Three-Dimensional Analysis and Ultrastructural Design of Mitotic Spindles from the *cdc20* Mutant of *Saccharomyces cerevisiae*

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The three-dimensional organization of mitotic microtubules in a mutant strain of *Saccharomyces cerevisiae* has been studied by computer-assisted serial reconstruction. At the nonpermissive temperature, *cdc20* cells arrested with a spindle length of $\sim 2.5 \mu\text{m}$. These spindles contained a mean of 81 microtubules (range, 56–100) compared with 23 in wild-type spindles of comparable length. This increase in spindle microtubule number resulted in a total polymer length up to four times that of wild-type spindles. The spindle pole bodies in the *cdc20* cells were ~ 2.3 times the size of wild-type, thereby accommodating the abnormally large number of spindle microtubules. The *cdc20* spindles contained a large number of interpolar microtubules organized in a "core bundle." A neighbor density analysis of this bundle at the spindle midzone showed a preferred spacing of ~ 35 nm center-to-center between microtubules of opposite polarity. Although this is evidence of specific interaction between antiparallel microtubules, mutant spindles were less ordered than the spindle of wild-type cells. The number of noncore microtubules was significantly higher than that reported for wild-type, and these microtubules did not display a characteristic metaphase configuration. *cdc20* spindles showed significantly more cross-bridges between spindle microtubules than were seen in the wild type. The cross-bridge density was highest between antiparallel microtubules. These data suggest that spindle microtubules are stabilized in *cdc20* cells and that the *CDC20* gene product may be involved in cell cycle processes that promote spindle microtubule disassembly.

INTRODUCTION

The budding yeast, *Saccharomyces cerevisiae*, has served as a useful model system for the study of cell cycle control and the mechanisms of mitosis (reviewed in Murray and Hunt, 1993; Page and Snyder, 1993). Much is known about its molecular biology, and large numbers of cell division cycle (*cdc*) mutants are available for analysis (Hartwell *et al.*, 1973; Hartwell and Smith, 1985). These approaches have been informative about some of the molecules involved in mitosis and

in cell cycle control, but little has yet been discovered about the way these molecules contribute to the spatial organization of the division machinery. The small size of yeast spindles and of their essential components, such as spindle pole bodies (SPBs), dictates that electron microscopy (EM) be used to obtain the resolution necessary to examine spindle architecture and aberrations in mutant strains. Here, we describe the results of an EM study of the three-dimensional (3-D) structure of the mitotic spindle of the *cdc20* mutant strain of *S. cerevisiae*.

The *cdc20* mutant was originally described by Hartwell *et al.* (1973). As with many other *cdc* mutants, this

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strain becomes arrested in mitosis as cells with large buds (Byers and Goetsch, 1974, 1975). Mutations in the *CDC20* gene affect a number of microtubule-related events, including chromosome segregation (Hartwell and Smith, 1985), nuclear movements prior to anaphase (Palmer *et al.*, 1989), and nuclear fusion during mating (Sethi *et al.*, 1991). The most dramatic effect of this mutation, however, is on mitotic spindle structure. Light microscopy of *cdc20* cells grown at restrictive temperature revealed a sixfold increase in spindle staining by immunofluorescence with antitubulin, yet the measurable pool of tubulin was not detectably different from that of wild-type cells (Sethi *et al.*, 1991). These investigators hypothesized that the intense staining observed might arise from changes in microtubules (MTs) that make them more accessible to antibodies, or from an increase in the number of spindle MTs, or both. These issues, however, could not be resolved by light microscopy.

The *CDC20* gene has recently been found to be homologous to the mammalian *p55^{CDC}* gene (Weinstein *et al.*, 1994) and the *fzy* gene of *Drosophila* (Dawson *et al.*, 1995), both of which are implicated in cell cycle control. In *Drosophila*, as in *S. cerevisiae*, mutations in this gene result in the arrest of mitotic progression with the formation of aberrant spindles (Sethi *et al.*, 1991; Dawson *et al.*, 1995). This gene encodes a protein with sequence similarity to the β subunit of mammalian transducin, a protein implicated in intracellular signaling (Sethi *et al.*, 1991;). A *fzy* mutant fails to degrade cyclins A and B and cells arrest in metaphase, suggesting that cell cycle-regulated cyclin proteolysis is dependent on the *fzy*⁺ gene. Recent studies have shown that the exit from mitosis (Holloway *et al.*, 1993; Surana *et al.*, 1993), as well as sister chromatid separation (Irniger *et al.*, 1995), is dependent on ubiquitin-dependent proteolysis. Thus, the increase in spindle MTs observed in *fzy* mutants may be explained by a failure to degrade protein(s) necessary for anaphase onset (Dawson *et al.*, 1995). Defects in spindle structure in turn signal feedback control processes to ensure that late events in mitosis do not occur before the spindle is properly assembled. Such feedback control mechanisms have been described in *S. cerevisiae*, where they are thought to ensure the proper ordering of events in the cell cycle (as reviewed in Hartwell and Weinert, 1989; Murray, 1992). In particular, the *BUB* and *MAD* genes of *S. cerevisiae* are thought to be involved in spindle assembly checkpoints (Hoyt *et al.*, 1991; Li and Murray, 1991; Roberts *et al.*, 1994; Wang and Burke, 1995; Wells and Murray, 1996).

Our recent studies describing the 3-D structure of mitotic spindles in *S. cerevisiae* have provided a benchmark for comparison with mitotic mutants (Winey *et al.*, 1995). Wild-type spindles are formed from arrays of MTs that emanate from two SPBs. Early in mitosis,

spindles consist of a core bundle of interpolar MTs and enough additional MTs to attach to each of the 32 kinetochores. Anaphase B results from an elongation of the core bundle and constitutes the bulk of chromosome movement in this organism. Anaphase A may be asynchronous, a feature of mitosis found in other fungi (Heath, 1980; Heath and Bauchop, 1985).

In this article, the methods and programs used for 3-D analysis of mitotic spindles (McDonald *et al.*, 1991; Ding *et al.*, 1993; Winey *et al.*, 1995) have been used to determine the ultrastructural design of the mitotic spindle in *cdc20* cells grown at the restrictive temperature. Reconstructions of seven spindles show that the number of MTs is up to four times greater than in wild-type spindles of similar length. These spindle MTs emanate from SPBs that are ~2.3 times the size of their wild-type counterparts. In addition, there is an increase in the number of cross-bridge structures detected between adjacent spindle MTs. The presence of a large number of stabilized polar MTs suggests that the *CDC20* gene product may be involved in the regulation of dynamic MT behavior.

MATERIALS AND METHODS

Cell Culture

The yeast strain used in this study was 402-2-9 (MATa, *cdc20-1*, *ade3*, *his7*, *leu2-3*, 112, *cyh2*, and *ura3-52* supplied by Dan Burke). The ploidy of this strain was confirmed with flow cytometry and genetic analysis. The cells were cultured at 25°C in rich medium (YEPD; Sherman, 1981) to 1.9×10^6 cells/ml and then collected by centrifugation. Cells were resuspended in fresh YEPD that had been prewarmed to 36°C and incubated in this medium for 4 h before harvest.

Preparation of Cells for EM

Cells were high-pressure frozen (HPF) using methods previously described for yeast (Ding *et al.*, 1993; Winey *et al.*, 1995). Briefly, aliquots (7–10 ml) of cells from log phase cultures were harvested by vacuum filtration on a 0.45- μ m Millipore filter. The resulting cell paste was transferred to sample holders and frozen using a Balzers HPM010 high-pressure freezer (Bal-Tec Corp., Middlebury, CT). The HPF cells were then freeze substituted for 3 days in 2% OsO₄ and 0.5% uranyl acetate in acetone at -90°C. Freeze-substituted cells were gradually warmed to room temperature and embedded in Spurr's resin. Serial thin (50 or 70 nm) sections were cut using a Reichert Ultracut E Microtome (Leica Instruments, Deerfield, IL) and placed on 1 $\times$ 2-mm Formvar-coated slot grids. Sections were poststained with 2% uranyl acetate in 70% methanol for 5 min and Reynold's lead citrate for 3 min.

EM

Serial sections were imaged in a Philips CM10 electron microscope operating at 80 kV and equipped with a rotating grid holder and a ± 60 -degree goniometer stage. Cells containing spindle microtubules in cross-section were rotated and tilted to maximize the circular profiles of the microtubules and serially imaged. All tilt data were recorded for each image to correct for apparent section thickness. Micrographs at 21,000 $\times$ or 28,000 $\times$ magnification were captured on Kodak 4489 film. Magnification was calibrated using a carbon replica grating.

3-D Reconstruction and Image Analysis

Microtubule tracking and analysis were carried out as previously described (McDonald *et al.*, 1991; Winey *et al.*, 1995). Briefly, negatives were digitized in serial order using a Dage 81 video camera and a Parallax 1280 graphics device in a Microvax III computer. The digitized images were then displayed in series and a model point was placed in the center of each microtubule along its length. Structures resembling bridges between adjacent MTs, as described in Winey *et al.*, 1995, were also marked in the models. 3-D display of the completed models and quantitative analysis were performed using the programs described in McDonald *et al.* (1991) and Winey *et al.* (1995).

RESULTS

General Organization of the *cdc20* Spindle

cdc20 cells grown at the restrictive temperature for 4 h arrested with an average spindle length of approximately 2.5 μm . Seven spindles with a range of lengths from 1.83 to 4.21 μm were analyzed (Table 1). Figure 1 shows cross-sectional images at various points along the length of a spindle and a 3-D model that represents all of the MTs projected onto a single plane (cell 1, Table 1). At the SPB, the MT cross-sections were tightly packed (Figure 1, A and E). This packing is lost as the MTs splay out to interdigitate with MTs from opposite poles (Figure 1, B–D). Cross-bridge structures (Figure 1, A–E, arrowheads) were seen between MTs throughout the length of the spindle. Multiple patches of electron-dense material were often visible in the nucleoplasm (Figure 1D, asterisk), situated adjacent to the spindle.

Figure 2 shows the length distributions of spindle MTs from seven *cdc20* cells and two wild-type cells at comparable lengths. The *cdc20* spindles contain mostly polar MTs (96%, $n = 485$, Table 1), which emanate from each of the two SPBs and end in the nucleo-

plasm. Only a small fraction (4%) of the MTs in the *cdc20* spindles were continuous, in that both MT ends were so near the two SPBs that their polarity could not be established. The spindles contained roughly equal numbers of polar microtubules emanating from each SPB, a feature commonly seen in wild-type spindles (Winey *et al.*, 1995), although three spindles (Figure 2, cells 4, 5, and 7) showed asymmetric distributions. Only one spindle (Figure 2, cell 7) consisted of polar MTs originating almost exclusively from one SPB (Table 1). This spindle was considered anomalous and was not used in the quantitative analyses described below. All of the cells studied displayed large buds, consistent with observations from the light microscope (Hartwell *et al.*, 1973; Byers and Goetsch, 1974, 1975).

MT Length, Distribution, and Organization in the *cdc20* Spindle

When compared with wild-type spindles, the *cdc20* spindles have many more MTs, which are on average somewhat longer and therefore contain considerably more total tubulin polymer (Figure 2 and Table 1). The lengths of most of the *cdc20* spindles fell in a range where we have no comparable wild-type spindles; therefore, it was necessary to make comparisons with a diverse range of wild-type spindles, particularly with the six medial spindles (1.3–1.55 μm) and four longer spindles (2.7, 3.1, 3.7, and 3.8 μm) of Winey *et al.* (1995). The spindles of *cdc20* grown at the restrictive temperature contained a mean of 81 MTs (range, 56–100). For wild-type cells, our published mean number of MTs was 44 in medial spindles, which fell to 22.5 in 2.7–3.1- μm spindles and 8.5 in 3.7–3.8- μm spindles (Winey *et al.*, 1995). *cdc20*

Table 1. Distribution of MTs in *cdc20* spindle reconstructions

Spindle	Spindle length (μm)	Total MTs	Total polymer length (μm)	MT no.	
				Polar ^a	Continuous
1	1.8	93	71.5	48/43	2
2	2.1	80	68.5	33/41	6
3	2.4	91	74.9	43/47	1
4	2.5	56	65.4	16/31	9
5	2.51	100	87.5	60/38	2
6	4.21	65	102.3	30/34	1
7	3.2	48	77.3	35/1	12
Wild-type ^b					
Medial	1.3–1.5	44	21.8	20.5/21	1–4
Long	2.7–3.1	22.5	16.8	9.5/10.5	2–3
	3.7–3.8	8.5		4.5/3.5	1

^a The two entries are the number of MTs emanating from each SPB.

^b Wild type values calculated from Winey *et al.* (1995). Values representing spindle lengths and numbers of continuous MTs are represented as ranges; other values are means.

Figure 1. Representative 3-D reconstruction from a *cdc20* spindle (cell 1, Table 1). Selected electron micrographs (A–E) show the morphology of the spindle cross-section at positions near each spindle pole body (A and E) and along the length of the spindle (arrows). The polarity of each microtubule is identified by the shape of the model point that marks it: microtubules originating from the left SPB (•), the right SPB (○), or continuous between the two SPBs (△). Structures defined as cross-bridges between adjacent MTs were present throughout the spindle (A–E, arrowheads). Bar, 0.1 μ m.

spindles thus contain twice as many MTs as do shorter wild-type spindles and four to nine times as many as do the longer wild-type spindles, which have eliminated numerous polar MTs.

Mean MT length varied strongly with spindle length; therefore, to facilitate comparison, we expressed mean MT length as a fraction of spindle length. This fraction was 0.40 ± 0.05 for the *cdc20* spindles compared with 0.34 ± 0.03 for medial and 0.36 ± 0.12 for longer wild-type spindles. The relative lengths of MTs in *cdc20* spindles were thus only slightly greater than in the wild type. However, when total polymer length was calculated, *cdc20* cells contained a mean of 78.2 μ m (range, 56–100 μ m), which is up to four times higher than in wild-type spindles (means, 21.8 μ m for medial and 16.8 μ m for longer spindles, respectively, overall range, 10–32 μ m).

Organization of the Spindle Midzone

During spindle elongation in wild-type cells, an orderly arrangement of MTs develops at the midzone; the primary component of this order is close associations between anti-parallel MTs (Winey *et al.*, 1995). We therefore used a neighbor density analysis (NDA; McDonald *et al.*, 1992) to see if MTs in *cdc20* spindles have a preferred spacing in the midzones. An NDA of all microtubules shows a peak at ~ 40 nm (Figure 3a). No peak appears in an analysis of neighboring microtubules of like polarity (Figure 3b), whereas MTs of opposite polarity have a preferred spacing of ~ 35 nm center-to-center (Figure 3c). The peaks in Figure 3 are intermediate between the weaker peaks found in the NDA of short or medial wild-type spindles and the sharper ones found for longer spindles (Winey *et al.*, 1995). These

Figure 2. Microtubule overlap displays from seven *cdc20* cells and two wild-type (wt) cells of comparable length (cells 10 and 11 from Winey *et al.*, 1995). The MTs are presented as single straight lines whose lengths and positions relative to each half spindle are represented from top to bottom in order of increasing length.

results are evidence for a specific interaction between antiparallel MTs in the *cdc20* spindle midzone.

Identification of Functional Classes of MTs: *cdc20* Spindles Contain a Core Bundle of MTs

One goal of this study was to identify functional classes of MTs, such as kinetochore MTs (kMTs), that might be present in the spindles of temperature-arrested *cdc20* cells. In wild-type cells, this was accomplished by examining the distance over which each MT was close to (within 45 nm of) either a continuous MT or a MT of opposite polarity. These "pairing lengths" for medial or longer spindles showed a bimodal distribution, with a distinct population of MTs having pairing lengths ≥ 240 nm. For *cdc20* cells, such distributions were generally unimodal; the MTs with high pairing lengths, although numerous, failed to form a distinct population. Nevertheless, we find it useful to make a distinction between core bundle and noncore MTs using the same pairing length criterion (240 nm) as was used for wild-type cells. Figure 4 shows a spindle (cell 5) separated into its core bundle (Figure 4b) and noncore MTs (Figure 4c).

This core bundle analysis has been applied to *cdc20* spindles 1–6 (Table 2). In each cell, nearly all of the longest MTs were in the core bundle, and the mean length of core bundle MTs was typically two to three times higher than that of noncore MTs. These features are similar to those seen in the wild type, although the overlap between core and noncore MT length distributions was greater for *cdc20*. As shown in Table 2, *cdc20* spindles had many more core bundle MTs than the wild type: the mean number was 28 versus 8 or 6 for medial or longer wild-type spindles (Winey *et al.*, 1995). However, the majority (65%) of MTs were noncore; consequently, 4 of the cells had many more than 32 noncore MTs. This is in contrast to the situation for the wild type, where the number of noncore MTs in short and medial spindles approximated the number expected for the 32 kMTs in this organism. It is thus not possible to identify kMTs in the *cdc20* spindles by this analysis.

Although the identity of the kMTs is unknown, the models do allow some statements about three possibilities. The first is that the separation between sister kinetochores is no greater than in short wild-type spindles, in which it was estimated to be 0.23 μm (Winey *et al.*, 1995). For those spindles, it was possible to find over 10 pairs of plus ends of MTs from opposite poles, with separations of 0.1–0.25 μm . We used the same methods to pair up MT plus ends in the *cdc20* models. For three of the cells, only a few such pairings could be made. For the other three, the midpoints between paired ends were spread widely along the length of the spindle, oc-

Figure 3. NDA for the MTs in the mid-zone of six *cdc20* spindles showing the frequency with which MTs are found at a given spacing. The densities of neighboring MTs are plotted as a function of distance from any MT (A), from parallel MTs (B), or from antiparallel MTs (C). Dashed lines, indicate baseline densities.

cupying a range of 0.76, 1.8, and 3.5 μm compared with 0.36 μm for short wild-type spindles (Winey *et al.*, 1995). A second possibility is that the kMTs are similar in length or no longer than the kMTs in wild-type spindles prior to anaphase A. To assess this point, we measured the lengths of the 16 shortest noncore MTs from each pole. Their mean length was $0.36 \pm 0.22 \mu\text{m}$, quite comparable to the $0.34 \pm 0.21 \mu\text{m}$ reported for the noncore MTs (presumptive kMTs) in wild-type medial spindles (Winey *et al.*, 1995). It is thus possible that kMTs are frozen at their wild-type length in the *cdc20* spindles. The third possibility is that the sister chromatids may be unseparated and attached to only one pole. In any event, these findings indicate that the *cdc20* spindles

do not assume a classic metaphase configuration with closely apposed sister kMTs aligned in a relatively narrow midzone.

cdc20 Cells Contain Large SPBs

We have asked whether the SPBs of the *cdc20* spindle accommodate an abnormally large number of MTs by being bigger or by packing the MTs more densely than in wild-type spindles. The area of the electron-dense matrix that defines the inner surface of the SPB (Byers, 1981) was measured in a plane perpendicular to the spindle axis. For all but long wild-type spindles, this area was estimated adequately by finding the smallest convex polygon that contained all of the MTs at the

Figure 4. Complete 3-D reconstruction of cell 5 (Table 1) presented as projections onto a plane that contains the pole-to-pole axis. A core bundle analysis was applied that first identified MTs that paired to within 45 nm of a continuous MT or a MT of opposite polarity. Those MTs that had pairing lengths ≥ 240 nm were considered members of a core bundle. The spindle is deconstructed to display the core bundle MTs (B) and noncore MTs (C). Bar, 0.1 μm .

Table 2. Distribution of core vs. noncore MTs in *cdc20* spindle reconstructions

Spindle	Total MTs	Number of MT		No. of Cross-bridges
		Noncore ^a	Core ^b	
1	93	39/30	9/13;2	34
2	80	29/35	6/4;6	30
3	91	31/32	14/13;1	35
4	56	7/12	9/19;9	20
5	100	44/26	16/12;2	34
6	65	16/16	14/18;1	68

^a Number of noncore MTs emanating from each pole.^b Number of polar core MTs from each pole; number of continuous MTs.

SPB. Wild-type SPBs had a mean area of $0.026 \pm 0.008 \mu\text{m}^2$ ($n = 28$), with no dependence on spindle length (means were 0.026, 0.026, and $0.027 \mu\text{m}^2$ for short, medial, and long spindles, respectively). The *cdc20* SPBs had a 2.3 times higher mean area, $0.061 \pm 0.027 \mu\text{m}^2$ ($n = 12$). The density of MTs at wild-type SPBs was $883 \pm 128 \text{ MTs}/\mu\text{m}^2$ ($n = 20$, including only the short and medial spindles, in which MTs occupy the full area of the SPB). This density was no higher, and in fact slightly lower, in *cdc20* cells ($744 \pm 210 \text{ MTs}/\mu\text{m}^2$, $n = 12$, $p < 0.05$ in a two-tailed *t* test.) However, the region of the SPB occupied by the core bundle, which happened to be the same size as the entire SPB in wild-type cells ($0.025 \mu\text{m}^2$), was just as densely packed as in wild-type cells ($886 \text{ MTs}/\mu\text{m}^2$). A detailed examination of the spindle MTs emerging from the SPBs revealed a tendency for the core MTs to cluster. Figure 5, A–D, shows a representative collection of MT cross-section distributions near the SPBs; core bundle MTs (filled circles) are distinguished from noncore MTs (open circles). This organization was seen in all but one data set (cell 2, Table 1) in which the spindle contained few core MTs (Table 2). In sum, SPBs in *cdc20* cells accommodate more MTs by being larger than in the wild type, and the MTs that form the core bundle tend to be associated closely with one another.

Stabilization of Spindle MTs in *cdc20* Mutants

Cross-bridges between adjacent MTs have been observed in the spindles of many organisms, including wild-type *S. cerevisiae* (Winey *et al.*, 1995). In normal spindles, these structures were predominantly between MTs of the core bundle and were thought to stabilize this MT population. Cross-bridges were also observed between MTs in all *cdc20* spindles modeled (Table 2 and Figure 1, A–E, arrowheads). The structures were similar in morphology to those detected in wild-type spindles. Figure 6a shows a model of the

Figure 5. The packing arrangement of MTs at the SPBs from representative cells, where core bundle MTs are identified as closed circles and noncore MTs are identified as open circles. The SPBs are from cells 2, 3, 5, and 6 (A–D, respectively). Bar, 50 nm.

distribution of cross-bridges in cell 3 (Table 1). The cross-bridges were found throughout the spindle, but

Figure 6. (A) shows a 3-D reconstruction of cell 3 with the position of cross-bridges represented by the filled circles. Bar, 0.1 μm . (B) Cross-bridge density as a function of relative pairing length for *cdc20* and wild-type (WT) spindles (modified from Winey *et al.*, 1995).

were predominantly distributed along the central spindle. The number of cross-bridges per spindle ranged from 19 to 68 (Table 2). To determine the identity of the MTs bearing cross-bridges, we analyzed the distribution of cross-bridges on the MTs identified in the core bundle analysis. Of the 221 cross-bridges identified in the six spindles, 66% were between core bundle MTs and 21% were between a core bundle MT and a noncore MT. The smallest fraction of cross-bridges (13%) was between two noncore MTs.

Because 87% of the cross-bridge structures involved at least one core bundle MT, we looked to see if the frequency of cross-bridges correlated with the pairing length of MTs. The cross-bridge density as a function of relative pairing length for all of the MT pairs in the six *cdc20* spindles and for wild-type cells, as reported in Winey *et al.* (1995), is shown in Figure 6b. Overall, densities are quantitatively similar to the wild type, but there are two major differences. First, in wild-type spindles, cross-bridge density was essentially zero at short pairing lengths and increased with increasing pairing length for all kinds of pairs, whereas in *cdc20* spindles the cross-bridge density is substantial even for low pairing lengths and does not increase from there. Second, in wild-type spindles, polar-continuous pairs had a higher density than parallel and antiparallel pairs, whereas in *cdc20*, antiparallel pairs have a higher density than parallel and polar-continuous pairs.

The longest spindle examined (cell 6, Table 1) was 4.21 μm . This cell contained the greatest number of cross-bridges ($n = 68$, Table 2); 93% of which involved a core MT. Figure 7a shows the model of this spindle with the location of its cross-bridges identified by single model points, Figure 7b shows the MTs that

form the core bundle, and Figure 7c is the 3-D distribution of noncore MTs. This spindle contains 16 noncore MTs associated with each SPB. The SPBs are separated by a long core bundle of MTs that are in turn connected along their length by multiple cross-bridges. These data suggest that the cross-bridges may act to stabilize and perhaps recruit MTs to form a core bundle that is important in spindle elongation. Alternatively, this spindle may represent a cell that has escaped arrest to enter anaphase and reenter the cell cycle.

DISCUSSION

We have used serial reconstruction by EM to model the 3-D trajectories of MTs in seven spindles from *cdc20* cells grown at the nonpermissive temperature. This technique has proved valuable for understanding the architecture of mitotic spindles in *Schizosaccharomyces pombe* (Ding *et al.*, 1993), mammalian tissue culture cells (McDonald *et al.*, 1992; Mastronarde *et al.*, 1993), as well as wild-type *S. cerevisiae* (Winey *et al.*, 1995). Our results confirm and extend the observations of aberrant spindle structure in *cdc20* cells reported by others (Byers and Goetsch, 1973, 1975; Sethi *et al.*, 1991). The increase in antitubulin immunofluorescence reported by Sethi *et al.* (1991) is shown here to correlate with an increase in MT number. This increase was due mostly to an increase in polar MTs, not continuous ones. In addition, the mutant spindles differ in structure and organization from wild-type spindles of comparable lengths. For example, the spindles of the temperature-arrested cells do not exhibit the packing order found in wild-type spindles of comparable length. In wild-type cells, a short ($<0.8 \mu\text{m}$)

Figure 7. (A) A 3-D model of cell 6 (Table 1) showing the distribution of cross-bridge structures ($\bullet$). The model is deconstructed to show 16 noncore MTs from each SPB (B) and the central core bundle of MTs (C). Bar, 0.1 μm .

unorganized mitotic spindle is formed, which in turn gives rise to medial spindles of 1.3–1.6 μm in length (Winey *et al.*, 1995). These medial spindles contain approximately the same number of spindle MTs as the short spindles, but have a core bundle of MTs that runs between the two SPBs. In both the short and medial spindles, there are enough noncore MTs to attach to each of the 32 kinetochores. In wild-type cells, spindles $>3 \mu\text{m}$ in length are of markedly different structure. These spindles are situated in the bud neck and show a sharp decrease in the number of polar MTs. There is also a marked reorganization of MTs comprising the core bundle, which is associated with anaphase spindle elongation (Winey *et al.*, 1995). The transition from a medial to a long spindle does not occur in *cdc20* cells grown at the nonpermissive temperature. In fact, the 3-D design of the temperature-arrested *cdc20* spindle resembles an exaggerated medial spindle from a wild-type cell containing an interpolar bundle of interdigitating core MTs and numerous polar MTs emanating from the SPBs. Thus, in the absence of Cdc20p function, the events of spindle assembly appears to be normal (e.g., polymerization of polar MTs and establishment of a core MT bundle), but subsequent mitotic events that promote spindle rearrangements are blocked.

The SPBs of *cdc20* cells grown at the restrictive temperature for 4 h were ~ 2.3 times larger than wild-type SPBs and could thus accommodate the increase in spindle MTs. Enlarged SPBs have been described for other mutants but always for those that form monopolar spindles (Winey and Byers, 1992). For example, in *cdc31* cells grown at the restrictive temperature, SPB material is assembled, yet SPB duplication does not occur. The resulting SPB is twice the size of those found in wild-type, and this SPB nucleates a monopolar spindle that contains about twice the normal number of MTs (Byers, 1981). Similarly, in the strains *ndc-1* (Winey and Byers, 1992) and *mps2-1* (Winey *et al.*, 1991), an enlarged SPB is formed to accommodate the total number of spindle MTs in the resulting monopolar spindle. In the temperature-arrested *cdc20* cells described here, both SPBs were enlarged and both nucleated an abnormally large number of MTs. The MTs forming the interpolar core bundle often formed a central cluster surrounded by the noncore MTs, suggesting that there is specific MT organization at the SPB. Neither the dynamics of SPB enlargement during temperature arrest nor the involvement of the Cdc20p in SPB growth is known. Nevertheless, this is the first study to describe enlarged SPBs in a mutant strain that forms a bipolar spindle. Our observations suggest that the SPB is a dynamic structure that can either accommodate an increase in spindle MTs or can grow to initiate extra MTs at either a single pole or in bipolar spindles.

The increase in spindle MT number in temperature-arrested *cdc20* cells correlated with an increase in the number of cross-bridge structures between MTs. In wild-type cells, these structures were not randomly distributed but were found predominantly between MTs that were members of a core bundle. The high axial density of cross-bridge structures between core bundle MTs in wild-type spindles was thought to stabilize populations of MTs that would serve as a scaffolding for spindles during anaphase B elongation (Winey *et al.*, 1995). The majority of cross-bridges identified in *cdc20* cells grown at the nonpermissive temperature was again in the core bundle. In the *cdc20* spindle, however, cross-bridges were found even between MTs with short pairing lengths, and they were predominantly between antiparallel MTs. MTs in the spindle of *S. cerevisiae* are presumably subject to dynamic instability (Mitchison and Kirschner, 1984; Mitchison *et al.*, 1986), just like the spindles of other organisms. MTs stabilized by some physical interaction, e.g., by a kinetochore bound to their plus end or by a cross-bridge, would likely remain in the spindle. A failure to degrade proteins may lead to an increase in the number of cross-bridges between MTs in *cdc20* spindles and, in turn, serve to stabilize populations of MTs that would normally display dynamic instability. The Cdc20p may therefore be important in the regulation of the dynamic MT behavior.

Studies of mitosis in *S. cerevisiae* must confront the challenge of identifying kMTs. This is difficult since chromosomes in this organism do not condense extensively during mitosis (Heath, 1980). Because *cdc20* mutants arrest prior to anaphase, we attempted to identify kMTs in our models. As with their wild-type counterpart, the *cdc20* spindle contains a population of core bundle MTs that associate over long distances, to form the bundle of interpolar MTs. Yet these spindles had many more noncore MTs than the 32 found in wild-type cells. A pairing analysis of these noncore MTs failed to detect MTs in a uniform metaphase configuration like that seen in higher eukaryotes and in *S. pombe* (Ding *et al.*, 1993). Analysis of the shortest 16 MTs from each pole, irrespective of pairing length or MT association, revealed a mean MT length of 0.34 μm . This mean length correlates well with the mean length of the putative kMTs present in the short and medial spindles of wild-type (Winey *et al.*, 1995), suggesting that kMTs in the *cdc20* spindle may be arrested at their wild-type lengths. If these do represent unseparated centromeres, the chromatin of sister chromatids would have to span a distance of 1.7 μm . This length exceeds the size of a centromere in *S. cerevisiae*, suggesting that other parts of the chromosome serve to hold sister chromatids together prior to anaphase onset. Alternatively, there may be unipolar attachments of unseparated sister chromatids. This interpretation is supported by the analysis of sister chromatid

pairing in *cdc20* mutants using fluorescent in situ hybridization (Guacci *et al.*, 1994). In *cdc20* diploids only two fluorescent signals were detected, instead of the four expected targets, suggesting that the sister chromatids were tightly paired. Therefore, in *cdc20* cells grown at the restrictive temperature, there may be defects in one kinetochore such that only one sister is attached to a pole. This notion is supported by the fact that *cdc20* cells recovering from arrest have their DNA asymmetrically distributed (Palmer *et al.*, 1989).

The observation of more MTs in the *cdc20* spindle than in the wild type is consistent with the effects reported for mutations in the homologous *fzy* gene of *Drosophila* (Dawson *et al.*, 1995). Both mutations result in mitotic arrest prior to anaphase with an enrichment of MTs in the spindle. Although the *fzy* gene product does not localize to the spindle, its activity is thought to be required both for metaphase-to-anaphase transition and for cyclin degradation. It is therefore thought to be linked to ubiquitin-dependent proteolytic events during mitosis. Dawson *et al.* (1995) hypothesized that with the presence of cyclins A and B, maturation-promoting factor activity would remain high, and thus early events in spindle formation, such as MT initiation and polymerization, would continue leading to the increase in spindle MTs and metaphase arrest.

In *cdc20* cells, a similar phenotype results from growth at nonpermissive temperature, and the defects lead to arrest in mitosis prior to anaphase with an enrichment of spindle MTs. These consequences could be explained by at least two mechanisms. The first is that Cdc20p is important for the anaphase-promoting complex (APC), a recently discovered protein assembly that is thought to play an important role in the ubiquitination of cyclins and other proteins whose degradation is important for the metaphase to anaphase transition in mammalian cells (Tugendreich *et al.*, 1995), *Xenopus* (King *et al.*, 1995), and *S. cerevisiae* (Irniger *et al.*, 1995). The second explanation is that there is a regulatory checkpoint that is dependent on the dynamic activity of spindle MTs. In this model, the checkpoint would regulate the activity of the APC to ensure that later events in mitosis, such as anaphase elongation, do not occur if the spindle is disrupted. Such feedback control mechanisms in *S. cerevisiae* have been described in the identification of *MAD* (Li and Murray, 1991) and *BUB* (Hoyt *et al.*, 1991; Roberts *et al.*, 1994) genes whose role in mitosis is apparent in the spindle assembly checkpoint (Wang and Burke, 1995; Wells and Murray, 1996). Spindle disruption could be defined as an event that disrupts the normal dynamic behavior of MTs, e.g., depolymerization with MT-destabilizing drugs or a stabilization of spindle MTs described here for *cdc20*. In fact, the double mutant *cdc20, bub2* does not arrest in the cell cycle (Hoyt *et al.*, 1991; Tavormina and Burke, unpublished observations), suggesting that *CDC20* acts in a *BUB2*-depen-

dent manner. In mitosis, the absence of functional Cdc20p may cause MTs to become stabilized. This could signal a checkpoint that inhibits the APC; therefore, sister chromatids do not separate and Cdc28p kinase activity remains high.

Although the location of the Cdc20p gene product is not known, mutations in the gene have effects at multiple cell cycle stages, suggesting that the role of this protein may be regulatory in nature. Many but not all MT-related events are affected, including the formation of a normal mitotic spindle and thus normal chromosome segregation (Hartwell *et al.*, 1973; Byers and Goetsch 1974; Sethi *et al.*, 1991) and karyogamy (Sethi *et al.*, 1991). Movement of the nucleus to the bud neck, which depends on the presence of astral MTs (Palmer *et al.*, 1992), is not affected in *cdc20* cells, but this role of MTs may not rely on dynamic MT behavior. Although it is unlikely that the Cdc20p is associated directly with MTs, its role may be to promote MT disassembly through a mechanism that also affects the proteolysis of proteins in other pathways. Future studies that combine immunoelectron microscopy with computer-generated serial reconstruction should be useful in identifying the spatial distribution of important gene products relative to the mitotic spindle.

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