The Cell Surface Protein Gene ecm33+ Is a Target of the Two Transcription Factors Atf1 and Mbx1 and Negatively Regulates Pmk1 MAPK Cell Integrity Signaling in Fission Yeast

Hirofumi Takada,* Aiko Nishida,* Mitsuhiro Domae,* Ayako Kita,* Yuki Yamanouchi,* Atsushi Uchida,* Shunji Ishiwata,* Yue Fang† Xin Zhou† Takashi Masuko,‡ Mitsuhiro Kinoshita,§ Kazuaki Kakehi,§ and Reiko Sugiura*

*Laboratory of Molecular Pharmacogenomics, ‡Laboratory of Molecular and Cellular Biology, and §Laboratory of Bioinformatics, School of Pharmaceutical Sciences, Kinki University, Higashi-Osaka, 577-8502, Japan; and †Division of Molecular Pharmacology and Pharmacogenomics, Department of Biochemistry and Molecular Biology, Kobe University Graduate School of Medicine, Chuo-ku, Kobe 650-0017, Japan

The highly conserved fission yeast Pmk1 MAPK pathway plays a key role in cell integrity by regulating Atf1, which belongs to the ATF/cAMP-responsive element-binding (CREB) protein family. We identified and characterized ecm33+, which encodes a glycosyl-phosphatidylinositol (GPI)-anchored cell surface protein as a transcriptional target of Pmk1 and Atf1. We demonstrated that the gene expression of Ecm33 is regulated by two transcription factors Atf1 and a MADS-box-type transcription factor Mbx1. We identified a putative ATF/CREB-binding site and an RLM1-binding site in the ecm33 promoter region and monitored the transcriptional activity of Atf1 or Mbx1 in living cells using a destabilized luciferase reporter gene fused to three tandem repeats of the CRE and six tandem repeats of the Rlm1-binding sequence, respectively. These reporter genes reflect the activation of the Pmk1 pathway by various stimuli, thereby enabling the real-time monitoring of the Pmk1 cell integrity pathway. Notably, the Δecm33 cells displayed hyperactivation of the Pmk1 signaling together with hypersensitivity to Ca2+ and an abnormal morphology, which were almost abolished by simultaneous deletion of the components of the Rho2/Pck2/Pmk1 pathway. Our results suggest that Ecm33 is involved in the negative feedback regulation of Pmk1 cell integrity signaling and is linked to cellular Ca2+ signaling.

INTRODUCTION

The mitogen-activated protein kinase (MAPK) pathway is one of the most important intracellular signaling pathways that play a crucial role in cell proliferation, cell differentiation, and cell cycle regulation (Nishida and Gotoh, 1993; Marshall, 1994; Herskowitz, 1995; Levin and Errede, 1995). MAPKs deliver extracellular signals from activated receptors to various cellular compartments, especially, the nucleus, where they regulate eukaryotic gene expression at the transcriptional and posttranscriptional levels (Fouyssegur, 2000; Sugiura et al., 2003; Edmunds and Mahadevan, 2004; Satoh et al., 2009). In the budding yeast Saccharomyces cerevisiae, the Slt2/Mpk1 MAPK pathway mediates cell cycle-regulated cell wall synthesis and responds to different signals, including cell cycle regulation, growth temperature, changes in external osmolarity, and mating pheromones (Gustin et al., 1998). Signaling proteins involved in the pathway include the GTP-binding protein Rho1, the protein kinase C homologue Pkc1, the MEKK Bck1p/Slk1p, the redundant pair of MAP/ERK kinases (MEKs) Mkk1 and Mkk2, the MAPK Slt2/Mpk1, and the transcription factor targets Rlm1 and SBF (Gustin et al., 1998). Moreover, signaling via Mpk1/Slt2-Rlm1 regulates the expression of at least 25 genes, most of which have been implicated in cell wall biogenesis (Jung and Levin, 1999; Jung et al., 2002).

We have been studying the Pmk1 MAPK signaling pathway in the fission yeast Schizosaccharomyces pombe. The Pmk1 MAPK, a homologue of the mammalian ERK/MAPK plays a central role in cell integrity in fission yeast (Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1997). The Pmk1 MAPK pathway is composed of MAPKKK Mkh1 (Sengar et al., 1997), MAPKK Pek1 (Sugiura et al., 1999), and MAPK Pmk1/Spm1. The Pmk1 MAPK pathway also regulates ion homeostasis and morphogenesis (Satoh et al., 2009) and is activated under multiple stresses, including heat shock, hyper- or hypotonic stresses, cell wall damage, or glucose deprivation (Toda et al., 1996; Sugiura et al., 1999; Madrid et al., 2006).

We have previously demonstrated that calcineurin and Pmk1 MAPK play antagonistic roles in Cl− homeostasis (Sugiura et al., 1998, 2002) and genetic screening on the basis of the functional interaction between calcineurin and Pmk1 MAPK has resulted in the isolation of negative regulators of the Pmk1 MAPK pathway, including pmp1+, encoding a...
dual-specificity MAPK phosphatase (Sugiura et al., 1998; pck1+, encoding a MAPK kinase (MAPKK; Sugiura et al., 1999); and ren1+, encoding a novel KH-type RNA-binding protein that stabilizes Pmp1 mRNA (Sugiura et al., 2003, 2004). Moreover, genetic screening for vic (viable in the presence of immunosuppressant and chloride ion) mutants revealed that the cpi1+ gene, encoding a β subunit of the protein farnesyltransferase, and its target Rh2 GPase (Ma et al., 2006) act as upstream regulators of the Pmk1-signaling pathway.

Most recently, we have identified the Atf1 transcription factor as a downstream target of the Pmk1 MAPK pathway and demonstrated that Atf1 is involved in cell integrity in addition to its well-established role in the stress responses mediated by the Slt2/Rlm1 signaling pathway (Takada et al., 2007). Mbx2, an Rlm1 homologue in fission yeast, unlike in budding yeast, displayed only a modest sensitivity to cell wall–damaging agents, suggesting that Mbx2 plays a minor role in this process (Takada et al., 2007). Moreover, the intermediate phenotypes of the Δatf1 cells in the cell integrity response suggest that other unidentified target(s) of Pmk1 must play a significant role in the cell integrity pathway in fission yeast.

To identify novel genes involved in cell integrity signaling pathway, we searched for S. pombe homologues of the cell wall biogenesis genes regulated by the Mpk1-Rlm1 pathway. In budding yeast, and characterized the expression mechanism of the Pom1-mediated cell integrity signaling. Of these genes, PST1 was particularly interesting because its gene expression was induced upon wall biogenesis genes regulated by the Mpk1-Rlm1 pathway.

\[ \text{(Takada et al., 2007).} \]

\[ \text{MATERIALS AND METHODS} \]

\text{Strains, Media, and Genetic and Molecular Biology Methods} 

\text{S. pombe strains used in this study are listed in Table 1. The complete medium YPD (yeast extract-peptone-dextrose) and the minimal medium EMM (Edinburgh minimal medium) have been described previously (Toda et al., 1996). Standard genetic and recombinant DNA methods (Moreno et al., 1991) were used, where specified. FK506, developed by Astellas Pharma (Osaka, Japan). An S. pombe haploid strain in which the \text{styg1+/styg1−} gene (geneID \text{SPAC24B11.06c}) had been deleted was purchased from Bioneer (Daejeon, Korea).} 

\text{Table 1. Schizosaccharomyces pombe strains used in this study} 

| Strain   | Genotype               | Reference  |
|----------|------------------------|------------|
| HM123    | h− leu1-32             | Our stock  |
| KP456    | h− leu1-32 ura4-D18    | Our stock  |
| KP208    | h− leu1-32 ura4-D18 pmkl::ura4+ | Our stock  |
| KP119    | h− leu1-32 ura4-D18 pph1::ura4+ | Our stock  |
| KP2163   | h− leu1-32 pck2::KanMX6 | Our stock  |
| KP471    | h− leu1-32 ura4-D18 spc1::ura4+ | Our stock  |
| KP495    | h− leu1-32 ura4-D18 atf1::ura4+ | Our stock  |
| KP278    | h− leu1-32 pmkl::KanMX6 | Satoh et al. (2009) |
| KP2118   | h− leu1-32 ura4-D18 pmkl::KanMX6 | Satoh et al. (2009) |
| SP655    | h− leu1-32 ura4-D18 ecml33::ura4+ | This study |
| SP652    | h− leu1-32 ura4-D18 meul10::ura4+ | This study |
| SP550    | h− leu1-32 ura4-D18 mbm2::ura4+ | This study |
| SP587    | h− leu1-32 mbm2::KanMX6  | This study |
| SP674    | h− leu1-32 ura4-D18 mbm2::KanMX6 | This study |
| SP977    | h− leu1-32 ura4-D18 ecml33::ura4+ pmkl::KanMX6 | This study |
| SP1220   | h− leu1-32 ura4-D18 adet-M210 sty1::KanMX4  | Bioneer |

\text{Cloning and Knockout of the \text{ecm33+} Gene} 

The \text{ecm33+} gene was amplified by PCR using the genomic DNA of S. pombe as a template. The sense primer used for PCR was 5′-GAA GAT CTC ATG TTG TTC AAA TCA TTC GCT CTC ACT CAC AAT TC-3′ (BglII site and start codon are underlined), and the antisense primer was 5′-GGA GAT CGT GGC CCC ATA GCA GGA GCA ACC AAA AGA G-3′ (BglII and NotI site are underlined). The amplified product was digested with BglII/NotI, and the resulting fragment was subcloned into Bluescript SK+ (+) to create pBS-ecm33.

To knockout the \text{ecm33+} gene, a one-step gene disruption by homologous recombination was performed (Rothstein, 1983). The \text{ecm33−} null mutants were obtained by entire deletion of the corresponding coding sequence and its replacement with the \text{ura4+} cassette by PCR-mediated strategy using plasmid pFA6α-urad as the template (Bialek et al., 1998).

\text{The \text{ecm33+} Promoter Assay} 

Firefly luciferase was chosen as a reporter, because the assay is simple to perform and has a high signal-to-noise ratio (Leskinen et al., 2003). A 133-kb DNA fragment (P0.5, 500/2 base pairs) in the 5′ flanking region of the \text{ecm33+} gene was amplified by PCR primers (forward primer 170, 5′-AA CTG CAG CAA CCT CTT GGT TGT GGG-3′; reverse primer 126, 5′-CCG TTC CTC GAG ATT GAC TTT AGA CTA TAT AAT GTA GAA ATA TG-3′). Similarly, the 5′-end deletion mutants of P0.5 (P0.45, 450/2 base pairs; P0.4, 402/2 base pairs; P0.37, 369/2 base pairs; and P0.3, 300/2 base pairs) were prepared using the reverse primer 126 and the following forward primers: 228 (5′-AA CTG CAG CAT TG TTT GAA GTA CAA TTA AAT GAA ATA TG-3′), 228 (5′-AA CTG CAG CAT TT TAT CTA ACA AGT CAC AAT TC-3′), 192 (5′-AA CTG CAG CTT CCC CTA ATG TAT AGA TAT GGT CTT TTC CGC-3′), and 171 (5′-AA CTG CAG ACA ATC TT TAT TAT TAC TAC CCC-3′). The 5′-end deletion mutants of P0.5 (P0.2, 200/2 base pairs; and P0.1, 100/2 base pairs) were prepared using the forward primer 170 and the following reverse primers: 230 (5′-CCG TTC CTC GATA TTA ACC AAA ATG TCT ATG CGC-3′) and 257 (5′-CCG TTC CTC GAG AAA GGA GCC CAA ACT TAT ATC GGG-3′). The 5′- and 3′-end deletion mutant of P0.5 (P0.07, 69/2 base pairs) was prepared using the forward primer 192 and the reverse primer 230. The various fragments of the P0.5 promoter region of ecm33− were subcloned into the Plsf1/Xhol-digested pKB5723 (Deng et al., 2006), a multi-copy vector that contains the destabilized luciferase gene from pGL3 (R2.2; Promega, Madison, WI).

Cells transformed with these reporter plasmids were cultured at 27°C in EMM to midlog phase. The \text{ecm33+} promoter activity was measured as described by Deng et al. (2006), with minor modifications. Briefly, the cells were diluted with fresh medium to OD600 = 0.2, and the cells were grown for 3 h at 27°C. Cells were incubated with 0.5 mM m-toturcin for 10 min at 27°C. Aliquots of the cell culture were pipetted into a 96-well plate, and NaCl was added to a final volume and concentration of 100 μl and 500 mM, respectively. Dye-dissolved water, which was used as control, was added to each well of the wells. The mixture was incubated at 27°C for 2 h, and light emission levels expressed as relative light units were measured using a luminometer (AB-2300; Atto, Tokyo, Japan) at 12-s intervals.

\text{Live-Cell Monitoring of Pmk1-mediated Transcriptional Activity} 

A 1.2-kb Pslf1/Xhol fragment of pKB5721 was replaced with the \text{ecm33−} derived CRE oligonucleotide (sense 259: 5′-GCG TTT TAC AGT AAA TAC ATG GAA GAT ACA AGT AAT TC-3′, antisense 260: 5′-GGC TTT TAC AGT AAA TAC ATG GAA GAT ACA AGT AAT TC-3′) by PCR-mediated strategy using plasmid pFA6α-urad as the template (Bialek et al., 1998).
5'-TGCGTTGCCCAG or CTGGGCCCAC, underlined). The expression of the ecm33 gene (SPAC1705.03c) encoding a putative GPI-anchored cell surface protein (De Groot et al., 2003), which is similar to PST1 (30% identity) and ECM33 (28% identity) in budding yeast (Pardo et al., 2004).

Northern blot analysis demonstrated that under unstrained conditions, the ecm33 mRNA level was significantly reduced in Δatf1 cells and Δmpk1 cells compared with that in wild-type cells (Figure 1A), suggesting that the expression of ecm33 is regulated by the Pmk1-Atf1 signaling. Here, we characterized the ecm33 gene as a transcriptional target of Pmk1 and Atf1. The Δecm33 cells, like Δmpk1 cells and Δatf1 cells, were highly sensitive to calcofluor, a cell wall–damaging agent (Figure 1B, 1.4 μg/ml calcofluor). Notably, the sensitivity of Δecm33 cells to calcofluor was higher than that of Δmpk1 cells and Δatf1 cells to this agent (Figure 1B, 1.2 μg/ml calcofluor). The cell integrity defect associated with the Δecm33 cells was further confirmed using β-glucanase, another cell wall–damaging agent. As shown in Figure 1C, the Δecm33 cells showed hypersensitivity to β-glucanase as did Δmpk1 cells. The Δatf1 cells showed intermediate response to β-glucanase compared with the responses of the wild-type cells and Δmpk1 cells (Figure 1C). Disruption of the neul1 gene (Tougan et al., 2002), which also shows significant amino acid similarity to PST1 and ECM33, did not result in cell wall defects (Figure 2E); therefore, we focused on the ecm33 gene.

To examine the expression and regulation of the ecm33 gene in more detail, we developed a reporter construct containing a 0.5-kb sequence upstream of ATG of the ecm33 gene fused to the destabilized version of luciferase (Pascual-Ahuir et al., 2002). As shown in Figure 1D, the ecm33 promoter analysis using [ecm33-205] (2R2) yielded similar results as obtained by Northern blot analysis under unstrained conditions (Figure 1D, basal). We further investigated whether the expression of the ecm33 reporter gene was Pmk1- and Atf1-dependent under stress conditions. For this, we examined the effects of various stimuli, which have been reported to activate Pmk1 MAPK (Madrid et al., 2006), on the ecm33 reporter expression. As expected, NaCl (500 mM), CaCl2 (400 mM), KCl (400 mM), calcofluor (2 μg/ml), and miconafungin (4 μg/ml) induced the expression of the ecm33 reporter gene in wild-type cells (Figure 1D, wt). In contrast, this induction was almost completely abolished in Δmpk1 cells and Δatf1 cells (Figure 1D). Moreover, the overexpression of Pek1 (Hirayama et al., 2000), the constitutively active version of MAPKK for Pmk1, increased the levels of the Ecm33 protein.
Figure 1. Identification of Ecm33 as a target of Pmk1 and Atf1. (A) Northern blot analysis of total RNA from the wild-type (wt), Δatf1 cells, and Δpmk1 cells. Cells were incubated in YPD medium and collected after culture. Total RNA (20 μg) was subjected to Northern blot analysis using a digoxigenin (DIG)-labeled Ecm33 or Leu1 cRNA. (B) The Δecm33 cells showed hypersensitivity to calcofluor. Wild-type, Δecm33, Δpmk1, and Δatf1 cells were streaked onto the plates as indicated and incubated for 4 d at 27°C. (C) The Δecm33 cells showed hypersensitivity to β-glucanase. Cell lysis was measured at different times during treatment with β-glucanase by determining the OD600. The strains examined were wt, Δecm33, Δpmk1, and Δatf1. (D) Ecm33 (P0.5R2.2) reporter expression is dependent on Pmk1-Atf1 signaling. Ecm33 expression was monitored using the luciferase reporter construct containing the 0.5-kb sequence upstream of ATG of the ecm33+ gene [ecm33 P(0.5)(R2.2)] transformed in wild-type (wt), Δpmk1, and Δatf1 cells. Cells were grown in YPD (basal) or subjected to various stimuli as indicated for 30 min at 27°C, and the assay was performed as described in Deng et al. (2006); data from at least three independent experiments are expressed as mean ± SD. (E) Overexpression of constitutively active Pek1 MAP kinase kinase stimulates Ecm33 expression. Wild-type or Δpmk1 cells harboring [ecm33 P(0.5)(R2.2)] transformed with either pREP41-Pek1DD (Pek1DD OP) or the control vector (vector) and P(0.5)(R2.2) were transformed with either pREP41-Pek1DD were transformed as indicated of the addition of NaCl (Figure 1E, wt, Δatf1Δpmk1, basal). Notably, the effect of overexpressing Pek1DD OP, 500 mM NaCl). Knockout of the pmk1 gene (Δecm33) showed almost no detectable promoter activity in response to a variety of Pmk1-activating stimuli as shown in Figure 1D, whereas the same stresses failed to induce the promoter activity of the pmk1Δecm33 (Figure 2A, pKD1952, pKD1361). The luciferase reporter construct containing the 0.3-kb sequence upstream of ATG of the ecm33+ gene (P0.3R2.2; Figure 2A, pKD1952, pKD1361) showed almost no detectable promoter activity (Figure 2B, 300 –1, basal). Moreover, the luciferase reporter constructs containing the region from −500 to −450 had little effect on the promoter activity (Table 2). Deletion of the region from −450 to −402 reduced the promoter activity by −54% (Figure 2B, pKD1361). The luciferase reporter construct containing the 0.3-kb sequence upstream of ATG of the ecm33+ gene is important for its regulated expression.

Deletion Analysis of the ecm33+ Promoter

To determine the promoter region involved in the Pmk1-dependent ecm33 expression, the 5′ deletion mutants of the 0.5-kb DNA fragment (P0.5) of the ecm33+ gene promoter were generated and subcloned into the multicopy luciferase vector (Figure 2A). These plasmids were transformed into a wild-type strain, and the promoter assay was performed under basal conditions (Figure 2B, basal, and Table 2). Deletion of the 5′-flanking sequences from −500 to −450 had little effect on the reporter activity (Table 2). Deletion of the region from −450 to −402 reduced the promoter activity by −54% (Figure 2B, pKD1361). The luciferase reporter construct containing the 0.3-kb sequence upstream of ATG of the ecm33+ gene is important for its regulated expression.

A database search (TESS SEARCH) revealed a CRE-like sequence motif TTACAGTAA at position −444 to −436 (Figure 2, A and C, CRE, underlined) and a sequence similar to the RLMI1-binding motif GTATATAG at position −362 to −353 (Figure 2, A and C, RLMI1, underlined) of the ecm33+ gene. The presence of putative consensus elements
Figure 2. Promoter analysis of ecm33+ gene. (A) Deletion analysis of the ecm33+ promoter. Segment from the ecm33+ upstream region indicated at the left was inserted into the multicopy plasmid containing the luciferase reporter gene. The positions of the CRE (*) and the RLM1 (**) sequences are shown. The numbers refer to the position of the deletion end point relative to the first base of the initiation codon of the gene, which are designated as +1. (B) The upstream region from −500 to −300 of the ecm33+ gene regulates Pmk1-responsive expression of the ecm33+ gene. The luciferase fusion plasmids as indicated were transformed into wild-type cells. Cells were either untreated (basal) or treated with various stimuli as indicated, and the assay was performed as described in Figure 1E. (C) Identification of CRE and RLM1 in the promoter region of the ecm33+ gene. The sequences of the CRE-like motif (TTACAGTAA) and the RLM1-like motif (GTATATATAG) identified in the ecm33+ promoter are underlined. The numbers refer to the first and last nucleotides of the displayed sequences. (D) The Mbx1 transcription factor is involved in the Ecm33 expression. The luciferase fusion plasmid Ecm33 (P0.5R2.2) was transformed into various strains as indicated. Cells were either untreated (basal) or treated with various stimuli as indicated, and the assay was performed as described in Figure 2B. (E) The Δmbx1, but not the Δmbx2 cells, showed hypersensitivity to calcifluor. The cells as indicated were streaked onto the plates and then incubated for 4 d at 27°C.

Table 2. Promoter analysis of the ecm33+ gene

| Promoter | No NaCl | +500 mM NaCl | Fold activation |
|----------|---------|--------------|----------------|
| −500/−1 | 1.00 ± 0.07 | 2.60 ± 0.20 | 2.6 |
| −450/−1 | 0.90 ± 0.16 | 2.16 ± 0.15 | 2.4 |
| −402/−1 | 0.49 ± 0.07 | 1.24 ± 0.09 | 2.5 |
| −300/−1 | 0.01 ± 0.01 | 0.01 ± 0.11 | 0.9 |
| −500/−301 | 1.04 ± 0.21 | 2.52 ± 0.09 | 2.4 |

A 0.5-kb DNA fragment (P (2.2)) of the ecm33+ gene promoter, its 5′-end deletion mutants and 3′-end deletion mutants were subcloned into the multicopy plasmid containing the wild-type luciferase reporter gene and the assay was performed as described in Materials and Methods. Values from at least three independent experiments are expressed as mean ± SD.

**Real-Time Monitoring of Atf1 Activity in Living Cells**

Atf1 activity in living cells was monitored by 3xCREECM33 fused to R2.2 destabilized luciferase [3xCREECM33::luc (R2.2)]. As shown in Figure 3A, wild-type cells harboring the multicopy 3xCREECM33::luc (R2.2) reporter were stimulated by the addition of 500 mM NaCl, a hyperosmotic stress that is reported to stimulate Atf1 activity (Wilkinson et al., 1996). Elevated extracellular NaCl caused an extremely rapid increase in the 3xCREECM33::luc (R2.2) reporter response within 3 min, followed by a rapid decrease to reach its lowest value at around 30 min, then again showed a second increase, and finally approached a constant level (Figure 3A, wt). In contrast, the Δafl1 cells harboring the same reporter showed minimal responses to the same stimuli, indicating that multicopy 3xCREECM33::luc (R2.2) reporter appears to be a reliable reporter of Atf1 activity (Figure 3A, Δafl1). To examine whether this CRE site was of functional relevance, we used PCR primers to mutate the −444/−436 element. Compared with the wild-type promoter 3xCREECM33::luc (R2.2) reporter, mutation in the CRE site caused a marked reduction of the promoter activity with (500 mM NaCl) or without (0 mM NaCl) the stimuli [Figure 3A, 3xCREEmECM33::luc (R2.2)].

We also examined whether the 3xCREECM33::luc (R2.2) reporter expression was dependent on two upstream MAPK pathways that phosphorylate and regulate Atf1, namely, the Sty1/Spc1 MAPK and the Pmk1 MAPK pathways. As shown in Figure 3B, the reporter expression of the 3xCREECM33::luc (R2.2) in Δafl1 cells was barely detectable both in the absence and presence of a hyperosmotic stress. In addition, the 3xCREECM33::luc (R2.2) promoter activity was very low in Δpml1 cells compared with that in the wild-type cells and responded only weakly to the hyperosmotic stress

for the binding of the Rlm1-like transcription factor as well as the ATF1/cAMP-responsive element-binding (CREB) family protein in the promoter region of the ecm33+ gene from −500 to −300 upstream of ATG prompted us to examine the involvement of Mbx1-like transcription factors in the regulation of Ecm33 expression. Next, we examined the promoter activity of p0.5(R2.2) in deletion mutant cells of Mbx1-like transcription factor genes, namely, Δmbx1 and Δmbx2, and compared them with the promoter activities of the Δafl1, Δpml1, or wild-type cells. Notably, disruption of the mbx1+ gene, but not the Rlm1-homologous gene mbx2+, resulted in a significant reduction of the Ecm33 promoter activity compared with that of the wild-type cells (Figure 2D). The relative promoter activity of the Δmbx1 cells was almost equivalent to that of the Δpml1 cells, but was slightly higher than that of the Δafl1 cells (Figure 2D). Moreover, deletion of Mbx1, but not Mbx2, abrogated the induction of promoter response by various stimuli, which activity by the Pmk1 pathway (Figure 2D). Similarly, disruption of the mbx1+ gene, but not the mbx2+ gene, resulted in the hyper-sensitivity to calcifluor (1.4 μg/ml) as observed in Δpml1, Δafl1, or Δecm33 cells (Figure 2E). Thus, we concluded that the Mbx1 transcription factor is also involved in the cell integrity pathway by regulating Ecm33 expression.
Moreover, when constitutively active MAPKK Pek1DD was overexpressed in the wild-type cells, a significantly higher level of 3xCRECM33::luc (R2.2) reporter response was observed even without any stimulation (Figure 3C, wt-Pek1DD OP), and addition of NaCl to the medium further stimulated the response (Figure 3C, wt-Pek1DD OP, 500 mM NaCl). This induction by Pek1DD and the addition of NaCl was almost abolished in pmk1 cells (Figure 3C, pmk1), indicating the Pmk1-dependent response of the 3xCRECM33::luc (R2.2) reporter. It should be noted that the relative light units in pmk1 cells were ~20% of that of the wild-type cells and were relatively higher than those of the Δatf1 cells and Δsty1 cells. Thus, 3xCRECM33::luc (R2.2) indicates both Sty1 and Pmk1 activation. The biphasic activation of the signal upon NaCl stimulation may reflect the intracellular Ca^{2+} concentration, i.e., the first sudden increase reflects the Ca^{2+} influx from the plasma membrane-localized Ca^{2+} channels, and the second increase indicates a mechanism similar to that of Ca^{2+}-induced Ca^{2+} release from the intracellular Ca^{2+} store.

To determine the cross-talk between Pmk1 and Sty1/Spc1 MAPK cascade in the regulation of Ecm33 transcription, we examined whether Pek1DD overexpression can overcome the Δsty1 defect in the transcription from 3xCRE even after NaCl stimulation (Figure 3C, Δsty1). This might be because of instability of the Atf1 protein in the absence of Sty1. Atf1 is a target for the ubiquitin-proteasome system (Lawrence et al., 2009), and Sty1 phosphorylation of Atf1 is required for modulating Atf1 stability and is vital for a robust response to certain stresses (Lawrence et al., 2007). Therefore, in the absence of the Sty1 protein, the Atf1 protein may be easily degraded and may fail to respond to Pmk1 activation by Pek1DD overexpression.

**Real-Time Monitoring of Mbx1 Activity in Living Cells**

We next created the reporter construct 6xRLM ECM33 fused to R2.2 destabilized luciferase [6xRLM ECM33::luc (R2.2)]. As shown in Figure 4A, wild-type cells harboring the multicopy
6xRLMECM33::luc (R2.2) reporter were stimulated by the addition of 500 mM NaCl. Elevated extracellular NaCl also caused a rapid increase in the 6xRLMECM33::luc (R2.2) reporter response within 3 min, followed by a rapid decrease to reach its lowest value at around 30 min, then again showed a second increase, and finally approached a constant level (Figure 4A, wt). In contrast, the \( \Delta \text{mbx}1 \) cells harboring the same reporter showed minimal responses to the same stimuli, indicating that multicopy 6xRLMECM33::luc (R2.2) reporter could reflect the Mbx1 activity (Figure 4A, \( \Delta \text{mbx}1 \)).

Disruption of \( \text{mbx}2 \) did not affect the promoter response (data not shown). Moreover, mutation at a consensus RLM site dramatically reduced the 6xRLMECM33::luc (R2.2) promoter activity with or without the stimuli (Figure 4A, wt, 6xRLMmECM33).

Notably, disruption of the \( \text{pmk}1^+ \) gene reduced the basal promoter activity and almost abolished the induction of the reporter by NaCl addition (Figure 4A, \( \Delta \text{pmk}1 \)). \( \Delta \text{sty}1 \) deletion did not significantly affect the rapid response of the 6xRLMECM33::luc (R2.2) reporter, whereas the second increase in the reporter response to NaCl stimulation in \( \Delta \text{sty}1 \) cells was distinct from that in the wild-type cells (Figure 4B, \( \Delta \text{sty}1 \)). Thus, the rapid phase of the 6xRLMECM33::luc (R2.2) reporter was transformed with either pREP2-Pek1DD (Pek1DD OP) or the control vector (vector) and cultured for 24 h in the absence of thiamine. Cells were either untreated (basal) or treated with 500 mM NaCl and analyzed as in Figure 1D.

Moreover, overexpression of constitutively active Pek1DD induced reporter expression in wild-type cells even in the absence of stimulation (Figure 4C, wt-Pek1DD OP, basal), and addition of NaCl to the medium further increased this response (Figure 4C, wt-Pek1DD OP, +500 mM NaCl). This induction by Pek1DD and its enhancement with the addition of NaCl were almost completely abolished in \( \Delta \text{pmk}1 \) cells, thereby indicating a Pmk1-dependent response of the 6xRLMECM33::luc (R2.2) reporter (Figure 4C, \( \Delta \text{pmk}1 \)). Moreover, the induction by Pek1DD and its enhancement with the addition of NaCl were also abolished in \( \Delta \text{mbx}1 \) cells (Figure 4C, \( \Delta \text{mbx}1 \)). Thus, the Pek1DD-induced transcription from 6xRLMECM33 observed in wild-type cells depends on the Mbx1 transcription factor.
Results showed that Pmk1 is hyperphosphorylated in the Δecm33 cells using anti-phospho Pmk1 antibodies that recognize only phosphorylated and hence activated Pmk1 (Sugiura et al., 1999). The results revealed that Δecm33 cells showed increased Pmk1 phosphorylation level compared with that of the wild-type cells under normal conditions (Figure 5B, 0 min). Moreover, upon treatment with CaCl₂, the phosphorylation of Pmk1 was greatly induced in Δecm33 cells than in wild-type cells (Figure 5B, left panel). Further, the addition of 500 mM NaCl induced a higher-normal level of Pmk1 phosphorylation in Δecm33 cells (Figure 5B, right panel). Consistently, the vic-negative phenotype associated with Δecm33 cells was rescued by Pmk1 deletion, because the Δecm33Δpmk1 double mutant cells grew well in the presence of the immunosuppressant FK506 and 0.12 M MgCl₂ as did Δpmk1 cells (Figure 5A, Δecm33Δpmk1). In addition, disruption of the rho2 gene, an upstream activator of the Pmk1 pathway, also rescued the vic-negative phenotype of Δecm33 cells (data not shown). Thus, loss of Ecm33 function induced hyperactivation of the Rho2/Pmk1 cell integrity pathway.

We next examined the effect of the overexpression of Ecm33 on the chloride ion hypersensitivity of calcineurin deletion (Δppb1). Our previous data showed that overexpression of the dual-specificity phosphatase Pmp1 or the type 2C phosphatases Ptc1 or Ptc3 suppressed the chloride ion hypersensitivity of Δppb1 cells by inhibiting Pmk1 activation (Sugiura et al., 1998; Takada et al., 2007). If Ecm33 were considered to play a role in the negative regulation of Pmk1 signaling, it would be expected that Ecm33 overexpression would also suppress Δppb1 cells. As expected, Δppb1 cells overexpressing the ecm33+ gene could grow in the presence of 0.12 M MgCl₂ whereas those bearing the control vector alone failed to grow (Figure 5C). Moreover, the overexpression of the ecm33+ gene almost abolished the stimulation of Pmk1 phosphorylation both before and after CaCl₂ treatment (Figure 5D, Ecm33 OP, left panel). The inhibitory effect of Ecm33 overproduction on Pmk1 phosphorylation was also observed when cells were treated with 500 mM NaCl (Figure 5D, right panel).

Knockout of the pmk1+ Gene Rescued Phenotypes of Δecm33 Cells

Another striking feature of Δecm33 cells is their hypersensitivity to CaCl₂. As shown in Figure 6A, Δecm33 cells grew poorly in the media supplemented with 150 mM CaCl₂, whereas wild-type cells grew normally. In contrast, Δecm33 cells grew well in the media supplemented with 150 mM MgCl₂ or 300 mM NaCl, suggesting that Ca²⁺ homeostasis is altered in Δecm33 cells (Figure 6A). Moreover, the Ca²⁺-hypersensitive phenotype observed in Δecm33 cells was rescued by Pmk1 deletion (Figure 6A, Δecm33Δpmk1), suggesting that this phenotype somehow results from Pmk1 hyperactivation in Δecm33 cells. In addition, morphologically, the Δecm33 cells were abnormally enlarged and swollen compared with the wild-type cells (Figure 6B, Δecm33). Notably, simultaneous deletion of Pmk1 almost rescued the morphological abnormality observed in Δecm33 cells (Figure 6B, Δecm33Δpmk1). Interestingly, the above finding that Pmk1 deletion rescued Δecm33 phenotypes clearly contrasts the re-

Role of Ecm33 in Pmk1 Signaling

We previously demonstrated that mutations in the components of the Pmk1 pathway result in the vic phenotype (Ma et al., 2006). These components include Pmk1 MAPK, Pek1 MAPKK, Mkh1 MAPKKK, Pck2 protein kinase C, and Rho2. We further examined the functional relationship between Ecm33 and Pmk1 signaling by analyzing whether the disruption of the ecm33+ gene affected the chloride ion hypersensitivity induced by the inhibition of the protein phosphatase calcineurin by using the immunosuppressant FK506, a specific inhibitor of calcineurin (Sugiura et al., 1998). The results showed that Δecm33 cells, like wild-type cells, failed to grow in the presence of the immunosuppressant FK506 and 0.12 M MgCl₂ whereas Δpmk1 cells grew well under these conditions (Figure 5A, +0.12 M MgCl₂, +FK506). Moreover, Δecm33 cells failed to grow in the presence of the immunosuppressant FK506 and 0.08 M MgCl₂, whereas wild-type cells grew slowly (Figure 5A, +0.08 M MgCl₂, +FK506). Thus, Ecm33 deletion exacerbated the chloride ion hypersensitivity induced by calcineurin inhibition. In our previous study, we showed that the hyperactivation of Pmk1 MAPK by the overexpression of the constitutively active Pek1DD exacerbated the chloride ion hypersensitivity of calcineurin deletion (Sugiura et al., 1999). This suggested that Ecm33 deletion, like Pek1DD, induced hyperactivation of Pmk1 signaling. To investigate this possibility, we examined the level of Pmk1 phosphorylation in Δecm33 cells using anti-phospho Pmk1 antibodies that recognize only phosphorylated and hence activated Pmk1 (Sugiura et al., 1999). The results revealed that Δecm33 cells showed increased Pmk1 phosphorylation level compared with that of the wild-type cells under normal conditions (Figure 5B, 0 min). Moreover, upon treatment with CaCl₂, the phosphorylation of Pmk1 was greatly induced in Δecm33 cells than in wild-type cells (Figure 5B, left panel). Further, the addition of 500 mM NaCl induced a higher-normal level of Pmk1 phosphorylation in Δecm33 cells (Figure 5B, right panel). Consistently, the vic-negative phenotype associated with Δecm33 cells was rescued by Pmk1 deletion, because the Δecm33Δpmk1 double mutant cells grew well in the presence of the immunosuppressant FK506 and 0.12 M MgCl₂ as did Δpmk1 cells (Figure 5A, Δecm33Δpmk1). In addition, disruption of the rho2 gene, an upstream activator of the Pmk1 pathway, also rescued the vic-negative phenotype of Δecm33 cells (data not shown). Thus, loss of Ecm33 function induced hyperactivation of the Rho2/Pmk1 cell integrity pathway.

We next examined the effect of the overexpression of Ecm33 on the chloride ion hypersensitivity of calcineurin deletion (Δppb1). Our previous data showed that overexpression of the dual-specificity phosphatase Pmp1 or the type 2C phosphatases Ptc1 or Ptc3 suppressed the chloride ion hypersensitivity of Δppb1 cells by inhibiting Pmk1 activation (Sugiura et al., 1998; Takada et al., 2007). If Ecm33 were considered to play a role in the negative regulation of Pmk1 signaling, it would be expected that Ecm33 overexpression would also suppress Δppb1 cells. As expected, Δppb1 cells overexpressing the ecm33+ gene could grow in the presence of 0.12 M MgCl₂ whereas those bearing the control vector alone failed to grow (Figure 5C). Moreover, the overexpression of the ecm33+ gene almost abolished the stimulation of Pmk1 phosphorylation both before and after CaCl₂ treatment (Figure 5D, Ecm33 OP, left panel). The inhibitory effect of Ecm33 overproduction on Pmk1 phosphorylation was also observed when cells were treated with 500 mM NaCl (Figure 5D, right panel).
port in budding yeast where simultaneous deletion of ECM33 and SLT2 results in synthetic lethality (Pardo et al., 2004).

**Altered Calcium Homeostasis in Δecm33 Cells**

To further characterize the Ca\(^{2+}\)-related phenotypes associated with Δecm33 cells, we used the 3xCDRE::luc(R2.2) reporter system that was developed to monitor the real-time activity of the Ca\(^{2+}\)/calcineurin signaling pathway (Deng et al., 2006). We assumed that if Ca\(^{2+}\) homeostasis is compromised in Δecm33 cells, the CDRE reporter response would be altered from that of the wild-type cells. As shown in Figure 6C, the Δecm33 cells showed an enhanced 3xCDRE::luc(R2.2) reporter response in the presence of various concentrations of extracellular CaCl\(_2\) (0–200 mM), compared with that of the wild-type cells. Notably, compared with the wild-type cells, Δecm33 cells showed a continuous increase in the 3xCDRE::luc(R2.2) response (Figure 6C, 100 mM CaCl\(_2\), 200 mM CaCl\(_2\)). The enhanced calcineurin activity as evidenced by the CDRE response and Ca\(^{2+}\) hypersensitivity of Δecm33 cells is reminiscent of that observed in Δpmk1 cells, which lack the vacuolar Ca\(^{2+}\)-ATPase (Deng et al., 2006). On the other hand, the overproduction of Ecm33 (Ecm33 OP) lowered the CDRE response even in the presence of 200 mM CaCl\(_2\) (Figure 6D).

We further examined the effect of Pmk1 deletion on the CDRE reporter response. In the presence of 100 mM CaCl\(_2\), the Δpmk1 cells exhibited a slightly lower peak response of the CDRE reporter than the wild-type cells. In the presence of 200 mM CaCl\(_2\), the peak response of the Δpmk1 cells was approximately half that of the wild-type cells (Figure 6E). Importantly, the peak responses of the CDRE reporter in the Δecm33Δpmk1 cells were almost similar to those in the Δpmk1 cells suggesting that the increased Ca\(^{2+}\)/calcineurin signaling observed in the Δecm33 mutant is dependent on Pmk1. However, the calcineurin activity remains higher in the Δecm33Δpmk1 cells than in the Δpmk1 cells, after the peak response to CaCl\(_2\) stimulation has been attained (Figure 6E).

---

Figure 6. Altered Ca\(^{2+}\) homeostasis in Δecm33 cells. (A) Δecm33 cells exhibited Ca\(^{2+}\) hypersensitivity. Wild-type cells, Δecm33, Δpmk1, or Δecm33Δpmk1 cells were streaked onto the plates as indicated and then incubated for 4 d at 27°C. (B) Δecm33 cells exhibited abnormal morphology. Morphology of the wild-type cells, Δecm33, Δpmk1, or Δecm33Δpmk1 cells incubated in YPD liquid medium. Scale bar, 10 μm. (C) Δecm33 cells displayed an enhanced calcineurin activity. Wild-type cells, Δecm33, or Δppbl cells harboring the multicopy plasmid [3xCDRE::luc(R2.2)] reporter vector were incubated with β-luciferin and treated with various concentrations of CaCl\(_2\) and then were analyzed as in Figure 1D. (D) Wild-type cells harboring the multicopy plasmid [3xCDRE::luc(R2.2)] reporter vector were transformed with either the control vector (vector) or the ecm33\(^{3+}\) gene (Ecm33 OP) and then analyzed as in Figure 6C. (E) Pmk1 deletion suppressed the enhanced calcineurin activity in Δecm33 cells. Wild-type, Δpmk1, or Δecm33Δpmk1 cells harboring the multicopy plasmid [3xCDRE::luc(R2.2)] reporter vector were analyzed for calcineurin activity as in Figure 6C.
Figure 7. Ecm33 regulates Ca\textsuperscript{2+} influx. The peak response of intracellular Ca\textsuperscript{2+} monitoring after the addition of CaCl\textsubscript{2}. Wild-type, Δecm33, and cells overproducing Ecm33 (Ecm33 OP) were transformed with pREP1-AEQ, and their intracellular Ca\textsuperscript{2+} levels were monitored during the first 10 min. Cells were either untreated or treated with 100 mM CaCl\textsubscript{2} or 200 mM CaCl\textsubscript{2}. The aequorin assay was performed as described in Materials and Methods. The data were averaged from peak heights of three independent experiments, and each sample was done in duplicate. Error bars, SD.

DISCUSSION

In the present study, the identification of Ecm33 as a novel component of the Pmk1 MAPK cell integrity signaling has led to the discovery that two transcription factors, namely, Atf1 and MBX1, are involved in the Pmk1-dependent expression of Ecm33. We also developed a reporter system to monitor the real-time activity of these transcription factors and hence the activation of the Pmk1 pathway.

Here, we show that Ecm33 is involved in the cell integrity signaling. First, mutants lacking ecm33\textsuperscript{+} displayed hypersensitivity to two typical cell wall–damaging agents, calcofluor white and β-glucanase. Second, Δecm33 cells exhibited the vic-negative phenotype and hyperphosphorylation of the Pmk1 MAPK, which is a strong indication of the negative regulation of the Pmk1 signaling (Ma et al., 2006). Third, Ecm33 overproduction suppressed calcineurin deletion and inhibited Pmk1 MAPK phosphorylation upon treatment with CaCl\textsubscript{2} and NaCl. Fourth, the mRNA levels of ecm33\textsuperscript{+} were Pmk1/Atf1– as well as Pmk1-Mbx1– dependent. Thus, Ecm33 is a novel component of the Pmk1 MAPK pathway.

In budding yeast, signaling via Mpk1-Rlm1 regulates the expression of several genes implicated in cell wall biogenesis, including PST1 (Jung and Levin, 1999; Jung et al., 2002). Unexpectedly, the deletion mutants of Mbx2, an Rlm1 homologue in fission yeast, displayed only modest sensitivity to cell wall–damaging agents, such as calcofluor (Figure 2E), suggesting that unlike budding yeast, the Rlm1 homolog only plays a minor role in cell wall integrity in fission yeast. Here, we identified Ecm33, a cell surface GPI-anchor protein homologous to PST1, as a target of Pmk1 and Atf1. Moreover, Mbx1, but not Mbx2, was found to be involved in the regulation of Ecm33 expression (Figure 2D). Mbx1 has been shown to be involved in gene expression in the M-G1 phase as a component of PBF (Pombe cell cycle box binding factor) transcriptional complex (Buck et al., 2004). Although Mbx1 genetically and functionally interacts with two forkhead transcription factors Fkh2 and Sep1 together with Plo1, the direct target of Mbx1 and its physiological role have not yet been elucidated; this is because the deletion of mbx1\textsuperscript{+} has little effect on M–G1 transcription (Papadopoulou et al., 2008). In this study, we showed that Mbx1 is involved in cell integrity in fission yeast via the regulation of Ecm33 in a Pmk1-dependent manner. Recently, Papadopoulou et al. (2008) reported that the Polo kinase Plo1, a key regulator of cell cycle, binds and phosphorylates Mbx1. It would be intriguing to speculate that Plo1 and Pmk1 kinases coordinately regulate cell cycle and/or cell integrity signaling via the phospho-regulation of Mbx1 activity.

Another important finding of this study is the role of Ecm33 in the MAPK cell integrity signaling. The finding that Ecm33 deletion and overproduction affect Ca\textsuperscript{2+}/calcineurin signaling and Ca\textsuperscript{2+} homeostasis suggested the possibility that some Ca\textsuperscript{2+}-mobilizing mechanism(s) might be involved in the Ecm33-mediated suppression of Pmk1 signaling. Carnero et al. (2000) reported that ehs1\textsuperscript{+}/gam8\textsuperscript{+}, encoding a homologue of the budding yeast Mid1, is involved in Ca\textsuperscript{2+} accumulation and cell wall integrity. Interestingly, high extracellular levels of Ca\textsuperscript{2+} as well as pck2\textsuperscript{+} overexpression suppressed all the phenotypes of ehs1/gam8 mutants, suggesting that the cell integrity defects of ehs1/gam8 mutant
result from inadequate calcium levels in the cell (Carnero et al., 2000). Similarly, our recent study showed that Pmk1 is required for the stimulation of calcineurin via Yam8/Cch1-mediated Ca\(^{2+}\) influx and that knockout of pck2 gene markedly diminished the Yam8/Cch1-dependent stimulation of calcineurin activity, suggesting that Pck2 acts upstream of Pmk1 in this signaling pathway (Deng et al., 2006). Thus, exogenous Ca\(^{2+}\) activates the Pck2/Pmk1 signaling, which in turn leads to Yam8/Cch1-mediated Ca\(^{2+}\) influx. The hyperactivation of Pck2/Pmk1 signaling induces lethality associated with strong Ca\(^{2+}\) accumulation (Carnero et al., 2000; Deng et al., 2006). One way to reverse this effect is by the dephosphorylation and inactivation of Pmk1 via the overexpression of Pmp1 or PP2C phosphatases as evidenced in our previous studies (Sugiyama et al., 1998; Takada et al., 2007). Alternatively, inactivation of the Ca\(^{2+}\)-influx machinery, such as Yam8/Cch1 complex, and maintenance of the normal Ca\(^{2+}\) homeostasis within the cell would also rescue cells from the lethal effect.

The molecular mechanism underlying Ecm33-mediated modulation of Pmk1 signaling is currently unknown. Given the plasma membrane localization of Ecm33, we hypothesize that Ecm33, a GPI-anchored protein, like the sensor protein Wsc1 in budding yeast (Philip and Levin, 2001), might interact with some component(s) of the plasma membrane localizing MAPK signaling molecules and/or the components of Ca\(^{2+}\)-influx machinery to inhibit protein function (Figure 8). Our results showed that Ecm33 deletion and overproduction affects Pmk1 phosphorylation upon treatment with CaCl\(_2\) and NaCl, two independent stimuli that activate Pmk1 (Figure 5, B and D). Moreover, Pmk1 deletion markedly suppressed the increased Ca\(^{2+}\)/calcineurin signaling observed in Δecm33 cells (Figure 6E). These results favor the possibility that Ecm33 impinges on the Pmk1 MAPK cascade via a Ca\(^{2+}\)-independent mechanism and that Pmk1 then regulates Ca\(^{2+}\) influx. However, Δecm33/pmk1 cells exhibited a higher CDRE reporter activity than the Δpmk1 cells after the peak response (Figure 6E), and Ecm33 overproduction resulted in a lower CDRE response than that in Δpmk1 cells upon in the CaCl\(_2\) treatment (Figure 6D). Thus, it does not exclude the possibility that Ecm33 may exert its effect on Ca\(^{2+}\)/calcineurin signaling largely via Pmk1 pathway and partly via other Ca\(^{2+}\)-influx machineries (Figure 8). Further studies will be required to clarify the precise role of Ecm33 and its involvement in the MAPK signaling.

In conclusion, to our knowledge, this article provides the first evidence for the involvement of a GPI-anchored cell surface protein in the negative regulation of cell wall integrity Pmk1 MAPKs signaling. Furthermore, we also discovered a novel functional link between Ecm33 and cellular Ca\(^{2+}\) signaling. Given the high similarity between the MAPK pathways of fission yeast and the mammals, this study may provide the basis of understanding the regulatory mechanisms underlying MAPK signaling in higher eukaryotes.

ACKNOWLEDGMENTS

We thank Drs. M. Yanagida, and T. Toda and Yeast Resource Centre (YGRC/ NBRP, http://yeast.lab.nig.ac.jp/pig) for providing strains and plasmids, Kazue Masuko for able technical assistance; and Susie O. Sio and Yukiko Fujimoto for critical reading of the manuscript. We are grateful to the members of the Laboratory of Molecular Pharmacogenomics for their support. This work was supported by Grant-in-Aid for Scientific Research on Innovative Areas and research grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (R.S.). This work was also financially supported by the Antaging Center Project for Private Universities from Ministry of Education, Culture, Sports, Science, and Technology, 2008–2012. H.T. is a Research Fellow of the Japan Society for the Promotion of Science.

REFERENCES

Agarwal, A. K., Rogers, P. D., Baer, S. R., Jacob, M. R., Barker, K. S., Cleary, J. D., Walker, L. A., Nagle, D. G., and Clark, A. M. (2003). Genome-wide expression profiling of the response to polyene, pyrimidine, azole, and echi- nocandin antifungal agents in Saccharomyces cerevisiae. J. Biol. Chem. 278, 34998–35015.

Bahler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A., III, Steever, A. B., Wach, A., Philippsen, P., and Pringle, J. R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast 14, 943–951.

Buck, V., Ng, S. S., Ruiz-Garcia, A. B., Papadopoulou, K., Bhatti, S., Samuel, J. M., Anderson, M., Millar, J. B., and Mcnemey, C. J. (2004). Fkh2p and Sep1p regulate mitotic gene transcription in fission yeast. J. Cell Sci. 117, 5623–5632.

Carnero, E., Ribas, J. C., Garcia, B., Duran, A., and Sanchez, Y. (2000). Schizo-saccharomyces pombe ehh1p is involved in maintaining cell wall integrity and in calcium uptake. Mol. Gen. Genet. 264, 173–183.

De Groot, P. W., Hellingwerf, K. J., and Klis, F. M. (2003). Genome-wide identification of fungal GPI proteins. Yeast 20, 781–796.

Deng, L., Sugiyama, S., Takeuchi, M., Suzuki, M., Ebina, H., Takami, T., Koike, A., Iba, S., and Kuno, T. (2006). Real-time monitoring of calcineurin activity in living cells: evidence for two distinct Ca\(^{2+}\)-dependent pathways in fission yeast. Mol. Biol. Cell 17, 4790–4800.

Edmunds, J. W., and Mahadevan, L. C. (2004). MAP kinases as structural adaptors and enzymatic activators in transcription complexes. J. Cell Sci. 117, 3715–3723.

Gustin, M. C., Albertyn, J., Alexander, M., and Davenport, K. (1998). MAP kinase pathways in the yeast Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. 62, 1246–1300.

Herskovitz, I. (1995). MAP kinase pathways in yeast: for mating and more. Cell 80, 187–197.

Hirayama, S., Sugiyama, R., Lu, Y., Maeda, T., Kawagishi, K., Yokoyama, M., Tobida, H., Hama, Y. G., Shuntoh, H., and Kuno, T. (2003). Zinc finger protein Prz1 regulates Ca\(^{2+}\) but not Cl\(^{-}\) homeostasis in fission yeast: identification of distinct branches of calcineurin signaling pathway in fission yeast. J. Cell Biol. 278, 18078–18084.

Jung, U. S., and Levin, D. E. (1999). Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. Mol. Microbiol. 34, 1049–1057.

Jung, U. S., Sobering, A. K., Romeo, M. J., and Levin, D. E. (2002). Regulation of the yeast Rim1 transcription factor by the Mpk1 cell wall integrity MAP kinase. Mol. Microbiol. 46, 781–789.

Kohrer, K., and Domdey, H. (1991). Preparation of high molecular weight RNA. Methods Enzymol. 194, 398–405.

Lawrence, C. L., Jones, N., and Wilkinson, C. R. (2009). Stress-induced phosphorylation of S. pombe Atl1 abrogates its interaction with F box protein Fbh1. Curr. Biol. 19, 1907–1911.

Lawrence, C. L., Maekawa, H., Worthington, J. L., Reiter, W., Wilkinson, C. R., and Jones, N. (2007). Regulation of Schizosaccharomyces pombe Atl1 protein levels by Sty1-mediated phosphorylation and heterodimerization with Per1. J. Biol. Chem. 282, 5160–5170.

Leskinen, P., Virta, M., and Karp, M. (2003). One-step measurement of firefly luciferase activity in yeast. Yeast 19, 128–131.

Ma, Y., Kuno, T., Kita, A., Asayama, Y., and Sugiyama, R. (2006). Rho2 is a target of the farnesyltransferase Cpp1 and acts upstream of Pmk1 mitogen-activated protein kinase signaling in fission yeast. Mol. Biol. Cell 17, 5026–5037.

Madrid, M., Soto, T., Khong, H. K., Franco, A., Vicente, J., Perez, P., Cacho, M., and Cansado, J. (2006). Stress-induced response, localization, and regulation of the Pmk1 cell integrity pathway in Schizosaccharomyces pombe. J. Biol. Chem. 281, 2033–2043.

Marshall, C. J. (1994). MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr. Opin. Genet. Dev. 4, 82–89.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol. 194, 795–823.

Nishida, E., and Gotob, Y. (1993). The MAP kinase cascade is essential for diverse signal transduction pathways. Trends Biochem. Sci. 18, 128–131.
