Midzone organization restricts interpolar microtubule plus-end dynamics during spindle elongation

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To study the dynamics of interpolar microtubules (iMTs) in *Saccharomyces cerevisiae* cells, we photobleached a considerable portion of the middle region of anaphase spindles in cells expressing tubulin-green fluorescent protein (GFP) and followed fluorescence recovery at the iMT plus-ends. We found that during anaphase, iMTs show phases of fast growth and shrinkage that are restricted to the iMT plus-ends. Our data indicate that iMT plus-end dynamics are regulated during mitosis, as fluorescence recovery was faster in intermediate anaphase (30 s) compared with long (100 s) and pre-anaphase (80 s) spindles. We also observed that deletion of Cin8, a microtubule-crosslinking kinesin-5 motor protein, reduced the recovery rate in anaphase spindles, indicating that Cin8 contributes to the destabilization of iMT plus-ends. Finally, we show that in cells lacking the midzone organizing protein Ase1, iMTs are highly dynamic and are exchangeable throughout most of their length, indicating that midzone organization is essential for restricting iMT dynamics.

Keywords: anaphase B; Ase1; Cin8; interpolar microtubule dynamics; midzone

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INTRODUCTION

One of the main factors that controls mitotic spindle morphogenesis is the dynamics of microtubules, polymers composed of $\alpha\beta$ -tubulin heterodimers. Microtubules show dynamic instability, which is manifested as rapid switches between phases of slow growth and fast shrinkage; their plus-ends, which terminate with β -subunits, are more dynamic than their minus-ends (Mitchison, 1989). During anaphase B spindle elongation, interpolar microtubules (iMTs) are stabilized at their minus-ends in *Saccharomyces cerevisiae* and in higher eukaryotic cells (Mallavarapu *et al*, 1999; Maddox *et al*, 2000; Brust-Mascher *et al*, 2004; Higuchi & Uhlmann, 2005; Dhonukshe *et al*, 2006; Cheerambathur *et al*,

2007). To facilitate spindle elongation, iMTs are polymerized at their plus ends. Owing to the absence of appropriate experimental approaches, limited data are available regarding the dynamics of iMT plus-ends during anaphase B. For example, it is not clear whether iMT growth at the plus-end occurs at a constant rate or whether it oscillates between transient phases of growth and shrinkage.

Antiparallel iMTs that emanate from the opposite poles overlap in the centre of the spindle, creating the spindle midzone. Numerous proteins that contribute to anaphase spindle stabilization and elongation localize to the midzone, including members of the conserved Ase1-like microtubule-associated proteins (MAPs). These proteins have been shown to bundle microtubules, and to contribute to anaphase midzone organization in fungi (Schuyler *et al*, 2003; Khmelinskii *et al*, 2007), as well as in mammalian (Fu *et al*, 2007) and plant cells (Smertenko *et al*, 2000). Another class of midzone-localized proteins is the kinesin-5 family members (Sharp *et al*, 1999; Fink *et al*, 2006; Tytell & Sorger, 2006). These bipolar mitotic motors (Kashina *et al*, 1996) are highly conserved among the eukaryotes (Barton & Goldstein, 1996; Kashina *et al*, 1997). They bundle (Gheber *et al*, 1999) and slide microtubules *in vitro* (Kapitein *et al*, 2005), and have been shown to perform important functions during anaphase spindle elongation in numerous organisms (Saunders *et al*, 1995; Straight *et al*, 1997; Sharp *et al*, 1999; Fink *et al*, 2006). So far, the functions of midzone organization and its components, such as Ase1-like MAPs and kinesin-5 motor proteins, in controlling anaphase iMT plus-end dynamics have not been studied. Here, we investigate the dynamics of iMT plus-ends in wild-type, *cin8* Δ and *ase1* Δ cells. Using high-resolution imaging and fluorescence recovery after photobleaching (FRAP), we characterize the iMT plus-end dynamics during anaphase spindle elongation, and show that Ase1 and kinesin-5 Cin8 influence these dynamics.

RESULTS AND DISCUSSION

Dynamics of iMT plus-ends in wild-type cells

To characterize the dynamics of iMTs during anaphase, we analysed FRAP of the spindle midzone region in *S. cerevisiae* cells expressing tubulin-green fluorescent protein (GFP). We followed spindles of various lengths; namely short pre-anaphase (1–2 μ m) spindles, intermediate (3.5–5.5 μ m) spindles or long (>5.5 μ m) anaphase spindles. We photobleached a region in the middle of the spindle, which spanned 40–60% of its length and

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exceeding the size of the midzone (Fig 1A). We then followed the recovered fluorescence intensity as a function of time ('FRAP traces') in a central region, approximately 80% of the initial photobleached region (Fig 1A,B). To minimize the effect of spindle elongation on the FRAP measurements, we performed short-term experiments (~ 200 s) with high temporal resolution (supplementary information online). During this time, the spindles elongated by $0.55 \pm 0.25 \mu\text{m}$ (s.d., $n = 15$), at an average rate of $0.17 \pm 0.7 \mu\text{m/min}$, which corresponds to the previously reported rates for the slow phase of anaphase B (Straight et al, 1998). Control experiments revealed that photobleaching of the midzone region by this protocol did not interfere with the progression of mitosis, as anaphase spindle elongation proceeded normally (supplementary Movies S2,S3 online, supplementary Fig S1A,B online).

Following photobleaching of the midzone region of intermediate anaphase spindles, fluorescence recovery was apparent within 1–2 s. In most cases, initial recovery emerged as two bright spots separated in the middle of the spindle (supplementary Movie S4 online; Fig 1B, arrowheads). As photobleaching of a small area outside the midzone, near the pole, did not result in the recovery of fluorescence (supplementary Fig S1C,D online; Higuchi & Uhlmann, 2005), we conclude that these two fluorescent spots reflect the incorporation of tubulin-GFP dimers into the plus-ends of the overlapping iMTs. The two bright spots merged into one continuous mid-spindle fluorescence signal within 40–60 s following photobleaching (Fig 1B, asterisk). At 60–100 s following photobleaching, two distinct dark regions were observed at the sides of the spindle (Fig 1B, arrows), which flanked the mid-spindle fluorescence signal. These dark regions correspond to a non-recoverable fraction of the iMTs (Maddox et al, 2000) and show that anaphase iMTs are exchangeable only in a portion of their length.

Photobleaching of the midzone region allowed us to follow the signal of newly incorporated tubulin-GFP at the iMT plus-ends, and thus to monitor the growth and shrinkage of single iMTs (Fig 2A,C,D). The growth and shrinkage events that we observed following photobleaching probably reflect the normal dynamics of iMTs, as such dynamics were also occasionally observed in unbleached spindles (Fig 2E,F). From kymographs of bleached and unbleached wild-type spindles obtained in our experiments, we determined the rates of iMT growth ($1.3 \pm 0.5 \mu\text{m/min}$; s.d. $n = 9$) and shrinkage ($7.3 \pm 2.3 \mu\text{m/min}$; s.d. $n = 11$; Fig 2A,F; supplementary Movie S6 online; supplementary Fig S2 online). The iMT growth rate is comparable with previously reported rates for cytoplasmic microtubule growth in wild-type *S. cerevisiae* cells, whereas the shrinkage rate is much faster (Tirnauer et al, 1999; Kosco et al, 2001; Gupta et al, 2002, 2006). We attribute the fast shrinkage rates to the high temporal resolution of our measurements (1–2 s), which allowed us to monitor rapid iMT shrinkage events that last 7–10 s. Alternatively, these fast shrinkage rates might reflect an inherent property of the anaphase iMTs.

To validate our characterization of the iMT plus-end dynamics, we followed the spindle localization of Bim1, a midzone-localizing and plus-end tracking protein (Tirnauer et al, 1999; Gardner et al, 2008; Fig 2G,H; supplementary Fig S3 online). We found that in intermediate spindles, the Bim1-GFP signal spans almost the entire length of the spindle and shows fluctuations in fluorescence intensity, size and location. As the spindles elongate, Bim1 becomes more restricted to the middle region, with it

Fig 1 | Measurements of microtubule dynamics during anaphase using FRAP. (A) Schematic presentation of FRAP measurement. Two antiparallel microtubules (solid lines) emanating from spindle poles are shown. Images before and immediately following photobleaching are shown on the left; bleached GFP-microtubules are indicated by the dotted lines. Regions of photobleaching (solid frame) and of the following FI measurement (dashed frame) are indicated. At initial recovery, fluorescence at the microtubule plus-end is indicated (arrowheads). At the later recovery, the fluorescence region increases owing to continuous microtubule dynamics and spindle elongation. (B,C) FRAP two-dimensional projection time-lapse images of (B) intermediate and (C) long anaphase spindles in wild-type cells expressing tubulin-GFP. Pre: 2 s before photobleaching. The solid line frame represents the photobleached region. $t = 0$: image acquired immediately after photobleaching. Time intervals: 4 s. The dashed line frame indicates the FI measured region. In (B), the arrowheads point toward the initially emerging two bright fluorescent spots. The asterisk indicates the merging of two initially emerging bright spots into one continuous fluorescence line ($t = 60$ s); the arrows indicate the two dark regions flanking the bright middle of the spindle ($t = 140$ s). Scale bar, 1 μm . FI, fluorescence intensity; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; MT, microtubule; SPB, spindle pole body; WT, wild type.

Fig 2 | Interpolar microtubule plus-end dynamics. (A–F) Tubulin-GFP and (G,H) Bim1-GFP. (A) Kymograph of the intermediate spindle shown in Fig 1B. Growth (asterisk) and shrinkage (arrow) of an iMT are indicated. (B) Kymograph of the long spindle shown in Fig 1C. No significant dynamics of the newly labelled iMTs is observed. (C) Two-dimensional time-lapse images of an intermediate spindle (5.1 μm) at various time points following photobleaching (as indicated). Pre: 2 s before photobleaching. (D) Kymograph of the spindle shown in (C). In (C,D), periods of rapid increase in the size of the iMT overlapping region (asterisks) and its translocation toward one pole (arrows) are shown. (E) Two-dimensional time-lapse images of an unbleached long spindle (8.2 μm); time intervals: 2 s. (F) Kymograph of the spindle shown in (E). In (E,F) microtubule growth (asterisk) and shrinkage (arrow) are indicated. (G,H) Kymographs of (G) intermediate (3.2 μm) and (H) long (6.5 μm) anaphase spindles in wild-type cells expressing Bim1-GFP (shown in supplementary Fig S3 online). Growth (thin arrows) and shortening (bold arrows) of single iMTs are indicated. (C,E) Scale bars, 2 μm . In kymographs, bars: vertical—40 s, horizontal—1 μm . GFP, green fluorescent protein; iMT, interpolar microtubule; WT, wild type.

being partly released from microtubules in the long spindles (supplementary Fig S3B online). Consistent with Bim1 being a microtubule plus-end tracking protein, we occasionally observed that it extended towards one of the poles. Such extensions are likely to be driven by the dynamics of single iMTs (Fig 2G,H). The iMT shrinkage events observed with Bim1-GFP were shorter than those observed with tubulin-GFP (Fig 2G,H), probably because Bim1 attaches less persistently to the plus-ends of shrinking microtubules. Thus, by monitoring the Bim1-GFP signal, we were able to follow iMT growth (1.9 ± 0.7 ; s.d. $n = 14$) and shrinkage (6.9 ± 3.8 ; s.d. $n = 8$) events, which occurred with rates similar to those obtained with tubulin-GFP.

In agreement with previous reports (Maddox *et al*, 2000; Higuchi & Uhlmann, 2005), we observed that FRAP kinetics of pre-anaphase spindles follow first-order exponential kinetics (supplementary Movie S1 online; Fig 3A), with an average rate constant of 0.018 1/s and 65% recovery (Table 1). In anaphase spindles, FRAP traces also showed first-order kinetics (Fig 3B–J). However, the first-order rate constant for intermediate anaphase spindles was 0.046 1/s, which is more than twofold that of pre-anaphase spindles (Table 1). This result indicates that microtubule dynamics are altered with the onset of anaphase. Additional observations suggest that iMT dynamics are also altered during the progression of anaphase. First, the FRAP kinetics of long spindles

Fig 3 | Representative FRAP traces of wild-type spindles. (A) Pre-anaphase, (B–E) intermediate and (F–J) long. Time intervals: 2 s; in (E) intervals: 1 s. Solid lines are fitting curves to first-order kinetics. The values of the spindle length (L , μm), the first-order rate constant (k , $1/\text{s}$) and the spindle elongation rate (V , $\mu\text{m}/\text{min}$) are indicated. In (A), selected images and the corresponding acquisition times (s) are shown. FRAP, fluorescence recovery after photobleaching.

were considerably slower than that of intermediate spindles, whereas spindle elongation rates were similar (Fig 3B–J; Table 1). Second, the intensity of the emerging fluorescence following photobleaching and the fluctuations of the midzone fluorescence were less pronounced in the long spindles (supplementary Movies S4,S5,S7 online; Figs 1B,C, 2A,B). Finally, the maximal recovery in the long spindles was significantly lower than in the intermediate spindles (Table 1). As the photobleached areas in all the anaphase spindles were similar (supplementary information online), this smaller recovery indicates that a smaller portion of the iMT length is exchangeable in the long spindles. Therefore, our results directly show that iMTs become stabilized at their plus-ends as the spindles elongate.

Effect of Cin8 and Ase1 on iMT plus-end dynamics

Next, we examined the iMT dynamics in cells lacking the kinesin-5 motor protein Cin8, which provides most of the spindle-pole separation function during anaphase in *S. cerevisiae* cells

(Hoyt *et al*, 1992; Saunders *et al*, 1995). Our analysis of pre-anaphase spindles revealed that the FRAP kinetics in *cin8Δ* cells were similar to those in wild-type cells (Fig 3A; Table 1; supplementary Fig S4A online).

To follow FRAP kinetics in *cin8Δ* anaphase spindles, we photobleached a larger portion of the spindles (70–80%) compared with that of wild-type cells, as we observed that in *cin8Δ* cells there is a larger region of overlapping iMTs (data not shown). Our experiments revealed that in *cin8Δ* anaphase spindles, the incorporation of tubulin-GFP was significantly slower than in wild-type anaphase spindles (supplementary Movie S9 online; Fig 4A,B, arrowheads), resulting in a persistent appearance of a dark area in the middle of the spindles (Fig 4A,B, arrows). The kinetics of the resulting FRAP traces in *cin8Δ* intermediate spindles was twofold slower than that of wild-type intermediate spindles, and showed a smaller magnitude of recovery (Table 1). In addition, unlike in wild-type cells, growth and shrinkage of iMTs were rarely observed in *cin8Δ* cells. To eliminate the possibility that these

Fig 4 | Microtubule dynamics in anaphase spindles of *cin8Δ* cells expressing tubulin-GFP. (A) Two-dimensional time-lapse images of an intermediate spindle (4.8 μm). Time interval: 6 s; scale bar, 1 μm. (B) Kymograph of the spindle shown in (A). Vertical bar: 70 s; horizontal bar: 1 μm. (A,B) Arrowheads: iMT plus-ends; arrows: the dark area in the middle of the spindle; asterisk: the merging of two plus-end bright spots into one continuous fluorescence midzone line. (C–G) Representative FRAP traces of *cin8Δ* anaphase spindles. L : spindle length (μm); k : the first-order rate constant (1/s); V : spindle elongation rate (μm/min). FI, fluorescence intensity; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; iMT, interpolar microtubule.

differences were the result of a larger photobleaching area in *cin8Δ* cells, we photobleached a smaller (~30%) region of anaphase spindles in these cells and obtained similar results (supplementary Fig S4B,C online). In addition, the differences in FRAP characteristics did not result from different spindle elongation rates in *cin8Δ* and wild-type cells, as in both cell types these rates were similar (Figs 3,4C–G). These rates were comparable with the rates of the slow phase of anaphase B (Movshovich et al, 2008), which have been shown to be unaffected by Cin8 deletion (Straight et al, 1998). Thus, our results strongly indicate that during anaphase, the plus-ends of iMTs in *cin8Δ* cells are stabilized, suggesting that, in addition to the well-established role of Cin8 in facilitating spindle elongation (Saunders et al, 1995; Straight et al, 1998), it contributes to the destabilization of iMT plus-ends.

The influence of Cin8 on iMT plus-end dynamics might be indirect. As Cin8 bundles microtubules (Gheber et al, 1999), the

midzone in *cin8Δ* cells might be disorganized, resulting in imbalanced activities of midzone-binding microtubule-stabilizing/destabilizing factors (Khmelinskii et al, 2007). Our recent finding that mutations in kinesin-5 proteins disrupt anaphase midzone organization in *S. cerevisiae* cells (Movshovich et al, 2008) supports this idea. Another possibility is that Cin8 directly destabilizes the plus-ends of iMTs by its microtubule-crosslinking activity. This possibility should be examined using *in vitro* experiments.

Next, we examined the effect of microtubule-bundling and the midzone-organizing protein Ase1 (Schuyler et al, 2003) on iMT dynamics. First, we performed FRAP experiments on pre-anaphase spindles in *ase1Δ* cells and found that recovery followed first-order kinetics (supplementary Fig S5A online), with a significantly slower recovery compared with wild-type cells (Table 1). This finding indicates that Ase1 deletion stabilizes pre-anaphase spindle microtubules. Although the mechanism of this effect is

Fig 5 | Microtubule dynamics in *ase1Δ* cells expressing tubulin-GFP and *nuf-2*-GFP. (A) Two-dimensional time-lapse images of an intermediate spindle (3.6 μm). Time interval: 4 s; scale bar, 1 μm. (B–E) Representative FRAP traces of *ase1Δ* anaphase spindles. Spindle lengths (*L*) are indicated. FI, fluorescence intensity; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; MT, microtubule.

Table 1 | Tubulin-GFP FRAP characteristics of pre-anaphase and anaphase spindles

	Spindle length*	<i>n</i>	<i>κ</i> —first order (1/s)	<i>t</i> _{1/2} [†] (s)	Recovery (%)
WT	Short	8	0.018 ± 0.003	39	64 ± 5
	Intermediate	8	0.046 ± 0.008 ^{##,§}	15	29 ± 2
	Long	7	0.014 ± 0.005	49	20 ± 1 ^{##,}
<i>ase1Δ</i>	Short	7	0.011 ± 0.001 ^{*,§}	63	52 ± 5
	Intermediate	5	ND [†]	ND	84 ± 25
<i>cin8Δ</i>	Short	7	0.015 ± 0.002	46	65 ± 1
	Intermediate	7	0.021 ± 0.004 ^{*,}	34	16 ± 2 ^{###,}
	Long	3	0.014 ± 0.0003	50	19 ± 2

FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; ND, not determined; WT, wild type.
*Short: 1–2 μm; intermediate: 3.5–5.5 μm; long: > 5.5 μm. [†]*t*_{1/2} = ln(2)/*κ*. [§]*t*-test compared with WT pre-anaphase spindles. ^{||}*t*-test compared with WT intermediate spindles. ^{††}FRAP did not follow first-order kinetics. ^{*}*P* < 0.05; ^{##}*P* < 0.01; ^{###}*P* < 0.001. Values are average ± s.e.m.

not clear, we suggest that, in addition to the well-established role of Ase1 in spindle stabilization during anaphase, Ase1 has a microtubule-destabilizing function during metaphase. To examine the function of Ase1 during anaphase, we followed Ase1-GFP localization. We found that the Ase1-GFP signal at the middle region of the spindles was uniform and did not fluctuate in size or location (supplementary Fig S5B online). This finding indicates that Ase1 does not track microtubule plus-ends, but rather bundles iMTs in the centre of the iMT-overlapping region. It is likely that this localization allows the iMT plus-ends to undergo the growth and shrinkage events that we observed (Fig 2).

Examination of the FRAP kinetics revealed that, in contrast to wild-type cells, spindles in *ase1Δ* cells show a recovery of 85% (supplementary Movie S8 online; Fig 5; Table 1). The FRAP traces in these cells did not follow first-order kinetics, but rather showed pronounced fluctuations in fluorescence intensity (Fig 5B–E). These results indicate that in anaphase *ase1Δ* cells, iMTs are not stabilized and are exchangeable throughout most of their length. Thus, we conclude that Ase1 is one of the main factors that controls iMT dynamics during anaphase. It has recently been shown that correct localization of Ase1 to the anaphase midzone in *S. cerevisiae* cells is dependent on its dephosphorylation by Cdc-14 (Khmelniskii et al, 2007), a conserved phosphatase that performs essential functions during spindle elongation and mitotic exit in *S. cerevisiae* and higher eukaryotes (reviewed in D’Amours & Amon, 2004). It has also been shown that when anaphase is initiated in the absence of Cdc-14 activation, spindle microtubules remain highly dynamic (Higuchi & Uhlmann, 2005), which is similar to our observation in *ase1Δ* cells. Thus, activation of Cdc-14 in early anaphase and the subsequent recruitment of Ase1 are essential to maintain correct iMT plus-end dynamics during anaphase. Furthermore, we suggest that the Ase1 bundling of midzone iMTs and the Ase1-mediated recruitment of microtubule-stabilizing proteins to the midzone (Bratman & Chang, 2007) restrict iMT dynamics to their far plus-ends, thereby preventing the ‘unzipping’ of the spindle and thus allowing correct anaphase spindle elongation. The finding that in *ase1Δ* cells anaphase spindles are fragile and do not elongate above an intermediate length of 4–5 μm (Schuyler et al, 2003) supports this idea. Finally, as Ase1-like microtubule-bundling proteins are important for midzone organization in fungal, mammalian and plant cells (Smertenko et al, 2000; Schuyler et al, 2003; Bratman & Chang, 2007; Fu et al, 2007; Khmelniskii et al, 2007), we propose that the Ase1-mediated control of

anaphase iMT plus-end dynamics shown here might represent a conserved mechanism that ensures correct spindle elongation in eukaryote cells.

METHODS

Yeast strains, the imaging setup, photobleaching and data analysis are described in detail in the supplementary information online. In brief, we used the Nipkow spinning disc confocal microscope (Perkin Elmer, Waltham, MA, USA) equipped with a FRAP module. For photobleaching of anaphase spindles, a rectangular region was bleached in the middle of the spindle, spanning 40–60% of the spindle length (Fig 1A). In wild-type cells, the size of this rectangle was $3.0 \times 1.5\text{--}4.5 \times 1.5 \mu\text{m}^2$. In *cin8Δ* cells, the bleaching area was longer by approximately 15%. Laser power was adjusted to yield 10–40% fluorescence intensity following photobleaching (Figs 3 and 4). In *ase1Δ* cells, the photobleaching laser power was reduced to ensure that no damage was inflicted on the fragile spindles in these cells (Schuyler *et al*, 2003), resulting in 30–55% fluorescence intensity following photobleaching (Fig 5). Images were acquired every 1–4 s. At each time point, a z-stack was acquired with 0.1–0.2 μm separation between planes. Image analysis was performed using the MetaMorph and ImageJ softwares.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>)

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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