The V260I Mutation in Fission Yeast α -Tubulin Atb2 Affects Microtubule Dynamics and EB1-Mal3 Localization and Activates the Bub1 Branch of the Spindle Checkpoint[□]

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We have identified a novel temperature-sensitive mutant of fission yeast α -tubulin Atb2 (*atb2-983*) that contains a single amino acid substitution (V260I). Atb2-983 is incorporated into the microtubules, and their overall structures are not altered noticeably, but microtubule dynamics is compromised during interphase. *atb2-983* displays a high rate of chromosome missegregation and is synthetically lethal with deletions in a subset of spindle checkpoint genes including *bub1*, *bub3*, and *mph1*, but not with *mad1*, *mad2*, and *mad3*. During early mitosis in this mutant, Bub1, but not Mad2, remains for a prolonged period in the kinetochores that are situated in proximity to one of the two SPBs (spindle pole bodies). High dosage *mal3*⁺, encoding EB1 homologue, rescues *atb2-983*, suggesting that Mal3 function is compromised. Consistently, Mal3 localization and binding between Mal3 and Atb2-983 are impaired significantly, and a *mal3* single mutant, such as *atb2-983*, displays prolonged Bub1 kinetochore localization. Furthermore in *atb2-983* back-and-forth centromere oscillation during prometaphase is abolished. Intriguingly, this oscillation still occurs in the *mal3* mutant, indicating that there is another defect independent of Mal3. These results show that microtubule dynamics is important for coordinated execution of mitotic events, in which Mal3 plays a vital role.

INTRODUCTION

The microtubule (MT) possesses intrinsic polarity, the dynamic plus end, and the less dynamic minus end. In many, if not all, cell types, the minus end is embedded in a specialized structure, called the microtubule organizing center (Pickett-Heaps, 1969), whereas the plus end interacts with a distinct class of MT accessory factors, collectively called plus-end-tracking proteins (Schuyler and Pellman, 2001). In both interphase and mitotic spindle MTs, it is the plus end that explores spatial cues through alternating cycles of growth and shrinkage, MT mechanics termed dynamic instability (Mitchison and Kirschner, 1986). This search-and-capture process facilitates efficient

attachment of MT plus end to appropriate subcellular sites such as the cell cortex and the kinetochore, which in turn results in altering dynamics of the connected MTs (Desai and Mitchison, 1997).

The mitotic bipolar spindle is a highly organized apparatus that is required for accurate chromosome segregation. The plus end of MTs that emanate from opposite poles is thought to capture sister kinetochores in a stochastic manner during prometaphase. This mechanism ensures spindle bipolarity and chromosome biorientation, which are vital for bidirectional sister chromatid segregation at anaphase. Errors in these attachment and segregation processes result in producing aneuploid progenies, which can lead to lethality, various genetic disease, and tumorigenesis. To maintain accuracy of chromosome segregation, cells have developed a mitotic surveillance mechanism, termed the spindle assembly checkpoint. This checkpoint monitors the integrity of spindle–kinetochore interaction and delays anaphase onset until bipolar attachment of all the kinetochores to spindles is secured (Millband *et al.*, 2002; Cleveland *et al.*, 2003; Bharadwaj and Yu, 2004). Spindle checkpoint proteins include Mad1, Mad2, Mad3 (in yeast and BubR1 in vertebrates), Bub1, Bub3, and Mps1. These proteins localize to mitotic kinetochores when bipolar attachment is not achieved and monitor the physical status of these kinetochores. It seems that the checkpoint pathway bifurcates, Mad1/Mad2 (/Mad3) and Bub1/Bub3/Mps1(/Mad3), although the two branches may not be functionally separable (Hard-

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Abbreviations used: MT, microtubule; SPB, spindle pole body; ts, temperature sensitive.

wick *et al.*, 1996; Lew and Burke, 2003). Although Mad2 monitors and localizes to unattached kinetochores in most, if not all, organisms, Bub1 localizes to not only unattached kinetochores but also to those that show weaker attachment or lack tension (Waters *et al.*, 1998; Skoufias *et al.*, 2001; Zhou *et al.*, 2002). In addition, Bub1 plays a role in chromosome segregation independent of its role in the spindle checkpoint (Tang *et al.*, 2004; Vanoosthuyse *et al.*, 2004; Kitajima *et al.*, 2005; Meraldi and Sorger, 2005).

Inside the cell, a number of regulatory factors are involved in MT morphogenesis and dynamism. Among these, plus-end-tracking proteins interact with and localize to the plus end of the MT and regulate MT dynamics, thereby participating in a number of cellular processes (Schuyler and Pellman, 2001). The conserved EB1 protein belongs to this MT-binding family. It was originally identified as an interacting partner for vertebrate tumor suppressor protein Adenomatous Polyposis Coli (APC) (Su *et al.*, 1995) and is thought to be crucial, together with APC, for cell polarity and chromosome stability control (Tirnauer and Bierer, 2000; Fodde *et al.*, 2001; Kaplan *et al.*, 2001). It is shown that chromosome instability phenotypes (CIN) observed in APC⁻ tumor cells are associated with defects in MT plus end attachments (Green and Kaplan, 2003), although causal involvement of EB1 in these tumor cells remains to be determined (Smits *et al.*, 1999).

In both fission and budding yeast, EB1 homologues (Mal3 and Bim1, respectively) play a vital role in cell polarity and chromosome stability (Beinhauer *et al.*, 1997; Schwartz *et al.*, 1997; Muhua *et al.*, 1998; Tirnauer *et al.*, 1999; Chen *et al.*, 2000; Browning *et al.*, 2003; Hwang *et al.*, 2003; Busch and Brunner, 2004; Busch *et al.*, 2004; Mayer *et al.*, 2004). Recent work in fission yeast indicates that Mal3 interacts with the kinetochore component Spc7 (homologue of budding yeast Spc105 and human KIAA1570) during mitosis and is important for accurate chromosome segregation (Kerres *et al.*, 2004). We have recently shown that in the absence of Mal3, mitotic delay is induced, in which the Bub1 branch of the spindle checkpoint is activated, and Bub1-localizing kinetochores are associated for a prolonged period of time with only one of two separating spindle pole bodies (SPBs) (Asakawa *et al.*, 2005; Asakawa and Toda, 2006).

In this study, we screened for temperature-sensitive mutants that are defective in chromosome segregation, in which the Bub1-dependent spindle checkpoint is activated. One of mutants identified was a novel allele of α -tubulin gene (*atb2-983*) that contained a missense mutation in the highly conserved valine residue (V261I). We isolated the *mal3*⁺ gene as a multicopy suppressor of *atb2-983*. Consistent with this, Mal3 loading to the MT is compromised in this mutant. Characterization of *atb2-983* and *mal3* mutants provides a novel insight into roles of microtubule dynamics in chromosome stability and spindle checkpoint control.

MATERIALS AND METHODS

Strains, Media, and Genetic Methods

Strains used in this study are listed in Table 1. The standard methods were followed as described previously (Moreno *et al.*, 1991; Bähler *et al.*, 1998). The *kan^r-nmtP3-GFP* cassette (Bähler *et al.*, 1998) was integrated in front of the initiator methionine to create a strain containing *nmtP3-GFP-atb2-983* (GFP-Atb2-983, KZ176). The C-terminal tagging of Mal3 with Myc epitope was performed using the nourseothricin-resistance gene as a selectable marker (*mal3⁺-myc-nat^r*) as described previously (Sato *et al.*, 2005).

Isolation of *cin* Mutants

A parental strain (KZ2; Table 1), which contains minichromosome Ch16 (Niwa *et al.*, 1989) and Bub1-GFP was mutagenized with nitrosoguanidine as described previously (Radcliffe *et al.*, 1998), spread on rich YE5S plates and incubated at 27°C. Colonies were replica-plated onto YE5S at 36°C and YE plates (rich medium lacking exogenously added supplements) at 32°C, on which cells that had lost Ch16 turned red because of the chromosomal *ade6-210* mutation (Niwa *et al.*, 1989). On this procedure, colonies that were temperature sensitive (ts) and also displayed red or sector appearance on YE plates were selected; 221 isolates out of 500,000 mutagenized colonies showed both minichromosome-loss and ts phenotypes. On backcrossing with a marker strain (containing Ch16), 39 mutants showed cosegregation of minichromosome loss and ts phenotypes. These 39 mutants were subsequently classified into 31 complementation groups. Among these isolates, mutations in 10 complementation groups (*cin1-cin10*) showed hypersensitivity to thiabendazole (TBZ) compared with a wild-type strain (Wang *et al.*, 2005; Supplemental Table 1).

Nucleic Acids Preparation and Manipulation

Enzymes were used as recommended by the suppliers (New England Biolabs, Beverly, MA; Takara Shuzo, Kyoto, Japan; and Stratagene, La Jolla, CA).

Identification of *cin3* as α -Tubulin-encoding *atb2*

The genomic library was introduced into ts *cin3-983* cells, and Ts⁺ transformants were isolated by replica plating. Whole DNAs were prepared from these transformants, and plasmid DNAs were recovered via transformation into *Escherichia coli*, which yielded in total 97 plasmids. DNA sequencing and restriction enzyme mapping of these plasmids revealed that seven different genes were able to suppress a ts phenotype of *cin3-983*. Two of them were α -tubulin-encoding genes, *nda2*⁺ (28 plasmids) and *atb2*⁺ (36 plasmids), respectively (Toda *et al.*, 1984; Adachi *et al.*, 1986). The third gene (25 plasmids) was *mal3*⁺. Cloning and characterization of the other four genes, and their products will be described elsewhere.

Genetic linkage analysis showed that the *cin3-983* mutation is tightly linked to the *atb2* locus. Identity between *cin3* and *atb2* was confirmed, because nucleotide sequencing of the *atb2* gene prepared from a *cin3-983* strain shows that it contains a point mutation, which results in amino acid replacement at position 260 from valine to isoleucine (see Figure 2A).

Construction of Plasmids Containing *atb2-983* Allele

Site-directed mutagenesis was performed using pAL-*atb2*⁺ (*LEU2*-carrying multicopy plasmids) as a template with QuikChange kit (Stratagene) to introduce the same point mutation as that of *atb2-983*, where plasmids were designated p(*atb2-V260I*). p(*atb2-V260I*) was used to transform an *atb2*-deletion strain (KZ115), and temperature sensitivity of transformants was tested.

Replacement of the *atb2*⁺ Gene with the *atb2-V260I* Allele at Its Genomic Locus

The *atb2-V260I* allele was integrated into the genomic *atb2* locus in the following manner. First, ~90% of the *atb2*⁺ open reading frame (ORF) (corresponding to 27–422 amino acid residues) was deleted and replaced with the *ura4*⁺ gene (*atb2:ura4*⁺, KZ115). Then, the 2-kb fragments containing the entire *atb2-V260I* ORF and each 300 base pairs of 5' and 3' flanking sequences were PCR-amplified from the p(*atb2-V260I*) plasmid DNA and used to transform KZ115. Uracil auxotroph colonies were selected on 5-fluoroorotic acid-containing plates. Correct replacement was verified with colony PCR (KZ217).

Immunocytochemistry

The following antibodies were used as primary antibodies for immunofluorescence microscopy: mouse monoclonal anti- α -tubulin antibody (TAT-1; provided by Dr. Keith Gull, The University of Oxford, Oxford, United Kingdom) and affinity-purified rabbit polyclonal anti-Sad1 antibody (gift from Dr. Mizuki Shimanuki, Kazusa DNA Research Institute, Chiba, Japan). For immunoblotting, mouse monoclonal anti-Myc antibody (9E10; BAbCO, Richmond, CA), anti-green fluorescent protein (GFP) antibody (1814 460; Roche Diagnostics, Indianapolis, IN), anti- α -tubulin antibody (TAT-1), and anti-Cdc2 antibody (PSTAIR, sc-53; Santa Cruz Biotechnology, Santa Cruz, CA) were used. For immunoprecipitation of GFP-Atb2 (–983) and Mal3-Myc or Mal3-GFP and Atb2 (–983), mouse monoclonal anti-Myc antibody or rabbit polyclonal anti-GFP antibody (A-11122; Molecular Probes, Eugene, OR) was used for a primary antibody, respectively, followed by addition of protein G-coupled Dynabeads (100.04; Dynal Biotech, Lake Success, NY). Immunoprecipitates were run on SDS-PAGE, transferred to Immobilon-P filter paper (IPVH00010; Millipore, Billerica, MA), and immunoblotted with rabbit polyclonal anti-Myc antibody (PRB-150C; BAbCO), mouse monoclonal anti-GFP antibody (8372-2; Clontech, Mountain View, CA), or TAT-1 antibody. Two micrograms of total protein extracts was used.

Table 1. Strains used in this study

Strain	Genotype	Derivation
513	<i>h⁻ leu1 ura4</i>	Our stock
393	<i>h⁻ leu1-32 ura4 his1-102 ade6-216 bub1::ura4⁺</i>	Dr. J.-P. Javerzat (Centre National de la Recherche Scientifique Institut, Bordeaux, France)
AE249	<i>h⁻ leu1 ura4 mad1::ura4⁺</i>	Dr. T. Matsumoto (Kyoto University, Kyoto, Japan)
MA145	<i>h⁻ leu1 ura4 mal3⁺-GFP-kan^r</i>	Our stock
mad2	<i>h⁻ leu1 ura4 mad2::LEU2</i>	Dr. T. Matsumoto
NK113	<i>h⁺ leu1 his2 ura4 mad3::ura4⁺</i>	Our stock
P3-GFP-atb2	<i>h⁻ leu1 kan^r-nmtP3-GFP-atb2⁺</i>	Our stock
YP350	<i>h⁻ leu1 ura4 his3 ade6-210 mal3::his3⁺</i>	Dr. Ursula Fleig (Institut für Mikrobiologie, Düsseldorf, Germany)
ts983	<i>h⁺ leu1 ade6-210 atb2-983 bub1⁺-GFP-kan^r</i>	This study
KZ2	<i>h⁺ leu1 ade6-210 bub1⁺-GFP-kan^r containing Ch16</i>	This study
KZ35	<i>h⁺ leu1 ura4 nda3-1828 bub1⁺-GFP-kan^r</i>	This study
KZ54	<i>h⁺ leu1 nda3-1828 mad2⁺-GFP-LEU2</i>	This study
KZ85	<i>h⁻ leu1 ura4 sad1⁺-RFP-LEU2 cen2-kan^r-ura4⁺-lacOp his7⁺-lacI-GFP</i>	This study
KZ94	<i>h⁺ leu1 atb2-983</i>	This study
KZ96	<i>h⁻ leu1 ade6-216 his7 atb2-983 bub1-K762M</i>	This study
KZ102	<i>h⁺ leu1 ura4 atb2-983</i>	This study
KZ109	<i>h⁻ leu1 ura4 atb2-983 mad3::ura4⁺</i>	This study
KZ110	<i>h⁻ leu1 ura4 atb2-983 mad1::ura4⁺</i>	This study
KZ115	<i>h⁻ leu1 ura4 atb2::ura4⁺</i>	Our stock
KZ118	<i>h⁻ leu1 ade6-704 atb2-983 bub1-Δkinase-GFP-kan^r</i>	This study
KZ124	<i>h⁻ leu1 atb2-983 mad2⁺-GFP-LEU2</i>	This study
KZ135	<i>h⁻ leu1 ade6-216 atb2-983 bub1::ura4⁺ containing p(mal3⁺)</i>	This study
KZ137	<i>h⁺ leu1 his3 atb2-983</i>	This study
KZ139	<i>h⁻ leu1 ura4 atb2-983 sad1⁺-RFP-LEU2 cen2-kan^r-ura4⁺-lacOp his7⁺-lacI-GFP</i>	This study
KZ141	<i>h⁻ leu1 his3 atb2-983 mal3::his3⁺</i>	This study
KZ144	<i>h⁻ leu1 ade6-210 atb2-983 bub1⁺-GFP-kan^r nuf2⁺-CFP-kan^r</i>	This study
KZ165	<i>h⁻ leu1 atb2-983 mal3-GFP-kan^r</i>	This study
KZ176	<i>h⁺ leu1 kan^r-nmtP3-GFP-atb2-983</i>	Our stock
KZ181	<i>h⁻ leu1 ade6-210 bub1⁺-GFP-kan^r sad1⁺-RFP-LEU2</i>	This study
KZ182	<i>h⁻ leu1 ade6-210 atb2-983 bub1⁺-GFP-kan^r sad1⁺-RFP-LEU2</i>	This study
KZ191	<i>h⁻ leu1 mal3::kan^r sad1⁺-RFP-LEU2 bub1⁺-GFP-kan^r</i>	This study
KZ194	<i>h⁻ leu1 mal3⁺-GFP-kan^r sad1⁺-RFP-LEU2</i>	This study
KZ195	<i>h⁻ leu1 atb2-983 mal3⁺-GFP-kan^r sad1⁺-RFP-LEU2</i>	This study
KZ211	<i>h⁻ leu1 ura4 mal3⁺-myc-nat^r</i>	This study
KZ214	<i>h⁻ leu1 kan^r-nmtP3-GFP-atb2⁺ mal3⁺-myc-nat^r</i>	This study
KZ216	<i>h⁻ leu1 kan^r-nmtP3-GFP-atb2-983 mal3⁺-GFP-nat^r</i>	This study
KZ217	<i>h⁻ leu1 ura4 atb2-V260I</i>	This study

Fluorescence Microscopy, Time-Lapse Live Analysis, and Image Processing

For GFP-fluorescence observation, cells were fixed with 3.7% formaldehyde. For indirect immunofluorescence with TAT-1, cells were fixed with methanol at -80°C , and then TAT-1 (1/50 dilution) was applied, followed by Cy3-conjugated goat anti-mouse IgG (C2128; Sigma-Aldrich, St. Louis, MO). Still images were viewed with a Zeiss Axioplan equipped with a chilled video charge-coupled device camera (C4742-95; Hamamatsu Photonics, Shizuoka, Japan) and a PC computer containing kinetic image AQM software (Kinetic Imaging, Durham, NC) and processed by use of Adobe Photoshop version 7.0 (Adobe Systems, Mountain View, CA).

For time-lapse live imaging, a 35-mm glass-bottomed culture dish (P35G-1.5-10-C; MatTek, Ashland, MA) was coated with 200 $\mu\text{g}/\text{ml}$ soybean lectin (L1395; Sigma-Aldrich). The culture of logarithmically growing cells (100 μl) was deposited in the well for a couple of minutes and then removed. The dish was filled with 3 ml of YE55 medium, and the cells that were attached to the bottom of the well were subjected to microscopic analysis at the room temperature. Images were taken using an Olympus IX70 wide-field inverted epifluorescence microscope with an Olympus PlanApo 100 $\times$, numerical aperture 1.4, oil immersion objective. DeltaVision image acquisition software (softWoRx 3.3.0; Applied Precision, Issaquah, WA) equipped with CoolSNAP HQ (Roper Scientific, Tucson, AZ) was used for capture of live images, which were processed with Adobe Photoshop version 7.0. The sections of images at each time point were compressed into a two-dimensional (2D) projection using the DeltaVision maximum intensity algorithm. Kymograph pictures derived from 2D-projected time-lapse images were constructed using softWoRx 3.3.0. All time-lapse analyses were performed at the room temperature (22 or 25°C).

Imaging Conditions

Still images were reconstructed from 10 to 14 sections of Z-stacks ($\Delta z = 0.3 \mu\text{m}$), which included the whole cell volume, by Lucida software. For the simultaneous observation of Sad1-red fluorescent protein (RFP) and *cen2*-GFP (Figures 4, B and C, and 9), eight to 10 sections of images ($\Delta z = 0.4 \mu\text{m}$) were taken at every 30 or 60 s. For the simultaneous observation of Mal3-GFP and Sad1-RFP (Figure 8B), four sections of images ($\Delta z = 0.4 \mu\text{m}$) were taken at every 10 s. The SPBs were included in these four sections at every time point. For the simultaneous observation of Sad1-RFP and Bub1-GFP (Figures 6A and 8F), 8–10 s of images ($\Delta z = 0.4 \mu\text{m}$) were taken every 30- or 60-s interval.

RESULTS

Isolation of Fission Yeast Mutants with Defects in Spindle-Kinetochore Interaction

To identify factors involved in a proper attachment of the kinetochore to the spindle, we performed a large-scale screening for fission yeast mutants with defects in the spindle-kinetochore interaction. The screening procedures consisted of three steps as briefly described below. First, ts mutants that displayed minichromosome loss at the permissive temperature (*cin* mutants) were isolated. Next, sensitivity to the MT depolymerizing drug TBZ was tested in these *cin* mutants. Finally, the cellular localiza-

Figure 1. Isolation of the *cin3-983* mutant. (A) Temperature and TBZ sensitivity. Wild-type (513; Table 1) or *cin3 (atb2)-983* mutants (KZ94) were spotted on rich medium (5×10^3 cells in the far-left spots for each plate and then diluted 10-fold in each subsequent spot rightward) in the presence (middle plate) or absence (left and right) of 10 μg/ml TBZ and incubated at 27°C (left and middle) or at 36°C (right) for 3 d. (B) Localization of Bub1-GFP. *nda3-1828* (KZ35) or *cin3 (atb2)-983* cells (ts983) were incubated at 36°C for 4 h and Bub1-GFP and DNA were visualized. The top panels show Bub1-GFP signals, whereas the bottom panels show merged pictures (Bub1-GFP in green and DAPI in orange). Bar, 10 μm.

tion of the Bub1 spindle checkpoint protein was examined. To facilitate mutant screening described above, we used a parental strain (KZ2; Table 1) that contained chromosome III-derived minichromosome Ch16 (Niwa *et al.*, 1989) and *bub1⁺-GFP*, in which GFP was integrated at the C terminus of the *bub1⁺* ORF under the native promoter. Among 31 *cin* complementation groups, 10 loci (*cin1-cin10*) showed hypersensitivity to TBZ compared with a parental strain (Figure 1A and Supplemental Table 1).

In wild-type cells, Bub1 is recruited to the kinetochore at early mitosis for a short time such that in asynchronously growing cultures, only a small population of cells (<2%) displays Bub1 localization to the kinetochore as a single nuclear dot (Bernard *et al.*, 1998; Toyoda *et al.*, 2002). On the other hand, in the ts β -tubulin mutant (*nda3-1828*), which loses the MT structure at the restrictive temperature (Radcliffe *et al.*, 1998), and is therefore expected to contain unattached kinetochores, 60% of cells showed Bub1 at the kinetochore at 36°C and even at the permissive temperature (26°C), Bub1 localized to the kinetochore in a higher proportion of cells (6.2%; Figure 1B). In the case of the *rad21-K1* mutant defective in cohesion, which is also required for bipolar attachment of the kinetochore to the spindle (Tatebayashi *et al.*, 1998; Tanaka *et al.*, 2000), a higher value of Bub1 dots (3.0% at 26°C and 5.6% at 36°C) was obtained. Having established the experimental system in which to assess the state of the spindle-kinetochore interaction, Bub1 localization in *cin1-cin10* mutants was investigated at both permissive and restrictive temperatures. It was found that all the *cin*

mutants displayed increased Bub1 localization to the kinetochore (Figure 1B and Supplemental Table 1).

cin3 Is Allelic to atb2, a Locus Encoding a Nonessential α 2-Tubulin

We cloned the *cin1⁺-cin4⁺* genes and identified their corresponding gene products (Supplemental Table 1). Among these, *cin3-983* was of our particular interest, because it was suppressed by plasmids containing the *atb2⁺* gene and tightly linked to *atb2*, which encodes α 2-tubulin (Toda *et al.*, 1984). Fission yeast contains two α -tubulin-encoding genes, *atb2⁺* and *nda2⁺*, in which *atb2⁺* is non-essential, whereas *nda2⁺* is essential for cell viability (Adachi *et al.*, 1986). This implied that if *cin3-983* were allelic to *atb2*, this mutation must be dominant over chromosomal *nda2⁺*. To confirm identity between *cin3* and *atb2*, nucleotide sequence of the *atb2* gene was determined using genomic DNA prepared from a *cin3-983* strain. This analysis demonstrated that *cin3-983* indeed contains a point mutation in *atb2* at position +778 from guanine to adenine (adenine for initiator ATG is denoted as +1), which results in amino acid substitution at position 260 from valine (GTA) to isoleucine (ATA). Comparison of amino acid residues among α -tubulin molecules from different species indicated that V260 is invariant (Figure 2A). Close inspection of the three-dimensional structure of the $\alpha\beta$ -tubulin heterodimer (Nogales *et al.*, 1998) shows that V260 is situated in a linker region between helix 8 (H8) and β -sheet 7 (B7), which locates in proximity to the interacting interface between α - and β -tubulin (Figure 2B). This residue also faces toward the outer surface of MT protofilaments.

To verify the V260I mutation results in temperature sensitivity of *atb2-983*, plasmids carrying *atb2-V260I* that contain the V260I mutation were introduced into an *atb2*-deleted strain. As shown in Figure 2C (top), this transformed strain indeed became ts, and even at the low temperature (27°C), introduction of the *Atb2-V260I* mutation compromised cell growth. Furthermore, to confirm that ts phenotypes of *cin3-983* are ascribable to an *atb2-V260I* single mutation, fragments carrying the mutated *atb2-V260I* gene were integrated into the corresponding *atb2* locus, which resulted in replacement of wild-type *atb2⁺* with this mutation at its endogenous locus (see *Materials and Methods*). This newly constructed strain indeed displayed ts growth (Figure 2C, middle). Heterozygous diploids containing *atb2-983* and wild-type *atb2⁺* alleles could form colonies at 36°C (Figure 2C, bottom), indicating that strictly speaking, *atb2-983* is not a dominant mutation. From these results, we concluded that *cin3* is allelic to *atb2*, in which its ts phenotype is derived from a single amino acid substitution (V260I) of *Atb2* (hereafter, *cin3-983* is referred to as *atb2-983*).

The Atb2-983 Protein Is Incorporated into the Microtubule

To examine the cellular localization of *Atb2-983*, the GFP cassette (under the control of the thiamine-repressible *nmtP3* promoter) (Bähler *et al.*, 1998) was inserted in front of the initiator methionine of the *atb2-983* gene (GFP-*Atb2-983*). Then, localization patterns of GFP-*Atb2-983* were examined in exponentially growing cells under repressed conditions. As shown in Figure 2D, like wild-type GFP-*Atb2*, GFP-*Atb2-983* could localize to both interphase MTs (top) and mitotic spindles (bottom). Although *Atb2-983* is incorporated into the MTs, it is possible that the *atb2-983* mutation results in down-regulation of essential *Nda2*, which might, at least in part, account for its ts

Figure 2. The *cin3-983* mutant is allelic to *atb2*. (A) The mutation site of Atb2-983. The valine 260 residue (white letters in black boxes) was substituted with isoleucine (red) in the *atb2-983* mutant. The regions around the valine 260 in α -tubulins from various species were aligned. The corresponding regions in fission yeast β -tubulin (Nda3) and γ -tubulin (Tug1/Gtb1) are also shown. Sp, *Schizosaccharomyces pombe*; Hs, *Homo sapiens*; Mm, *Mus muscalis*; Dm, *Drosophila melanogaster*; At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; and Sc, *Saccharomyces cerevisiae*. (B) Predicted microtubule structure. The structure of fission yeast α/β -tubulin dimers was predicted by RasMol software and aligned longitudinally based on bovine α/β -tubulin protofilament (Nogales *et al.*, 1998). Structure is oriented with the plus end upward (+), the minus end downward (-), the inside leftward (i), and the outside rightward (lat). The location of the *atb2-983* mutation was indicated by arrow with red circle in a magnified view of the interdimer interface (right). (C) The *atb2-V260I* mutation confers temperature sensitivity. p(*atb2*⁺), p(*atb2-V260I*) or vector plasmids were introduced into an *atb2*-deletion strain (KZ115, top). A strain containing the integrated *atb2-V260I* allele at its genomic locus (KZ217, middle), wild-type diploids (*atb2*⁺/*atb2*⁺, bottom) and heterozygous diploids (*atb2-983/atb2*⁺) were also tested for temperature sensitivity. Each strain was spotted on rich medium and incubated at 27 or 36°C for 3 d. (D) Incorporation of GFP-Atb2-983 into the microtubules. A strain containing wild-type GFP-*atb2*⁺ (P3-GFP-*atb2*) or mutant GFP-*atb2-983* (KZ176) was grown at 27°C in the liquid YE55 medium, and GFP signals were recorded. Images of interphase (top) and mitosis (bottom) are shown. Bar, 10 μ m.

phenotypes. However, protein levels of Nad2 and Atb2 (-983) did not alter significantly between wild type and *atb2-983* mutants (Supplemental Figure S1), suggesting that this mutation does not affect the quantity of total α -tubulin levels or a ratio between Nda2 and Atb2. Thus the Atb2-983 protein is incorporated into MTs, which might be directly related to the apparent dominance of *atb2-983* over the endogenous *nda2*⁺ gene.

atb2-983 Cells Display Less Dynamic Microtubules and a High Rate of Unequal Chromosome Segregation

As a first step to characterize the defective phenotypes of *atb2-983*, the MT structure was examined with indirect immunofluorescence microscopy using anti- α -tubulin antibody (TAT-1). We found that, unlike other *ts atb2* mutants previously isolated, which show reduced staining resulting from destabilized MTs (Radcliffe *et al.*, 1998), *atb2-983* cells retained apparently intact MT structures during both inter-

phase and mitosis at the restrictive temperature (Figure 3A). Interphase *atb2-983* cells showed a filamentous MT array that extended along the cell axis (top, wild type in the left and *atb2-983* in the right), and mitotic cells also showed seemingly normal spindle structure with distinct astral MTs like wild-type cells (second and third panels). Furthermore, telophase cells displayed long spindles with a medial ring (fourth panels), and postmitotic cells showed characteristic postanaphase array of interphase MT structures (bottom). This result suggested that MTs are not destabilized in the *atb2-983* mutant. We then measured MT dynamics using GFP-Atb2 and GFP-Atb2-983 described previously. Compared with wild type, both the growth and shrinkage rates were decreased down to 33 and 60%, respectively, in the *atb2-983* mutant (Table 2). Thus, it seems that the V260I mutation of Atb2 renders MTs less dynamic. It is likely that the efficiency of MT polymerization/depolymerization is somehow compromised by the V260I mutation in Atb2.

Figure 3. The *atb2-983* mutant shows unequal chromosome segregation. (A) Microtubule structure in *atb2-983* cells during the cell cycle. Wild-type (513) and *atb2-983* cells (KZ94) were incubated at 36°C for 4 h, fixed, and processed for immunofluorescence microscopy with anti- α -tubulin antibody (TAT-1; green) and DAPI (red). Representative images of wild-type (left) and *atb2-983* cells (right) in each cell cycle stage are shown (from the top, interphase, early mitosis, late anaphase, telophase, and postmitosis). (B) Unequal chromosome segregation in *atb2-983*. Wild-type (top) or *atb2-983* cells (bottom three panels) were processed for immunofluorescence microscopy as in described in A. Representative cells displaying chromosome missegregation in *atb2-983* are shown. Bars, 10 μ m. (C) Quantification of chromosome missegregation. Wild-type and *atb2-983* cells were grown at 25°C and shifted to 36°C. After 4 and 6 h, aliquots were taken, fixed, stained with DAPI, and the percentage of chromosome missegregation was counted ($n = 100$). We did not find any chromosome missegregation in wild-type cells under this condition.

Despite normal-looking MT architectures in fixed samples, consistent with minichromosome loss phenotypes, we often observed mitotic cells that undergo unequal chromo-

Table 2. Quantification of microtubule dynamics

Parameter	Wild type	<i>atb2-983</i>
Growth rate (μ m/min)	2.4 ± 0.3	0.8 ± 0.3
Shrinkage rate (μ m/min)	3.2 ± 0.6	1.4 ± 0.3
Catastrophe frequency (events/min)	0.39	0.31

Wild-type (GFP-Atb2) and *atb2-983* strains (KZ176 containing GFP-Atb2-983) were used. MT dynamics were measured at 25°C, and in each case 10 live cells were recorded and analyzed.

some segregation at the restrictive temperature (Figure 3B). Even at the permissive temperature, 18% of binucleate cells showed chromosome missegregation, and the value was increased to 60% upon temperature shift-up (Figure 3C). This suggested that less dynamic MTs in the *atb2-983* mutant impose chromosome missegregation without impairing overall MT structural integrity.

Prolonged Mitosis in the *atb2-983* Mutant

Quantification of mitotic cells in fluorescence microscopy showed that in the *atb2-983* mutant, the population of mitotic cells was significantly augmented compared with wild-type cells ($n = 200$; Figure 4A). Simple immunofluorescence, however, did not show which mitotic stage was prolonged in the *atb2-983* mutant, because the percentage of mitotic cells containing either short ($<2 \mu$ m) or long ($>2 \mu$ m) spindles was increased in *atb2-983* (13 or 6% for *atb2-983* and 5 or 1% for wild type, respectively; Figure 4A).

To determine precisely the mitotic phases in which the *atb2-983* mutant shows delay, time-lapse imaging analysis was performed. To monitor the length of the mitotic spindle in each live cell, the SPB was visualized by RFP-tagged Sad1 (Sad1-RFP), which is a constitutive component of the SPB (Hagan and Yanagida, 1995). In addition, to visualize centromere movement, the centromere of chromosome 2 was marked by the GFP-Lac repressor (GFP-LacI)/*lacO* system (*cen2-GFP*), in which the *lacO* array was integrated within 5 kb of *cen2* in a strain carrying GFP-LacI (Straight *et al.*, 1996; Nabeshima *et al.*, 1998; Ding *et al.*, 2004). This assay system enabled us to distinguish each mitotic stage, including the initial stage of SPB separation (prophase equivalent), prometaphase, metaphase, and the subsequent later stages, including anaphase A and B. Observation and quantification of a number of independent mitotic cells showed that the *atb2-983* mutant was delayed at three mitotic stages. First, the duration between initiation of SPB separation and onset of anaphase A was extended by $\sim 40\%$ (16.4 ± 3.7 min in *atb2-983* and 11.6 ± 1.3 min in wild type; Table 3). Representative patterns of kinetics of mitotic phases are shown in Figure 4B). It is of note that this 40% increase in *atb2-983* is ascribable to the delay in both prophase and prometaphase (kymograph images with the duration of these phases are shown in Figure 4C). In addition, the period of anaphase B was also expanded twofold (31.3 ± 9.5 min in *atb2-983* and 15.6 ± 1.3 min in wild type). This extension of anaphase B is largely attributable to the decrease in the velocity of spindle elongation ($0.36 \pm 0.12 \mu$ m/min in *atb2-983* and $0.65 \pm 0.11 \mu$ m/min in wild type). Therefore, the *atb2-983* mutation results in slower passage in three distinct stages, prophase, prometaphase, and anaphase B, suggesting that in addition to during interphase, MT dynamics during mitosis is also impeded in this α -tubulin mutation.

Figure 4. Mitosis is slowed down in *atb2-983* cells. (A) Mitotic delay in *atb2-983* cells. Wild-type (513) and *atb2-983* cells (KZ94) were incubated at 36°C for 4 h, and MTs were visualized with immunofluorescence as in Figure 3. The population of cells with short (<2 μm) or long spindle (>2 μm) was counted from >300 cells. (B) Passage of mitotic phases in live *atb2-983* cells. Wild-type (KZ85) and *atb2-983* cells (KZ139) containing *cen2*-GFP and Sad1-RFP were grown, and time-lapse live analysis was performed at the room temperature (n = 10). Typical examples for the two strains were shown. The timing of anaphase A onset and completion is marked with yellow and white circles, respectively. (C) Kymograph images of the cells shown in B. Kymograph images were produced every 1-min interval. Yellow and white arrows indicate the duration of prophase and prometaphase/metaphase, respectively. Vertical bar, 5 μm.

Bub1, but Not *Mad2*, Spindle Checkpoint Is Activated in the *atb2-983* Mutant

Next, we addressed a relationship between the spindle checkpoint and the *atb2-983* mutant. First, kinetochore localization of *Bub1* was confirmed by colocalization between *Bub1*-GFP and *Nuf2*-CFP, which is a core component of the kinetochore (Nabetani *et al.*, 2001) (Figure 5A). To further investigate the state of spindle checkpoint activation, we examined the localization of another spindle checkpoint protein *Mad2*. Interestingly, unlike *Bub1*, *Mad2* was not recruited to the kinetochore during mitosis in the *atb2-983* mutant (~1%; Figure 5, B and C), suggesting that *Bub1*, but not *Mad2*, detects the defect in the spindle–kinetochore interaction in the *atb2-983* mutant.

Genetic analysis further supported this notion. It was found that the *atb2-983* mutant is lethal even at the permissive temperature when *bub1*⁺ is simultaneously deleted (Figure 5D). In clear contrast, the double mutant between *mad2*-deletion and *atb2-983* is viable. Growth property of the *atb2-983mad2* double mutant was almost indistinguishable from that of the *atb2-983* single mutant (Figure 5E). Having established the distinct requirement of *Bub1* and *Mad2* for maintaining viability of the *atb2-983* mutant, we then examined the necessity of other checkpoint genes. These include *mad1*⁺, *mad3*⁺, *bub3*⁺, and *mph1*⁺ (the *MPS1* homologue). Genetic cross between *atb2-983* and individual deletion mutants enabled us to classify these genes into two functional groups. One group, including *bub1*⁺, *bub3*⁺, and *mph1*⁺, is essential in the *atb2-983* background, whereas the other group, including *mad1*⁺, *mad2*⁺, and *mad3*⁺ is not required for viability, although a ts phenotype of the double mutant was slightly exaggerated particularly with *mad1* (Figure 5E and Table 4). The *atb2bub1* double deletion strain was viable, substantiating the dominant nature of *atb2-983* (Table 4).

It is of note that the protein kinase activity of *Bub1* seems not to be required for keeping viability of *atb2-983*, because, unlike *bub1*-deletion, double mutants between *atb2-983* and *bub1* mutants, which contain either amino acid substitution (K762M, *bub1*-KD) (Vanoosthuyse *et al.*, 2004) or a whole deletion in the protein kinase domain (*bub1*-Δkinase) (Yamaguchi *et al.*, 2003), are viable (Table 4). It is known that *Bub1* plays checkpoint-dependent and -independent roles in chromosome segregation, in which its protein kinase activity is not involved in spindle checkpoint signaling (Vanoosthuyse *et al.*, 2004; Kadura *et al.*, 2005). The fact that *atb2-982* is viable in *bub1* kinase-dead mutant suggested that it is a *Bub1*-checkpoint function that is required for survival of *atb2-983*. Dependence of *atb2-983* viability on the presence of other checkpoint proteins such as *Bub3* and *Mph1* also supported this notion. Together, these results suggest that the *atb2-983* mutant has a specific defect in the spindle–kinetochore interaction, which activates only a subset of checkpoint proteins, including *Bub1*, *Bub3*, and *Mph1*, and these checkpoint proteins play an essential role in keeping *atb2-983* viable at the permissive temperature.

Bub1 Localizes in Proximity to Only One of the Two SPBs

Given the essential role for *Bub1* in *atb2-983*, we performed time-lapse imaging of *Bub1*-GFP in this mutant (a strain was also tagged with *Sad1*-RFP). In wild-type cells, *Bub1*-GFP signals first occurred as a blob during very early mitosis in the vicinity of the SPB before its separation and were associated with one body of separating SPBs for a short time (~6 min). Then, signals disappeared some time during early mitosis (n = 20; Figure 6A, top). In *atb2-983* cells, on the other hand, the duration of the *Bub1*-GFP blob was extended three- to fivefold, in which *Bub1*-GFP was retained in the

Table 3. Duration of mitotic phases

Strain	Duration between SPB separation and anaphase A onset (min)	Spindle elongation	
		Duration (min)	Rate (μm/min)
Wild type	11.6 ± 1.3	15.6 ± 1.3	0.65 ± 0.11
<i>atb2-983</i>	16.4 ± 3.7	31.3 ± 9.5	0.36 ± 0.12

At least 10 independent cells were observed in each strain.

Figure 5. Bub1-, but not Mad2-, checkpoint is activated in *atb2-983* cells. (A) Kinetochore localization of Bub1 in *atb2-983* cells. Characteristic mitotic *atb2-983* cells that display Bub1-GFP dots (left) at the kinetochores (Nuf2-CFP; middle) are shown (KZ144; 4 h at 36°C). In the merged image (right), Bub1-GFP (green), Nuf2-CFP (red), and DAPI (blue) are shown. (B) Mad2 recruitment to the kinetochore is not enhanced by the *atb2-983* mutation. Mad2-GFP and DNA were visualized in *nda3-1828* cells (KZ54; left) or *atb2-983* cells (KZ156; right) after a 4-h incubation at 36°C. Bars, 10 μ m. (C) Quantification of Mad2-GFP and Bub1-GFP dots. The percentage of wild-type and *atb2-983* mutant cells (grown at 27°C) that display Bub1 or Mad2 dots ($n > 350$) is shown. (D) Synthetic lethality of an *atb2-983bub1* strain. Tetrads from genetic cross between an *atb2-983* strain (KZ102) and *bub1::ura4⁺* strain (393) were dissected and incubated at 27°C for 4 d. Segregants corresponding to *atb2-983bub1::ura4⁺* double mutants (open square) failed to form colonies. (E) Growth properties of *atb2-983mad* double mutants. Strains with indicated genotypes were spotted on rich medium (5×10^4 cells in the far-left spots for each plate and then diluted 10-fold in each subsequent spot rightward) and incubated at 27°C (left), 32°C (middle), or 36°C (right) for 3 d.

vicinity of one SPB ($n = 10$; Figure 6A, bottom). It is important to note that in both wild-type and *atb2-983* cells, Bub1-GFP signals were associated with only one SPB, and we have never observed strong Bub1 dots that were dissociated from

the SPB or located between the two SPBs. Because Mad2 does not localize to the kinetochore in *atb2-983* cells, Bub1-localizing kinetochores are likely to be attached to the spindle, but not in a bipolar manner.

Table 4. Genetic interaction between *atb2-983* and spindle checkpoint genes

<i>atb2</i> alleles	Deleted genes							
	<i>bub1</i>	<i>bub3</i>	<i>mph1</i>	<i>mad1</i>	<i>mad2</i>	<i>mad3</i>	<i>bub1-KD^a</i>	<i>bub1-Δkinase^b</i>
<i>atb2-983</i>	L ^c	L	L	+ ^d	+	+	+	+
<i>atb2-deletion</i>	+	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D., not determined.

^a The *bub1-KD* mutant contains amino acid substitution of Bub1 at the 762nd residue (Bub1K762M), which would inactivate protein kinase activity of Bub1 (obtained from Dr. Jean-Paul Javerzat).

^b The *bub1- Δ kinase* mutant contains a truncated Bub1, which lacks protein kinase domain of Bub1 (obtained from Dr. Satoko Yamaguchi, Cancer Research UK, London, United Kingdom).

^c Double mutants are inviable at 27°C.

^d Double mutants are viable at 27°C.

Figure 6. The Bub1 spindle checkpoint prevents unequal chromosome segregation in *atb2-983* cells. (A) Prolonged localization of Bub1 blob during mitosis. Time-lapse images of Bub1-GFP and Sad1-RFP localization during mitosis were recorded and converted to a kymograph every 1-min interval (top, wild type, KZ181; and bottom, *atb2-983*, KZ182). Vertical bar, 5 μ m. (B) Unequal chromosome segregation phenotype of *atb2-983bub1* cells. *atb2-983bub1* cells containing *cen2*-GFP were kept viable by expressing episomal *atb2*⁺, starved for nitrogen for 12 h, and released into the rich medium (26°C) to induce plasmid loss. At 8 h after release, patterns of chromosome segregation were observed with *cen2*-GFP (green) and DAPI staining (red). Representative images displaying 2:0 distribution of *cen2*-GFP (marked with arrows) are shown. Bar, 10 μ m. (C) The percentage of unequal chromosome segregation in *atb2-983* and *atb2-983bub1* cells.

The Bub1 Checkpoint Prevents *atb2-983* Cells from Lethal Chromosome Missegregation

We sought to examine the lethal phenotype of *atb2-983bub1* cells. To this end, an *atb2-983bub1* strain, which contains *cen2*-GFP and plasmids carrying the *atb2*⁺ gene, was constructed. This plasmid is mitotically stable because double mutants are lethal without the episomal *atb2*⁺ gene. To induce plasmid loss, this strain was starved for nitrogen for 12 h and then allowed to grow again in rich medium. Nitrogen starvation, which results in growth arrest at G₁, is known to induce loss of episomal plasmids (Garcia *et al.*, 2002a). After 8-h incubation upon release into rich medium at 26°C, *atb2-983bub1* cells displayed massive chromosome missegregation. As shown in Figure 6, B and C, >70% of binucleate cells (n = 200) showed unequal chromosome segregation, which was evident by staining of *cen2*-GFP and 4,6-diamidino-2-phenylindole (DAPI). Under the same conditions, a single *atb2-983* strain showed modest chromosome missegregation (7%). It should be noted that we hardly ever observed “cut” phenotypes, in which a septum is formed in the middle of unseparated chromosomes. This suggests that

atb2-983bub1 cells are capable of segregating chromosomes by the mitotic spindle, albeit unequally. This analysis, therefore, established a role for the Bub1 checkpoint in preventing *atb2-983* cells from lethal missegregation of sister chromatids.

EB1 Homologue-encoding *mal3*⁺ Gene Is a High-Dosage Suppressor

During screening for multicopy suppressor genes of *atb2-983*, we isolated the *mal3*⁺ gene (Figure 7A), which encodes the fission yeast homologue of MT-binding protein EB1 (Su *et al.*, 1995; Beinhauer *et al.*, 1997). The *mal3* deletion is not lethal (Beinhauer *et al.*, 1997), but when combined with *atb2-983*, the double mutant grew significantly slower even at the low temperature (27°C) than wild type or each single mutant (Figure 7B, boxed) and displayed noticeable chromosome missegregation phenotypes (Figure 7, C and F). In addition to suppression of temperature-sensitivity of the *atb2-983* single mutant (Figure 7A), increased dosage of *mal3*⁺ rescued synthetic lethality of *atb2-983bub1* double mutants (Figure 7D). Nonetheless, suppression of ts *atb2-983* or lethal *atb2-983bub1* cells by high-dosage *mal3*⁺ was incomplete, because chromosome missegregation was still observed (Figure 7, E and F). Also, *atb2-983bub1* mutants containing multicopy *mal3*⁺ grew slowly (Figure 7D). These results suggested a close functional linkage between Atb2 (-983) and Mal3.

Mal3 Localization to the Microtubule Is Reduced in the *atb2-983* Mutant

Mal3 localizes to both cytoplasmic MTs and mitotic spindles (Beinhauer *et al.*, 1997; Browning *et al.*, 2003; Busch and Brunner, 2004; Busch *et al.*, 2004; Asakawa *et al.*, 2005). Visualization of Mal3 by GFP tagging (Mal3-GFP) showed that localization signals of Mal3-GFP on the mitotic spindle were substantially reduced in *atb2-983* cells compared with wild type (Figure 8A, bottom, and B), although its total protein levels are not decreased (Figure 8C). In addition Mal3 localization to the cytoplasmic MTs also seemed reduced (Figure 8A, top). Multicopy plasmids carrying *atb2*⁺, *nda2*⁺ or *mal3*⁺ were capable of restoring reduced spindle localization of Mal3 in the *atb2-983* mutant (Figure 8D, representative Mal3-GFP localization pictures to the spindles in each transformant are shown).

To further address a physical interaction between Mal3 and Atb2 (-983), coimmunoprecipitation analysis was performed in wild-type and *atb2-983* cells. For this purpose, the chromosomal *mal3*⁺ gene was tagged with *myc* epitope (Mal3-Myc), which was crossed with strains containing GFP-Atb2 (-983). Immunoprecipitation using anti-Myc antibody as a primary antibody, followed by immunoblotting with individual antibodies showed that a binding between Mal3 and Atb2-983 is compromised in the mutant at 36°C (Figure 8E, lane 9). In addition, immunoprecipitation using wild-type and *atb2-983* mutants containing Mal3-GFP also showed that binding between Mal3-GFP and Atb2-983 is impaired at 36°C (Supplemental Figure S2).

We also found that in a *mal3* deletion strain, Bub1-GFP localized, like *atb2-983*, to the vicinity of one SPB for a prolonged period (Asakawa *et al.*, 2005) (representative Bub1-GFP singles were shown in Figure 8F and see Figure 6A for wild-type control). Furthermore, *mal3* mutants display extension of mitotic prophase, prometaphase, and anaphase B and show synthetic genetic interaction with *bub1*, *bub3*, or *mph1* but not *mad1* or *mad2* (Asakawa *et al.*, 2005), genetic interactions very similar to those of *atb2-983* shown earlier (see Table 4). Together, these results suggested that

Figure 7. Suppression of *atb2-983* by multicopy *mal3*⁺ gene encoding the EBI homolog. (A) Suppression of the ts *atb2-983* mutation by multicopy plasmids containing *mal3*⁺. wt, wild-type cells. (B) Genetic interaction between *atb2-983* and *mal3*. Tetrads from genetic cross between an *atb2-983* strain (KZ137) and a *mal3*-deleted strain (YP350) were dissected and incubated at 27°C for 4 d. Segregants corresponding to *atb2-983mal3* double mutants are marked with open squares. (C) Chromosome missegregation of *atb2-983mal3* cells. The *atb2-983mal3* double mutant (KZ141) was grown at 27°C and stained with DAPI. Cells displaying chromosome missegregation are pointed by arrows. (D) Suppression of the lethality of *atb2-983bub1* by a high dosage of *mal3*⁺. *atb2-983bub1* (p*mal3*⁺-LEU2) (KZ135) was obtained from the cross between an *atb2-983* strain carrying plasmids containing *mal3*⁺ (p*mal3*⁺-LEU2) and a *bub1::ura4*⁺ strain. (E) Chromosome missegregation of *atb2-983bub1* carrying multicopy *mal3*⁺. Bar, 10 μ m. (F) Quantification of chromosome missegregation. Indicated strains were grown in minimal liquid media lacking leucine at 27°C and shifted to 36°C. At 0-, 4-, and 6-h time points, aliquots were taken and stained with DAPI. The percentage of binucleate cells displaying chromosome missegregation was quantified (n = 100).

temperature sensitivity and mitotic defects observed in *atb2-983* might stem from, at least in part, compromised Mal3 localization to the mitotic spindle.

Sister Chromatid Oscillation during Prometaphase Is Less Dynamic in the *atb2-983* Mutant Independent of Mal3 Dysfunction

It is shown that during mid-mitosis, fission yeast kinetochores display, like animal cells, back-and-forth oscillation between the two poles, which is likely to correspond to the prometaphase state (Saitoh *et al.*, 1997; Garcia *et al.*, 2002b). We addressed whether the *atb2-983* mutation affects this phase using *cen2*-GFP and Sad1-RFP described earlier (see Figure 4). To follow *cen2*-GFP behavior in more detail, kymograph imaging was performed every 10 s (note that images in Figure 4C were processed every 1-min interval). We observed >30 samples of wild-type mitoses and created kymograph pictures. This analysis showed that *cen2*-GFP

signals indeed displayed rapid and complex patterns of oscillation between the two SPBs (a representative image is shown in Figure 9A, top, and the period of prometaphase is marked with solid arrow at the bottom). Back-and-forth movement of *cen2*-GFP became more evident when the distance between *cen2*-GFP and the two SPBs were measured and plotted against time (Figure 9B, left, representative images are shown; n = 12).

In addition to dynamic oscillation, *cen2*-GFP signals seemed to be often split into two before anaphase onset (Figure 9A, top). This suggested that sister kinetochores are captured by the spindle MTs from both poles at this stage, thereby oscillating between the SPBs via opposing poleward forces imposed at each sister kinetochore. This oscillation period was followed by segregation of *cen2*-GFP signals toward each pole (anaphase A, shown with bidirectional yellow arrows). As reported previously (Tournier *et al.*, 2004), there was a transient pause (1–2 min) between pro-

Figure 8. Mal3 loading onto the spindle and binding to Atb2-983 are reduced in the *atb2-983* mutant. (A) Reduced Mal3 localization. Wild-type (MA145) and *atb2-983* cells containing Mal3-GFP (KZ165) were grown at 27°C and GFP signals were taken. (B) Time-lapse analysis of mitotic Mal3-GFP. The live signals of mitotic Mal3-GFP in wild-type (KZ194; left) or *atb2-983* (KZ195; right) cells were obtained and converted to kymograph pictures (Mal3-GFP in green and Sad1-RFP in red). The length of the white arrow and the black bar represents 1 min and 2 μ m, respectively. (C) Protein levels of Mal3 in *atb2-983*. Extracts were prepared from exponentially growing wild-type (lane 2) or *atb2-983* cells containing Mal3-GFP (lane 3) and immunoblotted with indicated antibodies. An untagged wild-type strain (lane 1) was used as a control. (D) Suppression of compromised spindle localization of Mal3. *atb2-983* mutants carrying various multicopy plasmids (an empty vector, *atb2*⁺, *nda2*⁺, or *mal3*⁺) were grown at 27°C and shifted to 36°C for 4 h, and Mal3-GFP signals were taken. Representative Mal3-GFP signals localizing to the spindle are shown (n = 20). The exposure time of these pictures are the same (2 s). (E) Immunoprecipitation between GFP-Atb2 (-983) and Mal3-Myc. Wild-type (KZ214; lanes 3, 6, and 8) or *atb2-983* mutants containing GFP-Atb2 (-983) and Mal3-GFP (KZ216; lanes 4, 7, and 9) were grown at 25°C (lanes 2, 3, 6, and 7) and shifted to 36°C (lanes 8 and 9). Total cell extracts were prepared at 0 (lanes 3 and 4, 10 mg of extracts was loaded) and 4 h (lanes 5–8), and immunoprecipitation was performed using monoclonal anti-Myc antibody as a primary antibody. Immunoprecipitates were run on SDS-PAGE, and immunoblotting was performed with polyclonal anti-Myc antibody (top) or monoclonal anti-GFP antibody (bottom). As a control, immunoprecipitation was performed from extracts prepared from a strain containing only GFP-Atb2 (lanes 2 and 5). Extracts prepared from an untagged wild-type strain was also run as another control (lane 1). Precipitated bands were marked with arrowheads, and nonspecific bands, probably corresponding to IgG heavy chains, were shown with asterisk. (F) Prolonged localization of Bub1 blob during early mitosis in *mal3* cells. Time-lapse images (1-min intervals) of Bub1-GFP and Sad1-RFP localization during mitosis were recorded and converted to a kymograph. For patterns of wild-type and *atb2-983* cells, see Figure 6A. Vertical bars, 2 μ m.

metaphase and onset of anaphase A, in which *cen2*-GFP signals tended to be situated in the middle of the two SPBs, likely corresponding to fission yeast metaphase (marked by black arrowhead).

We then observed *atb2-983* mutant cells. Despite a prolonged delay during early mitosis as shown earlier (see Figure 4B), *cen2*-GFP signals were segregated equally under live imaging conditions (at the room temperature). However, unlike wild type, *atb2-983* cells did not display dynamic oscillation of *cen2*-GFP. Instead, in this strain, *cen2*-GFP stayed near the equator throughout mid-mitotic phase without back-and-forth movement (n = 10; Figure 9A, bottom; also see Figure 4C). Plotting of *cen2*-GFP positions

relative to the two SPBs clearly indicated less dynamic behavior of *cen2*-GFP compared with wild-type cells (Figure 9B, right, representative patterns are shown). These observations suggest that in the *atb2-983* mutant, antagonizing forces normally generated at the sister kinetochores during prometaphase are not functional, which renders centromere oscillatory movement more static.

We next asked whether static prometaphase behavior of *cen2*-GFP in *atb2-983* is ascribable to Mal3 malfunction. We, however, found that unlike *atb2-983*, sister centromeres still oscillate in the *mal3* mutant, although a mild reduction of oscillation seemed to occur (n = 10; a representative kymograph image is shown in Figure 9C). Together, our results

Figure 9. Lack of sister centromere oscillation in the *atb2-983* mutation. (A) Visualization of sister centromere movement in live cells. Kymograph images at high resolution (10-s intervals) are shown (*cen2*-GFP in green and *Sad1*-RFP in red) in wild-type (top; $n = 32$) and *atb2-983* cells (bottom; $n = 30$). *cen2*-GFP signals that were persistently associated with one SPB like Bub1-GFP (see Figure 6A), which probably represented syntelic attachment, were not analyzed in this assay. Yellow bidirectional arrows show the duration of anaphase A. Solid and dotted arrows indicate the duration of prometaphase and anaphase, respectively. Arrowhead indicates the timing of metaphase. (B) Prometaphase movement of *cen2*-GFP. The positions of *cen2*-GFP (green circles) are plotted relative to the two SPBs (red squares) every 20-s interval. The central position of the two SPBs is shown with purple lines (diamonds). When *cen2*-GFP signals seemed split, the middle position is plotted. The representative images are shown in wild-type ($n = 12$) and *atb2-983* ($n = 10$). (C) Visualization of sister centromere movement in *mal3* cells. Kymograph image in the *mal3* mutant is constructed as described in A. Vertical bars, 5 μm .

suggest that, in addition to compromised Mal3 localization and function, there are Mal3-independent deficiencies in *atb2-983* mutants, including the failure of sister centromere oscillation during prometaphase.

DISCUSSION

In this study, we have presented characterization of a novel mutant allele of the fission yeast α -tubulin encoding *atb2*⁺ gene. The *atb2-983* mutant displays a number of intriguing and somewhat unexpected phenotypes. Through our analysis, we have uncovered novel aspects of fission yeast mitosis, such as dynamic behavior of sister centromeres, surveillance of the mitotic kinetochore by the Bub1-branch checkpoint, and a role for MT dynamics in bipolar microtu-

bule attachment. We think that the results obtained in this study would be of direct relevance toward understanding of chromosome segregation in higher eukaryotes.

Reduced Mal3 Localization in the *atb2-983* Mutation

Unlike other *ts atb2* mutants previously isolated previously (Yaffe *et al.*, 1996; Radcliffe *et al.*, 1998), in the *atb2-983* allele (V260I) overall MT structures are not impaired noticeably, but instead the growth and shrinkage rates of interphase MTs are reduced. Also, mitosis is slowed down, including the velocity of anaphase spindle elongation. It is, therefore, possible that MT dynamics is in general impaired in this mutant. We show that increased dosage of *mal3*⁺ rescues *atb2-983* and Mal3 loading onto the MT is significantly compromised. Consistently, whereas Mal3 and Atb2 interact in wild-type cells, a binding between Mal3 and Atb2-983 is hampered. This suggests that the region around V260, which is situated close to the interphase of the α/β -tubulin heterodimer and also faces toward the lattice of MT protofilaments, is critical for an interaction of Atb2 with Mal3. Budding yeast Mal3 homologue Bim1 is also shown to interact with α -tubulin (Tub1), but the mutations analogous to V260I have not been analyzed in this organism (Schwartz *et al.*, 1997; Richards *et al.*, 2000).

It should be pointed out that amino acid substitution from valine to isoleucine, which results in addition of another methyl group to the side chain, is usually considered as a conservative replacement. However, the fact that this valine is invariant among all α -tubulin species in various organisms underscores the importance of valine at this site. The V260I mutation would induce subtle but deleterious conformational alterations in α -tubulin, thereby compromising an interaction with Mal3 without affecting either α/β -heterodimer formation or incorporation of these heterodimers into MTs. Indeed, we show that Atb2-983 is capable of being incorporated into MTs. Compromised Mal3 binding combined with the incorporation-competent properties of Atb2-983 would contribute to the apparent dominant nature of this mutant protein over essential α -tubulin isoform Nda2.

It is of note that in the *atb2-983* mutant, compared with its mitotic defects, interphase deficiencies are less noticeable. The *mal3*-deleted mutant displays, on the other hand, a number of interphase phenotypes such as shortened cytoplasmic MTs and resulting bent or branched cell morphology (Beinhauer *et al.*, 1997). It is thus possible that an interaction between Mal3 and its binding partners or Mal3 function is subject to cell cycle-dependent regulation. Mal3 is a phosphoprotein (Busch and Brunner, 2004) and analysis of Mal3's structural domains and modification during the cell cycle would address this question.

Phenotypic Similarities between *mal3* Mutants and *atb2-983*

Reduced localization of Mal3 to the spindle in *atb2-983* suggests that some of mitotic defects in this mutant are derived from compromised Mal3 function. Consistently, prolonged passage through distinct mitotic stages and longer lasting Bub1 kinetochore localization seem to be ascribed to Mal3 malfunction, because a *mal3* deletion strain also shows very similar phenotypes (Asakawa *et al.*, 2005; Asakawa and Toda, 2006). It should be noted that Mad2 localization to the kinetochores is not prolonged in either *mal3* or *atb2-983*. In both vertebrates and yeast, Mad2 localizes to, mostly, if not exclusively, unattached mitotic kinetochores (Waters *et al.*, 1998; Kapoor *et al.*, 2000; Skoufias *et al.*, 2001; Garcia *et al.*, 2002a; Ikui *et al.*, 2002; Gillett *et al.*, 2004), suggesting that the kinetochores of mitotic *atb2-983* or *mal3* cells are captured by

the spindle. However, subsequent establishment of bipolar attachment is substantially delayed or defective, in which Bub1 is retained in the kinetochores that are associated with only one of the two SPBs.

We have recently shown that most, if not all, of mitotic delay in the *mal3* mutant is ascribable to prolonged syntelic attachment of the kinetochore to the spindle, in which spindles emanated from one pole interact with both of the sister kinetochores (Asakawa *et al.*, 2005; Asakawa and Toda, 2006). Given the phenotypic similarities between *mal3* and *atb2-983* strains and the suppression of *atb2-983* by high dosage of *mal3*⁺, we envisage that *atb2-983* cells also display prolonged syntelic attachment during early mitotic stage, in which the Bub1 spindle checkpoint plays a role in preventing chromosome missegregation. It should be pointed out that a specific requirement of the Bub1 branch spindle checkpoint is analogous to that reported for the spindle orientation checkpoint (Rajagopalan *et al.*, 2004; Tournier *et al.*, 2004). *atb2-983* mutants might be defective in the positioning or rotation of the mitotic spindle, which would require spindle dynamics.

Sister Centromere Oscillation during Prometaphase

Mal3 dysfunction is not likely to be the sole reason for the *atb2-983* phenotype, because the *mal3* deletion is not ts. Time-lapse live analysis has revealed that whereas wild type or *mal3* mutant cells display dynamic sister centromere oscillation during prometaphase, *atb2-983* cells show sister centromeres situated in the middle of the spindle with very little oscillatory movement. This implies that mitotic spindles in *atb2-983* are defective in generation of pulling and/or pushing forces at the kinetochore during prometaphase, the stage in which Mal3 function is not involved. Disappearance of Mal3 from the tips of mitotic spindles during mid-mitosis in wild-type cells is in line with this notion (Asakawa *et al.*, 2005). Because neither Bub1 nor Mad2 localizes to these kinetochores during prometaphase in *atb2-983*, it is possible that amphitelic attachment has already been established at this stage, nonetheless anaphase onset being delayed mechanically by compromised spindle dynamics.

Another possibility is that the kinetochore attachment is merotelic, in which a single kinetochore is attached to the spindles emanating from both poles (Cimini *et al.*, 2001). It is shown that merotelic attachment occurs frequently during early mitosis in mammalian tissue cells (Cimini *et al.*, 2003). A single kinetochore is capable of interacting with multiple MTs in fission yeast as in animal systems (Ding *et al.*, 1993); therefore, merotelic configuration could occur in this organism. Indeed, lagging chromosomes that are frequently observed in a subset of chromosome segregation mutants are reported to represent merotelic attachment (Pidoux *et al.*, 2000). It is possible that the *atb2-983* mutant is hampered in correcting this attachment during prometaphase, possibly because of compromised dynamics of mitotic spindles. As in animal cells, the process of kinetochore–spindle attachment during fission yeast prometaphase is likely to be stochastic, which requires the dynamic nature of spindle MTs.

In conclusion, we show that in fission yeast dynamism of the spindle MT is crucial for accurate sister chromatid segregation. In this process, Mal3-EB1 interacts with α -tubulin and localizes to mitotic spindles, thereby ensuring establishment of stable bipolar MT attachment possibly by enhancing spindle dynamics. In the absence of Mal3 function, syntelic attachment is prolonged, which results in activation of the Bub1/Bub3/Mph1 checkpoint. In addition, MT dynamics is involved in sister chromatid oscillation during prometaphase, which is largely independent of Mal3 function. Fis-

sion yeast mitosis is, thus, remarkably similar to that in higher eukaryotes and further analysis will contribute to elucidation of the unified view on MT dynamics and accurate chromosome segregation at the molecular levels.

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