Current Biology, Vol. 13, 264–275, February 18, 2003, ©2003 Elsevier Science Ltd. All rights reserved. PII S0960-9822(03)00049-6

Conservation of Mechanisms Controlling Entry into Mitosis: Budding Yeast Wee1 Delays Entry into Mitosis and Is Required for Cell Size Control

Stacy L. Harvey and Douglas R. Kellogg*
Department of Molecular, Cellular,
and Developmental Biology
Sinsheimer Labs
University of California
Santa Cruz, California 95064

Summary

Background: In fission yeast, the Wee1 kinase delays entry into mitosis until a critical cell size has been reached; however, a similar role for Wee1-related kinases has not been reported in other organisms. *SWE1*, the budding yeast homolog of *wee1*, is thought to function in a morphogenesis checkpoint that delays entry into mitosis in response to defects in bud morphogenesis.
Results: In contrast to previous studies, we found that budding yeast *swe1*Δ cells undergo premature entry into mitosis, leading to birth of abnormally small cells. Additional experiments suggest that conditions that activate the morphogenesis checkpoint may actually be activating a G2/M cell size checkpoint. For example, actin depolymerization is thought to activate the morphogenesis checkpoint by inhibiting bud morphogenesis. However, actin depolymerization also inhibits bud growth, suggesting that it could activate a cell size checkpoint. Consistent with this possibility, we found that actin depolymerization fails to induce a G2/M delay once daughter buds pass a critical size. Other conditions that activate the morphogenesis checkpoint block bud formation, which could also activate a size checkpoint if cell size at G2/M is monitored in the daughter bud. Previous work reported that Swe1 is degraded during G2, which was proposed to account for failure of large-budded cells to arrest in response to actin depolymerization. However, we found that Swe1 is present throughout G2 and undergoes hyperphosphorylation as cells enter mitosis, as found in other organisms.
Conclusions: Our results suggest that the mechanisms known to coordinate entry into mitosis in other organisms have been conserved in budding yeast.

Introduction

Cells show extraordinary diversity in size. The molecular mechanisms underlying the control of cell size are likely to be complex and dynamic since single-celled organisms are able to maintain a fairly constant size over widely varying conditions. In addition, multicellular organisms are composed of cells of many different sizes and include cells that are able to grow without dividing (e.g., oocytes) and others that are able to divide without growing (e.g., fertilized embryos).

Maintenance of a specific cell size in dividing cells requires coordination of cell growth and cell division. In fission yeast, the Wee1 and Cdc25 proteins have been shown to play critical roles in coordinating cell growth and cell division at the G2/M transition [1, 2]. Wee1 is a protein kinase that phosphorylates and inhibits Cdc2 on a conserved tyrosine, thereby preventing entry into mitosis until cells reach a critical size [3, 4]. Cdc25 is a phosphatase that removes the inhibitory phosphate added by Wee1, thereby promoting entry into mitosis [5–8]. Loss of Wee1 function causes cells to undergo premature entry into mitosis before sufficient growth has occurred, leading to the formation of abnormally small cells [9]. Conversely, loss of Cdc25 function causes delayed entry into mitosis, leading to growth of abnormally large cells [5].

Although Wee1 has a well-defined role in the coordination of cell growth and cell division in fission yeast, no such role has been demonstrated in other organisms. Loss of function of Swe1, the budding yeast homolog of Wee1, has not been found to cause a small cell phenotype or premature entry into mitosis [10–12]. Instead, it has been proposed that Swe1 has evolved to function in a checkpoint that delays cell cycle progression in response to defects in morphogenesis [11, 13, 14]. Activation of the morphogenesis checkpoint occurs in response to depolymerization of the actin cytoskeleton or other conditions that inhibit the formation of a daughter cell, and it is thought that Swe1 specifically monitors the status of the actin cytoskeleton [11, 14, 15].

The finding that budding yeast Swe1 is not involved in coordination of cell growth and cell division is surprising since Wee1-related kinases have been highly conserved in evolution. In addition, Swe1 can fully complement a loss of Wee1 function in fission yeast, suggesting that the basic functions of the fission yeast Wee1 have been conserved in the budding yeast Swe1 [10]. We therefore carried out new experiments to determine whether Swe1 plays a role in coordinating cell growth and cell division in budding yeast.

Results

*swe1*Δ Cells Enter Mitosis Prematurely

Previous studies concluded that *swe1*Δ cells do not enter mitosis prematurely. One study utilized flow cytometry and analyzed the relative sizes of 1N and 2N DNA peaks in wild-type and *swe1*Δ cells [10]. However, this approach may lack the sensitivity to detect a shortening of G2, especially if there is a compensatory lengthening of G1, as observed in fission yeast *wee1*− mutants [9]. Other studies used DNA staining to assay nuclear division in wild-type and *swe1*Δ cells at 15-min intervals during the cell cycle after release from a G1 arrest [11, 12]. We analyzed the timing of entry into mitosis by determining the percentage of cells with short or long spindles at 10-min intervals as cells progressed through the cell cycle after release from a G1 arrest. Previous work has shown that formation of a short spindle is

*Correspondence: kellogg@darwin.ucsc.edu

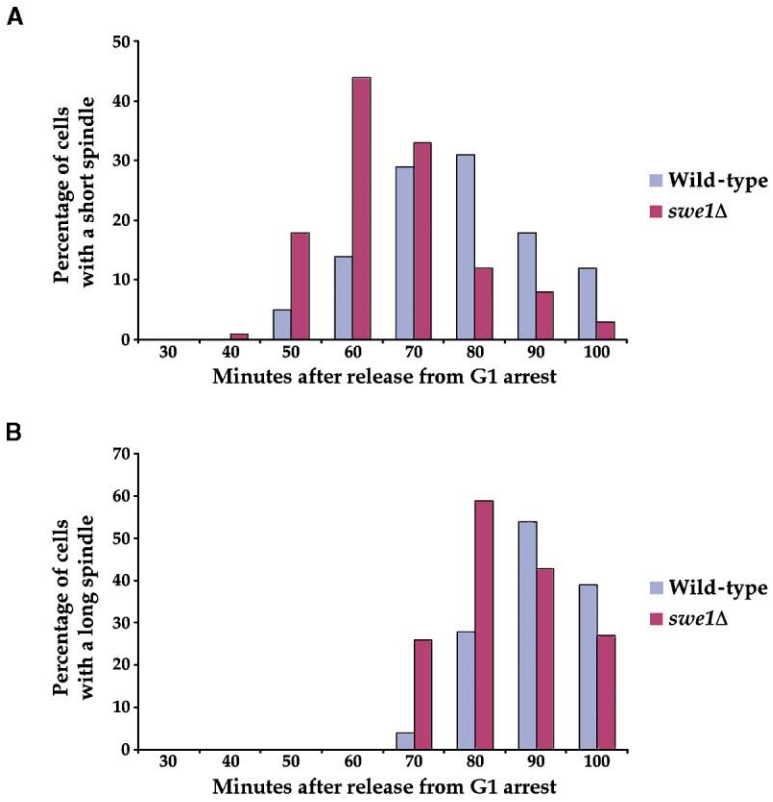

Figure 1. *swe1*Δ Cells Enter Mitosis Prematurely

(A and B) Wild-type and *swe1*Δ cells were arrested in G1 by the addition of α factor. After release from the arrest, samples were taken every 10 min, and cells were stained with an anti-tubulin antibody. The percentage of cells with (A) short spindles or (B) long spindles was determined and plotted as a function of time.

dependent upon synthesis of the mitotic cyclins, which indicates that spindle assembly is a good marker for entry into mitosis [16]. We found that *swe1*Δ cells enter mitosis prematurely, as expected if the functions of fission yeast Wee1 have been conserved in budding yeast (Figure 1A). Formation of long spindles, which serves as a marker for nuclear division, also occurs prematurely (Figure 1B). Repeated counts of the same samples revealed a maximum standard deviation of ±2%. We obtained the same results in six independent experiments.

While carrying out these experiments, we noticed that a fraction of *swe1*Δ cells have multiple nuclei and multiple microtubule-organizing centers. Examples of cells with three and eight nuclei are shown in Figure 2. Cells with multiple nuclei were always much larger than cells with one nucleus, which is consistent with the fact that cell size increases with ploidy. We determined that 7.5% ± 1% of *swe1*Δ cells grown at 30°C have multiple nuclei that can be detected by fluorescence staining; however, this is likely to be an underestimate since cells with only one additional nucleus may be difficult to identify. Fission yeast *wee1*− mutants also accumulate cells with a greater than 1N DNA content, indicating another similarity in the phenotypes caused by loss of function of *wee1* or *SWE1* [17].

Swe1 and Mih1 Are Required for Cell Size Control

Since *swe1*Δ cells enter mitosis prematurely, one would expect newly born daughter cells to be smaller than normal, as observed in fission yeast *wee1*− mutants. We therefore measured the volume of cells in early G1 in rapidly growing cultures. To identify this population of cells, we stained with a septin antibody. Formation of a polarized ring of septins is one of the first events of bud emergence in late G1, and unbudded cells that lack polarized septin staining are therefore in early G1 [18–20]. We measured the volumes of cells in early G1

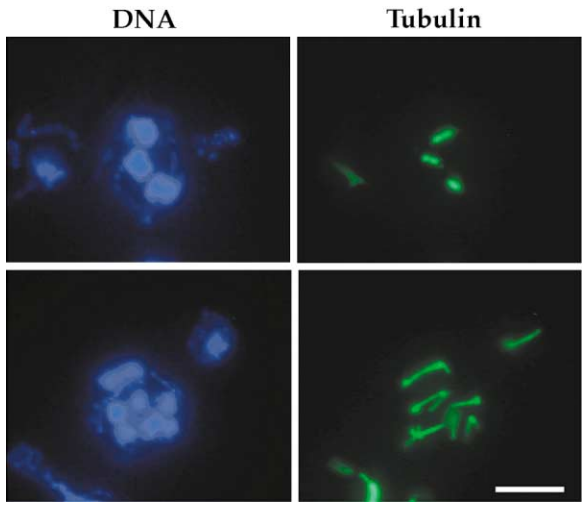

Figure 2. A Fraction of *swe1*Δ Cells Are Multinucleate

Examples of *swe1*Δ cells with three and eight nuclei. *swe1*Δ cells were grown to log phase at 30°C and were stained with an anti-tubulin antibody and with a DNA stain. The scale bar represents 5 μm.

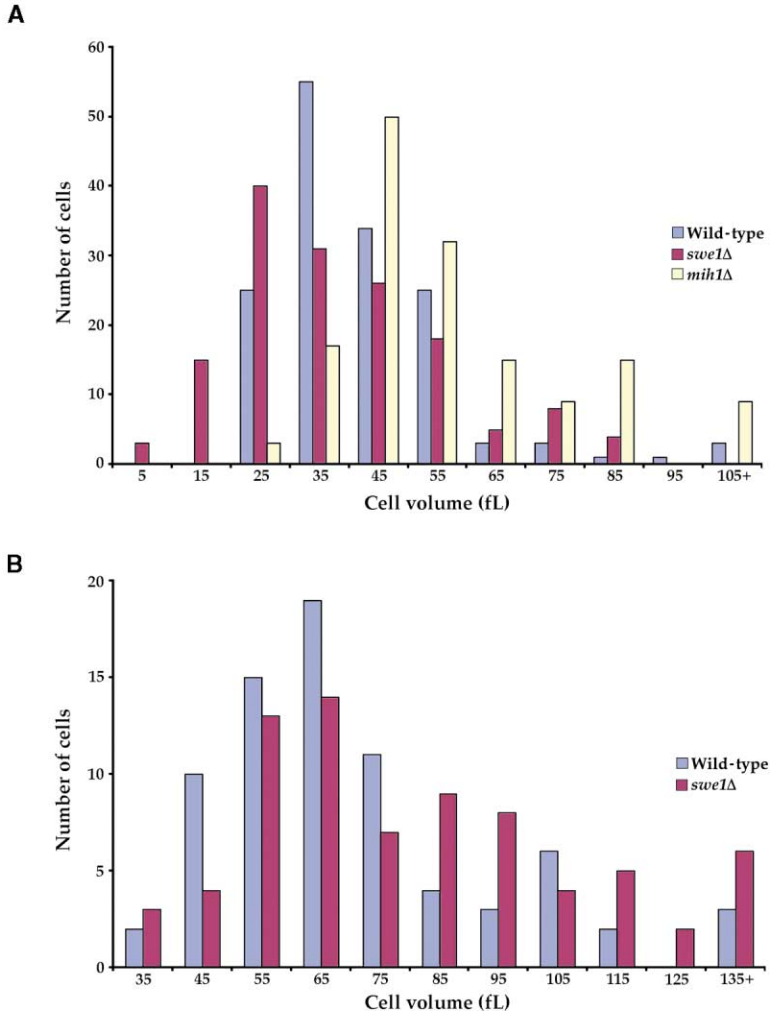

Figure 3. *swe1*Δ Cells Are Born at a Smaller Cell Volume and Undergo Compensatory Growth during G1

Cells of the indicated strains were grown in YPD at 30°C to 2 × 10^7 cells/ml and were fixed with formaldehyde. Cells were stained with an anti-Cdc11 antibody to allow identification of cells in early or late G1 and with a general cell stain to allow measurement of cell size. Unbudded cells that do not show polarized septin staining are in early G1, whereas unbudded cells with polarized septins are in late G1 (see text).

(A) The volumes of 150 cells in early G1 were measured as described in the Experimental Procedures, and cell volume was plotted as a function of the number of cells.

(B) The volumes of 75 cells in late G1 were measured, and cell volume was plotted as a function of the number of cells.

and then generated a histogram showing numbers of cells as a function of cell volume. For comparison, we also measured the volume of cells lacking *MIH1*, the budding yeast homolog of fission yeast *cdc25*. We found that *swe1*Δ cells show a smaller cell size distribution than wild-type cells, while *mih1*Δ cells are larger (Figure 3A). Importantly, in five independent experiments, we consistently observed a fraction of *swe1*Δ cells that were significantly smaller than the smallest wild-type cells. We did not include cells that had multiple nuclei in these measurements; however, it is likely that our size measurements included some *swe1*Δ cells with multiple nuclei that we could not discern by microscopy. Since these cells are larger than cells with a single nucleus, it is likely that the cell size measurements for *swe1*Δ cells are skewed toward a larger size than would be measured for a population of cells that was completely haploid. A recent study utilizing a Coulter Channelizer to systematically measure cell sizes in the yeast gene deletion collection also found that *swe1*Δ cells are smaller than wild-type cells [21]. Since these experiments were carried out in a different strain background than ours, it is unlikely that the cell size phenotype we have observed is dependent upon strain background.

*swe1*Δ Cells Undergo Compensatory Growth during G1

If *swe1*Δ cells are born at a smaller volume, one would expect them to spend more time in G1 undergoing compensatory growth [22]. Similarly, *mih1*Δ cells should spend less time in G1 since they are born at a larger size. To test whether this is true, we first determined the fraction of unbudded cells in rapidly growing populations of each strain. We found that wild-type cells were 32% ± 0.5% unbudded, *mih1*Δ cells were 27% ± 0.2% unbudded, and *swe1*Δ cells were 39% ± 0.4% unbudded, consistent with the idea that *swe1*Δ and *mih1*Δ cells are born at different sizes. A recent study independently reported that *swe1*Δ cells show a higher percentage of unbudded cells [21].

To further test whether *swe1*Δ cells undergo compensatory growth during G1, we measured the size of *swe1*Δ cells in late G1. To identify these cells, we stained with a septin antibody and looked for cells that had a polar-

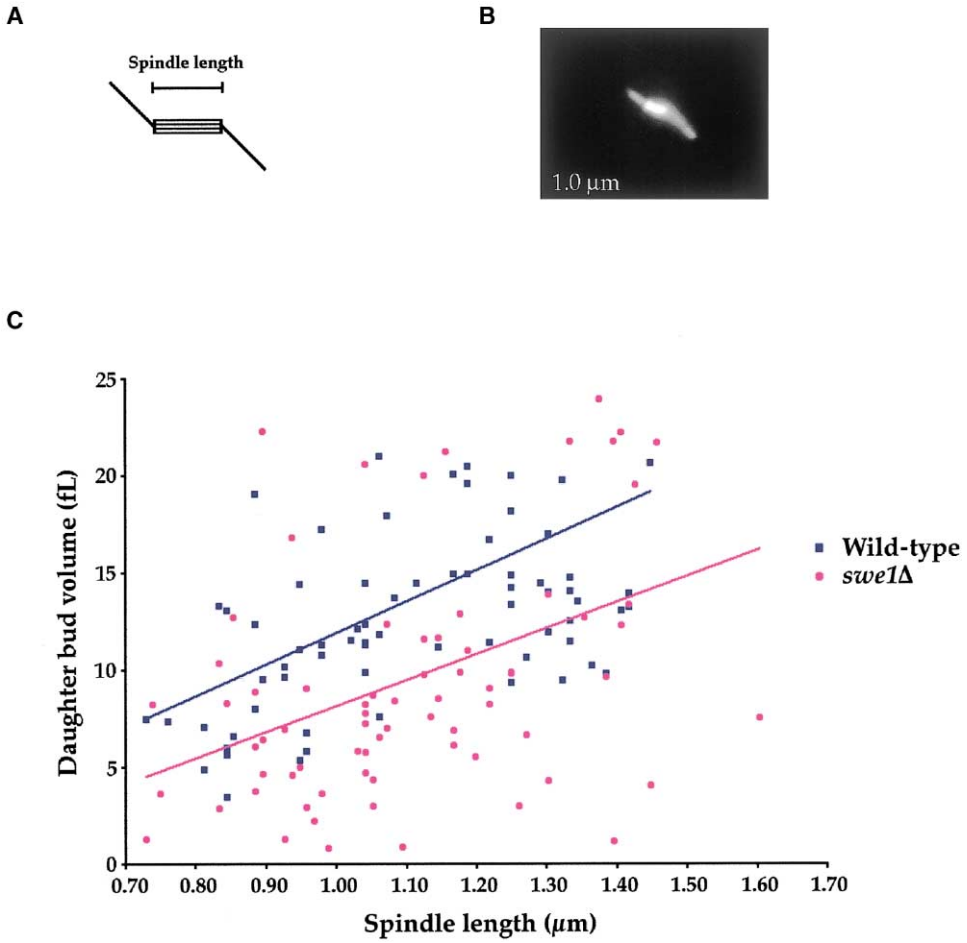

Figure 4. *swe1*Δ Cells Enter Mitosis at a Smaller Cell Size

Wild-type and *swe1*Δ cells were grown in YPD at 30°C to 1.2 × 10^7 cells/ml and were fixed with formaldehyde. Cells were stained with an anti-tubulin antibody to allow measurement of spindle length and with rhodamine phalloidin to allow measurement of bud size.

(A) A diagram showing how the length of short spindles was measured.

(B) An example of a short spindle and the measured spindle length.

(C) Bud volume and spindle length were measured for 75 cells and were plotted against each other. Linear trend lines were calculated by using the least squares fit for a line.

ized ring of septin staining, but no daughter bud, indicating that they were just initiating bud emergence in late G1 [18–20]. Although *swe1*Δ cells in early G1 were smaller than wild-type cells (Figure 3A), we found that *swe1*Δ cells in late G1 were slightly larger than wild-type cells (Figure 3B). Note that the size distributions of both wild-type and *swe1*Δ cells in late G1 are larger than in early G1 (compare Figures 3A and 3B), indicating that growth occurred during G1, as expected. In addition, *swe1*Δ cells in early G1 show a broader size distribution than wild-type cells (see Figure 3A). This finding is consistent with the idea that they are born at a smaller size and then grow to approximately the same size as wild-type cells. These results demonstrate that *swe1*Δ cells undergo compensatory growth during G1, which would explain why only a fraction of cells in early G1 are smaller than wild-type cells (Figure 3A). The fact that *swe1*Δ cells initiate bud emergence at a slightly larger size may be due to the presence of cells with multiple nuclei that skew the size distribution toward larger cells. Alternatively, Swe1 may participate in late G1 events required for timely bud emergence. In Figure 1, note that cells arrested in G1 with α factor have time to undergo growth, which explains why premature entry into mitosis in *swe1*Δ cells is not obscured by a prolonged G1 delay.

*swe1*Δ Cells Enter Mitosis at a Smaller Bud Size

We next determined whether *swe1*Δ cells enter mitosis at a smaller size, as observed for fission yeast *wee1*− mutants. After bud emergence, cell growth occurs almost entirely in the daughter bud [23]. We therefore measured the volume of daughter buds in cells entering mitosis. To do this, we used immunofluorescence to identify cells with short spindles, indicating that they were in early mitosis. In addition, we stained the actin cytoskeleton to reveal the outline of the daughter bud. We then measured spindle length and the volume of the daughter bud in the same cells. The dimension of the

short spindle that we measured is shown in Figure 4A, and an example of a short spindle is shown in Figure 4B. We plotted bud volume as a function of spindle length and found that *swe1*Δ cells enter mitosis at a smaller bud size, as would be expected if they enter mitosis prematurely (Figure 4C). As in Figures 3A and 3B, we did not include cells with multiple nuclei in these measurements. However, note that a fraction of *swe1*Δ cells (10/75) entered mitosis at a bud volume larger than most wild-type cells. These may represent cells with multiple nuclei that we were not able to exclude by fluorescence staining.

Depolymerization of the Actin Cytoskeleton Does Not Induce a Cell Cycle Delay Once Daughter Buds Have Reached a Critical Size

Work on the morphogenesis checkpoint has demonstrated that depolymerization of the actin cytoskeleton with latrunculin A induces a Swe1-dependent G2/M delay [15]. Since actin is required for bud emergence, these results have led to the conclusion that Swe1 monitors bud morphogenesis. However, actin is also required for growth of the daughter bud [23, 24]. In addition, recent work in fission yeast has shown that actin depolymerization causes a G2/M delay, but only in cells that have not reached a critical size [25]. It therefore seemed possible that depolymerization of the actin cytoskeleton in budding yeast may actually activate a cell size checkpoint by blocking growth and preventing cells from reaching a critical size. To examine this issue further, we determined when during the cell cycle actin depolymerization is capable of inducing a Swe1-dependent delay. We first released cells from a G1 arrest and added latrunculin A at 10-min intervals to depolymerize actin. All samples were incubated until 130 min after release from the arrest, when cells would normally have exited mitosis. We then assayed Cdc28 tyrosine phosphorylation and Swe1 protein levels (Figure 5A). We also determined the fraction of cells with buds and the average bud size at the time that latrunculin A was added (Figures 5B and 5C). We found that depolymerization of actin early in the cell cycle caused cells to arrest with high levels of Swe1 and tyrosine-phosphorylated Cdc28. However, when daughter buds reached an average size of 7 fL at 70 min, the majority of the cells no longer arrested. A small fraction of cells did arrest in response to actin depolymerization at 70 min, as revealed by the presence of small amounts of Swe1 and phosphorylated Cdc28 at this time point. This is consistent with the fact that the bud size distribution at 70 min overlaps somewhat with the size distribution at 60 min. The variability in bud size at each time point is due to imperfect synchrony.

We used a second approach to determine when actin depolymerization results in a cell cycle delay that did not rely on synchronization with α factor. For this approach, we treated log phase cells with latrunculin A for 1 hr and then fixed the cells. Since latrunculin A completely stops the growth of the daughter bud [23], the size of the bud in the fixed cells can be used to determine the size of the bud when latrunculin A was added. We therefore measured bud sizes and determined whether cells underwent nuclear division, which allowed us to correlate bud size at the time of latrunculin A addition with whether cells went into a checkpoint arrest. To view the data, we plotted whether cells had undergone nuclear division as a function of bud size at the time of latrunculin A addition (Figure 6A). We found that cells with small buds always arrested before nuclear division, while cells with large buds always failed to arrest. To estimate the bud size at which cells were no longer sensitive to actin depolymerization, we determined the average bud size defined by the upper size that arrested in response to actin depolymerization and the lower size that failed to arrest (dashed lines in Figure 6A). The calculated size of 9 fL (solid line in Figure 6A) agrees well with the size of 7 fL determined in Figure 5. Examples of bud sizes are shown in Figure 6B. In addition to the data shown in Figure 6A, we determined whether nuclear division occurred in an additional 100 cells with small buds (i.e., smaller than 5 fL) and found that nuclear division was arrested in 98% of the cells. These results suggest that actin depolymerization causes a Swe1-dependent delay when buds have already formed and are increasing in size. In addition, these results demonstrate that cells with buds that have grown beyond a certain size no longer undergo a G2/M delay in response to actin depolymerization.

We also carried out this experiment in *swe1*Δ and *mih1*Δ cells. Almost all of the *swe1*Δ cells failed to arrest at G2/M, which is consistent with previous work showing that the G2/M delay induced by actin depolymerization is *SWE1* dependent (Figure 6C) [15]. In *mih1*Δ cells, the average volume at which cells became insensitive to actin depolymerization was larger than that in wild-type cells (13 fL) (Figure 6D). We found that mother cell size had no influence on whether cells arrested in response to actin depolymerization (Figure 6E).

The Swe1 Protein Appears before Bud Emergence and Is Present throughout G2 and Mitosis

Previous work concluded that the Swe1 protein is degraded during G2, accounting for failure of cells with large buds to arrest in response to actin depolymerization [12, 15, 26]. These experiments were carried out by using Swe1 tagged with 12 copies of the myc epitope and did not utilize molecular markers of cell cycle progression. Other experiments studying the behavior of the endogenous Swe1 protein found that it is present throughout G2 and does not disappear until Clb2 levels begin to decline as cells exit mitosis [27, 28]. To more carefully determine when the Swe1 protein is made relative to the events of bud formation and mitosis, we released cells from a G1 arrest and monitored bud emergence, spindle assembly, and levels of the Swe1 and Clb2 proteins by using affinity-purified polyclonal antibodies that recognize the endogenous proteins (Figure 7). We found that Swe1 first appears during late G1, just before bud emergence. Swe1 is initially in a hypophosphorylated form, but it undergoes dramatic hyperphosphorylation as Clb2 appears and cells enter mitosis. Swe1 remains present until Clb2 levels begin to decline as cells form long spindles and exit mitosis. Thus, Swe1 is present throughout G2, and degradation of Swe1 can-

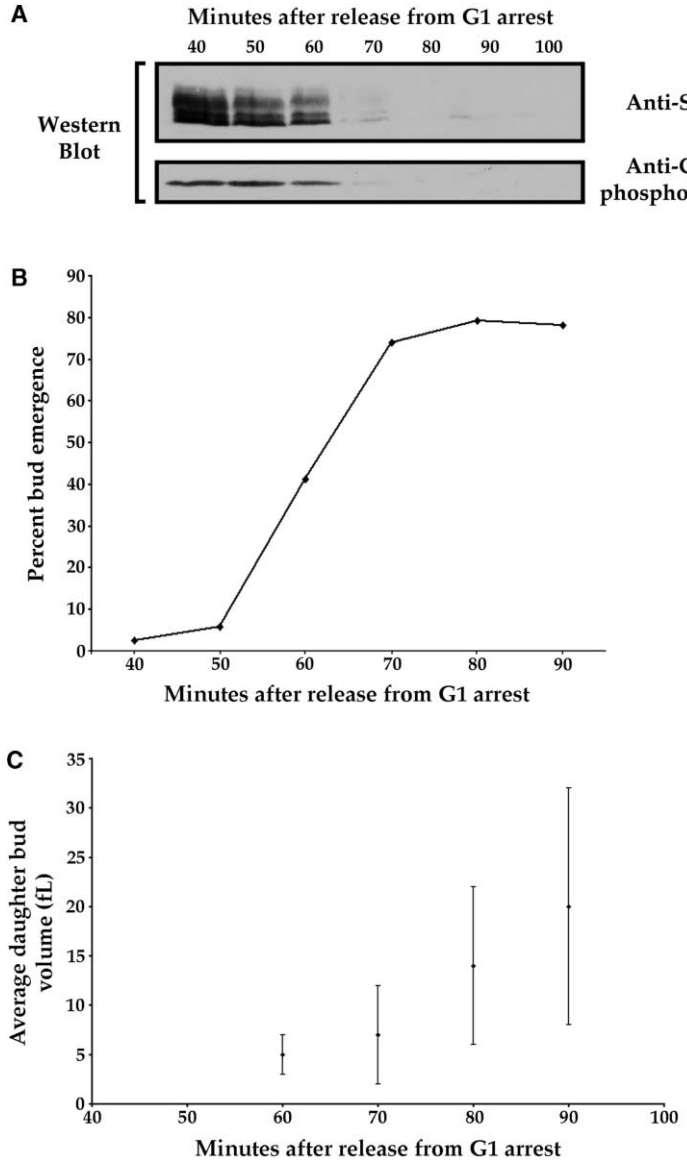

Figure 5. Depolymerization of the Actin Cytoskeleton Does Not Induce a Cell Cycle Delay Once Daughter Buds Have Reached a Critical Size

(A) Wild-type cells were arrested in G1 by the addition of α factor. After release from the arrest, samples were taken at 10-min intervals starting at 40 min, and latrunculin A was added to each sample to depolymerize actin. At 130 min after release from G1 arrest, all samples were harvested and used for Western blotting to assay Swe1 protein levels and Cdc28 tyrosine phosphorylation.

(B) Budding index at the time of latrunculin A addition.

(C) Mean bud size and standard deviation at the time of latrunculin A addition.

not account for the failure of cells with medium-sized buds to arrest in response to actin depolymerization. Note that assembly of a short mitotic spindle is strongly correlated with Clb2 protein levels, which is consistent with the idea that spindle assembly is a mitotic event.

Discussion

Conservation of Mechanisms Controlling Entry into Mitosis

A number of experiments have suggested that the mechanisms known to control entry into mitosis in other organisms have not been conserved in budding yeast. For example, although loss of Wee1 function causes premature entry into mitosis in fission yeast and *Xenopus* [9, 29], budding yeast *swe1*Δ cells have not been found to enter mitosis prematurely [10–12]. It has also been reported that the Swe1 protein is degraded during G2 [12, 15, 26], whereas in *Xenopus*, humans, and fission yeast, Wee1 is present throughout G2 and mitosis [30–32]. A final peculiarity of mitosis in budding yeast is that it has been reported that spindle assembly is initiated during S phase, rather than in G2/M [33].

In contrast to previous studies, we have found that *swe1*Δ cells do enter mitosis prematurely and show a small cell phenotype, while *mih1*Δ cells show a large cell phenotype, suggesting that the well-characterized functions of fission yeast Wee1 and Cdc25 have been conserved in budding yeast. The fact that Swe1 can functionally replace fission yeast Wee1 further supports the idea that the functions of Wee1 have been conserved in budding yeast [10]. We also found that Swe1 is not degraded during G2. Rather, Swe1 is present throughout G2, undergoes hyperphosphorylation as cells enter mitosis, and remains present until cyclin levels begin to decline as cells exit mitosis. Finally, assembly of the mitotic spindle requires mitotic cyclin synthesis [16] and is strongly correlated with mitotic cyclin levels (Figure

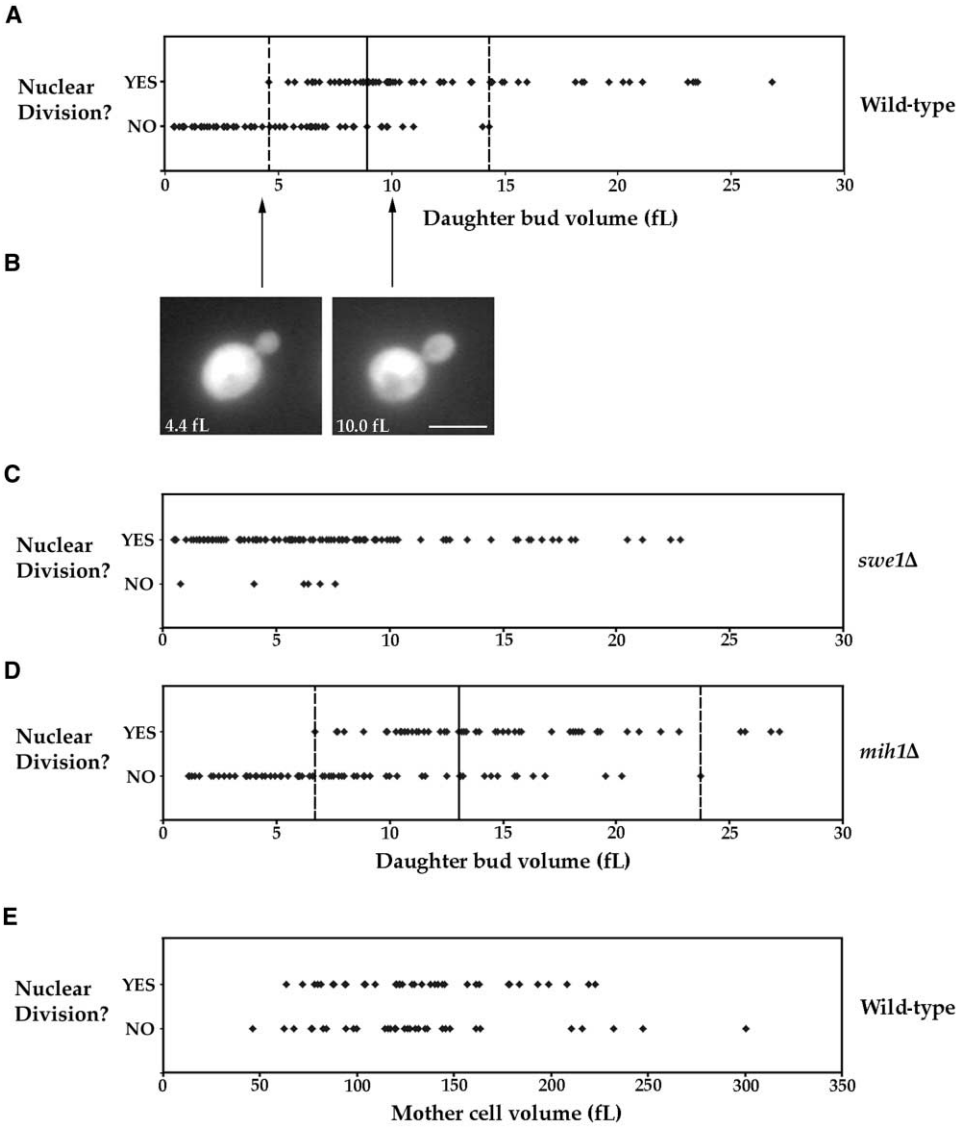

Figure 6. Actin Depolymerization Induces a G2/M Arrest Only in Small-Budded Cells

Cells of the indicated strains were grown to log phase in YPD at 30°C, treated with latrunculin A for 1 hr, and fixed with formaldehyde. The cells were stained with a general cell stain to allow measurement of bud volume and with an anti-tubulin antibody and a DNA stain to determine whether nuclear division occurred.

(A) A plot showing whether wild-type cells underwent nuclear division as a function of bud volume. The dashed lines define the upper bud size that arrested in response to actin depolymerization and the lower size that failed to arrest. The solid line is the average bud size between these two limits.

(B) Examples of bud sizes. The scale bar represents 5 μm.

(C) A plot showing whether *swe1*Δ cells underwent nuclear division as a function of bud volume.

(D) A plot showing whether *mih1*Δ cells underwent nuclear division as a function of bud volume.

(E) A plot showing whether wild-type cells underwent nuclear division as a function of mother cell volume.

For [A], [C], and [D], a total of 135 cells were counted. For [E], a total of 75 cells were counted.

7), suggesting that spindle assembly is a mitotic event. Taken together, these observations argue that the basic mechanisms that coordinate entry into mitosis in other organisms have been conserved in budding yeast, as one might expect for so fundamental a process.

Reevaluation of the Role of Swe1 in Budding Yeast

Previous studies reached the conclusion that Swe1 has evolved to mediate a morphogenesis checkpoint in budding yeast [11, 13–15]. In these experiments, it was found that actin depolymerization inhibits bud morphogenesis and causes a Swe1-dependent G2 delay. It is important to note, however, that actin depolymerization also completely blocks daughter bud growth and could therefore be activating a size checkpoint [23]. The morphogenesis checkpoint is also thought to be activated by loss of function of *CDC24* or *CDC42* [11, 14, 15]. In these cases, mutant cells fail to form a daughter bud

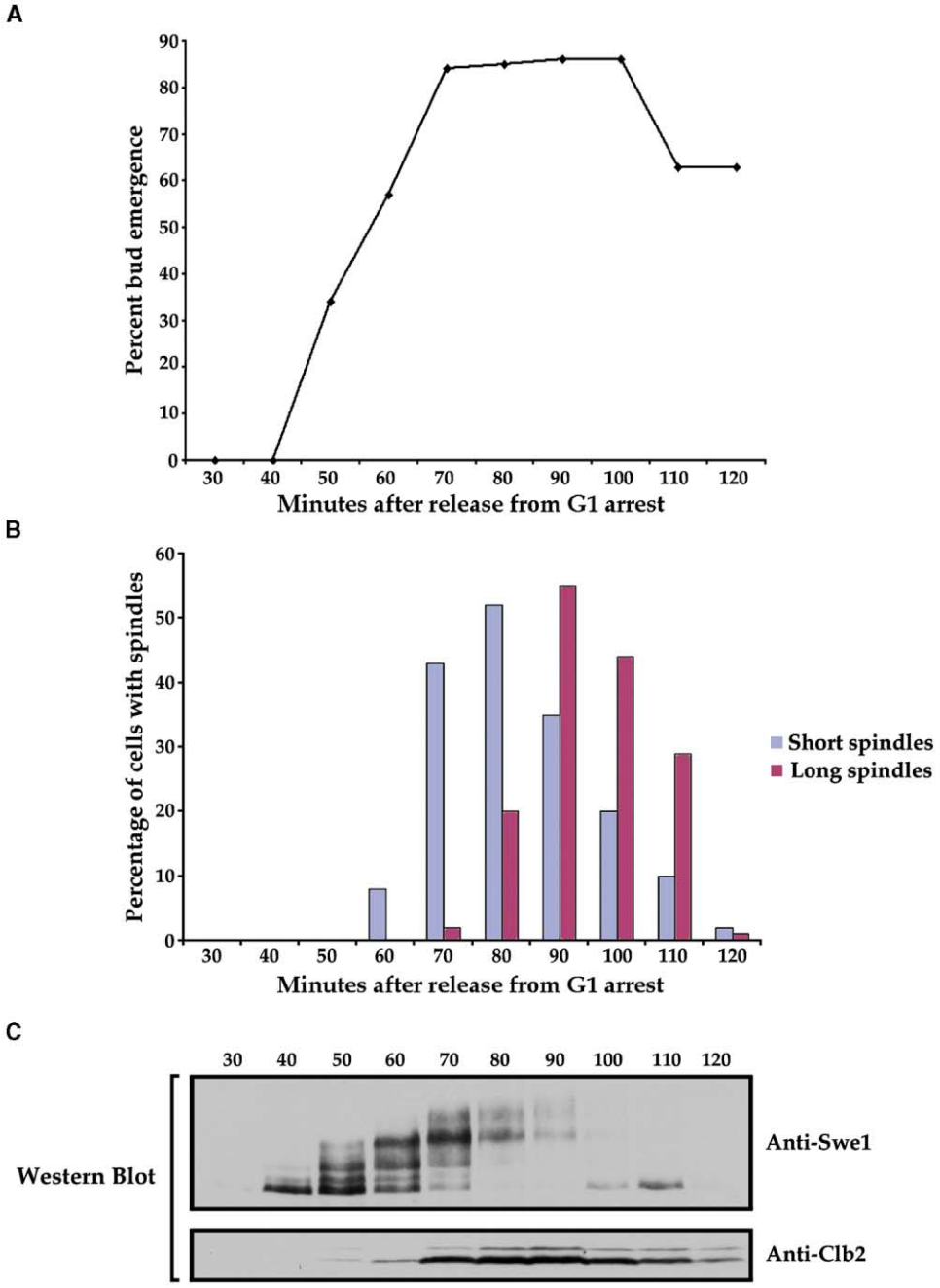

Figure 7. The Swe1 Protein Is Present throughout G2 and Mitosis

Wild-type cells were arrested in G1 by the addition of α factor. After release from the arrest, samples were taken every 10 min at the indicated times.

(A) Budding index as a function of time during the cell cycle.

(B) Percentage of cells with short or long spindles as a function of time.

(C) Western blot analysis of Swe1 and Clb2 protein levels as a function of time.

and undergo a Swe1-dependent G2 arrest as large unbudded cells. However, if cell size at G2/M is monitored in the daughter cell, the signal indicating that a critical size has been reached would never be sent in mutants that fail to form a daughter cell, and *CDC24* and *CDC42* mutants could therefore be activating a cell size checkpoint. Since cell growth during the budded phase of the cell cycle occurs almost entirely in the daughter cell [22, 23], one might expect that cell size at G2/M is monitored in the daughter cell. Our finding that mother cell size has no influence on whether cells arrest in response to actin depolymerization is consistent with the possibility that size at G2/M is monitored in the daughter cell.

Experiments reported here are consistent with the

possibility that actin depolymerization activates a size checkpoint rather than a morphogenesis checkpoint. First, we found that actin depolymerization causes a Swe1-dependent G2/M arrest in cells with small- and medium-sized buds. Importantly, this result demonstrates that actin depolymerization causes an arrest when bud morphogenesis is largely complete and the bud is primarily undergoing growth. We also found that cells with buds that have grown beyond a certain size no longer arrest in response to actin depolymerization, which is consistent with the existence of a checkpoint that monitors bud size. Finally, we found that *mih1*Δ cells become insensitive to actin depolymerization at a larger bud size, which would not be predicted by a checkpoint that simply monitors bud morphogenesis.

Experiments in fission yeast also argue that actin depolymerization activates a size checkpoint rather than a morphogenesis checkpoint [25]. In these experiments, it was found that actin depolymerization blocks cell growth and causes a G2 delay that is dependent upon tyrosine phosphorylation of Cdc2, as observed in budding yeast. However, by manipulating cell size, it was possible to clearly demonstrate that actin depolymerization has no effect upon entry into mitosis once a critical cell size has been reached.

Taken together, experiments in both fission yeast and budding yeast argue that Wee1-related kinases function in a conserved checkpoint that coordinates cell size and cell division at G2/M. An alternative view is that Wee1-related kinases simply control the timing of entry into mitosis, rather than mediating a cell size checkpoint. According to this view, the cell size defects observed in *wee1*− or *swe1* mutants are due simply to premature entry into mitosis, rather than to a failure in a cell size checkpoint. However, the effects of actin depolymerization on G2/M progression indicate that Wee1-related kinases link cell cycle progression to the successful completion of an event that is dependent upon actin filaments. Since cell growth is dependent upon actin filaments, this result is consistent with the possibility that Wee1-related kinases monitor cell size.

It should be noted that our experiments do not rule out the possibility that Swe1 functions both in a cell size checkpoint and in a checkpoint that functions earlier to monitor bud emergence. In addition, it is possible that Swe1 monitors the total amount of growth that occurs, rather than absolute cell size.

Control of Swe1 Protein Levels

Previous work led to the conclusion that Swe1 is degraded during G2 in an SCF complex-dependent manner, and that the morphogenesis checkpoint induces a G2 arrest at least in part by stabilizing the Swe1 protein via a pathway that works through Hsl7 [12, 15, 26]. In contrast, we found that the Swe1 protein is present throughout G2 and undergoes dramatic hyperphosphorylation as cells enter mitosis (Figure 7 and [27, 28]). Furthermore, a recent study failed to find any evidence that Swe1 is degraded in an SCF complex-dependent manner [34]. We did find that treatment of small budded cells with latrunculin A causes an arrest with high Swe1 levels and tyrosine-phosphorylated Cdc28 (Figure 5). However, the cells are arrested in G2/M, when the Swe1 protein is normally present, and it is therefore possible that the high Swe1 protein levels are a consequence of the arrest, rather than the cause. The proposed role of Hsl7 in Swe1 stabilization might also be explained by the fact that *hsl7* mutants delay in G2 when Swe1 is stable, rather than by a direct role for Hsl7 in the regulation of Swe1 stability [35].

Work in *Xenopus* has suggested that Wee1 is inactivated by hyperphosphorylation as cells enter mitosis, rather than by degradation [31]. It is therefore interesting to note that the Swe1 protein is in the hypophosphorylated form during the latrunculin A-induced arrest (compare Figures 5A and 7C) and that Swe1 undergoes hyperphosphorylation as cells enter mitosis. We favor the idea that Swe1 is regulated by hyperphosphorylation at G2/M, as in *Xenopus*.

Coordination of Cell Growth and Cell Division at G2/M in Budding Yeast

The finding that Swe1 delays entry into mitosis and is required for cell size control has important implications for the mechanisms that coordinate cell growth and cell division in budding yeast. Previous work has shown that an intricate signaling network is required for proper coordination of cell growth and cell division at G2/M [27, 28, 35–42]. This signaling network includes the kinases Gin4, Cla4, Elm1, and Hsl1, as well as a number of proteins that are required for regulation of these kinases, including Nap1, Cdc42, Hsl7, and the septins. Loss of function of any of these proteins can cause cell growth to continue during a prolonged G2 delay, leading to the formation of highly elongated cells that are significantly larger than wild-type cells. Deletion of the *SWE1* gene largely reverses this phenotype [27, 35, 40, 41]. In addition, the signaling network is required for full hyperphosphorylation of Swe1 in vivo [27, 28]. Taken together, these observations argue that the signaling network is required for normal regulation of Swe1 activity.

It has been argued that inactivation of this signaling network causes a G2 delay and excessive cell growth by activating the morphogenesis checkpoint, leading to activation of Swe1 and inhibition of Cdc28 activity [40, 41]. However, the finding that Swe1 regulates entry into mitosis and is required for cell size control suggests that the signaling network may regulate Swe1 to coordinate cell growth and cell division. Many of the proteins that function in the signaling network have been highly conserved, suggesting that similar networks function in all eukaryotic cells. Gin4, for example, is the budding yeast homolog of fission yeast Cdr2, which was identified in a screen for mutants unable to alter G2/M size control in response to nitrogen limitation [43]. A number of observations suggest that the budding yeast signaling network may also play a role in responding to nutrient limitation, since mutations in proteins that regulate Swe1 lead to increased filamentous growth on low-nitrogen media, while loss of Swe1 leads to a slight decrease in filamentous growth characteristics [42, 44, 45]. The existence of intricate signaling networks that coordinate cell growth and cell division at G2/M seems likely since cells must regulate their growth and size over widely varying conditions.

A Bud Size Checkpoint?

Our results show that G2/M progression becomes insensitive to actin depolymerization once daughter buds pass a critical size. In addition, mother cell size has no influence on when cells commit to enter mitosis. These results suggest that there may be a size checkpoint that operates specifically in the daughter bud. The existence of such a checkpoint is perhaps expected since cells must ensure that nuclear division does not occur before the bud is large enough to accommodate the nucleus.

How might cells monitor the size of the daughter bud? A commonly invoked model for measuring cell size postulates the existence of a diffusable cytoplasmic molecule that is gradually diluted as the cell grows larger. This kind of model, however, could not specifically monitor bud size without a mechanism to prevent diffusion into the mother cell. A more attractive model is that cells monitor the concentration of a membrane-associated molecule. This model is particularly appealing because recent work has shown that the septins are required for maintenance of a specialized membrane domain in the daughter cell [46, 47]. In addition, loss of septin function causes cells to arrest at G2/M while growth continues, resulting in the formation of highly elongated daughter buds. Thus, loss of septin function might cause mislocalization of molecules that are normally restricted to the daughter bud. This, in turn, could result in a failure to sense that a critical size has been reached and in continued growth of the bud. This model could also explain the phenotypes caused by loss of function of proteins like Elm1, Gin4, and Nap1, since these proteins are required for proper septin localization. Other models are possible, however, and it is clear that additional work needs to be done to better understand how cell growth and cell division are coordinated at G2/M.

Experimental Procedures

Yeast Strains and Culture Conditions

Except where noted, all yeast strains were grown in yeast extract-peptone-dextrose (YPD) media. All yeast strains are in the W303 strain background (*leu2-3,112 ura3-52 can1-100 ade2-1 his3-11 trp1-1*). The following yeast strains were used: DK186: *MATa, bar1*Δ; HT179: *MATa, bar1*Δ*, mih1*Δ*::URA3*; and SH24: *MATa, bar1*Δ*, swe1*Δ*::URA3*.

Cell Cycle Arrests, Immunofluorescence, and Western Blotting

Arrest with α factor was carried out for 3–3.5 hr at room temperature at a final concentration of 0.5 μg/ml. Fixation and staining of mitotic spindles and DNA were carried out as previously described [48]. For Cdc28 phosphotyrosine detection, Western blotting was carried out as previously described [49], except that the blot was blocked in P-Tyr buffer containing 5% BSA and was incubated overnight at 4°C in blocking buffer containing anti-phospho-Cdc2 (Tyr15) antibody (New England Biolabs) diluted 1:500. After washing in TBST (10 mM Tris-Cl [pH 7.5], 100 mM NaCl, 0.1% Tween 20), the blot was incubated in blocking buffer containing HRP-conjugated anti-rabbit antibody at a 1:5000 dilution for 45 min at 25°C. For Swe1 and Clb2 detection, Western blotting was carried out as previously described [50, 51].

Spindle Assembly Assays, Cell Volume Measurements, and Determination of Budding Index

The percentage of cells with short or long spindles was determined by scoring over 200 cells for each time point. Cell volume was measured essentially as previously described [52]. To measure the size of cells in early G1 (Figure 3A), cells were grown overnight in YPD media at 30°C to a cell density of 2×10^7 cells/ml and were then fixed for 1.5 hr in 3.7% formaldehyde. We stained the cells with an antibody against the Cdc11 septin to identify cells that had not yet polarized their septins and were therefore in early G1. To reveal the outline of the cell, we also stained the cells with tetramethylrhodamine succinimidyl ester (Molecular Probes). Briefly, after removing the cell wall with zymolyase, cells from 0.5 ml of culture were resuspended in phosphate-buffered saline (PBS) containing 0.05% Tween 20, and tetramethylrhodamine succinimidyl ester was added to 0.25 μM from a 25 μM stock made in methyl sulfoxide and stored at −80°C. After 10 min at room temperature, cells were washed twice with PBS containing 0.05% Tween 20 and were then stained for septins with an anti-Cdc11 polyclonal antibody and a fluorescein-conjugated secondary antibody. Cells were also stained with DAPI to visualize nuclei, and cells with multiple nuclei were not measured. The length and width of cells without polarized septins were measured in digital images, and cell volume was determined by using the formula for a prolate spheroid ($[\pi/6]lw^2$) [52]. To determine the size of cells in late G1 (Figure 3B), we measured the volumes of cells that had polarized septins but no detectable bud.

The percentage of unbudded cells was determined by staining cells with an anti-Cdc11 antibody and counting the number of cells without polarized septins. The reported numbers represent an average of 3 independent counts of over 300 cells. Cells in each strain were grown to the same density (2×10^7 cells/ml).

To measure the size of buds in cells entering mitosis, cells were grown overnight at 30°C to a density of 1.2×10^7 cells/ml. Cells were fixed and stained with an anti-tubulin antibody and a fluorescein-labeled secondary antibody. The cells were also stained with rhodamine phalloidin to stain the actin and reveal the outlines of the daughter bud. Cells with short spindles were identified, and spindle length and bud volume were determined for 75 cells. We only measured cells that were lying on their side, such that the outlines of the bud were clear. Bud volume was plotted as a function of spindle length, and trend lines were calculated by using the least squares fit for a line.

Latrunculin A Treatment

For Figure 5, cells were grown overnight at room temperature to 1.2×10^7 cells/ml, arrested with α factor, and then released into fresh YPD media at 30°C. After 60 min, α factor was added back to the culture to prevent a second round of cell division. Starting at 40 min after release from α factor arrest, 1.6 ml samples were taken every 10 min, and latrunculin A was added to each sample to a final concentration of 100 μM. All samples were incubated at 30°C with gentle mixing until 130 min after release from α factor, and the samples were then used for Western blotting. A second sample was taken at each time point when latrunculin A was added, and cells were fixed and stained with tetramethylrhodamine succinimidyl ester to allow measurement of bud size and budding index.

For Figure 6, cells were grown overnight at 30°C to an optical density of 1.0×10^7 cells/ml. Latrunculin A was added to 350 μl of each culture to a final concentration of 150 μM, and the cultures were mixed at 30°C for 1 hr. The samples were then fixed with formaldehyde and were stained with tetramethylrhodamine succinimidyl ester, anti-tubulin antibody, and DAPI.

Acknowledgments

We thank Paul Young, Ivan Rupes, David Morgan, Jeff Ubersax, Topher Carroll, Tin Tin Su, Derek McCusker, Eric Mortensen, and Yolanda Sanchez for critical reading of the manuscript or for helpful discussions. We also thank Phil Crews for providing latrunculin A. This work was supported by grants from the National Institutes of Health and the Pew Charitable Trusts Biomedical Scholars program.

Received: May 29, 2002
Revised: December 4, 2002
Accepted: December 10, 2002
Published: February 18, 2003

References

1. Nurse, P. (1990). Universal control mechanism regulating cell cycle timing of M-phase. Nature *344*, 503–508.
2. Norbury, C., and Nurse, P. (1992). Animal cell cycles and their control. Annu. Rev. Biochem. *61*, 441–470.
3. Gould, K.L., and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast cdc2^{+} protein kinase regulates entry into mitosis. Nature *342*, 39–45.
4. Russell, P., and Nurse, P. (1987). Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. Cell *49*, 559–567.
5. Russell, P., and Nurse, P. (1986). cdc25+ functions as an inducer in the mitotic control of fission yeast. Cell *45*, 145–153.
6. Strausfeld, U., Labbé, J.C., Fesquet, D., Cavadore, J.C., Picard, A., Sadhu, K., Russell, P., and Dorée, M. (1991). Dephosphorylation and activation of a p34^{cdc2}/cyclin B complex *in vitro* by human CDC25 protein. Nature *351*, 242–245.
7. Gautier, J., Solomon, M.J., Booher, R.N., Bazan, J.F., and Kirschner, M.W. (1991). cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. Cell *67*, 197–211.
8. Dunphy, W.G., and Kumagai, A. (1991). The cdc25 protein contains an intrinsic phosphatase activity. Cell *67*, 189–196.
9. Nurse, P. (1975). Genetic control of cell size at cell division in yeast. Nature *256*, 547–551.
10. Booher, R.N., Deshaies, R.J., and Kirschner, M.W. (1993). Properties of *Saccharomyces cerevisiae* wee1 and its differential regulation of p34^{CDC28} in response to G1 and G2 cyclins. EMBO J. *12*, 3417–3426.
11. Sia, R.A., Herald, H.A., and Lew, D.J. (1996). Cdc28 tyrosine phosphorylation and the morphogenesis checkpoint in budding yeast. Mol. Biol. Cell *11*, 1657–1666.
12. Sia, R.A.L., Bardes, E.S.G., and Lew, D.J. (1998). Control of Swe1p degradation by the morphogenesis checkpoint. EMBO J. *17*, 6678–6688.
13. Lew, D.J. (2000). Cell cycle checkpoints that ensure coordination between nuclear and cytoplasmic events in *Saccharomyces cerevisiae*. Curr. Opin. Genet. Dev. *10*, 47–53.
14. Lew, D., and Reed, S.I. (1995). A cell cycle checkpoint monitors cell morphogenesis in budding yeast. J. Cell Biol. *129*, 739–749.
15. McMillan, J.N., Sia, R.A.L., and Lew, D.J. (1998). A morphogenesis checkpoint monitors the actin cytoskeleton in yeast. J. Cell Biol. *142*, 1487–1499.
16. Fitch, I., Dahmann, C., Surana, U., Amon, A., Nasmyth, K., Goetsch, L., Byers, B., and Futcher, B. (1992). Characterization of four B-type cyclin genes of the budding yeast *Saccharomyces cerevisiae*. Mol. Biol. Cell *3*, 805–818.
17. Thuriax, P., Nurse, P., and Carter, B. (1978). Mutants altered in the control co-ordinating cell division with cell growth in the fission yeast *Schizosaccharomyces pombe*. Mol. Gen. Genet. *161*, 215–220.
18. Ford, S.K., and Pringle, J. (1991). Cellular morphogenesis in the *S. cerevisiae* cell cycle: localization of the CDC11 gene product and the timing of events at the budding site. Dev. Genet. *12*, 281–292.
19. Haarer, B.K., and Pringle, J.R. (1987). Immunofluorescence localization of the *Saccharomyces cerevisiae* CDC12 gene product to the vicinity of the 10-nm filaments in the mother-bud neck. Mol. Cell. Biol. *7*, 3678–3687.
20. Kim, H.B., Haarer, B.K., and Pringle, J. (1991). Cellular morphogenesis in the *S. cerevisiae* cell cycle: localization of the Cdc3 gene product and the timing of events at the budding site. J. Cell Biol. *112*, 535–544.
21. Jorgensen, P., Nishikawa, J.L., Breitkreutz, B.J., and Tyers, M. (2002). Systematic identification of pathways that couple cell growth and cell division in yeast. Science *297*, 395–400.
22. Hartwell, L.H., and Unger, M.W. (1977). Unequal division in *Saccharomyces cerevisiae* and its implications for the control of cell division. J. Cell Biol. *75*, 422–435.
23. Karpova, T.S., Reck-Peterson, S.L., Elkind, N.B., Mooseker, M.S., Novick, P.J., and Cooper, J.A. (2000). Role of actin and Myo2p in polarized secretion and growth of *Saccharomyces cerevisiae*. Mol. Biol. Cell *11*, 1727–1737.
24. Ayscough, K.R., Stryker, J., Pokala, N., Sanders, M., Crews, P., and Drubin, D.G. (1997). High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A. J. Cell Biol. *137*, 399–416.
25. Rupes, I., Webb, B.A., Mak, A., and Young, P.G. (2001). G2/M arrest caused by actin disruption is a manifestation of the cell size checkpoint in fission yeast. Mol. Biol. Cell *12*, 3892–3903.
26. McMillan, J.N., Longtine, M.S., Sia, R.A.L., Theesfeld, C.L., Bardes, E.S.G., Pringle, J.R., and Lew, D.J. (1999). The morphogenesis checkpoint in *Saccharomyces cerevisiae*: cell cycle control of Swe1p degradation by Hsl1p and Hsl7p. Mol. Cell. Biol. *19*, 6929–6939.
27. Sreenivasan, A., and Kellogg, D. (1999). The Elm1 kinase functions in a mitotic signaling network in budding yeast. Mol. Cell. Biol. *19*, 7983–7994.
28. Shulewitz, M.J., Inouye, C.J., and Thorner, J. (1999). Hsl7 localizes to a septin ring and serves as an adapter in regulatory pathway that relieves tyrosine phosphorylation of Cdc28 protein kinase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *19*, 7123–7137.
29. Walter, S.A., Guadagno, S.N., and Ferrell, J.E.J. (2000). Activation of Wee1 by p42 MAPK in vitro and in cycling *Xenopus* egg extracts. Mol. Biol. Cell *11*, 887–896.
30. McGowan, C., and Russell, P. (1995). Cell cycle regulation of human Wee1. EMBO J. *14*, 2166–2175.
31. Mueller, P.R., Coleman, T.R., and Dunphy, W.G. (1995). Cell cycle regulation of a *Xenopus* wee1-like kinase. Mol. Biol. Cell *6*, 119–134.
32. Aligue, R., Wu, L., and Russell, P. (1997). Regulation of *Schizosaccharomyces pombe* Wee1 tyrosine kinase. J. Biol. Chem. *272*, 13320–13325.
33. Byers, B., and Goetsch, L. (1975). Behavior of spindle and spindle plaques in the cell cycle and conjugation of *Saccharomyces cerevisiae*. J. Bacteriol. *124*, 511–523.
34. McMillan, J.N., Theesfeld, C.L., Harrison, J.C., Bardes, E.S., and Lew, D.J. (2002). Determinants of Swe1p degradation in *Saccharomyces cerevisiae*. Mol. Biol. Cell *13*, 3560–3575.
35. Ma, J.X., Lu, Q., and Grunstein, M. (1996). A search for proteins that interact genetically with histone H3 and H4 amino termini uncovers novel regulators of the Swe1 kinase in *Saccharomyces cerevisiae*. Genes Dev. *10*, 1327–1340.
36. Kellogg, D.R., and Murray, A.W. (1995). NAP1 acts with Clb2 to perform mitotic functions and suppress polar bud growth in budding yeast. J. Cell Biol. *130*, 675–685.
37. Altman, R., and Kellogg, D.R. (1997). Control of mitotic events by Nap1 and the Gin4 kinase. J. Cell Biol. *138*, 119–130.
38. Carroll, C., Altman, R., Schieltz, D., Yates, J., and Kellogg, D.R. (1998). The septins are required for the mitosis-specific activation of the Gin4 kinase. J. Cell Biol. *143*, 709–717.
39. Tjandra, H., Compton, J., and Kellogg, D.R. (1998). Control of mitotic events by the Cdc42 GTPase, the Clb2 cyclin and a member of the PAK kinase family. Curr. Biol. *8*, 991–1000.
40. Barral, Y., Parra, M., Bidlingmaier, S., and Snyder, M. (1999). Nim1-related kinases coordinate cell cycle progression with the organization of the peripheral cytoskeleton in yeast. Genes Dev. *13*, 176–187.
41. Longtine, M.S., Theesfeld, C.L., McMillan, J.N., Weaver, E., Pringle, J.R., and Lew, D.J. (2000). Septin-dependent assembly of a cell cycle-regulatory module in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *20*, 4049–4061.
42. Edgington, N.P., Blacketer, M.J., Bierwagen, T.A., and Myers, A.M. (1999). Control of *Saccharomyces cerevisiae* filamentous growth by cyclin-dependent kinase Cdc28. Mol. Cell. Biol. *19*, 1369–1380.
43. Young, P.G., and Fantes, P.A. (1987). *Schizosaccharomyces pombe* mutants affected in their division response to starvation. J. Cell Sci. *88*, 295–304.
44. La Valle, R., and Wittenberg, C. (2001). A role for the Swe1 checkpoint kinase during filamentous growth of *Saccharomyces cerevisiae*. Genetics *158*, 549–562.
45. Ahn, S.H., Acurio, A., and Kron, S.J. (1999). Regulation of G2/M progression by the STE mitogen-activated protein kinase pathway in budding yeast filamentous growth. Mol. Biol. Cell *10*, 3301–3316.

46. Barral, Y., Mermall, V., Mooseker, M.S., and Snyder, M. (2000). Compartmentalization of the cell cortex by septins is required for maintenance of cell polarity in yeast. Mol. Cell *5*, 841–851.
47. Takizawa, P.A., DeRisi, J.L., Wilhelm, J.E., and Vale, R.D. (2000). Plasma membrane compartmentalization in yeast by messenger RNA transport and a septin diffusion barrier. Science *290*, 341–344.
48. Pringle, J.R., Adams, A.E.M., Drubin, D.G., and Haarer, B.K. (1991). Immunofluorescence methods for yeast. Methods Enzymol. *194*, 565–602.
49. Rudner, A.D., and Murray, A.W. (2000). Phosphorylation by Cdc28 activates the Cdc20-dependent activity of the anaphase-promoting complex. J. Cell Biol. *149*, 1377–1390.
50. Anderson, C.W., Baum, P.R., and Gesteland, R.F. (1973). Processing of adenovirus 2-induced proteins. J. Virol. *12*, 241–252.
51. Harlow, E., and Lane, D. (1988). Antibodies. A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).
52. Johnston, G.C., Pringle, J.R., and Hartwell, L.H. (1977). Coordination of growth with cell division in the yeast *Saccharomyces cervisiae*. Exp. Cell. Res. *105*, 79–98.