# A role for the Pkc1p/Mpk1p kinase cascade in the morphogenesis checkpoint

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**In many cells the timing of entry into mitosis is controlled by the balance between the activity of inhibitory Wee1-related kinases (Swe1p in budding yeast) and the opposing effect of Cdc25-related phosphatases (Mih1p in budding yeast) that act on the cyclin-dependent kinase Cdc2 (Cdc28p in budding yeast)[1]. Wee1 and Cdc25 are key elements in the G2 arrest mediated by diverse checkpoint controls[2]. In budding yeast, a 'morphogenesis checkpoint' that involves Swe1p and Mih1p delays mitotic activation of Cdc28p[3]. Many environmental stresses (such as shifts in temperature or osmolarity) provoke transient depolarization of the actin cytoskeleton, during which bud construction is delayed while cells adapt to environmental conditions. During this delay, the morphogenesis checkpoint halts the cell cycle in G2 phase until actin can repolarize and complete bud construction, thus preventing the generation of binucleate cells[4]. A similar G2 delay can be triggered by mutations or drugs that specifically impair actin organization[5], indicating that it is probably actin disorganization itself, rather than specific environmental stresses, that triggers the delay. The G2 delay involves stabilization of Swe1p in response to various actin perturbations[6], although this alone is insufficient to produce a long G2 delay[7].**

Conserved protein-kinase cascades involving mitogen-activated protein kinases (MAPKs) are commonly involved in signal-transduction pathways that link intracellular responses (including decisions to halt or proceed with the cell cycle) to extracellular signals[8]. These cascades involve sequential phosphorylation and activation of three kinases (MAPK kinase kinase (MAPKKK), MAPKK and MAPK), which culminates in phosphorylation of transcription factors and other targets[9]. The yeast genome encodes five MAPK homologues, four of which are expressed during vegetative growth (reviewed in ref. 10). We tested strains lacking each of these MAPKs to determine whether they function in the morphogenesis-checkpoint pathway. To monitor checkpoint function, we inoculated G1-arrested stationary-phase cells into fresh medium (stimulating them to re-enter the cell cycle) in the presence of the actin-depolymerizing drug Latrunculin-B (to prevent bud formation). Under these circumstances, wild-type cells arrested in G2 whereas *swe1Δ* mutants failed to arrest, proceeded through mitosis without forming buds, and became binucleate[5,11]. We then used the proportion of binucleate cells to calculate a 'percentage checkpoint defect' (*swe1Δ* mutants were designated as 100% defective; see Methods).

Deletion of *FUS3*, *KSS1* or *HOG1* had no effect on checkpoint function, but deletion of *MPK1*(also called *SLT2)* severely impaired the G2 arrest of cells exposed to Latrunculin-B (Fig. 1a). *MPK1* acts in the 'cell-integrity' pathway, and *mpk1* mutants exhibit a temperature-dependent cell-lysis phenotype[12]. Activation of Mpk1p has been detected in a variety of situations (for example, upon heat shock or hypotonic shock) that are thought to stress the plasma membrane or cell wall[13,14]. Increased external osmolarity (which is thought to help to protect the plasma membrane by reducing the turgor pressure pushing it against the cell wall) reduces or eliminates Mpk1p activation in response to such stresses, and protects *mpk1* mutants from lysis[12]. Cells lacking Mpk1p were not completely checkpoint-defective, as they could still induce partial Cdc28p tyrosine phosphorylation (to about half of wild-type levels; Fig. 1b) and still exhibited a small residual G2 delay (Fig. 1c) upon actin perturbation.

Mpk1p is activated by dual phosphorylation at threonine and tyrosine residues, which can be monitored using a phosphospecific antibody that recognizes only doubly phosphorylated (active) Mpk1p[14]. Mpk1p phosphorylation was markedly induced upon actin depolymerization, to a level comparable to or greater than that induced by heat shock (Fig. 2a). This effect was only partially suppressed when cells were grown in medium containing osmotic stabilizers (Fig. 2a). Mpk1p phosphorylation was accompanied by Mpk1p kinase activation, and was not due to an increase in the overall abundance of Mpk1p (Fig. 2b). Activation by Latrunculin-B occurred in a dose-dependent manner that paralleled the dose-dependent checkpoint response[5] (Fig. 2c). Because Mpk1p activity can change as a function of cell-cycle progression[15,16], we considered the possibility that Mpk1p activation is a secondary consequence of the checkpoint-induced cell-cycle arrest. However, Mpk1p was induced in a similar manner in both *swe1Δ* cells (which fail to delay the cell cycle[5]) and wild-type cells (Fig. 2d), showing that Mpk1p is induced in response to actin stress and not as a consequence of cell-cycle arrest. Together, these results show that Mpk1p is activated in response to actin depolymerization, and is required to trigger the G2 arrest induced by the morphogenesis checkpoint.

Previous studies have identified many components of the cell-integrity pathway (Fig. 3a), including the putative plasma-membrane 'stress sensors' Wsc1p–Wsc3p and Mid2p, which are thought to act through the guanine nucleotide exchange factor Rom2p to promote GTP loading of the small G protein Rho1p, which binds to and activates the kinase Pkc1p (homologous to vertebrate protein kinase C). Pkc1p then activates the MAPK module that consists of the MAPKKK Bck1p, the redundant MAPKKs Mkk1p and Mkk2p, and finally Mpk1p. Mpk1p promotes cell integrity by switching on a range of cell-wall regulatory genes through the transcription factors Rlm1p and Swi4p/Swi6p (reviewed in refs 10, 12). We tested the ability of mutants lacking elements at every step of the pathway to arrest the cell cycle in response to actin depolymerization (Fig. 3b). The pathway components from Rho1p to Mpk1p were all required for an effective checkpoint response (Fig. 3b), and were essential for activation of Mpk1p in response to actin depolymerization (Fig. 3c). However, neither the upstream

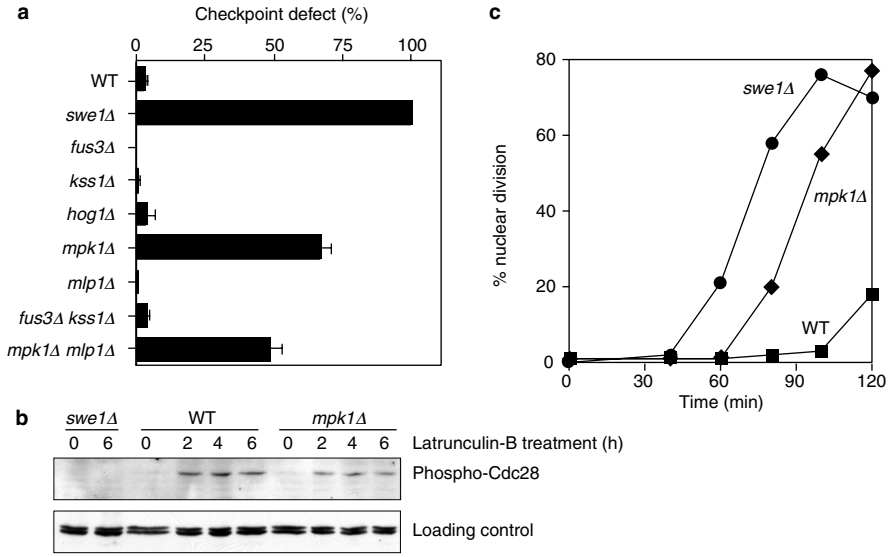


Figure 1 **Checkpoint defect in *mpk1Δ* cells. a**, The effectiveness of the morphogenesis-checkpoint-mediated G2 delay in response to actin perturbation was assayed (see Methods) in the following strains: WT, DLY1; *swe1Δ*, DLY4021; *fus3Δ*, K2297; *kss1Δ*, K2306; *hog1Δ*, E929-6C-79; *mpk1Δ*, DLY4070; *mlp1Δ*, DLY1040; *fus3Δ kss1Δ*, K2314; *mpk1Δ mlp1Δ*, DLY1041. Cells lacking both Fus3p and Kss1p were tested because these kinases can have redundant roles in the pheromone response[10]. Mlp1p (YKL161c) is 53% identical to Mpk1p but contains several altered residues that make it highly unlikely to possess intrinsic kinase activity[32]. Deletion of *MLP1* did not impair checkpoint function or further exacerbate the checkpoint defect of *mpk1Δ* mutants. **b**, Cells of the indicated strains were treated with 100 μM Latrunculin-B for the indicated times, and were then lysed and processed to monitor tyrosine-19 phosphorylation of Cdc28p. **c**, Cells of the indicated strains were synchronized in G1 phase using α-factor and released into fresh medium containing 100 μM Latrunculin-B. Nuclear division was scored by microscopic analysis (see Methods). The 20-min cell-cycle delay observed in *mpk1Δ* cells compared with *swe1Δ* cells is partly the result of a 10-min delay in recovery from pheromone arrest (data not shown) and partly of a 10-min residual checkpoint delay.

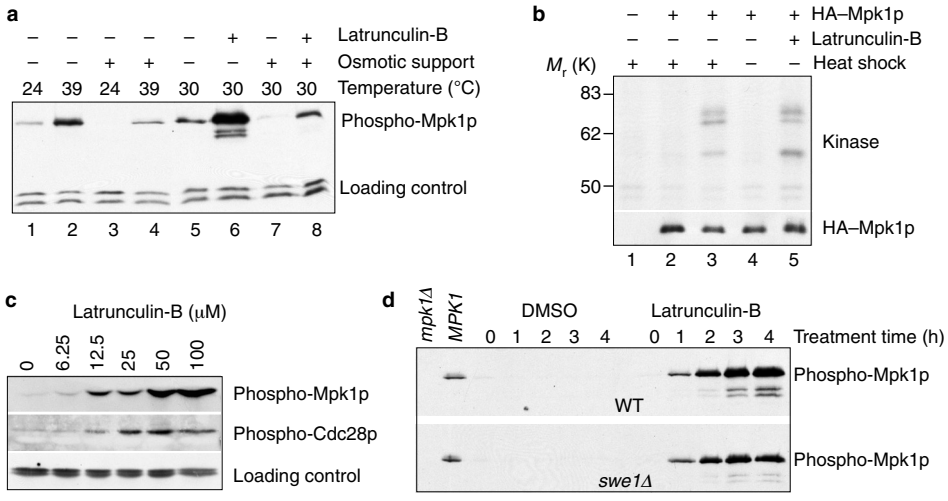


Figure 2 **Activation of Mpk1p in response to actin perturbation. a**, Asynchronous cultures of wild-type cells (DLY1) were grown in YEPD in the absence (lanes 1, 2, 5 and 6) or presence (lanes 3, 4, 7 and 8) of 0.4 M NaCl at 24 °C (lanes 1–4) or 30 °C (lanes 5–8). Samples were subjected to heat shock (40 min at 39 °C, lanes 2 and 4) or treatment with Latrunculin-B (100 μM for 2 h, lanes 6 and 8). Activating Mpk1p phosphorylation was monitored by western blotting (see Methods). **b**, Cells of strains DLY1 (lane 1), DLY4671 (*mkk1Δ mkk2Δ MPK1::HA*; lane 2) and DLY4233 (*MPK1::HA*; lanes 3–5) were subjected to heat shock (40 min at 39 °C, lanes 1–3) or treatment with vehicle (1% DMSO, lane 4) or Latrunculin-B (100 μM for 2 h, lane 5); they were then lysed and Mpk1p–HA was immunoprecipitated. Mpk1p-associated kinase activity against co-precipitated substrates[16] was assayed and total Mpk1p–HA levels were monitored (see Methods). **c**, Asynchronous cultures of wild-type cells (DLY1) were grown in YEPD supplemented with 0.4 M NaCl at 30 °C and treated with the indicated concentrations of Latrunculin-B for 2 h. Mpk1p phosphorylation (upper panel) and Cdc28p tyrosine phosphorylation (middle panel) were monitored using appropriate phospho-specific antibodies (see Methods). Loading control: anti-PSTAIRE antibody (recognizes Pho85p and Cdc28p). **d**, Wild-type (DLY1; upper panel) and *swe1Δ* (DLY4021; lower panel) strains were grown to exponential phase in YEPD supplemented with 0.4 M NaCl, and treated with 1% DMSO or 100 μM Latrunculin B as shown for the indicated times; Mpk1p phosphorylation was monitored as above. Left-most lanes contain lysates from *mpk1Δ* (DLY4070; lane 1) or wild-type (DLY1; lane 2) strains grown to exponential phase in YEPD with no added osmotic support at 30 °C, and provide controls for the mobility of Mpk1p.

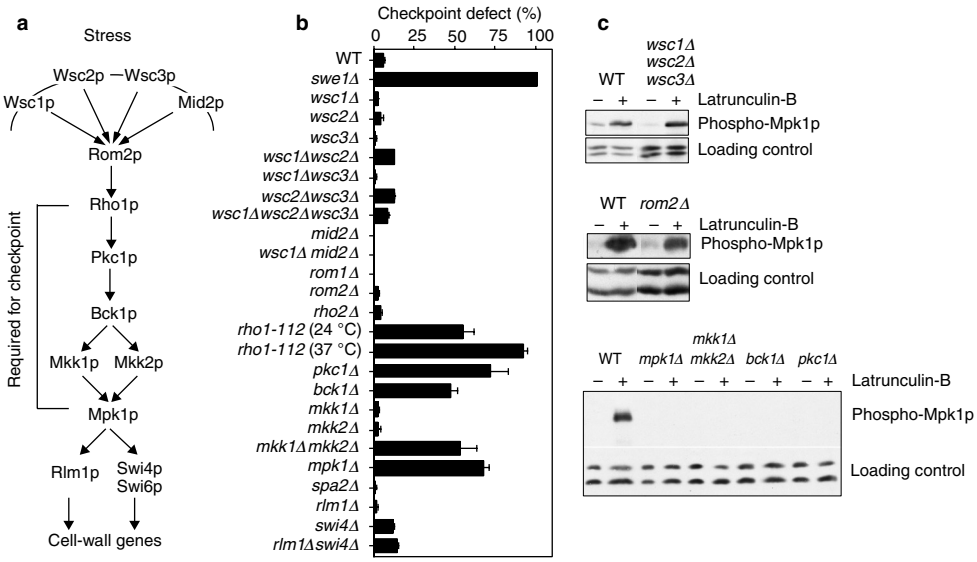


Figure 3 **Role of components of the cell-integrity pathway in the morphogenesis checkpoint. a**, Schematic representation of the cell-integrity pathway. Links between putative membrane sensors (particularly Mid2p) and Rom2p are speculative. **b**, Checkpoint defects were assayed (see Methods) in the following strains: WT, DLY1; *swe1Δ*, DLY4021; *wsc1Δ* ALH7; *wsc2Δ*, ALH18; *wsc3Δ*, ALH15; *wsc1Δ wsc2Δ*, ALH718; *wsc1Δ wsc3Δ*, ALH715; *wsc2Δ wsc3Δ*, ALH1518; *wsc1Δ wsc2Δ wsc3Δ*, ALH758; *mid2Δ*, DLY4533; *wsc1Δ mid2Δ*, DLY4545; *rom1Δ*, DLY4653; *rom2Δ*, DLY4500; *rho2Δ*, DLY4497; *rho1-112*, YOC756; *pkc1Δ*, DLY3962; *bck1Δ*, DLY3994; *mkk1Δ*, DLY468; *mkk2Δ*, DLY469; *mkk1Δ mkk2Δ*, DLY 4351; *mpk1Δ*, DLY4070; *spa2Δ*, JMY1030; *rlm1Δ*, DLY4505; *swi4Δ*, DSY12; *rlm1Δ swi4Δ*, DLY3992. The *rho1-112* allele (which contains a C112R substitution) was generated by random mutagenesis[33]. The published *rho1-5* allele[33] was also tested and did not exhibit a checkpoint defect. This list also includes mutants that lack Rom1p (an exchange factor that is homologous to Rom2p[34]), Rho2p (a small GTPase that is thought to act downstream of Rom1p and Rom2p but which has no known effectors) and Spa2p (which is thought to act as a scaffold for Mpk1p-pathway kinases[35]), which are not shown in **a** because their relationship (if any) to the cell-integrity pathway remains unclear. The list does not include *wsc1Δ wsc2Δ wsc3Δ mid2Δ* or *rom1Δ rom2Δ* mutants because these segregants were not obtained from the appropriate crosses (indicating the synthetic lethality of these mutant combinations). **c**, The indicated strains were grown to exponential phase in YEPD supplemented with 0.4 M NaCl and treated with 1% DMSO (–) or 100 μM Latrunculin-B (+) for 2 h. Mpk1p phosphorylation was monitored (see Methods).

putative membrane stress sensors nor Rom2p was required for checkpoint function (Fig. 3b) or Mpk1p activation (Fig. 3c). A caveat to this is that certain combinations of mutations were lethal even in osmotically supplemented medium, precluding analysis of those strains (see Fig. 3). In addition, Rlm1p and Swi4p were not required, either singly or in combination, for checkpoint-mediated cell-cycle arrest (Fig. 3b). Thus, whereas the core cell-integrity pathway from Rho1p to Mpk1p is required for checkpoint signalling, the known elements that are thought to act upstream of Rho1p and downstream of Mpk1p seem to be dispensable. This indicates that the core pathway may be accessible through distinct upstream components in response to actin depolymerization, and may promote cell-cycle arrest through distinct downstream targets.

The most obvious potential downstream target of Mpk1p is Swe1p, which is stabilized in response to actin perturbation and mediates the checkpoint delay through phosphorylation and inhibition of Cdc28p[6]. If Mpk1p were specifically required for Swe1p stabilization during a checkpoint response (Fig. 4, hypothesis I), then cells in which Swe1p had been stabilized by other means should no longer require Mpk1p for checkpoint function. The *HSL1* and *HSL7* genes are required to target Swe1p for degradation (so that Swe1p is stabilized in cells that lack either of these genes even when actin is not perturbed)[7]. However, stabilizing Swe1p by deletion of *HSL1* or *HSL7* did not restore checkpoint function to *mpk1Δ* mutants (Fig. 4), showing that Mpk1p must function in a parallel pathway to Swe1p stabilization to promote G2 arrest.

Whereas neither stabilization of Swe1p nor elimination of Mih1p (the phosphatase that counteracts Swe1p by dephosphorylating Cdc28p) is itself sufficient to promote a significant G2 delay, simultaneous stabilization of Swe1p and elimination of Mih1p causes G2 arrest[7], indicating that downregulation of Mih1p is an

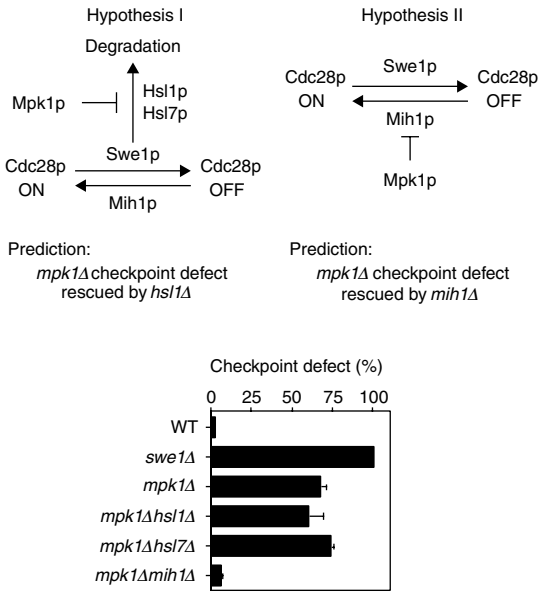


Figure 4 **Genetic interaction of *MPK1* with cell-cycle control elements.** Hypotheses (left and middle) indicate potential ways in which Mpk1p could affect Cdc28p regulators, and predictions from these hypotheses are indicated below. Right, checkpoint defect were assayed (see Methods) in the following strains: WT, DLY1; *swe1Δ*, DLY4021; *mpk1Δ*, DLY4070; *mpk1Δ hsl1Δ*, DLY4075; *mpk1Δ hsl7Δ*, DLY4077; *mpk1Δ mih1Δ*, DLY4074.

effective mechanism when it occurs in parallel with Swe1p stabilization. If Mpk1p acted specifically to downregulate Mih1p (Fig. 4, hypothesis II), cells in which *MIH1* had been deleted would no longer require Mpk1p for checkpoint function. This prediction was confirmed by the fact that the *mpk1Δ mih1Δ* double mutant showed no checkpoint defect (Fig. 4). Thus, although other alternative hypotheses are not excluded, Mpk1p may function to downregulate Mih1p during the checkpoint response.

We have investigated the roles of MAPKs in the morphogenesis checkpoint of budding yeast. We have shown that kinases of the cell-integrity pathway are all required for full checkpoint function, whereas other yeast MAPKs are not. In principle, Mpk1p could be constitutively required for the synthesis or activity of checkpoint components. However, the morphogenesis checkpoint was fully functional in cells that were grown in medium with increased osmolarity, which suppresses the constitutive activity of Mpk1p to undetectable levels. In addition, Mpk1p itself was activated in response to actin depolymerization. Thus, it is likely that Mpk1p has a direct role in transmitting a signal from the actin cytoskeleton to the cell-cycle machinery. □

## Methods

### Yeast media and cell synchrony.

Yeast media (YEPD rich medium, synthetic medium lacking specific nutrients, and sporulation medium) have been described[17]. Synchronous release of cells from α-factor arrest was carried out as described[11].

### Strains, plasmids and PCR manipulations.

Standard media and methods were used for plasmid manipulations[18] and yeast genetic manipulations[17]. The *Saccharomyces cerevisiae* strains and oligonucleotides used in this study are listed in Supplementary Information.

The *hog1-Δ1::TRP1* (ref. 19), *mih1::LEU2* (ref. 20), *hsl1Δ1::URA3* (ref. 21), *hsl7Δ1::URA3* (ref. 21), *swi4::LEU2* (ref. 22), *mkk1Δ::LEU2* (ref. 23), *mkk2Δ::URA3* (ref. 24) and *spa2-Δ3::URA3* (ref. 25) alleles were generated by one-step gene replacement as described. Strains DLY468, DLY469 and DLY4351 are all descended from strain BB108 (ref. 24; a gift from B. Errede).

The *bck1ΔLEU2* (primers bck1Δ5′ and bck1Δ3′), *mid2Δ::kan* (primers mid2Δ5′ and mid2Δ3′), *mlp1Δ::kan* (primers mlp1Δ5′ and mlp1Δ3′), *mpk1ΔTRP1* (primers mpk1Δ5′ and mpk1Δ3′), *rho2Δ::kan* (primers rho2Δ5′ and rho2Δ3′), *rlm1Δ::kan* (primers rlm1Δ5′ and rlm1Δ3′) *rom1Δ::kan* (primers rom1Δ5′ and rom1Δ3′), *rom2Δ::hyg* (primers OJ128 and OJ129)) and *swe1ΔTRP1* (primers OJ43 and OJ44) alleles were constructed by polymerase chain reaction (PCR)[26], using plasmids pRS304 (*TRP1*; ref. 27), pRS305 (*LEU2*; ref. 27), pFA6a-kanMX6 (*kan*; ref. 28) or pAG32 (*hyg*; ref. 29) as a template. Gene disruptions were confirmed by whole-cell PCR of transformant colonies. Primers BCK1-1 and BCK1-2 generate a 0.5-kilobase (kb) PCR product from genomic DNA of wild-type, but not *bck1ΔLEU2*, cells. Primers mid2Δ3′ and MID2 tester generate a 1.8-kb PCR product from genomic DNA of *mid2Δ::kan* but not wild-type cells. Primers mlp1Δ3′ and MLP1-200 generate a 1.7 kb PCR product from genomic DNA of *mlp1Δ::kan*, but not wild-type, cells. Primers MPK1 5′ untranslated region and OJ55 (internal to *TRP1*) generate a 0.8-kb PCR product from genomic DNA of *mpk1ΔTRP1*, but not wild-type, cells. Primers rho2Δ3′ and RHO2 tester generate a 1.8-kb PCR product from genomic DNA of *rho2Δ::kan*, but not wild-type, cells. Primers rlm1Δ3′ and RLM1 tester generate a 1.8-kb PCR product from genomic DNA of *rlm1Δ::kan*, but not wild-type, cells. Primers rom1Δ3′ and ROM1 tester generate a 1.6-kb PCR product from genomic DNA of *rom1Δ::kan*, but not wild-type, cells. Primers OJ129 and ROM2 tester generate a 2.5-kb PCR product from genomic DNA of *rom2Δ::hyg*, but not wild-type, cells. Primers OJ21 (upstream of *SWE1*) and OJ55 generate a 1.1-kb PCR product from genomic DNA of *swe1ΔTRP1*, but not wild-type, cells.

The *pkc1ΔLEU2* construct was made by replacing the 1.3-kb internal *Stu*I fragment of *PKC1* with a 2-kb *Hpa*I *LEU2* fragment in YIplac128 (ref. 30) carrying the 5-kb *Sph*I fragment of the *PKC1* locus. This construct was introduced into the diploid strain DLY5 to create the strain DLY3960 (*pkc1ΔLEU2/PKC1*, a gift from N. Marini (Gene Soft Inc., San Francisco, California, USA)).

To create strains with regulatable expression of *PKC1*, the integrating vector YIpG2 (ref. 31) was used to express *PKC1* under control of the *GAL1* promoter. The *LEU2* gene in YIpG2 was disrupted and replaced with *TRP1* by inserting a blunt-ended *TRP1* fragment into the *Eco*RV site in *LEU2*, generating the plasmid YIpG2(*TRP1*). The 2.3-kb *Eco*RI fragment of *PKC1* was amplified by PCR with 5′-*Mlu*I and 3′-*Bgl*II ends. This fragment was cloned into the *Mlu*I–*Bam*HI sites of YIpG2(*TRP1*). The resulting construct was transformed into DLY3960, and transformants were subjected to sporulation. A $Trp^+$ $Leu^+$ spore was selected, which grows only on galactose; this strain is DLY3962 (a gift from N. Marini).

To monitor expression of Mpk1p, a 2.3-kb *Eco*RI–*Sal*I fragment carrying *MPK1::HA* and the *MPK1* promoter from YEp352*MPK1::HA* (ref. 13) was ligated into the *Eco*RI and *Sal*I sites of YIplac128 (ref. 30). The resulting plasmid was ligated with *Bst*EII to target integration at the chromosomal *leu2* locus.

### Morphogenesis-checkpoint assay.

Cells were grown for 2 days at 24 °C in YEPD in the presence or absence of osmotic stabilizers (1 M Sorbitol or 0.4 M NaCl) as indicated. Stationary-phase cells were counted using a haemacytometer and diluted to $10^7$ cells per ml with fresh medium containing either 100 μM Latrunculin B (BioMol Research Laboratories Inc., Plymouth Meeting, Pennsylvania) or 1% dimethylsulphoxide (DMSO; vehicle control). After growth at 24 °C for roughly two cell cycles (6–7 h), cells were fixed in 70% ethanol and stained with 4′,6′-diamidino-2-phenylindole (DAPI) for visualization of DNA as described[11]. One hundred unbudded cells were scored as premitotic (single nucleus) or postmitotic (two or more nuclei). Each experiment included a *swe1Δ* strain, and the relative checkpoint defect of each strain was calculated by dividing the proportion of postmitotic cells in that strain by the proportion of postmitotic cells in the *swe1Δ* internal control. This method is based on the finding that *swe1Δ* cells do not significantly delay the cell cycle in response to Latrunculin-B and can therefore be considered to be completely checkpoint-defective[11]. Several strains were tested either with or without osmotic support, and no difference in relative checkpoint defects was observed, although in all strains emergence from stationary phase was somewhat slower in the presence of osmotic support (data not shown).

### Cell lysates, electrophoresis and western blotting.

After exposure to the indicated stresses, cells were rapidly pelleted and stored at –80 °C. Cell lysis, SDS–PAGE and transfer to nitrocellulose membrane (Schleicher and Scheull, Keene, New Hampshire) were carried out as described[7] except that 20 mM NaF were added to the lysis buffer. For detection of diphospho-Mpk1p, a three-antibody protocol was used to enhance sensitivity — rabbit anti-phospho-p44/p42 MAP kinase antibody (New England Biolabs) was used at 1:1000 dilution, followed by mouse anti-rabbit immunoglobulin G (IgG; Jackson ImmunoResearch Laboratories) at 1:5000 dilution, and finally horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson Laboratories) at 1:3000 dilution. Tyrosine-phosphorylated Cdc28p was detected using the same three-antibody protocol, starting with rabbit anti-phospho-Cdc2 (New England Biolabs) at 1:500 dilution. For the loading control, filters were probed using mouse anti-PSTAIRE (which recognizes Pho85p and Cdc28p) as described[6]. Mpk1p–HA was detected using mouse anti–HA (12CA5; Boehringer) at 1:1000 dilution, followed by HRP-conjugated goat anti-mouse IgG (Jackson Laboratories; see above). Antibody incubations, washes, blot development and visualization using enhanced chemiluminescence (NEN Life Sciences Products Inc., Boston, Massachusetts) were carried out as described[6], except that Tris–HCl buffer was used rather than phosphate buffer during antibody incubations.

### Immunoprecipitation and kinase assay.

Lysates were prepared as above, and Mpk1p–HA was immunoprecipitated using the 12CA5 antibody (Boehringer) and Protein A–sepharose beads (Sigma). Immunoprecipitates were washed five times with lysis buffer lacking added protease inhibitors, and kinase assays were carried out essentially as described[11], but without added substrate and with a 10 μM final ATP concentration. Ten per cent of the immunoprecipitate was used for anti-HA western blotting (see above).

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**TABLE 1: Yeast strains used in this study.**

| Strain | Relevant Genotype | Source |
|---|---|---|
| ALHWT[a] | **a** *leu2 ura3 his3 trp1 ade8* | 1 |
| ALH7[a] | **a** *wsc1::ADE8* | 1 |
| ALH15[a] | **a** *wsc3::TRP1* | 1 |
| ALH18[a] | **a** *wsc2::URA3* | 1 |
| ALH715[a] | **a** *wsc1::ADE8 wsc3::TRP1* | 1 |
| ALH718[a] | **a** *wsc1::ADE8 wsc2::URA3* | 1 |
| ALH758[a] | **a** *wsc1::ADE8 wsc2::URA3 wsc3::TRP1* | 1 |
| ALH1518[a] | **a** *wsc2::URA3 wsc3::TRP1* | R. Ballester |
| BF264-15Du | *MAT***a** *ade1 his2 leu2-3,112 trp1-1 ura3Δns* | 2 |
| DLY1 | **a** *bar1* | 3 |
| DLY5 | **a**/a | 3 |
| DLY468 | a *mkk1Δ::LEU2* | This study |
| DLY469 | **a** *mkk2*D*::URA3* | This study |
| DLY1040 | **a** *mlp1Δ::kan* | This study |
| DLY1041 | **a** *mlp1Δ::kan mpk1ΔTRP1* | This study |
| DLY3960 | **a**/a *pkc1ΔLEU2/PKC1* | N. Marini |
| DLY3962 | a *pkc1ΔLEU2 GAL1p-PKC1::TRP1* | N. Marini |
| DLY3992 | **a** *rlm1Δ::kan swi4::LEU2* | This study |
| DLY3994 | **a** *bck1ΔLEU2* | This study |
| DLY4021 | **a** *bar1 swe1ΔTRP1* | This study |
| DLY4070 | **a** *bar1 mpk1ΔTRP1* | This study |
| DLY4074 | a *mpk1ΔTRP1 mih1::LEU2* | This study |
| DLY4075 | **a** *mpk1ΔTRP1 hsl1Δ1::URA3* | This study |
| DLY4077 | **a** *mpk1ΔTRP1 hsl7Δ1::URA3* | This study |
| DLY4233 | **a** *MPK1::HA::LEU2* | This study |
| DLY4351 | **a** *mkk1Δ::LEU2 mkk2Δ::URA3* | This study |
| DLY4497 | **a** *rho2Δ::kan* | This study |
| DLY4499 | a *wsc1ΔURA3* | This study |
| DLY4500 | **a** *rom2Δ::hyg* | This study |
| DLY4505 | **a** *rlm1Δ::kan* | This study |
| DLY4533 | **a** *mid2Δ::kan* | This study |
| DLY4545 | a *wsc1ΔURA3 mid2Δ::kan* | This study |
| DLY4653 | **a** *rom1Δ::kan* | This study |
| DLY4671 | **a** *mkk1Δ::LEU2 mkk2Δ::URA3 MPK1::HA::LEU2* | This study |
| DSY12 | **a** *swi4::LEU2* | Ref. 22 in text |
| E929-6C[b] | **a** *cyc1 CYC7-H2 can1 leu2-3,112 trp1Δ1 ura3-52* | 4 |
| E929-6C-79[b] | **a** *hog1-Δ1::TRP1* | B. Errede |
| JMY1030 | **a** *spa2-Δ3::URA3* | This study |
| K1107[c] | **a** *bar1::HisG ura2-52 ade2-1 can1-100 his2 leu2-3,112 trp1-1* | 5 |
| K2297[c] | **a** *bar1::HisG fus3::LEU2* | 5 |
| K2306[c] | **a** *bar1::HisG kss1::URA3* | 5 |
| K2314[c] | **a** *bar1::HisG fus3::LEU2 kss1::URA3* | 5 |
| YOC756[d] | a *rho1Δ::HIS3 rho1-112::LEU2* | This study |
| YOC784[d] | **a** *ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 RHO1::LEU2* | Ref. 33 in text |

All strains are congenic to BF264-15Du except for [a] which are congenic with each other, [b] which are congenic with each other, [c] which are congenic with each other, and [d] which are congenic with each other.

## Table 2: Oligonucleotides used in this study

bck1Δ5' 5'-ATGCCCTTTTGGAGGAAAATAGCGGGGACAGCA-GATACACATTCTAGGTCTTTCCTGATGCGGTATTTTCTCCT-3'
bck1Δ3' 5'-TCACTAAAAGGATGAGAAAGAAGCTCGTTAGCG-GTTGGCCTTTTCTCTGGGCGCGTTTCGGTGATGAC-3'
BCK1-1 5'-GCTCTAGAGCCAATCACTGAAGGAATAGC-3'
BCK1-2 5'-GGGCACCTCAACCTGCCTAACGGCCATCAT-3'
mid2Δ5' 5'-TTCGTTGAAGATTGGACATATAAAATACGCAAAT-CATAGTCAGCTGAAGCTTCGTACGC -3'
mid2Δ3' 5'-AAAAGTAGCCATAAGCACTAAATGATATGAAT-GATATGAATGGATATGAGCATAGGCCACTAGTGGATCTG-3'
MID2 tester 5'-GCTGACTTTGCGTTTTAGG-3'
mlp1Δ5' 5'-GAAGAAAGATCCACAATTAAATGGCGACTGA-CACCGAGAGGTGTATTTCAGCTGAAGCTTCGTACGC-3'
mlp1Δ3' 5'-GAAATGAATTAGAGCTTATTCCCATAAG-GTTTTGATATGGCTGGGTTGGCATAGGCCACTAGTGGATCTG-3'
MLP1-200 5'-GAGACTGGATGAAAAGGGAC-3'
mpk1Δ5' 5'-ATGGCTGATAAGATAGAGAGGCATACTTTCAAG-GTCTTCAATCAAGATTTCAGTGTAGATCAGATTGTACTGA-GAGTGC-3'
mpk1Δ3' 5'-GTGCCGTTATCATTCTGAGGCGGAATATCTGCG-GTATTTCCAGTGGCAGGTCTCATCTCCCCTTACGCATCTGT-GCGG-3'
MPK1 5'UTR 5'-GAACTGTGCATTCAGTCAGC-3'
OJ21 5'-ATCATCTTGCGCAGTTAGTCCA-3'
OJ43 5'-GGACTTCGAAATGCTGGACACGGA-GAACCTCCAGTTTATGGCGCGTTTCGGTGAATGAC-3'
OJ44 5'-GTCGTCTTCCTGGATAATAGCACCTG-CATTGCGTGTCATTTTTCCTGATGCGGTATTTTCTCCT-3'
OJ55 5'-TTCTGTGAAGCTGCACTGAG-3'
OJ87 5'-CCATGCCACGGTTCTGCTC-3'
OJ128 5'-TGCTGACTTAATTGGA-CAATTCATCTCTTTTCCTGCGGTTCAGCTGAAGCTTCG-TACGC-3'
OJ129 5'-CTAAAGAAAATAAGGAAAGTCTATAT-ACGTTGCTATCTTAGCATAGGCCACTAGTGGATCTG -3'
ROM2 tester 5'-CAGTACCTAACTGGGGTAATAAG-3'
rho2Δ5' 5'-CTGAAACGTTCTGCTTTGGTTGTGCTTTTGATC-CCGTACTCAGCTGAAGCTTCGTACGC-3'
rho2Δ3' 5'-TTGCTAAAAAGATAATGTATCATTTCAGTG-TAAGTTTTTTGCATAGGCCACTAGTGGATCTG-3'
RHO2 tester 5'-TATTCAAAGCGCAACTCTCC-3'
rlm1Δ5' 5'-TAAATATTAAAGTGTCGCAAAACTATACTATAGAT-ACAACCAGCTGAAGCATCGTACGC-3'
rlm1Δ3' 5'-TCTTATGCTTGGAATATTCATACTGGT-CAAATTTTTTGGTGCATAGGCCACTAGTGGATCTG-3'
RLM1 tester 5'-GAACTGCCTACTCCTGATAG-3'
rom1Δ5' 5'-CCCCTACTCAGTTGTTCTCCGGCTC-CAAAGTTCAGACAAACATCAATGCAGCTGAAGCTTCG-TACGC-3'
rom1Δ3' 5'-GGGCGGTCAACTCAAAAATTTAACAGTTC-GATAATTTCAAATCCTTGAGGGCATAGGCCACTAGTG-GATCTG-3'
ROM1 tester 5'-CCAAGTCGTTGGTAAGAATTTGG-3'

The underlined portions anneal to the plasmid carrying the knockout marker, while the remaining portions target homologous recombination of the PCR product to the specific locus.


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