The Roles of Fission Yeast Ase1 in Mitotic Cell Division, Meiotic Nuclear Oscillation, and Cytokinesis Checkpoint Signaling[□] [▽]

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Submitted October 4, 2004; Revised December 23, 2004; Accepted December 29, 2004
Monitoring Editor: Tim Stearns

The Ase1/Prc1 proteins constitute a conserved microtubule-associated protein family that is implicated in central spindle formation and cytokinesis. Here we characterize a role for fission yeast Ase1. Ase1 localizes to microtubule overlapping zones and displays dynamic alterations of localization during the cell cycle. In particular, its spindle localization during metaphase is reduced substantially, followed by robust appearance at the spindle midzone in anaphase. *ase1* deletions are viable but defective in nuclear and septum positioning and completion of cytokinesis, which leads to diploidization and chromosome loss. Time-lapse imaging shows that elongating spindles collapse abruptly in the middle of anaphase B. Either absence or overproduction of Ase1 results in profound defects on microtubule bundling in an opposed manner, indicating that Ase1 is a dose-dependent microtubule-bundling factor. In contrast microtubule nucleating activities are not noticeably compromised in *ase1* mutants. During meiosis astral microtubules are not bundled and oscillatory nuclear movement is impaired significantly. The Aurora kinase does not correctly localize to central spindles in the absence of Ase1. Finally Ase1 acts as a regulatory component in the cytokinesis checkpoint that operates to inhibit nuclear division when the cytokinesis apparatus is perturbed. Ase1, therefore, couples anaphase completion with cytokinesis upon cell division.

INTRODUCTION

The mitotic spindle is a highly organized apparatus that is required for chromosome alignment at metaphase and sister chromatid segregation during subsequent anaphase. Its orientation and positioning are also vital for cell polarity and cytokinesis, to ensure the completion of cell division. Structurally the mitotic spindle consists of two types of microtubules, in which each class plays a distinct role. The pole-to-kinetochore microtubules capture at their plus ends the kinetochore that is formed around centromeric DNA regions and function to pull sister chromatids toward the opposite poles during anaphase A. The pole-to-pole microtubules come from each pole and meet at the spindle equator, thereby forming microtubule-overlapping region on the central zone of the spindle. This interdigitating structure, consisting of antiparallel microtubules (referred to as the spindle midzone), is believed to be required for the maintenance of overall spindle architecture and acts as a backbone plat-

form for chromosome congression and segregation. The spindle midzone plays a central role particularly during anaphase B and telophase. The absence or malfunctioning of the midzone results in a physical collapse of elongating spindles in a corresponding middle region, leading to a disastrous mis-segregation of sister chromatid (Mitchison and Salmon, 2001).

On the completion of anaphase B, elongated spindles are disassembled and replaced by cytoplasmic interphase microtubules, followed by cytokinesis, which requires the deposition of a medial actomyosin ring and its subsequent constriction. These sequential events, consisting of spindle elongation, spindle disassembly, actomyosin ring formation and its resolution, must be spatially and temporally coordinated, to achieve successful cell division. Premature or mislocated cytokinesis would result in the breakage of segregating chromosomes, whereas a delay or bypass of cytokinesis would produce binucleated cells, leading to polyploid or aneuploid daughter cells. These types of events can often be observed in various human disease and cancerous cells (Lengauer *et al.*, 1998; Carmena and Earnshaw, 2003; Dechant and Glotzer, 2003).

As the temporal ordering of mitotic exit and cytokinesis is so vital for cell reproduction, one might imagine that a surveillance mechanism that monitors this final step of cell division would be operational. Indeed, at least in fission yeast *Schizosaccharomyces pombe*, the existence of such a surveillance mechanism has been reported (Le Goff *et al.*, 1999; Liu *et al.*, 2000). This checkpoint, called the cytokinesis or

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-10-0859>) on January 12, 2005.

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

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contractile ring checkpoint, monitors formation and integrity of medial actomyosin ring and septum, and thereby it delays subsequent nuclear division. The checkpoint signaling results in inhibition of Cdc2/Cdk1, leading the cell to two G2 nuclei arrest upon perturbation of cytokinetic structures (Krapp *et al.*, 2004; Mishra *et al.*, 2004). For this checkpoint to be functional, the Wee1 protein kinase and the SIN (septation initiation network) are indispensable. In addition protein phosphatase Clp1/Flp1, a homologue of budding yeast Cdc14, is necessary for this checkpoint signaling (Cueille *et al.*, 2001; Trautmann *et al.*, 2001). In budding yeast *S. cerevisiae*, a similar, if not identical, mechanism also exists, called the morphogenesis checkpoint, which restrains activation of Cdc28/Cdk1 via Wee1-homologue Swe1 when bud emergence is inhibited (Lew, 2003).

Ase1, first identified in budding yeast, comprises a conserved family of microtubule-associated proteins (MAPs). Depending upon how their homologues were identified and characterized, individual members from different species were designated different names. For instance, budding yeast Ase1, a founding member of this family, was identified as a mutation that displayed synthetic lethality when combined with the *bik1* mutation, which is defective in a structural homologue of another MAP, CLIP-170 (Berlin *et al.*, 1990; Pierre *et al.*, 1992; Pellman *et al.*, 1995). As its name represents, Ase1 is required for anaphase spindle elongation and is proposed to comprise midzone-specific spindle matrix (Schuyler *et al.*, 2003). In fly and worm, its homologue Feo (fascetto) and SPD-1, respectively are required for central spindle formation. (Verni *et al.*, 2004; Verbrugghe and White, 2004). In human cells, Prc1 (protein required for cytokinesis) was identified as a mitotic CDK substrate (Jiang *et al.*, 1998) and required for spindle midzone formation and cytokinesis (Jiang *et al.*, 1998; Mollinari *et al.*, 2002). In addition to yeast and metazoans, the Ase1 family is also conserved in plants and called MAP65. The plant homologue also localizes to microtubule overlapping zones (Smertenko *et al.*, 2000; Lloyd and Hussey, 2001). One member MAP65-3/PLE was recently shown to be required for cytokinetic phragmoplast function (Müller *et al.*, 2004). What is common to this family of proteins is their involvement in microtubule bundling, and therefore these proteins are often referred to as microtubule-bundling factors.

In this study we characterize the fission yeast homologue of Ase1/Prc1, which has not been studied before in this organism. Simple rod-shaped morphology directed by tip growth makes this organism an ideal system by which to study cell morphogenesis and cytokinesis (Nurse, 1994; Chang and Verde, 2003; Wang *et al.*, 2003; Mishra *et al.*, 2004). Furthermore detailed knowledge of molecular networks that are involved in mitotic exit and septum formation (Simanis, 2003) is advantageous for understanding mechanisms underlying the termination of cell division. We show that fission yeast Ase1 is not essential for cell viability, but is required for genome stability and, like other members of this family, the integrity of the spindle midzone. Notably we present evidence that in addition to its structural role, fission yeast Ase1 plays a regulatory role in cytokinesis, in which it is a controlling element of the cytokinesis checkpoint, required for inhibiting nuclear division when the cell encounters perturbation of actomyosin ring formation.

MATERIALS AND METHODS

Strains, Media, and Genetic Methods

Strains used in this study are listed in Table 1. Rich YES5 and minimal EMM2 media were used. Unless otherwise stated, 30°C was used for most of the

experiments. For live imaging analysis, room temperature was used. The standard methods were followed as described (Moreno *et al.*, 1991).

Nucleic Acids Preparation and Manipulation

Enzymes were used as recommended by the suppliers (New England Biolabs, Beverly, MA and Takara Shuzo Co., Kyoto, Japan). An *S. pombe* expression vector pREP1, which carried the strong thiamine-repressible promoter, was used for overexpression of *ase1*⁺. The *ase1*⁺ ORF was amplified by PCR with a pair of primers carrying an *NdeI* site and a *BamHI* site. PCR products were digested with *NdeI* and *BamHI* and cloned into pREP1.

Gene Disruption and Construction of C-terminally Tagged Strains

A PCR-based gene targeting method (Bähler *et al.*, 1998) was used for constructing gene deletion or C-terminally tagged strains under the natural promoter. All the tagged strains used in this study behaved like nontagged parental strains, indicating that tagging does not interfere with protein function.

Construction of Plasmid Containing the RFP-tubulin (RFP-*atb2*⁺) Gene

The entire ORF encoding a monomeric RFP (Campbell *et al.*, 2002) was PCR-amplified with *NdeI* and *BamHI* sites at its 5' and 3' end, respectively. The amplified fragment was cloned into *NdeI*-*BamHI* sites of pDQ105 (Ding *et al.*, 1998) to replace GFP with the mRFP fragment. This plasmid was designated pREP1-mRFP-*atb2*⁺.

Indirect Immunofluorescence Microscopy

Cells were fixed with methanol. Primary antibody (TAT-1, 1/50, provided by Dr. Keith Gull, Oxford University, United Kingdom, or polyclonal anti-GFP antibody, 1/50, 8372-2, Clontech, Palo Alto, CA) was applied, followed by Cy3-conjugated goat anti-mouse IgG (AP124C, CHEMICON, Temecula, CA) or BODIPY-conjugated goat anti-rabbit IgG (B-2766, Molecular Probes, Eugene, OR). Fixed cells were imaged with a fluorescence microscope (Axioplan 2, Carl Zeiss, Oberkochen, Germany) equipped with a chilled CCD camera (Photometrics, Quantix, Tucson, AZ) and MetaMorph software (Universal Imaging Corporation, Downingtown, PA) or a confocal microscope (LSM5, Carl Zeiss). Captured images were processed by use of Adobe Photoshop (version 6.0; San Jose, CA).

Time-lapse Live Imaging Analysis

CFP- α -tubulin was expressed from pRL72 (*nmt*-CFP-*atb2*⁺ plasmid) and used as previously described (Glynn *et al.*, 2001). To observe GFP- α -tubulin, *atb2*⁺ was expressed from the weak thiamine-repressible promoter on pREP81. To observe RFP- α -tubulin, the procedures previously described (Zimmerman *et al.*, 2004b) were followed. Briefly, cells carrying pREP1-mRFP-*atb2*⁺ were grown on minimal plates containing thiamine (5 μ g/ml) for 1–2 d and inoculated into liquid minimal culture in the presence of thiamine for 3–5 h.

For live analysis using Metamorph systems, cells were cultured in minimal medium and then mounted on a thin layer of 1% agarose containing minimal medium, which was attached to a glass slide. For analysis of cells in meiosis, homothallic haploid cells were cultured in minimal medium until the midlog phase, washed, shifted to nitrogen-free minimal medium, and incubated for 4–6 h. Live images (stained with GST-GFP-NLS) were viewed with fluorescence microscopy (Niccoli *et al.*, 2004). For live imaging analysis using the DeltaVision microscope (Applied Precision, Issaquah, WA), cells were stuck by lectin to the slide glass at the bottom of a plastic dish, which was then filled with 2–3 ml of minimal media with appropriate supplements. Imaging and processing were performed by SoftWoRx software (Applied Precision). For cold treatment, a dish carrying the cells was chilled with ice, and time-lapse analysis was performed afterward.

RESULTS

Identification of the Fission Yeast Ase1/Prc1 Homologue

Homology search against the fission yeast genome sequence (Wood *et al.*, 2002) using budding yeast Ase1 and human Prc1 as queries showed that one ORF (SPAPB1A10.09) encodes a potential homologue, which we call *ase1*⁺ hereafter. The *ase1*⁺ gene contains one intron and codes for a protein of 731 amino acid residues with stretches of several coiled-coil regions (Figure 1A). As reported previously, Ase1 is a conserved protein among all eukaryotic species (see Supplementary Figure S1), in which overall identity is ~20% (and ~40% similarity). Homology is seen in the entire protein

Table 1. Strain list used in this study

Strains	Genotypes	Derivations
513	<i>h⁻ ura4</i>	Our stock
JY450	<i>h⁹⁰ ade6-M216</i>	Our stock
JW327	<i>h⁹⁰ dhc1::ura4⁺ ura4 ade6-M216</i>	Y. Hiraoka
JW819	<i>h⁹⁰ cdc4-8 ade6-M210</i>	Our stock
JV188	<i>h⁺ cdc7-24 ade6-M216</i>	Our stock
PZ123	<i>h⁹⁰ ark1⁺-GFP-FLAG-His- kan^r ade6-M216</i>	This study
JV155	<i>h⁹⁰ ase1::kan^r cdc4-8 ade6-M216</i>	This study
JV167	<i>h⁹⁰ ase1::kan^r cdc7-24 ade6-M216</i>	This study
JV413	<i>h⁻ ase1::kan^r cps1-191 ura4 lys1 ade6-M216</i>	This study
JV615	<i>h⁹⁰ ase1::kan^r ark1⁺-GFP-FLAG-His- kan^r ade6-M216</i>	This study
JW706	<i>h⁹⁰ ase1::kan^r ade6-M216</i>	This study
JW997	<i>h⁹⁰ ase1⁺-GFP-kan^r ade6-M216</i>	This study
MS50	<i>h⁻ ura4 ase1-YFP-kan^r cut12-CFP-kan^r</i>	This study
HM248	<i>h⁻ his2 ade6-M210 Ch16</i>	Our stock
MS93	<i>h⁻ ase1::kan^r his2 ade6-M210 Ch16</i>	This study
MS61	<i>h⁻ ase1::kan^r his2 ura4 ade6-M216</i>	This study
3251	<i>h⁻ mbo1::ura4⁺ ura4 ade6-M210</i>	K. Gould
MS820	<i>h⁻ ura4 ase1-YFP-kan^r cut12-CFP-kan^r mbo1::ura4⁺ ade6-M210</i>	This study
MS71	<i>h⁹⁰ nuc2-663 ase1-GFP-kan^r mis6-3HA-kan^r ura4</i>	This study
KS819	<i>h⁺ mod20-GFP-kan^r ade6-M216 ura4</i>	K. Sawin
MS697	<i>h⁻ mod20-GFP-kan^r sad1-dsRed-kan^r ura4</i>	This study
MS825	<i>h⁻ mod20-GFP-kan^r sad1-dsRed-kan^r ase1::kan^r ura4</i>	This study
MS618	<i>h⁻ alp4-GFP-kan^r sad1-dsRed-LEU2 ura4</i>	This study
MS826	<i>h⁻ alp4-GFP-kan^r sad1-dsRed-LEU2 ase1::kan^r ura4</i>	This study
MS842	<i>h⁻ cdc3-124 ase1::kan^r ade6-M210 ura4</i>	This study
MBY15	<i>h⁻ cdc3-124 ade6-M210 ura4</i>	M. Balasubramanian
MBY53	<i>h⁻ myo2-E1 ade6 ura4</i>	M. Balasubramanian
MBY159	<i>h⁻ cps1-191 ura4 lys1 ade6-M210</i>	M. Balasubramanian

All the strains (except for HM248, MS93, and MBY15) listed in this table contain *leu1-32*. *ura4* used is *ura4-D18*.

among different species, but the most homologous region is the central coiled-coil domain.

The Cellular Localization of Ase1

As a first step to address whether Ase1 colocalizes to microtubules, its intracellular localization was examined. For this purpose, the C-terminus of Ase1 and Cut12, a component of the SPB (spindle pole body, the centrosome equivalent; Bridge *et al.*, 1998), was tagged with YFP and CFP, respectively, under the natural promoter. The C-terminal tagging did not interfere with Ase1 or Cut12 function, as a strain containing these tags (Ase1-YFP Cut12-CFP) behaved like wild-type cells in terms of growth properties and cell morphology (see below as to phenotypes of *ase1*-deleted cells). In addition, to visualize microtubules simultaneously, plasmids carrying RFP-tubulin (*RFP-atb2⁺*, see *Materials and Methods*) were constructed and introduced into an Ase1-YFP Cut12-CFP strain. As shown in Figure 1B, Ase1-YFP colocalized to microtubules during both interphase and mitosis. In interphase, Ase1 localized along cytoplasmic microtubule arrays (top two rows). It was observed that the middle regions of interphase microtubule filaments, which are believed to represent overlapping zones of individual microtubules (Drummond and Cross, 2000; Tran *et al.*, 2000), were most brightly stained with Ase1-YFP. Consistent with this notion, intensities of signals from RFP-tubulin were also stronger in these regions. On entry into mitosis, Ase1-YFP partially colocalized to mitotic spindles (bottom two rows). During this stage, Ase1 localized to mainly three regions, two dots or arrays coincident with the position of the SPBs and the area of the spindle midzone.

Time-lapse live imaging of a strain containing Ase1-GFP (GFP tagging was performed in a manner similar to Ase1-

YFP) revealed dynamic alterations of Ase1 localization upon mitotic exit. As shown in Figure 1C, at the end of anaphase B, midzone localization started to fade out and Ase1-GFP signals were seen as medial dots (8 min and see Supplementary Movie S1). Then these dots displayed sequential structural alterations, which would correspond to spindle disassembly and reconstruction of interphase microtubules as reported previously (Heitz *et al.*, 2001; Zimmerman *et al.*, 2004b). At this stage Ase1 appeared to colocalize to a new MTOC, called eMTOC (equatorial microtubule organizing center, 8–11 min). In summary fission yeast Ase1 preferentially localizes to overlapping zones of both interphase microtubules and mitotic spindles. In addition Ase1 is found in the regions to which the mitotic SPBs and the postmitotic eMTOC are situated.

Ase1 Is Not a Core Component of the SPB

Data of subcellular localization presented above suggested that Ase1 localizes to the mitotic SPBs, the centrosome equivalent structures. This apparent colocalization appears inconsistent with the current view that the Ase1/Prc1 family is a microtubule-bundling factor, whereas the centrosomes (and the SPBs) are believed to be involved in microtubule nucleation, but not bundling (Jiang *et al.*, 1998; Smertenko *et al.*, 2000; Lloyd and Hussey, 2001; Schuyler *et al.*, 2003; Tolic-Norrelykke *et al.*, 2004). Also budding yeast Ase1 was not copurified as SPB components (Wigge *et al.*, 1998) and Prc1/Ase1 localization to the centrosomes has not been reported in higher eukaryotes. Given these facts, we set up two experiments to address whether Ase1 is a component of the SPBs. First experiment was depolymerization of microtubules by cold treatment. If Ase1 is a core component of the SPB, Ase1 should remain in the vicinity of the SPBs after

Figure 1. Structural comparison of Ase1 protein family and its cellular localization in fission yeast. (A) Schematic comparison between yeast Ase1 and human Prc1. Coiled-coil domains are denoted by filled boxes and the percentage of amino acid identity is shown. (B) Live images showing Ase1-YFP, RFP-tubulin, and Cut12-CFP. Wild-type cells containing *ase1⁺-YFP* and *cut12⁺-CFP* (an SPB marker) expressed from the native promoter and ectopically expressed *RFP-atb2⁺* (encoding α 2-tubulin) were grown in the presence of thiamine and signals from YFP, CFP, and RFP were recorded at room temperature. Images of Ase1-YFP (the leftmost panels), RFP-tubulin (the second panels), and Cut12-CFP (the third panels) and merged images (the rightmost panels, Ase1-YFP in green, RFP-tubulin in red and Cut12-CFP in blue) are shown. (C) Time-lapse analysis of Ase1-GFP during mitosis. Serial pictures (1-min interval) are shown. Bars, 5 μ m.

microtubule depolymerization. Live cells containing Ase1-YFP, Cut12-CFP, and RFP-tubulin were placed on plastic dish plates precoated with lectin and liquid media were added into the dish, followed by in situ cold treatment. Alterations of localization patterns of these proteins were

then examined in live cells with fluorescence microscopy. As shown in Figure 2A, it was clear that Ase1 proteins apparently colocalizing to the SPBs before cold treatment (time 0) disappeared after 20 and 45 min (the left panels, the positions of the SPBs are marked with arrows). Microtubule

Figure 2. Ase1 is not an intrinsic component of the SPB. (A) Disappearance of Ase1-YFP signals upon microtubule depolymerization. A strain containing Ase1-YFP, RFP-tubulin, and Cut12-CFP described in Figure 1B was placed on ice to depolymerize microtubules (time 0, top row), and alterations of localization patterns of each protein were observed afterward in live. Images after 20-min (middle) and 45-min treatment (bottom) are shown. Merged images are also shown in the rightmost panels (Ase1-YFP in green, RFP-tubulin in red, and Cut12-CFP in blue). (B) Absence of SPB-like Ase1-YFP dots in *mbo1/mod20* mutants. Wild-type or *mbo1/mod20* mutants that contain Ase1-YFP and Cut12-CFP were grown, and anaphase B cells were observed. Two representative images in each strain are shown. Arrows indicate the position of the separating SPBs in *ase1* mutants. The merged images are shown in the rightmost panels (Ase1-YFP in green and Cut12-CFP in red). Bars, 5 μ m.

arrays were indeed depolymerized from these cells (the second panels). Note that SPB signals were retained during cold treatment (Cut12-CFP, the third panels). In fact not only these SPB-like spots but also almost all of other Ase1 signals including those in the spindle midzones (shown with arrowheads) and eMTOC disappeared after 45 min cold treatment. This result strongly suggested that a specific subcellular localization of Ase1 is dependent on microtubule structures and that Ase1 is not a structural component of the SPBs.

Second experiment performed was the usage of mutants defective in astral microtubule function. MboI/Mod20 is a nonessential component of the fission yeast γ -tubulin complex and is required for nucleation of cytoplasmic astral microtubules during mitosis (Sawin *et al.*, 2004; Venkatram *et al.*, 2004). As a result, in the *mbo1/mod20* mutant, no astral microtubules were formed from the SPB during anaphase B. Observation of Ase1-YFP and Cut12-CFP in *mbo1/mod20* mutants showed unambiguously lack of SPB localization of Ase1 during anaphase B (Figure 2B, arrows). Apparent localization of Ase1 to eMTOC is also diminished in this mutant (Sato and Toda, unpublished results). In clear con-

trast Ase1 was capable of localizing to the spindle midzones in the absence of MboI/Mod20. This result substantiated the notion that Ase1 is not a component of the SPB and its apparent colocalization to the mitotic SPBs is likely to correspond to overlapping astral microtubules that emanated from these structures. In summary Ase1 localizes to overlapping zones of interphase microtubules, mitotic spindles, and anaphase astral microtubules.

Spindle Localization of Ase1 Is Lessened during Midmitosis

In vertebrates, it is reported that mitotic phosphorylation, possibly performed by Cdk, down-regulates microtubule-bundling activity of the Prc1 protein (Mollinari *et al.*, 2002). As a first step to address the cell cycle-dependent regulation of fission yeast Ase1, mitotic localization of Ase1 was examined in more detail using an Ase1-YFP Cut12-CFP strain. On mitotic entry, Ase1 first appeared around the SPB (Figure 3A, row 1), and then moved to the spindle midzone and two distinct areas, which colocalized to the separating SPBs as shown earlier (see Figures 1, B and C, and 2). Careful obser-

Figure 3. Reduced spindle localization of Ase1 during midmitosis. (A) Ase1 localization during wild-type mitosis. Representative mitotic localization of Ase1-YFP and Cut12-CFP is shown. Images of Ase1-YFP (the leftmost panels), Cut12-CFP (the second left panels), doubly merged (the third panels, Ase1-YFP in green and Cut12-CFP in red), DAPI (the fourth panels), and triply merged images (the rightmost panels, Ase1-YFP in green, Cut12-CFP in red, and DAPI in blue) are shown for each mitotic stage (1, early mitosis; 2, midmitosis; 3, late mitosis). Note that Ase1-GFP signals in midmitosis (row 2) are weaker than those in anaphase B cell (row 3). (B) Reduced Ase1-GFP signals in a metaphase-arrest cell cycle mutant. A *nuc2-663* mutant containing Ase1-GFP and RFP-tubulin was shifted to 36°C and incubated for 2 h. Metaphase spindles and reduced Ase1-GFP signals are marked with arrows. Merged image is shown in the rightmost panel (Ase1-GFP in green and RFP-tubulin in red). Note that merged color of metaphase spindles is red instead of yellow because of low signals from Ase1-GFP. Bars, 5 μ m.

vation showed that during midmitosis, Ase1-YFP signals at the middle region of the mitotic spindle appeared to be reduced (row 2 in Figure 3A). Note that sister chromosomes did not separate at this stage.). On initiation of anaphase B, Ase1 signals in the spindle midzone became much stronger (row 3 in which DAPI showed two separated bodies).

To substantiate the notion of decreased Ase1 localization to preanaphase A spindles, we used the metaphase-arrested *nuc2-663* mutant, which is defective in the APC/C ubiquitin ligase (anaphase promoting complex/cyclosome; Hirano *et al.*, 1988; Yamashita *et al.*, 1996; Peters, 2002). A strain was made that contained Ase1-GFP and RFP-tubulin in the *nuc2-663* background and GFP and RFP signals were observed under the restrictive conditions (2 h at 36°C). As evident with RFP-tubulin patterns, more than 70% of cells showed metaphase spindles (Figure 3B, the middle panel). Under this condition, Ase1-GFP signals were substantially decreased (the left panel, arrows). Thus, like other members of this family, fission yeast Ase1 appears to be down-regulated during midmitosis, in which Ase1 targeting to the spindle midzone is significantly inhibited before anaphase onset.

Genome Instability in *ase1*-deleted Mutants

To clarify a physiological role for Ase1, the whole *ase1*⁺ ORF was deleted. *ase1*⁺ was not an essential gene, but cells deleted for *ase1*⁺ showed a number of defective phenotypes as described below. First of all we realized that during propagation of *ase1* mutant strains on plates containing phloxine B, the red dye removed from diploid cells less efficiently

than haploid cells (Moreno *et al.*, 1991), two types of colonies appeared, one was pink and the other red (Figure 4A). Flow cytometry analysis of the DNA content of cells derived from a single colony displaying each color showed that pink colonies consisted of haploid cells (2C), whereas red colonies were diploid (4C, Figure 4B). Thus the *ase1*-deleted fission yeast cells are prone to diploidization, which is a rare and unfavorable event in this organism (Broek *et al.*, 1991; Komiyama and Toda, 1997). This suggested that *ase1* mutants suffer genome instability. To assess the fidelity of chromosome segregation, a strain was constructed in the *ase1* deletion background that contained chromosome III-derived nonessential minichromosome Ch16 (Niwa *et al.*, 1989). It was found that in addition to spontaneous diploidization, *ase1* mutants displayed a high rate of minichromosome loss, as red or sectorized colonies appeared at a high frequency (indicative of minichromosome loss, ~3.2%; Figure 4C). These results suggest that although Ase1 is dispensable for cell viability, it is important for maintenance of genome ploidy and stability.

ase1 Mutants Are Defective in Cytokinesis and Septation

Microscopic observation of *ase1*-deleted cells hinted at the reason for diploidization and genome instability. As shown in Figure 4D, many of septated *ase1* cells displayed abnormal septa, multiple and/or misoriented (right panels). DNA staining with DAPI showed that separated chromosomes in these septated cells often existed in only one of the two compartments, whereas the other part was anucleated, or

Figure 4. Genome instability and septation and cytokinesis defects in *ase1* mutants. (A and B) Spontaneous diploidization of *ase1* deletion mutants. An *ase1* mutant strain was streaked on rich YES5 plate containing phloxine B and incubated at 30°C for 3 d. Examples of light pink (arrow) and dark red (arrowhead) are shown in A. In B, flow cytometry analysis of haploid wild-type control (top), pink colonies (middle), or red colonies of *ase1* mutants (bottom) is shown. Patterns of DNA content (left panels) and forward scattering (right) are shown. Note that cells derived from red colonies of *ase1* mutants have 4C DNA content with larger cell size compared with wild-type and pink *ase1* cells. (C) Minichromosome loss. Wild-type (top) or *ase1* mutant cells (bottom) carrying Ch16 minichromosome were grown in minimal medium without adenine (selective conditions for minichromosomes), streaked on rich YE plates lacking adenine and incubated at 30°C for 4 d. Sector colonies are marked with arrows. (D) Defective septation and cytokinesis. Exponentially growing wild-type (left) or *ase1* mutant cells (right) were doubly stained with Hoechst 33342 and Calcofluor. Bottom two cells in *ase1* mutants are probably diploid, because both width and length of these cells are larger than wild-type cells (left panels) or *ase1* haploid cells (top two cells). Bar, 5 μ m.

the septa appeared to cut through the middle of the chromosomes (top cell). Quantitative measurement of these abnormal cytokinesis and nuclear division showed that in the absence of Ase1, more than 50% of septated cells displayed

defects. It is most likely that this defect in septum formation and cytokinesis leads to the appearance of cells that contain two pairs of separated chromosome sets (diploids) or unequal sets (aneuploids). Consistent with this notion, we of-

Figure 5. The *ase1* mutant shows normal progression during early mitosis but displays abrupt anaphase spindle collapse. (A) Hypersensitivity of *ase1* mutants to microtubule depolymerizing drugs. Wild-type (top) or *ase1* mutant cells (bottom) were spotted on rich YES5 plates (10^5 cells in the far-left spots for each plate and then diluted 10-fold in each subsequent spot rightwards) in the absence (left) or presence (right) of 5 $\mu\text{g}/\text{ml}$ MBC and incubated at 30°C for 3 d. (B) No additive growth defects in double mutants between *ase1* and spindle checkpoint mutant *mad2*. Four strains (wild-type, *mad2*, *ase1*, and *ase1mad2*) were spotted on rich YES5 plates as in A and incubated at 30°C (left) or 36°C. (C) Time-lapse analysis of anaphase spindle elongation. Kinetics of spindle behavior during anaphase, in

ten found that cultures started originally from haploid cells contain a population (20–30%) of larger cells, both cell length and width, which would correspond to diploidized *ase1* mutant cells (bottom two cells in Figure 4D, right). Ase1, therefore, plays an important part in genome stability via spatial and temporal regulation of septation and cytokinesis and thereby it ensures equal partition of segregating sister chromatids.

ase1 Mutants Are not Defective in Progression of Early Mitosis, but Display Abrupt Collapse of Elongating Anaphase B Spindles

Mutants defective in chromosome segregation often show hypersensitivity to microtubule-depolymerizing drugs. Given the severe defects in sister chromatid segregation in the absence of Ase1, we next examined sensitivity of this mutant to MBC (methyl benzimidazole-2-yl carbamate). As shown in Figure 5A, it was found that *ase1* mutant cells were hypersensitive to this drug. The sensitivity to microtubule-depolymerizing drugs is sometimes ascribable to failure of or compromised attachment of the kinetochore to the mitotic spindle, which is monitored by the spindle assembly checkpoint (Shah and Cleveland, 2000; Millband *et al.*, 2002). To examine the potential effect derived from simultaneous elimination of Ase1 and the spindle checkpoint, double mutants were constructed between deletions of *ase1*⁺ and *mad2*⁺, which encodes a conserved component of the spindle checkpoint (Shah and Cleveland, 2000; Millband *et al.*, 2002) and their growth characteristics was assessed. No obvious reduction of colony forming abilities was observed between *ase1* single and *ase1mad2* double mutants (Figure 5B). This suggested that Ase1 is not involved in progression of early mitosis, the process that ensures bipolar attachment of the kinetochore to the spindle (Waters *et al.*, 1998). Further genetic analysis indicated that, as is the case for budding yeast *ase1* mutants (Pellman *et al.*, 1995), fission yeast *ase1* deletion strains were synthetically lethal with mutations in cold-sensitive β -tubulin *nda3-311* (Hiraoka *et al.*, 1984). On the other hand, unlike budding yeast, in fission yeast, the double deletions of *ase1*⁺ and CLIP-170 homologue-encoding *tip1*⁺ (Brunner and Nurse, 2000) were viable. These results implied that some mitotic steps other than early stages are impaired in *ase1* mutants. Also it is suggested that Ase1 function is similar, but not identical, between these two yeasts.

Given the Ase1 localization at the spindle midzone and the septation and cytokinesis defects in the absence of Ase1, we carried out live analysis of anaphase spindles in *ase1* mutants. GFP-tagged tubulin (GFP-Atb2) was expressed in wild-type and *ase1* mutants and spindle behavior at anaphase was recorded. Wild-type cells showed characteristic spindle elongation during anaphase B (velocity is 0.75 $\mu\text{m}/\text{min}$ at room temperature, Figure 5C, left panels, and Table 2). In clear contrast, we found that many *ase1* mutants (8 of 10 samples) showed defects in the completion of anaphase, in which the anaphase spindle abruptly collapsed in the middle

wild-type (left) or *ase1* mutant cells (two panels on the right-hand side), which expressed ectopic *nmtP81-GFP-atb2*⁺ (encoding α -tubulin), was recorded at room temperature. Points when anaphase spindle collapsed in *ase1* mutants are marked with arrowheads. Bar, 5 μm . (D) Velocity of spindle elongation. Spindle length was measured in each time point and plotted against time. The time point when spindle collapsed in the *ase1* mutant is shown with arrows. See Table 2 for quantitative data.

Table 2. Duration of mitotic progression in *ase1* mutants

	Phase 1	Phase 2	Phase 3	Phase 3	Maximal length
	Length (min)	Length (min)	Length (min)	Velocity ($\mu\text{m}/\text{min}$)	(μm)
Wild type	2.3 ± 0.7	8.7 ± 2.4	10.1 ± 2.1	0.75 ± 0.14	11.0 ± 1.3
<i>ase1</i>	2.2 ± 0.5	8.8 ± 2.0	5.3 ± 2.1	0.67 ± 0.26	5.1 ± 1.9

Definition of mitotic phases 1, 2, and 3 is followed according to Nabeshima *et al.* (1998), in which phase 1 corresponds to prophase (the period of initial spindle elongation); phase 2 consists of prometaphase, metaphase, and anaphase A (the period of constant spindle length); and phase 3 is equivalent to anaphase B. The sample numbers of each parameter is $n = 9$ (phases 1 and 2 of wild type), $n = 10$ (phase 3 of wild type), and $n = 12$ (phases 1, 2, and 3 of *ase1*).

region (two representative live images are shown in Figure 5C, right panels; see also Supplementary Movies S2–S4). Although the spindle length at telophase reaches $11 \mu\text{m}$ in wild-type cells, in the absence of Ase1, anaphase spindle stopped elongating in the half way ($5.1 \mu\text{m}$, Table 2), followed by the physical breakage in the middle region (Figure 5D, marked with arrowheads). The rate of spindle elongation itself did not appear to be compromised, although it was slightly slower than wild-type ($0.75 \mu\text{m}/\text{min}$ in wild-type vs. $0.67 \mu\text{m}/\text{min}$ in the *ase1* mutant; Table 2). It should be noted that we also measured the duration of early mitotic stages, phases 1 and 2, corresponding to the period of prophase and that from prometaphase to anaphase A, respectively (Nabeshima *et al.*, 1998). Consistent with previous results, we did not see any significant delay during early mitosis in *ase1* mutants (Table 2). Ase1 is, therefore, required for structural maintenance of elongating anaphase B spindles.

Ase1 Is a Dose-dependent Microtubule Bundling Factor

We next examined the degree of microtubule bundling in the absence of Ase1 or under Ase1 overproduction conditions. Microtubules staining showed that interphase microtubules in *ase1* mutants showed overall weaker staining than in wild type and the number of microtubule filaments per cell was increased (Figure 6, A and B). In wild-type cells, we saw on average 3 microtubules per cell, whereas in *ase1* mutants the number was increased to 5 or more (Figure 6B). Given the localization of Ase1 to medial microtubule overlapping regions (see Figures 1B and 2), this suggests that Ase1 plays a connecting role in bundling cytoplasmic microtubules. In contrast to deletion phenotypes, ectopic overproduction of Ase1 was lethal (Figure 6C) and resulted in apparent hyper-bundling of microtubules as shown in Figure 6D (1–2 per cell, top right panels). Furthermore these cells showed branched cell morphology, which is a hallmark of compromised cytoplasmic microtubules (Toda *et al.*, 1983; Mata and Nurse, 1997; Brunner and Nurse, 2000). Ase1 overproduction also resulted in mitotic defects, as mitotic spindles looked shorter and thicker (bottom two panels), suggesting that the excess amount of Ase1 blocks spindle elongation. It is of note that these mitotic cells often displayed chromosome segregation defects. Taken together, Ase1 is required for microtubule bundling in both interphase and mitosis and its deletion or overproduction leads to bundling defects in an opposed manner. Thus it appears that Ase1 is a dosage-dependent microtubule-bundling factor.

Ase1 Is Not Involved in Cytoplasmic MTOC Function

Given the requirement of Ase1 for microtubule bundling, we next asked whether Ase1 is also involved in nucleation of interphase cytoplasmic microtubules. It is known that in fission yeast, two non-SPB MTOCs, eMTOC, and iMTOC

(interphase MTOC), play a role in morphogenesis of interphase microtubules (Heitz *et al.*, 2001; Zimmerman *et al.*, 2004b). eMTOC plays a vital role in reorganization of cytoplasmic microtubules after mitotic exit, whereas iMTOC is required for nucleation of interphase microtubules that emanate from the nuclear periphery (Heitz *et al.*, 2001; Sato and Toda, 2004; Sawin *et al.*, 2004; Zimmerman *et al.*, 2004b). Wild-type and *ase1* strains were constructed that contained either MboI/Mod20-GFP or Alp4-GFP, an essential component of the γ -tubulin complex homologous to budding yeast Spc97 and human Gcp2 (Vardy and Toda, 2000; Fujita *et al.*, 2002; Sawin *et al.*, 2004). To show the position of SPBs, the SPB marker Sad1 protein (Hagan and Yanagida, 1995) was also tagged with dsRed in these strains. It is known that eMTOC resides in the medial region of postanaphase cells before cytokinesis as either dot or ring-like structures to which MboI/Mod20 and Alp4 localize (Vardy and Toda, 2000; Heitz *et al.*, 2001; Sawin *et al.*, 2004; Venkatram *et al.*, 2004). Close examination of postanaphase cells in wild-type and *ase1* mutants showed that both MboI/Mod20 and Alp4 proteins exist in a similar pattern, and no obvious differences were observed between these two strains (Figure 7A, dots and rings are marked with arrows and arrowheads, respectively). Quantification data are shown in the right-hand side).

To confirm the functional integrity of eMTOC in the absence of Ase1, time-lapse live analysis was performed in strains containing MboI/Mod20-GFP, CFP-tubulin, and Sad1-dsRed. It was found that, like wild-type cells, *ase1* mutants displayed microtubule-nucleating activities originated from eMTOC in postanaphase cells, characterized by elongation of reorganized microtubules (arrowheads in Figure 7B). Note that, as shown earlier (see Figure 6A), in *ase1* mutants (lower three rows), the number of microtubule filaments, which were thinner in width than those in wild-type cells (upper three rows), was increased. We also examined microtubule nucleation and elongation from iMTOC in *ase1* mutants using the same strains described above. As is the case for eMTOC, *ase1* mutants appeared to show functional iMTOC activities, as cytoplasmic microtubules emanated from MboI/Mod20 spots elongated toward the cell cortex, though again as in postanaphase cells, cytoplasmic microtubules in *ase1* mutants were increased in number (Supplementary Figure S2). Taking these results together, Ase1 is required for anaphase spindle elongation and proper cytokinesis, thereby maintaining genome ploidy, but this protein appears to play no major role in eMTOC and iMTOC functions that involve nucleation and elongation of cytoplasmic microtubules from non-SPB MTOCs.

Figure 6. Defective microtubule bundling in the absence or excess amount of Ase1. (A) Unbundling of interphase microtubules in the *ase1* mutant. Interphase cells of wild-type (left) or *ase1* mutant (right) were stained with anti- α -tubulin antibody (TAT-1) and confocal images were taken. (B) The number of microtubule bundles per interphase cell was also counted ($n = 50$) and plotted. (C) Toxicity of *ase1*⁺ overexpression. A wild-type strain was transformed with an empty vector (the left part of the plate) or a plasmid containing the *ase1*⁺ gene under the thiamine-repressible *mtl1* promoter (the right half). Two independent transformants were streaked on minimal medium in the presence (left) or absence of thiamine (middle) and incubated at 30°C for 3 d. (D) Hyper-bundling of microtubules upon overexpression of *ase1*⁺. Transformants prepared in C were grown in the liquid minimal culture (in the absence of thiamine) at 30°C for 16 h and processed for immunofluorescence microscopy with anti- α -tubulin antibody (green). Chromosomes were stained with DAPI (blue). Representative images of wild-type cells (two interphase cells in the top left two panels and one mitotic cell in the bottom) and *ase1* mutants (two interphase cells in the top right panels and two mitotic cells in the bottom two panels) are shown. Note branched morphology of interphase cells and chromosome segregation defects in mitotic cells when *ase1*⁺ is overexpressed. Bars, 5 μ m.

Figure 7. *Ase1* is not involved in either formation or function of equatorial MTOC. (A) Localization of components of the γ -tubulin complex to the medial region (eMTOC). Wild-type or *ase1* mutants containing *MboI*/Mod20-GFP (top two rows) or Alp4-GFP (bottom two rows) and Sad1-dsRed were grown at room temperature, and postanaphase cells were pictured without prior fixation. Characteristic structures of eMTOC are marked with either arrows (dots) or arrowheads (rings). Merged images are shown in the third panels (*MboI*/Mod20-GFP and Alp4-GFP in green and Sad1-dsRed in red). Quantification data are also shown in the rightmost part. (B) Live observation of microtubule elongation derived from eMTOC. Wild-type (top three rows) or *ase1* mutants (bottom two rows) that contain *MboI*/Mod20-GFP, CFP-tubulin, and Sad1-dsRed were grown at room temperature, and growth of cytoplasmic microtubules that emanated from eMTOC during postanaphase were recorded. Time represents seconds. Merged images are shown in the leftmost panels (*MboI*/Mod20-GFP in green, CFP-tubulin in red, and Sad1-dsRed in blue). Growing microtubules are marked with arrowheads. Bars, 5 μ m.

Ase1 Is Required for Oscillatory Nuclear Movement during Meiotic Prophase and for Production of Viable Spores

Microtubules play an important role in nuclear positioning in many eukaryotes including fission yeast (Reinsch and Gönczy, 1998; Tran *et al.*, 2001). In fission yeast, the nucleus displays dynamic back and forth oscillatory movement during meiotic prophase, which is often referred to as horse tail movement (Robinow, 1977; Chikashige *et al.*, 1994). This oscillatory movement is driven by astral microtubules and

believed to ensure pairing of homologous chromosomes before meiotic recombination (Ding *et al.*, 1998). Dynein and dynactin proteins are absolutely required for this oscillatory movement (Yamamoto and Hiraoka, 2001; Niccoli *et al.*, 2004); however, whether other microtubule binding regulatory proteins also play a role in this process remains to be determined. Given the requirement of *Ase1* for mitotic microtubule bundling and anaphase completion, we addressed the role of this protein in meiotic nuclear oscillation. Homothallic wild-type, *dhc1*- and *ase1*-deleted cells were allowed

Figure 8. Compromised meiotic nuclear movement in the *ase1* mutant. (A) Nuclear dynamics in meiotic horse tail period. Time-lapse images of the nucleus (1.5-min intervals) of wild-type (left), *dhc1* (middle), and *ase1* mutant cell (right) are shown. (B) The position of the nuclear edges in cells of A was plotted every 30 s. The vertical bars denote the central position in each zygote. (C) Meiotic astral microtubules. Two representative images of astral microtubules (GFP-Atb2 in green) are shown with DNA (blue) for wild-type (left) and *ase1* mutants (right). Bars, 5 μ m.

to proceed into mating and meiosis by nutrient starvation, nuclear movement was followed using GFP fused to a nuclear localization signal. Nuclear movement was substantially compromised in the absence of Ase1, though, unlike *dhc1* mutants defective in dynein heavy chain (Yamamoto *et al.*, 1999), some marginal oscillation was still observed. One representative example of live images in each strain is shown in Figure 8A (see

Supplementary Movies S5 and S6). We traced the edges of the nucleus, which highlighted compromised nuclear movement in the *ase1* mutant (Figure 8B).

To examine the morphology of astral microtubules in meiotic prophase cells, GFP-Atb2 was expressed and costained with DNA. As shown in Figure 8C, although wild-type cells mostly contain a single astral microtubule that traverses through the

Figure 9. Mislocalization of the Aurora kinase Ark1 in anaphase *ase1* mutants. Wild-type (A) or *ase1* deleted cells (B) that contained Ark1-GFP and CFP-tubulin were grown, fixed, and observed with fluorescence microscopy. Representative merged images of anaphase cells are shown (Ark1-GFP in green, CFP-tubulin in red, and DAPI in blue). Bar, 5 μ m.

nucleus (left), in the absence of Ase1 many cells (31%) displayed astral microtubules consisting of multiple arrays (right). This suggested that Ase1 plays a role in bundling of meiotic astral microtubules, which is necessary for microtubule-dependent nuclear oscillation during prophase. Consistent with these meiotic defects, we found that spore viability derived from homozygous *ase1* deletions was low, because 97% viability for wild-type and 33% viability for *ase1* mutants were obtained. Thus Ase1 is necessary for microtubule bundling not only in mitotic cycles but also during meiosis.

Midzone Localization of the Aurora Kinase Is Dependent on Ase1

In vertebrates, it is known that the conserved protein kinase Aurora is required for the completion of cytokinesis (Severson *et al.*, 2000; Speliotes *et al.*, 2000). Although it has not been established whether the fission yeast Aurora kinase (Ark1) is involved in cytokinesis (Petersen and Hagan, 2003), Ark1, like all the members of Aurora kinases in other species, localizes to the spindle midzone (Morishita *et al.*, 2001; Petersen *et al.*, 2001). Accordingly we asked whether Ase1 is involved in Ark1 localization at this site during anaphase. A strain, which contained C-terminally GFP tagged Ark1 (Ark1-GFP) and episomally expressed CFP-Atb2, was constructed in the *ase1* mutant and its localization during the cell cycle was examined. Although Ark1 still localized to the spindle in the absence of Ase1, we noticed that the pattern of localization during anaphase is altered in the *ase1* mutant compared with wild-type. Instead of localizing to the middle region of anaphase spindles in wild-type cells (Figure 9A), in the absence of Ase1, Ark1 localized to either the region displaced from the center or multiple discrete regions along the spindle (Figure 9B). This result indicates that Ase1 is required for midzone integrity, such that in the absence of Ase1 the midzone protein Ark1 is no longer capable of localizing properly to this region.

Ase1 Is Required for Cytokinesis Checkpoint Signaling

Having established the role for Ase1 in microtubule bundling and cytokinesis, we next sought to identify a func-

tional network in which Ase1 is involved during late mitosis and mitotic exit. We crossed an *ase1* deletion strain with various mutants defective in septation and cytokinesis. During this genetic analysis, we found that *ase1* shows a strong genetic interaction with temperature-sensitive *cdc4-8* and *cps1-191*, defective in myosin light chain and (1,3) β -D-glucan synthase, respectively (McCollum *et al.*, 1995; Ishiguro *et al.*, 1997; Cortes *et al.*, 2002). As shown in Figure 10A, the *ase1cdc4-8* or *ase1cps1-191* double mutant did not form colonies at 32°C, the semirestrictive temperature at which a single *cdc4-8* or *cps1-191* mutant could grow. It is known that mutants, defective in cytokinesis, including *cdc4-8* and *cps1-191*, trigger activation of the cytokinesis checkpoint (Trautmann *et al.*, 2001; Mishra *et al.*, 2004). To substantiate this genetic interaction, we also tested synthetic growth properties in double mutants between *ase1* and *cdc3-124* or *myo2-E1*, defective in profilin and myosin II heavy chain, respectively (Balasubramanian *et al.*, 1994, 1998; Kitayama *et al.*, 1997). These two mutations are also known to activate the cytokinesis checkpoint (Mishra *et al.*, 2004). As is the case for *cdc4-8* and *cps1-191*, *ase1cdc3-124* double mutants could not grow at 32°C (Figure 10A). In the case of *ase1myo2-E1*, we could manage to obtain colonies of double mutants; however, these strains grew extremely slowly even at 26°C and fast growing revertants accumulated during propagation of the double mutant, which hampered further analysis. Taken together, *ase1* deletions display synthetic growth defects when combined with mutants in the pathway leading to actomyosin ring formation and cytokinesis.

Activation of the cytokinesis checkpoint leads to inhibition of successive nuclear division when the completion of cytokinesis is perturbed, which results in the appearance of cells containing two G2 nuclei at the restrictive temperature with compromised actomyosin ring. Intriguingly DAPI staining of *ase1cdc4-8* double mutants showed that these cells, instead of arresting as binucleates like the single *cdc4-8* mutant, nuclear division continued at the restrictive temperature (Figure 10B) and 70% of cells showed more than 4 nuclei after 4-h incubation at 36°C (Figure 10C). To follow

Figure 10. The *ase1* mutant is defective in cytokinesis checkpoint signaling. (A) Genetic interaction between *ase1* and cytokinesis mutants. Wild-type (top row), *ase1*-deleted (second), *cdc4-8* (third), *ase1cdc4-8* (fourth), *cps1-191* (fifth), *ase1cps1-191* (sixth), *cdc3-124* (seventh), or *ase1cdc3-124* cells (eighth) were spotted on rich plates (10^5 cells in the far-left spots for each plate and then diluted 10-fold in each subsequent spot rightwards), incubated at 25, 32, and 37°C for 3 d (top panels). *cdc7-24* (ninth) or *ase1cdc7-24* cells (bottom) were also spotted in the same way and incubated at 25, 30, 32 and 34°C. (B) Multinucleated phenotypes of the *ase1cdc4-8* double mutant. *cdc4-8* single (left) or *ase1cdc4-8* double mutants (right) were shifted to 36°C for 4 h and stained with DAPI upon fixation. Fluorescence microscopy was performed combined with DIC images. (C) Quantification of the number of the nuclei at each time point after temperature shift-up. Wild-type, *clp1/flp1*, *ase1*, *cdc4-8*, *clp1cdc4-8*, or *ase1cdc4* cells were grown at 25°C and shifted to 36°C at time 0. Every 2-h interval, the number of the nuclei in each strain was counted and plotted. At least 400 cells were counted for each strain. (D) Multinucleated phenotypes of the *ase1cdc3-124* double mutant. *cps1-191* single (left) or *ase1cps1-191* double mutants (right) were shifted to 36°C for 4 h and stained with DAPI as in B. Bar, 5 μ m.

the kinetics of nuclear division in these mutants, the number of the nuclei was counted at each time point upon temperature shift-up. As a control, *clp1* single and *clp1cdc4-8* double

mutants were included in this assay. As shown in Figure 10C, it was found that nuclear division continued in *ase1cdc4-8* mutants and furthermore the kinetics of nuclear

division was indistinguishable between *ase1cdc4-8* and *clp1cdc4-8* mutants. This continual nuclear division was reminiscent of cytokinesis checkpoint-deficient cells, as evident in *clp1cdc4-8* cells. In addition to *ase1cdc4-8*, the *ase1cps-191* mutant also displayed the increased population of 4 nuclei-cells after 4-h incubation at 36°C (Figure 10D). These results suggested that Ase1 has a regulatory function in inhibiting nuclear division when actomyosin ring formation is perturbed. Checkpoint-deficient mutant cells also include combinations of cytokinesis mutants with *wee1* or mutations in the SIN (Le Goff *et al.*, 1999; Liu *et al.*, 2000; Cueille *et al.*, 2001; Trautmann *et al.*, 2001). We then constructed double mutants between *ase1* and *cdc7-24*, in which the SIN signaling is defective, thereby the cytokinesis checkpoint being inactivated (Cueille *et al.*, 2001; Trautmann *et al.*, 2001). In clear contrast to cytokinesis mutations shown above, no reduction of the restrictive temperature was observed in *ase1cdc7-24* double mutants (Figure 10A). Taken together analysis described here shows that Ase1 is, in addition to its structural role in microtubule morphology and cytokinesis, an integral component of cytokinesis checkpoint signaling.

DISCUSSION

In this study we have characterized the roles of fission yeast Ase1, a conserved MAP. Like other members of this protein family, the fission yeast homologue colocalizes to microtubule overlapping zones, and displays cell cycle-dependent dynamic alterations of its subcellular localization. Furthermore we have present evidence that in addition to its structural role Ase1 is involved in cytokinesis checkpoint signaling that restrains nuclear division when the cell division apparatus is perturbed.

Role for Ase1 in Microtubule Bundling

During both interphase and mitosis, the predominant localization site of Ase1 is at the microtubule overlapping zones. In fission yeast during interphase, medial overlapping regions of cytoplasmic microtubule arrays consist of minus ends with the plus ends extending out toward the cell tip, whereas during mitosis the spindle midzone comprises the overlapping plus ends (Tran *et al.*, 2001). This apparent polarity-independent association of Ase1 with microtubules is in clear contrast to other MBPs, such as plus-end tracking proteins including conserved EB1 and CLIP-170 proteins (Schuyler and Pellman, 2001) and conventional MAPs that bind microtubules along longitudinal lattices (Drewes *et al.*, 1998). Thus Ase1 constitutes a unique family of microtubule-bundling proteins that specifically localize to microtubule overlapping regions irrespective of microtubule directionality.

In budding yeast Ase1 overproduction results in forced spindle elongation under conditions of HU treatment. Usually HU addition leads to cell cycle arrest at early S phase with short spindles. This suggests that in addition to a microtubule-bundling activity, Ase1 may play a direct role in promoting spindle elongation (Schuyler *et al.*, 2003). Consistently it is known that elongation of anaphase spindles in fission yeast, as in other systems, takes place at the overlapping zones by polymerization and subsequent sliding (Masuda and Cande, 1987; Masuda *et al.*, 1990; Mallavarapu *et al.*, 1999), pointing toward the possibility that fission yeast Ase1 also promotes spindle elongation at the midzone. Despite this notion, overproduction of fission yeast Ase1 results in bundling and thickening of both interphase microtubules and mitotic spindles and clearly inhibits microtubule elongation instead. The appearance of branched

(T-shaped) cell morphology and chromosome segregation defects support this notion, as these phenotypes are reminiscent of malfunctioning of interphase microtubules and mitotic spindles, respectively (Radcliffe *et al.*, 1998; Sawin and Nurse, 1998). Conversely the absence of Ase1 leads to unbundling of interphase microtubules and a physical collapse of anaphase spindles without affecting the velocity of spindle elongation. All these data suggest that fission yeast Ase1's major, if not sole, function lies in a structural connector at microtubule overlapping zones and it is not directly involved in promotion of spindle elongation reaction per se. It is worth pointing out that phenotypic consequences caused by either deletion or overproduction of Ase1 are in fact very similar to those reported for plant MAP65, fly Feo, and human Prc1 (Mollinari *et al.*, 2002). This suggests a conservation of protein function between fission yeast and higher eukaryote Ase1/Prc1.

Mitotic Localization of Ase1

Ase1 shows dynamic cell-cycle-dependent localization. Particularly the appearance of Ase1 signals in the vicinity of the SPBs upon initiation of anaphase B is intriguing. Recent analysis indicates that cytoplasmic astral microtubules are formed at the SPBs coincident with anaphase B onset (Zimmerman *et al.*, 2004a). Importantly these cytoplasmic astral microtubules grow toward both directions. Given that Ase1 localizes to the microtubule overlapping regions including the spindle midzones at this stage, it is most likely that cytoplasmic astral microtubules make contact at the SPBs in an interdigitating manner. Almost complete disappearance of Ase1 from these sites upon microtubule depolymerization supports this notion. At present, a physiological significance of Ase1 localization to these sites remains to be determined, as astral microtubules appear to be formed in the absence of Ase1, though more careful cytological characterization would be needed.

Another interesting point is reduced localization of Ase1 to the spindle during midmitosis (metaphase) when Cdk activity remains high. Mammalian Prc1 and budding yeast Ase1 are CDK substrates and it is shown that Cdk-phosphorylation of Prc1 suppresses microtubule-bundling activity of this protein (Jiang *et al.*, 1998; Mollinari *et al.*, 2002; Ubersax *et al.*, 2003). Given the reduction of Ase1 levels to metaphase spindles, it appears that, at least in fission yeast, bundling of pole-to-pole microtubules via Ase1 during metaphase may not be required for structural maintenance of bipolar spindles, and instead hyper-bundling might be deleterious for formation and establishment of bipolar spindles. Fission yeast Ase1 contains four consensus Cdk phosphorylation sites at the C-terminal region, and we have been currently pursuing a significance of these sites (see below).

Novel Role for Ase1 in Meiotic Nuclear Oscillation

We show that in the absence of Ase1, meiotic nuclear movement is significantly compromised. Meiotic nuclear oscillation is thought to be orientated by the SPB and telomeres and driven by the force generated by dynein-dynactin motor complex (Yamamoto and Hiraoka, 2001). The requirement of microtubule-bundling factor Ase1 for this process implies that these astral microtubules consist of a multitude of overlapping microtubules rather than a single microtubule bundle. In *ase1* mutant, horse tail movement is impaired, but not as severely as in dynein or dynactin mutants, which display complete abolishment of oscillatory movement. This suggests that dynein motors retain the activity to drive the nuclear oscillation even in the absence of Ase1 but do not exert its power satisfactorily due to unbundled microtu-

bules. We also show that Ase1 is required to produce fully viable spores. The impairment of horse tail movement probably accounts for the low spore viability, as this oscillatory movement promotes homologous chromosome pairing, thereby ensuring subsequent chromosome segregation (Yamamoto and Hiraoka, 2001). In addition to this, in *ase1* mutants precocious breakdown of meiotic spindles might occur, as in mitotic anaphase, which might also contribute to the reduction of spore viability. It is worth noting that Ase1 is the first MAP (except for dynein motors) that is essential for nuclear oscillation during fission yeast meiosis. Other MAPs that are involved in this process still await further analysis.

Ase1 as a Regulatory Component of the Cytokinesis Checkpoint

One of the unexpected findings in this study is the involvement of Ase1 in the cytokinesis checkpoint. This checkpoint has evolved in fission yeast to maintain viability when the cell division apparatus is perturbed (Mishra *et al.*, 2004). This checkpoint acts through down-regulation of ubiquitous Cdc2/CDK activity and requires the Clp1/Flp1 phosphatase (Le Goff *et al.*, 1999; Liu *et al.*, 2000). Budding yeast Cdc14, a homologue of Clp1/Flp1, dephosphorylates CDK substrates, thereby antagonizing CDK activity (Visintin *et al.*, 1998). Interestingly in addition to the involvement of cytokinesis checkpoint, there are other phenotypic similarities between deletions of Clp1/Flp1 and Ase1. These include impairment of cytokinesis and septation, spontaneous diploidization and the requirement of the SIN for phenotypic appearance (Cueille *et al.*, 2001; Trautmann *et al.*, 2001). The simplest interpretation, which explains the involvement of Ase1 in cytokinesis checkpoint signaling, is that Ase1 is a downstream CDK substrate, critical for inhibiting nuclear division and that dephosphorylation of Ase1 is required for initiating and/or maintaining checkpoint activation. As shown in this study, spindle localization of Ase1 is substantially suppressed under the conditions of high Cdk activities. This suggests that Cdk phosphorylation of Ase1 results in reduced activities of microtubule bundling, which somehow results in inhibition of the checkpoint signaling. We have been addressing this point experimentally by mutating potential Cdk sites to nonphosphorylatable or phosphorylation-mimicking residues (Yamashita and Toda, unpublished results).

The alternate possibility, albeit not mutually exclusive with the first possibility, is that Ase1 is required for localization of other proteins that are involved in this checkpoint signaling. An obvious primary candidate would be Clp1/Flp1. However, we have found that this phosphatase is still capable of localizing to central spindles in the absence of Ase1 (Sato and Toda, unpublished results). Instead we have shown that Ark1 localization to the spindle midzone is altered, despite not abolished, in *ase1* mutants. It should be mentioned that recent analysis indicates that, in addition to its localization to the mitotic spindles, Clp1/Flp1 physically binds Ark1 (Trautmann *et al.*, 2004), pointing toward the possibility that Clp1/Flp1, Ark1, and Ase1 constitute a functional network involved in mitotic exit and the cytokinesis checkpoint. Although at this stage whether or not Ark1 is implicated in the cytokinesis checkpoint remains to be determined, it is relevant to note that in vertebrates, Prc1-knockdown results in, as in fission yeast cells, the altered discrete localization of Aurora B along spindles, instead of accumulating at the spindle midzone (Kurasawa *et al.*, 2004).

Link between the Microtubule and the Actomyosin upon Mitotic Exit

Microtubule and actomyosin pathways display a mutual interplay during mitosis and cytokinesis in many systems (Ban *et al.*, 2004; Mishima *et al.*, 2004; Yasuda *et al.*, 2004). In fission yeast a contractile actin ring is required to form eMTOC-dependent postanaphase microtubule arrays, which are in turn required for equatorial retention of the contractile ring (Heitz *et al.*, 2001; Pardo and Nurse, 2003). Given the defective cytokinesis accompanied with the displacement of actomyosin ring in *ase1* mutants, Ase1 may be a key player that links the microtubule cytoskeleton to actomyosin ring function at the end of the cell cycle. More work is needed to explore the point as to how Ase1 activity and localization are regulated and the underlying mechanism coupling anaphase completion to cell division.

ACKNOWLEDGMENTS

We thank Drs. Mohan Balasubramanian, Fred Chang, Kathy Gould, Keith Gull, Jacqueline Hayles, Yasushi Hiraoka, Dan McCollum, Takashi Morishita, Osami Niwa, Ken Sawin, Mizuki Shimanuki, Viestus Simanis, Ayumu Yamamoto, and Minoru Yoshida for providing materials used in this study. We thank Dr. Jacqueline Hayles for critical reading of the manuscript and useful suggestions. Special thanks are given to Dr. Roger Y. Tsien who generously provided plasmid constructs containing a gene encoding monomeric RFP. This work is supported by the Cancer Research UK and the HFSP research grant (T.T.) and Grants-in-Aid for Scientific Research and Specially Promoted Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (M.Y.).

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