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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, revealing a mechanism for the integration of multiple signals to a single endpoint. Finally, the Kap120 β-importin binds the Mpk1-regulated Swi6 NLS but not the Cdc28-regulated 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 (MAP) kinase cascade (reviewed in Levin, 2005). The MAP kinase cascade is a linear pathway comprised of Pkc1, a mitogen-activated protein kinase kinase kinase (Bck1), a pair of redundant mitogen-activated protein kinase kinases (MEKs) (Mkk1/2) and an MAP kinase (Mpk1/Slt2). Mpk1 is a functional homolog of human extracellular signal-regulated kinase 5 (Truman *et al*., 2006), a mitogen-activated protein 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 Rlm1 (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*., 2000), 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 *mpk1*Δ mutant is suppressed by overexpression of Swi4 (Madden *et al*., 1997). Second, both *swi4*Δ and *swi6*Δ

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Table 1. *S. cerevisiae* strains

Strain	Relevant genotype (and strain background)	Reference or source
1788	*MAT***a**/*MATα leu2-3,112 trp1-1 ura3-52 his4 can1*^{r} *(EG123)*	I. Herskowitz, (University of California, San Francisco); Silicianoand Tatchell (1984)
DL100	*MAT*a *leu2-3,112 trp1-1 ura3-52 his4 can1*^{r} *(EG123)*	I. Herskowitz; Siliciano and Tatchell (1984)
DL456	*MATa/MATα mpk1*Δ*::TRP1/mpk1*Δ*::TRP1* (EG123)	Kamada *et al*. (1995)
DL3145	*MAT***a**/*MATα swi4*Δ*::TRP1/swi4*Δ*::TRP1* (EG123)	Kim *et al*. (2008)
DL3148	*MAT***a**/*MATα swi6*Δ*::LEU2/swi6*Δ*::LEU2* (EG123)	Kim *et al*. (2008)
DL3187	*MAT*a *his3*Δ *leu2*Δ *ura3*Δ *lys2*Δ (S288c; BY4741)	Research Genetics
DL3195	*MAT***a**/*MATα mpk1*Δ*::KanMX/mpk1*Δ*::KanMX* (S288c)	Kim *et al*. (2008); Research Genetics (Huntsville, AL)
DL3196	*MAT***a**/*MATα mpk1*Δ*::KanMX/mpk1*Δ*,::KanMX mlp1*Δ*::KanMX/mlp1*Δ*::KanMX* (S288c)	Kim *et al*. (2008); Research Genetics
DL3233	*MAT***a** *swi6*Δ*::KanMX* (S288c)	Research Genetics
DL3327	*MAT***a**/*MATα mpk1*Δ*::TRP1/mpk1*Δ*::TRP1 mlp1*Δ*::ura3/mlp1*Δ*::ura3* (EG123)	This study
DL3809	*MAT***a** *kap108*Δ*::KanMX* (S288c)	Research Genetics
DL3810	*MAT***a** *kap114*Δ*::KanMX* (S288c)	Research Genetics
DL3811	*MAT***a** *kap120*Δ*::KanMX* (S288c)	Research Genetics
DL3812	*MAT***a** *kap122*Δ*::KanMX* (S288c)	Research Genetics
DL3813	*MAT***a** *kap123*Δ*::KanMX* (S288c)	Research Genetics
DL3814	*MAT***a** *trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3* (SWY518)	S. Wente; Ryan *et al*. (2007)
DL3816	*MAT***a** *kap95-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	*MAT***a** *ura3-52 trp1-63 leu2-1 GAL+* (PSY580)	P. Silver; Seedorf and Silver (1997)
DL3822	*MAT***a** *pse1-1 ura3-52 trp1-63 leu2-1 GAL+* (PSY580)	P. Silver; Seedorf and Silver (1997)
DL3823	*MAT***a** *ade2-1 his3-11,15 ura3-52 leu2-3,112 trp1-1 can1-100 Gal+* (RS453)	E. Hurt, Senger *et al*. (1998)
DL3824	*MAT***a** *mtr10(kap111)::HIS3 ade2-1 his3-11,15 ura3-52 leu2-3,112 trp1-1 can1-100 Gal+* (RS453)	E. Hurt; Senger *et al*. (1998)
DL3835	*MAT***a** *KAP120-TAP* S288c	Open Biosystems (Huntsville, AL)
DL3878	*MAT***a** *KAP120-TAP swi6*Δ*::LEU2* (S288c)	This study

mutants are hypersensitive to calcofluor white, supporting a role for SBF in cell wall biogenesis (Igual *et al*., 1996). Third, Mpk1 associates with SBF in vivo (Madden *et al*., 1997) and with Swi4 (but not Swi6) in vitro (Baetz *et al*., 2001). Fourth, Swi6 is phosphorylated in vivo and in vitro by Mpk1 in response to cell wall stress (Madden *et al*., 1997).

We found recently that CWI signaling drives expression of the *FKS2* gene through SBF in a manner that is independent of the role of this factor in G1-specific transcription (Kim *et al*., 2008; Truman *et al*., 2009). Mpk1 and its pseudokinase paralogue Mlp1, which is also activated by the MEKs of the CWI signaling pathway, use a noncatalytic mechanism to activate transcription of *FKS2* that is dependent on SBF and on activating signal. Activated (phosphorylated) Mpk1, or Mlp1, form a complex with Swi4 that associates with an SBF-binding site in the *FKS2* promoter independently of Swi6 (Kim *et al*., 2008). In this context, Mpk1 (or Mlp1) relieves the autoinhibitory Swi4 interaction by binding to a MAPK docking site on Swi4 that neighbors the C-terminal Swi6-binding site (Truman *et al*., 2009). Mutational ablation of the MAPK interaction site on Swi4 specifically blocks cell wall stress-induced *FKS2* transcription, without interfering with the cell cycle functions of Swi4. Although Swi6 is not required for Swi4 to bind to the *FKS2* promoter, it must still be recruited to the Mpk1–Swi4 complex to activate transcription. Because transcriptional activation of *FKS2* through SBF does not require Mpk1 catalytic activity, the significance of Swi6 phosphorylation by Mpk1 remains unknown.

Here, we find that Mpk1 phosphorylation of Swi6 is a negative regulatory event that interferes with Swi6 entry to the nucleus. We show that nucleocytoplasmic shuttling of Swi6 in response to CWI signaling is a biphasic process. First, upon activation of Mpk1, Swi6 is recruited to the nucleus in a manner dependent on formation of the Mpk1–Swi4 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 (*mpk1*Δ*::TRP1 mlp1*Δ*::ura3*) was constructed from DL3164 (*mpk1*Δ*::TRP1 mlp1*Δ*::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 (Ho *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 Xho1 and Sac1 to liberate the fragment bearing various point

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	pLGΔ-178; *CYC1-lacZ*	Guarente and Mason (1993)
p1366	*PRM5-lacZ*	Jung *et al*. (2002)
p2022	YEp351 *MLP1-3×HA*	Kim *et al*. (2008)
p2052	*FKS2*(−540 to −375)-*CYC1-lacZ* with *URA3* marker	Kim *et al*. (2008)
p2066	*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	pRS304 *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 *swi4-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	YCpGAL-2GFP-*SWI6-NLS2*	This study
p2861	YCpGAL-2GFP-*SWI6-NLS2-K231A*	This study
pGS840	YCpGAL-2GFP	Maurer *et al*. (2001)

mutants in the N-terminal domain of Swi6. These fragments were cloned into p2391 (by Xho1/Sac1) to generate p2557, p2558, p2729-p2733, and p2773-p2774. 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/Kpn1) 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-GAL-2GFP (pGS840) by using double-overlap mutagenesis. A 0.7-kb BamH1–Pvu1 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 (horseradish 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 [Ig]G-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, Litttle Chalfont, Buckinghamshire, United Kingdom). Immunoprecipitates were subjected to Swi6 detection using 20% of the precipitated protein, as described above. An isogenic wild-type strain (DL3187) 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 $MgCl_2$ and 3 mM KCl. Sample treated for release of Swi6 were washed with immunoprecipitation buffer before immunoblot analysis for Kap12-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_{600} of ~0.5 before nocodozole 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

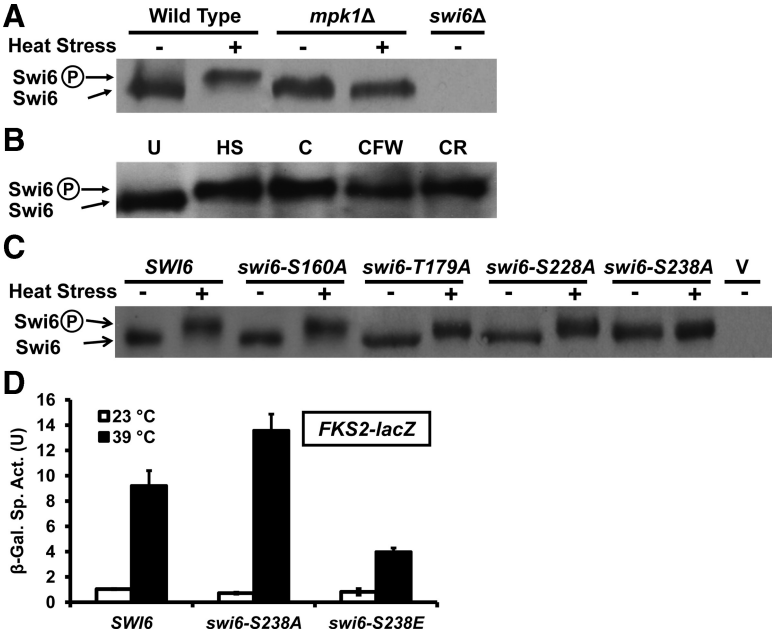

Figure 1. Cell wall stress-induced phosphorylation of Swi6. (A) Mild heat shock induces an Mpk1-dependent band shift in Swi6. Wild-type yeast (1788), an *mpk1*Δ mutant (DL456), and a *swi6*Δ (DL3148) mutant were grown to mid-log phase in YPD + 2% sorbitol at 23°C and subjected to heat shock (39°C) for 2 h before extract preparation and immunoblot detection of Swi6. (B) Other cell wall stresses 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 *swi6*Δ 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 *swi6*Δ 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 (A_{600} 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.

temperature shift to 39°C. Samples were subjected to fluorescence microscopy using an Axioplan II microscope (Carl Zeiss , Jena, Germany) with a 100× objective and fitted with a GFP filter. For experiments in which cycling yeast cells were subjected to heat shock, cultures were grown to mid-log phase in selective medium before heat shock by dilution with selective medium.

Reverse Transcriptase (RT)-PCR

For RT-PCR measurement of *FKS2* mRNA, cells growing in YPD were treated with nocodozole for 4 h at 23°C to synchronize cells as described above and with FK506 (0.2 mg/ml; Cayman Chemical, Ann Arbor, MI) for the final 1 h to inhibit calcineurin. This was done because the endogenous *FKS2* gene is a target for calcineurin-induced transcription in response to cell wall stress (Zhao *et al*., 1998). After heat stress for various times, RNA was isolated using a RiboPure-Yeast kit (Ambion, Austin, TX). Total RNA (5 μg) was used for the preparation of cDNA with reverse transcriptase (SuperScript II; Invitrogen, Carlsbad, CA), primed with random hexamers (QIAGEN, Valencia, CA), as recommended by the manufacturer. Fragments of *FKS2* and 18S cDNA were amplified by PCR using 0.1% of the total cDNA from these reactions and separated by agarose gel electrophoresis. DNA bands were quantitated from the gel image (Gel Doc 1000; Bio-Rad Laboratories, Hercules, CA) using ImageJ software (National Institutes of Health, Bethesda, MD). *FKS2* mRNA induction was normalized to 18S RNA in each reaction.

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 alanyl 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 predominantly in the cytoplasm from late G1 until late M phase, at which time it relocalizes 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 (Srp1) for the NLS, thereby diminishing the rate of Swi6 import relative to export, with the net effect

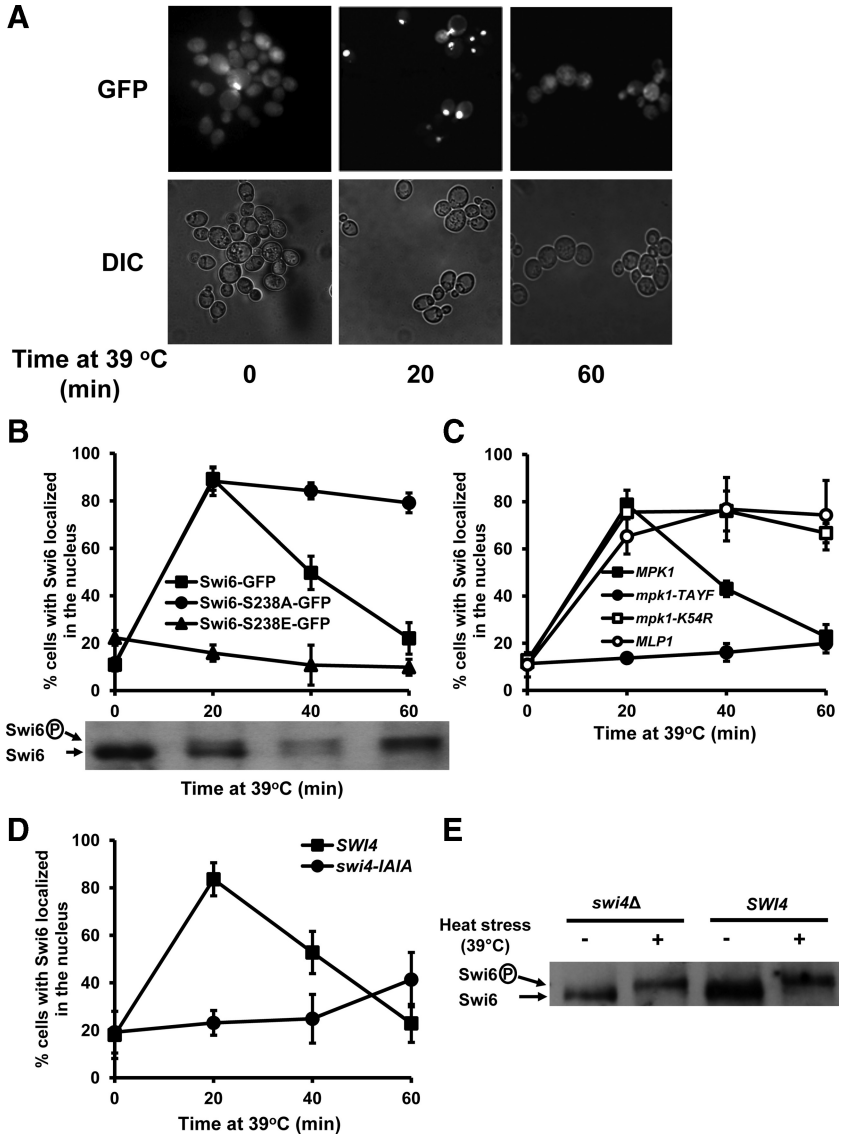

Figure 2. Nucleocytoplasmic shuttling of Swi6. (A) Representative fluorescent (GFP) and differential interference contrast (DIC) images of a *swi6*Δ mutant (DL3148) transformed with a centromeric plasmid expressing wild-type Swi6-GFP (p2391). Transformants were cultivated at 23°C in the presence of nocodozole to synchronize them in G2 phase before exposure to cell wall stress by heat shock for the indicated times. (B) Top, a *swi6*Δ mutant (DL3148) was transformed with plasmids expressing wild-type Swi6-GFP (p2391), Swi6-S238A-GFP (p2557), or Swi6-S238E-GFP (p2558). Cells were treated as described in A, except that populations of cells were scored visually for Swi6-GFP localization. Each value represents the mean and SD from three experiments in which at least 100 cells were scored. Bottom, a wild type strain (DL100) was treated as described above and subjected to immunoblot detection of Swi6. (C) An *mpk1*Δ *mlp1*Δ (DL3327) strain was cotransformed with centromeric plasmids expressing wild-type Mpk1 (p2188), Mpk1-T190A, Y192F (Mpk1-TAYF; p2190), 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 *swi4*Δ mutant (DL3145) was cotransformed with centromeric plasmids expressing wild-type Swi6-GFP (p2647) and either wild-type Swi4 (p2713) or Swi4-I913A, I915A (Swi4-IAIA; p2714). Cells were treated as described in B. (E) A *swi4*Δ 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 Swi6.

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 nocodozole. 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 2, A 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), is blocked for phosphorylation by its upstream activating kinases (Mkk1/2) and, as a consequence, is devoid of detectable protein kinase activity (Kamada *et al*., 1995). Because this mutant cannot be shifted to an active conformation, it does not bind to Swi4 and therefore does not drive *FKS2* transcription (Kim *et al*., 2008).

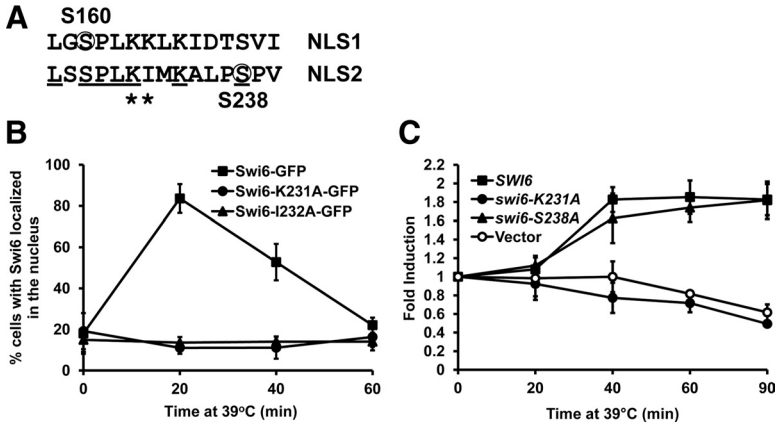

Figure 3. Swi6 possesses two NLS sequences. (A) Amino acid sequence alignment of Swi6 NLS1 and NLS2. Residues phosphorylated by Cdc28 (Ser160) and Mpk1 (Ser238) are circled. Asterisks indicate the residues (K231 or I232) mutated to Ala. (B) Mutations in NLS2 block CWI signaling-induced nuclear import of Swi6. A *swi6*Δ mutant (DL3148) was transformed with a centromeric plasmid expressing wild-type Swi6-GFP (p2391), or mutants in the putative NLS2 sequence, Swi6-K231A-GFP (p2730), or Swi6-I232A-GFP (p2731) and treated as in Figure 2 for assessment of Swi6 localization. (C) A mutation in NLS2 blocks *FKS2* transcription in response to cell wall stress in cells arrested in G2. A *swi6*Δ mutant (DL3233) was transformed with a centromeric plasmid expressing wild-type Swi6 (p2542), mutants in the putative NLS2 sequence, Swi6-S238A (p2547), Swi6-K231A (p2832), or vector (pRS313). Transformants were treated as described in B, but *FKS2* mRNA levels were measured by RT-PCR at the indicated time points and normalized against 18S RNA. Each value represents the mean and SD from three experiments.

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-IAIA) and is therefore blocked for *FKS2* transcription but carries out cell cycle transcription normally (Truman *et al*., 2009). 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 was required for Mpk1 phosphorylation of Swi6. Swi6 phosphorylation in response to heat shock was not impaired in a *swi4*Δ strain (Figure 2E) or the *swi4-IAIA* mutant (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 Clb6/Cdc28 (Harreman *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 *swi6*Δ 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)

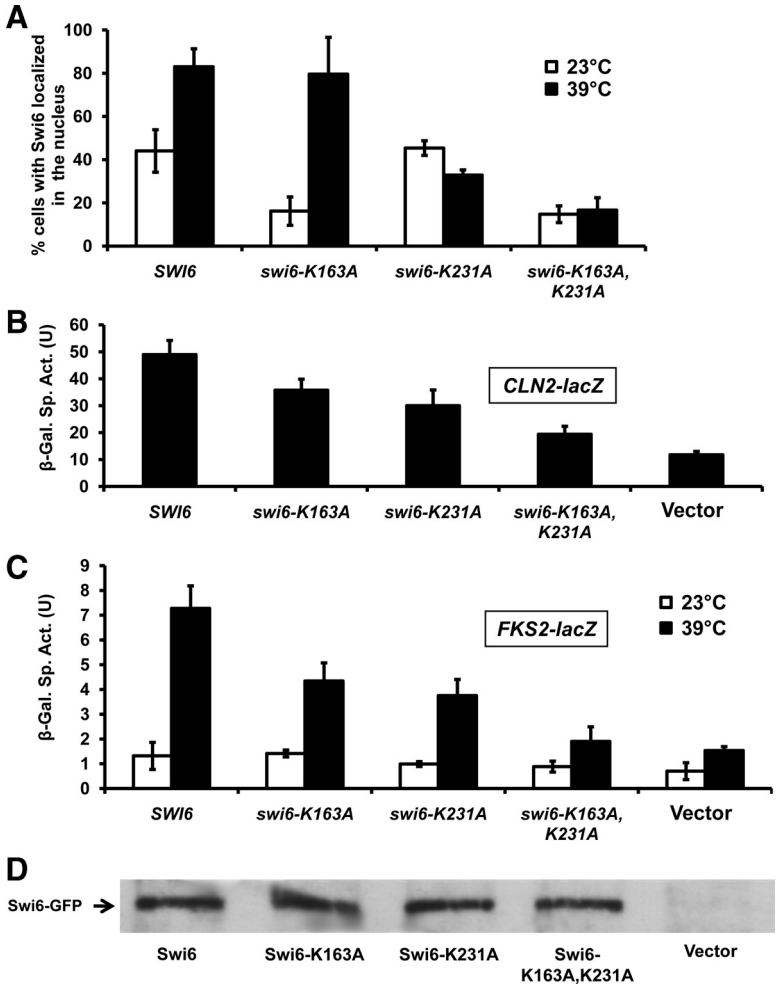

Figure 4. Two NLS sequences in Swi6 display partial overlap of function. (A) Nuclear localization of Swi6 mutant forms in cycling cells. A *swi6*Δ mutant (DL3148) was transformed with a centromeric plasmid expressing wild-type Swi6-GFP (p2391), mutants in the putative NLS1 (Swi6-K163A-GFP; p2729), or NLS2 (Swi6-K231A-GFP; p2730), the double mutant (Swi6-K163A, K231A-GFP; p2733), or vector control (pAC242). Transformants were grown to mid-log phase at 23°C in YPD, maintained at that temperature or subjected to heat shock for 20 min at 39°C, and assessed for Swi6 localization. Each value represents the mean and SD from three independent experiments in which at least 100 cells were scored. (B and C) NLS1 and NLS2 have partially overlapping functions for the cell cycle and cell wall stress roles of Swi6. The transformants in A were further transformed with either a *CLN2-lacZ* reporter plasmid (p2066; B), or an *FKS2-lacZ* reporter plasmid (p2052; C). Transformants bearing the *CLN2-lacZ* reporter were grown to mid-log phase at 23°C. Transformants bearing the *FKS2-lacZ* reporter were grown at 23°C or subjected to heat stress at 39°C, as described in Figure 1D, except that 2% sorbitol was added to the medium for osmotic support. 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. (D) Mutant Swi6 proteins are stably maintained. Cell extracts from DL3148 (*swi6*Δ) expressing the indicated forms of Swi6-GFP were subjected to SDS-PAGE and immunoblot detection of Swi6.

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 relocalizing 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

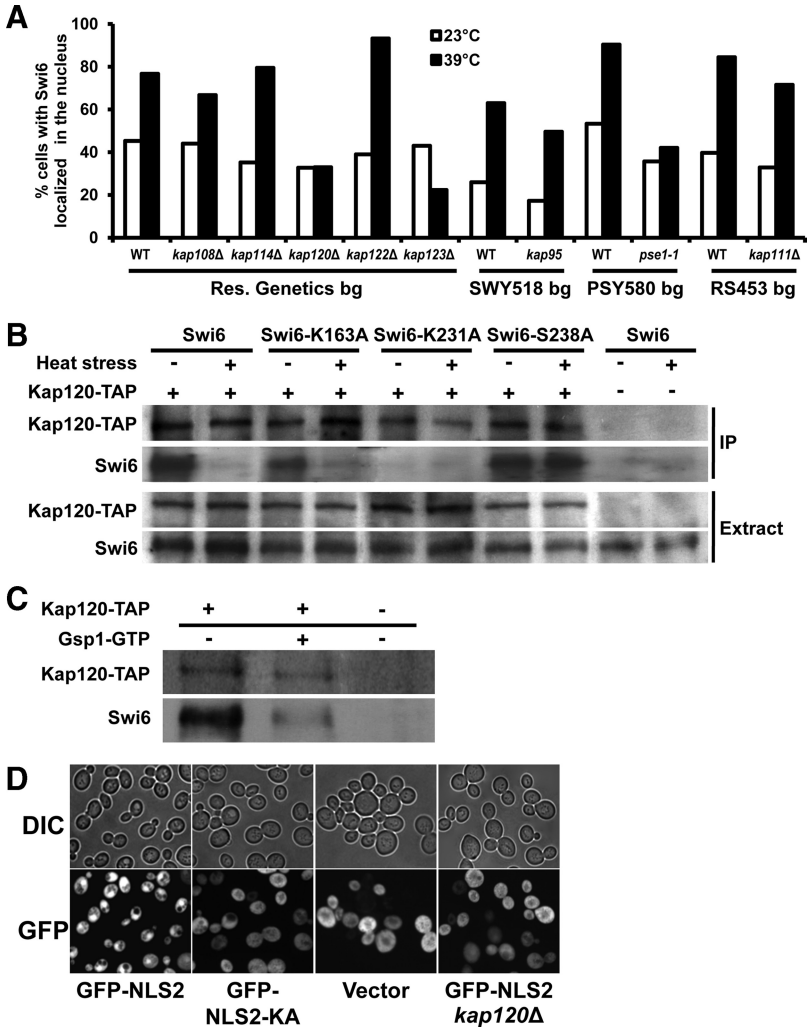

Figure 5. Identification of the β-importin that binds to Swi6 in response to cell wall stress. (A) Cell wall stress-induced Swi6 nuclear localization in β-importin mutants. The indicated β-importin mutants and their isogenic wild-type strains, expressing Swi6-GFP from p2647, were assessed for Swi6 localization as in Figure 4A. Each value represents the mean of at least two experiments in which at least 100 cells were scored. (B) Binding of β-importin Kap120 to Swi6. A yeast strain expressing TAP-tagged Kap120 and deleted for the endogenous *SWI6* gene (*KAP120-TAP*; DL3878) and its isogenic wild-type (DL3187) were transformed with centromeric plasmids expressing wild-type Swi6 (p2542), mutants in NLS1 (Swi6-K163A; p2831), NLS2 (Swi6-K231A; p2832), or the Mpk1 phosphorylation site (Swi6-S238A; p2547). 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 and Kap120, as described above. (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 10-amino acid Swi6-NLS2 sequence (p2860) or its K231A mutant form (p2861). GFP expression was induced in wild-type (DL3187) and the *kap120*Δ mutant (DL3811) transformed with the indicated plasmids by growth on galactose.

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 (*sxm1*Δ [*kap108*], *kap114*Δ, *kap120*Δ, *kap122*Δ [*pdr6*], *kap123*Δ [*yrb4*], and *mtr10*Δ [*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 (*kap120*Δ, *pse1-1*, and *kap123*Δ) 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) treatment to induce release of Swi6 from Kap120. Nuclear GTP-bound Ran is responsible for releasing cargo proteins from their associated importins after transport to the nucleus (Görlich *et al*., 1996). The Kap120 immunoprecipitates 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–

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 *kap120*Δ 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*., 2004). 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/Swi4 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 stress-induced 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 Mlp1 pseudokinase paralogue of Mpk1. Mlp1 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 Mlp1. 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 (Scrimale *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 Rpf1 (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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