出芽酵母の細胞壁チェックポイントにおける Bzz1p の働きに関する研究

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Possible involvement of Bzz1p in the cell wall integrity checkpoint

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Table of contents

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List of Figures

Figure 10. *FKS1 Δbzz1* cells do not achieve bud-maturation in the presence of EchB.

Figure 11. Deletion of *BZZ1* in *FKS1* cells overrides the cell wall integrity checkpoint induced by EchB treatment.

Figure 12. Cells with a small bud are accumulated in *fks1-1154 Δvrp1*.

Figure 13. *fks1-1154 Δvrp* cells override the cell wall integrity checkpoint.

List of tables

Abbreviations

EchB	: Echinocandin B
DAPI	: 4',6-Diamidino-2-phenylindole
DMSO	: Dimethyl Sulfoxide
FITC	: Fluorescein isothiocyanate
PCR	: Polymerase chain reaction
ts	: temperature-sensitive

Summary

Cell cycle checkpoints ensure that events during the cell cycle are completed with fidelity in the correct order. In budding yeast, several checkpoint pathways have been identified. Previously, we have identified a novel checkpoint system that inhibits entry of mitosis in response to cell wall synthesis perturbation and components of the dynactin complex (Arp1p, Jnm1p and Nip1001p) are required to achieve the G2 arrest. However, some components of the cell wall integrity checkpoint pathway have been revealed, the pathway remains obscure. Searching for new components is required for elucidating the signaling pathway. To search for new components, I focused on proteins which interact with Arp1p, Nip100p or Jnm1p and localized in or near plasma membrane. Among of them, I revealed Bzz1p localized in actin cortical patches has a role in the cell wall integrity checkpoint pathway. Moreover, I found Vrp1p which is also localized in actin cortical patches is involved in the checkpoint. Bzz1p and Vrp1p are members of Las17p complex, it is suggested that Las17p complex plays role in the checkpoint. These new components of the checkpoint provide important insights into the regulation of the cell wall integrity checkpoint.

Introduction

Cell cycle is a fundamental process to various cells in all-living organisms. The cell cycle includes the set of processes, duplication of all cell constituents, segregation each other into different part of the cell and division into two daughter cells. To ensure that each division produces two viable progeny with the right genetic information, cells must coordinate all events of cell cycle with fidelity in correct order at the appropriate time. Control mechanism of the cell cycle events is called "checkpoint". It restrains cell cycle progression until key events have been completed (Hartwell and Weinert, 1989). Several checkpoint pathways have been identified (Fig. 1). For instance, the DNA replication and DNA damage checkpoint monitor DNA fidelity, the spindle assembly and positioning checkpoint monitor microtubule attachment or tension of the kinetochore and the morphogenesis checkpoint monitors the actin cytoskeleton and septin assembly (Murray, 1992; Lew and Reed, 1995; Elledge, 1996; Muhua *et al.*, 1998).

The cell wall of *Saccharomyces cerevisiae* is a rigid structure, which provides osmotic and physical protection and determines the shapes of the cell. The cell wall is synthesized in the appropriate place depending on the phase of the cell cycle and surroundings (Fig. 2; Klis *et al.*, 2002). The yeast cells do not begin nuclear division until the remodeling of the cell wall components has been completed. This led us to the question how a checkpoint system controls to couple synthesis of cell wall components with cell cycle progression. To investigate the mechanism, we analyzed a *fks1*^{ts} strain,

which is a temperature sensitive mutant defective in 1,3-β-glucan synthase activity. 1,3-β-glucan is a main component of the cell wall. As a result, we identified the cell wall integrity checkpoint. It stops the cell cycle progression in response to the perturbation of the cell wall synthesis and maintains viability. Moreover, we isolated the *wac1* (*wa*ll *c*heckpoint deficient) mutant by screening of mutants which are sensitive to *FKS1* shut-off. Cloning and genetic linkage analyses demonstrated that *wac1* resides in the *ARP1* gene (Suzuki *et al.*, 2004). Arp1p is a core subunit of a dynactin complex which is required for the nuclear migration process after anaphase. The dynactin complex in *Saccharomyces cerevisiae* is proposed to associate with the cytoplasmic dynein motor, resulting in the pulling force to promote nuclear migration (Hidebrandt and Hoyt, 2000). In *Saccharomyces cerevisiae*, it is known that a dynactin complex contains, at least, three proteins Arp1p, Nip100p and Jnm1p (McMillan and Tatchell, 1994; Geiser *et al.*, 1997; Kahana *et al.*, 1998). We identified that Nip100p and Jnm1p are also needed to achieve the G2 arrest, and M-phase cyclin Clb2p and transcription factor Fkh2p are regulated by the cell wall integrity checkpoint (Fig. 3; Suzuki *et al.*, 2004).

Although several components of the cell wall integrity checkpoint pathway have been revealed, the pathway remains obscure. To describe the signaling pathway in more detail, searching for new components is required. Then, we focused on the proteins interacting dynactin complex. Protein-protein interactions play crucial roles in various biological functions. With the completion of the genome sequence of the budding yeast *Saccharomyces cerevisiae* in 1996, screens had been performed to

identify protein-protein interactions among the predicted ORFs. To date, it has been reported that dynactin complex (Arp1p, Nip100p, Jnm1p) physically interacts with 25 proteins (Uetz *et al.*, 2000; Ito *et al.*, 2001; Hazbun *et al.*, 2003; Helen *et al.*, unpublished). Among of them, it has been reported that Srp1p is involved in the cell wall integrity checkpoint (Igarashi Doctor Thesis 2006).

In this study, I focused on proteins physically interacting dynactin complex and localized at cell surface. It was supposed the checkpoint signaling caused by perturbation of cell wall synthesis transmits from cell surface to nuclear. Therefore, these proteins are most likely to involve in the checkpoint. Surprisingly, I found that Bzz1p is involved in the cell wall integrity checkpoint. Bzz1p is a member of the PCH (pombe Cdc15 homology) family proteins, which have two Src homology 3 (SH3) domains. Bzz1p is localized in actin patches and isolated as a protein which physically interacts with Las17p (Soulard, *et al.*, 2002). Las17p, associates Vrp1p, Myo3p and Myo5p to form Las17p complex and regulates actin polymerization at the cell cortex (Lechler *et al.* 2000). From the information, it was suggested Las17p complex is involved in the cell wall integrity checkpoint. To clarify whether Bzz1p plays a role in the checkpoint through Las17p complex, I examined involvement of Vrp1p in the checkpoint, since Vrp1p is a major component in the Las17p complex. As a result, it was demonstrated that Vrp1p is required for the checkpoint. It suggests Bzz1p plays a role in the checkpoint in Las17p complex-dependent-manner. This study provides important insights into the regulation of the cell wall integrity checkpoint.

Results

Strategy for isolation of new components of the cell wall integrity checkpoint

Although several components has been revealed in the cell wall integrity checkpoint pathway, this pathway remains obscure (Suzuki *et al.*, 2004). To elucidate the signaling pathway in more detail, it is necessary to search for new components in the checkpoint. Since protein-protein interactions play crucial role in various biological functions, I focused on proteins interacting with dynactin complex. To date, it has been reported 25 proteins that have physical interaction with Arp1p, Nip100p or Jnm1p (Uetz *et al.*, 2000, Ito *et al.*, 2001, Hazbun *et al.*, 2003, Helen *et al.*, unpublished). Among of them I focused on 6 proteins, Bzz1p, Sro77p, Rho2p, End3p, Smi1p and Gon7p, since localization of these proteins are predicted in or near plasma membrane. These proteins are likely to involve in the checkpoint which monitors cell wall defect.

Since the cell wall integrity checkpoint couples cell wall remodeling with mitosis, bipolar spindles formation during inhibition of glucan synthesis is a landmark of the checkpoint defect (Suzuki *et al.*, 2004). To clarify whether Bzz1p, Sro77p, Rho2p or End3p is involved in the cell wall integrity checkpoint, I deleted these genes in *fks1-1154* strain, which is a temperature sensitive mutant defective in 1,3-β-glucan synthase activity and shows G2 arrest under the restrictive temperature due to the checkpoint activation. *wac1* (wall checkpoint defect) mutant was used as a checkpoint defective control. Although cells of *fks1-1154 wac1* can not form a mature bud, they form bipolar

spindles at the restrictive temperature and show decrease of viability (Suzuki *et al.*, 2004). If Bzz1p, Sro77p, Rho2p or End3p is involved in the checkpoint, deletion of these genes induces bipolar spindle formation under perturbation of cell wall synthesis. I tested this idea in *fks1-1154 Δsro77*, *fks1-1154 Δend3*, *fks1-1154 Δbzz1* and *fks1-1154 Δrho2*.

Cells of *fks1-1154 Δbzz1* are defective in the cell wall integrity checkpoint.

To clarify whether Bzz1p, Sro77p, Rho2p or End3p is involved in the cell wall integrity checkpoint, I observed bipolar spindles formation in *fks1-1154 Δsro77*, *fks1-1154 Δend3*, *fks1-1154 Δbzz1* and *fks1-1154 Δrho2* at the restrictive temperature. Then, Small G1 cells of *FKS1*, *fks1-1154*, *fks1-1154 wac1*, *fks1-1154 Δsro77*, *fks1-1154 Δend3*, *fks1-1154 Δbzz1* and *fks1-1154 Δrho2* were isolated by centrifugal elutriation, and incubated at 37°C. Cells of *FKS1* formed a mature bud at 240 min (Fig. 4). Whereas cells with a small bud are accumulated in *fks1-1154*, *fks1-1154 wac1*, *fks1-1154 Δsro77*, *fks1-1154 Δend3*, *fks1-1154 Δbzz1* and *fks1-1154 Δrho2* cells due to inhibition of 1,3-β-glucan synthase activity (Fig. 4b). Since cells with a small bud were not saturated at 240 min in *fks1-1154 Δsro77*, *fks1-1154 Δend3*, *fks1-1154 Δbzz1* and *fks1-1154 Δrho2*, I observed cell cycle progression until 320 minutes in these mutants (Fig. 4a). I fixed cells at each time point and stained with anti-tubulin and DAPI for visualization of spindles and DNA, then determined the percentage of cells with bipolar spindles (Fig. 5). At the restrictive temperature, most of *fks1-1154* cells were arrested cell cycle in G2 with a

monopolar spindle due to the checkpoint activation, whereas cells of *FKS1* formed bipolar spindles. However, most of *fks1-1154 wac1* cells overrode the G2 arrest and formed bipolar spindles under perturbation of glucan synthesis. This phenotype indicats *fks1-1154 wac1* cells are defective in the cell wall integrity checkpoint and is identical previous report (Suzuki *et al.*, 2004). Most of *fks1-1154 Δbzz1* cells could not arrest cell cycle in G2 phase, and assembled bipolar spindles. And *fks1-1154 Δrho2* cells did not form bipolar spindles. In *fks1-1154 Δend3* and *fks1-1154 Δsro77* cells, about 30% of them formed bipolar spindles. It is suggested Bzz1p is involved in the cell wall integrity checkpoint, and Rho2p is not. And *fks1-1154 Δend3* and *fks1-1154 Δsro77* cells are slightly defective in the checkpoint.

SMI1 or _GON7_ is not involved in the cell wall integrity checkpoint.

Smi1p and Gon7p are proteins which physically interact with Jnm1p and Nip100p, respectively. Then, I examined whether Smi1p and Gon7p are involved in the checkpoint. I tried to delete their genes in *fks1-1154* strain, but I could not. Therefore, I used Echinocandin B (EchB), a glucan synthase inhibitor, to activate the checkpoint. It has been reported that 4 μg/ml of EchB causes the G2 arrest to WT cells (Suzuki *et al.* 2004)

To confirm the condition, I observed bud-growth of *FKS1* cells with 4 μg/ml EchB. Small G1 cells of *FKS1* were isolated by centrifugal elutriation, and incubated in YPD medium with or without EchB. However, 4 μg/ml EchB did not inhibit

accumulation of cells with a medium to large bud sufficiently (Fig. 6). According to our unpublished data, treatment of 8 μg/ml EchB greatly decreased accumulation of cells with a medium to large bud. Then, I used this condition. As a result, *FKS1* cells with a medium to large bud were largely decreased (Fig. 6). At indicated times, cells were fixed and stained with anti-tubulin and DAPI, then and determined the percentage of cells which have bipolar spindles (Fig. 7). In the presence of 8 μg/ml EchB, *FKS1* cells were arrested cell cycle in G2 with a monopolar spindle, while *FKS1* cells treated with DMSO formed a mature bud and bipolar spindles. This result showed that 8 μg/ml EchB is sufficient to cause the cell wall integrity checkpoint. The same experiment was carried out in *FKS1 wac1* cells. Although *FKS1 wac1* cells treated with 8 μg/ml EchB did not form a mature bud due to inhibition of glucan synthesis (Fig. 6), they formed bipolar spindles at 180 min-240 min (Fig 7). This indicates that *wac1* cells are defective in the checkpoint, which is an identical result previous report (Suzuki *et al.*, 2004). Whereas *FKS1 wac1* cells treated with DMSO formed a mature bud and bipolar spindles.

To test whether Smi1p and Gon7p are involved in the checkpoint, I observed these mutants in the same conditions. Small G1 cells of *FKS1 Δsmi1* and *FKS1 Δgon7* were isolated by centrifugal elutriation, and incubated in YPD medium with 8 μg/ml EchB. Since *FKS1 Δgon7* cells appeared slow-growth-phenotype, I monitored cell cycle progression until 320 minutes. Under treatment of EchB, both mutants did not form a mature bud (Fig. 8) At indicated times, cells were stained with anti-tubulin and DAPI. I

found these mutants do not show bipolar spindles formation (Fig 9). It suggested that Smi1p and Gon7p do not involved in the cell wall integrity checkpoint.

BZZ1 deletion cells override the cell wall integrity checkpoint under Echinocandin B treatment.

Phenotype of *fks1-1154 Δbzz1* suggested that Bzz1p has a role in the cell wall integrity checkpoint. Well-characterized method causing the checkpoint is inhibition of glucan synthesis, using a $fks1^{ts}$ strain under restrictive temperature or EchB treatment. By the former method, it was found that *BZZ1* deletion induced defect in the cell wall integrity checkpoint. To confirm involvement of Bzz1p in the checkpoint, I tested the latter method with *FKS1 Δbzz1* cells. Small G1 cells of *FKS1 Δbzz1* were isolated by centrifugal elutriation, and incubated at 25°C in YPD medium with 8 μg/ml EchB. In *FKS1 Δbzz1*, accumulation of cells with a medium to large bud was inhibited as well as WT cells (Fig. 10). At indicated times, cells were determined the percentage of cells which have bipolar spindles (Fig. 11). Despite the presence of EchB, *FKS1 Δbzz1* cells formed bipolar spindles. It was strongly supported that Bzz1p is a component of the cell wall integrity checkpoint system.

Vrp1p, a component of Las17p complex, is involved in the cell wall integrity checkpoint.

It was suggested that Bzz1p is involved in the cell wall integrity checkpoint from the

above-described. It was reported Bzz1p localizes near plasma membrane and also actin cortical patches, and physically interacts with Las17p (Soulard, *et al.*, 2002). Las17p forms high-molecular-weight protein complex and regulates actin polymerization at the cell cortex. To investigate whether Las17p complex was concerned in the cell wall integrity checkpoint, I investigated involvement of Vrp1p, a major component of Las17p complex, in the checkpoint. I deleted *VRP1* gene in *fks1-1154* cells and observed spindle formation under the restrictive temperature.

Small G1 cells of *fks1-1154 Δvrp1* were isolated by centrifugal elutriation, and incubated at 37°C. Then, cells with a small bud are accumulated in *fks1-1154 Δvrp1* cells (Fig. 12). Under the restrictive temperature, accumulation of cells with a medium to large bud was greatly inhibited. I fixed cells at each time point and stained with anti-tubulin and DAPI, then determined the percentage of cells with bipolar spindles (Fig. 13). Most of *fks1-1154 Δvrp1* cells formed bipolar spindles. It indicated that they are defective in the cell wall integrity checkpoint. It was suggested Vrp1p is involved in the checkpoint.

Discussion

Possible involvement of Bzz1p in the cell wall integrity checkpoint

To clarify the signaling pathway of the cell wall integrity checkpoint in more detail, I searched new components in the checkpoint. As a result, a new component, Bzz1p was found in this study. Since proteins localized on or near plasma membrane are likely to be involved in the checkpoint which monitors cell wall defect, I focused on proteins located on or near plasma membrane. I performed microscopic analysis in each mutant, and found that *fks1-1154 Δbzz1* appears severe defect in the cell wall integrity checkpoint. Moreover, I demonstrated that *FKS1 Δbzz1* also does not achieve G2/M arrest under treatment of EchB. These results strongly suggested Bzz1p is involved in the cell wall integrity checkpoint.

Bzz1p has a role in the checkpoint in Las17p complex-dependent-manner.

Bzz1p seems to be involved in the cell wall integrity checkpoint. Bzz1p is regarded as a member of the Las17p complex (Soulard *et al.* 2002), which gives us the anticipation that Las17p complex is concerned with the checkpoint. To investigate whether Las17p complex is concerned with the cell wall integrity checkpoint, *VRP1* was deleted in *fks1-1154* cells, since Vrp1p is a major component in the Las17p complex and binds strongly to Las17p (Lechler, *et al.* 2001; Vaduva G, *et al.* 1997). Deletion of *VRP1* caused defect in the cell wall integrity checkpoint. This result indicates Vrp1p is also involved in the

cell wall integrity checkpoint. It is suggested Bzz1p is involved in the checkpoint in Las17p complex-dependent-manner.

Speculation to the signaling pathway of the cell wall integrity checkpoint

In this study, I revealed that Bzz1p and Vrp1p are involved in the cell wall integrity checkpoint. It suggested Bzz1p is involved in the checkpoint in Las17p complex-dependent-manner. However, it has not been elucidated yet whether Bzz1p has a direct function in the cell wall integrity checkpoint or not. Therefore, from these results, two possibilities are suggested; 1) Bzz1p directly functions in the checkpoint through Las17p complex; 2) deletion of *BZZ1* and *VRP1* indirectly influences some effects in the checkpoint. The details are described as follows.

1) It is suggested Bzz1p and Vrp1p directly transmit signal to the dynactin complex. Igarashi reported that Jnm1p moves to cell cortex from nuclear according to the cell cycle in WT cells. Moreover, Jnm1p does not move to the cell cortex in *wac1* mutant treated with EchB, which suggests movement of Jnm1p is required to achieve the G2 arrest (Igarashi Doctor thesis 2006). However, anchoring proteins for localization of dynactin complex to the cell cortex are not required for the checkpoint (Suzuki *et al.* 2004; Hidebrandt and Hoyt 2000). Then, the question remains how localization of dynactin complex is regulated. I think that Bzz1p plays a role in localization of dynactin complex with Las17p complex at cell cortex. In this case, in *Δbzz1* cells dynactin complex does not anchor to the cell cortex, which causes inhibition

of the checkpoint signaling to nuclear. Observation of Jnm1p localization in *FKS1 Δbzz1* cells treated with EchB will elucidate it.

2) On the other hand, it is remained that deletion of *BZZ1* or *VRP1* provokes some indirect effects to the checkpoint. Previous studies suggested Las17p, Vrp1p and End3p are required for endocytosis (Naqvi *et al.* 1998; Tang *et al.* 1997; Pruyne and Bretscher 2000). In addition, cells of *Δmyo5* has temperature sensitive defect in endocytosis and deletion of *BZZ1* in *Δmyo5* strain causes additive phenotype in endocytosis, which suggests Bzz1p has a role in endocytosis (Soulard *et al.*, 2005). From the information, it is suggested that proteins involved in endocytosis reflect some indirect effects in the checkpoint. Indeed, cells deleted *END3* were slightly defective in the checkpoint (Fig 5). This result suggests that defect of endocytosis causes defect in the checkpoint. This idea can be demonstrated if the checkpoint signaling overlaps with endocytic pathway. To elucidate it, it should be tested whether two functions can be separated in the molecular structure of Las17p or Vrp1p. It has been shown that functions of the cell wall integrity checkpoint and nuclear migration of Arp1p are separated in its molecular structure (Igarashi *et al.* 2005).

Materials and Methods

Yeast Media

The rich yeast medium (YPD) consisted of 1% Bacto yeast extract (DIFCO), 2% polypeptone, and 2% glucose. YPD with the sugar component replaced by 2% galactose and 0.1% sucrose was designated as YPGS. YPA medium consisted 1% Bacto yeast extract (DIFCO), 2% polypeptone, 1% K-Acetate (Wako) and 20 μg/ml of adenine. Yeast synthetic medium (SD) contained 0.67% Bacto yeast nitrogen base without amino acids (DIFCO), 2% glucose and appropriate nutritional supplements. SGS medium was identical to SD except for the use of galactose and sucrose in place of glucose. Addition of 1 μg/ml 5-fluoroorotic acid to SD produced FOA medium. 2% K-acetate was designed as SPO medium. Plate media were made with addition of 2% agar (Shouei Chemicals, Tokyo).

Yeast Strains, Plasmids and Oligonucleotides.

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1, The plasmids and oligonucleotides are catalogued in Table 2 and Table 3, respectively.

All the strains are derivatives of YPH499 and YPH500 (Sikorski and Hieter, 1989). The *fks1* mutant strains used in the study harbored an *fks1* temperature-sensitive allele integrated into the Δ*fks1* Δ*fks2* background, as the *FKS1* and *FKS2* products have overlapping functions (Inoue *et al.*, 1995; Mazur *et al.*, 1995).

Deletion constructs, Δ*sro77*, Δ*end3*, Δ*bzz1*, Δ*rho2*, Δ*smi1* and Δ*vrp1* were created by the PCR-mediated gene disruption method as described previously (Sakumoto *et al.*, 1999); the *Candida glabrata* (*Cg*) *LEU2* gene (compatible with *Saccharomyces cerevisiae LEU2*) was amplified with a pair of primers with flanking sequence derived from the upstream and downstream regions of the corresponding genes. PCR products were introduced into *FKS1* (YOC1001) or *fks1-1154* (YOC1087). Gene disruption and integration was confirmed by PCR. Deletion of *GON7* by the PCR-mediated gene disruption method was described previously (Sakumoto *et al.*, 1999); the *Candida glabrata* (*Cg*) *URA3* gene (compatible with *Saccharomyces cerevisiae URA3*) was amplified with a pair of primers with flanking sequence derived from the upstream and downstream regions of the corresponding gene. PCR products were introduced into *FKS1/FKS1* (YOC3916) diploid cells. Gene disruption and integration was confirmed by PCR. To obtain *FKS1* Δ*gon7* haploid cells, *FKS1/FKS1* Δ*gon7* cells were grown on YPA plate and transferred to SPO plate for sporulation. *FKS1* Δ*gon7* haploid cells were confirmed by PCR.

Cell Synchrony

100 ml of cells was grown at 25°C to a density of 10^7 cells/ml, and then sonicated to disperse clumps. The cells were pelleted using an elutriator rotor (R5E; Hitachi). The pump speed was adjusted so that the smallest cells flowed through the elutriator and the selected cells were collected in tubes placed on ice. The collected cells were

concentrated by centrifugation, resuspended in a fresh medium at < 10^7 cells/ml and incubated at the specified temperature. In cell wall checkpoint assay, *fks1* temperature-sensitive mutants were used to cause perturbations in cell wall synthesis, because four out of 10 *fks1* strains examined (*fks1-1114*, *fks1-1125*, *fks1-1144*, and *fks1-1154*) had significant defects in glucan synthesis activity both *in vitro* and *in vivo* (Sekiya-Kawasaki *et al.*, 2002; our unpublished data). *fks1* mutant cells, which have a low glucan synthesis activity, failed to form mature (medium to large) buds and most were with no bud or with a tiny projection after release of small G1 cells at the restrictive temperature (Sekiya-Kawasaki *et al.*, 2002).

Fluorescence Microscopy

For fluorescence microscopy, the cell fixation method was used (Rout and Kilmartin, 1990). Fixed cells were washed with SP buffer, and treated with 28 μg/ml zymolyase at a final concentration for 30 minutes at 30°C. The cells were corrected and suspended in SP buffer containing 0.1% of TritonX-100. Then, cells were incubated 10 minutes at room temperature. After that, cells were washed and suspended with appropriate volume of SP buffer. To visualize spindle, cells were spread on a poly-L-lysine-coated slide, and stained with a rat monoclonal antibody (YOL 1/34; Oxford Biotechnology Ltd.) and a FITC-conjugated α-rat IgG antibody as the first and the second antibody, respectively. For visualization of DNA, 4',6-Diamidino-2-phenylindole (DAPI; Sigma) was utilized at a final concentration of 100-200 ng/ml. The stained cells were observed using a Leica

DMRE with a x100 oil-immersion objective lens.

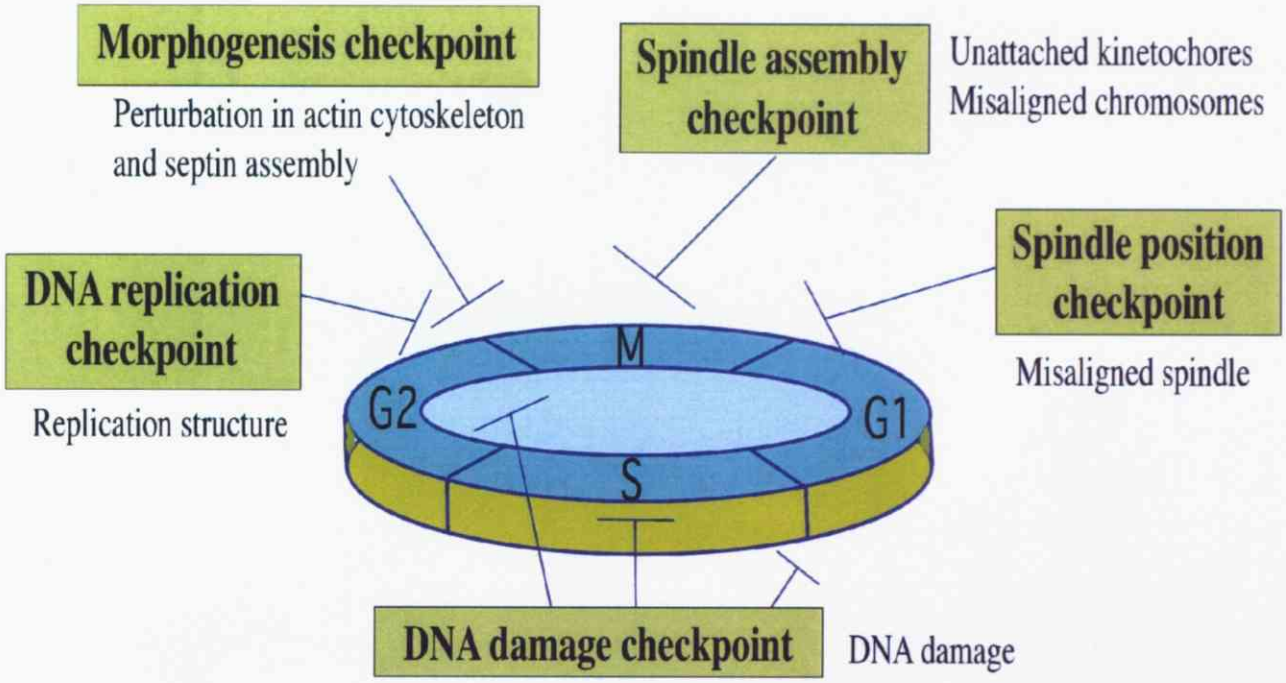


Figure 1. Cell cycle checkpoints in the budding yeast.

Checkpoint controls key processes of cell cycle, and acts to delay cell cycle progression when those processes are defective. In the budding yeast, various cell cycle checkpoints have been found out. For example, if DNA is incompletely replicated or damaged, these checkpoint control delay chromosome segregation.

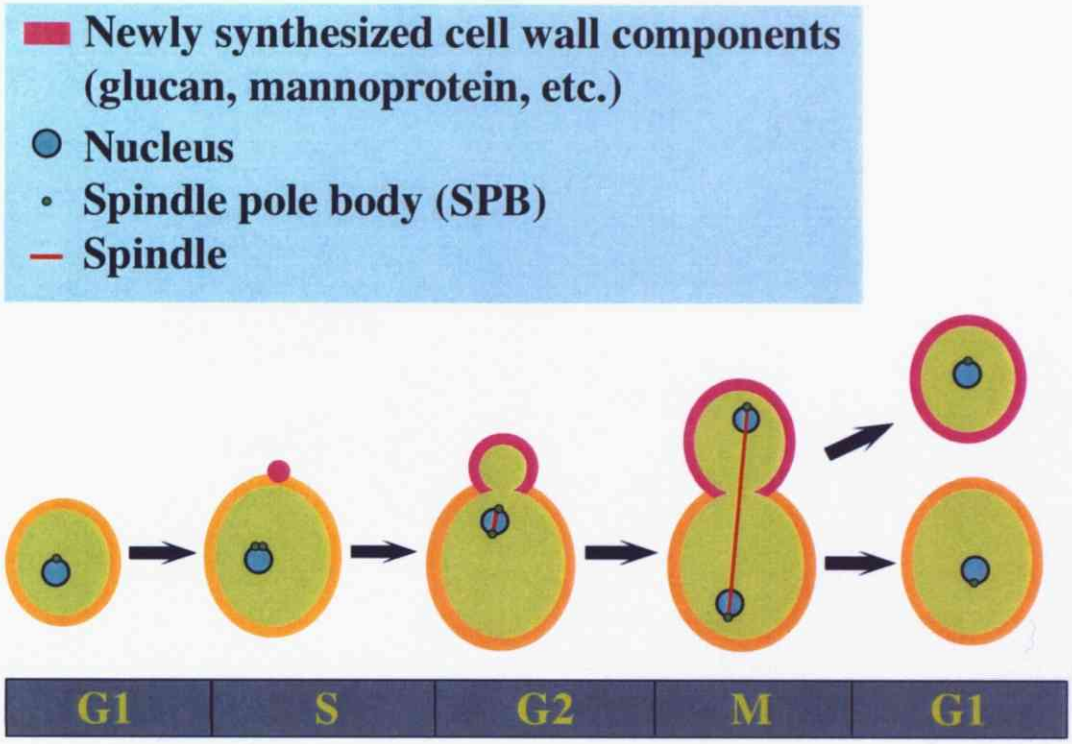


Figure 2. Cell wall remodeling during the cell cycle in the budding yeast.

Cell wall components are incorporated into the site of growth. During the normal cell cycle in budding yeast, cell wall components are duplicated at the appropriate time. Cell wall remodeling seems to be temporally coincident with DNA replication and spindle assembly, and it completes before nuclear division.

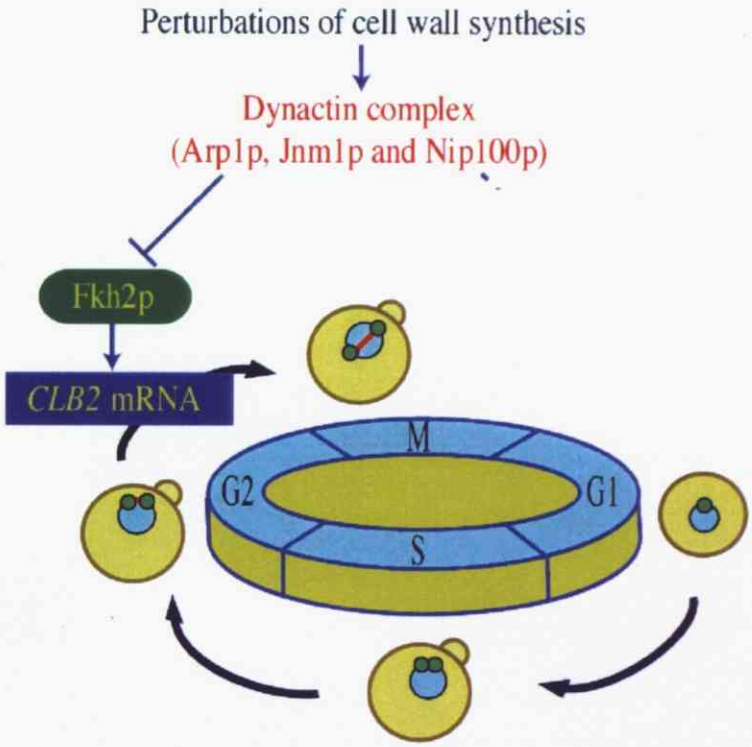
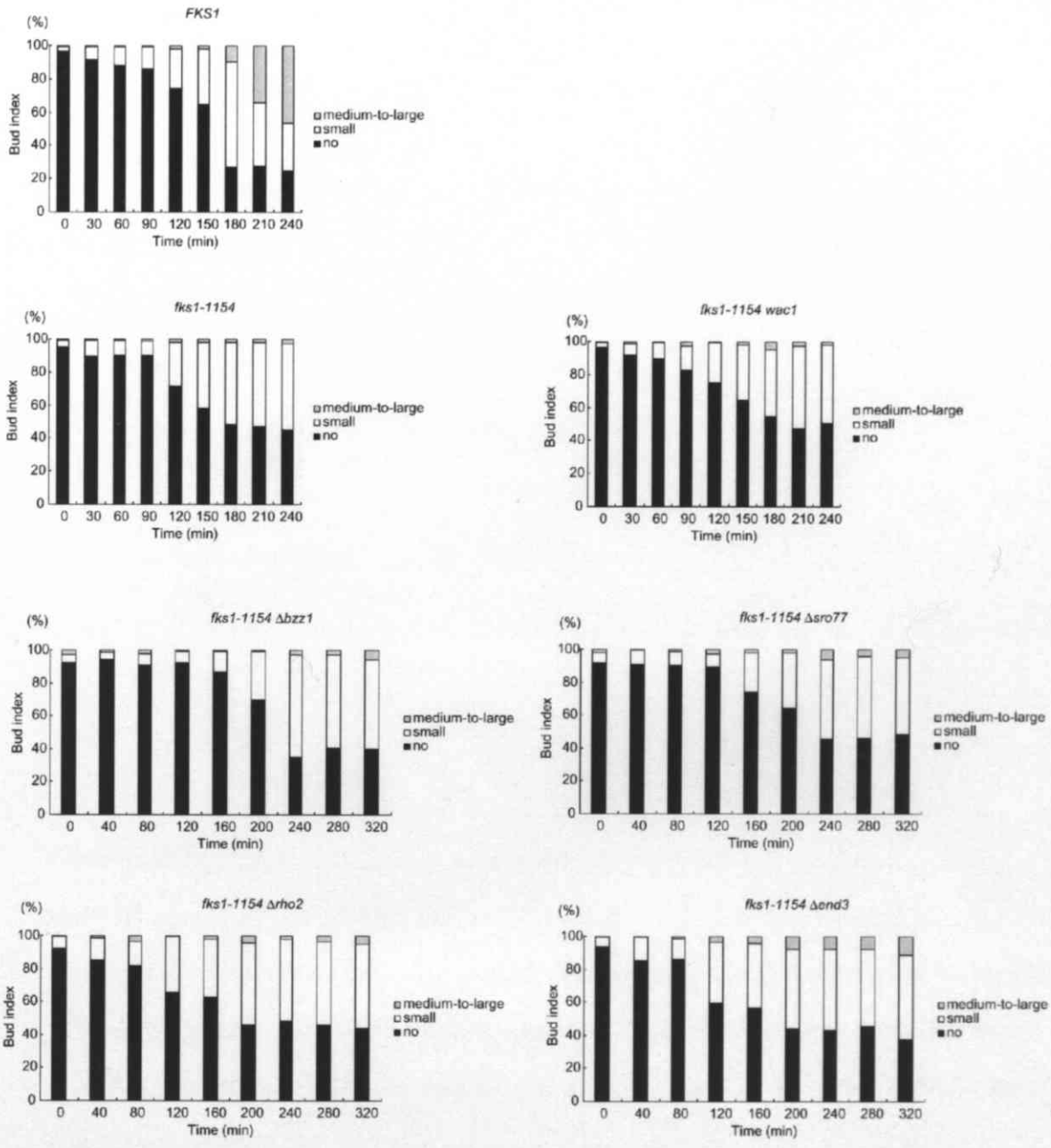


Figure 3. Model of the cell wall integrity checkpoint

The cell wall integrity checkpoint ensures that the cell wall is sufficiently synthesized before entry into mitosis. When cell wall synthesis is perturbed, *CLB2* expression is negatively regulated at the transcriptional level through Fkh2p. A dynactin-dependent mechanism including at least Arp1p, Jnm1p and Nip100p is involved in regulation of cell cycle progression. This causes cell cycle arrest after DNA replication and SPB duprication, but before bipolar spindle formation.

(a)


FKS1
fks1-1154
fks1-1154 wac1
fks1-1154 Δbzz1
fks1-1154 Δsro77
fks1-1154 Δrho2
fks1-1154 Δend3
(%)
Bud index
Time (min)
medium-to-large
small
no

(b)

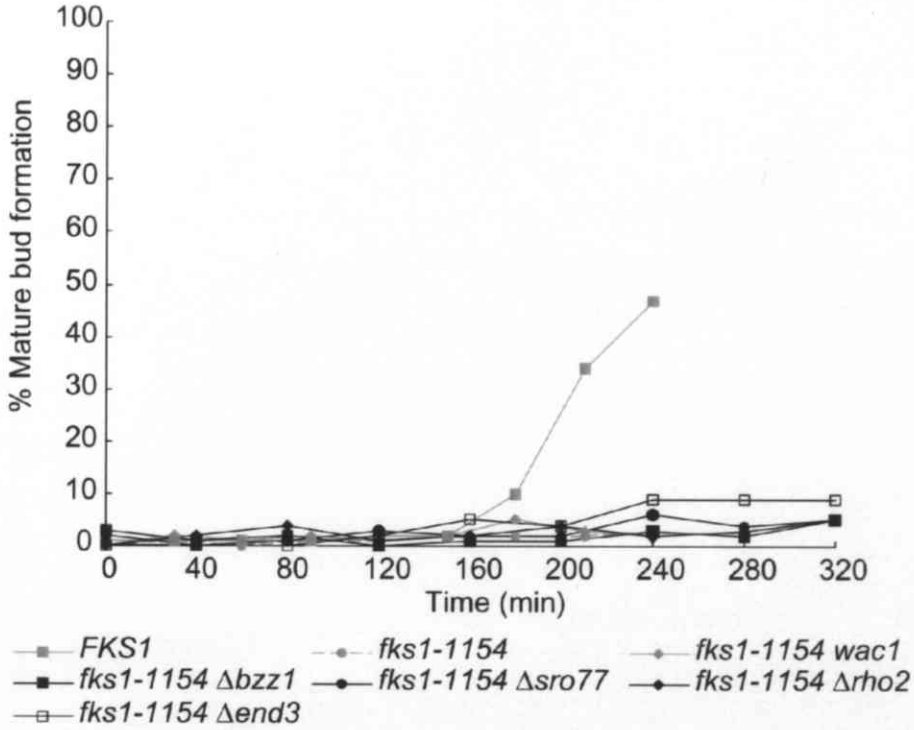
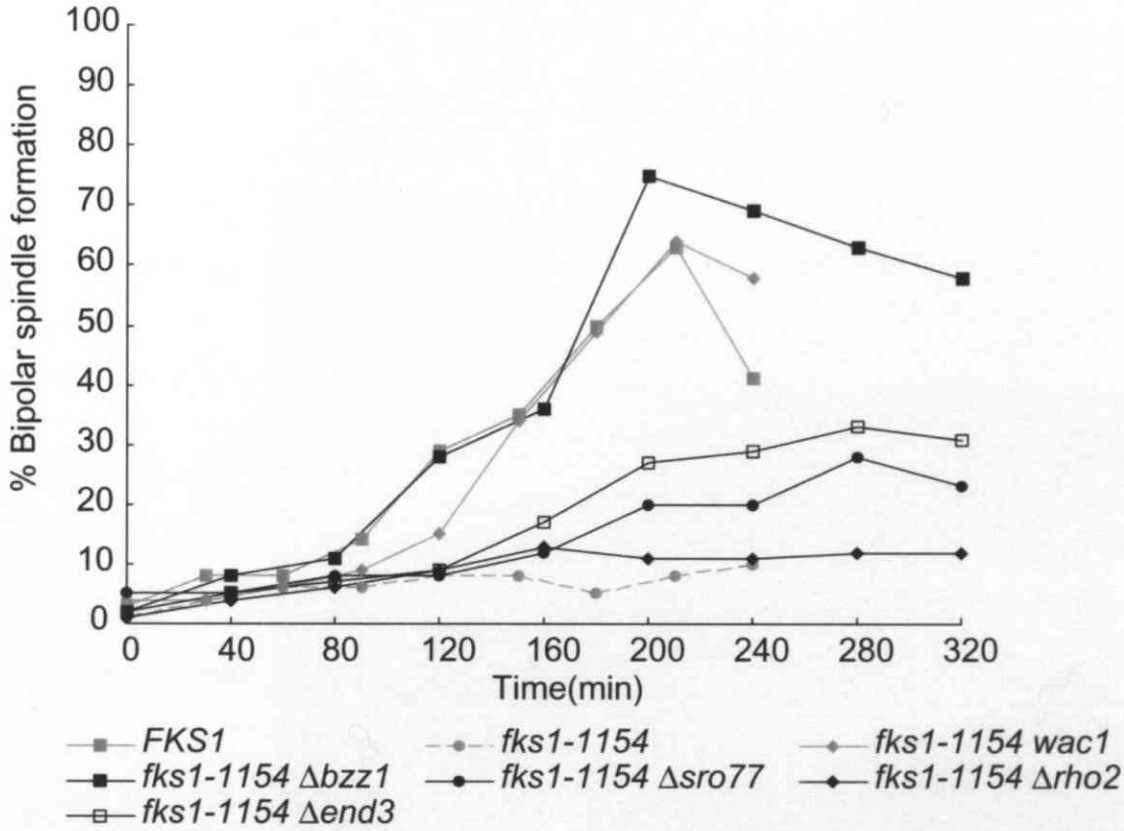


Figure 4. Cells with a small bud are accumulated in *fks1-1154* background.

Small G1 cells of *FKS1*, *fks1-1154*, *fks1-1154 wac1*, *fks1-1154 Δsro77*, *fks1-1154 Δend3*, *fks1-1154 Δbzz*1 and *fks1-1154 Δrho2* were isolated by centrifugal elutriation, and incubated in YPD at 37 °C. At the indicated times, cells were fixed. Time course of the bud growth is shown in (a). The percentage of cells with a mature bud was quantitated (b). More than 200 cells were counted in each strain.

(a)


% Bipolar spindle formation
100
90
80
70
60
50
40
30
20
10
0
0
40
80
120
160
200
240
280
320
Time(min)
FKS1
fks1-1154
fks1-1154 wac1
fks1-1154 Δbzz1
fks1-1154 Δsro77
fks1-1154 Δrho2
fks1-1154 Δend3

(b)

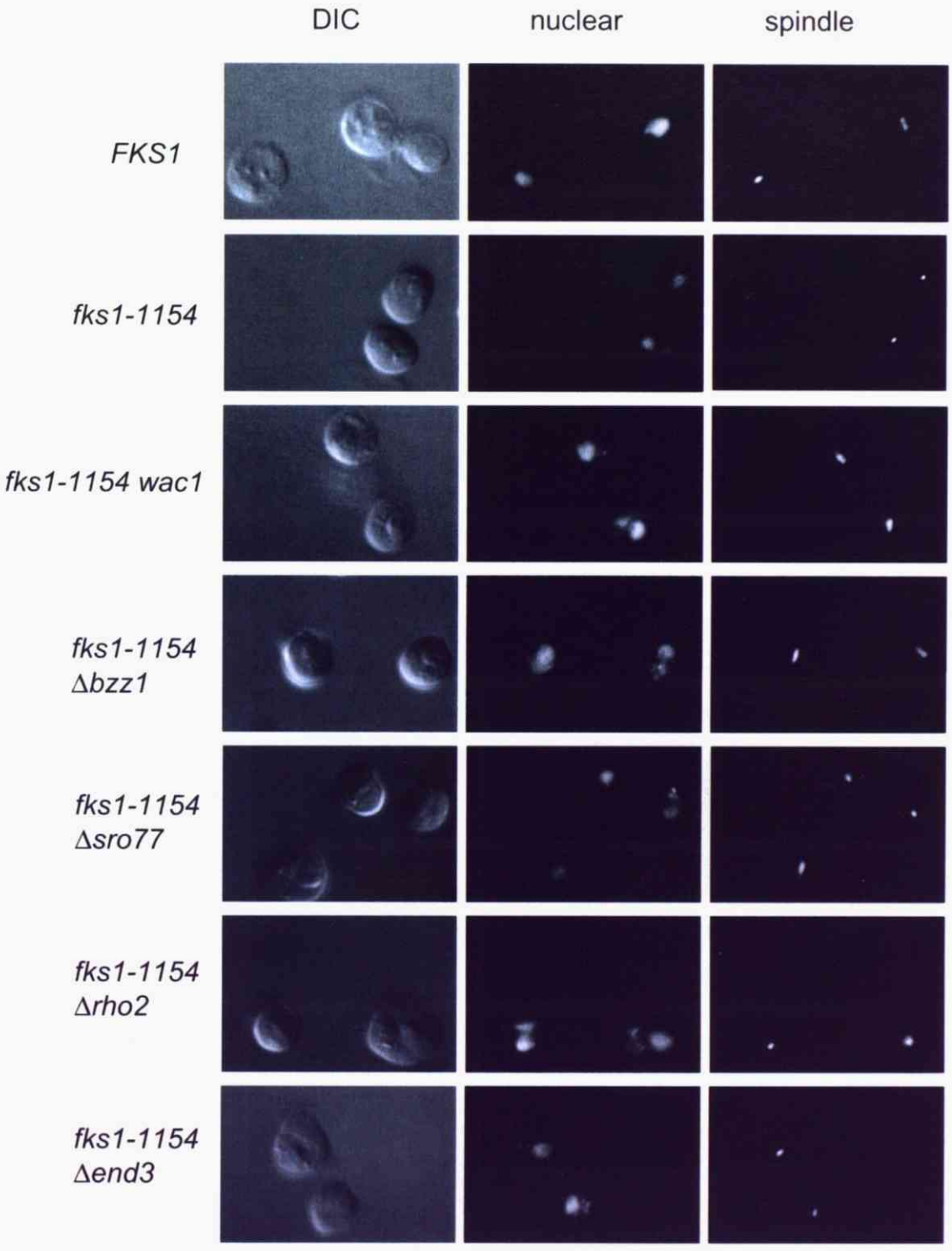

DIC
nuclear
spindle
FKS1
fks1-1154
fks1-1154 wac1
fks1-1154 Δbzz1
fks1-1154 Δsro77
fks1-1154 Δrho2
fks1-1154 Δend3

Figure 5. Deletion of the *BZZ1* gene causes defect on the cell wall integrity checkpoint.

Small G1 cells of *FKS1*, *fks1-1154*, *fks1-1154 wac1*, *fks1-1154 Δsro77*, *fks1-1154 Δend3*, *fks1-1154 Δbzz1* and *fks1-1154 Δrho2* were isolated by centrifugal elutriation, and incubated in YPD at 37 °C. At the indicated times, cells were fixed and stained with DAPI and the anti-tubulin antibody. The percentage of cells with bipolar spindles was quantitated (a). More than 200 cells were counted in each strain. Nuclear and spindle morphologies of *FKS1*, *fks1-1154* and *fks1-1154 wac1* cells at 210 min after release into YPD, and nuclear and spindle morphologies of *fks1-1154 Δsro77*, *fks1-1154 Δend3*, *fks1-1154 Δbzz1* and *fks1-1154 Δrho2* cells at 240 min are shown in (b).

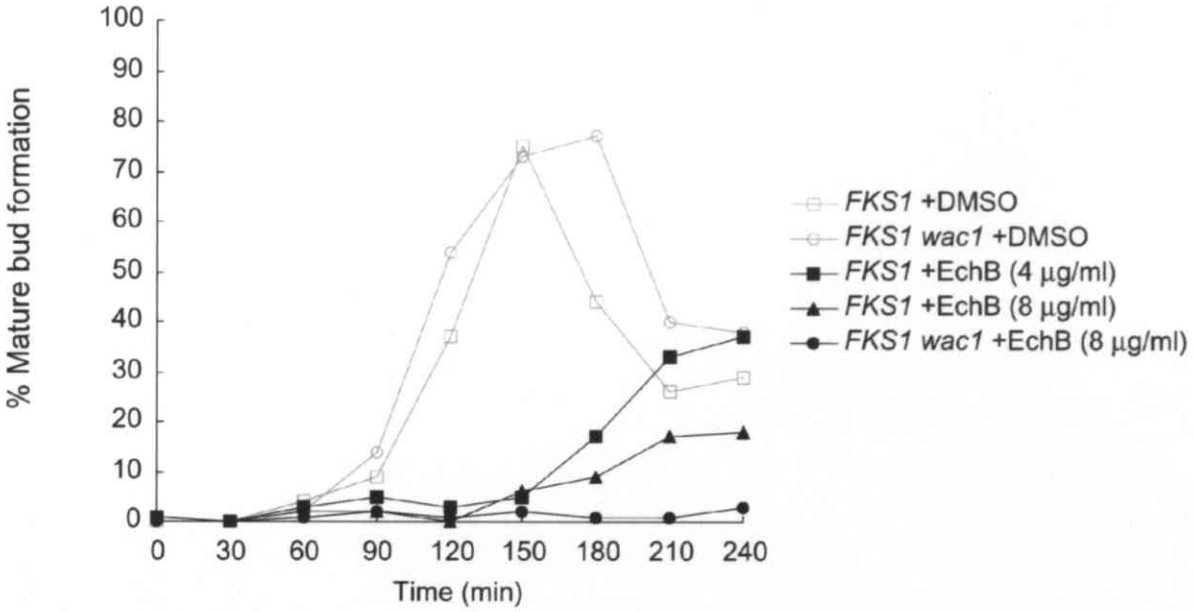
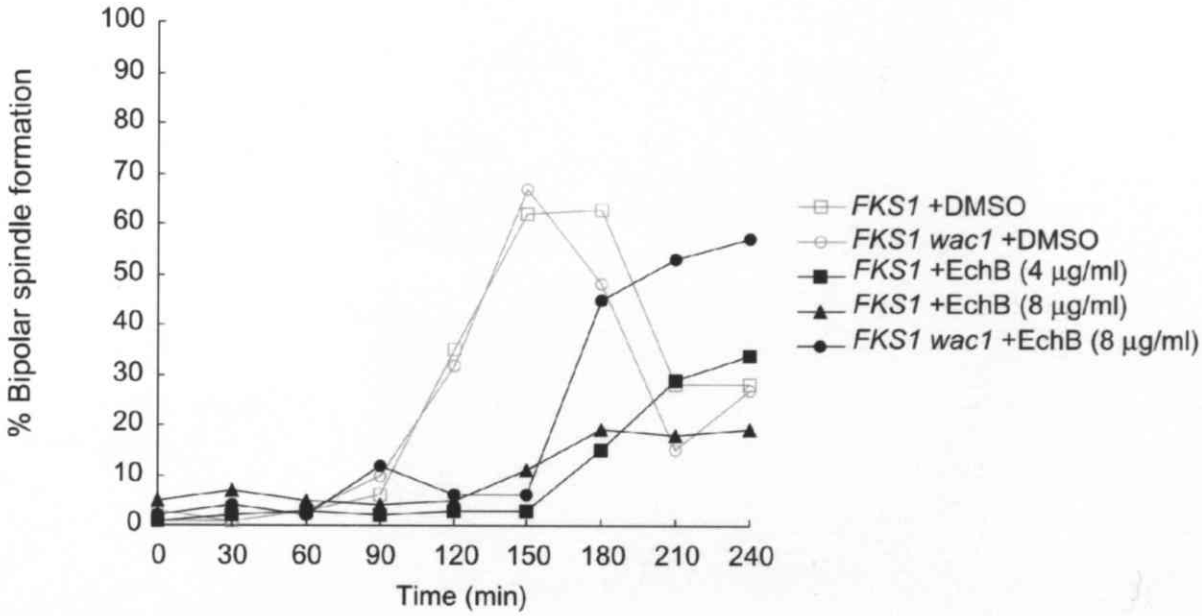


Figure 6. Treatment of 8 µg/ml of EchB inhibits mature bud formation.

Small G1 cells of *FKS1* and *FKS1 wac1* were isolated by centrifugal elutriation, and incubated in YPD at 25°C with 4 µg/ml or 8 µg/ml of EchB or DMSO. At the indicated times, cells were fixed. The percentage of cells with a mature bud was quantitated. More than 200 cells were counted in each strain.

(a)


% Bipolar spindle formation
100
90
80
70
60
50
40
30
20
10
0
0
30
60
90
120
150
180
210
240
Time (min)
FKS1 +DMSO
FKS1 wac1 +DMSO
FKS1 +EchB (4 µg/ml)
FKS1 +EchB (8 µg/ml)
FKS1 wac1 +EchB (8 µg/ml)

(b)

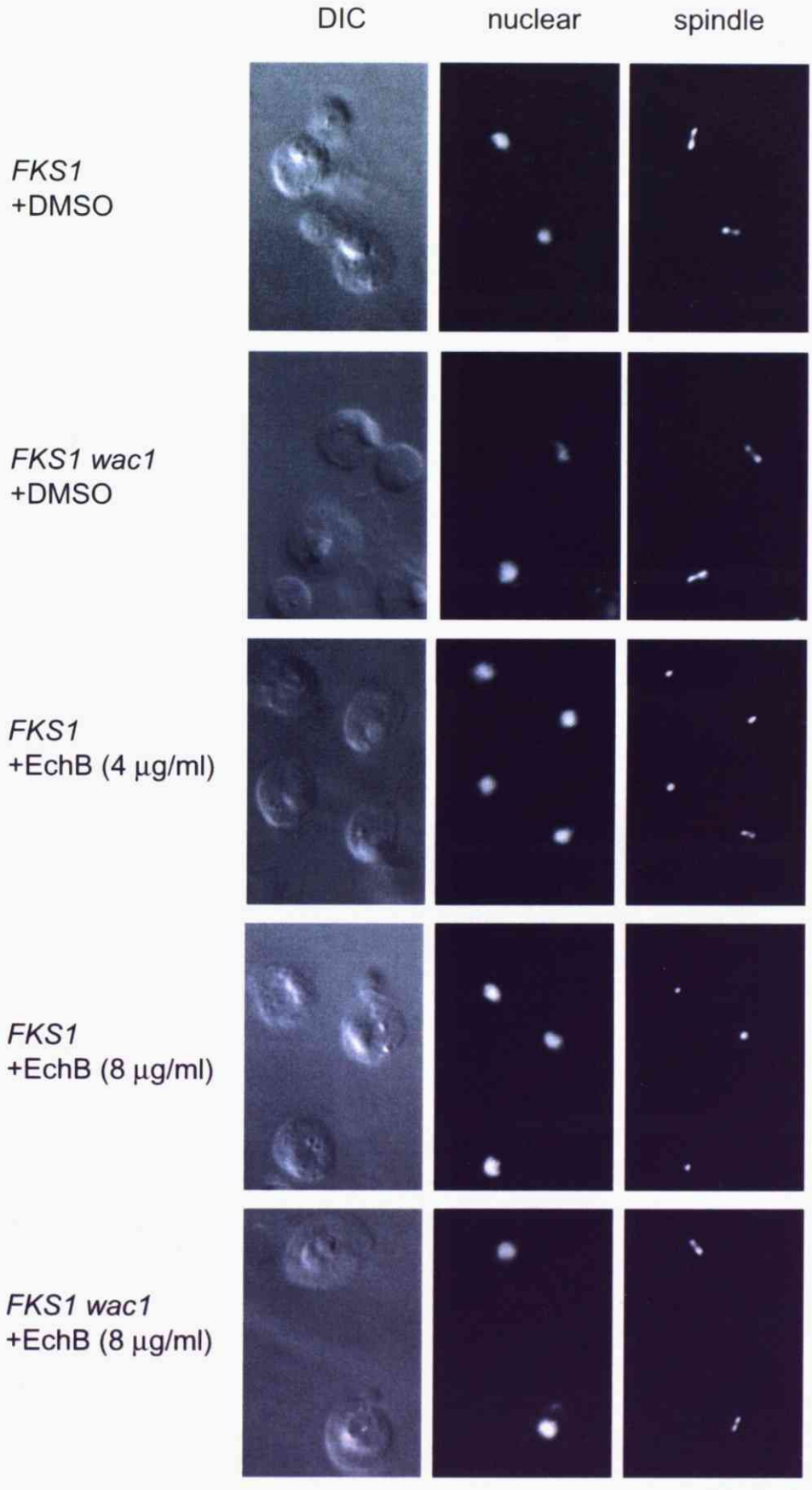

DIC
nuclear
spindle
FKS1
+DMSO
FKS1 wac1
+DMSO
FKS1
+EchB (4 μg/ml)
FKS1
+EchB (8 μg/ml)
FKS1 wac1
+EchB (8 μg/ml)

Figure 7. Treatment of 8 μg/ml of EchB is sufficient to cause the cell wall integrity checkpoint.

Small G1 cells of *FKS1* and *FKS1 wac1* were isolated by centrifugal elutriation, and incubated in YPD at 25°C with 4 μg/ml or 8 μg/ml of EchB or DMSO. At the indicated times, cells were fixed and stained with DAPI and the anti-tubulin antibody. The percentage of cells with bipolar spindles was quantitated (a). More than 200 cells were counted in each strain. Nuclear and spindle morphologies of cells of indicated strains at 180 min after release into YPD are shown in (b).

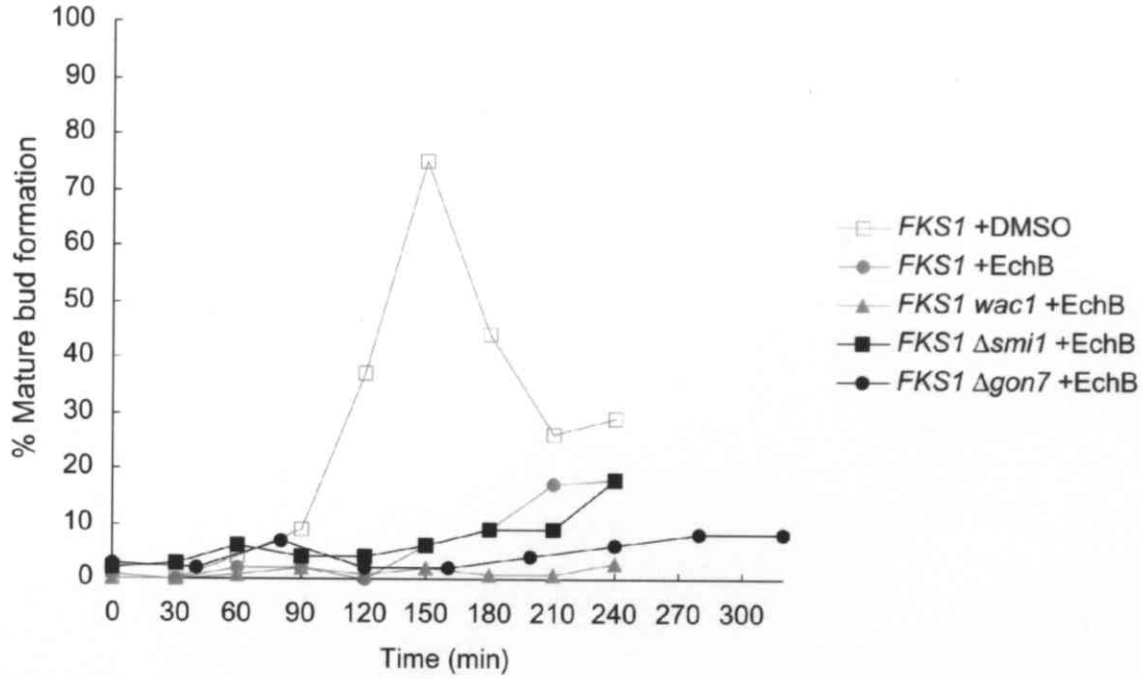
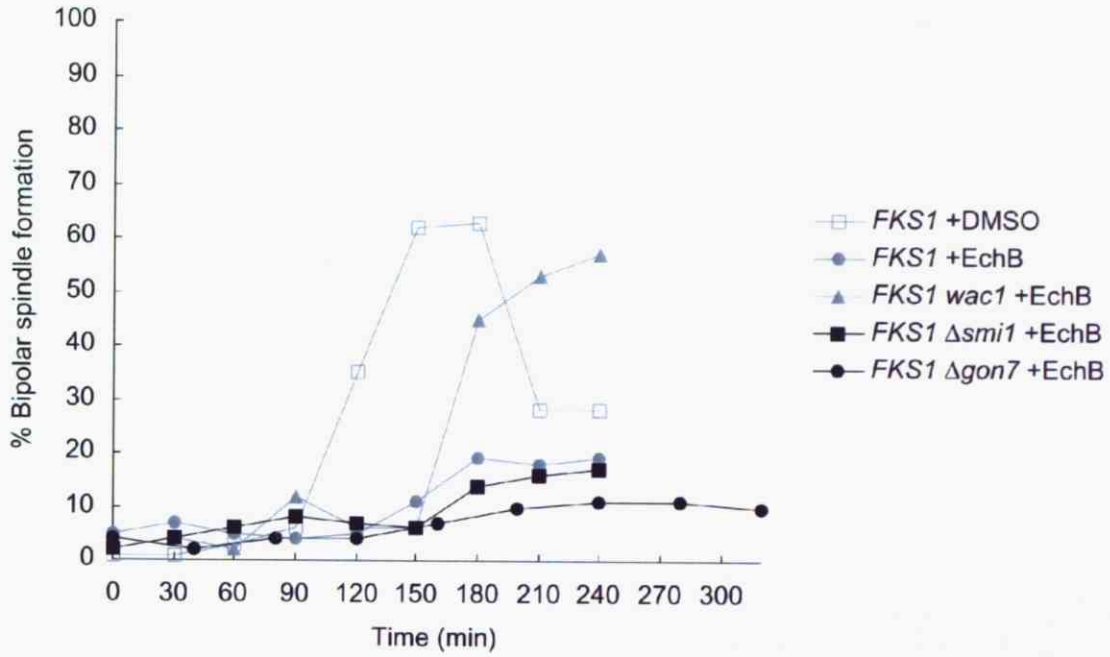


Figure 8. *FKS1 Δsmi1* and *FKS1 Δgon7* cells are inhibited mature bud formation by Echinocandin B.

Small G1 cells of *FKS1, FKS1 wac1, FKS1 Δsmi1* and *FKS1 Δgon7* were isolated by centrifugal elutriation, and incubated in YPD at 25°C with 8 μg/ml of EchB. At the indicated times, cells were fixed. The percentage of cells with a mature bud was quantitated. More than 200 cells were counted in each strain.

(a)


% Bipolar spindle formation
100
90
80
70
60
50
40
30
20
10
0
0 30 60 90 120 150 180 210 240 270 300
Time (min)
FKS1 +DMSO
FKS1 +EchB
FKS1 wac1 +EchB
FKS1 Δsmi1 +EchB
FKS1 Δgon7 +EchB

(b)

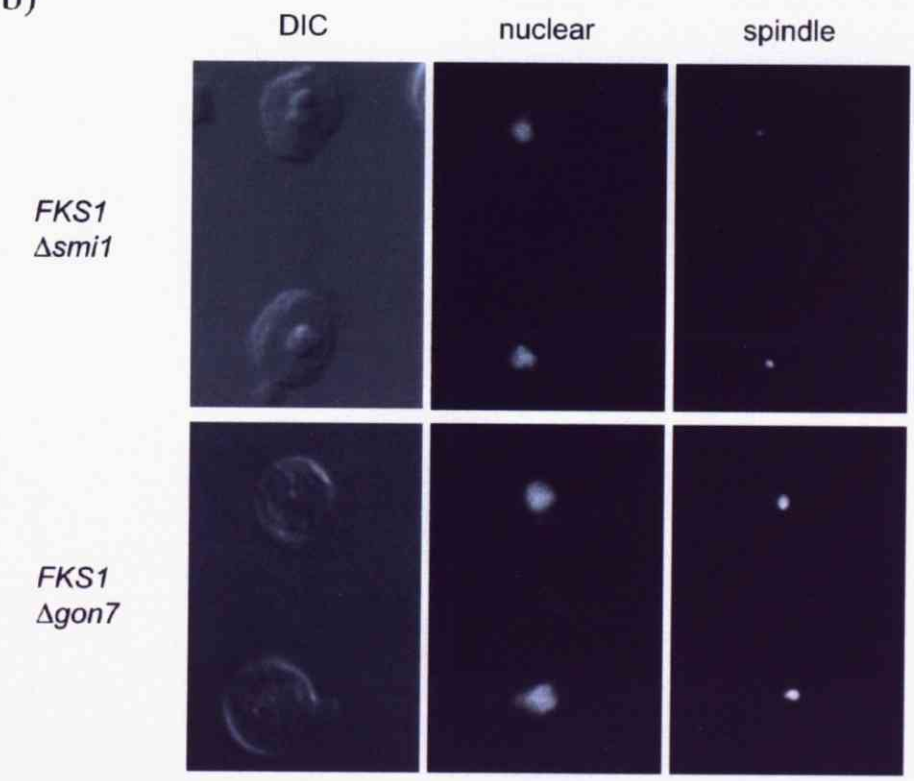

DIC
nuclear
spindle
FKS1
Δsmi1
FKS1
Δgon7

Figure 9. Deletion of *SMI1* and *GON7* does not induce defect in the cell wall integrity checkpoint.

Small G1 cells of *FKS1*, *FKS1 wac1*, *FKS1 Δsmi1* and *FKS1 Δgon7* were isolated by centrifugal elutriation, and incubated in YPD at 25°C with 8 μg/ml of EchB. At the indicated times, cells were fixed and stained with DAPI and the anti-tubulin antibody. The percentage of cells with bipolar spindles was quantitated (a). More than 200 cells were counted in each strain. Nuclear and spindle morphologies of cells of *FKS1 Δsmi1* or *FKS1 Δgon7* at 180 min or 280 min after release into YPD are shown in (b).

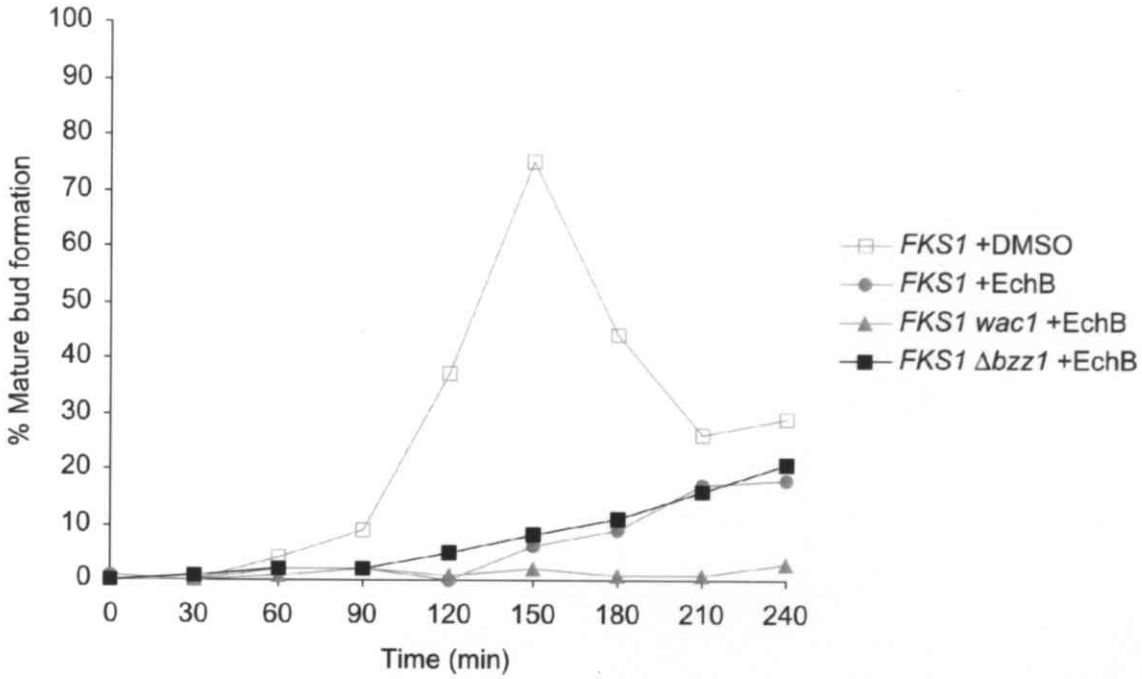


Figure 10. *FKS1 Δbzz1* cells do not achieve bud-maturation in the presence of EchB.

Small G1 cells of *FKS1*, *FKS1 wac1* and *FKS1 Δbzz1* were isolated by centrifugal elutriation, and incubated in YPD at 25 °C with 8 µg/ml of EchB. At the indicated times, cells were fixed. The percentage of cells with a mature bud was quantitated. More than 200 cells were counted in each strain.

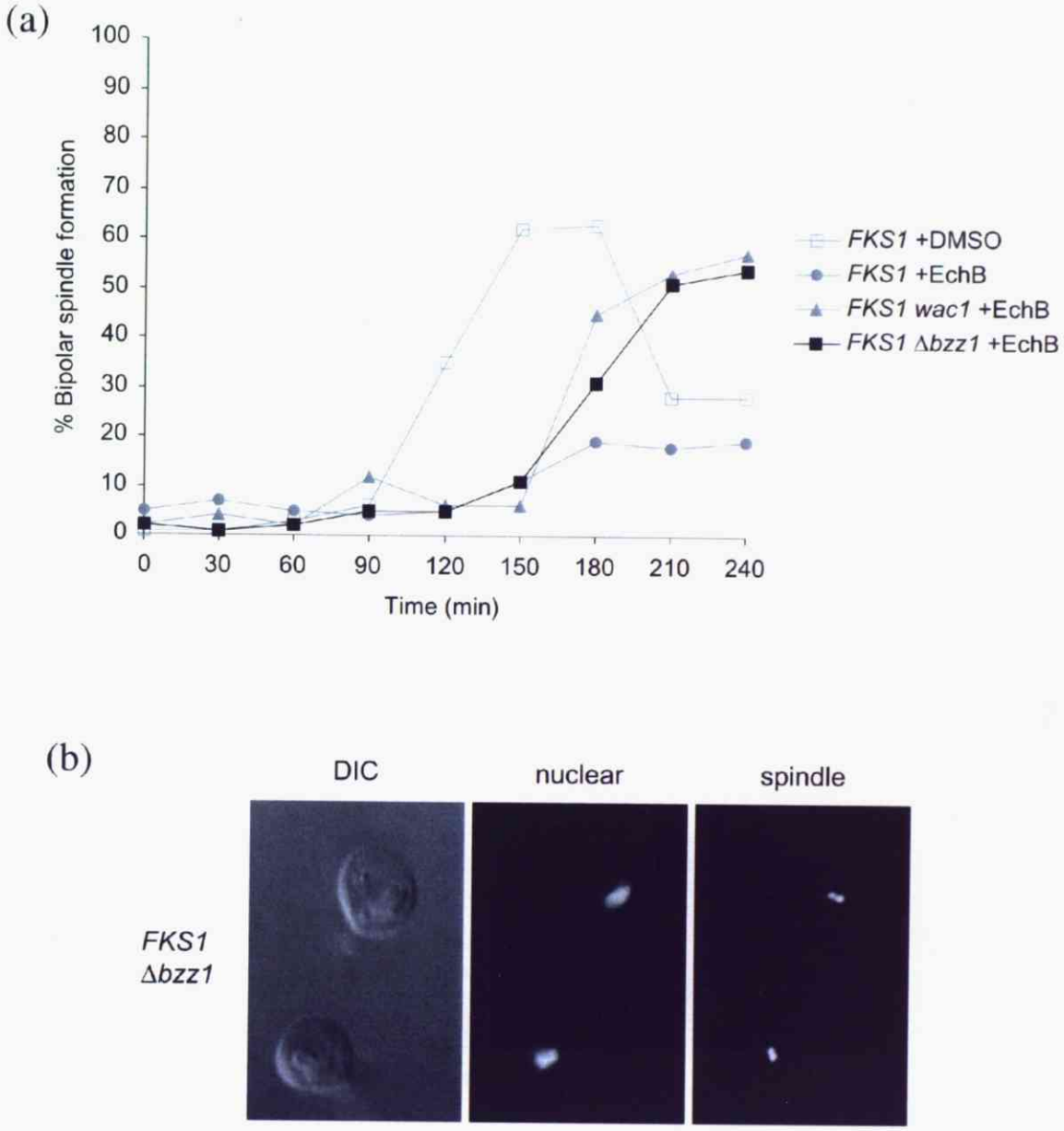


Figure 11. Deletion of *BZZ1* in *FKS1* cells ovverrides the cell wall integrity checkpoint induced by EchB treatment.

Small G1 cells of *FKS1*, *FKS1 wac1* and *FKS1 Δbzz1* were isolated by centrifugal elutriation, and incubated in YPD at 25 °C with 8 μg/ml of EchB. At the indicated times, cells were fixed and stained with DAPI and the anti-tubulin antibody. The percentage of cells with bipolar spindles was quantitated (a). More than 200 cells were counted in each strain. Nuclear and spindle morphologies of cells of indicated strains at 210 min after release into YPD are shown in (b).

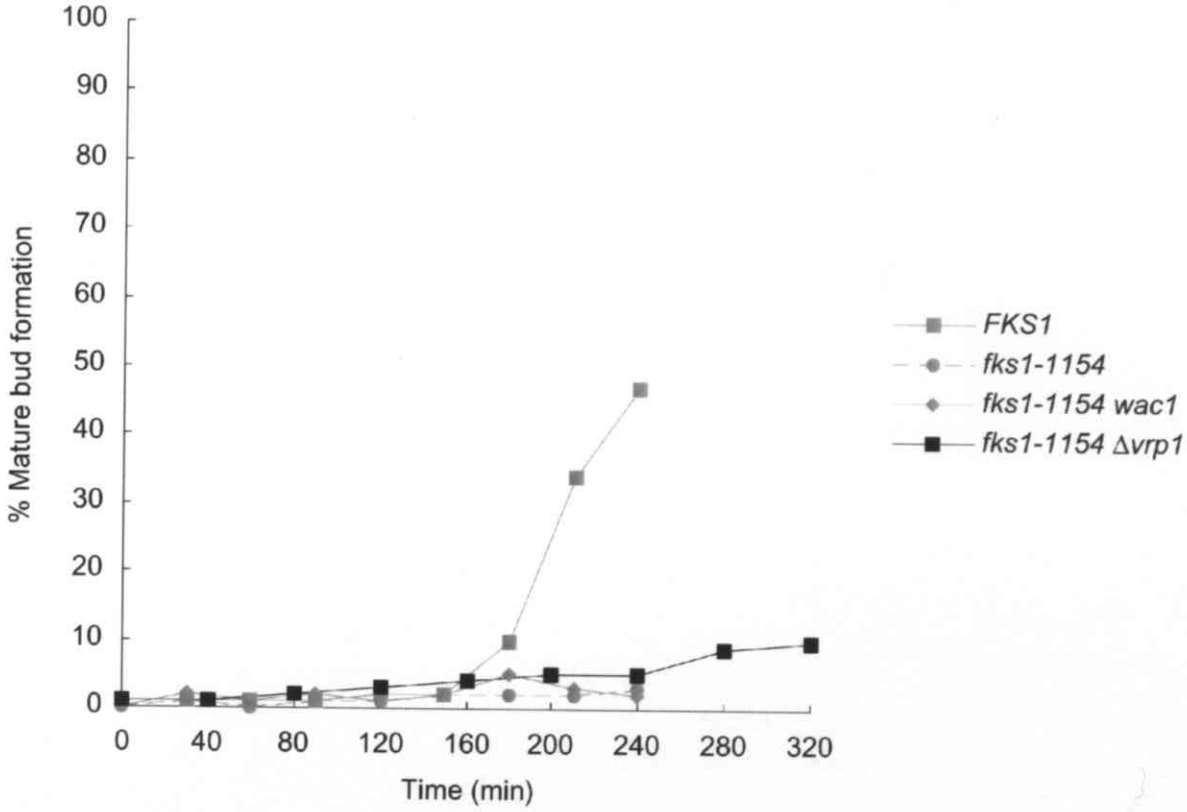
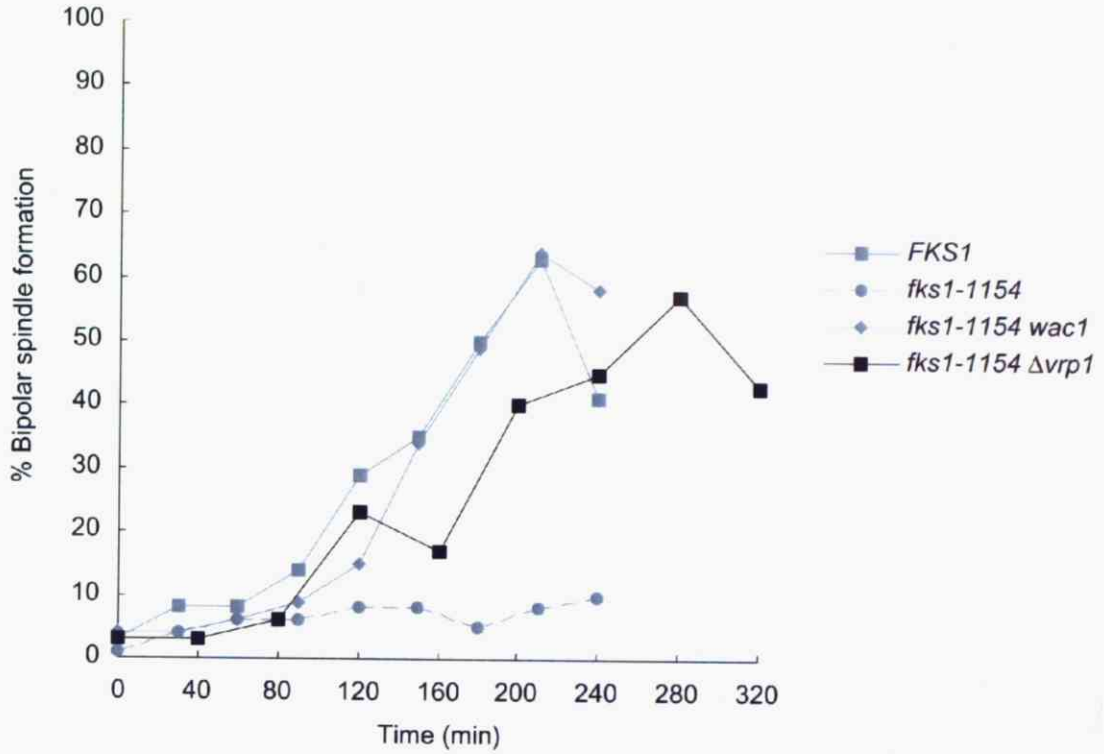


Figure 12. Cells with a small bud are accumulated in *fks1-1154 Δvrp1*.

Small G1 cells of *FKS1*, *fks1-1154*, *fks1-1154 wac1* and *fks1-1154 Δvrp1* were isolated by centrifugal elutriation, and incubated in YPD at 37 °C. At the indicated times, cells were fixed. The percentage of cells with a mature bud was quantitated. More than 200 cells were counted in each strain.

(a)



(b)

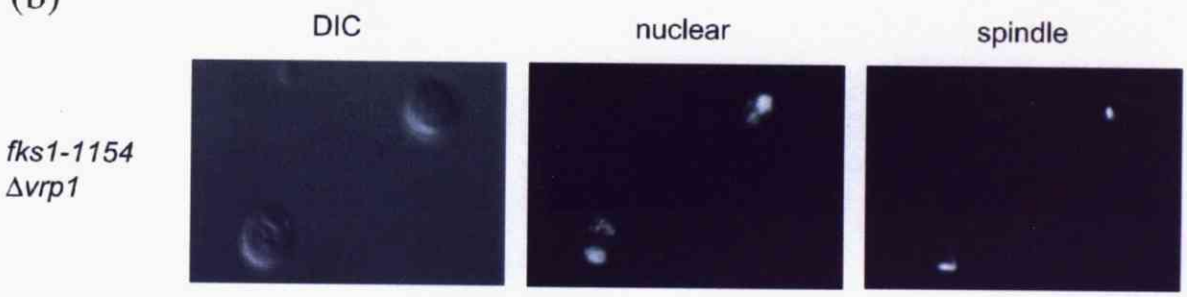


Figure 13. Cells of *fks1-1154 Δvrp1* override the cell wall integrity checkpoint.

Small G1 cells of *FKS1*, *fks1-1154*, *fks1-1154* wac1 and *fks1-1154 Δvrp1* were isolated by centrifugal elutriation, and incubated in YPD at 37 °C. At the indicated times, cells were fixed and stained with DAPI and the anti-tubulin anti-body. The percentage of cells with bipolar spindles was quantitated (a). More than 200 cells were counted in each strain. Nuclear and spindle morphologies of cells of indicated strains at 280 min after release into YPD are shown in (b).

Table 1. Strains used in this study

Strain	Genotype	Description	Reference
YOC1001	*FKS1*	*MAT***a** *fks1::HIS3 fks2::LYS2 ade3::FKS1::TRP1*	Sekiya-Kawasaki *et al.*
YOC1087	*fks1-1154*	*MAT***a** *fks1::HIS3 fks2::LYS2 ade3::fks1-1154::TRP1*	Sekiya-Kawasaki *et al.*
YOC2857	*FKS1 wac1*	*MAT***a** *fks1::HIS3 fks2::LYS2 ade3::FKS1::TRP wac1*	Suzuki *et al.*
YOC2858	*fks1-1154 wac1*	*MAT***a** *fks1::HIS3 fks2::LYS2 ade3::fks1-1154::TRP wac1*	Suzuki *et al.*
YOC3916	*FKS1/FKS1*	*MAT***a/α** *fks1::HIS3 fks2::LYS2 ade3::FKS1::TRP1*	This study
YOC3924	*fks1-1154 Δsro77*	*MAT***a** *fks1::HIS3 fks2::LYS2 ade3::fks1-1154::TRP1 sro77::CgLEU2*	This study
YOC3926	*fks1-1154 Δend3*	*MAT***a** *fks1::HIS3 fks2::LYS2 ade3::fks1-1154::TRP1 end3::CgLEU2*	This study
YOC3927	*FKS1 Δbzz1*	*MAT***a** *fks1::HIS3 fks2::LYS2 ade3::FKS1::TRP1 bzz1::CgLEU2*	This study
YOC3928	*fks1-1154 Δbzz1*	*MAT***a** *fks1::HIS3 fks2::LYS2 ade3::fks1-1154::TRP1 bzz1::CgLEU2*	This study
YOC3930	*fks1-1154 Δrho2*	*MAT***a** *fks1::HIS3 fks2::LYS2 ade3::fks1-1154::TRP1 rho2::CgLEU2*	This study
YOC4133	*FKS1 Δsmi1*	*MAT***a** *fks1::HIS3 fks2::LYS2 ade3::FKS1::TRP1 smi1::CgLEU2*	This study
YOC4134	*FKS1 Δgon7*	*MAT***a** *fks1::HIS3 fks2::LYS2 ade3::FKS1::TRP1 gon7::CgLEU2*	This study
YOC4135	*fks1-1154 Δvrp1*	*MAT***a** *fks1::HIS3 fks2::LYS2 ade3::FKS1::TRP1 vrp1::CgLEU2*	This study

Table 2. Plasmids used in this study

Name	Description	Source
PBS-Cg-LEU2	**Candida Glablatag LEU2**	S. Nogami
PBS-Cg-URA3	*Candida Glablatag URA3*	S. Nogami

Table 3. Oligonucleotides used in this study

Name	Sequence (5'→3')
sro77-F	AAAAGTTACAAAATTGTCAATTGAACAGATACAAAAATTTTATAATCGAGGTCG ACGGTATC
sro77-R	CGAGCAAGGGCAACCTGATCGAATTTTATAAAAACTATTTATGGACGCTCTAGA ACTAGTGGATC
SRO77-OF	TCCGGAAGATACATCATATT
SRO77-OR	CTTTCCTATAGTTGTTTAAT
end3-F	AGTGGGTATTGGAAAGGCCGGTAAAGATAACAGGGATCTCTGAAAATCGAGGT CGACGGTATC
end3-R	ACAAACAGTAAATATTACACATTCATGTACATAAAATTAATTATCCGCTCTAGAA CTAGTGGATC
END3-OF	TCTTGAAATCGCTTAAATCA
END3-OR	TAATCGATGAAAACGTGTTT
bzz1-F	AATCTATCCTTAAACGCCAACTACTACATTACTTGCAATAAAAATGTCGAGGTCG ACGGTATC
bzz1-R	CGGCCAGGGAAAATATTTAATAGTTTCAGTTCATTCCTTCGTTCCGCTCTAGAAC TAGTGGATC
BZZ1-OF	TCCCTTTGCAATTGTTTGTC
BZZ1-OR	ATTGAACAAGTCTTGGAAAT
gon7-F-3	ATATATACAGCCGATAGTGCACTGGAACCTTATCCTAATATCTCACCATGATTACG CCAAGCTCG
gon7-R-3	TCTTTTCTGTTTGTATATACACTCTCTAGCGCTACATATCGTTGCGAGCTCCACCG CGGTGGCGG
GON7-OF	TCAACCTGGTAATTGTTCAC
GON7-OR	AATCAACCGGCTGGAGGCAG
rho2-F	AATTGCTGAAACGTTCTGCTTTGGTTGTGCTTTTGATCCCGTACTTCGAGGTCGA CGGTATC
rho2-R	TCCCTTGCTAAAAAGATAATGTATCATTTCAGTGTAAGTTTTTTCGCTCTAGAAC TAGTGGATC
RHO2-OF	TCAAGGCCTCATACCTCCAC
RHO2-OR	GGTTTGCTCTACGACGTGGT
smi1-F	CATTCGTTCTATAGGCGACAACTTGGCAGAATTTTTTAGTATAAATCGAGGTCGA CGGTATC
smi1-R	ATTGTCCAAATTTCTTTTTGGTTGTAGAATGTTAATTGTGATATTCGCTCTAGAAC TAGTGGATC

(continued)

Name	Sequence (5'→3')
SMI1-OF	AATATTGAAATATTTGCAAA
vrp1-F	ACCGTTTGTTGAAGAAGCCTACCATATATATAACGAATTGTTGAATCGAGGTCGA CGGTATC
vrp1-R	AATGCTCACGTAAATAATGTTAAGTCCAATGGCACACTACTACCCCGCTCTAGAA CTAGTGGATC
VRP1-OF	TAAATGTAGATATTGCCCTG
VRP1-OR	TCCTCTTTTCGGACGTTGGT

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Possible involvement of Bzz1p in the cell wall integrity checkpoint

（出芽酵母の細胞壁チェックポイントにおける Bzz1p の働きに関する研究）

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序論

細胞が正確に自己複製をおこない、生存可能な次世代の細胞を生み出すためには、細胞周期の事象が適切な時期に正確に起きる必要がある。細胞周期の事象の適切な順序を保証し監視するための機構は細胞周期チェックポイントと呼ばれ、細胞周期のある事象に異常が起こった時、異常が修正されその事象が完了するまで細胞周期の進行を停滞させることによって細胞の生存を保証している。出芽酵母では DNA 損傷や DNA 複製、アクチン骨格、スピンドル形成などのチェックポイントが明らかにされてきたが、当研究室はこれら既知のチェックポイントとは異なる、細胞壁の異常をモニターする新規チェックポイント機構の存在を明らかにした (Suzuki *et al.*, 2004)。

細胞壁チェックポイントは、細胞壁の合成阻害が起きると細胞周期を G2 期で停止し、生存率を維持する。このとき、M 期に進行したことを示す目印ともなる紡錘体（スピンドル）の形成が阻害される。一方、チェックポイントに欠損を持つ株では細胞壁合成阻害下でも紡錘体が形成され細胞周期が異常に進行する結果として生存率の低下が起きることをすでに報告している。この細胞壁チェックポイント機構にはダイナクチン複合体 (Arp1p, Jnm1p, Nip100p) がフォークヘッド転写因子 Fkh2p を介しサイクリン Clb2p の発現を制御していることが確認されている。こうして断片的にチェックポイントに関与する因子は明らかにされつつあるが、シグナル伝達経路の全体像を解明するためには新たな構成因子の発見が必要である。そこで本研究では、ダイナクチン複合体と物理的に作用し、かつ細胞膜など細胞表層に局在するタンパクに注目し、これらが細胞壁チェックポイントに関与するかを解析した。

結果と考察

1. 細胞壁チェックポイントにおける新規因子探索のためのストラテジー

我々はチェックポイントの構成因子の一部であるダイナクチン複合体と物理的に相互作用するタンパク質に注目した。ダイナクチン複合体の構成因子 Arp1p, Jnm1p, Nip100p と物理的に相互作用するタンパク質は、網羅的な研究から 22 個が現在までに報告されている (Uetz *et al.*, 2000; Ito *et al.*, 2001; Hazbun *et al.*, 2003; Helen *et al.*, unpublished)。これらの中には細胞膜付近に局在する Bzz1p, Sro77p, Rho2p, End3p, Smi1p, Gon7p が含まれていた。私はこれらの中に細胞壁チェックポイントに関与する因子が含まれている可能性が高いと考え、まずこれらの遺伝子の欠損株が細胞壁チェックポイントに欠損を示すか検証した。

2. Bzz1p は細胞壁チェックポイントに関与する。

1.で注目したタンパク質が細胞壁合成チェックポイントに関与するかを調べるために、*fks1-1154*株（細胞壁の主要な構成成分である1,3-β-グルカン合成酵素の触媒サブユニット、*FKS1* 遺伝子の温度感受性変異株）のバックグラウンドで*BZZ1*, *SRO77*, *RHO2*, *END3*遺伝子をそれぞれ破壊した。*fks1-1154*株を制限温度下におくと芽の形成が抑えられ、チェックポイントが働いて紡錘体形成前で細胞周期を停止する。*fks1-1154 Δrho2*株はこれまでに報告されている*fks1-1154*株の表現型と同様に制限温度下で成熟した芽を形成せず、紡錘体の形成も見られなかった。これに対して、*fks1-1154 Δbzz1*株は制限温度下で成熟した芽を形成できないのにも

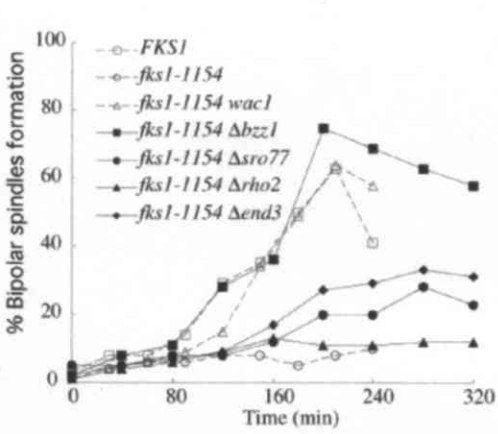
関わらず紡錘体を形成した。このことは*bzz1*変異株では細胞壁チェックポイントが欠損していることを示すものである。(図1)。また*fks1-1154 Δend3*と*fks1-1154 Δsro77*株は30%程度の細胞が紡錘体を形成し、細胞壁チェックポイントに部分的な欠損を示す可能性があることが明らかになった。

*SMI1*と*GON7*については、*fks1-1154*株中で遺伝子を破壊することができなかったので、野生型である*FKS1*株中で遺伝子を破壊し、細胞壁合成阻害剤であるEchinocandin Bを用いることで細胞壁合成を停止させ、細胞壁チェックポイントに欠損を示すか調べた。その結果、*SMI1*と*GON7*は細胞壁の合成阻害下で紡錘体を形成せず細胞壁合成チェックポイントに関与しないことが示された。(図２)

fks1-1154 Δbzz1 株の表現型をさらに確認するために、*BZZ1* 遺伝子を *FKS1* 株中で破壊し、Echinocandin B を用いて同様の実験を行った。その結果、*FKS1 Δbzz1* 株では Echinocandin B 存在下でも紡錘体を形成し、細胞壁チェックポイントに欠損を示した。この結果から、Bzz1p が細胞壁チェックポイントに関与していることが強く示唆された。(図２)

3. Las17p 複合体も細胞壁チェックポイントに関与する可能性がある。

出芽酵母の Bzz1p は Las17p と物理的に相互作用し、Las17p 複合体を介してアクチンの重合を正に制御していると考えられている（Soulard, *et al.*, 2002 & 2005)。そこで私は Bzz1p が Las17p 複合体に依存的に細胞壁チェックポイントに関与しているのかを調べるため、Las17p 複合体の主要な構成因子である *VRP1* (verprolin)遺伝子を *fks1-1154* 株中で破壊し、細胞壁チェックポイントに関与するかを解析した。この結果、*fks1-1154 Δvrp1* 株は制限温度下で紡錘体を形成し、細胞壁チェックポイントに欠損を示すことが明らかになった。この結果は、Las17p 複合体も細胞壁チェックポイントに関与していることを示唆している。



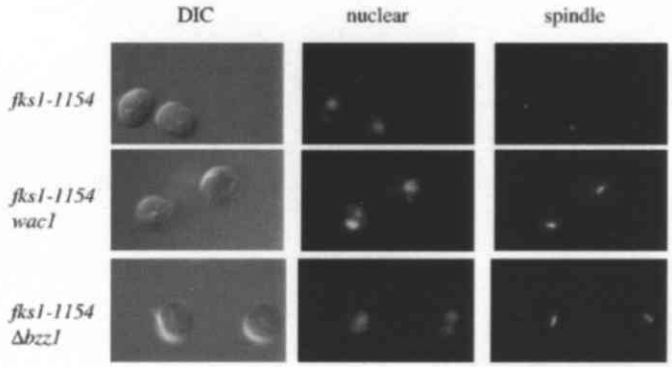
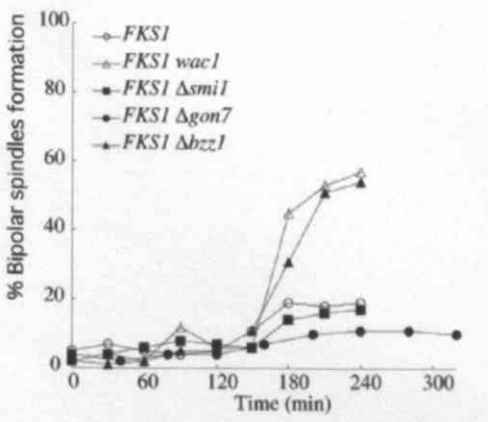


図１. 各変異株の制限温度下における紡錘体を形成した細胞の割合（左）と*fks1-1154*, *fks1-1154 wac1*株は同調後210分、*fks1-1154 Δbzz1*株は同調後240分の写真（右）。



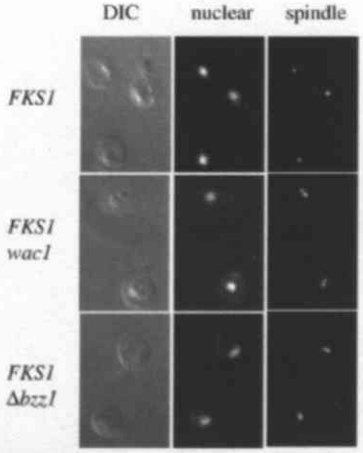


図２. Echinocandin Bを終濃度 8 μg/mlで処理したときの各変異株の紡錘体を形成した細胞の割合（左）と*FKS1*, *FKS1 wac1*株は同調後180分、*FKS1 Δbzz1*株は同調後210分の写真（右）。