Yeast Mpk1 Cell Wall Integrity Mitogen-activated Protein Kinase Regulates Nucleocytoplasmic Shuttling of the Swi6 Transcriptional Regulator

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The yeast SBF transcription factor is a heterodimer comprised of Swi4 and Swi6 that has a well defined role in cell cycle-specific transcription. SBF serves a second function in the transcriptional response to cell wall stress in which activated Mpk1 mitogen-activated protein kinase of the cell wall integrity signaling pathway forms a complex with Swi4, the DNA binding subunit of SBF, conferring upon Swi4 the ability to bind DNA and activate transcription of FKS2. Although Mpk1–Swi4 complex formation and transcriptional activation of FKS2 does not require Mpk1 catalytic activity, Swi6 is phosphorylated by Mpk1 and must be present in the Mpk1–Swi4 complex for transcriptional activation of FKS2. Here, we find that Mpk1 regulates Swi6 nucleocytoplasmic shuttling in a biphasic manner. First, formation of the Mpk1–Swi4 complex recruits Swi6 to the nucleus for transcriptional activation. Second, Mpk1 negatively regulates Swi6 by phosphorylation on Ser238, which inhibits nuclear entry. Ser238 neighbors a nuclear localization signal (NLS) whose function is blocked by phosphorylation at Ser238 in a manner similar to the regulation by Cdc28 of another Swi6 NLS.

INTRODUCTION

The cell wall of the budding yeast *Saccharomyces cerevisiae* is required to maintain cell shape and integrity (Klis, 1994; Cid et al., 1995). The cell must remodel this rigid structure during vegetative growth and during pheromone-induced morphogenesis. Wall remodeling is monitored and regulated by the cell wall integrity (CWI) signaling, which activates a mitogen-activated protein kinase (MAPK) kinase cascade (reviewed in Levin, 2005). The MAP kinase cascade is a linear pathway comprised of Pkc1, a mitogen-activated protein kinase kinase (MAPK) that is activated in response to growth factors as well as physical and chemical stresses (Abe et al., 1996; Yan et al., 2001).

CWI signaling is induced in response to a variety of cell wall stressors. First, signaling is activated persistently in response to growth at elevated temperatures (e.g., 37–39°C; Kamada et al., 1995), consistent with the finding that null mutants in many of the pathway components display cell lysis defects only when cultivated at high temperature. Second, hypo-osmotic shock induces a rapid but transient activation of signaling (Davenport et al., 1995; Kamada et al., 1995). Third, treatment with mating pheromone stimulates signaling at a time that is coincident with the onset of morphogenesis (Buehrer and Errede, 1997). Finally, CWI signaling is also stimulated by agents that interfere with cell wall biogenesis, such as the chitin antagonist calcofluor white (CFW) (Ketela et al., 1999), Congo red, caffeine, or Zymolyase (de Nobel et al., 2000; Martin et al., 2000).

CWI signaling pathway induces activation of two known transcription factors. One of these factors is Rim1 (Dodou and Treisman, 1997; Watanabe et al., 1997), which is activated through phosphorylation by Mpk1 (Jung et al., 2002). A second transcription factor that plays a role in CWI signaling is SBF (Madden et al., 1997; Baetz et al., 2001). SBF is a dimeric transcriptional regulator, made up of Swi4 and Swi6, which is essential to normal regulation of G1-specific transcription (reviewed in Breeden, 2003). Swi4 is the sequence-specific DNA-binding subunit (Taylor et al., 1997), but Swi6 is required for binding to cell cycle-regulated promoters (Andrews and Moore, 1992; Sidorova and Breeden, 1993; Baetz and Andrews, 1999). Swi6 allows Swi4 to bind DNA by relieving an autoinhibitory intramolecular association of the Swi4 C terminus with its DNA-binding domain. In addition, Swi6 is the transcriptional activation component of SBF (Sedgwick et al., 1998).

That SBF has a second function related to CWI signaling was suggested with several findings. First, the cell lysis defect of an *mpk1Δ* mutant is suppressed by overexpression of *swi4* (Madden et al., 1997). Second, both *swi4Δ* and *swi6Δ*
Although Swi6 is not required for Swi4 to bind to the nucleus in a manner dependent on formation of the Mpk1–Swi6 complex, but independently of Mpk1 catalytic activity. Swi6 subsequently exits the nucleus in response to phosphorylation by Mpk1 on Ser238, which interferes with the function of an adjacent nuclear localization signal (NLS). Finally, we identified Kap120 as the β-importin that binds to Swi6 through this NLS.

### MATERIALS AND METHODS

#### Strains, Growth Conditions, and Transformations

The *S. cerevisiae* strains used in this study are listed in Table 1. Yeast cultures were grown in YEPD (1% Bacto yeast extract, 2% Bacto Peptone, and 2% glucose) with or without 2% sorbitol for osmotic support, or in SD (0.67% yeast nitrogen base and 2% glucose) supplemented with the appropriate nutrients to select for plasmids and gene replacements. *Escherichia coli DH5α* was used to propagate all plasmids. *Escherichia coli* cells were cultured in Luria broth medium (1% Bacto Tryptone, 0.5% Bacto yeast extract, and 1% NaCl) and transformed to carbenicillin resistance by standard methods. Promoter-lacZ expression experiments were carried out as described previously (Kim et al., 2008), with methods for β-galactosidase assays described in Zhao et al. (1998). DL3327 (mpk1Δ::TRP1 mpk1Δ::URA3) was constructed from DL3164 (mpk1Δ::TRP1 mpk1Δ::URA3) by selection for loss of URA3 function on 5-fluoro-orotic acid.

#### Plasmids

Plasmids used in this study are listed in Table 2. Double-overlap mutagenesis (Hs et al., 1989) was used to construct point mutants of *Swi6* in p2391 (pAC1202; CEN TRP1 SWI6-GFP; a gift from Anita Corbett, Emory University). Polymerase chain reaction (PCR) products from p2391 as template were treated with XhoI and SacI to liberate the fragment bearing various point

### Table 1. *S. cerevisiae* strains

| Strain | Relevant genotype (and strain background) | Reference or source |
|--------|------------------------------------------|---------------------|
| 1788   | MATa/MATa leu2-3,112 trp1-1 ura3-52 his4 can1* (EG123) | I. Herskowitz, (University of California, San Francisco); Siliciano and Tatchell (1984) |
| DL100  | MATa leu2-3,112 trp1-1 ura3-52 his4 can1* (EG123) | Kamada et al. (1995) |
| DL456  | MATa/MATa mpk1Δ::TRP1/mpk1Δ::TRP1 (EG123) | Kim et al. (2008); Kim et al. (2008) |
| DL3145 | MATa/MATa swi6Δ::LEU2/swi6Δ::LEU2 (EG123) | Kim et al. (2008); Research Genetics (Huntsville, AL) |
| DL3187 | MATa his4Δ leu2Δ ura3Δ his3Δ (S288c; BY4741) | Kim et al. (2008); Research Genetics (Huntsville, AL) |
| DL3195 | MATa/MATa mpk1Δ::KanMX/mpk1Δ::KanMX (S288c) | Kim et al. (2008); Research Genetics (Huntsville, AL) |
| DL3196 | MATa/MATa mpk1Δ::KanMX/mpk1Δ::KanMX mpk1Δ::KanMX/mpk1Δ::KanMX (S288c) | This study |
| DL3233 | MATa swi6Δ::KanMX (S288c) | P. Silver; Seedorf and Silver (1997) |
| DL3277 | MATa/MATa mpk1Δ::TRP1/mpk1Δ::TRP1 mpk1Δ::ura3::mpl1Δ::ura3; a gift from Anita Corbett, Emory University |
| DL3809 | MATa kapi10Δ::KanMX (S288c) | Research Genetics (Huntsville, AL) |
| DL3810 | MATa kapi114Δ::KanMX (S288c) | Research Genetics (Huntsville, AL) |
| DL3811 | MATa kapi120Δ::KanMX (S288c) | Research Genetics (Huntsville, AL) |
| DL3812 | MATa kapi122Δ::KanMX (S288c) | Research Genetics (Huntsville, AL) |
| DL3813 | MATa kapi123Δ::KanMX (S288c) | Research Genetics (Huntsville, AL) |
| DL3814 | MATa trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2 ura3 (SWY518) | S. Wente; Ryan et al. (2007) |
| DL3816 | MATa kap5-E126K trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2 ura3 (SWY518) | S. Wente; Ryan et al. (2007) |
| DL3821 | MATa ura3-52 trp1-63 leu2-1 GAL+ (PSY580) | P. Silver; Seedorf and Silver (1997) |
| DL3822 | MATa pse1-1 ura3-52 trp1-63 leu2-1 GAL+ (PSY580) | P. Silver; Seedorf and Silver (1997) |
| DL3823 | MATa ade2-1 his3-11,15 ura3-52 trp1-1 can1-100 GAL+ (RS543) | E. Hurt; Senger et al. (1998) |
| DL3824 | MATa mtr10(kap1111); H153 ade2-1 his3-11,15 ura3-52 trp1-1 can1-100 GAL+ (RS543) | E. Hurt; Senger et al. (1998) |
| DL3835 | MATa KAPI20-TAP S288c | Open Biosystems (Huntsville, AL) |
| DL3878 | MATa KAPI20-TAP swi6Δ::LEU2 (S288c) | This study |
Table 2. Plasmids

| Plasmid | Description | Reference of source |
|---------|-------------|---------------------|
| pRS313  | HIS3-based centromeric plasmid | Sikorski and Hieter (1989) |
| pRS315  | LEU2-based centromeric plasmid | Sikorski and Hieter (1989) |
| pRS316  | URA3-based centromeric plasmid | Sikorski and Hieter (1989) |
| pAC242  | TRP1-based centromeric plasmid with GFP | A. Corbett |
| p904    | pLG4-178; CYC1-lacZ | Guarente and Mason (1993) |
| p1366   | PRM3-lacZ | Jung et al. (2002) |
| p2022   | Yep351 MLPI-3×HA | Kim et al. (2008) |
| p2057   | PKS2-R340 to -375-CYC1-lacZ with URA3 marker | Kim et al. (2008) |
| p2069   | CLN2-600 to -400-CYC1-lacZ with URA3 marker | Kim et al. (2008) |
| p2120   | Yep13 SWI6 | B. Andrews |
| p2188   | pRS315 MPK1-3×HA | Kim et al. (2008) |
| p2190   | pRS315 mpk1-T190A, Y192F-3×HA | Kim et al. (2008) |
| p2193   | pRS315 mpk1-K54R-3×HA | Kim et al. (2008) |
| p2344   | swi6-Δ::LEU2 | Kim et al. (2008) |
| p2391   | pAC1202, CEN TRP1 SWI6-GFP | A. Corbett |
| p2393   | CEN TRP1 swi6-S160A-GFP | A. Corbett |
| p2542   | pRS313 SWI6 | This study |
| p2543   | pRS313 swi6-S160A | This study |
| p2545   | pRS313 swi6-T179A | This study |
| p2546   | pRS313 swi6-S228A | This study |
| p2547   | pRS313 swi6-S238A | This study |
| p2548   | pRS313 swi6-S238E | This study |
| p2557   | CEN TRP1 swi6-S238A-GFP | This study |
| p2558   | CEN TRP1 swi6-S238E-GFP | This study |
| p2647   | pRS316 SWI6-GFP | This study |
| p2713   | pRS315 SWI4 | Truman et al. (2009) |
| p2714   | pRS315 swi6-I913A, I915A | Truman et al. (2009) |
| p2729   | CEN TRP1 swi6-K163A-GFP | This study |
| p2730   | CEN TRP1 swi6-K231A-GFP | This study |
| p2731   | CEN TRP1 swi6-I232A-GFP | This study |
| p2733   | CEN TRP1 swi6-K163A, K231A-GFP | This study |
| p2773   | CEN TRP1 swi6-T179A-GFP | This study |
| p2774   | CEN TRP1 swi6-S228A-GFP | This study |
| p2831   | pRS313 swi6-K163A | This study |
| p2832   | pRS313 swi6-K231A | This study |
| p2860   | YCpGal2-2GFP-SWI6-NLS2 | This study |
| p2861   | YCpGal2-2GFP-SWI6-NLS2-K231A | This study |
| pGS840  | YCpGal2-2GFP | Maurer et al. (2001) |

Mutants in the N-terminal domain of Swi6. These fragments were cloned into p2391 (by XhoI/Sacl) to generate p2397, p2558, p2729-p2733, and p2773-p2777. To create untagged mutant alleles of SWI6, the wild-type allele was first subcloned from p2120 (Yep13 SWI6; a gift from Brenda Andrews, University of Toronto) into pRS313 using a Kpn1/Nde1 fragment that bears the entire coding sequence with the promoter and terminator to yield p2542. Next, potential phosphorylation site mutant swi6 alleles were cloned into p2542 from the green fluorescent protein (GFP)-tagged constructions using Xho1 and Sac1, as described above, to yield p2543-p2548, p2831, and p2832. The marker was changed in p2391 from TRP1 to URA3 by subcloning the entire SWI6-GFP into pRS316 (by vector sites EcoRV/KpnI) to create p2647. The 10-amino acid Swi6-NLS2 sequence (residues 228–237), or its K231A mutant form, were fused to the C terminus of a tandem GFP-GFP in YCp-TRP1::LEU2-K231A-GFP (pC8840) by using double-overlap mutagenesis. A 0.7-kb BamHI–PvuI fragment from the amplified region was cloned into the corresponding sites in the vector so as to fuse the NLS sequence in frame with GFP. This yielded 2xGFPs fused either to wild-type NLS2 (p2860) or to the K231A mutant form (p2861). All PCR-amplified sequences were confirmed by DNA sequence analysis across the entire amplified region. Primer sequences are available upon request.

Immunoblot Detection of Swi6

Protein extracts were made as described previously (Kamada et al., 1995). After separation of proteins (20 μg) by SDS-polyacrylamide gel electrophoresis (PAGE) (7.5%), Swi6 was detected by immunoblot analysis with goat polyclonal anti-Swi6 (yN-19) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:2000 dilution. Secondary antibodies (horse-radish peroxidase-conjugated rabbit anti-goat; Jackson ImmunoResearch Laboratories, West Grove, PA) were used at a 1:25,000 dilution.

Coprecipitation of Swi6 with Kap120-TAP

An OPEN Biosystems strain expressing tandem affinity purification (TAP)-tagged Kap120 (bearing a calmodulin-binding domain and two immunoglobulin [IgG]-binding domains from Staphylococcus aureus protein A; DL3835) was transformed with a swi6-Δ::LEU2 cassette (p2344; Kim et al., 2008) to delete the endogenous SWI6 gene. The resultant strain (DL3878) was transformed with centromeric plasmids (pRS313) bearing wild-type or point mutant forms of SWI6. TAP-tagged Kap120 was immunoprecipitated from protein extracts (100 μg) of cells grown at room temperature or heat shocked for 2 h at 39°C, with IgG-Sepharose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Immunoprecipitates were subjected to Swi6 detection using 20% of the precipitated protein, as described above. An isolectric wild-type strain (DL3817) was used as an untagged control. Whole-cell extract (20 μg) from each time point was used as loading controls. The antibodies used to detect Swi6 also detected Kap120-TAP by virtue of the ZZ (protein A)-tag. For the Gsp1-guanosine triphosphate (GTP) release experiment, Swi6 coprecipitated with Kap120-TAP was released by incubation of the immunoprecipitates for 2 h on ice with 29 μg Gsp1-GTP (6His-Gsp1-Q71L; Maurer et al., 2001) in 50 μl of immunoprecipitation buffer (Kamada et al., 1995), replacing EDTA and EGTA with 2 mM MgCl2 and 3 mM KCl. Sample treated for release of Swi6 was washed with immunoprecipitation buffer before immunoblot analysis for Kap120-TAP and Swi6.

Detection of Swi6 Nuclear Localization

For experiments in which yeast strains were synchronized in G2 phase, cells were grown in selective medium to mid-log phase, washed, and resuspended in YPD (with 2% sorbitol where indicated) at a density of 0.5 before nocodazole treatment (15 μg/ml; Sigma-Aldrich, St. Louis, MO) for 4 h at 23°C to synchronize cells. Cultures were then subjected to heat stress by 2.1 dilution with fresh medium prewarmed to 75°C, which resulted in an immediate heat synchronization.
Sidorova region of Swi6, were mutated as a group (Swi6-SA4; of these sites, which occur in a cluster in the N-terminal sites (S/T-P; S160, T179, S228, S238, and T320). The first four

Mpk1 by other cell wall stressors, including CFW, caffeine, phosphatase treatment (Madden et al., 1997). Activation of Mpk1 by other cell wall stressors, including CFW, caffeine, and Congo red also results in phosphorylation of Swi6 (Figure 1B).

Swi6 contains five potential MAP kinase phosphorylation sites (S/T-P; S160, T179, S228, S238, and T320). The first four of these sites, which occur in a cluster in the N-terminal region of Swi6, were mutated as a group (Swi6-SA4; Sidorova et al., 1995) in experiments aimed at identifying the

Cell wall stress-induced phosphorylation of Swi6. (A) Mild heat shock induces an Mpk1-dependent band shift in Swi6. Wild-type yeast (1788), an mpk1Δ mutant (DL456), and a Swi6Δ (DL3148) mutant were grown to mid-log phase in YPD + 2% sorbitol at 23°C and subjected to heat shock (39°C) for 2 h before extract preparation and immunoblot detection of Swi6. (B) Other cell wall stressors induce phosphorylation of Swi6. Wild-type yeast strain (1788) was grown to mid-log phase in YPD at 23°C and subjected to the indicated cell wall stress for 2 h before extract preparation and immunoblot detection of Swi6. U, unstressed; HS, heat shock (as in A); C, caffeine (8 mM for 2 h); CFW (40 μg/ml for 2 h); and CR, Congo red (50 μg/ml for 2 h). (C) Identification of Ser238 of Swi6 as a cell wall stress-induced phosphorylation site. A Swi6Δ strain was transformed with centromeric plasmids expressing wild-type Swi6 (p2542), the indicated point mutants, or the parent vector (V; pRS313). Transformants were grown to mid-log phase in YPD at 23°C and subjected to heat shock as described above before detection of Swi6. (D) Effects of Swi6 phosphorylation site mutations on FKS2 transcription. An FKS2-lacZ reporter plasmid (p2052) was cotransformed with a centromeric plasmid expressing wild-type Swi6 (p2542), Swi6-S238A (p2547), or Swi6-S238E (p2548) into a swi6Δ strain (DL3233). Transformants were grown to saturation at 23°C in selective medium. Cultures were diluted into 3 ml of YPD so that subsequent incubation at 23 or 39°C for 15 h resulted in mid-log phase cultures (ΔA600 of 1.0–1.5). β-Galactosidase activity was measured in crude extracts. Each value represents the mean and SD from three independent transformants. Sp. Act., specific activity; U, unit.

**RESULTS**

**Mpk1 Phosphorylates Swi6 on Ser238 in Response to Cell Wall Stress**

Madden et al. (1997) demonstrated that Swi6 is phosphorylated in vivo and in vitro by heat stress-activated Mpk1. This phosphorylation can be detected as an MPK1-dependent shift in Swi6 mobility to a more slowly migrating form (Figure 1A) that is collapsed to the faster migrating form by phosphatase treatment (Madden et al., 1997). Activation of Mpk1 by other cell wall stressors, including CFW, caffeine, and Congo red also results in phosphorylation of Swi6 (Figure 1B).

Swi6 contains five potential MAP kinase phosphorylation sites (S/T-P; S160, T179, S228, S238, and T320). The first four of these sites, which occur in a cluster in the N-terminal region of Swi6, were mutated as a group (Swi6-SA4; Sidorova et al., 1995) in experiments aimed at identifying the Cdc28 phosphorylation site (also S/T-P), now known to be S160 (Geymonat et al., 2004). Baetz et al. (2001) noted that the Swi6-SA4 mutant could not be phosphorylated by Mpk1 in vivo, suggesting that at least one of the mutated S/T-P sites was a target for Mpk1. Therefore, we mutated each of these S/T sites individually to alanine residues to prevent phosphorylation and tested the ability of each mutant to undergo mobility shift in response to mild heat shock. Only the Swi6-S238A mutant failed to shift its mobility after a 2-h heat shock (Figure 1C), indicating that Ser238 is an Mpk1 phosphorylation site. We have shown previously that FKS2-lacZ is a reliable reporter for CWI-induced transcription through SBF that responds to heat shock, Congo red (Kim et al., 2008), and calcofluor white (Kim, unpublished data). We chose to use heat shock rather than another cell wall stress for these and subsequent experiments, because heat shock activates Mpk1 rapidly (within 20 min; Kamada et al., 1995). Interestingly, the swi6-S238A mutant was hyperresponsive to heat stress-induced FKS2-lacZ transcription (Figure 1D), suggesting that Mpk1 phosphorylation of Swi6 on Ser238 is a negative regulatory event. Consistent with this conclusion, the swi6-S238E phosphomimic mutant was hyporesponsive to this stress.

**Mpk1 Regulation of Swi6 Nucleocytoplasmic Shuttling**

Swi6 undergoes nucleocytoplasmic shuttling in a cell cycle-regulated manner (Sidorova et al., 1995). It resides predominately in the cytoplasm from late G1 until late M phase, at which time it relocates to the nucleus in response to dephosphorylation at Ser160, where it remains throughout most of G1. The Clb6/Cdc28 S phase cell cycle kinase is responsible for phosphorylation of Swi6 at Ser160, an event that is not detectable through a mobility shift (Geymonat et al., 2004). This site resides immediately N-terminal to an NLS so that phosphorylation of Ser160 reduces the affinity of the α-importin (Sr1p) for the NLS, thereby diminishing the rate of Swi6 import relative to export, with the net effect...
being a reduction in nuclear accumulation of Swi6 (Harreman et al., 2004).

To determine whether cell wall stress regulates Swi6 through a similar mechanism, we examined the effect of Mpk1 activation on the localization of Swi6-GFP and Swi6-S238A-GFP. To avoid interference from cell cycle-dependent nucleocytoplasmic shuttling, progression through the cell cycle was arrested in G2 phase by treatment with the microtubule antagonist nocodazole. This resulted in a starting population of predominantly G2 cells (90%) with cytoplasmic Swi6 phosphorylated on Ser160. Mpk1 was then activated by mild heat shock (39°C) and the location of Swi6-GFP was followed by fluorescence microscopy. Swi6-GFP localized to the nucleus within 20 min of shift to high temperature and returned slowly to the cytoplasm over the next 40 min at high temperature (Figure 2A and B). Swi6 became phosphorylated on Ser238 gradually over this time course such that the resulting band-shift was complete after 60 min (Figure 2B, bottom). Significantly, Swi6-S238A-GFP entered the nucleus normally in response to heat shock, but it failed to return subsequently to the cytoplasm (Figure 2B). This suggested that phosphorylation of Swi6 on Ser238 by Mpk1 blocks nuclear import in a manner similar to that of Cdc28 phosphorylation at Ser160. This conclusion was supported by the observation that the Swi6-S238E-GFP phospho-mimic form of Swi6 failed to enter the nucleus at all (Figure 2B). In addition, these results revealed that nucleocytoplasmic shuttling of Swi6 in response to CWI signaling is a biphasic process—cell wall stress-stimulated Swi6 nuclear import, followed by phosphorylation-dependent return of Swi6 to the cytoplasm. Because net localization of Swi6 to the cytoplasm or the nucleus is a consequence of the balance between the rates of nuclear import and export, phosphorylation of Swi6 by Mpk1 might either inhibit its import or stimulate its export. We return to this issue below.

To dissect the requirements for cell wall stress-induced nucleocytoplasmic shuttling of Swi6, we first examined the impact of inactivating mutations in Mpk1. One mutant form, Mpk1-T190A, Y192F (Mpk1-TAYF; p2190), or Mpk1-K54R (p2193), or 2-μ Mlp1 (p2022), and wild-type Swi6-GFP (p2647). For this experiment, cells were treated as described in B, except that 2% sorbitol was added to the medium for osmotic support. (D) A swi4Δ mutant (DL3145) was cotransformed with centromeric plasmids expressing wild-type Swi4 (p2647) and either wild-type Swi4 (p2713) or Swi4-I913A, I915A (Swi4-IAIA; p2714). Cells were treated as described in B. (E) A swi4Δ mutant (DL3145) was transformed with a centromeric plasmid expressing wild-type Swi4 (p2713) or vector (pRS315). Transformants were subjected to heat shock for 2 h, and cell extracts were processed for immunoblot detection of endogenous Swi4.
The second mutation, Mpk1-K54R, resides within the ATP-binding site and blocks catalytic activity by interfering with ATP positioning. Although this mutant form of Mpk1 is also devoid of detectable protein kinase activity (Zarzov et al., 1996; Madden et al., 1997), it can be shifted to an active conformation through phosphorylation by Mkk1/2, and can therefore bind to Swi4 and drive FKS2 transcription through a noncatalytic mechanism (Kim et al., 2008). Figure 2C shows that, in the absence of the Mlp1 pseudokinase paralogue of Mpk1, the Mpk1-TAYF mutant was blocked for cell wall stress-induced nuclear entry of Swi6-GFP, indicating that activating signal to Mpk1 is required for Swi6 translocation to the nucleus. By contrast, the catalytically inactive Mpk1-K54R mutant was able to drive nuclear entry of Swi6-GFP normally but was blocked for subsequent nuclear exit. This latter behavior mimics that of the Swi6-S238A mutant, supporting the conclusion that Mpk1 stimulates return of Swi6 to the cytoplasm by phosphorylation at Ser238. Expression of Mlp1, which is naturally devoid of catalytic activity, induced Swi6-GFP behavior that was identical to that of the catalytically inactive Mpk1-K54R mutant (Figure 2C).

Although both the Mpk1-TAYF and Mpk1-K54R mutant forms are catalytically inactive, the observation that they behave differently with regard to nucleocytoplasmic shuttling of Swi6-GFP indicates that activated (phosphorylated) Mpk1 has a noncatalytic function in the recruitment of Swi6 to the nucleus. We know of only one noncatalytic function of Mpk1—formation of a complex with Swi4 that is competent to bind the FKS2 promoter (Kim et al., 2008). Therefore, we asked whether preventing the association between phosphorylated Mpk1 and Swi4 would inhibit nuclear entry of Swi6. To address this question, we used a form of Swi4 that is mutated at its Mpk1 docking site (swi4-I913A, I915A; Swi4-1A1A) and is therefore blocked for FKS2 transcription but carries out cell cycle transcription normally (Truman et al., 2004). This mutant was blocked for Swi6 nuclear import in response to heat shock (Figure 2D), indicating that formation of the Mpk1–Swi4 complex is required for nuclear recruitment of Swi6 in response to cell wall stress. Therefore, we asked whether recruitment of Swi6 to the nucleus by the Mpk1–Swi4 complex is required for Mpk1 phosphorylation of Swi6. Swi6 phosphorylation in response to heat shock was not impaired in a swi4Δ strain (Figure 2E) or the swi4Δ I915A1A mutation (data not shown), revealing that negative regulation of Swi6 by Mpk1 is independent of its nuclear recruitment.

Swi6 Possesses a Cell Wall Stress-regulated NLS

The sequence immediately N-terminal to the Mpk1 phosphorylation site on Swi6 (Ser238) is similar to the NLS immediately C-terminal to Ser160 (Figure 3A). To determine whether this sequence is important for nuclear import of Swi6, we constructed two mutations within the region immediately N-terminal to Ser238. One alteration (swi6–K231A) eliminates a lysyl residue that aligns with one shown previously to be critical for cell cycle-regulated nuclear import of Swi6 (Lys163; Harreman et al., 2004). The second alteration is at an adjacent isoleucyl residue (swi6–I232A). Both of these mutations blocked heat shock-induced nuclear import of Swi6 (Figure 3B). Taken in the aggregate, these data suggest that Swi6 possesses two NLS sequences that are both regulated negatively through phosphorylation by different protein kinases in response to different signals. Specifically, the NLS adjacent to Ser160 (NLS1) is inhibited by cell cycle-dependent phosphorylation by Cdc28 (Truman et al., 2004; Geymonat et al., 2004) and the NLS adjacent to Ser238 (NLS2) is inhibited by cell wall stress-induced phosphorylation by Mpk1. To determine whether regulation of Swi6 nucleocytoplasmic shuttling by Mpk1 has an impact on FKS2 transcription under the same conditions as described above, we used RT-PCR to examine the induction of endogenous FKS2 expression by cell wall stress in cells arrested in G2. In wild-type cells, modest induction of FKS2 was observed over the first 90 min (Figure 3C). By contrast, the swi6–K231A mutant was completely blocked for transcriptional activation of FKS2 in this setting, behaving identically to a swi6Δ mutant, consistent with its failure to enter the nucleus. The swi6–S238A phosphorylation site mutant, however, behaved indistinguishably from wild-type over this short time course. This is in contrast to the enhanced FKS2-lacZ transcription we observed for this mutant after a 15-h induction. That the short time course was not sufficient to bring out the effect of Swi6 phosphorylation on FKS2 transcription suggests that this modification may be most important during long-term cell wall stress.

To test the importance of Swi6 nucleocytoplasmic shuttling in the context of cells progressing normally through the cell cycle, we compared the behavior of GFP-tagged mutant forms of Swi6 that were defective in one or both of the NLS sequences. Approximately 45% of cycling cells with wild-type Swi6 growing in the absence of cell wall stress (23°C)
were scored with Swi6-GFP in the nucleus (Figure 4A), reflecting the fraction of cells in G1 phase. By contrast, the Swi6-NLS1 mutant (Swi6-K163A-GFP) was largely defective for nuclear localization through the cell cycle (~15% of cells scored with nuclear GFP), as reported previously (Harreman et al., 2004). The Swi6-NLS2 mutant (Swi6-K231A-GFP) was similar to wild-type in the fraction of cells that displayed nuclear localized Swi6 under this condition, indicating that NLS2 is not important for cell cycle-regulated nuclear localization. However, upon exposure to cell wall stress (20-min heat shock at 39°C), the situation was reversed. The fraction of cells with wild-type Swi6 in the nucleus increased from ~45–80% in response to cell wall stress, indicating that cells with cytoplasmic Swi6 (outside of G1 phase) responded to wall stress by relocating Swi6 to the nucleus. The NLS1 mutant was recruited to the nucleus normally in response to cell wall stress, whereas the NLS2 mutant failed to relocalize to the nucleus, even displaying a slight reduction in the fraction of cells with nuclear Swi6 from that in unstressed cycling cells. These results reveal the importance of NLS2, but not NLS1, in cell wall stress-induced Swi6 nuclear localization. The double NLS1 NLS2 mutant (Swi6-K163A, K231A-GFP) remained mostly cytoplasmic under both conditions (~15% of cells scored with nuclear GFP), indicating that both signals are blocked in this mutant. It is not clear why Swi6-K163A, K231A-GFP was detected in the nucleus of any cells. Perhaps Swi6 possesses a third, as yet undiscovered, NLS that is responsible for the remaining nuclear localization.

It was reported previously that interfering with the NLS adjacent to Swi6 Ser160 diminishes, but does not completely eliminate, SBF-driven cell cycle transcription (Sidorova et al., 1995). We confirmed this conclusion with the swi6-K163A NLS1 mutant. In fact, this mutant was only modestly reduced for SBF-dependent CLN2-lacZ expression (Figure 4B). The NLS1 mutant was also slightly impaired for FKS2-lacZ induction in response to cell wall stress (Figure 4C). Similar results were observed for the swi6-K231A NLS2 mutant. However, the double swi6-K163A, K231A mutant displayed an additive defect for both CLN2-lacZ expression and FKS2-lacZ induction, revealing a partial overlap of function between the two NLS sequences that the binary scoring of Swi6-GFP localization failed to capture. All of the mutant forms of Swi6 were maintained at levels comparable with wild-type as judged both by fluorescence signal and immunoblot detection (Figure 4D), indicating that their functional defects are not the consequence of Swi6 destabilization.

Kap120 Is the β-Importin That Recognizes Swi6 NLS2

Classical nuclear import signals are recognized by an α-importin in heterodimeric complex with a β-importin (Sorokin et al., 2007; Terry et al., 2007). Although yeast has only a single α-importin (Srp1), it possesses 10 β-importins (Fried and Kutay, 2003; Caesar et al., 2006). Srp1 forms a dimeric
complex with the β-importin Kap95 (Enenkel et al., 1995) and is known to recognize Swi6 NLS1 (Harreman et al., 2004). However, other β-importins are capable of binding nonclassical NLS signals in the absence of α-importin.

To identify the β-importin responsible for recognizing the Swi6 NLS2, we examined a collection of six deletion mutants (swm1Δ, kap114Δ, kap120Δ, kap122Δ [pdr6], kap123Δ [yrb4], and mtr10Δ [kap111]) and two temperature-sensitive mutants (kap95-E126K and pse1-1 [kap121]) in β-importin genes (Sorokin et al., 2007) for their ability to accumulate Swi6 in the nucleus in response to cell wall stress. Three of the mutants (kap120Δ, pse1-1, and kap123Δ) were blocked for Swi6 nuclear accumulation after a 20-min heat shock at 39°C (Figure 5A). To determine which, if any, of these β-importins recognizes Swi6 NLS2, we carried out coprecipitation experiments using epitope-tagged forms of all three β-importins. In preliminary experiments, only Kap120 coprecipitated Swi6 (data not shown), suggesting that Kap121 and Kap123 are important for nuclear localization of other factors required for Swi6 nuclear recruitment (perhaps Swi4 and Mpk1). Therefore, we focused on the Kap120/Swi6 interaction. Swi6 coprecipitated with Kap120 in extracts from cells grown at low temperature (Figure 5B). This association was diminished in response to heat shock for 1 h, suggesting that phosphorylation of Swi6 at Ser238 by Mpk1 decreases its affinity for Kap120.

To test this idea, we examined the binding of Kap120 with various mutant alleles of Swi6. A form of Swi6 that is blocked for function of NLS1 (Swi6-K163A) behaved like wild-type Swi6 for Kap120 binding (Figure 5B). By contrast, a form of Swi6 that is blocked for function of NLS2 (Swi6-K231A) was not found in association with Kap120. Finally, a form of Swi6 that cannot be phosphorylated by Mpk1 at Ser238 (Swi6-S238A) not only bound to Kap120, but this binding was not diminished in response to heat shock. This result suggests that phosphorylation of Swi6 at Ser238 reduces its affinity for Kap120 and explains how Mpk1 catalytic activity restores Swi6 to the cytoplasm after its nuclear recruitment in response to cell wall stress.

As a direct test of the conclusion that Kap120 engages Swi6 as an import cargo, we examined the ability of Ran-GTP (Gsp1-GTP) to induce release of Swi6 from Kap120. Nucleolar GTP-bound Ran is responsible for releasing cargo proteins from their associated importins after transport to the nucleus (Görlich et al., 1996). The Kap120 importin precipitates described above released bound Swi6 when incubated in the presence of Gsp1-GTP (Figure 5C), revealing that Kap120 and Swi6 engage in a true importin−cargo interaction.

As a final test of the ability of NLS2 to drive nuclear import in a Kap120-dependent manner, we fused the 10 amino acid sequence surrounding Swi6 NLS2 (residues 228–238) with a form of Swi6 that is blocked for function of NLS2 (Swi6-S238A; p2832), or the Mpk1 phosphorylation site (Swi6-S238A; p2834). Transformants were grown to mid-log phase in YPD at 23°C and subjected to mild heat shock (39°C) for 1 h. Kap120-TAP was precipitated from protein extracts, subjected to SDS-PAGE and immunoblot detection of coprecipitated Swi6 (top). Whole-cell extract was used as a loading control (bottom). The Swi6 antibody also detected the IgG-binding epitope of the TAP tag. (C) TAP-tagged Kap120 was immunoprecipitated from yeast strain DL3878 expressing wild-type Swi6 from “B” and the precipitate was treated with Gsp1-GTP to catalyze release of Swi6 from Kap120. After 2h on ice, samples were washed and processed for immunoblot detection of Swi6 as an import cargo. (D) The NLS2 sequence directs GFP to the nucleus in a Kap120-dependent manner. Representative fluorescent (GFP) and differential interference contrast (DIC) images of cells expressing 2xGFP fused at its C terminus to the nonclassical NLS signals in the absence of α-importin.

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237) to the C terminus of GFP-GFP. This sequence directed the fluorescent protein to the nucleus (Figure 5D) in wild-type cells, but not in a kap120a mutant. Moreover, a version of this fusion bearing the Swi6-K231A mutation failed to direct the fluorescent signal to the nucleus.

**DISCUSSION**

This study was concerned with the role of the Mpk1 CWI pathway MAP kinase in regulating the dimeric SBF transcription factor made up of Swi4 and Swi6, best known for its role in cell cycle-regulated transcription, in response to cell wall stress. We demonstrated previously that activated Mpk1 binds to the Swi4 DNA-binding subunit of SBF and, through a noncatalytic mechanism, renders it competent to bind DNA and drive transcriptional activation of the FKS2 gene (Kim et al., 2008). However, the Mpk1-Swi4 complex also requires Swi6 for FKS2 induction. Moreover, Madden et al. (1997) demonstrated that Mpk1 phosphorylates Swi6 in response to cell wall stress induced by heat shock. Therefore, we sought to understand the consequences of Mpk1 phosphorylation of Swi6. We found here that Mpk1 phosphorylates Swi6 on Ser238 in response to cell wall stress. A mutant blocked for phosphorylation of Swi6 on Ser238 (swi6-S238A) displayed elevated transcription of an SBF-dependent cell wall stress reporter (FKS2-lacZ), indicating that Mpk1 phosphorylation of Swi6 is a negative regulatory event.

**Mpk1 Regulates Nucleocytoplasmic Shuttling of Swi6 through a Biphasic Process**

The Clb6/Cdc28 S phase cell cycle kinase regulates Swi6 nuclear localization by interfering with the function of an NLS sequence neighboring its phosphorylation site at Ser160 (Sidorova et al., 1995; Geymonat et al., 2004; Harreman et al., 2002). Noting that Ser238 neighbors another potential NLS sequence in Swi6, we examined the effect of cell wall stress on nucleocytoplasmic shuttling of this protein. We found that Mpk1 regulates Swi6 localization in a biphasic manner. In the first phase (after 20-min heat shock), cytoplasmic Swi6 was relocalized to the nucleus concurrent with Mpk1 activation. In the second phase (over the next 40 min of heat stress), nuclear Swi6 returned to the cytoplasm. The first phase required Mpk1 to be activated (phosphorylated) but did not require its catalytic activity. The only noncatalytic function of Mpk1 of which we are aware is its ability to bind Swi4 and form a complex on the FKS2 promoter (Kim et al., 2008). We found that Swi6 nuclear localization during the first phase of its regulation was dependent on the ability of Mpk1 to bind Swi4, because a mutant in Swi4 that is specifically defective in this association was also blocked for Swi6 nuclear localization in response to cell wall stress. This suggests that nuclear Mpk1–Swi4 complex serves to retain Swi6 in the nucleus. Although Mpk1/Swi6 must be bound to the FKS2 promoter for recruitment of Swi6 to the DNA (Kim et al., 2008), it is not clear whether this is also a requirement for recruitment of Swi6 to the nucleus. This is unlikely, because the number of Mpk1/Swi4 molecules in complex with promoters must be much smaller than the number of Swi6 molecules that are recruited to the nucleus. Regardless, it seems likely that the nuclear pool of Mpk1/Swi4 complex shifts the kinetics of Swi6 nucleocytoplasmic shuttling through a direct interaction.

The second phase of Swi6 regulation, nuclear exit, was dependent both on Mpk1 catalytic activity and phosphorylation of Swi6 on Ser238, supporting the conclusion that Mpk1 phosphorylation of Swi6 is a negative regulatory event. A Swi6 mutant that was blocked for phosphorylation at Ser238 (swi6-S238A) entered the nucleus normally in response to Mpk1 activation (phase 1) but failed to return to the cytoplasm during phase 2. Similarly, a catalytically inactive, but phosphorylatable, mutant of Mpk1 recruited Swi6 to the nucleus normally, but failed to promote its subsequent exit. The kinetics of Swi6 phosphorylation by Mpk1, which required a full hour to complete, fit well with this biphasic model for Swi6 regulation.

**Swi6 Possesses a Cell Wall Stress-regulated NLS**

The finding that Mpk1 phosphorylation of Swi6 on Ser238 promotes its net nuclear exit suggested the possibility that Ser238 resides near an NLS sequence. We identified a putative NLS neighboring Ser238 (Figure 3A) that shares sequence similarity with a classical Swi6 NLS (NLS1), which is inhibited by cell cycle-dependent phosphorylation. Mutations in this putative NLS (NLS2) blocked cell wall stress-induced Swi6 nuclear localization, but not cell cycle regulated Swi6 nucleocytoplasmic shuttling. By contrast, mutation of NLS1, which is known to block nuclear localization of Swi6 through the cell cycle, had no effect on cell wall-stressed Swi6 nuclear localization. Mutation of both NLS sequences blocked nuclear localization of Swi6 nearly completely. Therefore, we conclude that Swi6 possesses two NLS sequences—NLS1, which is inactivated periodically through the cell cycle by the Clb6/Cdc28 S phase kinase; and NLS2, which is inactivated by Mpk1 in response to cell wall stress. The independent regulation of these NLS sequences allows cells under persistent cell wall stress, during which NLS2 is inactive, to still recruit Swi6 to the nucleus during G1 phase through the use of NLS1.

The dual positive and negative regulation of Swi6 by Mpk1 observed here suggests a temporal shift in which the initial stress signal mobilizes Swi4 and Swi6 for transcriptional activation. Thereafter, further transcriptional activation would be muted by the effect of Mpk1 phosphorylation of Swi6. One implication of this model is that the transcriptional response of the FKS2 gene to cell wall stress should be transient. However, the situation is more complex than this. We found that the two Swi6 NLS sequences share partial overlap of function, such that inactivating mutations in either NLS reduced FKS2 transcription in response to cell wall stress and CLN2 transcription through the cell cycle only modestly. However, mutation of both sequences completely blocked transcription from both promoters. Therefore, although FKS2 transcription is subject to some down-regulation in response to phosphorylation of Swi6 by Mpk1, this effect is mitigated by the function of NLS1, which allows Swi6 nuclear entry, albeit only during G1 phase, even when NLS2 has been phosphorylated by Mpk1.

Another factor expected to mitigate the down-regulation of Swi6 is the Mpl1 pseudokinase parologue of Mpk1. Mpl1 is capable of carrying out the noncatalytic function of Mpk1—transcriptional activation of FKS2 through recruitment of Swi4 and Swi6 to the FKS2 promoter (Kim et al., 2008). However, because it lacks catalytic activity, it cannot down-regulate Swi6 by phosphorylation of Ser238. We speculate that the evolutionary advantage of retaining a catalytically inactive form of this protein is to maintain high levels of FKS2 transcription in response to continuous cell wall stress. S. cerevisiae may modulate FKS2 transcription in part by controlling relative amounts of Mpk1 and Mpl1. In this regard, it is interesting to note that MLP1 expression is tightly regulated by Mpk1 through the activity of another transcription factor (Rlm1; Jung and Levin, 1999; Jung et al., 2002).
It is also possible that Swi6 has a cytoplasmic function that is promoted upon phosphorylation by Mpk1, which blocks its nuclear import. This possibility is supported by the recent observation that Swi6, but not Swi4, is required for activation of the unfolded protein response at the endoplasmic reticulum in cells challenged by cell wall stress (Srimale et al., 2009). In this regard, it is also noteworthy that Mpk1 phosphorylation of Swi6 does not require the recruitment of Swi6 to the Mpk1–Swi4 complex, or even into the nucleus, because this phosphorylation occurs in the absence of Swi4. The observation that Mpk1 can phosphorylate Swi6 outside of the Mpk1–Swi4–Swi6 transcriptional complex indicates that the two phases of Swi6 regulation by Mpk1 are not interdependent.

In addition to phosphorylation-inhibited nuclear import of Swi6, negative regulation of transcription by other yeast MAPKs has been reported to result through different mechanisms. For example, phosphorylation of the filamentous growth transcription factor Tec1 by the Fus3 mating-specific MAPK induces its ubiquitin-mediated degradation as a mechanism for preventing cross-talk between two signaling pathways with shared components (Chou et al., 2004; Bao et al., 2004). In addition, the Kss1 filamentous growth MAPK functions in the inactive state as a transcriptional repressor of filamentous growth genes through association with the Ste12 transcription factor, which recruits the Dig1/2 transcriptional repressors to the DNA (Cook et al., 1997; Madhani et al., 1997; Bardwell et al., 1998).

Kap120 Is the β-Importin That Recognizes Swi6 NLS2 Swi6 possesses a classical NLS (NLS1) that is recognized by the α/β-importin dimer Srp1/Kap95 (Enenkel et al., 1995; Harreman et al., 2004). This NLS is regulated through the cell cycle by Cdc28 phosphorylation (Geymonat et al., 2004). We found that Swi6 NLS2 is recognized by Kap120 and that this interaction is disrupted in response to phosphorylation of Swi6 at Ser238 by Mpk1. The only other cargo protein identified previously for Kap120 is the ribosome assembly factor Rpr1 (Caesar et al., 2006). It is interesting that two distinct signal transduction pathways—cell cycle control and cell wall stress—impinge upon Swi6 function by controlling its nuclear import through separate NLSs. Our results reveal a mechanism for the integration of disparate signals directed toward a single consequence.

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REFERENCES

Abe, J., Kusuhara, M., Ulevitch, R. J., Berk, B. C., and Lee, J. D. (1996). Big mitogen-activated protein kinase 1 (BMM1) is a redox-sensitive kinase. J. Biol. Chem. 271, 16586–16590.

Andrews, B. J., and Moore, L. A. (1992). Interaction of the yeast Swi4 and Swi6 cell cycle regulatory proteins in vitro. Proc. Natl. Acad. Sci. USA 89, 11852–11856.

Baetz, K., and Andrews, B. (1999). Regulation of the cell cycle transcription factor Swi4 through auto-inhibition of DNA binding. Mol. Cell. Biol. 19, 6729–6741.

Baetz, K., Mofrad, J., J Haynes, Chang, M., and Andrews, B. (2001). Transcriptional coregulation by the cell integrity mitogen-activated protein kinase Slt2 and the cell cycle regulator Swi4. Mol. Cell. Biol. 21, 6515–6528.

Bao, M. Z., Schwartz, M. A., Cantin, G. T., Yates, J. R., 3rd, and Madhani, H. D. (2004). Pheromone-dependent destruction of the Tec1 transcription factor is required for MAP kinase signaling specificity in yeast. Cell 119, 991–1000.

Bardwell, L., Cook, J. G., Voora, D., Baggott, D. M., Martinez, A. R., and Thorner, J. (1998). Repression of yeast Ste12 transcription factor by direct binding of unphosphorylated Kss1 MAPK and its regulation by the Ste7 MEK. Genes Dev. 12, 2887–2898.

Breeden, L. L. (2003). Periodic transcription: a cycle within a cycle. Curr. Biol. 13, R31–R38.

Buehrer, B. M., and Errede, B. (1997). Coordination of the mating and cell integrity mitogen-activated protein kinase pathways in Saccharomyces cerevisiae. Mol. Cell. Biol. 17, 6517–6525.

Caesar, S., Greiner, M., and Schlienseht, G. (2006). Kap120 functions as a nuclear import receptor for ribosome assembly factor Rpr1 in yeast. Mol. Cell. Biol. 26, 3170–3180.

Cid, V. J., Duran, A., Rey, F., Snyder, M. P., Nombela, C., and Sanchez, M. (1995). Molecular basis of cell integrity regulation in Saccharomyces cerevisiae. Microbiol. Rev. 59, 345–386.

Chou, S., Huang, L., and Liu, H. (2004). Fus3-regulated Tec1 degradation through SCFCdc4 determines MAPK signaling specificity during mating in yeast. Cell 119, 981–990.

Cook, J. G., Bardwell, L., and Thorner, J. (1997). Inhibitory and activating functions for MAPK Kss1 in the S. cerevisiae filamentous growth signaling pathway. Nature 380, 85–88.

Davenport, K. R., Schaskey, M., Kamada, Y., Levin, D. E., and Gustin, M. C. (1995). A second osmosensing signal transduction pathway in yeast. Hypotonic shock activates the PKC1 protein kinase-regulated cell integrity pathway. J. Biol. Chem. 270, 30157–30161.

de Nobel, H., Ruiz, C., Martin, H., Morris, W., Brul, S., Molina, M., and Klis, F. M. (2000). Cell wall perturbation in yeast results in dual phosphorylation of the Shl2/Mpk1 MAP kinase and in an Shl2-mediated increase in PKS2-laZ expression, glucanase resistance and thermostolerance. Microbiology 146, 2121–2132.

Dodou, E., and Treisman, R. (1997). The Saccharomyces cerevisiae MADS-box transcription factor Rim1 is a target for the Mpk1 mitogen-activated protein kinase pathway. Mol. Cell. Biol. 17, 1848–1859.

Enenkel, C., Blobel, G., and Resch, M. (1997). Identification of a yeast karyopherin heterodimer that targets import substrate to mammalian nuclear pore complexes. J. Biol. Chem. 270, 16499–16502.

Fried, H., and Kutay, U. (2003). Nucleocytoplasmic transport: taking an inventory. Cell. Mol. Life Sci. 60, 1659–1688.

Geymonat, M., Spanos, A., Wells, G. P., Smardon, S. J., and Sedgwick, S. G. (2004). Cld6/Cdc28 and Cdc14 regulate phosphorylation status and cellular localization of Swi6. Mol. Cell. Biol. 24, 2277–2285.

Görlich, D., N. Panté, U. Kutay, U. Aebi, and F. R. Bischoff. (1996). Identification of different roles for RanGDP and RanGTP in nuclear protein import. EMBO J. 15, 5884–5894.

Guarente, L., and Mason, T. (1983). Heme regulates transcription of the CYCl gene of S. cerevisiae via an upstream activation site. Cell 32, 1279–1286.

Harreman, M. T., Kline, T. M., Milford, H. G., Harben, M. B., Hodel, A. E., and Caesar, S., Greiner, M., and Schlienseht, G. (2006). Kap120 functions as a nuclear import receptor for ribosome assembly factor Rpr1 in yeast. Mol. Cell. Biol. 26, 3170–3180.
catalytic mechanism that requires upstream signal. Mol. Cell. Biol. 28, 2579–2589.

Klis, F. M. (1994). Review: cell wall assembly in yeast. Yeast 10, 851–869.

Levin, D. E. (2005). Cell wall integrity signaling in Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. 69, 262–291.

Madden, K., Sheu, Y. J., Baetz, K., Andrews, B., and Snyder, M. (1997). SBF cell cycle regulator as a target of the yeast PKC-MAP kinase pathway. Science 275, 1781–1784.

Madhani, H. D., Styles, C., and Fink, G. R. (1997). MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation. Cell 91, 673–684.

Martin, H., Rodriguez-Pachon, J. M., Ruiz, C., Nombela, C., and Molina, M. (2000). Regulatory mechanisms for modulation of signaling through the cell integrity Slt2-mediated pathway in Saccharomyces cerevisiae. J. Biol. Chem. 275, 1511–1519.

Maurer, P., Redd, M., Solsbacher, J., Bischoff, F. R., Greiner, M., Podtelejnikov, A. V., Mann, M., Stade, K., Weis, K., and Schlenstedt, G. (2001). The nuclear export receptor Xpo1p forms distinct complexes with NES transport substrates and the yeast Ran binding protein 1 (Yrb1p). Mol. Biol. Cell 12, 539–549.

Ryan, K. J., Zhou, Y., and Wente, S. R. (2007). The karyopherin Kap95 regulates nuclear pore complex assembly into intact nuclear envelopes in vivo. Mol. Biol. Cell 18, 886–898.

Scrimale, T., L. Didone, de Mesy Bentley, K. L., and Krysan, D. J. (2009). The unfolded protein response is induced by the cell wall integrity mitogen-activated protein kinase signaling cascade and is required for cell wall integrity in Saccharomyces cerevisiae. Mol. Biol. Cell 20, 164–175.

Sedgwick, S. G. et al. (1998). Structural and functional architecture of the yeast cell-cycle transcription factor Swi6. J. Mol. Biol. 281, 763–775.

Seedorf, M., and Silver, P. A. (1997). Importin/karyopherin protein family members required for mRNA export from the nucleus. Proc. Natl. Acad. Sci. USA 94, 8591–8595.

Senger, B., Simos, G., Bischoff, F. R., Podtelejnikov, A., Mann, M., and Hurt, E. (1998). Mtr10p functions as a nuclear import receptor for the mRNA-binding protein Npl3p. EMBO J. 17, 2196–2207.

Sidorova, J. M., and Breeden, L. L. (1993). Analysis of the SWI4/SWI6 protein complex, which directs G1/S-specific transcription in Saccharomyces cerevisiae. Mol. Cell. Biol. 13, 1069–1077.

Sidorova, J. M., Mikesell, G. E., and Breeden, L. L. (1995). Cell cycle-regulated phosphorylation of Swi6 controls its nuclear localization. Mol. Biol. Cell 6, 1641–1658.

Silkorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122, 19–27.

Siliciano, P. G., and Tatchell, K. (1984). Transcription and regulatory signals at the mating type locus in yeast. Cell 37, 969–978.

Sorokin, A. V., Kim, E. R., and Ovchinnikov, L. P. (2007). Nucleocytoplasmic transport of proteins. Biochemistry 47, 1439–1457.

Taylor, I. A., McIntosh, P. B., Pala, P., Treiber, M. K., Howell, S., Lane, A. N., and Smerdon, S. J. (2000). Characterization of the DNA-binding domains from the yeast cell-cycle transcription factors Mbp1 and Swi4. Biochemistry 39, 3943–3954.

Terry, L. I., Showes, E. B., and Wente, S. R. (2007). Crossing the nuclear envelope: hierarchical regulation of nucleocytoplasmic transport. Science 318, 1412–1416.

Truman, A. W., K-Kim, Y., and Levin, D. E. (2009). Mechanism of Mpk1 MAPK binding to the Swi4 transcription factor and its regulation by a novel caffeine-induced phosphorylation. Mol. Cell. Biol. 29, 2649–2661.

Truman, A. W., Millson, S. H., Nuttall, J. M., King, V., Mollapour, M., Prodromou, C., Pearl, L. H., and Piper, P. W. (2006). Expressed in the yeast Saccharomyces cerevisiae, human ERK5 is a client of the Hsp90 chaperone that complements loss of the Slt2 (Mpk1) cell integrity stress-activated protein kinase. Eukaryot. Cell 5, 1914–1924.

Yan, C., Luo, H., Lee, J. D., Abe, J., and Berk, B. C. (2001). Molecular cloning of mouse ERK5/BMK1 splice variants and characterization of ERK5 functional domains. J. Biol. Chem. 276 10870–10878.

Watanabe, Y., Irie, K., and Matsumoto, K. (1995). Yeast RLM1 encodes a serum response factor-like protein that may function downstream of the Mpk1 (Slt2) mitogen-activated protein kinase pathway. Mol. Cell. Biol. 15, 3740–3749.

Zhao, C., Jung, U. S., Garrett-Engele, P., Roe, T., Cyert, M. S., and Levin, D. E. (1998). Temperature-induced expression of yeast FKS2 is under the dual control of protein kinase C and calcineurin. Mol. Cell. Biol. 18, 1013–1022.

Zarzov, P., Mazzoni, C., and Mann, C. (1996). The SLT2 (MPK1) MAP kinase is activated during periods of polarized cell growth in yeast. EMBO J. 15, 83–91.