The microtubule-based motor Kar3 and plus end-binding protein Bim1 provide structural support for the anaphase spindle

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Introduction

Mitotic chromosome segregation requires the formation of a stable bipolar spindle. Interpolar microtubules (ipMTs) from opposing spindle pole bodies (SPBs) form an organized array by cross-linking with each other (Winey et al., 1995). ipMTs may be cross-linked by MT-based motor proteins and/or MT-associated proteins. This arrangement contributes to the structural stability of the two halves of the mitotic spindle during metaphase and provides the means by which SPBs are rapidly separated from each other during anaphase. To dissect the requirements for anaphase spindle stability, we introduced a conditionally functional dicentric chromosome into yeast. When centromeres from the same sister chromatid attach to opposite poles, anaphase spindle elongation is delayed and a DNA breakage-fusion-bridge cycle ensues that is dependent on DNA repair proteins. We find that cell survival after dicentric chromosome activation requires the MT-binding proteins Kar3p, Bim1p, and Ase1p. In their absence, anaphase spindles are prone to collapse and buckle in the presence of a dicentric chromosome. Our analysis reveals the importance of Bim1p in maintaining a stable ipMT overlap zone by promoting polymerization of ipMTs during anaphase, whereas Kar3p contributes to spindle stability by cross-linking spindle MTs.

In budding yeast, the mitotic spindle is comprised of 32 kinetochore microtubules (kMTs) and ~8 interpolar MTs (ipMTs). Upon anaphase onset, kMTs shorten to the pole, whereas ipMTs increase in length. Overlapping MTs are responsible for the maintenance of spindle integrity during anaphase. To dissect the requirements for anaphase spindle stability, we introduced a conditionally functional dicentric chromosome into yeast. When centromeres from the same sister chromatid attach to opposite poles, anaphase spindle elongation is delayed and a DNA breakage-fusion-bridge cycle ensues that is dependent on DNA repair proteins. We find that cell survival after dicentric chromosome activation requires the MT-binding proteins Kar3p, Bim1p, and Ase1p. In their absence, anaphase spindles are prone to collapse and buckle in the presence of a dicentric chromosome. Our analysis reveals the importance of Bim1p in maintaining a stable ipMT overlap zone by promoting polymerization of ipMTs during anaphase, whereas Kar3p contributes to spindle stability by cross-linking spindle MTs.

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In addition to Kar3p generating inwardly directed spindle forces, it could be that Kar3p functions passively as an ipMT cross-linker, thus resisting spindle collapse (Hoyt et al., 1993). For example, the MT plus end-binding protein Ase1p acts to bundle MTs and thus stabilize the anaphase spindle (Pellman et al., 1995; Schuyler et al., 2003; Janson et al., 2007). Similarly, Bim1p, the yeast EB1 homologue, may contribute to ipMT interactions through its function at MT plus ends (Tirnauer et al., 1999). It has been difficult to test the function of various MT-binding proteins during mitosis because of the complexity of forces generated between parallel and antiparallel MTs and the forces generated between oppositely oriented sister chromatids.

The structure of the anaphase spindle in yeast provides a unique system to address the function of MT-binding proteins in the maintenance of spindle stability. By anaphase, sister

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Abbreviations used in this paper: Chr III, chromosome III; ipMT, interpolar MT; kMT, kinetochore MT, MT, microtubule, SPB, spindle pole body.
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chromatids have separated and kinetochore MTs (kMTs) have shortened to the spindle poles (kMTs, ~50 nm in length; Winey et al., 1995). Thus, the ipMTs are responsible for maintaining spindle integrity throughout anaphase.

In this work, we used a conditionally functional dicentric chromosome to test the structural stability of the anaphase spindle. When centromeres from sister chromatids attach to opposite poles, tension across the DNA strand satisfies the spindle checkpoint (Dewar et al., 2004). Cohesin is degraded, anaphase onset ensues, sister centromeres migrate to opposite poles (anaphase A), and sister chromatids segregate. However, segregation of centromeres on sister chromatids of a dicentric chromosome are restrained via their covalent linkage. Anaphase spindle elongation is delayed until the error is resolved. In wild-type cells, resolution is characterized by DNA breakage, with >70% survival. Alternatively, if spindle breakage precedes DNA breakage, the entire chromosome is missegregated. Missegregation of the entire chromosome leads to aneuploidy and subsequent cell death.

Using the conditionally functional dicentric assay, we find that cell survival after dicentric chromosome activation requires the MT-binding proteins Kar3p, Bim1p, and Ase1p. In their absence, anaphase spindles have poor structural stability and therefore collapse and break in the presence of a dicentric chromosome. Our analysis reveals the importance of Bim1p in maintaining a stable ipMT overlap zone by promoting growth of ipMT plus ends during anaphase, whereas Kar3p contributes to spindle stability by acting to cross-link spindle MTs.

**Results**

Viability in response to activation of a dicentric chromosome requires spindle-associated proteins

Strains with an activated dicentric chromosome that lacked the spindle-associated proteins Bim1p, Kar3p, Cik1p, Ase1p, or Bik1p were 20–300-fold reduced in viability as compared with wild-type cells with an activated dicentric chromosome (Fig. 1, A and B; and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200710164/DC1). The most severe defect was observed in bim1Δ and kar3Δ, followed by bik1Δ, cik1Δ, and ase1Δ. The loss of viability in these mutants is comparable to mutants in DNA repair (rad52Δ), indicating that an essential function is abrogated in their absence.

Although several of these MT-binding proteins have been implicated in kinetochore function (Tytell and Sorger, 2006), two pieces of evidence argue that kinetochore defects are not the cause of the decreased viability in the presence of a dicentric chromosome. First, our findings with mutations in spindle-associated proteins are different than those previously reported for kinetochore mutants. Specifically, kinetochore mutant cells with activated dicentric chromosomes do not have decreased viability (Fig. 1 B; Doheny et al., 1993; Brock and Bloom, 1994; Mythreye and Bloom, 2003). Second, the MT-associated binding protein Ase1 has no known kinetochore function but is required for ipMT function during anaphase (Pellman et al., 1995; Schuyler et al., 2003).

Dicentric chromosome breakage is reduced in cells lacking these spindle-associated proteins

If the structural integrity of the anaphase spindle is compromised, dicentric chromosome activation may result in spindle breakage and/or failure rather than dicentric chromosome breakage. To test whether dicentric chromosomes break and rearrange in bim1Δ mutants, wild-type and bim1Δ cells were transformed with the conditionally dicentric minichromosome pGAL-CENF. DNA breakage and rearrangement products can be detected by transformation into bacteria and subsequent gel electrophoresis (Hill and Bloom, 1987). Monocentric rearrangements occurred in 50 out of 50 minichromosomes recovered from wild-type cells (Fig. 2 A, wt), with loss of one of the two centromeres (CEN3 or CEN4). In contrast, ~50% of dicentric minichromosomes recovered from bim1Δ cells contained both centromeres (Fig. 2 A, bim1Δ, first two lanes, rearrangement products in 23 out of 50; last six lanes, unarranged 27 out of 50). Thus, the reduced viability of bim1Δ mutants is accompanied by an increase in propagation of the intact dicentric chromosome. The appearance of rearrangement products in 23 out of 50 cells indicates that reduced viability in bim1Δ mutants is not caused by a deficiency in DNA repair.

To examine the rearrangement frequency of a dicentric chromosome, we introduced GAL-CEN3 into HIS4 on chromosome III (Chr III) in wild-type and bim1Δ mutants (Brock and Bloom, 1994). In wild-type cells, a 1.1-kb repair product diagnostic of breakage and recombination between GAL-CEN3 and CEN3 on Chr III (Hill and Bloom, 1989) was observed within 2.5–5 h after dicentric chromosome activation (Fig. 2 B). The 1.1-kb repair product was not apparent until 12–24 h after switching bim1Δ cells to glucose (Fig. 2 B). Quantitative analysis indicated 7.4% of GAL-CEN3 was intact after wild-type cells were switched to glucose for 30 h, whereas 36% of GAL-CEN3 remained in bim1Δ cells (Fig. 2 C). Intact GAL-CEN3 was elevated fivefold and the 1.1-kb rearrangement product decreased fourfold in bim1Δ cells, which is indicative of reduced chromosome breakage (Fig. 2, C and D). A decrease in the 1.1-kb repair product was also observed in ase1Δ and kar3Δ cells (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200710164/DC1). These data reveal that dicentric chromosomes are physically stable in bim1Δ, ase1Δ, and kar3Δ mutants.

Dicentric chromosomes missegregate in spindle-associated protein mutants

To determine if the loss of viability after dicentric chromosome activation in bim1Δ mutants reflects missegregation of the intact dicentric chromosome, we used the LacO–LacI–GFP system to visualize the dicentric chromosome in live cells (Straight et al., 1997; Thrower and Bloom, 2001). The LacO coding sequence was integrated between the two centromeres on Chr III at LEU2. LacI fused to GFP was expressed to image the dicentric chromosome. In wild-type cells with an activated dicentric chromosome, chromosome spots at LEU2 remain as a single focus of fluorescence between the spindle poles until sister chromatids separate in anaphase and two spots are visible (Fig. 3, A and B). However, in bim1Δ cells the dicentric chromosome is...
Spindle-associated protein mutants have unstable spindles in the presence of a dicentric chromosome

We imaged MTs (GFP-Tub1p) in mitotic spindles to assess the structural stability of wild-type and mutant cells in the presence of a dicentric chromosome. In ~50% of cells with an activated dicentric chromosome, the kinetochores will orient toward opposite SPBs. After anaphase onset, the bioriented dicentric chromosome will stretch between both SPBs, resulting in a midanaphase pause. To resume anaphase, the dicentric chromosome breaks and the DNA lesion is repaired to generate a monocentric derivative (blue, chromosomes; green, MTs; orange, SPBs). Thus, the frequency of missegregation of the intact dicentric chromosome is highly elevated in bim1Δ mutants.

Spindle-associated protein mutants have unstable spindles in the presence of a dicentric chromosome

We imaged MTs (GFP-Tub1p) in mitotic spindles to assess the structural stability of wild-type and mutant cells in the presence of a dicentric chromosome. We found that in ~50% of cells, the kinetochores orient toward opposite SPBs after anaphase onset, due to the activated dicentric chromosome. The bioriented dicentric chromosome stretches between both SPBs, causing a midanaphase pause. To resume anaphase, the dicentric chromosome breaks and the DNA lesion is repaired to generate a monocentric derivative. Thus, the frequency of missegregation of the intact dicentric chromosome is highly elevated in bim1Δ mutants.
Figure 2. bim1Δ suppresses dicentric chromosome breakage. (A) Restriction analysis of dicentric plasmid pGAL-CEN^3 DNA (Hill and Bloom, 1989) recovered from wild-type or bim1Δ cells maintained on galactose or glucose. Plasmid DNA derived from wild-type and bim1Δ cells was digested as previously described (Hill and Bloom, 1989). In wild-type cells, 0 out of 50 cells had both centromeres intact after plasmid recovery and analysis. 27 out of 50 dicentric plasmids derived from bim1Δ cells had both centromeres intact. Molecular masses of DNA fragments after restriction digestion of the unrearranged plasmid are indicated to the right of each gel. CEN4 and CEN3 indicate the fragments containing the centromere fragment from Chr IV or III, respectively. (B) Time course for monocentric derivatives generated by recombination between the two centromeres on Chr III. Wild-type and bim1Δ cells were cultured and the time course was performed as previously described (Brock and Bloom, 1994). Time (in hours) indicates the time points after activating the dicentric chromosome (switch to glucose). At each time point, the chromosomal DNA was prepared and analyzed by Southern analysis (see Materials and methods). The Southern blot was also probed for MET14 that served as a loading control. Molecular masses for each of the respective fragments are indicated to the right of each gel (kb). (C) Histogram of the percentage of radioactive GAL-CEN3 relative to MET14 over the time course for wild-type and bim1Δ cells as determined by ImageQuant analysis. There was a 13.5-fold decrease in the GAL-CEN3 band in wild-type cells but only a 2.8-fold decrease in bim1Δ. (D) Histogram of the percentage of radioactive 1.1-kb rearrangement product relative to MET14 over the time course for wild-type and bim1Δ cells as determined by ImageQuant analysis. There was a 13.6-fold increase in the 1.1-kb rearrangement product in wild-type cells but only a 3.8-fold increase in bim1Δ cells.
an activated dicentric chromosome. In >95% of wild-type cells with an activated dicentric chromosome, GFP-Tub1p was continuous along the length of the spindle in both preanaphase and anaphase (Fig. 3 C; n = 57 cells). However, 38% of mitotic bim1Δ cells displayed discontinuous GFP-Tub1p fluorescence that appeared as two foci (Fig. 3 C; n = 166 cells). The two GFP-Tub1p fluorescent foci could separate >5 μm before moving back together (unpublished data). Thus, when the anaphase spindle is constrained by a dicentric chromosome bridge the bim1Δ ipMTs cannot maintain a continuous overlapping array. The gap in GFP-Tub1p could result from spindle destabilization, possibly related to a change in ipMT length (Tirnauer et al., 1999) or cross-linking defects. In either case, ipMT function is required in anaphase for cells to resolve the dicentric chromosome into monocentric derivatives. In contrast, weakening of mitotic spindle structural stability via loss of ipMT function favors chromosome missegregation and/or spindle breakage rather than dicentric chromosome breakage and rearrangement.

**ipMT-associated tubulin polymer is reduced in bim1Δ cells but not in kar3Δ cells**

If both Bim1p and Kar3p contribute to spindle stability, as is predicted by the dicentric assay, then the loss of these proteins should result in a quantifiable defect in anaphase spindle morphology. To test this prediction we examined the distribution of GFP-Tub1p in anaphase spindles of wild-type and mutant cells.
using quantitative microscopy and model convolution. In wild-type cells, there is a uniform distribution of GFP-Tub1p fluorescence along the entire length of the anaphase spindle (Fig. 4 A). Anaphase GFP-Tub1 fluorescence levels in kar3Δ mutants are comparable to those in wild-type spindles (Fig. 4 A), suggesting that reduced spindle stability upon KAR3 deletion is not caused by improper length regulation of ipMTs. In contrast, there is a sharp drop in GFP-Tub1p fluorescence in bim1Δ anaphase spindles (Fig. 4 A). These results suggest that the tubulin polymer associated with ipMTs during anaphase may be reduced in bim1Δ mutants as compared with wild-type spindles. In contrast, another spindle defect may prevail in the absence of Kar3p.

Bim1-GFP is concentrated at the spindle midzone in anaphase (Fig. 4 B and Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200710164/DC1), suggesting that Bim1p is bound near to ipMT plus ends during anaphase. Bim1p promotes growth of astral MTs in G1 (Tirnauer et al., 1999) and is likely to have a similar mechanism of action when bound to ipMT plus ends in anaphase.

During anaphase, bim1Δ mutant ipMTs are shorter than wild-type ipMTs

To quantify ipMT length, we used electron tomography to reconstruct MT length distributions in wild-type anaphase spindles. By normalizing all MT lengths to the individual spindle lengths of two anaphase spindles, we found one class of abundant, short MTs. These MTs exhibit an exponential length distribution, with their plus ends clustered near to the spindle poles (Fig. 5 A, bottom left; mean ± SD, 7 ± 7.7% of spindle length [absolute length, 151 ± 213 nm]; n = 64 MTs; probability of fit to exponential model (p-value) = 0.59 [see Materials and methods for calculation procedure]). In contrast, GFP-Tub1p fluorescence has decreased intensity in bim1Δ cells (top right; white arrows show approximate minimum intensity location; note comparison to wild-type spindle in similar location), suggesting that ipMTs have reduced length as compared with wild-type spindles (spindle length 5.15 ± 1.21 μm). Quantification of GFP-Tub1 fluorescence by normalized spindle position shows reduced GFP-Tub1p fluorescence in bim1Δ anaphase spindles as compared with wild-type spindles (bottom, spindle position normalized to total spindle length). Error bars represent SEM. Bar, 1,000 nm. (B) Bim1-GFP (green) is localized within the spindle midzone (blue arrows; spindle lengths 4.66 ± 0.97 μm; for quantification see Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200710164/DC1). Bar, 1,000 nm.

To use model convolution to statistically compare tubulin distributions in different populations, we sampled the exponential fit curves from the tomograms (Fig. 5 A) to generate simulated GFP-Tub1p distributions of anaphase spindles. By convolving the experimentally observed microscope point-spread function with the simulated GFP-Tub1p distributions (Gardner et al., 2007; see Materials and methods), we could directly compare simulated images (Fig. 5 B, bottom) to GFP-Tub1p experimental images (Fig. 5 B, top). Simulated images based on electron tomography data qualitatively match experimental fluorescence images (Fig. 5 B, graph; P = 0.07). Thus, the exponential wild-type ipMT length distribution obtained via quantitative analysis of electron tomography data is a reasonable approximation of wild-type anaphase spindles (Fig. 5 B, animation).

The theoretical length distribution of anaphase spindle MTs that most closely reproduced the experimentally observed GFP-Tub1p fluorescence distribution in the absence of Bim1p is shown in Fig. 5 C. The best fit to experimental data could be obtained using a Gaussian ipMT length distribution with mean ± SD of...
52 ± 7% of spindle length (Fig. 5C; P = 0.06). Thus, we predict that the ipMT plus ends in \( \text{bim}^1 \Delta \) mutants are located very near to the spindle equator, which results in a significant decrease in the antiparallel ipMT overlap zone as compared with wild-type spindles (Fig. 5C, animation, compare with Fig. 5B, animation).

The role of Bim1p in regulating MT length is consistent with previous observations (Tirnauer et al., 1999). The decrease in overlap of antiparallel MTs in \( \text{bim}^1 \Delta \) mutants would reduce the number of productive binding sites available for the kinesin-5 motors Cin8p and Kip1p, preventing \( \text{bim}^1 \Delta \) spindles from achieving the outwardly directed force threshold that is required to break dicentric chromosomes. Interestingly, cell viability after activation with a dicentric chromosome was increased ~12-fold in a \( \text{bim}^1 \Delta \text{kip}^3 \Delta \) double mutant as compared with \( \text{bim}^1 \) mutants with an activated dicentric chromosome (\( \text{bim}^1 \Delta \text{kip}^3 \Delta \) dicentric viability, 6.8 ± 0.73%; \( n = 4 \) trials). This result suggests that an increase in ipMT length (in the absence of the MT depolymerizing motor Kip3p) is sufficient to stabilize the anaphase spindle, thus increasing viability after activation of a dicentric chromosome.
Kar3p regulates bundling of ipMTs during anaphase

Because deletion of KAR3 did not have a significant effect on anaphase spindle tubulin polymer (Fig. 4), we predicted that another spindle defect, such as the proper bundling of ipMTs, may prevail in the absence of Kar3p. To test this prediction, we examined mutant spindle morphology in kar3Δ mutants expressing GFP-Tub1p. As shown in Fig. 6 A, 47% of kar3Δ spindles showed splaying of MTs in anaphase. Thus, ipMTs are poorly bundled in kar3Δ mutants as compared with wild-type spindles (8% MT separation). Disrupting the organization of ipMTs may also disturb proper antiparallel cross-linking of Cin8p and Kip1p and suggests that Kar3p is required for efficient attachment of the kinesin-5 motors Cin8p and Kip1p to oppositely oriented ipMTs. Therefore, deletion of KAR3 results in reduced outwardly directed spindle forces as mediated by kinesin-5 motors, even though Kar3p itself may act to antagonize Cin8p/Kip1p function in wild-type spindles.

Kar3p is known to interact with either Cik1p or Vik1p light chains in vivo and in vitro (Manning et al., 1999; Sproul et al., 2005; Allingham et al., 2007). Because cik1Δ mutants have low cell viability in response to activation of a dicentric chromosome, we predicted that the Kar3p–Cik1p complex may regulate spindle integrity via the ipMT bundling mechanism, as shown in Fig. 6 A, and thus localize near to ipMT plus ends in the anaphase spindle. Indeed, although Kar3-GFP is localized at the SPB in wild-type anaphase spindles it is also present in punctuate locations within the spindle (Fig. 6 B, left). To assess Kar3p–Cik1p binding exclusively, we examined Kar3-GFP localization in vik1Δ spindles (Fig. 6 B, middle and Fig. S1 B). Here, there is reduced fluorescence of Kar3-GFP near to the SPB, with increased fluorescence in punctuate spots near putative ipMT plus ends. Simulations in which fluorescent proteins are distributed at the plus ends of MTs also resulted in punctuate spots along the length of the spindle (Fig. 6 C).

In contrast, Kar3-GFP that is complexed with Vik1p (in cik1Δ cells) is focused at the SPBs with little fluorescence along the length of the spindle (Fig. 6 B, right; and Fig. S1 B). These results demonstrate that the light chains Cik1p and Vik1p differentially regulate the localization and function of Kar3p to either the ipMT plus ends (Cik1p) or spindle poles (Vik1p). In addition, our results suggest that Kar3p–Cik1p regulates ipMT bundling along the length of the spindle and that poor ipMT bundling is the dominant mechanism for low cell viability in response to activation of a dicentric chromosome in kar3Δ mutants.

Discussion

There are two pathways required for cell survival in the presence of a dicentric chromosome. Dicentric chromosomes are physically unstable and undergo a breakage-fusion-bridge cycle that is required for cell survival (Fig. 1 A). We found a second pathway required for cell survival, which is the maintenance of spindle integrity. If the spindle is compromised, spindle breakage results in chromosome loss and the subsequent loss of cell viability.

Using the activated dicentric chromosome assay, we find that Bim1p and Kar3p both contribute to structural stability of
of benomyl. Strains were maintained at 25 °C, except for some wild-type midlogarithmic phase and diluted into YPD (glucose; dicentric) to analyze binding of the kinesin-5 motors Cin8p and Kip1p, so reducing the size of the anaphase antiparallel overlap zone (Fig. 7, right). The ipMT overlap zone is necessary for efficient antiparallel binding of the kinesin-5 motors Cin8p and Kip1p, so reducing its length likely has a direct effect on the ability of the kinesin-5 motors to provide the outwardly directed forces that are required to stabilize the elongating anaphase spindle.

In contrast, deletion of KAR3 does not have a significant effect on the level of anaphase tubulin polymer (Fig. 4). Rather, kar3Δ mutants show splaying of anaphase ipMTs, likely because of improper ipMT bundling as mediated by Kar3p–Cik1p complexes at the plus ends of ipMTs (Fig. 6). The differential localization of Kar3-GFP in cik1Δ mutants and vik1Δ mutants (Fig. 6 B) is consistent with the difference in activated dicentric lethality between the mutants (Fig. 1 B), suggesting that spindle-bound Kar3p acts to promote ipMT bundling. Similar to the bim1Δ mutants, poor ipMT bundling in kar3Δ mutants also likely prevents proper antiparallel binding of the kinesin-5 motors and therefore results in reduced outwardly directed spindle forces (Fig. 7, middle). This effect explains the long-standing enigma of how spindle lengths could be shorter in kar3Δ mutants even though Kar3p could act to resist outwardly directed spindle forces when ipMTs are properly bundled.

These results highlight the importance of the spindle midzone structure in maintaining anaphase spindle integrity. An important future effort will involve mechanistically linking the spindle localization of Kar3p and Bim1p to their specific functions within the anaphase spindle.

### Materials and methods

#### Media, growth conditions, and strain construction

Strains and plasmids used in this study are listed in Table S2 (available at http://www.jcb.org/cgi/content/full/jcb.200710164/DC1). Media and strains, including gene deletions and GFP fusions, were prepared as described previously (Molk et al., 2004) and confocal (Maddock et al., 2003) images were obtained as previously detailed. The microscope used for widefield imaging was a TE2000E stand (Eclipse; Nikon) with a 100 NA 1.4 objective (PlanApo) with a camera (Orca ER; Hamamatsu). Images were acquired at room temperature with MetaMorph imaging software (MDS Analytical Technologies). Differential interference contrast image exposure times were for 150–200 ms and epifluorescence exposure times were 300–400 ms. LacI-GFP expression was induced as described previously (Thrower and Bloom, 2001). Images were processed as previously detailed (Molk et al., 2004). To quantify the dicentric Chr III missegregation events, cells were grown overnight in glucose media and induced for LacI-GFP expression for 2 h and five-plane Z series of the population were acquired. The percentage of mitotic cells in the population was also recorded at this time. Fluorescence intensity measurements by spindle position were performed as previously described (Gardner et al., 2005). All measurements were exported to Excel (Microsoft) for further analysis.

#### Electron tomography

Cells were prepared for electron microscopy using methods previously described (Winery et al., 1995). In brief, log-phase cultures were collected by vacuum filtration and high-pressure frozen using a high-pressure freezer (BalTech). The frozen cells were freeze substituted in 1% OsO4 and 0.1% uranyl acetate in acetone and embedded in epon resin. Serial 250-nm-thick sections were collected onto formvar-coated slot grids and stained with lead citrate and uranyl acetate.

Dual axis tomography was performed as described previously (O’Toole et al., 2002). 15 nm colloidal gold was affixed to each end of the sections to serve as fiducial markers for tomography. The specimens were imaged using a microscope (TECNAI F30; FEI) operated at 300 kV. Images were captured every 1° over a ±60° range at a pixel size of 1 nm around two orthogonal axes. The serial tilted views were then aligned and dual axis tomograms, computed using the IMOD software package (Kremer et al., 1996; Mastronarde, 1997). Tomograms were computed from adjacent serial sections to reconstruct complete mitotic spindles.
In total, we reconstructed two wild-type anaphase spindles. Individual MIUs were modeled from the tomographic volumes and a projection of the 3D model was then displayed to study its 3D geometry. MT lengths were extracted from the model contour data using the program IMODINFO.

Modeling of MT length distributions

MT length distributions obtained from electron tomography were fit to exponential models as follows. It was assumed that the 32 shortest MTs in each spindle were kMTs. The cumulative distribution of kMT lengths were then fit to an exponential distribution according to $F_l = 1 - e^{-l/\lambda}$ where $F_l$ is the cumulative distribution function for MT length, $l$ is MT length normalized to spindle length, and $\lambda_{exp}$ is the mean normalized MT length for the sample. All measured MTs longer than the 32 shortest in each spindle were assumed to be iPMTs. The cumulative distribution of iPMT lengths were fit to an exponential distribution according to $F_l = 1 - e^{-l/\lambda_{iPMT}}$ where $\lambda_{iPMT}$ is the maximum observed normalized MT length, which is 0.905. This correction takes into account an experimentally observed iPMT plus end–exclusion zone near to the SPBs, possibly caused by entropic or steric exclusion originating from the high density of kMTs near to the poles. A bootstrap method was used for calculating fit probability of experimental data to exponential models, as previously described (Sprague et al., 2003; Gardner et al., 2007). Here, a set of 100 simulated sum-of-squares error (SSE$_{exp}$) values were calculated by comparing each simulated MT length distribution curve (generated via sampling from the exponential fit curve) to the mean curve over all 100 simulations. The experimental SSE value (SSE$_{exp}$) was then calculated by comparing the experimental data to the mean simulation curve. The SSE$_{exp}$ was then ranked in the list of 100 simulated SSE$_{exp}$ values to calculate the probability of fit (p-value).

Model-convolution simulations

Model convolution of simulated fluorescence distributions was completed by convolving the experimentally observed microscope point spread function with the simulated distribution of fluorescent proteins, as previously described (Sprague et al., 2003; Gardner et al., 2007). Calculation of p-values for fit of simulated model-convolution images to experimental images was completed as previously described (Sprague et al., 2003; Gardner et al., 2007).

Online supplemental material

Table S1 lists dicentric viability in plus end–binding proteins. Table S2 lists products after dicentric activation in mutant and wild-type cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/

The mean simulation curve. The SSE$_{exp}$ was ranked in the list of 100 simulated SSE$_{exp}$ values to calculate the probability of fit (p-value).

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