

The Surveillance Mechanism of the Spindle Position Checkpoint in Yeast[Ⓢ]

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Abstract. The spindle position checkpoint in *Saccharomyces cerevisiae* delays mitotic exit until the spindle has moved into the mother–bud neck, ensuring that each daughter cell inherits a nucleus. The small G protein Tem1p is critical in promoting mitotic exit and is concentrated at the spindle pole destined for the bud. The presumed nucleotide exchange factor for Tem1p, Lte1p, is concentrated in the bud. These findings suggested the hypothesis that movement of the spindle pole through the neck allows Tem1p to interact with Lte1p, promoting GTP loading of Tem1p and mitotic exit. However, we report that deletion of *LTE1* had little effect on the timing of mitotic exit. We also examined several mu-

tants in which some cells inappropriately exit mitosis even though the spindle is within the mother. In some of these cells, the spindle pole body did not interact with the bud or the neck before mitotic exit. Thus, some alternative mechanism must exist to coordinate mitotic exit with spindle position. In both wild-type and mutant cells, mitotic exit was preceded by loss of cytoplasmic microtubules from the neck. Thus, the spindle position checkpoint may monitor such interactions.

Key words: checkpoint • mitosis • dynein • EBI • *Saccharomyces cerevisiae*

Introduction

During cell division in eukaryotes, the mitotic spindle separates each duplicated pair of chromosomes to opposite poles of the cell. Cytokinesis then occurs between the poles, ensuring that each cell receives a full complement of chromosomes. In most eukaryotes, the position of the cleavage plane is determined by the position of the spindle (Oegema and Mitchison, 1997). In budding yeasts, such as *Saccharomyces cerevisiae*, cell division occurs at the mother–bud neck. Therefore, budding yeast must move the spindle into the neck before dividing.

S. cerevisiae has two partially redundant mechanisms to move the spindle into the neck (Heil-Chapdelaine et al., 1999). A major mechanism for spindle movement into the neck is dynein-dependent sliding of microtubule sides along the bud cortex (Yeh et al., 1995; Adames and Cooper, 2000). A backup mechanism for spindle movement into the neck involves capture of microtubule ends at the bud cortex by Kar9p (Miller and Rose, 1998) and subsequent microtubule shrinkage to pull the nucleus toward the bud and help orient the spindle (Shaw et al., 1997; Adames and Cooper, 2000). Both mechanisms require cytoplasmic microtubules to pass through the neck and interact with the bud cortex.

In cells lacking functional dynein or dynactin, spindle movement into the neck can be delayed (Eshel et al., 1993;

Li et al., 1993; Yeh et al., 1995). When this happens, cytoplasmic microtubules passing through the neck continue to probe the bud cortex and grow longer, often pushing the spindle further from the neck (Adames and Cooper, 2000). When spindle movement into the neck is delayed, cytokinesis and mitotic exit is also delayed (Yeh et al., 1995). Inappropriate mitotic exit, defined here as mitotic exit when the spindle is not positioned in the neck, is extremely rare. Therefore, a checkpoint mechanism appears to monitor spindle position and prevents mitotic exit. Previously, we found that some mutations caused a failure of the tight coupling between spindle position and mitotic exit (Muhua et al., 1998).

Recent work has shed light on how the spindle position checkpoint might work. Exit from mitosis is controlled by the small G protein Tem1p, which is an essential protein that activates proteins of the mitotic exit network (Shirayama et al., 1994; Shou et al., 1999). The major function of the mitotic exit network is to promote degradation of the mitotic cyclins and to inhibit the mitotic cyclin–cdk complex (Visintin et al., 1998; Shou et al., 1999). Tem1p is located at the spindle pole body (SPB)¹ destined for the bud (Bardin et al., 2000; Bloecher et al., 2000). Lte1p has homology to guanine–nucleotide exchange factors, is presumed to activate Tem1p, and is located in the bud (Bardin et al., 2000; Pereira et al., 2000). Thus Tem1p and

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¹Abbreviations used in this paper: SEP, standard error of the proportion; SPB, spindle pole body.

Lte1p are in the same compartment of the cell only after the spindle moves into the neck, suggesting that movement of the spindle into the neck signals mitotic exit (Hoyt, 2000). Consistent with this model, multinucleate and anucleate cells were formed when overexpression of Lte1p caused some Lte1p to mislocalize in the mother (Bardin et al., 2000).

Bub2p and Bfa1p (Byr4p), which form a presumed GTPase-activating protein thought to downregulate Tem1p, are necessary for the spindle position checkpoint (Bardin et al., 2000; Bloecher et al., 2000; Pereira et al., 2000). Bub2p and Bfa1p are located at the same SPB as Tem1p, and the localization of Tem1p depends on Bub2p and Bfa1p (Pereira et al., 2000), at least until late anaphase. These results suggest that Bub2p–Bfa1p maintains Tem1p in an inactive state on the SPB while the SPB is in the mother cell (Hoyt, 2000). Thus, the model predicts that delayed mitotic exit in cells with mispositioned spindles is due to the absence of a positive signal for mitotic exit.

Here, we test this model through direct observation of mitotic exit in *lte1Δ* mutants and show that loss of Lte1p has little effect on the kinetics of mitotic exit, at least at elevated temperatures. Also, we confirm that the spindle position checkpoint is completely abolished in *bub2Δ* and *bfa1Δ* mutants. Mitotic exit in *bub2Δ* and *bfa1Δ* mutants occurs regardless of spindle position. Hence, Bub2p–Bfa1p inhibition of Tem1p is crucial to preventing mitotic exit but is somehow relieved upon spindle movement into the neck, even in the absence of Lte1p.

To further examine how the spindle position checkpoint works, we examined several other mutants in which exit from mitosis can occur despite mispositioning of the spindle—*bim1Δ* (BIM1 is also known as YEB1 for yeast EB1 [Muhua et al., 1998], after the first identified member of this protein family. The name Bim1, for binds to microtubules, denotes how the yeast gene was first identified [Schwartz et al., 1997]. The Bim nomenclature should not be confused with the Bim genes (blocked in mitosis) identified in *Aspergillus* [Morris, 1975].), *tub2-401*, and *cnm67Δ*. In these mutants, cells with mispositioned spindles generally delayed mitotic exit, suggesting that the spindle position checkpoint mechanism was intact. However, many mutant cells with mispositioned spindles did exit mitosis inappropriately, generating binucleate and anucleate cells. These instances of failure of the checkpoint were associated with one of two events: transient (but ultimately abortive and ineffective) passage of an SPB into the neck, or transient loss of cytoplasmic microtubule interactions with the neck while both SPBs remained within the mother. Deletion of *LTE1* did not prevent inappropriate mitotic exit in these cases. Thus, the observed features of the coupling between spindle position and mitotic exit are largely unaffected by Lte1p. We propose that a parallel checkpoint pathway exists in which the interaction of cytoplasmic microtubules with the neck prevents mitotic exit through activation of Bub2p–Bfa1p.

Materials and Methods

Yeast Strains and Plasmids

With the exception of the *tub2-401* mutant, all strains were isogenic with the wild-type strain YJC1687 (*MATa ade2-1 ade3 lys2-801 his3-Δ200 leu2-3, 112 ura3-52::GFP::TUB1*) as described (Adames and Cooper, 2000). Strains differed from wild type as indicated: YJC1658 (*trp1-1*

bim1Δ::HIS3), YJC1567 (*stt4-7*), YJC1659 (*leu2-3,112::LEU2-ts-arp1 arp1Δ::HIS3*), YJC1655 (*bim1Δ::HIS3 leu2-3,112::LEU2-ts-arp1 arp1Δ::HIS3*), YJC1571 (*stt4-7 leu2-3,112::LEU2-ts-arp1 arp1Δ::HIS3*), and YJC2228 (*trp1-1 cnm67Δ::kanMX*). Hof1–GFP on pLP14 (a gift from John Lippincott and Rong Li, Harvard University, Cambridge, MA) was integrated into the above strains to make YJC2161 (*ura3::URA3-HOF1-GFP*), YJC2162 (*bim1Δ ura3::URA3-HOF1-GFP*), YJC1888 (*ts-arp1 ura3::URA3-HOF1-GFP*), YJC1889 (*ts-arp1 bim1Δ ura3::URA3-HOF1-GFP*), and YJC2329 (*cnm67Δ ura3::URA3-HOF1-GFP*). YJC1888 was used to make YJC2456 (*ts-arp1 lte1Δ::kanMX HOF1-GFP*), YJC2327 (*ts-arp1 mad2Δ::kanMX HOF1-GFP*), YJC2288 (*ts-arp1 bub2Δ::kanMX HOF1-GFP*), YJC2290 (*ts-arp1 bfa1Δ::kanMX HOF1-GFP*), and YJC 2327 (*ts-arp1 mad2Δ::kanMX HOF1-GFP*). YJC2161 was used to make YJC2443 (*lte1Δ::kanMX*). YJC1889 was used to make YJC2444 (*ts-arp1 bim1Δ lte1Δ::kanMX*). YJC2228 was used to make YJC2446 (*cnm67Δ lte1Δ::TRP1*). To make the *tub2-401* mutant with GFP–tubulin and Hof1–GFP (YJC2318), we integrated pAFS92 (a gift from Aaron Straight, Harvard University, Cambridge, MA) and pLP14 into CUY66 (*MATa ade2-101 ura3-52 tub2-401*; a gift from Tim Huffaker, Cornell University, Ithaca, NY). YJC2318 was used to make YJC2445 (*tub2-401 lte1Δ::kanMX*). All deletion strains were analyzed by PCR to confirm replacement of the open reading frame with the appropriate selection marker. Deletion strains showed the appropriate phenotypes, including cold-sensitive growth in *lte1Δ* mutants. Strains expressing Myo1–GFP were also made but are not shown. There were no differences in the timing of spindle breakdown, cytokinesis, and cell separation between cells expressing Myo1–GFP or Hof1–GFP or cells expressing neither (data not shown). Media, genetic manipulations, and lithium acetate transformation were as described (Kaiser et al., 1994).

Assay for Mitotic Exit

Strains expressed GFP–tubulin to allow visualization of mitotic spindles (Straight et al., 1997). Some strains also expressed Myo1–GFP (not shown) or Hof1–GFP to visualize the process of cytokinesis (Bi et al., 1998; Lippincott and Li, 1998a,b; Vallen et al., 2000).

The use of Myo1–GFP or Hof1–GFP to monitor cell division provides a more accurate determination of when cell division occurs than does cell separation. Both proteins constrict with the actomyosin ring during cytokinesis and disappear from the neck thereafter. Cells within the population expressed these GFP fusion proteins to different extents, and strong uniform expression was seen only in slowly growing cells in dense cultures, in cells that delay spindle movement into the neck, or at low temperatures.

Using GFP–tubulin allowed us to visualize spindle breakdown directly. Spindle disassembly is dependent on the same proteolytic events involved in the degradation of mitotic cyclins (Juang et al., 1997). Spindle breakdown and constriction of the actomyosin ring always coincided, regardless of whether the spindle was in the neck or entirely in the mother (59/59 wild-type cells, 46/46 *bim1Δ* cells, 20/20 *ts-arp1* cells, and 80/80 *ts-arp1 bim1Δ* cells). Spindles were completely disassembled 1.6 ± 0.2 min after the start of actomyosin ring constriction in the wild-type strain (58 cells), 2.7 ± 0.4 min after constriction in *ts-arp1* cells (19 cells), and 1.7 ± 0.3 min after constriction in *ts-arp1 bim1Δ* cells (65 cells). In the strains examined, cell separation occurred at the same time interval after spindle breakdown or actomyosin ring constriction (24.3 ± 1.6 min, 29 cells, for wild type; 23.8 ± 2.5 min, 30 cells, for *bim1Δ*; 29.3 ± 7.5 min, 6 cells, for *stt4-7*; 29.7 ± 2.6 min, 50 cells, for *ts-arp1*; 26.7 ± 2.8 min, 79 cells, for *ts-arp1 bim1Δ*). In some experiments, cells expressed only GFP–Tub1p, and cytokinesis was presumed to occur at the time of spindle breakdown. Scoring of SPB interactions with the neck in cells that exited mitosis inappropriately was confirmed in blind tests.

Movies and Analysis

GFP–tubulin expression was induced as described (Adames and Cooper, 2000). For low-temperature experiments, the ambient temperature was maintained $<14^{\circ}\text{C}$. An Air Therm heater (World Precision Instruments) warmed the system to the appropriate temperature. At the semipermissive temperature of 18°C used by Sullivan and Huffaker (1992), spindle microtubules were affected, as indicated by abnormal spindle elongation, abnormal spindle breakdown, and the failure of some cells to enter anaphase, suggestive of spindle assembly checkpoint activity (data not shown). Therefore, *tub2-401* experiments were done at 20°C or the permissive temperature of 30°C . All other experiments were at 37°C . Cells were incubated at 37°C for 1 h to inactivate Arp1p before movie collection.

Movies were collected as described (Waddle et al., 1996). Z-series of 10 focal planes $0.5 \mu\text{m}$ apart were collected and projected onto a single two-dimensional image. The timing of nuclear movements and cell cycle events

were analyzed with NIH Image 1.62 (written by Wayne Rasband at National Institutes of Health). Comparisons of statistical significance were by *t* test.

Immunofluorescence

For microtubule staining, *tub2-401* cells were grown to $OD_{600} = 0.5$ and synchronized with 0.2 M hydroxyurea for 2 h to arrest the cells in G2/M. The culture was washed and released from arrest, and split into two; one culture was grown at 20°C for 60 min and the other at 30°C for 30 min to allow the cells to enter anaphase. Very few cells showed anaphase spindles in the mother at 30°C. However, at 20°C, many anaphase spindles were in the mother. Immunofluorescence was performed as described (Amatruda and Cooper, 1992) using a mouse α -*Drosophila* α -tubulin primary antibody (1:10) and a rhodamine-conjugated donkey α -mouse secondary antibody (1:100).

Online Supplemental Material

Videos of cells depicted in Figs. 1, 2, 4, 6, and 7 are available at <http://www.jcb.org/cgi/content/full/153/1/159/DC1>. Supplemental Videos 1–14 correspond to Figs. 1 A, 2 C, 2 D, 4 A–E, 6 D, and 7, respectively.

Results

Mitotic Exit in the Absence of *Lte1p*

The model proposed by Bardin et al. (2000) and Pereira et al. (2000) invokes a failure of Tem1p to interact with *Lte1p* as the basis for the delayed mitotic exit in cells with mispositioned spindles. *lte1Δ* mutants are cold sensitive (Shirayama et al., 1994) but are able to grow at higher temperatures. Whether the dynamics of mitotic exit are affected at higher temperatures is unknown.

Using long-term (3 h) movies of cells, we analyzed progression through mitosis in *lte1Δ* cells. Most experiments in this study were done at 37°C for comparison with strains carrying a temperature-sensitive allele for dynactin (*ts-arp1*; Muhua et al., 1998). We found an excellent correlation between the timing of spindle disassembly (detected using GFP–Tub1p) and the timing of cytokinesis (detected using either Hof1p–GFP or Myo1p–GFP; Materials and Methods), and these events were used interchangeably to determine the timing of mitotic exit. Selected time points from movies of representative cells are shown.

We found that *Lte1p* was not required for mitotic exit at 37°C. All of the 112 anaphase *lte1Δ* cells we observed proceeded through anaphase and divided (Video 1; Fig. 1 A). Furthermore, loss of *Lte1p* had little effect on the timing of mitotic exit compared with wild-type cells (Fig. 1 B). Thus, Tem1p can apparently be activated with near normal kinetics without *Lte1p* under these conditions. *Lte1p* was also not necessary for mitotic exit at lower temperatures (20°C), although mitotic exit appeared slow compared with *LTE1* cells at the same temperature (see below).

Although *Lte1p* was not necessary for mitotic exit, there was a remarkable consistency in the timing of mitotic exit relative to spindle movement into the neck, even in mutants that delay spindle movement. In *ts-arp1 lte1Δ* mutants, when the spindle did eventually move into the neck, the time until mitotic exit was similar to that observed in the wild-type strain or *ts-arp1* single mutant (Fig. 1 B). Mitotic exit always occurred within ~20 min of spindle movement into the neck regardless of how long it took to move the spindle into the neck (compare the interval from spindle movement into the neck to mitotic exit in Figs. 1 B, 2 A, and 3 A). This observation implies the existence of a *Lte1p*-independent mechanism that couples spindle movement into the neck with exit from mitosis.

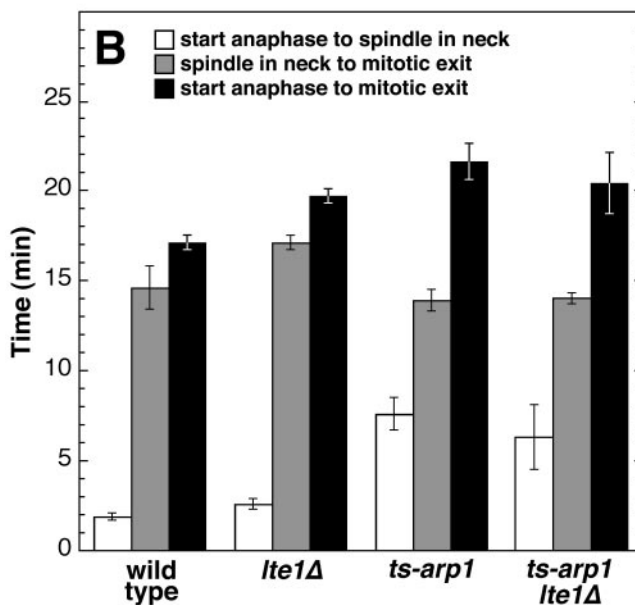
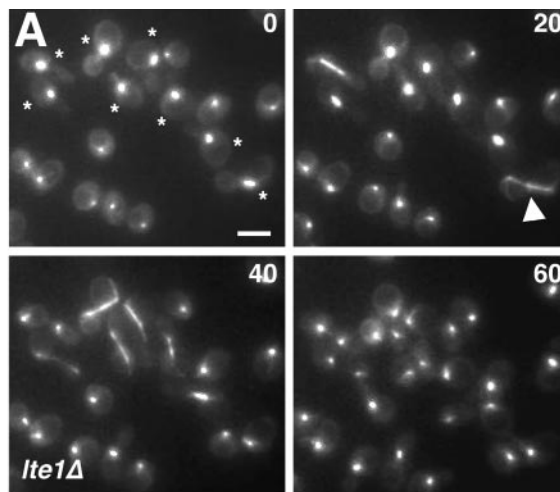
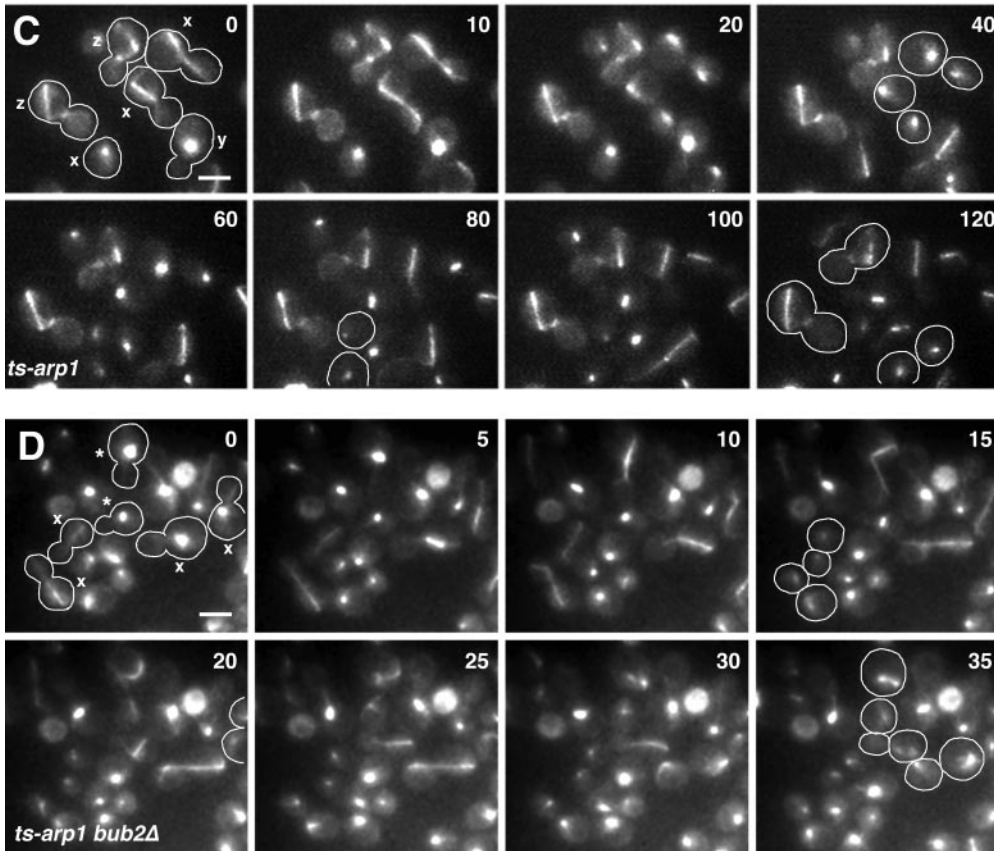
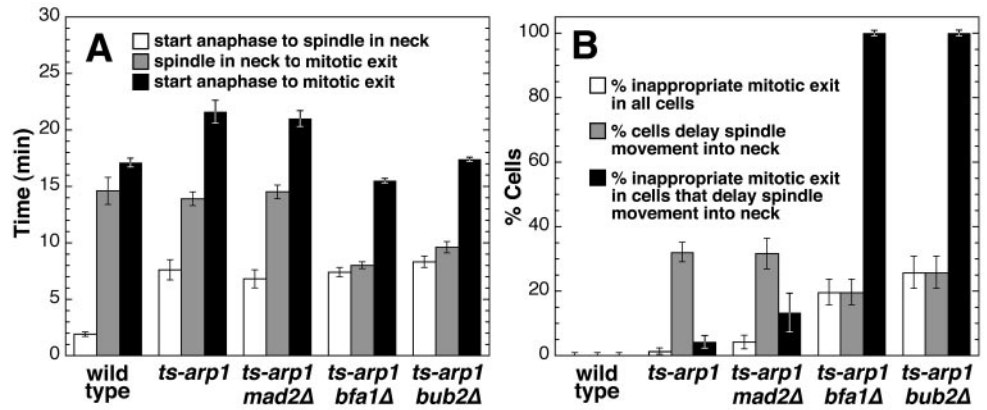


Figure 1. *Lte1p* is not necessary for mitotic exit. (A) Still images from Video 1. Representative field showing mitosis in *lte1Δ* cells. Cells expressed GFP–tubulin to allow visualization of the spindle (bright thick bar of fluorescence) and cytoplasmic microtubules. Cells also expressed Hof1–GFP to allow visualization of mitotic exit. Hof1–GFP initially localizes to the septin ring structures at the neck (arrowhead). During mitotic exit, Hof1–GFP moves to the constricting actomyosin ring and then disappears from the neck (Lippincott and Li, 1998a). Not all cells in the population expressed Hof1–GFP to the same extent. Time (min) is indicated in the upper right. (B) Loss of *Lte1p* has little effect on the kinetics of mitotic exit. Shown are the mean time intervals from the start of anaphase (spindle elongation) to spindle movement into the neck (white columns), from spindle movement into the neck to mitotic exit (spindle break down and constriction of the actomyosin ring; gray columns), and the total time for anaphase to mitotic exit (black columns). The *lte1Δ* mutant was slightly slower than the wild-type strain ($P < 0.01$). Number of events for each column from left to right on the chart: wild type, 108, 83, 83; *lte1Δ*, 115, 112, 112; *ts-arp1*, 120, 116, 114; *ts-arp1 lte1Δ*, 48, 66, 48. Error bars represent SEM. Supplemental videos are available at <http://www.jcb.org/cgi/content/full/153/1/159/DC1>. Bar, 5 μ m.

In all of the 1,082 cells we observed in which the spindle did successfully move into the neck (after variable delays), mitotic exit always occurred within 25 min of the initial interaction of the SPB with the neck. When spindles failed to



cells that delayed spindle movement is also shown (black columns). Cell numbers: wild type, 217; *ts-arp1*, 287; *ts-arp1 mad2Δ*, 95; *ts-arp1 bfa1Δ*, 117; *ts-arp1 bub2Δ*, 93. Error bars represent standard error of the proportion (SEP). (C) Still images from Video 2. Mitosis in representative cells of a *ts-arp1* mutant. The cells of interest are outlined in the first frame and again after they divide, or they are outlined in the last frame. Some cells (x) move the spindle into the neck within the normal time for mitotic exit. Other cells (y) delay spindle movement into the neck beyond the normal time for mitotic exit, but then correct the spindle position and divide. A third type of cell (z) does not move the spindle into the neck or exit mitosis during the course of the movie. Time (in min) is indicated. (D) Still images from Video 3. Mitosis in representative cells of a *ts-arp1 bub2Δ* mutant. Most cells (x) move the spindle into the neck within the normal time for mitotic exit. Those cells that do not move the spindle into the neck in time (*), exit mitosis at the normal time producing a binucleate mother and an anucleate daughter. Time (in min) is indicated. Supplemental videos are available at <http://www.jcb.org/cgi/content/full/153/1/159/DC1>. Bars, 5 μ m.

move into the neck within 25 min of the initial interaction of the SPB with the neck, inappropriate exit from mitosis occurred (57/57 incidents in all aforementioned strains). Therefore, mitotic exit appears to be activated within ~25 min of SPB interaction with the neck, regardless of whether the spindle subsequently elongates successfully into the bud.

Inappropriate Mitotic Exit in *bim1Δ* Mutants

To test any model for how the spindle position checkpoint monitors the position of the spindle, we needed to identify mutants that would affect the surveillance mechanism of the checkpoint while leaving intact the signaling pathway leading to Bub2p–Bfa1p down regulation of Tem1p. Such mutants

Figure 2. The spindle position checkpoint is completely abolished in *bub2Δ* and *bfa1Δ* mutants, but not in *mad2Δ* mutants. (A) Cytokinesis was delayed in *ts-arp1* and *ts-arp1 mad2Δ* cells, but not in the *ts-arp1 bfa1Δ* and *ts-arp1 bub2Δ* mutants. Shown are the mean time intervals from the start of anaphase to spindle movement into the neck (white columns), from spindle movement into the neck to mitotic exit (gray columns), and the total time for anaphase (black columns). In the *bfa1Δ* and *bub2Δ* mutants, mitotic exit occurred in about half the time ($P < 0.001$), after spindle movement into the neck, compared with the wild-type strain. Number of events for each column from left to right on the chart: wild type, 108, 83, 83; *ts-arp1*, 120, 116, 114; *ts-arp1 mad2Δ*, 65, 71, 64; *ts-arp1 bfa1Δ*, 94, 91, 114; *ts-arp1 bub2Δ*, 68, 66, 92. Error bars represent SEM. (B) In the *ts-arp1* and *ts-arp1 mad2Δ* strains, only a small fraction of cells that delayed spindle movement into the neck exited mitosis inappropriately. In the *ts-arp1 bfa1Δ* and *ts-arp1 bub2Δ* strains, all cells that delayed spindle movement into the neck underwent inappropriate mitotic exit. Comparison of the percentage of cells that underwent inappropriate mitotic exit (white columns) to the percentage of cells that delayed spindle movement into the neck by >20 min (gray columns) is shown. The frequency of inappropriate mitotic exit in

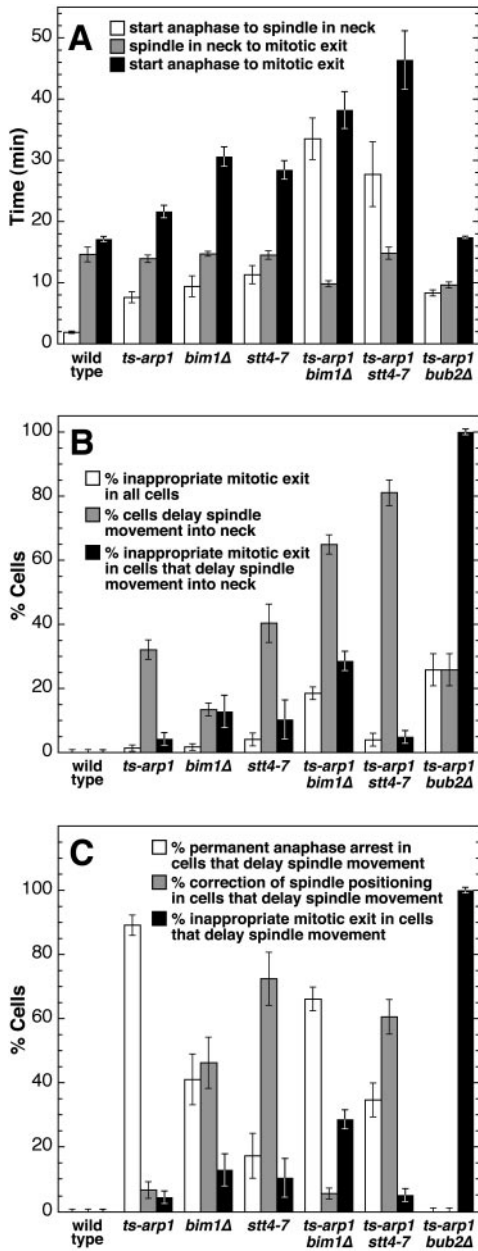


Figure 3. Inappropriate mitotic exit in *bim1Δ* and *stt4-7* mutants. (A) Exit from mitosis was delayed in the *ts-arp1 bim1Δ* and *ts-arp1 stt4-7* mutants. Shown are the mean time intervals from the start of anaphase to spindle movement into the neck (white columns), from spindle movement into the neck to mitotic exit (gray columns), and the total time for anaphase (black columns). Notice the near lack of variability in the time interval from spindle movement into the neck to cytokinesis. Number of events for each column from left to right on the chart: wild type, 108, 83, 83; *ts-arp1*, 120, 116, 114; *bim1Δ*, 129, 170, 150; *stt4-7*, 49, 49, 55; *ts-arp1 bim1Δ*, 147, 135, 127; *ts-arp1 stt4-7*, 27, 33, 29; *ts-arp1 bub2Δ*, 68, 66, 92. Error bars represent SEM. (B) In the *ts-arp1 bim1Δ* mutant, a large fraction of cells that delayed spindle movement into the neck exited mitosis inappropriately. However, inappropriate mitotic exit did not occur in all *ts-arp1 bim1Δ* cells that delayed spindle movement into the neck. Comparison of the percentage of cells that underwent inappropriate mitotic exit (white columns) with the percentage of cells that delayed spindle movement into the neck by >20 min (gray columns) is shown. The frequency of inappropriate mitotic exit in cells that delayed spindle movement is also

should show incomplete penetrance in their checkpoint phenotype and undergo inappropriate mitotic exit without moving the spindle into the neck only under certain conditions. We have previously reported that *ts-arp1* mutants carrying additional mutations in *BIMI1* or *STT4* sometimes undergo a checkpoint failure in which mitotic exit occurs while the spindle is confined to the mother cell, yielding binucleate and anucleate progeny (Muhua et al., 1998). More recently, it was shown that *BUB2* and *BFA1* (*BYR4*) are also required to prevent such inappropriate mitotic exit, whereas *MAD2* is not (Bloecher et al., 2000; Pereira et al., 2000). We examined spindle position and mitotic exit in these various mutants to determine the penetrance of their checkpoint phenotypes.

In wild-type cells, mitotic exit occurred within 20 min of anaphase start (spindle elongation) in nearly all cells (Fig. 2 A). Consistent with previous reports, most *ts-arp1* and *ts-arp1 mad2Δ* cells that delayed spindle movement into the neck also delayed mitotic exit (Video 2; Fig. 2, A–C). Only a small fraction of these cells exited mitosis without moving the spindle into the neck (Fig. 2 B). In contrast, *ts-arp1 bub2Δ* and *ts-arp1 bfa1Δ* cells that delayed spindle movement into the neck did not delay exit from mitosis, which almost always occurred within 20 min of anaphase onset (Fig. 2 A). In these strains, cells exited mitosis inappropriately when spindle movement into the neck was delayed (Video 3; Fig. 2, B and D), indicating that the *bub2Δ* and *bfa1Δ* mutants are completely defective for checkpoint function.

Next we examined *bim1Δ* and *stt4-7* single mutants and *ts-arp1 bim1Δ* and *ts-arp1 stt4-7* double mutants. As expected, many *ts-arp1 bim1Δ* and *ts-arp1 stt4-7* cells failed to move the spindle into the neck by 20 min (Fig. 3, A and B). However, in contrast to *bub2Δ* and *bfa1Δ*, inappropriate mitotic exit occurred in only a fraction of cells that delayed spindle movement (Figs. 3 B and 4 A; Video 4). Some cells that delayed spindle movement into the neck eventually succeeded in moving the spindle into the neck and divided (Figs. 3 C and 4, A and B; Videos 4–6). Other cells failed to move the spindle into the neck and remained arrested in anaphase (Fig. 3 C and 4 A; Video 4). Because many cells delayed spindle movement into the neck without undergoing mitotic exit, the *ts-arp1 bim1Δ* and *ts-arp1 stt4-7* mutants showed, on average, a delay in mitotic exit, as did the *ts-arp1* control cells (Fig. 3 A; $P < 0.001$). These results suggest that the spindle position checkpoint retains at least partial function in *bim1Δ* and *stt4-7* mutants.

What accounts for the occasional failure of the spindle position checkpoint in these mutants? Even *ts-arp1* single

shown (black columns). Cell numbers: wild type, 217; *ts-arp1*, 287; *bim1Δ*, 290; *stt4-7*, 72; *ts-arp1 bim1Δ*, 259; *ts-arp1 stt4-7*, 100; *ts-arp1 bub2Δ*, 93. (C) Fate of cells that delay spindle movement into the neck. In the *ts-arp1* mutant, most cells that delay spindle movement into the neck fail to move the spindle into the neck during the course of observation (white column). In the *ts-arp1 stt4-7* mutant, many cells delay spindle movement into the neck but then correct the spindle position and exit mitosis (gray column). In the *ts-arp1 bim1Δ* mutant, many cells exit mitosis inappropriately (black column) instead of correcting the spindle position. Cell numbers: wild type, 0; *ts-arp1*, 92; *bim1Δ*, 39; *stt4-7*, 29; *ts-arp1 bim1Δ*, 168; *ts-arp1 stt4-7*, 81; *ts-arp1 bub2Δ*, 24. Error bars represent SEP.

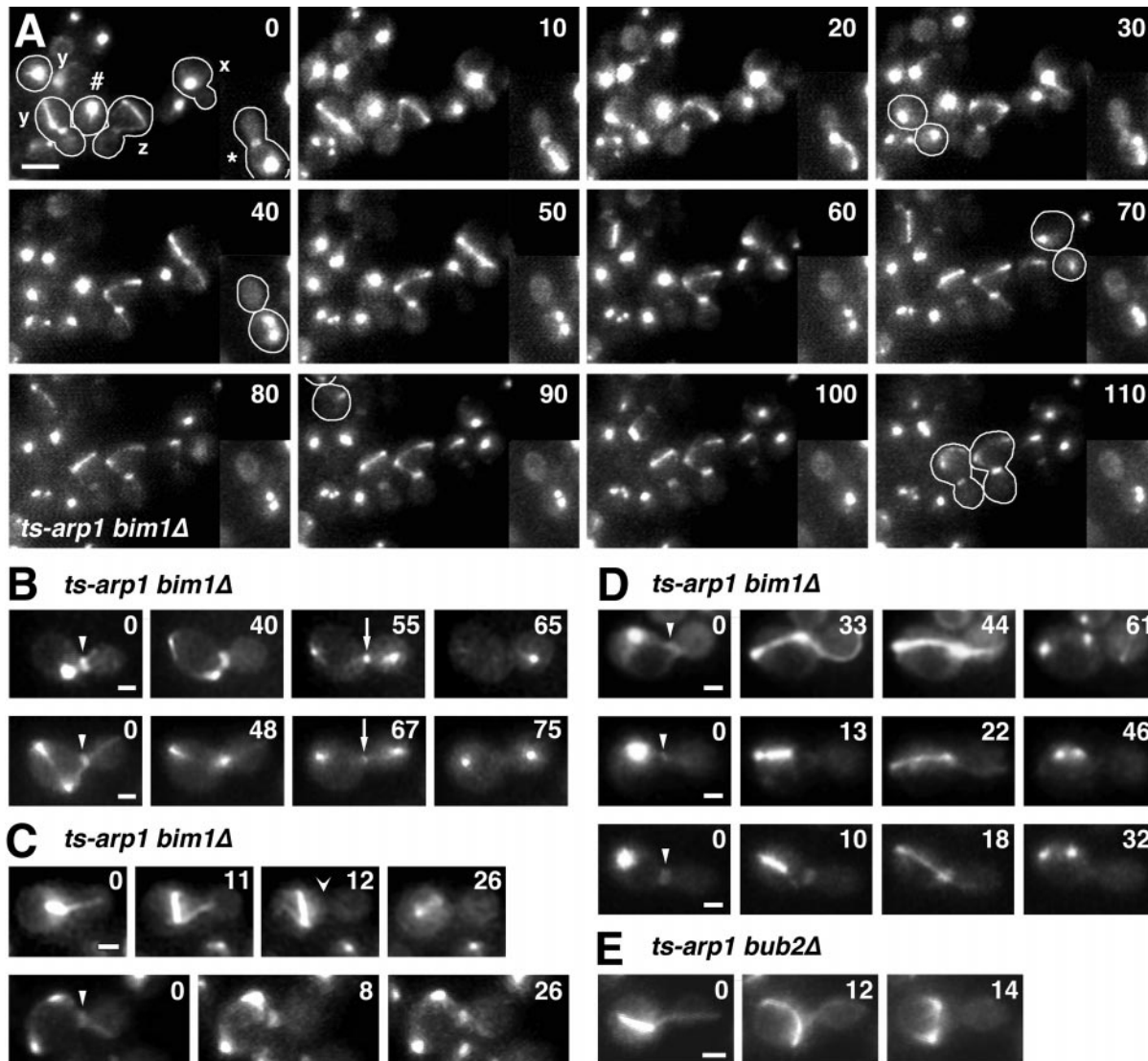


Figure 4. Events that precede inappropriate mitotic exit in *ts-arp1 bim1Δ* cells. (A) Still images from Video 4. A representative field of *ts-arp1 bim1Δ* cells. The cells of interest are outlined in the first frame and again after they exit mitosis or in the last frame. Some cells (x) move the spindle into the neck within the normal time for mitotic exit. Other cells (y) delay spindle movement into the neck beyond the normal time for mitotic exit, but then correct the spindle position and divide. A third type of cell (z) does not move the spindle into the neck or exit mitosis

during the course of the movie. Inappropriate mitotic exit occurs after abortive spindle movements into the neck (*; cell in inset taken from a different field in the same movie) or when cytoplasmic microtubules are lost from the neck (#). (B) Still images from Videos 5 and 6. In some *ts-arp1 bim1Δ* cells, spindle movement into the neck is severely delayed and so is exit from mitosis, as indicated by maintenance of Hof1-GFP on the septin ring (arrowhead). Once the spindle moves into the neck, mitotic exit and spindle disassembly proceed (arrow). (C) Still images from Videos 7 and 8. In *ts-arp1 bim1Δ* cells, the spindle delays movement into the neck and Hof1-GFP remains at the septin rings (arrowheads). Inappropriate mitotic exit occurs in the absence of SPB-neck interactions. Instead, cytokinesis and spindle disassembly (arrow) occur after the loss of cytoplasmic microtubules from the neck (indented arrowhead). In the bottom cell of the lower series, cytoplasmic microtubules fail to penetrate or touch the neck at any time during anaphase. (D) Still images from Videos 9–11. Examples of *ts-arp1 bim1Δ* cells, in which inappropriate mitotic exit occurred after the spindle moved toward and touched the neck without completely penetrating the neck. Spindle disassembly and cytokinesis occurred while the SPB was at the neck and often resulted in the shearing of a microtubule within the bud. The Hof1-GFP disappears from the neck after spindle disassembly. (E) Still images from Video 12. In *ts-arp1 bub2Δ* cells, inappropriate mitotic exit occurred without SPB-neck interactions or loss of cytoplasmic microtubules from the neck. Times are indicated in the upper right. Supplemental videos are available at <http://www.jcb.org/cgi/content/full/153/1/159/DC1>. Bars: (A) 5 μ m; (B–E) 2 μ m.

mutants undergo inappropriate exit from mitosis on occasion (albeit at very low frequency), suggesting that the checkpoint has a low but discernible error rate. The fraction of cells that show delayed spindle movement into the

neck is higher in *ts-arp1 bim1Δ* and *ts-arp1 stt4-7* cells than in *ts-arp1* cells (Fig. 3 B), so that on average there is a longer time between anaphase onset and spindle movement into the neck (Fig. 3 A). Thus, it could be that the

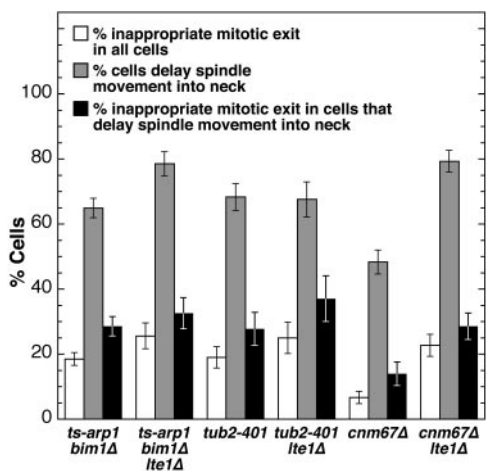


Figure 5. *Lte1p* is not required for inappropriate mitotic exit. Deletion of *LTE1* does not decrease the frequency of inappropriate mitotic exit in mutants affecting cytoplasmic microtubule function—*bim1Δ*, *tub2-401*, and *cnm67Δ*. Shown are the percentage of cells that exit mitosis inappropriately (white columns), the percentage of cells that delay spindle movement into the neck (gray columns), and the percentage of cells that delay spindle movement into the neck and exit mitosis inappropriately (black columns). Cell numbers for each column from left to right: *ts-arp1 bim1Δ*, 259, 259, 168; *ts-arp1 bim1Δ lte1Δ*, 121, 121, 95; *tub2-401*, 142, 142, 97; *tub2-401 lte1Δ*, 80, 80, 54; *cnm67Δ*, 178, 178, 86; *cnm67Δ lte1Δ*, 150, 150, 119. Error bars represent SEP.

checkpoint mechanism is simply unable to provide the longer delays necessary to maintain coordination with mitotic exit. However, many cells with mispositioned spindles appeared to delay mitotic exit indefinitely, arguing that the checkpoint has the capacity to arrest mitotic exit as long as is necessary (Fig. 3 C). In addition, inappropriate mitotic exit was much more frequent in *ts-arp1 bim1Δ* cells than in *ts-arp1 stt4-7* cells, despite the similar severity of spindle mispositioning in these mutants (Fig. 3, A and B). Thus, the frequency of inappropriate mitotic exit is not simply correlated with the frequency or duration of delayed spindle movement to the neck.

Inappropriate Mitotic Exit in the Absence of SPB Contact with the Bud

The SPB model for the spindle position checkpoint predicts that the SPB must interact with the bud to cause mitotic exit (Bardin et al., 2000; Bloecher et al., 2000). We asked whether SPB interactions with the neck always occurred before inappropriate mitotic exit by examining microtubule and spindle behavior in the cells that underwent inappropriate exit from mitosis.

We found that the SPB did not always interact with the neck before inappropriate mitotic exit. In 15% (7/48) of the *ts-arp1 bim1Δ* cells that underwent inappropriate exit from mitosis, we did not observe any spindle pole contacts with the neck (Videos 7 and 8; Fig. 4 C). Therefore, mitotic exit does not require interaction of the SPB with the neck. Mitotic exit in these cells was associated with transient or prolonged loss of cytoplasmic microtubule-neck contacts: either cytoplasmic microtubules failed to grow into the bud at all, or they transiently depolymerized out of the neck (7/7 cells; Videos 7 and 8; Fig. 4 C). Inappropriate mitotic exit followed the loss of microtubules from the neck if mi-

crotubules were absent from the neck for >1 min, and the spindle failed to move into the neck within ~20 min of microtubule loss from the neck. Short-term loss of cytoplasmic microtubules from the bud is frequent in *bim1Δ* and *ts-arp1 bim1Δ* cells because of defects in microtubule dynamics and microtubule capture at the bud cortex (Adames and Cooper, 2000).

In the remaining 85% (41/48) of *ts-arp1 bim1Δ* cells that underwent inappropriate exit from mitosis, the spindle first made an abortive movement to the neck, placing one SPB close to or inside the neck. The spindle then moved back out into the mother before or during mitotic exit. Microtubules that extended into the bud were frequently sheared off, indicating that once mitotic exit starts it is irreversible (Videos 9–11; Fig. 4 D). Abortive spindle movement to the neck also preceded inappropriate exit from mitosis in 5/5 *bim1Δ* cells, 4/4 *ts-arp1* cells, 3/3 *stt4-7* cells, and 4/4 *ts-arp1 stt4-7* cells.

In contrast to the *ts-arp1 bim1Δ* and *ts-arp1 stt4-7* mutants, inappropriate mitotic exit in the *ts-arp1 bub2Δ* and *ts-arp1 bfa1Δ* mutants frequently occurred without abortive spindle movements to the neck (10/24 *ts-arp1 bub2Δ* cells and 9/23 *ts-arp1 bfa1Δ* cells in which inappropriate mitotic exit occurred) and without the loss of microtubules from the neck (23/24 *ts-arp1 bub2Δ* cells and 23/23 *ts-arp1 bfa1Δ* cells in which inappropriate mitotic exit occurred; Video 12; Fig. 4 E). These results further underscore the difference between the *bub2Δ* or *bfa1Δ* mutants and the *bim1Δ* or *stt4-7* mutants. Although the former are essential for the spindle position checkpoint to function, the infrequent instances of checkpoint failure in the latter are associated with abnormal microtubule and/or spindle behaviors.

Inappropriate Mitotic Exit in the Absence of *Lte1p*

The frequent occurrence of abortive spindle movements into the neck before inappropriate exit from mitosis in *ts-arp1 bim1Δ* and other mutants is consistent with the possibility that interaction of Tem1p on the spindle pole with *Lte1p* in the bud occurred when the SPB transiently penetrated the neck, and that this triggered mitotic exit. However, inappropriate exit from mitosis occurred at the same frequency in *ts-arp1 bim1Δ lte1Δ* cells as in the *ts-arp1 bim1Δ* mutant (Fig. 5), indicating that *Lte1p* is not required for inappropriate mitotic exit. As in the *ts-arp1 bim1Δ* mutants, inappropriate mitotic exit in *ts-arp1 bim1Δ lte1Δ* cells was preceded by abortive SPB-neck interactions (19/31 cells) or, in cells where the spindle did not move to the neck (12/31 cells), by the loss of cytoplasmic microtubules from the neck (10/12 cells). Conceivably, some other Tem1p activator may be located within the bud, and the abortive spindle movement to the neck might provide access of this hypothetical activator to the Tem1p on the spindle pole. However, this type of model cannot easily explain why inappropriate exit from mitosis occurs in some cells that never move a spindle pole to the neck.

Inappropriate Mitotic Exit in Mutants Defective for Cytoplasmic Microtubules

The spindle positioning defects associated with inappropriate mitotic exit in *ts-arp1 bim1Δ* cells is likely due to changes in cytoplasmic microtubule dynamics and defects in capture of cytoplasmic microtubules at the bud cortex (Tirnauer et al., 1999; Adames and Cooper, 2000; Korinek et al., 2000;

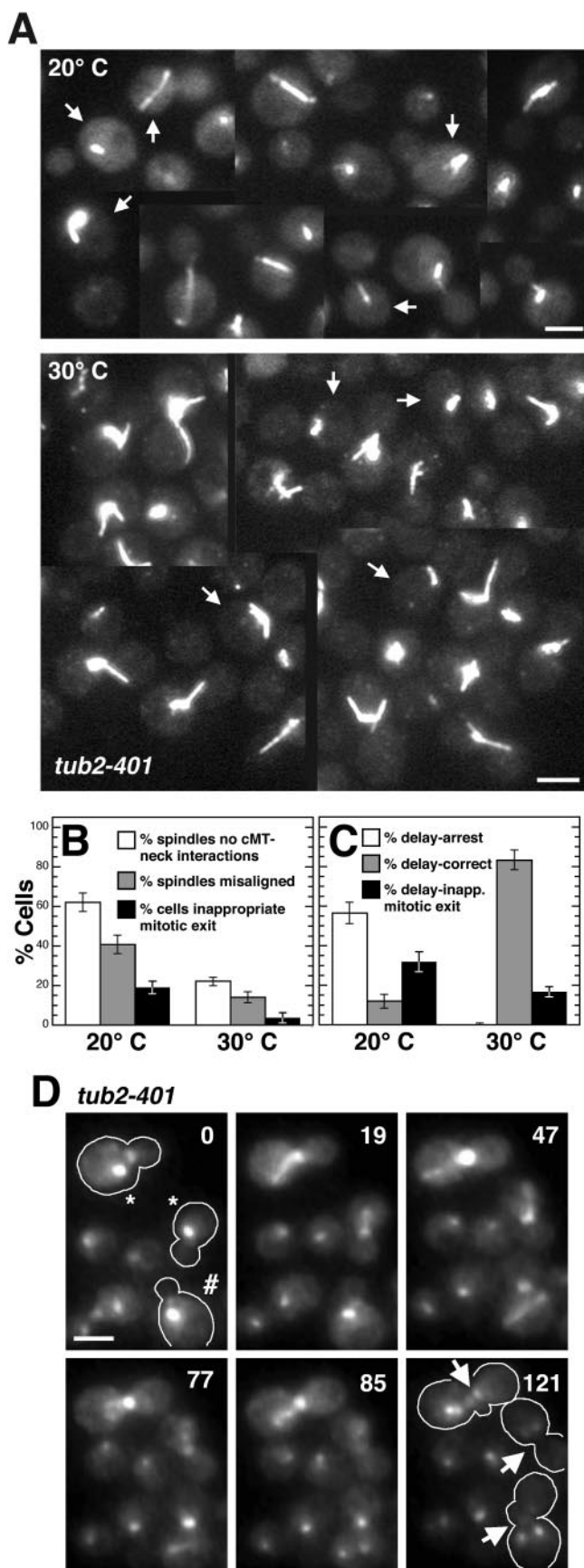


Figure 6. Loss of cytoplasmic microtubules and inappropriate mitotic exit in *tub2-401* cells. (A) At semipermissive temperatures, cytoplasmic microtubule staining is greatly reduced. At 20°C, many of the spindles are misaligned and lack cytoplasmic

microtubules (Lee et al., 2000). If so, other mutants affecting cytoplasmic microtubules should also show inappropriate mitotic exit while the spindle is in the mother. At least two mutants with cytoplasmic microtubule defects frequently give rise to binucleate and anucleate cells—*tub2-401* and *cnm67Δ* (Sullivan and Huffaker, 1992; Hoepfner et al., 2000). We tested whether these mutants underwent inappropriate mitotic exit with or without SPB contact with the neck.

We first examined the cold-sensitive *tub2-401* tubulin mutant, which undergoes a preferential loss of cytoplasmic microtubules at the semipermissive temperature of 18°C (Sullivan and Huffaker, 1992). We obtained the same results at 20°C, without adverse effects on the spindle (Fig. 6 A). In many of these cells, cytoplasmic microtubules did not appear to penetrate or touch the neck (Fig. 6, A and B). Cytoplasmic microtubules are responsible for alignment of the spindle along the mother–bud axis before spindle movement into the neck (Sullivan and Huffaker, 1992; Miller and Rose, 1998; Adames and Cooper, 2000). Accordingly, *tub2-401* cells grown at 20°C showed an increase in misaligned spindles compared with cells grown at 30°C (Fig. 6, A and B). As with *ts-arp1 bim1Δ* mutants, many *tub2-401* cells that delayed spindle movement into the neck also delayed exit from mitosis (Fig. 6 C).

Movie analysis of *tub2-401* cells revealed that inappropriate mitotic exit was frequent at 20°C but was rarely observed at 30°C (Fig. 6 B). When we examined the events leading up to mitotic exit at 20°C, we found that some cells (4/35 cells that exited mitosis inappropriately) exited mitosis without moving the spindle to the neck (Video 13; Fig. 6 D), whereas most (31/35 cells) exhibited abortive spindle movement into the neck before inappropriate mitotic exit (Video 13; Fig. 6 D). These events resemble those observed in the *ts-arp1 bim1Δ* strain.

We attempted to use GFP–tubulin to visualize cytoplasmic microtubules in the *tub2-401* mutant, but the fluores-

microtubules or show no interactions between short cytoplasmic microtubules and the neck (arrows). Misaligned spindles lacking cytoplasmic microtubule interactions with the neck are also present at 30°C but are much less frequent. Shown are collages of representative cells. (B) Frequency of the lack of cytoplasmic microtubule interactions with the neck (white columns), spindle misalignment (gray columns), and inappropriate mitotic exit (black columns) in *tub2-401* cells. Cell numbers for each column from left to right: 20°C, 108, 108, 142; 30°C, 150, 150, 54. (C) The spindle position checkpoint is intact in the *tub2-401* mutant. At 20°C, the majority of cells that delay spindle movement into the neck by >40 min arrest with an anaphase spindle at the neck on the mother side of the neck (white columns). At the lower temperature, few cells that delay spindle movement into the neck correct the defect and move the spindle into the neck (gray columns). Many of these cells undergo inappropriate mitotic exit (black columns). Cell numbers: 20°C, 85; 30°C, 12. (D) Still images from Video 13. Events preceding inappropriate mitotic exit in the *tub2-401* mutant. Cells of interest are outlined for clarity. In this series, two cells exit mitosis inappropriately after an SPB touches the neck (*). One cell exits mitosis without any SPB–neck interaction (#). Note the poor alignment of the spindle with respect to the mother–bud axis in this cell. After mitotic exit, Hof1–GFP disappears or immediately relocates to the necks of the new buds (arrows). Error bars represent SEP. Supplemental videos are available at <http://www.jcb.org/cgi/content/full/153/1/159/DC1>. Bars, 5 μm.

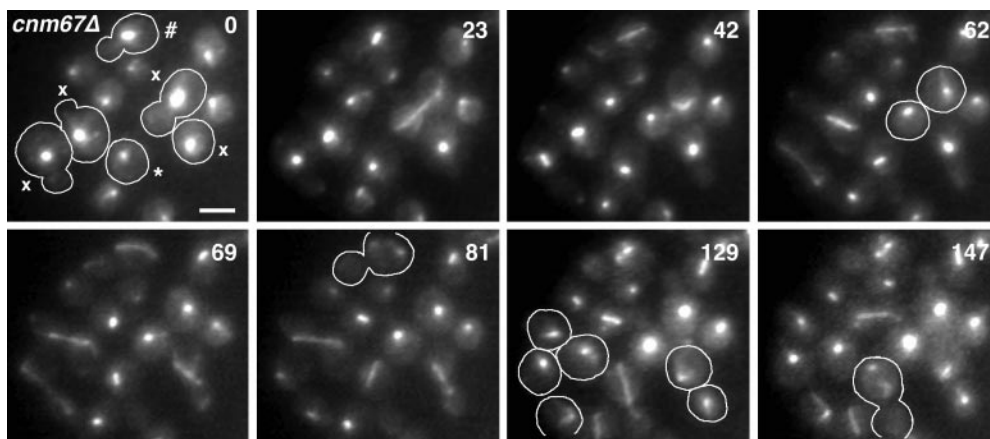


Figure 7. Inappropriate mitotic exit in *cnm67Δ* cells. Still images from Video 14. Representative cells showing two examples of inappropriate mitotic exit, one after the SPB touches the neck (*), and one without any SPB–neck interactions (#). Also shown are cells that move the spindle into the neck within the normal time for mitotic exit (x). Cells of interest are outlined. Supplemental videos are available at <http://www.jcb.org/cgi/content/full/153/1/159/DC1>. Bar, 5 μ m.

cence was weak, presumably due to inefficient incorporation of GFP–tubulin, and we could not detect cytoplasmic microtubules with certainty. However, we could see the spindle and noted that spindles were misaligned ($>45^\circ$ from the mother–bud axis) in 4/4 of the cells that underwent mitotic exit without spindle movement to the neck, suggesting that cytoplasmic microtubules had failed to align the spindle (presumably due to loss of contact with the bud) in these cells (Video 13; Fig. 6 D). In contrast, cells (68/164 anaphase cells) in which spindles were retained in the mother but properly aligned ($<45^\circ$) along the mother–bud axis never underwent inappropriate mitotic exit.

Another mutant that displays defects in cytoplasmic microtubules and produces binucleate and anucleate cells is *cnm67Δ* (Hoepfner et al., 2000). Cnm67p is an SPB outer plaque protein that is required for formation of the outer plaque to anchor cytoplasmic microtubules to the SPB. In *cnm67Δ* cells, cytoplasmic microtubules frequently detach from the SPB, becoming free in the cytoplasm (Hoepfner et al., 2000). Consequently, *cnm67Δ* mutants are unable to move the spindle into the neck efficiently (Hoepfner et al., 2000). We confirmed that some *cnm67Δ* cells underwent inappropriate mitotic exit, resulting in binucleate and anucleate cells (Video 14; Fig. 7). In 8/12 of these cells, the spindle never touched the neck. In *cnm67Δ* cells, the free cytoplasmic microtubules are stable and move along the cell cortex, sometimes moving through the neck. Thus, in some cases the number and movement of the free cytoplasmic microtubules made it difficult to determine if cytoplasmic microtubules were absent from the neck.

As shown above for *ts-arp1 bim1Δ* cells at 37°C, Lte1p was not required for inappropriate mitotic exit in *cnm67Δ* mutants at 37°C and *tub2-401* mutants at 20°C (Fig. 5). In 14/34 *cnm67Δ lte1Δ* cells and 2/20 *tub2-401 lte1Δ* cells that exited mitosis inappropriately, the spindle never directly interacted with the neck. Therefore, neither Lte1p nor spindle interaction with the neck are required for inappropriate mitotic exit in any of the mutants defective for cytoplasmic microtubule function.

Discussion

Recent work has suggested a model for how exit from mitosis is coordinated with partitioning of the nucleus between mother and bud. In the current model, Bub2p–Bfa1p is responsible for keeping Tem1p in an inactive

state on the SPB while the spindle is in the mother (Bardin et al., 2000; Bloecher et al., 2000; Pereira et al., 2000). Subsequent spindle movement into the neck promotes exit from mitosis by allowing Lte1p in the bud access to Tem1p at the SPB (Bardin et al., 2000; Hoyt, 2000; Pereira et al., 2000). This model predicts that Lte1p and spindle pole movement into the bud are essential for mitosis.

Our results here provide two conclusions that are not explained by this model. First, Lte1p is not necessary for mitotic exit (at least at high temperatures). Mitotic exit occurred at the normal time in all *lte1Δ* cells in which the spindle moved into the neck. Moreover, inappropriate mitotic exit in mutants lacking Lte1p occurred with at least the same frequency as in their *LTE1* counterparts, even at low temperatures. Also, in some mutants, mitotic exit occurred without spindle movement into the neck or interaction of an SPB with the neck. These cases occurred in three mutants—*bim1Δ*, *tub2-401*, and *cnm67Δ*—where cytoplasmic microtubules are defective. In these mutants, 47 of 196 instances of inappropriate mitotic exit occurred without any SPB–neck interactions.

The GTPase-activating protein activity of Bub2p–Bfa1p is thought to constitutively maintain Tem1p in the GDP-bound state in the current model (Bardin et al., 2000; Hoyt, 2000; Pereira et al., 2000). Lte1p activation of Tem1p in the bud then overrides inhibition by Bub2p–Bfa1p. However, the observation that loss of Bub2p–Bfa1p activity allows mitotic exit even when spindles are mispositioned in the mother supports the observation that Tem1p activation can occur without Lte1p, which resides in the bud. In fact, we found that in dynactin mutants lacking Bub2p or Bfa1p, the spindle poles often do not enter or even touch the neck, and yet the timing of mitotic exit was normal. Nevertheless, there was a remarkable constancy in the timing of mitotic exit relative to spindle movement into the neck in strains with functional Bub2p–Bfa1p. Together, these results suggest that Bub2p–Bfa1p is inactivated upon spindle movement into the neck, allowing the intrinsic activity of Tem1p to signal mitotic exit. In this model, the guanine–nucleotide exchange factor activity of Lte1p aids mitotic exit at low temperatures that possibly reduce the intrinsic rate of nucleotide exchange on Tem1p.

Whether Bub2p–Bfa1p inhibition of Tem1p is really regulated or not remains to be determined. However, we do have some clues to how such regulation could occur. The mutants we used to study inappropriate mitotic exit

all were defective in cytoplasmic microtubules. The spindle position checkpoint was not completely abrogated in these mutants, indicating that the lost proteins are not directly necessary for Bub2p–Bfa1p activity and checkpoint function. Our observations of the *ts-arp1 bim1Δ* and *ts-arp1 bim1Δ lte1Δ* mutants show that inappropriate mitotic exit was preceded by the loss of cytoplasmic microtubules from the neck in at least 17/19 cells that divided without any SPB–neck interactions. It therefore seems that certain aspects of cytoplasmic microtubule behavior in these mutants are responsible for inactivation of Bub2p–Bfa1p leading to inappropriate mitotic exit.

An attractive hypothesis that could potentially account for all of our observations is that interactions of cytoplasmic microtubules with the neck provide an inhibitory signal through Bub2p–Bfa1p that prevents exit from mitosis. In wild-type cells, penetration of the spindle through the neck leads to disappearance of cytoplasmic microtubule–neck interactions, allowing cells to trigger the mitotic exit pathway. However, if the spindle fails to enter the bud, cytoplasmic microtubules continue to traverse the neck and thereby inhibit exit from mitosis. In *bim1Δ* (and other) mutants, alterations in cytoplasmic microtubule behavior occasionally cause the spindle to fall back into the mother after penetrating the neck, or (less frequently) keep the spindle in the mother but fail to maintain cytoplasmic microtubule–neck interactions. The failure to maintain such contacts then triggers an inappropriate exit from mitosis.

If loss of microtubules from the neck allows inappropriate mitotic exit, and then the persistence of microtubules in the neck after spindle movement into the neck should prevent mitotic exit. In *kip3Δ* and *bud6Δ* mutants, cytoplasmic microtubules often protrude through the neck after the spindle has moved into the neck (Miller et al., 1998; Straight et al., 1998; Segal et al., 2000). Even after the spindle has moved into the neck, mitotic exit is delayed in these mutants (Miller et al., 1998; Straight et al., 1998; Segal et al., 2000). In the *bud6Δ* mutant, it is clear that the delay in mitotic exit occurs only in cells in which cytoplasmic microtubules remain in the neck after the spindle is properly positioned (Segal et al., 2000). Together, these observations suggest that the spindle position checkpoint might monitor the presence of cytoplasmic microtubules in the neck.

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