 min (Drummond and Cross, 2000; Tran *et al.*, 2001). In contrast, spindle microtubules appeared more stable, with some microtubules sustaining the growth state throughout the duration of spindle elongation, which last for ~45 min (Table 2).

To monitor the dynamics of ase1p throughout the cell cycle, we performed time-lapse 3D imaging of living fission yeast cells expressing ase1p-GFP. ase1p-GFP is highly dynamic throughout the cell cycle. During interphase, the multiple bars of ase1p-GFP can be seen to assemble near the nuclear region as a dot, which lengthens into a bar, and then

moves toward existing bars and incorporates into the existing bars (Figure 7C, yellow arrows, 3–7 min). Existing ase1p-GFP bars can shorten and disassemble (Figure 7C, yellow arrow, 11–12 min). All the interphase ase1p-GFP bars disassemble during the transition into mitosis (Figure 7C; Movie_4_ase1GFP.mov).

ase1p-GFP is associated with the mitotic spindle during all phases of mitosis. During phase I and II, a faint signal of ase1p-GFP was observed at the spindle (Figure 7C; Movie_4_ase1GFP.mov). During the transition from phase II to phase III, or anaphase A to anaphase B, ase1p-GFP redistributed to the overlapping microtubule midzone at the middle of the bipolar spindle (Figure 7C, red arrow, 41 min). Concurrent with the appearance of SPB-organized cytoplasmic astral microtubules during phase III, ase1p-GFP appeared at the SPBs (Figure 7C, red asterisks, 41 min). At the end of anaphase B, the PAA microtubules are organized at the site of the septum by the eMTOCs. At this time, ase1p-GFP also localized to the PAA microtubules at the site of the septum as dynamic bars of varying lengths (Figure 7C, blue arrows, 52–59 min).

To further quantify the dynamics of ase1p-GFP, we performed high temporal-resolution time-lapse imaging of ase1p-GFP in living cells. During interphase, ase1p-GFP were highly dynamic, exhibiting changes in their total lengths, either growing or shrinking, or assembling and disassembling (Figure 7D). We measured the average lengths of the ase1p-GFP bars to be 1.4 ± 0.5 μm (*n* = 100). Imaging also revealed multiple dots of ase1p-GFP moving from both cell tips in a linear, directed manner toward the central ase1p-GFP bars (Figure 7D). The movement of the ase1p-GFP dots during interphase has rate of 5.32 ± 1.21 μm/min (*n* = 10) and are consistent with directed motion from the microtubule plus end toward the microtubule minus end. In contrast, during mitosis, ase1p-GFP localizes to the spindle during phase II of spindle elongation and then concentrate at the spindle midzone during phase III. We did not observed motile ase1p-GFP dots during mitosis. These results suggest that ase1p-GFP is highly dynamic and that the dynamics may be regulated throughout the cell cycle.

FRAP Shows Two Populations of ase1p-GFP

To further probe the differential dynamics of ase1p during interphase compared with mitosis, we performed FRAP

Figure 7. *ase1p* colocalizes with sites of microtubule overlaps and are highly dynamic during interphase. (A) 3D projection images of wild-type (PT338) cells coexpressing CFP-tub1p (red) and *ase1p*-GFP (green). The merged images show that *ase1p*-GFP localizes to bright sites of microtubule overlaps throughout the cell cycle, i.e., microtubule overlaps associated with the iMTOCs (white arrows) during interphase, the SPBs (red arrows) and the eMTOCs (blue arrow) and with the spindle midzone (yellow arrow) during mitosis. Bar, 5 μ m. (B) Histogram of the number of microtubule bundles and *ase1p*-GFP bars per cell during interphase ($n = 100$). (C) 3D projection time-lapse images of a wild-type (PT306) cell expressing *ase1p*-GFP. During interphase, *ase1p*-GFP forms multiple bars that exhibit assembly and disassembly dynamics. Yellow arrows highlight a newly assembled *ase1p*-GFP dot, which increases in length to form an *ase1p*-GFP bar and then subsequently disassembled (3–7 and 11–12 min). During mitosis, the interphase *ase1p*-GFP bars disassembled (see the transition from 19 to 25 min), and new *ase1p*-GFP is recruited to the spindle midzone (41 min, red arrow) and the SPBs (red asterisks). During late mitosis, *ase1p*-GFP bars are recruited to the site of the septum, presumably to organize the PAA microtubules (52 and 59 min, blue arrows). Bar, 5 μ m. (D) High temporal resolution of interphase *ase1p*-GFP dynamics. The single optical section time-lapse images show a dynamic central bar (0 s, red asterisk) of *ase1p*-GFP. A single dot of *ase1p*-GFP appears (5 s, green arrows) and moves toward and incorporates into the central bar. At a later time (1060 s), the *ase1p*-GFP bar has moved to a new position, and a new dot of *ase1p*-GFP appears and moves toward the bar (1060 s, green arrow). The direction of movements of the *ase1p*-GFP dots are from the cell tips toward the cell center or from the microtubule plus end toward the microtubule minus end. Bar, 5 μ m.

analysis on cells expressing *ase1p*-GFP. FRAP analysis showed that the interphase *ase1p*-GFP bars can completely recover $86 \pm 12\%$ ($n = 9$) of the prebleached level (Figure 8, B and C), indicating that *ase1p*-GFP is completely mobile during interphase. In contrast, *ase1p*-GFP at the spindle midzone during anaphase B can only recover $25 \pm 5\%$ ($n = 8$) of its prebleached level (Figure 8, B and C), indicating that *ase1p*-GFP associated with the spindle midzone is highly immobile.

It has been reported that the cytoplasmic astral microtubule bundles, organized by the SPBs at the end of mitosis, behave similarly to the iMTOC-organized interphase microtubule bundles (Sagolla *et al.*, 2003). To confirmed this finding, and further delineate the differences between interphase and astral to that of mitotic *ase1p*, we simultaneously photobleached *ase1p*-GFP at the SPB region and the midzone region in mitotic cells. *ase1p*-GFP associated with the astral microtubules at the SPBs can recover $92 \pm 15\%$ ($n = 8$) of its prebleached level (Figure 8, B and C), similar to that of the interphase *ase1p*-GFP.

We measured the interphase *ase1p*-GFP recovery time $\tau_{1/2}$ to be 35 ± 14 s ($n = 9$) (Figure 8, B and C). The astral *ase1p*-GFP at the SPB showed a recovery time $\tau_{1/2}$ of 30 ± 9 s (Figure 8, B and C), similar to that of interphase *ase1p*-GFP. In contrast, FRAP analysis of the *ase1p*-GFP at the spindle midzone during anaphase B showed an ~ 10 -fold slower recovery, with an extrapolated $\tau_{1/2}$ of ~ 400 s ($n = 8$; Figure 8, B and C). These results suggest that *ase1p* may be highly dynamic in the cytoplasm and that its dynamic may be differentially regulated and attenuated inside the nucleus during mitosis.

To test whether *ase1p* is cell cycle regulated, we examined for possible modification of *ase1p* at different stages of the cell cycle. We detected *ase1p* in total cell extracts from *cdc25-22* cells expressing *ase1p*-GFP synchronized in the cell cycle by G_2 block and release. A gradual increase in a higher-molecular-weight form of *ase1p*-GFP was detected as cells entered mitosis (Figure 8D). This second form of *ase1p*-GFP gradually disappeared as cells exit mitosis and transit to the next cell cycle. These results are consistent with *ase1p*

Figure 8. ase1p-GFP has different mobility at interphase and mitosis. (A) Interphase and mitotic wild-type (PT306) cells expressing ase1p-GFP. A bar of ase1p-GFP is present in the interphase cell, and ase1p-GFP is present at the spindle midzone and the SPBs in the mitotic cell. The yellow dotted box shows the region of subsequent FRAP. (B) Time-lapse images of the FRAP experiments. During the time of FRAP (0 s, FRAP), almost 100% of the GFP signals are photobleached. Within 8 s of recovery, both the interphase ase1p-GFP bar and the mitotic SPB-organized ase1p-GFP dot appear as robust signals. At 50 s of recovery, both the interphase ase1p-GFP bar and the SPB-organized ase1p-GFP dot have almost fully recovered their fluorescence. In contrast, the spindle midzone does not recover as effectively during these times. Bar, 5 μ m. (C) Intensity plots of the FRAP experiment from B. (D) Western blot shows two forms of ase1p during mitosis (M) compared with interphase (G₂ and G₁/S), as assayed with the G₂/M cell cycle arrest mutant *cdc25-22* expressing ase1p-GFP (PT403).

being phosphorylated during mitosis and that phosphorylated ase1p may be inside the nucleus, whereas nonphosphorylated ase1p may be cytoplasmic.

DISCUSSION

The ASE1/PRC1/MAP65 Family of Microtubule-bundling Proteins

Fission yeast ase1p is a member of the conserved ASE1/PRC1/MAP65 family of microtubule-bundling proteins. In the budding yeast *Saccharomyces cerevisiae*, ase1p functions in anaphase spindle elongation by stabilizing the spindle midzone (Schuyler *et al.*, 2003). In *Caenorhabditis elegans*, SPD-1 functions in organizing a stable spindle midzone (Verbrugghe and White, 2004). In *Drosophila*, Feo functions in organizing the central spindle essential for subsequent cytokinesis (Verni *et al.*, 2004). Similarly, in human, PRC1 functions to organize the spindle midzone essential for sub-

sequent completion of cytokinesis (Mollinari *et al.*, 2002, 2004). To date, in these different organisms, no role has been implicated for ase1p during interphase. Fission yeast ase1p functions during mitosis to organize the spindle midzone, a role consistent with this family of protein. Interestingly, fission yeast ase1p also has a role during interphase to organize the interphase microtubule bundles. Furthermore, the differential dynamics and specific localization of ase1p on microtubule structures, i.e., microtubule minus ends during interphase and microtubule plus ends during mitosis at the midzone, suggests cell cycle-dependent regulation of ase1p. Fission yeast ase1p, therefore, performs dual roles during the cell cycle and shows complex regulation reminiscent of plants. In plant cells, MAP65 has multiple isoforms; some isoforms function during interphase to organize the cortical arrays of microtubules, and other isoforms function during mitosis to organize microtubules cytokinetic phragmoplast formation (Chan *et al.*, 1999, 2003; Muller *et al.*, 2004; Smertenko *et al.*, 2004; Wicker-Planquart *et al.*, 2004).

S. pombe ase1p Performs Dual Functions at Interphase and Mitosis

In the current studies, we have identified a dual function for ase1p. First, ase1p functions to organize microtubules nucleated by the multiple iMTOCs into bundles of antiparallel microtubule overlapping at their minus ends (Figure 7A). Each of the iMTOC microtubule bundles contains microtubules with their minus ends bundled together and connected to the nuclear membrane and their plus ends facing and dynamically interacting with the distal cell tips. This microtubule organization creates a bilaterally symmetric microtubule bundle, with equal microtubule numbers facing and interacting with opposing cell tips (Figure 4). This symmetry ensures that the microtubule-polymerization forces used to push the nucleus can achieve a balance, where pushing forces exerted from both sides of the nucleus creates frequent periodic nuclear oscillation to dynamically maintain the nucleus at the geometrical center of the growing cell (Figure 5B). In the loss-of-function *ase1Δ* mutant, microtubules failed to form bilaterally symmetric bundles (Figure 4). This asymmetry cannot generate a balance of opposing microtubule pushing forces, leading to infrequent microtubule pushing events, and failure to dynamically position the nucleus at the cell center (Figure 5B). The misplaced nucleus in *ase1Δ* mutant results in misplaced contractile ring and septum during cytokinesis (Figures 1A and 5C).

Second, ase1p functions during mitosis, where it localizes to the spindle midzone to stabilize the interdigitating microtubule plus ends that emanate from opposite SPBs (Figures 6 and 7A). This interaction stabilizes the bipolar spindle, preventing it from collapsing during metaphase and anaphase A, and from breaking prematurely during anaphase B. Our FRAP data are further evidence that ase1p is highly mobile during interphase (Figure 8). In contrast, ase1p is drastically less mobile during mitosis. Finally, our biochemical data revealed two forms of ase1p: one form is present constitutively throughout the cell cycle, and the other form appears only during mitosis as a higher molecular weight protein (Figure 8D), possibly because of increased phosphorylation level (our unpublished data).

Ase1Δ cells showed defects in spindle integrity and elongation during mitosis, but the overall duration time of mitosis is the same as wild-type cells (Table 2), and astral microtubules organized by the SPBs and PAA microtubules organized by the eMTOCs were still present in *ase1Δ* cells (Figure 6A and Movie_2_ase1Δ_MT.mov). Further, we have preliminarily examined the *ase1Δmad2Δ* double mutant and

Figure 9. A model for *ase1p* function in fission yeast cell cycle. (A) During interphase, the *mto1p* complex of proteins, which may be anchored to the nuclear membrane by *sad1p*, recruits γ -TuRC to the nuclear membrane. The γ -TuRC nucleates cytoplasmic microtubules. Nonphosphorylated *ase1p*, coupled to a minus end motor, is delivered to the microtubule minus ends, where *ase1p* can bundle two microtubules in minus end to minus end antiparallel manner, forming the iMTOC structures. (B) During mitosis, *ase1p* is phosphorylated by Cdk, and enters the nucleus where it is coupled to a plus end motor to move toward the microtubule plus ends, where it bundles microtubules in plus end to plus end antiparallel manner, forming the spindle midzone structure.

found little evidence of synthetic interaction, i.e., the *ase1 Δ mad2 Δ* double mutant has a cell length phenotype similar to that of the *ase1 Δ* single mutant alone (our unpublished data). These results indicate that fission yeast *ase1p* may not have a role in the Mad2p-dependent mitotic checkpoint nor the septation initiation network (SIN) pathway. In addition, fission yeast *ase1 Δ* showed an abnormally long cell length phenotype before entering mitosis (Figure 1, A and C). This would indicate that there is a cell cycle delay before entering mitosis in *ase1 Δ* cells. Interestingly, although PRC1 and Feo showed defects in completion of cytokinesis (Mollinari *et al.*, 2002, 2004; Verni *et al.*, 2004; Zhu and Jiang, 2005), fission yeast *ase1 Δ* appeared to complete cytokinesis and septation, although we cannot rule out the possibility of fission yeast *ase1p* playing a role at the end of cytokinesis. Fission yeast *ase1p* may function similarly to SPD-1 during mitosis to support spindle structural integrity, but does not affect the completion of cytokinesis (Verbrugghe and White, 2004).

A Model for Fission Yeast *ase1p*: Implications for the Dual Functions of *ase1p*

We present a model for the function of *ase1p* in fission yeast (Figure 9). Interesting implications abound for the dual functions of *ase1p*. At interphase, *ase1p* is cytoplasmic highly motile. The movement of *ase1p* dots from the plus toward

the minus end of the microtubule suggests a possible coupling of *ase1p* to a minus-end-directed microtubule motor (Figure 9A). At mitosis, *ase1p* is phosphorylated, is transported inside the nuclear membrane, and is less motile, and its localization to the spindle midzone suggests a possible coupling to a plus-end-directed microtubule motors (Figure 9B). There is evidence in support of the roles of microtubule motors in recruiting *ase1p* to the microtubule overlapping antiparallel regions. PRC1 has been found to bind to KIF4, CENP-E, and MKLP1, all different members of the kinesin families of motors (Kurasawa *et al.*, 2004; Zhu and Jiang, 2005). Further, cyclin-dependent kinase (Cdk) phosphorylation of PRC1 appeared to control the timing of KIF4 transport of PRC1 to the spindle midzone (Zhu and Jiang, 2005). The spatial-temporal regulation of motors and *ase1p* coupling and how *ase1p* can have different specificity for different polarity states of the microtubule ends will be interesting future questions.

The formation and stabilization of the spindle midzone in animal cells have been shown to require two classes of proteins: the kinesin-related motor proteins and the nonmotor microtubule-associated proteins. Of the kinesins, three subclasses are distinguished: 1) the plus-end-directed bipolar homotetramer BimC family, 2) the minus-end-directed C-terminal motor domain KAR3 family, and 3) the microtubule-bundling MKLP1/CHO1 family (Sharp *et al.*, 2000). Of the nonmotor microtubule-associated proteins, there are the ASE1/PRC1/MAP65 family and the Orbit/Mast family (Sharp, 2002). In the fission yeast, both kinesin-related motor proteins and the nonmotor microtubule-associated proteins have been shown to play a role in bipolar spindle formation and stabilization. Cut7p is a member of the BimC family of microtubule plus-end-directed kinesin (Hagan and Yanagida, 1992). Cut7p localizes to the spindle midzone, and mutants of *cut7* failed to achieve a bipolar spindle. Pkl1p and klp2p are members of the KAR3 family (Pidoux *et al.*, 1996; Troxell *et al.*, 2001). Pkl1p has been reported to be present in the nucleus throughout the cell cycle and binds to microtubules and localizes to the spindle at mitosis. *Pkl1 Δ* cells have abnormally short spindles. In contrast, *klp2p* is cytoplasmic during interphase and appears as dots moving in a microtubule minus-end-directed manner. At mitosis, *klp2p* localizes to the spindle midzone. *klp2 Δ* cells have abnormally long spindles. Coupling to motors would provide a mean for *ase1p* recruitment and specificity to specific microtubule ends. Future works will need to examine the interactions of the fission yeast *ase1p* with *cut7p*, *pkl1p*, and *klp2p* throughout the cell cycle.

It has been shown that an allele of PRC1 lacking the two putative Cdk phosphorylation sites caused an increase in microtubule bundling during mitosis (Mollinari *et al.*, 2002), suggesting that phosphorylation suppresses PRC1 microtubule bundling activity at the end of anaphase to allow for spindle breakdown and transition to cytokinesis. Cdk phosphorylation of PRC1 also controls the localization of PRC1 to the spindle midzone by KIF4 (Zhu and Jiang, 2005). We found that fission yeast *ase1p* is phosphorylated during the transition from G₂ to M (Figure 8D). We also observed suppressed *ase1p* protein mobility by FRAP during mitosis at the spindle midzone (Figure 8, B and C). Thus, it is interesting to speculate that *ase1p* phosphorylation may confer plus- or minus-ended motor specificity, plus- or minus-ended microtubule polarity specificity, and/or cytoplasmic or nuclear specificity.

ase1p and Bilaterally Symmetric Organization of Microtubule Structures

The centrosome, which organizes microtubules into a radially symmetric structure, represents a canonical MTOC in animal cells. However, there are numerous examples of linear microtubule structures in highly differentiated cell types, such as polarized epithelial cells, neurons, and myotubes, as well as many plant cells (Hussey and Hawkins, 2001; Dammermann *et al.*, 2003). Little is known about the formation and maintenance of these linear microtubule structures. The fission yeast iMTOCs organize microtubules into bilaterally symmetric linear bundles. A key determinant of symmetric linearity is *ase1p*. Thus, we propose that *ase1p* and the linear microtubule bundles of fission yeast may serve as a good model for understanding microtubule organization.

In fission yeast, the *mbo1p/mod20p* (*mto1p*) complex of proteins functions to recruit the γ -TuRC to the nuclear membrane (Sawin *et al.*, 2004; Venkatram *et al.*, 2004). γ -TuRC nucleates cytoplasmic microtubules from the nuclear region. *ase1p*, coupled to a minus-end motor, is transported on the microtubule toward its minus end at the nucleus. Here, *ase1p* bundles minus-end microtubules into a stable antiparallel structure called the iMTOC (Figure 9A). During mitosis, Cdk phosphorylates *ase1p*. The phosphorylated *ase1p* is transported into the nucleus, where it is coupled to a plus-end motor and move on spindle microtubules toward to distal microtubule plus ends. Here, phosphorylated *ase1p* bundles plus-end microtubules into a stable antiparallel structure called the midzone (Fig. 9B). There are some evidence supporting this model. In the *ase1 Δ* cells, microtubules are still nucleated and, although misorganized, have dynamics similar to that of wild-type cells. On the other hand, in the *mto1 Δ* cells, where iMTOC-organized microtubules are missing, we failed to see localization of *ase1p*-GFP at iMTOC sites (our unpublished data). Furthermore, we still detected *alp4*-GFP, a γ -TuRC marker, at all SPBs, iMTOCs, and eMTOCs in the *ase1 Δ* cells (our unpublished data).

Of the three structural determinants required to form an MTOC—nucleation, organization, and attachment—*ase1p* may perform the organization and attachment roles. For microtubule nucleation, it is well established that the γ -TuRC plays an essential and conserved role (Zheng *et al.*, 1991; Stearns and Kirschner, 1994; Moritz *et al.*, 2000). For organization of radially symmetric microtubule structures, in vitro evidence suggest that microtubule motors such as dynein and kinesin can interact with microtubules to spontaneously form stable radial arrays of microtubules (Nedelec *et al.*, 1997; Surrey *et al.*, 2001). For organization of linear arrays of microtubules, *ase1p* may play a key role in bundling opposite polarity microtubules into bilaterally symmetric structures. For attachment of the microtubule structures to specific cell targets such as the nucleus, the Hook family of membrane-cytoskeletal linking protein ZYG12 in *C. elegans* is the only reported protein implicated in attaching the centrosome to the nucleus (Malone *et al.*, 2003). Our findings that fission yeast cells lacking *ase1p* can repolymerize microtubules upon washout of microtubule depolymerizing drug at cytoplasmic sites away from the nucleus suggests that *ase1p* may play a role in attaching microtubules to the nuclear membrane.

In summary, we revealed a new function for *ase1p* of bundling antiparallel minus ends of microtubules during interphase. This function is in contrast to its reported role of bundling antiparallel plus ends of microtubules at the spindle midzone during mitosis. This dual function of *ase1p*

implies specific cell cycle regulation of the dynamics, mobility, and specificity of *ase1p*.

ACKNOWLEDGMENTS

We thank Erfei Bi, Marcel Janson, and Séverine Morizur (Institute Curie-CNRS) for helpful discussions. We thank our colleagues Chris Burd and Erfei Bi (University of Pennsylvania) for generously sharing lab equipments. We thank Michel Bornens (Institut Curie-CNRS) for his support and encouragement during the FRAP visits. We thank Raymond Lustig and Fred Chang (Columbia University), Da-Qiao Ding and Yasushi Hiraoka (Kansai Advanced Research Center), Kathy Gould (Vanderbilt University), Iain Hagan (University of Manchester), Paul Nurse (Rockefeller University), Shelley Sazer (Baylor College of Medicine), Ken Sawin (University of Edinburgh), and Takashi Toda (Cancer Research UK) for generously providing useful strains and plasmids. This work was supported by the ADF funds from the University of Pennsylvania Cell and Developmental Biology Department to P.T.T.

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