# Intracellular mechanism by which genotoxic stress activates yeast SAPK Mpk1

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**ABSTRACT** **Stress-activated MAP kinases (SAPKs) respond to a wide variety of stressors. In most cases, the pathways through which specific stress signals are transmitted to the SAPKs are not known. The *Saccharomyces cerevisiae* SAPK Mpk1 (Slt2) is a well-characterized component of the cell-wall integrity (CWI) signaling pathway, which responds to physical and chemical challenges to the cell wall. However, Mpk1 is also activated in response to genotoxic stress through an unknown pathway. We show that, in contrast to cell-wall stress, the pathway for Mpk1 activation by genotoxic stress does not involve the stimulation of the MAP kinase kinases (MEKs) that function immediately upstream of Mpk1. Instead, DNA damage activates Mpk1 through induction of proteasomal degradation of Msg5, the dual-specificity protein phosphatase principally responsible for maintaining Mpk1 in a low-activity state in the absence of stress. Blocking Msg5 degradation in response to genotoxic stress prevented Mpk1 activation. This work raises the possibility that other Mpk1-activating stressors act intracellularly at different points along the canonical Mpk1 activation pathway.**

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## INTRODUCTION

The cell-wall integrity (CWI) signaling pathway of the budding yeast *Saccharomyces cerevisiae* has been well characterized with regard to its regulation by cell-wall stress (reviewed in Klis *et al.*, 2002; Levin, 2005, 2011; Lesage and Bussey, 2006). This pathway regulates biosynthesis of cell-wall polymers, organization of the actin cytoskeleton, exocytosis, and the Pkc1-mediated stress-activated MAP kinase (SAPK) cascade through activation of the small GTPase, Rho1. The SAP kinase cascade is a linear pathway composed of Pkc1, a MEKK (Bck1), a pair of redundant MEKs (Mkk1/2), and a SAPK (Mpk1/Slt2). Activation of Mpk1 in response to cell-wall stress or hyperactivation of upstream pathway components, drives transcription in support of cell-wall biogenesis (Jung and Levin, 1999; Jung *et al.*, 2002; Roberts *et al.*, 2000; Boorsma *et al.*, 2004; Garcia *et al.*, 2004) through the serum-response factor–like transcription factor Rlm1 (Dodou and Treisman, 1997; Watanabe *et al.*, 1997) and the cell-cycle transcription factor Swi4:Swi6 complex (Madden *et al.*, 1997; Baetz *et al.*, 2001; Kim *et al.*, 2008, Kim and Levin, 2011). Moreover, loss-of-function mutants in the SAPK cascade display cell lysis defects that are suppressed by external osmotic support (Levin, 2005), highlighting the central role of this signaling pathway in the maintenance of cell-wall integrity. In addition to its central role in cell-wall biogenesis, Mpk1 also contributes to cell-cycle arrest triggered by the morphogenesis checkpoint, which blocks mitosis until cells have constructed a bud (Harrison *et al.*, 2001).

Beyond the importance of Mpk1 in the process of cell-wall maintenance, this SAPK is additionally stimulated by a wide array of seemingly unrelated stress signals. These include DNA damage (Queralt and Igual, 2005; Soriano-Carot *et al.*, 2012), heat shock (Kamada *et al.*, 1995), oxidative stress (Vilella *et al.*, 2005), actin perturbations (Harrison *et al.*, 2001), toxic metalloids arsenate (As[V]; Matia-Gozalez and Rodriguez-Gabriel, 2011) and arsenite (As[III]; Ahmadpour *et al.*, 2016), and endoplasmic reticulum stress (Chen *et al.*, 2005; Babour *et al.*, 2010). Mpk1 is functionally orthologous to mammalian ERK5/BMK1 (Truman *et al.*, 2006), which is similarly activated by a wide array of stress stimuli, including hyperosmotic shock, heat shock, oxidative stress, ischemia, and shear stress (Abe *et al.*, 1996; Kato *et al.*, 1997; Kamakura *et al.*, 1999; Takeishi *et al.*, 1999;

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Abbreviations used: ATP, adenosine triphosphate; CFW, calcofluor white; CWI, cell wall integrity; DSP, dual-specificity phosphatase; GFP, green fluorescent protein; HU, hydroxyurea; IP, immunoprecipitation; MEK, MAP kinase kinase; MEKK, MEK kinase; MMS, methyl methanesulfonate; SAPK, stress-activated MAP Kinase.

Yan *et al.*, 1999). However, the mechanisms by which various stress signals activate ERK5 are poorly understood (Keshet and Seger, 2010). This raises two important and related questions. First, do these various stresses activate their target SAPKs through a common pathway or through alternative inputs? Second, how does an activated SAPK mount a specific response appropriate to the particular stress experienced? We have begun to address these questions with an analysis of CWI pathway signaling in response to genotoxic stress.

It has been shown for nearly all of the stresses mentioned above that upstream components of the CWI signaling pathway, including the cell-surface sensors, are required for activation of the SAPK. However, such requirements do not distinguish between pathway components that play an active role in signaling a particular stress and components that merely provide basal activity that can be modulated by intracellular inputs to the pathway. Basal flux through a signaling pathway would be essential for intracellular stress inputs to modulate the signal. Inputs at various points along a pathway could provide mechanisms to distinguish among different stresses and perhaps mechanisms for directing the specificity of SAPK output. Indeed, there is evidence for an intracellular input to Mpk1 by heat shock that cannot be explained through activation of the CWI MAPK cascade and suggests the existence of "lateral" inputs to the pathway (Harrison *et al.*, 2004), although the mechanism and pathway details remain unknown.

We reasoned that it would be instructive to examine the mechanism by which genotoxic stress activates Mpk1 for three reasons. First, because this signal originates from within the cell rather than from the cell surface. Although genotoxic chemicals and UV or ionizing irradiation can damage other cellular components in addition to DNA, Soriano-Carot *et al.* (2012) presented an elegant demonstration that a double-stranded DNA break introduced by the homothallic switching endonuclease is sufficient to activate Mpk1. Second, genetic interactions have been identified between *MPK1* and DNA damage checkpoint genes that suggest Mpk1 plays a role in the response to genotoxic stress (Queralt and Igual, 2005; Enserink *et al.*, 2006; Truman *et al.*, 2009; Bandyopadhyay *et al.*, 2010). Third, the CWI pathway is required for survival of DNA damage and is important for the response to genotoxic stress. Defects in any component of the pathway from the cell-surface sensors to Mpk1 cause hypersensitivity to a variety of DNA damaging agents (Zu *et al.*, 2001; Leduc *et al.*, 2003; Queralt and Igual, 2005; Soriano-Carot *et al.*, 2012).

In this study, we show that genotoxic stress activates Mpk1 by driving ubiquitin-mediated degradation of Msg5, the dual specificity phosphatase that maintains Mpk1 in an inactive state in the absence of stress. In contrast to cell-wall stress, activation of Mpk1 by DNA damaging agents does not involve the activation of its immediately upstream protein kinase. Activation of SAPKs through different input nodes in response to various stressors may have broad implications for consequent SAPK target specificity.

## RESULTS

### Genotoxic stress signal enters the CWI pathway at the level of Mpk1

Activation of the CWI SAPK Mpk1 in response to treatment with a variety of DNA damaging agents suggests that genotoxic stress is signaled through an intracellular route that interfaces with the CWI pathway rather than through activation of the canonical cell-surface sensors of this linear pathway (Figure 1A). We chose two chemical agents, hydroxyurea (HU) and methyl methanesulfonate (MMS), which act by different means to induce genotoxic stress. Hydroxyurea inhibits ribonucleotide reductase (Yarbro, 1992; Koc *et al.*, 2004), thereby limiting deoxyribonucleotide triphosphate pools for DNA synthesis, whereas MMS damages DNA principally by purine methylation (Lundin *et al.*, 2005). We found that treatment of cells with either 250 mM HU for 4 h or 0.08% MMS for 2 h strongly activates Mpk1 (Figure 1B). Deletion of the gene encoding the MEKK of the CWI pathway (*BCK1*), or the genes encoding the redundant MEKs (*MKK1* and *MKK2*), blocked Mpk1 activation by HU or MMS (Figure 1B), revealing the importance of these protein kinases in the activation of Mpk1 by genotoxic stress. The requirement for upstream kinases in the activation of Mpk1 may indicate that the DNA damage signal enters the pathway above Bck1. However, an alternative explanation for their requirement is that genotoxic stress might amplify basal signal that flows through the pathway to Mpk1 without impacting the activity of protein kinases that act upstream of the SAPK. In the absence of the MEKK or the MEKs of the pathway, there would be no basal signal that could be subject to modulation. To test this possibility, we examined a CWI pathway mutant in which Mpk1 was mutationally severed from its upstream activators by deletion of *BCK1*, but with basal signal restored by a constitutive pathway mutation. We restored basal signal to Mpk1 below the *bck1*Δ block point by expression of a phosphomimetic mutant form of *MKK1* that provides some protein kinase activity that is not regulated by phosphorylation from Bck1 (*MKK1-DD*; Harrison *et al.*, 2004). Low-level expression of *MKK1-DD* from a centromeric plasmid did not strongly activate Mpk1 by itself, but Mpk1 was activated in this strain by HU treatment (Figure 1C), strongly suggesting that the DNA damage signal enters the pathway at a point below the MEKs. It is noteworthy that expression of wild-type *MKK1* failed to restore Mpk1 activation in the absence of *BCK1*, confirming the need for basal signal to Mpk1. To rule out potential involvement of the wild-type *MKK1* and *MKK2* genes in the context of the *MKK1-DD* allele, a triple *bck1*Δ*mkk1*Δ*mkk2*Δ mutant was tested. Mpk1 was activated similarly by HU treatment in this mutant when *MKK1-DD* was present (Figure 1C), supporting the conclusion that the activating signal enters the pathway at the level of Mpk1, without activation of the MEKs. Mpk1 was also activated by MMS treatment in the triple mutant expressing *MKK1-DD* (Figure 1D), confirming the general nature of the genotoxic stress signal to Mpk1. In contrast to these results, calcofluor white (CFW), a cell-wall stress agent well known to activate CWI signaling (Jung *et al.*, 2002), failed to stimulate Mpk1 in the *bck1*Δ*MKK1-DD* strain (Figure 1E), consistent with activation of the canonical CWI pathway through action of this agent at the cell surface.

As a final test of the hypothesis that genotoxic stress activates Mpk1 without activation of its immediately upstream kinase, we conducted an in vitro protein kinase assay for Mkk1 activity toward its substrate Mpk1. For this experiment, Mkk1–green fluorescent protein (GFP) was isolated from untreated cells or from cells treated with HU or CFW, and unphosphorylated Mpk1-6His was isolated from an *mkk1*Δ*mkk2*Δ strain. Mkk1 was activated in response to CFW treatment, as judged by a strong increase in its ability to phosphorylate Mpk1 in vitro (Figure 2A). This activity was dependent on the addition of Mpk1-6His to the protein kinase assays, ruling out contamination of the Mkk1-GFP preparations with endogenous Mpk1 as the source of P-Mpk1 signal. Importantly, no increase in Mkk1 activity was detected for Mkk1 isolated from cells treated with HU, despite the activation of Mpk1 in vivo in the same extracts from which the Mkk1-GFP was isolated (Figure 2C), confirming that Mpk1 is activated in response to HU treatment in the absence of activation of its immediately upstream protein kinase. Figure 2B shows that Mkk1 was activated ~12-fold by CFW treatment, whereas HU treatment did not activate Mkk1 in three independent experiments. We noted additionally that Mkk1-GFP from cells treated with CFW migrated

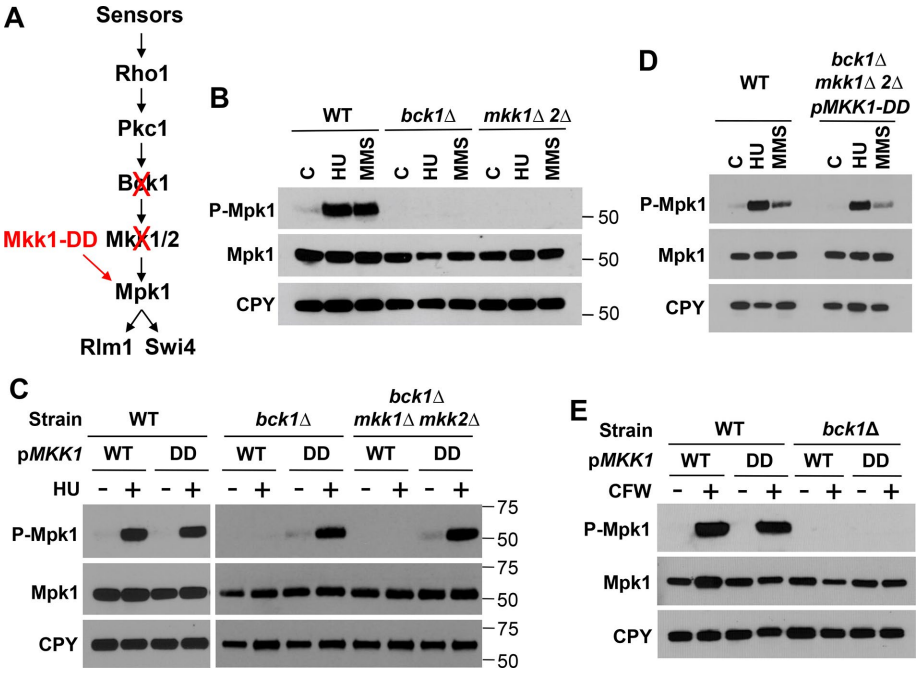


FIGURE 1: Genotoxic stress activates Mpk1 through a mechanism that acts below its MEKs, Mkk1 and Mkk2. (A) The CWI pathway from the cell-surface sensors to the transcription factors Rlm1 and Swi4. Strains were constructed to test the requirement for activation of protein kinases that function upstream of Mpk1 in the activation of Mpk1 by genotoxic stress. Basal signal to Mpk1 was restored in these strains, which lack the endogenous *BCK1* gene, with or without the endogenous *MKK1* and *MKK2* genes (indicated by red "X"), by expression of a phosphomimetic, Bck1-independent form of Mkk1 (Mkk1-DD). (B) Genotoxic stress activates Mpk1 in a manner that requires its upstream protein kinases. Cultures of wild-type yeast strain (DL100), a *bck1*Δ mutant (DL252), and an *mkk1*Δ *mkk2*Δ mutant (DL2673) were treated with 250 mM HU for 4 h, 0.08% MMS for 2 h, or untreated "C." Extracts were separated by SDS–PAGE prior to examination by immunoblot for activated Mpk1 (P-Mpk1), total Mpk1, and CPY as a loading control. Molecular mass markers (in kDa) are on the right. (C) HU treatment activates Mpk1 through an intracellular mechanism that acts below Mkk1 and Mkk2. Strains were wild-type (DL100), *bck1*Δ (DL252), and *bck1*Δ *mkk1*Δ *mkk2*Δ (DL4317) and carried a centromeric plasmid bearing either wild-type *MKK1* (WT; p3372) or *MKK1-DD* (DD; p3373). Cultures were treated with 250 mM HU for 4 h and extracts were processed as in B. (D) The genotoxic stress signal to Mpk1 acts below its MEKs. A wild-type strain (DL100) and the *bck1*Δ *mkk1*Δ *mkk2*Δ mutant (DL4317) expressing *MKK1-DD* were examined for activation of Mpk1 after treatment with HU or MMS, as above. (E) Mpk1 is not activated by cell-wall stress in a *bck1*Δ mutant expressing constitutive Mkk1. A wild-type strain (DL100) and a *bck1*Δ mutant (DL252) expressing either wild-type *MKK1* (WT) or *MKK1-DD* (DD) were examined for activation of Mpk1 after treatment with cell-wall stress agent calcofluor white (CFW; 40 μg/ml) for 2 h.

more slowly than that from untreated or HU-treated cells (Figure 2, A and C). This band shift is the consequence of retrophosphorylation of Mkk1 by Mpk1 that functions as negative feedback regulation of the MEKs (Jimenez-Sanchez *et al.*, 2007, and Supplemental Figure S1) and reveals distinct interactions between Mpk1 and its activators in response to cell-wall stress versus genotoxic stress.

### Genotoxic stress induces degradation of dual specificity protein phosphatase Msg5

A key negative regulator of Mpk1 is the dual specificity protein phosphatase (DSP) Msg5, which has been shown to associate stably with Mpk1 (Flandez *et al.*, 2004) and to maintain the SAPK in a low-activity state in the absence of stress (Martin *et al.*, 2000). A paralogue of Msg5, Sdp1, appears to play a minor role in the regulation of basal Mpk1 activity (Hahn and Theile, 2002). Because the genotoxic stress signal activates Mpk1 without activation of Mkk1, we considered the possibility that DNA damage inhibits Msg5 action on Mpk1 (Figure 3A). We examined Msg5 protein levels in response to treatment by HU or MMS and found that these treatments greatly diminished Msg5 levels (Figure 3B). We detected two distinct forms of Msg5, which result from alternative translational start sites that are separated by 44 codons (Flandez *et al.*, 2004). As can be seen in Figure 3B and subsequent figures, both forms of Msg5 were diminished in response to HU or MMS treatment. The diminution of Msg5 levels in response to HU or MMS treatment was not dependent on either the epitope tag used or the strain background tested, as we observed this effect with either Msg5-HA or Msg5-GFP (Supplemental data Figure S2, A and B) and in two different strain backgrounds (Supplemental Figure S2C). We also examined the level of Sdp1-HA in response to HU treatment and found that, unlike Msg5-HA, the level of this DSP was unaffected during the course of the experiment (Figure 3C). The activation of Mpk1 over a 4-h time course correlates strongly with the diminished Msg5 levels (Figure 3, D and E) and suggested the possibility that Mpk1 is activated as a consequence of Msg5 diminution. These results are in contrast to a time course of Mpk1 activation in response to CFW treatment, in which the Msg5 protein level remained stable throughout (Figure 3, D and E).

To test the relative roles of *MSG5* and *SDP1* in the activation of Mpk1 in response to HU treatment, we conducted a time course of Mpk1 phosphorylation in single and double mutants in these DSP-encoding genes. An *msg5*Δ mutant displayed a high basal level of Mpk1 phosphorylation, which was not increased further by HU treatment (Figure 4A), supporting the conclusion that Mpk1 activation by genotoxic stress results from inactivation of Msg5 rather than from activation of the protein kinases that function upstream of Mpk1. Indeed, the basal level of Mpk1 phosphorylation in the *msg5*Δ mutant was very similar to the level induced in wild-type cells after a 4-h HU treatment. In contrast to this, an *sdp1*Δ mutant behaved very much like wild-type cells with regard to Mpk1 activation state, indicating that it plays at most a minor role in the activation of Mpk1 in response to HU treatment. In further support of this conclusion, a double *msg5*Δ*sdp1*Δ mutant behaved like the *msg5*Δ mutant. These results contrasted with those observed in response to CFW treatment, which induced phosphorylation of Mpk1 in all strains, despite the increased level of basal Mpk1 phosphorylation in strains lacking *MSG5* (Figure 4B), consistent with a mechanism of Mpk1 activation through stimulation of its activating protein kinases. Collectively, our results suggest that HU treatment activates Mpk1 through an inhibitory effect specifically on Msg5, with little or no involvement of Sdp1.

Because our data suggest that Mpk1 is activated in response to genotoxic stress through inhibition of Msg5, we asked whether an *msg5*Δ mutant was preadapted for survival of genotoxic stress. As

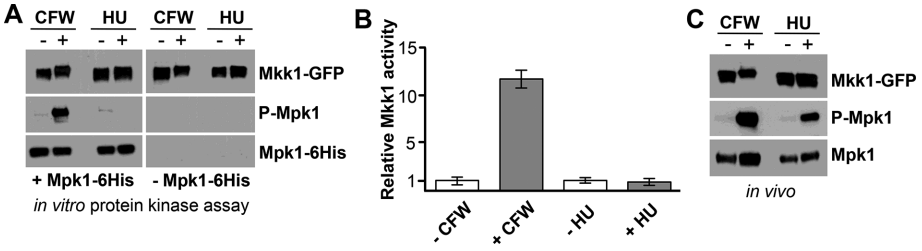


FIGURE 2: HU treatment does not activate Mkk1. (A) In vitro protein kinase assay for Mkk1-GFP using Mpk1-6His as substrate. Mkk1-GFP (from p1564) was immunoprecipitated from untreated wild-type cells (DL100) or from cells treated either with 250 mM HU for 4 h or 40 µg/ml CFW for 2 h. Isolated Mkk1 was tested by immunecomplex protein kinase assays for its ability to phosphorylate affinity-purified Mpk1, which was detected by immunoblot analysis. The left panels are from assays that included Mpk1-6His; the right panels are from assays that omitted Mpk1-6His. Unphosphorylated Mpk1-6His (from p3462) was isolated from an *mkk1*Δ *mkk2*Δ mutant (DL3539). (B) Mean and SD for three independent experiments as described in A. Values are expressed as relative Mkk1 activity with untreated controls set to 1. (C) In vivo phosphorylation of Mpk1. The same extracts from which Mkk1-GFP was isolated in A were tested by immunoblot analysis for activation of Mpk1 in vivo.

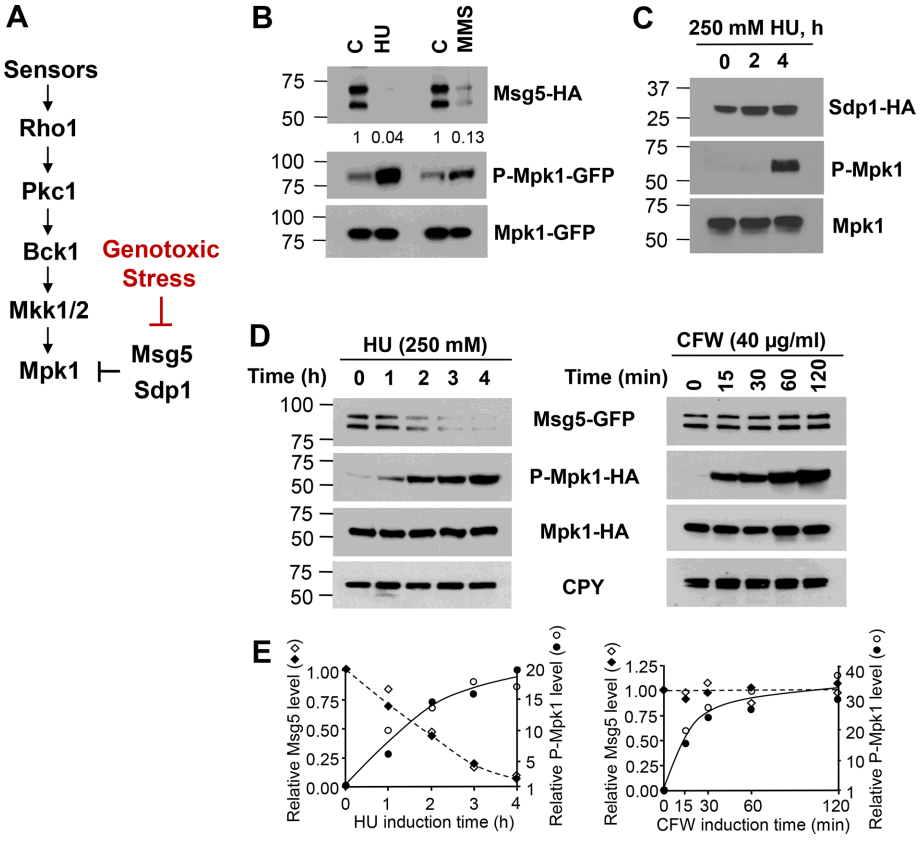


FIGURE 3: Genotoxic stress diminishes the level of DSP Msg5. (A) Model for genotoxic stress activation of Mpk1 by inhibition of the DSPs that down-regulate the SAPK. (B) Wild-type cells (DL100) expressing Msg5-HA (p3405) and Mpk1-GFP (p2283) were untreated (C) or treated with HU or MMS. Extracts were examined by immunoblot analysis for Msg5 level and activation of Mpk1. Values are for relative level of Msg5-HA remaining after treatment. Molecular mass markers (in kDa) are on the left. (C) Wild-type cells (DL2772) expressing Sdp1-HA (p3445) were treated with HU for the indicated times and extracts were examined by immunoblot analysis for Sdp1-HA level and activation of Mpk1. (D) Time course for treatment with HU or CFW. Wild-type cells (DL100) expressing Msg5-GFP (p3404) and Mpk1-HA (p672) were treated with HU (left panels) or CFW (right panels) for the indicated times. Extracts were examined by immunoblot analysis for Msg5 levels and activation of Mpk1. (E) Relative Msg5 levels (◆ ◇; broken trend lines) and P-Mpk1 levels (● ○; solid trend lines) from two independent experiments were plotted as a function of HU treatment time (left) and CFW treatment time (right) and normalized to loading control CPY.

observed previously (Soriano-Carot *et al.*, 2012), an *mpk1*Δ mutant was hypersensitive to treatment with DNA damaging agents HU, or MMS, as well as to cell-wall stress agent CFW (Figure 4C). However, an *msg5*Δ mutant displayed increased tolerance, relative to wild-type cells, to both HU and MMS treatment, consistent with the conclusion that this mutant is preadapted for these stresses through its elevated Mpk1 activity. In contrast, we observed decreased tolerance of the *msg5*Δ mutant to CFW treatment, suggesting that Msg5 regulation of Mpk1 activity is important in the adaptation to cell-wall stress.

The observed decrease in Msg5 protein level in response to genotoxic stress may result from a decrease in *MSG5* transcription or an increase in the rate of Msg5 turnover. A quantitative real-time (RT)-PCR experiment demonstrated that *MSG5* mRNA levels are unchanged across a 4-h HU treatment (Supplemental Figure S3), suggesting that the decrease in Msg5 protein level is a consequence of degradation. We therefore sought to stabilize Msg5 using the proteasome inhibitor, MG132 (Lee and Goldberg, 1998). For these experiments, we used a *pdr5*Δ mutant to enhance the intracellular retention of MG132 (Fleming *et al.*, 2002; Collins *et al.*, 2010). Cotreatment of cells with MG132 and HU resulted in considerable stabilization of Msg5 and, importantly, prevented activation of Mpk1 (Figure 5, A and B), revealing that Msg5 is targeted for ubiquitin-mediated proteasomal degradation in response to DNA damage and supporting the conclusion that activation of Mpk1 in this setting is caused by Msg5 degradation.

We next asked whether we could detect ubiquitination of Msg5 in response to HU treatment. Cells expressing Msg5-GFP were treated with HU together with MG132 to stabilize Msg5. Msg5-GFP was immunoprecipitated from cell extracts and tested by immunoblot analysis for the presence of ubiquitin (Figure 5C). We were able to detect a poly-ubiquitin smear above the Msg5-GFP bands in samples treated with MG132 or cotreated with MG132 and HU (left panels), suggesting that Msg5 is itself ubiquitinated. This smear was more prominent in samples cotreated with HU and MG132, suggesting that Msg5 ubiquitination is stimulated in response to genotoxic stress. To confirm that this smear is associated with Msg5-GFP, we compared the cotreated sample to a comparable extract in which GFP alone was expressed and immunoprecipitated (right panels). No poly-ubiquitin smear was detected in the control GFP lane.

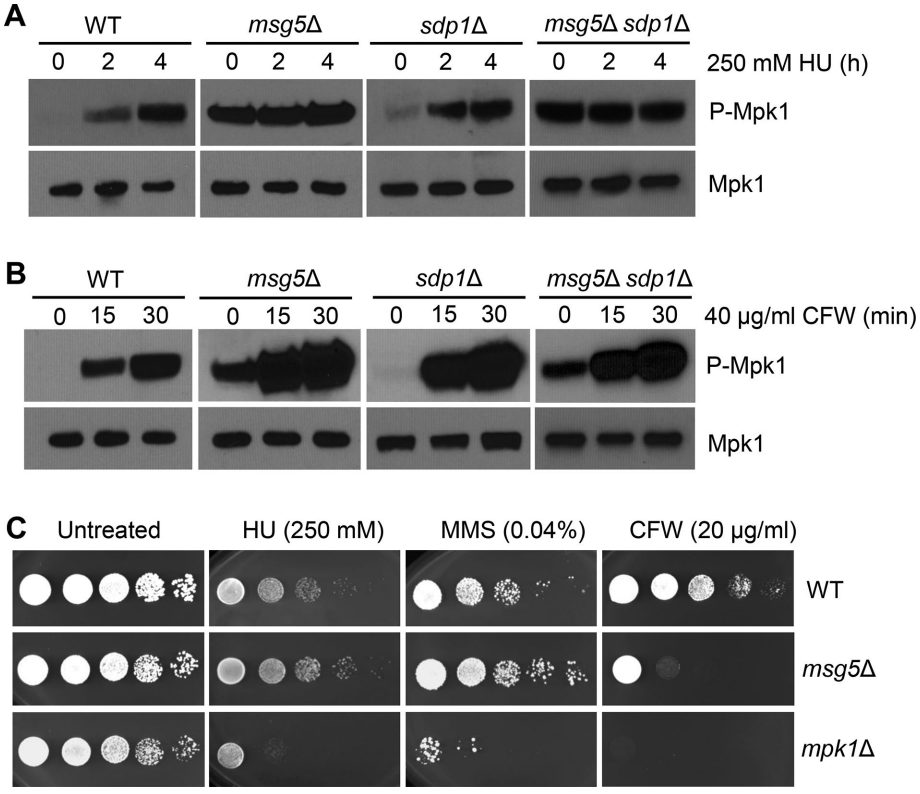


FIGURE 4: *MSG5* is essential for HU-induced activation of Mpk1. (A) HU treatment fails to activate Mpk1 in an *msg5*Δ mutant. Wild-type strain (DL2772), *msg5*Δ (DL4321), *sdp1*Δ (DL4345), and *msg5*Δ *sdp1*Δ (DL4346) mutants were subjected to HU treatment, as indicated, and extracts were tested for Mpk1 activation. Note the elevated basal phosphorylation of Mpk1 in strains that bear the *msg5*Δ mutation. (B) CFW treatment activates Mpk1 in the absence of *MSG5*. The same strains as in A were subjected to CFW treatment, as indicated, and extracts were tested for Mpk1 activation. (C) Loss of *MSG5* confers tolerance to genotoxic stress. Wild-type (DL2772), *msg5*Δ (DL4321), and *mpk1*Δ (DL3155) cells were spotted onto YPD plates with the indicated concentration of stressor at serial 10-fold dilutions (from left to right). Plates were incubated at 30°C for 2 d.

To identify the ubiquitin ligase(s) responsible for modification of Msg5 in response to genotoxic stress, we surveyed a collection of viable deletion mutants in genes encoding ubiquitin E2 and E3 ligases (and core components) implicated in the DNA damage response (Lee *et al.*, 2007; Findley *et al.*, 2012) for stabilization of Msg5 during treatment with HU. However, none of the 14 candidate deletion mutants displayed appreciable protection of Msg5 in response to HU treatment (Supplemental Figure S4), suggesting that the responsible E2 and E3 ligases are essential, are redundant with other ubiquitin ligases, or have not previously been implicated in the DNA damage response.

Finally, to determine whether Mpk1 is required for the genotoxic stress-induced degradation of Msg5, we examined an *mpk1*Δ mutant for Msg5 degradation in response to HU treatment. Interestingly, the Msg5 level was stable across a 4-h time course in an *mpk1*Δ mutant (Figure 6, A and B), indicating that the mechanism of Msg5 destruction requires Mpk1. To assess the requirement for Mpk1 protein kinase activity, we compared the *mpk1*Δ mutant expressing either wild-type *MPK1-HA* or a catalytically inactive form (*mpk1-K54R-HA*; Kim *et al.*, 2008). The catalytically inactive form of Mpk1 fully restored HU-induced degradation of Msg5 (Figure 6, A and B), revealing that although Mpk1 is important for this function, its protein kinase activity is not. This result raises the possibility that Mpk1 serves as a scaffold for a ubiquitin ligase to act on Msg5.

## DISCUSSION

There is a tendency to view SAPK pathways through the same lens as growth factor pathways in which signals are initiated from the cell surface. With the large and growing list of diverse stressors that activate a relatively small number of SAPKs, it seems increasingly likely that many of these stressors activate signaling through intracellular mechanisms rather than by top-down signaling through their canonical signaling pathways. This is particularly true for the yeast *S. cerevisiae*, which possesses only two SAPK activation pathways—the high osmolarity glycerol (HOG) pathway, which responds to hyperosmotic stress, and the CWI pathway, which responds to cell-wall stress—both of which are well characterized from their plasma membrane sensors to their kinase cascades and transcription factors. Yet both of these SAPKs are activated by a wide range of seemingly unrelated stressors.

### Mpk1 is activated by genotoxic stress through the proteolytic degradation of its negative regulator, Msg5

In this study, we found that genotoxic stress activates the CWI SAPK Mpk1 by inducing ubiquitin-mediated proteolysis of Msg5, the DSP that maintains Mpk1 in an inactive state in the absence of stress. We used two different treatments to induce genotoxic stress: HU, which causes nucleotide deprivation, and methylating agent MMS. Both of these treatments induced Msg5 degradation and Mpk1 activation, consistent with the generalized nature of the DNA damage signal sent to Mpk1 (Soriano-Carot *et al.*, 2012). Activation of Mpk1 in response to these treatments was dependent on protein kinases that act upstream of Mpk1 (i.e., Bck1, Mkk1, and Mkk2) but did not involve the activation of these protein kinases. Instead, upstream protein kinases were required to provide basal signal to Mpk1, which was modulated by elimination of Msg5.

Four lines of evidence support the above conclusion. First, expression of a constitutive phosphomimetic form of Mkk1 (Mkk1-DD), the protein kinase that functions immediately upstream of Mpk1, restored responsiveness of Mpk1 to genotoxic stress signals in strains in which Mpk1 had been severed from upstream components of the CWI MAPK cascade. Second, Mkk1 isolated from cells treated with HU was not activated as measured by the ability to phosphorylate Mpk1 in vitro, despite activation of Mpk1 in the extracts from which the Mkk1 was isolated. Third, deletion of *MSG5* increased the basal level of Mpk1 phosphorylation, but its phosphorylation level did not increase further in response to HU treatment. This would be expected if the mechanism of activation by HU was entirely through destruction of Msg5 and did not involve Sdp1, the other DSP known to act on Mpk1. Further, genetic ablation of *MSG5* had the effect of preadapting cells to DNA damage stress. Fourth, cotreatment of cells with HU and a proteasome inhibitor (MG132), which blocked degradation of Msg5 in response to the genotoxic stress signal, also prevented activation of Mpk1 and

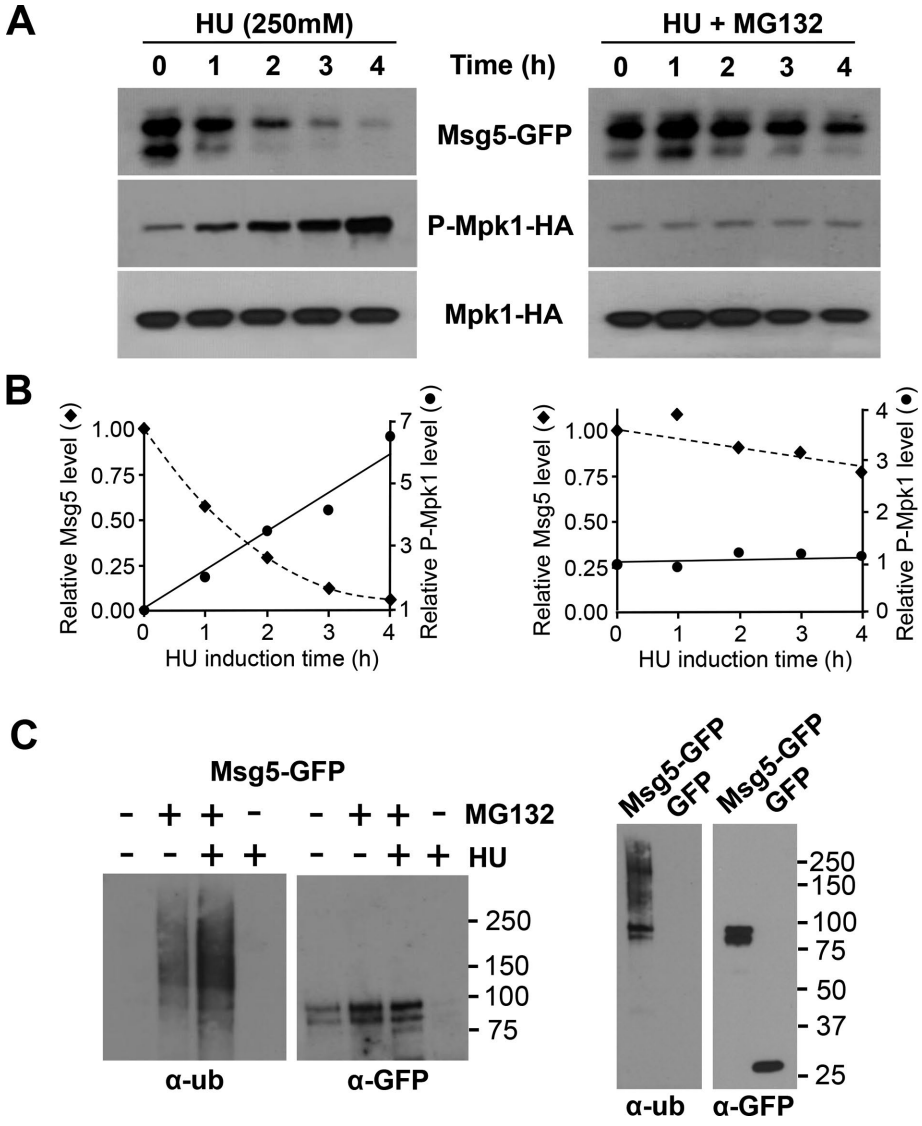


FIGURE 5: Proteasomal degradation of Msg5 in response to genotoxic stress causes Mpk1 activation. (A) Proteasome inhibitor MG132 blocks degradation of Msg5 in response to HU treatment and prevents activation of Mpk1. A *pdr5*Δ mutant (DL4334) was used to enhance intracellular retention of MG132. This strain, which expresses Msg5-GFP (p3404) and Mpk1-HA (p672), was treated with HU (left panels) or HU together with 50 μM MG132 (right panels) and tested for Msg5 levels and Mpk1 activation. (B) Quantitation of data from A. Relative Msg5 levels (◆; broken trend lines) and P-Mpk1 levels (●; solid trend lines) were plotted as a function of HU treatment time (left) and HU plus MG132 treatment time (right). (C) Msg5 is modified by ubiquitin in response to genotoxic stress. Strain DL4334, expressing Msg5-GFP (p3404) was treated with HU, MG132, or both for 3.5 h or incubated without treatment (left panels). The same strain expressing either Msg5-GFP or GFP alone (p3472) was double treated with HU and MG132 (right panels). Extracts were subjected to immunoprecipitation of GFP, followed by immunoblot analysis for GFP (α-GFP) and ubiquitin (α-ub). The samples in C were separated on 4–20% gradient gels. Molecular mass markers (in kDa) are on the right.

established the causal relationship between degradation of Msg5 and Mpk1 activation.

This mode of activation was in contrast to that observed for the cell-wall stress signal induced by the cell-wall antagonist CFW. Treatment of cells with CFW similarly required components of the CWI MAPK cascade to activate Mpk1, but activation of Mpk1 in mutants lacking upstream components was not restored by expression of Mkk1-DD. Consistent with the established model for transmission of cell-wall stress signals through the MAPK cascade, Mkk1 was activated in cells treated with CFW. Additionally, treatment with CFW did not induce the destruction of Msg5, and an *msg5*Δ mutant retained the ability to activate Mpk1 in response to this cell-wall stress.

It is interesting to note that Msg5 also down-regulates Fus3, the MAPK of the pheromone response pathway (Doi *et al.*, 1994). However, we did not detect phosphorylated Fus3 in response to genotoxic stress. The absence of activated Fus3 is likely the consequence of the different regulatory functions that Msg5 serves in the control of Mpk1 and Fus3. Msg5 forms a stable complex with Mpk1 and maintains the SAPK in a state of low basal activity. Therefore, a decrease in the level of Msg5, or deletion of *MSG5*, causes a strong increase in Mpk1 basal activity. In contrast to this, loss of Msg5 does not have a major impact on the basal activity of Fus3, because it acts primarily to down-regulate this MAPK after its activation by pheromone (Zhan *et al.*, 1997; Blackwell *et al.*, 2003).

Ubiquitination of Msg5 has not been reported previously. Although we were able to detect poly-ubiquitin modification of Msg5 stimulated in cells cotreated with HU and MG132, we were not able to identify the E3 ubiquitin ligase responsible for targeting Msg5. Because we tested deletion mutants in genes encoding a subset of nonessential ubiquitin ligases that had previously been implicated in the DNA damage response, our failure to identify the ligase(s) responsible for degradation of Msg5 may be explained by functional redundancy. Alternatively, the responsible ligase may not have been implicated previously in the response to DNA damage. It is also interesting to note that destruction of Msg5 required Mpk1 but not its protein kinase activity. Because Mpk1 and Msg5 normally reside in a complex together, it is possible that the E3 ligase responsible for the genotoxic stress-induced degradation of Msg5 uses Mpk1 as a scaffold from which to modify Msg5. Nevertheless, there remains a significant gap in our understanding of how genotoxic stress interfaces with the machinery of ubiquitin-mediated proteolysis specific to Msg5 degradation.

We recently described a similar intracellular mechanism for the activation of yeast SAPK Hog1 by arsenite (Lee and Levin, 2018). Arsenite is metabolically activated by the dimeric methyltransferase Mtq2:Trm112 to methylarsenite, which is a potent inhibitor of the protein tyrosine phosphatases (Ptp2 and Ptp3) that normally maintain Hog1 in an inactive state. One consequence of Hog1 activation by arsenite is the closure of the glycerol channel Fps1, the major port through which arsenite enters the cell (Thorsen *et al.*, 2006; Lee *et al.*, 2013; Lee and Levin, 2018). Another example of

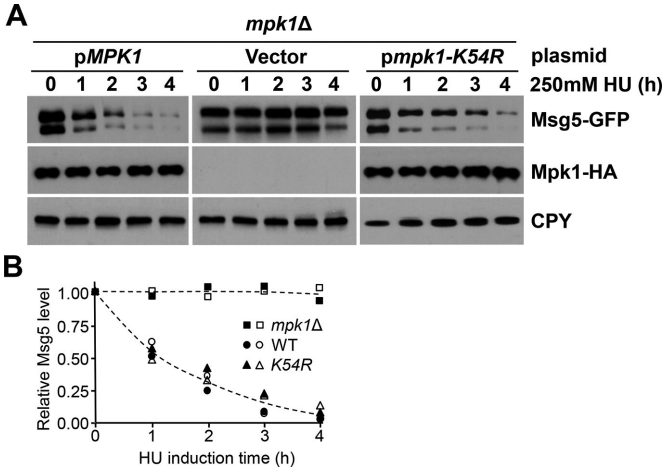


**FIGURE 6:** Mpk1, but not its protein kinase activity, is required for HU-induced Msg5 degradation. (A) An *mpk1*Δ mutant (DL454), transformed with a plasmid bearing wild-type *MPK1-HA* (p672), a mutant allele that encodes a catalytically inactive form (*mpk1-K54R-HA*; p1846), or vector alone (p121), was treated with HU and tested for degradation of Msg5-GFP. (B) Relative Msg5 levels in wild-type (● ○), *mpk1*Δ (□ ■), and *mpk1-K54R* (▲ △) from two independent experiments were plotted as a function of HU treatment time.

similar regulation has been reported for the *Saccharomyces pombe* SAPK Spc1, a homologue of *S. cerevisiae* Hog1 and human p38 (Nguyen and Shiozaki, 1999). Spc1 is activated by heat shock in a manner that is independent of the activation of its MEK Wis1. The mechanism of Spc1 activation, in the case of heat shock, appears to be inhibition of its interaction with the protein tyrosine phosphatase, Pyp1. Thus, three similar, but distinct, mechanisms by which different stresses activate SAPKs by interfering with the function of the protein phosphatase(s) that maintains them in low-activity states have now been described.

Although mammalian ERK5 is generally regarded to be a functional analog of yeast Mpk1, the former does not appear to be activated in response to DNA damage (Kato *et al.*, 1997). In this regard, Mpk1 is more like mammalian SAPKs p38 and Jun N-terminal kinase (JNK), which are both activated by genotoxic stressors (Hibi *et al.*, 1993; Derijard *et al.*, 1994; Kyriakis and Avruch, 1996; Benhar *et al.*, 2001; Zarubin and Han 2005). Several mechanisms by which DNA damage activates JNK have been described. The most recent is a circuitous autocrine path from DNA damage checkpoint kinase ataxia telangiectasia mutated (ATM) that goes through transcription factor nuclear factor (NF)-κB and cytokine tumor necrosis factor-α, which activates JNK3 through its cell-surface receptor (Biton and Ashenazi, 2011; Picco and Pages, 2013). Another mechanism involves ionizing radiation-induced generation of ceramide and clustering of death receptors on the plasma membrane (Ruvolo, 2003; Munshi and Ramesh, 2013). Finally, Brancho *et al.* (2003) detected activation of MEKs MKK3, MKK4, and MKK6 in mouse embryo fibroblasts treated with UV irradiation, supporting the conclusion that JNK activation by genotoxic stress involves signaling from above. The pathways by which genotoxic stress activate SAPK p38 remain largely unclear (Wood *et al.*, 2009; Corre *et al.*, 2017). However, because MKK3, MKK4, and MKK6 are all activated by UV irradiation and have been shown to play redundant roles in the activation of p38 under these conditions (Brancho *et al.*, 2003), it seems likely that the mechanism involved is rather different from the one described here for Mpk1.

### The relationship between CWI signaling and DNA damage checkpoint signaling

The Mec1 and Tel1 kinases of yeast are the major regulators of the response to DNA damage and are orthologues of mammalian ATR and ATM, respectively (Melo and Toczyski, 2002; Harrison and Haber, 2006). Their stimulation results in the activation of effector kinases Chk1 and Rad53 (Chk2 in mammals), which mediate cell-cycle arrest and induction of DNA repair genes (Harper and Elledge, 2007). As noted earlier, the entire CWI pathway is also required for survival of genotoxic stress. However, with regard to DNA metabolism, there is a bifurcation of pathway outputs at Pkc1, the protein kinase at the head of the CWI SAPK cascade. Mutants in *PKC1*, but not *MPK1*, are strikingly hyperrecombinogenic, similarly to mutants in many genes involved in DNA metabolism (Huang and Symington, 1994; Fasullo *et al.*, 2010). Thus, Pkc1 may play a housekeeping function in DNA metabolism that does not involve the CWI SAPK cascade, or it may be directed to targets involved in DNA repair in response to genotoxic stress. Additionally, Pkc1, but not Mpk1, was reported to be required for activation of the DNA damage checkpoint through Mec1 and Tel1 as judged by loss of a characteristic Rad53 phosphorylation band shift in *pkc1* mutants (Soriano-Carot *et al.*, 2012, 2014). However, we have not been able to validate these results using *pkc1*Δ mutants in three different strain backgrounds (Supplemental Figure S5), including the *pkc1*Δ strain used by Soriano-Carot *et al.* (2014). However, Pkc1 is hyperphosphorylated in response to genotoxic stress in a manner dependent on Tel1 (Soriano-Carot *et al.*, 2014). Although it is not clear whether Pkc1 hyperphosphorylation is a positive, negative, or targeting regulatory event, we have shown that the SAPK cascade under the control of Pkc1 is not activated in response to genotoxic stress. As for Mpk1, its activation by genotoxic stress is independent of the DNA damage checkpoint. In fact, basal Mpk1 activity is elevated in the absence of checkpoint kinases Tel1 and Mec1, or Rad53, and is further stimulated by DNA damage in these mutants (Enserink *et al.*, 2006; Soriano-Carot *et al.*, 2012). Thus, there appear to be two separate inputs to the CWI pathway in response to genotoxic stress—a signal of unknown consequence from the DNA damage checkpoint to Pkc1 and an activating signal to Mpk1 through Msg5 that is independent of the checkpoint. It is possible that targeting of Pkc1 activity to DNA repair functions by the DNA damage checkpoint kinases necessitates an alternative pathway to Mpk1 activation for its function in the response to genotoxic stress.

### Mpk1 activated by different stresses drives divergent outputs

When Mpk1 is activated in response to cell-wall stress, a well-characterized transcriptional program is activated in support of cell-wall biogenesis (Jung and Levin, 1999; Jung *et al.*, 2002; Roberts *et al.*, 2000; Lagorce *et al.*, 2003; Boorsma *et al.*, 2004; Garcia *et al.*, 2004; Klis, 2004; Rodriguez-Pena *et al.*, 2005; Kim *et al.*, 2008, Kim and Levin, 2011). Global gene expression analyses using a variety of genotoxic agents have similarly revealed the scope of gene expression changes in response to DNA damage (reviewed in Fu *et al.*, 2008). However, despite the activation of Mpk1 by genotoxic stress, regulation of its known transcriptional targets is not impacted by these stressors, suggesting that the cell-wall stress transcription factors, Rlm1 and Swi4, are not regulated by Mpk1 in this context. Additionally, a study of genetic network interactions among loss-of-function mutations in response to MMS treatment (Bandyopadhyay *et al.*, 2010) revealed that Mpk1 is required for proper regulation of the genes that encode ribonucleotide reductase (*RNR1* through *RNR4*). Induction of all four *RNR* genes in response to MMS treatment was strongly elevated in an *mpk1*Δ mutant relative to wild

type, suggesting that Mpk1 normally restrains the transcriptional response induced by the DNA damage checkpoint kinases.

It is not yet clear what factors dictate the differential output of Mpk1 activated in response to cell-wall stress as compared with genotoxic stress. However, different mechanisms of Mpk1 activation may be important in the specification of targets through the formation of distinct Mpk1-associated complexes. For example, Mpk1 that has been activated through destruction of Msg5, rather than by activation of its MEKs Mkk1 and Mkk2, may differ in its interactions with various pathway components in addition to its lost association with Msg5. In this regard, it is interesting to note that Mpk1 activated by HU treatment apparently does not engage in the retrophosphorylation of Mkk1 and Mkk2 (Figure 2 and Supplemental Figure S1) normally observed when Mpk1 is activated by cell-wall stress (Jimenez-Sanchez *et al.*, 2007).

The mechanism described in this study for the activation of Mpk1 in response to genotoxic stress suggests the possibility that other Mpk1-activating stressors may act at different points along the pathway. Detailed examination of the pathways by which other stressors activate Mpk1 may reveal a multitude of intracellular inputs to this SAPK. Moreover, how the variety of stress signal input mechanisms contribute to the specificity of SAPK output is an important question and may be one key to understanding the full range of SAPK signaling in yeast and humans.

## MATERIALS AND METHODS

### Strains, growth conditions, and transformations

The *S. cerevisiae* strains used in this study were derived from backgrounds of EG123 (Siliciano and Tatchell, 1984), S288c (BY4742; Research Genetics, Huntsville, AL), or SEY6210 (Scott Emr, Cornell University) and are listed in Table 1. Yeast cells were grown at 30°C (or 25°C) in YPD (1% Bacto yeast extract, 2% Bacto Peptone, 2% glucose) or minimal selective medium, SDM (0.67% yeast nitrogen base, 2% glucose) supplemented with the appropriate nutrients to select for plasmids. Yeast cells were transformed with DNA according to Gietz *et al.* (1995). Cell-wall stress was induced by treatment of calcofluor white (CFW, 40 µg/ml; Sigma-Aldrich) for 2 h or as indicated for time-course experiments. Genotoxic stress was induced by treatment with HU (250 mM; Fisher Scientific) for 4 h, or as indicated for time-course experiments, or MMS (0.08%; Sigma-Aldrich) for 2 h.

### Genomic deletions

A triple *bck1*Δ*mkk1*Δ*mkk2*Δ mutant (DL4317) was generated by crossing DL252 (*MAT***a***bck1*Δ::*URA3*) with DL2670 (*MATα mkk1*Δ::*LEU2 mkk2*Δ::*URA3*). Deletion alleles in segregants from this cross were confirmed by genomic PCR. A double *msg5*Δ*sdp1*Δ mutant was created by homologous recombination of *msg5*Δ-2::*LEU2* at the *MSG5* locus in an *sdp1*Δ::*KanMX* strain (DL4345). The *msg5*Δ-2::*LEU2* allele was amplified by PCR from genomic DNA of yeast strain DL1130 using Phusion high-fidelity DNA polymerase (ThermoFisher). Transformants were selected for leucine prototrophy and validated by genomic PCR across both integration junction sites. The resulting strain was *sdp1*Δ::*KanMX msg5Δ-2::LEU2* (DL4346). The *pkc1*Δ::*LEU2* allele was amplified by genomic PCR from DL376 and transformed into a wild-type diploid S288c strain and segregants were validated by genomic PCR for gene replacement of *PKC1*, yielding DL3040.

### Plasmid construction

The *MSG5* gene was epitope tagged on its C-terminus with GFP or the 3xHA epitope and expressed under its natural promoter. The promoter region of *MSG5* (from position –639) and its entire coding sequence was amplified from genomic yeast DNA (DL2772) by high-fidelity PCR (Phusion) using primers designed with a *Sac*II site for the GFP tag or an *Xho*I site for the 3xHA tag (upstream) and with a *Not*I site (downstream) for both tags and cloned into pRS425-GFP (p1202; Rajavel *et al.*, 1999) or pRS426-*3HA-ADT1*$^{T}$ (p3150; Lee *et al.*, 2013) to generate pRS425-*MSG5-GFP* (p3404) or pRS426-*MSG5-3HA* (p3405). The promoter region of *MSG5* (from position –639) without its coding sequence was amplified and cloned into the *Sac*II and *Not*I sites of p1202 to express GFP under the control of the MSG5 promoter (p3472). A similar method was employed for generation of a plasmid expressing C-terminally GFP-tagged Mkk1 in p1202 to yield pRS425-*MKK1-GFP* (p1564). GFP-tagged Mpk1 in YEp351 was generated from the 3xHA-tagged version (p777; Kamada *et al.*, 1995) in which the 3xHA-encoding sequence was removed by *Not*1 digestion and replaced by the GFP coding sequence to yield YEp351-*MPK1-GFP* (p2283).

For purification of Mpk1 used as a substrate in in vitro Mkk1 protein kinase assay, Mpk1 was tagged at its C-terminus with 6xHis ($His_6$) and expressed from its native promoter. A PCR fragment containing the promoter region of Mpk1 (from position –600) and its coding sequence was amplified from p672 using a primer that includes a *Bam*H1 site (upstream) and a primer that introduces a $His_6$ sequence followed by a stop codon and an *Xho*I site (downstream). The PCR fragment was inserted into pRS425 at *Bam*HI and *Xho*I sites to generate pRS425-*MPK1-6His* (p3462).

To detect Sdp1 expression from its native promoter, Sdp1 was epitope-tagged at its C-terminus with 3xHA. A PCR fragment containing the promoter region of *SDP1* (from position –581) and its coding sequence was amplified from genomic yeast DNA (DL2772) using a primer that includes a *Xho*I site (upstream) and a primer with a *Not*I site (downstream) and inserted into pRS425-*3HA-ADH1*$^{T}$ at the *Xho*I and *Not*I sites to generate pRS425-*SDP1-3HA* (p3445). The plasmids used in this study are listed in Table 2.

### SDS–PAGE and immunoblot analysis

For direct immunoblot experiments, cell lysates were prepared using the rapid boiling method (Kushnirov, 2000). Proteins were separated by SDS–PAGE (10% or 4–20% gradient gels, BioRad) followed by immunoblot analyses. Mouse monoclonal α-HA (16B12; Covance), α-GFP (Roche), α-carboxypeptidase Y (CPY) or goat polyclonal α-Mpk1 (yC-20; Santa Cruz) were used at a dilution of 1:10,000. Mouse monoclonal α-ubiquitin (P4D1; Cell Signaling), rabbit polyclonal α-phospho-p44/42 MAPK (Thr202/Tyr204; Cell Signaling), and rabbit polyclonal α-Rad53 (abcam) were used at a dilution of 1:2000. Secondary goat anti-mouse (Jackson ImmunoResearch), donkey anti-rabbit (GE Healthcare), and donkey anti-goat (Santa Cruz) antibodies were used at a dilution of 1:10,000.

### In vitro protein kinase assay

An in vitro protein kinase assay with Mkk1-GFP was performed with using Mpk1-6His as substrate. The unphosphorylated form of Mpk1 was isolated from an *mkk1*Δ*mkk2*Δ strain (DL3539) expressing Mpk1-6His (from p3462). This strain was grown to mid–log phase in 500 ml of selective medium at 30°C, and the cells were collected and lysed in 20 mM HEPES buffer containing 0.5% triton and protease inhibitors (20 µg/ml leupeptin, 20 µg/ml benzamidine, 10 µg/ml pepstatin A, 40 µg/ml aprotinin, and 1 mM phenylmethane sulfonyl fluoride). The protein extract (3 ml) was incubated with HisPur Ni-NTA Superflow Agarose (500 µl;

| Strain | Relevant genotype | Source or reference |
|---|---|---|
| DL100 | *MAT*a EG123 *ura3-52 leu2-3,112 trp1-1 his4 can1$^r$* | Siliciano and Tatchell (1984) |
| DL252 | *MAT*a EG123 *bck1Δ::URA3* | Lee and Levin (1992) |
| DL333 | *MAT*a SEY6210 *leu2-3,112 ura3-52 his3-200 trp1-901 ade2-101 lys2-801 suc2-9* | Scott Emr |
| DL376 | *MAT*a EG123 *pkc1Δ::LEU2* | Levin and Bartlett-Heubusch (1992) |
| DL454 | *MAT*a EG123 *mpk1Δ::TRP1* | Lee *et al.* (1993) |
| DL1130 | *MAT*a 15D *ade1 his2 leu2 trp1 ura3 msg5Δ-2::LEU2* | Doi *et al.* (1994) |
| DL1021 | *MAT*a SEY6210 *pkc1Δ::HIS3 leu2-3,112 ura3-52 his3-200 trp1-901 ade2-101 suc2-9* (GPY1115) | Paravicini *et al.* (1992) |
| DL2670 | *MATα* EG123 *mkk1Δ::LEU2 mkk2Δ::URA3* | B. Philip and D. E. Levin (unpublished) |
| DL2673 | *MAT*a EG123 *mkk1Δ::LEU2 mkk2Δ::URA3* | B. Philip and D. E. Levin (unpublished) |
| DL2772 | *MATα* S288c (BY4742) *his3Δ leu2Δ ura3Δ lys2Δ* | Research Genetics |
| DL3040 | *MATα* S288c *pkc1Δ::LEU2* | This study |
| DL3155 | *MAT*a S288c *mpk1Δ::KanMX* | Research Genetics |
| DL3539 | *MAT*a EG123 *mkk1Δ::KanMX mkk2Δ::KanMX* | Research Genetics |
| DL4106 | *MATα* S288c *bre1Δ::KanMX* | Research Genetics |
| DL4317 | *MAT*a EG123 *bck1Δ::URA3 mkk1Δ::LEU2 mkk2Δ::URA3* | This study |
| DL4319 | *MATα* S288c *rad6Δ::KanMX* | Research Genetics |
| DL4321 | *MATα* S288c *msg5Δ::KanMX* | Research Genetics |
| DL4334 | *MATα* S288c *pdr5Δ::KanMX* | Research Genetics |
| DL4335 | *MATα* S288c *rad18Δ::KanMX* | Research Genetics |
| DL4345 | *MATα* S288c *sdp1Δ::KanMX* | Research Genetics |
| DL4346 | *MATα* S288c *sdp1Δ::KanMX msg5-2Δ::LEU2* | This study |
| DL4369 | *MATα* S288c *ubc7Δ::KanMX* | Research Genetics |
| DL4370 | *MATα* S288c *ufd4Δ::KanMX* | Research Genetics |
| DL4389 | *MATα* S288c *ubc8Δ::KanMX* | Research Genetics |
| DL4390 | *MATα* S288c *rad16Δ::KanMX* | Research Genetics |
| DL4391 | *MATα* S288c *ubc13Δ::KanMX* | Research Genetics |
| DL4392 | *MATα* S288c *rtt101Δ::KanMX* | Research Genetics |
| DL4393 | *MATα* S288c *dia2Δ::KanMX* | Research Genetics |
| DL4395 | *MATα* S288c *rad5Δ::KanMX* | Research Genetics |
| DL4396 | *MATα* S288c *slx8Δ::KanMX* | Research Genetics |
| DL4397 | *MATα* S288c *uls1Δ::KanMX* | Research Genetics |
| DL4398 | *MATα* S288c *slx5Δ::KanMX* | Research Genetics |

**TABLE 1:** Yeast strains.

Thermo Scientific) at 4°C for 2 h. The resin was washed with 20 mM HEPES buffer with 0.1% Triton X-100 (Sigma-Aldrich) three times, and Mpk1-$His_6$ was eluted with 250 mM imidazole. The Mpk1-$His_6$ eluate was dialyzed against 50 mM Tris-HCl to a final concentration of 549 ng/μl protein (Bradford).

Mkk1 was isolated from a wild-type strain (DL100) expressing C-terminally GFP-tagged Mkk1 under the control of its own promoter (p1564). Cells were grown at 25°C in 25 ml of selective medium to mid–log phase and treated with 250 mM HU for 4 h, 40 μg/ml CFW for 2 h, or untreated. Cells were collected and fractured in lysis buffer with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 5 mM ethyleneglycol-bis tetraacetic acid, 0.5% Triton, protease inhibitors, and phosphatase inhibitors (PhosSTOP, Roche). The lysates (200 μl) were incubated with 10 μl of GFP trap beads (Chromotek) for 2 h at 4°C. The Mkk1-GFP immune complexes were washed three times with immunoprecipitation (IP) buffer (lysis buffer without protease inhibitors) and twice with kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol).

Each reaction contained 20 μl Mpk1-$His_6$ mixed with the Mkk1-GFP trap beads in a total volume of 30 μl. Kinase reactions were initiated with ATP at a final concentration of 300 μM. Reaction mixtures were incubated at 30°C for 30 min and stopped by addition of SDS sample buffer, followed by boiling for 5 min. Immunoblot analysis was performed as described above.

### Immunoprecipitation

For detection of ubiquitin modification of Msg5, cultures of a *pdr5Δ* strain (DL4334) expressing Msg5-GFP (from p3404) were

| Plasmid | Description | Source or reference |
|---|---|---|
| p121 | YEp352 | Hill *et al.* (1986) |
| p672 | YEp352-*MPK1-3HA* | Kamada *et al.* (1995) |
| p777 | YEp351-*MPK1-3HA* | Kamada *et al.* (1995) |
| p1105 | pRS425 | Sikorski and Hieter (1989) |
| p1106 | pRS426 | Sikorski and Hieter (1989) |
| p1202 | pRS425-*GFP* | Rajavel *et al.* (1999) |
| p1564 | pRS425-*MKK1-GFP* | This study |
| p1846 | YEp352-*mpk1-K54R-3HA* | Kim *et al.* (2008) |
| p2283 | YEp351-*MPK1-GFP* | This study |
| p3150 | pRS426-*3HA-ADT1*$^{T}$ | Lee *et al.* (2013) |
| p3372 | pRS314-*12HA-MKK1* | Harrison *et al.* (2004) |
| p3373 | pRS314-*12HA-MKK1-DD* | Harrison *et al.* (2004) |
| p3404 | pRS425-*MSG5-GFP* | This study |
| p3405 | pRS426-*MSG5-3HA* | This study |
| p3445 | pRS425-*SDP1-3HA* | This study |
| p3462 | pRS425-*MPK1-6His* | This study |
| p3472 | pRS425-*MSG5*$^{P}$*-GFP* | This study |

**TABLE 2:** Plasmids.

grown to mid–log phase in selective medium and treated with 50 µM proteasome inhibitor MG132 (Sigma), 250 mM HU, or both, for 3.5 h. Proteins were extracted using a glass beads lysis method, as described (Kamada *et al.*, 1995), but with the addition of 100 mM iodoacetamide to inhibit deubiquitinases (Emmerich and Cohen, 2015). Extracts (1 mg of protein) were incubated with 10 µl of GFP trap beads at 4°C for 2 h and the samples were washed with IP buffer, as above, three times and boiled for 5 min in SDS sample buffer.

### Quantitative real-time PCR

Quantitative real-time PCR was conducted to quantify the levels of Msg5 mRNA under DNA damage stress. Wild-type cells (DL100) bearing pRS426-*MSG5-3HA* (p3405) were treated with 250 mM HU for 0, 1, 2, 3, and 4 h. Total RNA was prepared using the RNeasy mini kit (Qiagen), and cDNA was generated using Superscript III (Invitrogen) First-Strand Synthesis System for RT-PCR, as per the manufactures' instructions, respectively. Real-time PCR was performed using a Bio-Rad CFX96 Real-Time system with Bio-Rad SYBR green mix. The data from biological triplicates were analyzed using Microsoft Excel and plotted using Prism (GraphPad).

### Notes on reproducibility

All immunoblots were reproduced at least once in independent experiments with representative images shown. Quantitation of signal intensity from immunoblots was done using ImageJ software, and data points from two independent experiments were plotted together as different symbols.

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