Dissociation of the H3K36 demethylase Rph1 from chromatin mediates derepression of environmental stress-response genes under genotoxic stress in *Saccharomyces cerevisiae*

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ABSTRACT **Cells respond to environmental signals by altering gene expression through transcription factors. Rph1 is a histone demethylase containing a Jumonji C (JmjC) domain and belongs to the C_2H_2 zinc-finger protein family. Here we investigate the regulatory network of Rph1 in yeast by expression microarray analysis. More than 75% of Rph1-regulated genes showed increased expression in the *rph1*-deletion mutant, suggesting that Rph1 is mainly a transcriptional repressor. The binding motif 5′-CCCCTWA-3′, which resembles the stress response element, is overrepresented in the promoters of Rph1-repressed genes. A significant proportion of Rph1-regulated genes respond to DNA damage and environmental stress. Rph1 is a labile protein, and Rad53 negatively modulates Rph1 protein level. We find that the JmjN domain is important in maintaining protein stability and the repressive effect of Rph1. Rph1 is directly associated with the promoter region of targeted genes and dissociated from chromatin before transcriptional derepression on DNA damage and oxidative stress. Of interest, the master stress-activated regulator Msn2 also regulates a subset of Rph1-repressed genes under oxidative stress. Our findings confirm the regulatory role of Rph1 as a transcriptional repressor and reveal that Rph1 might be a regulatory node connecting different signaling pathways responding to environmental stresses.**

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INTRODUCTION

Living organisms constantly encounter diverse environmental stress conditions. To maintain homeostasis and adapt to environmental changes, cells require a timely integration of multiple signaling pathways by mainly coordinating the expression of genes responsive to different stresses (Gasch *et al.*, 2000; Causton *et al.*, 2001; Weake and Workman, 2010; de Nadal *et al.*, 2011). The dynamic interplay between transcription factors (TFs) and chromatin modifiers plays a pivotal role in regulating gene expression on a whole-genome scale. In yeast, various stress conditions activate a common response in gene expression—the environmental stress response (ESR). Activation of the ESR is important to maintain cell viability under stress conditions and may provide cross-protection against different types of stress in yeast (Gasch, 2002; Berry *et al.*, 2011). Recent studies showed that a cross-stress protection mechanism is important in acquiring resistance because mild primary stress can increase cell resistance to severe secondary stress (Kandror *et al.*, 2004; Berry and Gasch, 2008; Slavov *et al.*, 2012). In multicellular organisms, cells can be primed by an initial stress that activates faster and stronger defense to subsequent stress stimuli and thus increases cell fitness and survival (Durrant and Dong, 2004; Kensler *et al.*, 2007).

ESR alters the gene expression of ~900 genes, of which nearly one-third are induced by stresses (Gasch *et al.*, 2000; Causton *et al.*, 2001). Several signaling pathways involved in the activation of ESR include DNA damage, high-osmolarity glycerol, oxidative stress signals, and inhibition of target of rapamycin (TOR; Gasch, 2002;

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Abbreviations used: CHX, cycloheximide; ChIP, chromatin immunoprecipitation; ESR, environmental stress response; Jmj, Jumonji; MMS, methyl methanesulfonate; Msn2/4, multicopy suppressor of SNf1 2 and 4; PHR1, photoreactivation repair deficient 1; STRE, stress response element; TF, transcription factor; TOR, target of rapamycin; UV, ultraviolet; ZF, zinc finger.

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Berry *et al.*, 2011). General stress-induced TFs such as multicopy suppressor of SNf1 2 and 4 (Msn2/4) and specific TFs such as heat shock factor (Hsf1), oxidative-stress factor (Yap1), and osmotic-stress factor (Sko1) are involved in a complex transcription process in ESR. In addition, transcription cofactors such as Tup1-Ssn6 and Rpd3 complexes help to regulate ESR gene expression (Gasch *et al.*, 2001; Alejandro-Osorio *et al.*, 2009; Verghese *et al.*, 2012). Little is known, however, about how different signal transduction pathways are integrated to control gene expression via various TFs.

Msn2/4 are the master regulators of stress-response genes and primarily govern the full activation of ~88% of ESR-induced genes under a broad range of stresses (Schmitt and McEntee, 1996; Gasch *et al.*, 2000; Causton *et al.*, 2001). Msn2/4 bind to the stress response element (STRE) via their zinc-finger (ZF) domains to activate transcription (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996; Berry and Gasch, 2008). Msn2/4 also mediate the expression of ESR-related genes triggered by DNA damage (Schmitt and McEntee, 1996; Gasch *et al.*, 2001). Whether similar signaling mechanisms are involved in transducing the DNA-damage response and the general stress response is not fully understood.

DNA lesions induced by exogenous agents or cellular metabolic processes result in genotoxic stresses that can be extremely detrimental to cell viability and growth. The checkpoint signal is highly conserved to ensure a timely and proper response to DNA damage (Harrison and Haber, 2006). The activation of a checkpoint leads to cell-cycle arrest, DNA repair, apoptosis, and large-scale alteration in transcription (Huen and Chen, 2008; Lord and Ashworth, 2012). In budding yeast, altered gene expression in response to DNA damage is mainly regulated by a checkpoint kinase cascade consisting of two key proteins, Mec1 (homologue of hATR) and Rad53 (homologue of hCHK2). In addition, Dun1 is the main kinase downstream of Rad53 kinase in regulating the DNA damage response (Zhou and Elledge, 1993). A well-documented transcription response to DNA damage is the derepression of Crt1 by Dun1. Crt1 binds to the X-box of *RNR2*, *RNR3*, *RNR4*, and *HUG1* promoters as a transcriptional repressor in the absence of DNA damage (Huang *et al.*, 1998; Basrai *et al.*, 1999). Another example is the repression of *PHR1*, encoding a photolyase, by Rph1 and Gis1, which is released on DNA damage (Sebastian and Sancar, 1991; Jang *et al.*, 1999). Thus both derepression and activation of gene expression play important roles in the coordinated regulation of gene networks crucial for DNA-damage response.

Rph1 and Gis1 are two paralogous ZF proteins that share high similarity (34.7%) in overall sequences and 100% identity in the ZF domains at the C-terminus. In addition, both the Jumonji N (JmjN) and JmjC domains in the N-terminus of Rph1 and Gis1 are evolutionarily conserved (Jang *et al.*, 1999; Tu *et al.*, 2007). The JmjC domain is highly conserved from yeast to humans and can catalyze the demethylation of lysine residues in histones (Klose *et al.*, 2006; Tsukada *et al.*, 2006). Rph1 has robust demethylase activity specific to H3K36, whereas Gis1 has only modest activity because of a mutation in the cofactor-binding site (Klose *et al.*, 2007; Tu *et al.*, 2007). Furthermore, Rph1 and Gis1 have overlapping and distinct functions in many of their targeted genes (Jang *et al.*, 1999; Zhang and Oliver, 2010; Orzechowski Westholm *et al.*, 2012). The most-characterized target of Rph1 and Gis1 is *PHR1*. Both Rph1 and Gis1 have a redundant role in the repression of *PHR1*, but the former is considered a major regulator (Jang *et al.*, 1999). The mechanistic role of Rph1 in regulating *PHR1* expression under the control of a checkpoint signal has been studied in detail. Rph1 is associated with the *PHR1* promoter via the ZF domain for recruiting the corepressor Rpd3. In response to DNA damage, activated Rad53 is required for dissociation of Rph1 from the *PHR1* promoter to efficiently induce *PHR1* expression (Liang *et al.*, 2011). The regulatory network of Rph1 responding to genotoxic stress remains to be explored.

Regulation of gene expression in stress response pathways has been extensively studied to elucidate the function of key transcriptional activators and the role of chromatin modifiers (Jenuwein and Allis, 2001; Lo *et al.*, 2005; Weake and Workman, 2010). We have considerably less knowledge about the interplay between transcriptional repressors and histone methylation/demethylation to mediate switches between repression and activation of gene expression. In this study, we demonstrate that Rph1 functions mainly as a transcriptional repressor to regulate gene expression in response to DNA damage and general stress. Rph1 occupies the promoters of stress-responsive genes by recognizing a motif resembling the STRE to repress transcription under physiological conditions. Derepression is achieved by Rph1 dissociating from the promoters of otherwise suppressed genes that respond to oxidative stress or DNA-damage signals mediated by Rad53. Oxidative stress and DNA damage both induce phosphorylation of Rph1, which dissociates Rph1 from the promoters of target genes. In turn, the master stress-activated regulator Msn2 binds to the promoter regions of several Rph1-repressed genes for transcriptional activation. Rph1 may be a transcriptional repressor downstream of multiple signaling pathways. Our results suggest a functional link of the histone demethylase Rph1 as a regulatory component in the integral networks of ESR.

RESULTS

Rph1 regulates the expression of genes associated with DNA damage and the ESR

To investigate the biological functions and effect of Rph1 on global gene expression, we used expression microarray analysis for genome-wide identification of Rph1 targets in yeast. Hierarchical clustering of data involved use of GeneSpring GX11 (Agilent) and Cluster programs (Eisen *et al.*, 1998); transcripts with differential expression (false discovery rate [FDR] <0.05 and fold change >1.3) were identified in the *rph1*-deletion mutant (*rph1*Δ) as compared with the wild type (WT; Figure 1A). The fold-change threshold was based on the incremental fold change in expression of *PHR1*, the best-characterized target of Rph1 in yeast (Liang *et al.*, 2011). Approximately 76% (158/208) of genes regulated by Rph1 showed increased expression in *rph1*Δ yeast (Figure 1A, dif., and Supplemental Table S1), so Rph1 functions primarily as a transcriptional repressor. This finding agrees with a recent microarray analysis showing that Rph1 mainly acts to repress gene expression in the exponential phase (Orzechowski Westholm *et al.*, 2012).

We next performed computational analyses to uncover the putative TFs and binding motifs associated with the expression of Rph1-regulated genes. By using the Pscan analysis program (Zambelli *et al.*, 2009), we discovered several TF-binding motifs that are overrepresented in the promoter regions of Rph1-repressed genes (Table 1). The putative Rph1 binding motif, 5′-CCCCTWA-3′ (W = T or A), was proposed by experimental study (Jang *et al.*, 1999) and bioinformatics studies (Badis *et al.*, 2008; Zhu *et al.*, 2009). Of interest, the core binding motif shared by the TFs in Table 1 is 5′-CCCCT-3′, as the STRE, which is essential for Msn2/4 to activate transcription of ESR genes (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996). In addition to Msn2/4, we found that transcriptional factors such as YER130C, Usv1, and Rgm1 (Table 1) might also be involved in regulating the expression of stress-related genes (Estruch, 1991; Hlynialuk *et al.*, 2008;

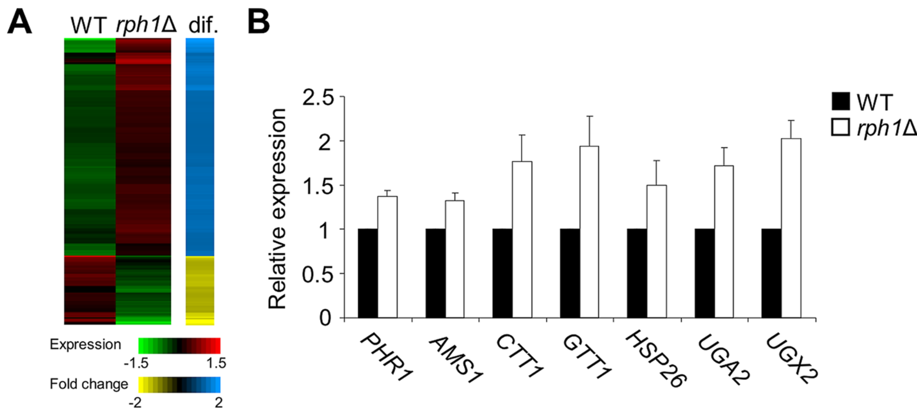

FIGURE 1: Microarray analysis reveals that Rph1 mainly functions as a transcriptional repressor under physiological conditions. (A) Heat map of the expression patterns of Rph1-regulated genes from an average of three biological repeats. The difference (dif.) in fold change between WT and *rph1*Δ strain is indicated by the color bar on the right. The color scales indicate the log 2 ratio of relative expression (top) and fold change (bottom) in transcript levels. (B) Expression of selected Rph1 targets verified by RT-qPCR. The relative expression of selected genes was normalized to that of *ACT1* in the WT and *rph1*Δ. Data are mean ± SD from three biological repeats.

Ramsdale *et al.*, 2008). Further, we used Yeast Search for Transcriptional Regulators and Consensus Tracking (www.yeastract.com; Teixeira *et al.*, 2006) to identify the potential transcription regulatory associations of Rph1. More than 65% of the promoters of Rph1-repressed genes contained a recognition motif shared by Gis1, Rph1, and Msn2/4 (Supplemental Table S2, $p < 0.0001$). These bioinformatics analyses suggest that Rph1 might negatively regulate the transcription of ESR genes by directly binding to promoters harboring the STRE. To investigate whether the genes repressed by Rph1 are induced by stresses such as the DNA-damage response and ESR, we compared the gene expression profiles in *rph1*Δ with those by different stress treatments (Gasch *et al.*, 2000, 2001; Wade *et al.*, 2009). Remarkably, the expression of 29.7, 24.6, and 28.5% of Rph1-repressed genes overlapped with that of genes responsive to ultraviolet (UV) irradiation, methyl methanesulfonate (MMS) treatment, and ionizing radiation, respectively (Table 2, $p < 0.0001$). Moreover, a significant proportion of Rph1-repressed genes were also involved in stress-induced ESR (Table 2, 25.9%, $p < 0.0001$). Thus a substantial number of Rph1-repressed genes are involved in genotoxic stress and ESR.

To examine the relationship between Rph1-mediated transcriptional repression and stress response and verify the results from expression microarray, we selected six stress-induced ESR genes (*AMS1*, *CTT1*, *GTT1*, *HSP26*, *UGA2*, and *UGX2*) that were among those repressed by Rph1 for further analysis using reverse transcription quantitative PCR (RT-qPCR) assay. *AMS1* encodes a vacuolar mannosidase that is involved in degradation of free oligosaccharide and is required for resistance to acidity stress (Chantret *et al.*, 2003; Lawrence *et al.*, 2004). *GTT1* encodes an endoplasmic reticulum–associated glutathione *S*-transferase (glutathione transferase 1) that is functional in maintaining cellular redox potential (Collinson and Grant, 2003). *UGA2* is involved in nonprotein amino acid γ-aminobutyric acid and oxidative stress tolerance (Coleman *et al.*, 2001). *UGX2* is accumulated under various stress conditions (Wu *et al.*, 2004). *HSP26* encodes a small heat shock protein with chaperone activity (Bossier *et al.*, 1989; Amoros and Estruch, 2001), and *CTT1* encodes a cytosolic catalase involved in the detoxification of H_2O_2 (Jamieson, 1998). *CTT1* and *HSP26* are well-known targets of the master stress-activated regulators Msn2/4 (Schmitt and McEntee, 1996). In agreement with our microarray data, the mRNA expression of all the selected ESR genes and *PHR1* was increased in *rph1*Δ (Figure 1B). We conclude that Rph1 acts to repress stress-associated genes.

Rph1-repressed genes are induced by DNA damage in a Rad53-dependent manner

We next determined whether the expression of selected ESR genes repressed by Rph1 was affected by DNA-damage signaling elicited by UV irradiation or MMS treatment. The expression of *PHR1* and *HUG1* served as the control for intact Rad53 signaling (Basrai *et al.*, 1999; Liang *et al.*, 2011), with the repression of the

Transcription factor	*p* value	Binding motif (JASPAR)
GIS1	3.39E-17	
RPH1	5.24E-12	
YER130C	8.53E-08	
USV1	1.24E-06	
RGM1	3.04E-05	
MSN4	1.05E-04	
TBF1	7.55E-04	

Genes up-regulated in *rph1*Δ were analyzed by Pscan (www.beaconlab.it/pscan; Zambelli *et al.*, 2009). Overrepresented TFs are ranked by *z*-test *p*-value.

TABLE 1: Transcription factors and their binding motifs overrepresented in the promoter regions of genes repressed by Rph1.

Stress	% of genes
UV[a]	29.7*
MMS[b]	24.6*
Ionizing radiation[b]	28.5*
ESR[c]	25.9*

[a]Gene list from Wade *et al.* (2009).
[b]Gene lists from Gasch *et al.* (2001).
[c]Gene list from Gasch *et al.* (2000).
*$p < 0.0001$ by Fisher's exact test.

TABLE 2: Percentage of Rph1-repressed genes with expression overlapping that of genes responding to DNA-damage treatment or induced by environmental stress response.

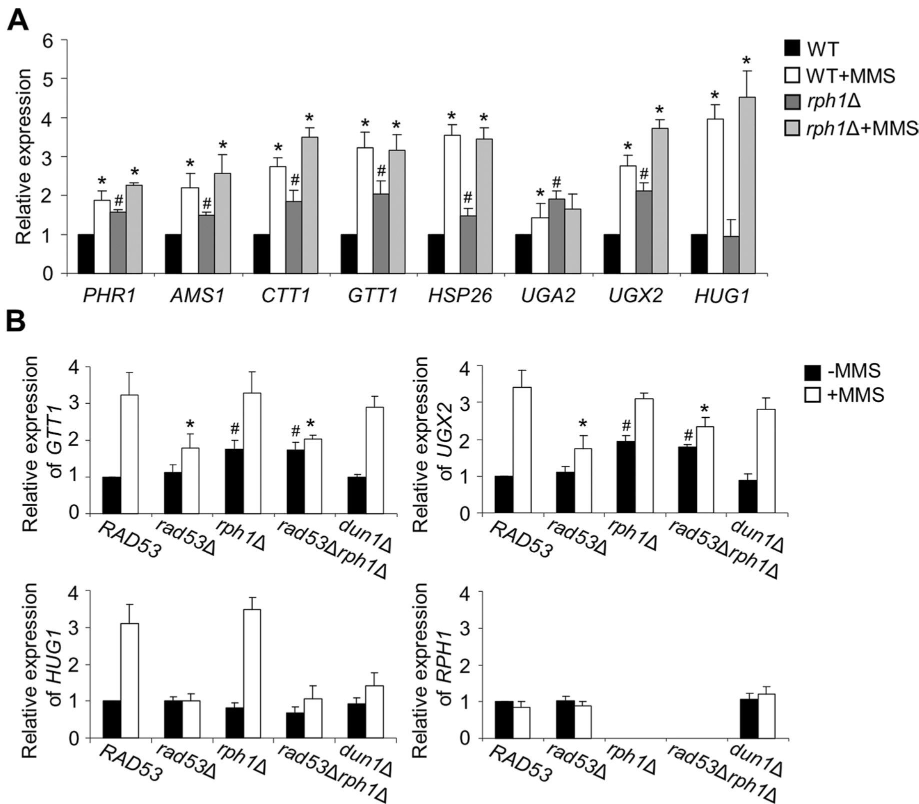

FIGURE 2: Rph1-repressed genes are induced by DNA damage in a Rad53-dependent manner. (A) RT-qPCR analysis of WT and *rph1*Δ with (+) and without (–) MMS (0.1%). (B) Rad53 is required for inducing *GTT1* and *UGX2* expression in response to MMS (0.1%). RT-qPCR analysis of the mRNA levels of *GTT1*, *UGX2*, *HUG1*, and *RPH1* in the wild type (*RAD53*) and *rad53*Δ, *rph1*Δ, *rad53*Δ*rph1*Δ, and *dun1*Δ with (+) or without (–) MMS. Data are mean ± SD from three biological repeats. $^{*,\#}p < 0.05$ as compared with WT by analysis of variance.

former dependent and that of the latter independent of Rph1 expression (Figure 2A). The expression of all genes except *HUG1* was elevated in *rph1*Δ as compared with the WT, and MMS treatment further enhanced the expression of genes in the WT and in *rph1*Δ yeast (Figure 2A, $p < 0.05$). Similar findings occurred with UV irradiation (Supplemental Figure S1). These results indicate that the selected ESR genes repressed by Rph1 also respond to DNA damage.

We next determined whether the checkpoint protein kinase Rad53 was involved in the derepression of gene expression through Rph1, as it acts on *PHR1* (Jang *et al.*, 1999; Liang *et al.*, 2011). As a control, the MMS induction of *HUG1* was decreased in both *rad53*Δ and *dun1*Δ strains (Figure 2B, bottom left). As compared with wild type (*RAD53*), the *rad53Δrph1Δ* and *rph1Δ* strains showed an increased basal level (–MMS), and the *rad53Δrph1Δ* and *rad53Δ* strains had reduced induction (+MMS) of Rph1-repressed genes (*GTT1* and *UGX2*) in response to MMS (Figure 2B, $p < 0.05$). These results suggest that Rad53 is dispensable for Rph1-mediated repression but is required for efficient induction of *GTT1* and *UGX2* upon DNA damage. Of interest, the transcript level of *RPH1* was similar in *RAD53*, *rad53*Δ, and *dun1*Δ with or without MMS treatment (Figure 2B, bottom right). Therefore the functional Mec1–Rad53 cascade is likely involved in relieving the Rph1-dependent transcriptional repression with selected ESR genes responding to genotoxic stress. Rad53 does not affect the transcript level of *RPH1*, however, so Rad53 may regulate Rph1 function independent of controlling *RPH1* transcription.

The checkpoint kinase Rad53 negatively regulates Rph1 protein

To determine whether the protein level and function of Rph1 are regulated under physiological and stress conditions, we generated a yeast strain carrying a Myc-tagged Rph1 driven by its own promoter. Rph1 is phosphorylated in response to DNA damage (Kim *et al.*, 2002; Liang *et al.*, 2011). Phosphatase treatment diminishes the band shift of Myc-tagged Rph1, which confirms the change in protein mobility by protein phosphorylation (Figure 3A). Of interest, we found that Rph1 was phosphorylated and its protein level decreased with MMS treatment (Figure 3, A and B). Rad53 kinase plays a pivotal role in the phosphorylation and dissociation of Rph1 from chromatin with DNA damage (Kim *et al.*, 2002; Liang *et al.*, 2011). Therefore we examined whether Rad53 regulates the protein level of Rph1. We first measured the Rph1 protein level in WT and *rad53*Δ cells. In the presence of *RAD53*, Rph1 was phosphorylated and protein levels decreased to 74 and 57% in response to MMS and UV irradiation, respectively (Figure 3B). Under physiological conditions, Rph1 protein level was 1.7-fold increased in *rad53*Δ as compared with the WT (Figure 3B). In addition, the phosphorylation of Rph1 was abolished in *rad53*Δ, which confirmed the role of Rad53 kinase in mediating Rph1 phosphorylation with DNA damage. Nevertheless, Rph1 levels in *rad53*Δ were decreased 32 and 49% in response to MMS and UV irradiation, respectively (Figure 3B), which implies that additional factors may be involved in the regulation of Rph1 protein responding to DNA damage. To further determine whether Rad53 regulates Rph1 protein stability, we measured the half-life of Rph1 in WT and *rad53*Δ by cycloheximide (CHX) treatment (Belle *et al.*, 2006). Rph1 is a labile protein, with a half-life of ~35 min (Figure 3C), and the basal level of Rph1 is higher in *rad53*Δ than the WT (Figure 3D). The half-life of Rph1 increases from ~34 min in the WT to 43 min in *rad53*Δ under physiological conditions, so Rad53 contributes modestly to regulate Rph1 degradation in the absence of DNA damage (Figure 3D). In addition, the slopes of Rph1 protein degradation kinetics are similar in *rad53*Δ and WT (Figure 3D, $p > 0.05$). These data suggest that Rad53 is involved in regulating Rph1 phosphorylation on DNA damage and the steady-state protein level of Rph1 in unstressed cells but has only a negligible effect, if any, on Rph1 protein degradation kinetics.

JmjN and ZF domains of Rph1 are required for its function

Rph1 contains several functional domains, including the JmjN, JmjC (catalytic domain), and two ZF (DNA-binding) domains at its C terminus. To determine whether these defined domains in Rph1 contribute to specific biological functions, we generated the following *rph1* mutants for further analyses: deletion of the JmjN domain (*JN*Δ), deletion of ZF domains (*ZF*Δ), and an alanine substitution at H235 (H235A) in the JmjC domain for disrupting Rph1 demethylase activity (Liang *et al.*, 2011). The native *RPH1* promoter was used to drive the expression of the WT and mutated *rph1* tagged with a

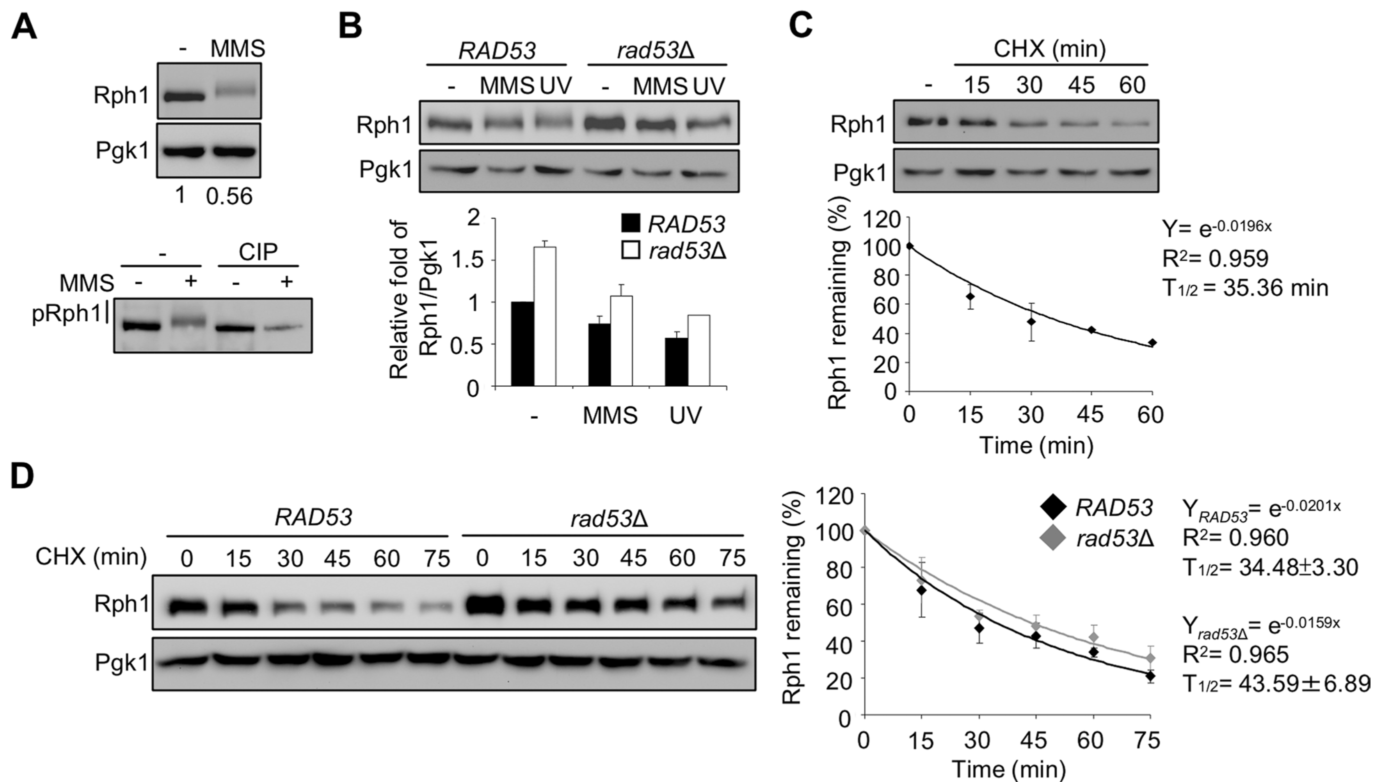

FIGURE 3: Rad53 negatively regulates Rph1 protein level. (A) The protein level and phosphorylation of Rph1 are modulated by DNA damage. Rph1 protein shows a band shift and reduced level after 30 min of MMS (0.1%) treatment (top). Calf intestine phosphatase (CIP) abolishes the retarded Rph1 (bottom). Rph1 immunoprecipitated from cells with (+) or without (–) MMS treatment was incubated with or without CIP. The band-shifted Rph1 is indicated as phosphorylated Rph1 (pRph1). (B) Immunoblotting of Rph1 protein levels in *RAD53* and *rad53*Δ strains in response to DNA damage by MMS (0.1%) and UV irradiation (25 mJ/cm^2). The bar graph represents the relative Rph1 levels with SD from three biological repeats. (C) The half-life of Rph1 was analyzed by CHX treatment (5 μg/ml). Bottom, quantification of Rph1 decay kinetics. (D) The kinetics of Rph1 degradation in *RAD53* and *rad53*Δ strains with CHX treatment. Right, quantification ($p = 0.11$, by Student's *t* test). Data are mean ± SD from three biological repeats; Pgk1 was the internal control, and the protein level of untreated samples (–) in C and CHX = 0 min in D was set to 100%.

c-Myc epitope in episomal plasmids in the *rph1Δ* strain. The transcript levels of WT *RPH1* and mutants did not differ (Supplemental Figure S2). The protein levels of *rph1*-H235A and *rph1-ZF*Δ were comparable to that of WT Rph1 with CHX treatment, so demethylase activity and DNA-binding ability are not involved in regulating Rph1 protein stability; of note, deletion of the JmjN domain reduced the steady-state protein level and stability of Rph1 (Figure 4A). Our result is consistent with previous findings that the JmjN domain is crucial for catalytic activity, substrate specificity, and protein folding for members of the KDM5 (H3K4 demethylase) and KDM4 (H3K9 and H3K36 demethylases) families (Chen *et al.*, 2006; Klose *et al.*, 2007; Huang *et al.*, 2010; Chang *et al.*, 2011).

We next evaluated the transcriptional repressive effect of *rph1* mutants on the expression of selected target genes. The transcriptional repression of examined genes by Rph1 required the JmjN and ZF domains (Figure 4, B and C, *JN*Δ and *ZF*Δ). Surprisingly, demethylase activity, which is disrupted in *rph1*-H235A, was required to suppress the transcription of *PHR1* (Figure 4B) but not *GTT1*, *UGX2*, *CTT1*, and *HSP26* (Figure 4C). Our results suggest that both demethylase activity–dependent and activity–independent regulatory mechanisms are present for Rph1 functions. Demethylase-independent regulation in transcription has been reported in other JmjC-domain–containing proteins, such as lysine(K) demethylase 4A (H3K36 and H3K9 demethylases) involved in *Drosophila* embryogenesis, JMJD3 (H3K27 demethylase) in mediating T-box TF-dependent gene expression, and UTX/KDM6 (H3K27 demethylase) in regulating development in worm (Miller *et al.*, 2010; Vandamme *et al.*, 2012; Crona *et al.*, 2013)

Rph1 binds to gene promoters and is dissociated with DNA damage

The ZF domains of Rph1 are required for DNA binding and thus may be responsible for recognizing the STRE. An STRE is present in the upstream repression sequence at the *PHR1* promoter, which was demonstrated to be a direct target of Rph1 (Jang *et al.*, 1999; Liang *et al.*, 2011). In this study, we found 32 of 158 Rph1-repressed genes containing at least one Rph1-binding motif at the promoter region (Supplemental Table S3). A study of the genomic binding map of chromatin regulators surveyed the regulator-enriched regions, including the upstream activation sequence (UAS), transcription start site (TSS), and open reading frame (ORF), by use of chromatin immunoprecipitation (ChIP)-on-chip technology (Venters *et al.*, 2011). We compared our microarray data with that from Venters *et al.* (2011) and found that 63 of 158 Rph1-repressed genes contained Rph1-enriched regions in the UAS, TSS, or ORF by ChIP-on-chip assay (Supplemental Table S4, $p < 0.0001$). Of note, the Rph1-ChIP signals in the 63 Rph1-repressed genes were enriched in the promoter-proximal region (UAS and/or TSS), so Rph1 may directly bind

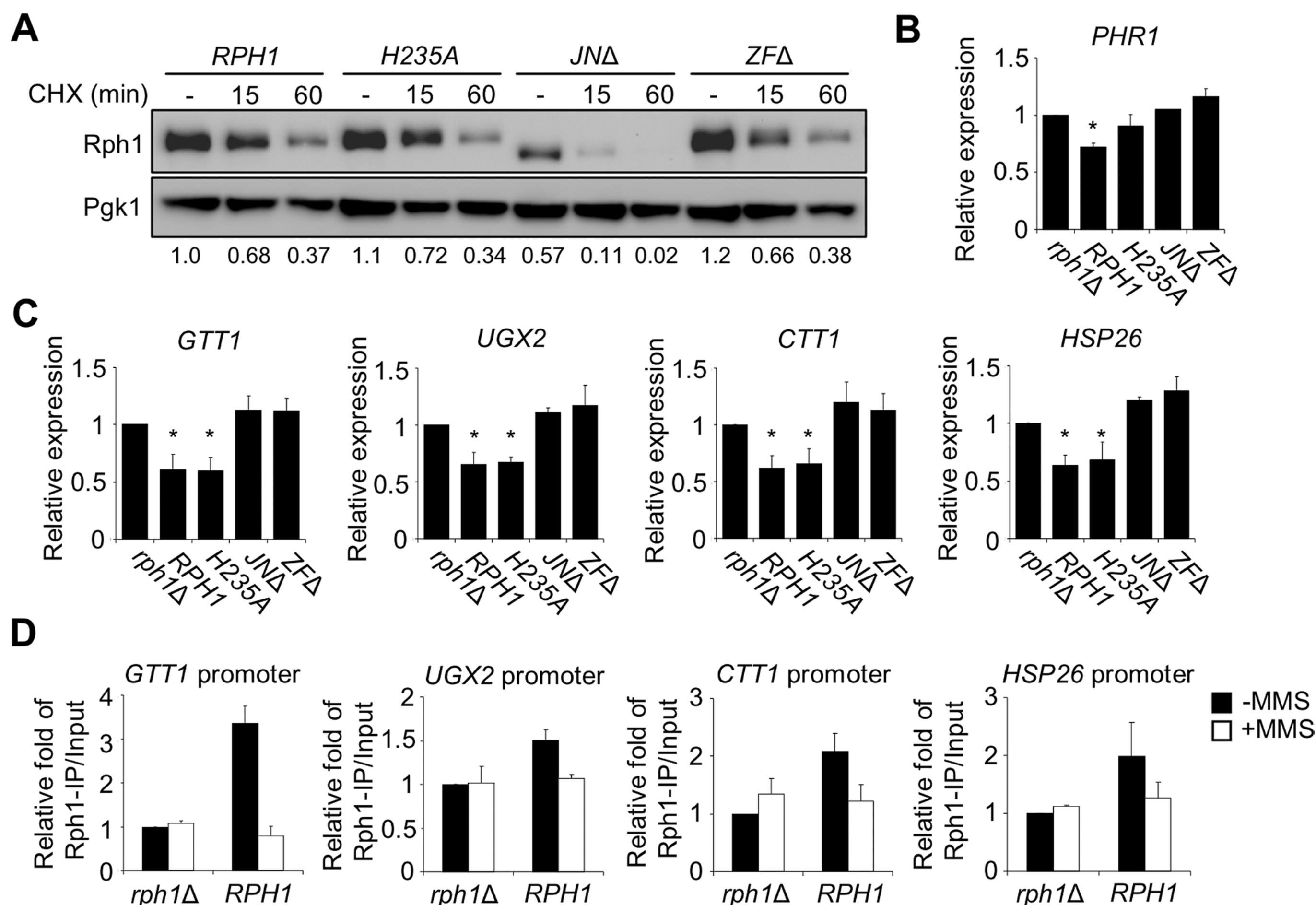

FIGURE 4: Mutations in the conserved domains of Rph1 affect its protein level and transcriptional repression. (A) Western blot analysis of the protein level of Rph1 in the WT (*RPH1*) and mutant strains (*H235A*, JmjN deletion [*JN*Δ] and ZF deletion [*ZF*Δ]) in response to CHX treatment (5 µg/ml). Pgk1 is the loading control. (B) RT-qPCR analysis of mRNA expression of *PHR1* and (C) *GTT1*, *UGX2*, *CTT1*, and *HSP26* in the WT and *rph1* mutants was performed with *rph1*Δ strains harboring an empty vector (*rph1*Δ), *RPH1*, *H235A*, JmjN deletion (*JN*Δ), or ZF deletion (*ZF*Δ). $*p < 0.05$ by Student's *t* test. (D) Quantified ChIP assay of Rph1 binding on the promoters of genes with (+) and without (–) MMS (0.1%) in WT (*RPH1*) and *rph1*Δ. Data are mean ± SD from three biological repeats in B–D.

to promoters through chromatin-binding ability. To verify this possibility, we examined the direct association of Rph1 with the promoter regions of *GTT1*, *UGX2*, *CTT1*, and *HSP26*, which all contain the Rph1-recognition motif and STRE (Supplemental Figure S3), by ChIP assay. Indeed, in the absence of MMS, Rph1 was enriched at the promoters of these genes (Figure 4D). With DNA damage induced by MMS, Rph1 was dissociated from chromatin, which resulted in derepression of target genes (Figures 2A and 4C). These findings support the role of Rph1 as a repressor to restrict chromatin accessibility for activators and transcriptional machinery. Chromatin recruitment to specific binding sequences may contribute significantly to the transcriptional repressive function of Rph1.

Rph1 is phosphorylated under oxidative stress, which leads to Rph1 dissociation and transcriptional activation

We showed that Rad53 is involved in regulation of Rph1 phosphorylation on DNA damage (Figure 3, A and B). We asked whether additional kinases are involved in ESR-regulated Rph1 phosphorylation and function. Rph1 was phosphorylated in the presence of H_2O_2 and MMS (Figure 5A), so both oxidative stress and DNA damage trigger Rph1 phosphorylation. Genome-wide proteomic studies revealed that stress-signaling pathways might coordinately control Rph1 phosphorylation; these include the Hog1–Hsl1 pathway (Brewster *et al.*, 1993; De Nadal *et al.*, 2004; Soufi *et al.*, 2009) and the protein kinase A–Rim15 pathway (Bodenmiller *et al.*, 2008; Huber *et al.*, 2009). In addition, elevated reactive oxygen species level induced by oxidative stress can initiate several signal cascades in yeast, including mitogen-activated protein kinase–Hog1, AMP-activated protein kinase–Snf1, phosphatidylinositol 3-kinase–TOR, and ataxia-telangiectasia mutated/ATM and Rad3-related pathways (Hamanaka and Chandel, 2010; Ray *et al.*, 2012). These proteomics data suggest that Rph1 phosphorylation induced by oxidative stress may be mediated by the aforementioned pathways alone or in combination. To test this possibility, we analyzed Rph1 phosphorylation in several oxidative stress–activated kinase mutants, including *rim15*Δ, *hog1*Δ, *hsl1*Δ, and *rad53*Δ. Of interest, Rph1 phosphorylation under oxidative stress was diminished in *rad53*Δ but not *rim15*Δ, *hog1*Δ, or *hsl1*Δ (Figure 5B). Rad53 may be the key kinase essential for Rph1 phosphorylation on oxidative stress with H_2O_2 treatment, which most likely evokes a DNA-damage signaling pathway to trigger the derepression of stress response genes.

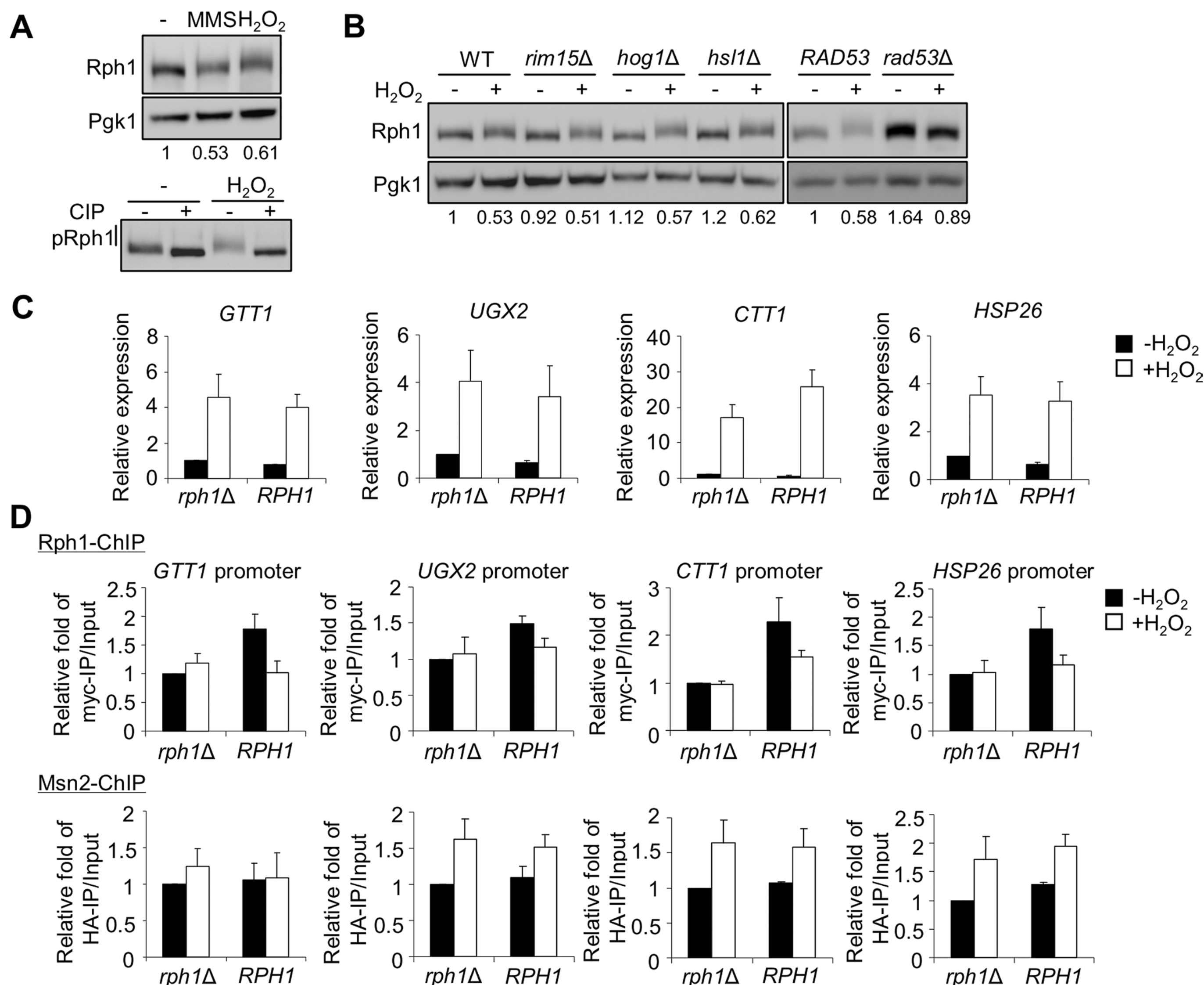

FIGURE 5: Rph1 is phosphorylated and dissociated from the gene promoters under oxidative stress. (A) Phosphorylation of Rph1 is induced by oxidative stress. Immunoblotting analysis of the protein level of Rph1 with (+) and without (–) MMS (0.1%) or H_2O_2 (0.5 mM; top). The band-shifted Rph1 is abrogated in the presence of calf intestine phosphatase (bottom). The retarded Rph1 protein is indicated as phosphorylated Rph1 (pRph1). (B) Phosphorylation of Rph1 was examined in various kinase mutants with (+) and without (–) H_2O_2 (0.5 mM). (C) RT-qPCR analysis of mRNA expression of *GTT1*, *UGX2*, *CTT1*, and *HSP26* with (+) and without (–) H_2O_2 (0.5 mM) in WT (*RPH1*) and *rph1*Δ. (D) ChIP assays show the chromatin binding of Rph1 and Msn2 at the promoters of genes with (+) and without (–) H_2O_2 (0.5 mM). A yeast strain coexpressing Rph1-Myc and Msn2-HA fusion proteins was used for ChIP assays. Data are mean ± SD from three biological repeats in C and D.

The core motif shared by Rph1 and Msn2/4 is enriched at the promoters of Rph1-repressed genes (Table 1). Furthermore, the expression of Rph1-repressed genes significantly overlaps that of genes induced by ESR (25.9%, Table 2, $p < 0.0001$). Given the expression profile and chromatin association results, Rph1 appears to occupy the promoters of stress-responsive genes under physiological conditions. To verify the interplay between Msn2 and Rph1 in mediating stress-induced ESR gene expression, we examined the transcript levels and chromatin dynamics of genes coregulated by Rph1 and Msn2, using RT-qPCR and ChIP assays under oxidative stress. We generated a yeast strain coexpressing Rph1-Myc and Msn2-hemagglutinin (HA) fusion proteins under the control of native promoters to examine chromatin associations responding to stresses. We first analyzed the data from a recent genome-wide mapping of Msn2 direct targets under oxidative stress (Huebert *et al.*, 2012) and found that in response to stress, Msn2 occupies the promoter regions of 39 of the 158 Rph1-repressed genes (Supplemental Table S5, $p < 0.0001$). The expression of *GTT1*, *UGX2*, *CTT1*, and *HSP26* was highly induced by H_2O_2 treatment (Figure 5C). Consistently, we detected the dissociation of Rph1 from the promoters of these genes by ChIP assay under oxidative stress (Figure 5D, top). In contrast, Msn2 was associated with the promoters of *UGX2*, *CTT1*, and *HSP26* but not *GTT1* with H_2O_2 treatment (Figure 5D, bottom). Msn2 does not bind directly to the promoter of *GTT1* but is required for its induction under oxidative stress (Huebert *et al.*, 2012; Supplemental Table S5). Results in Figure 5, C and D, indicate that Rph1 and Msn2 bind to the same promoter regions and Rph1 represses gene expression under physiological conditions. In

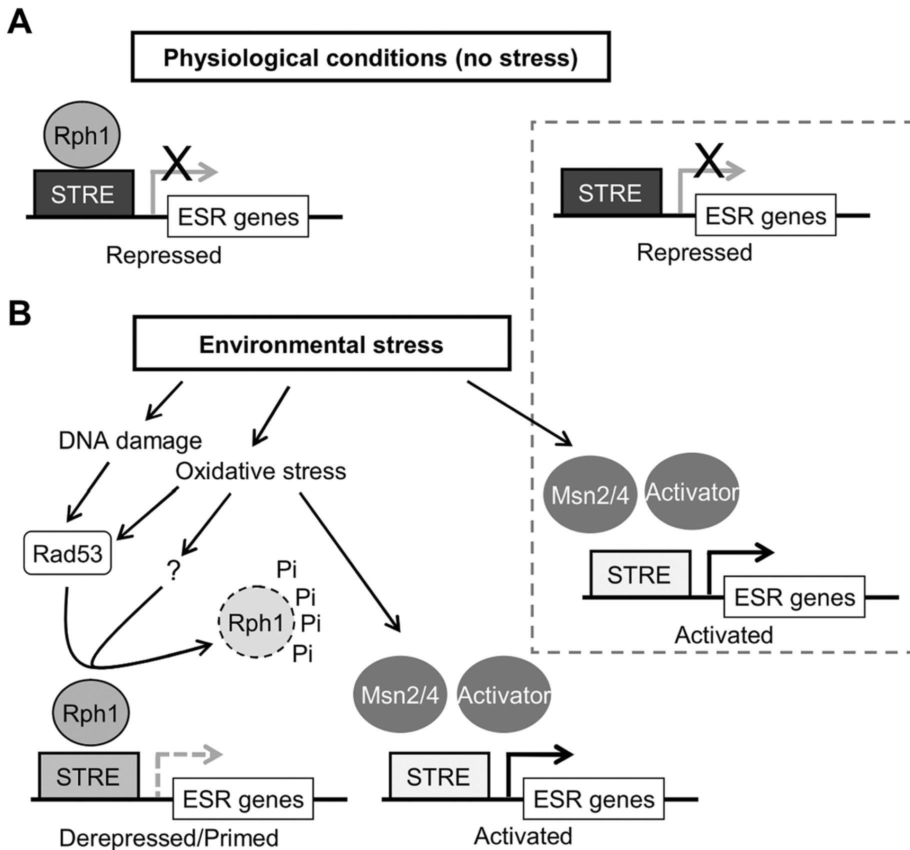

FIGURE 6: A hypothetical model of Rph1-mediated transcriptional regulation of ESR genes. (A) Under physiological conditions, Rph1 recognizes the 5′-CCCCTWA-3′ motif resembling the STRE and binds to the promoter of target genes to repress their transcription. (B) In response to stresses such as DNA damage and oxidative stress, derepression of Rph1 target genes is achieved by Rph1 dissociating from chromatin, which may require phosphorylation (Pi) and/or reduced Rph1 level (light gray with dashed line). The checkpoint kinase Rad53 is required for efficient induction of Rph1-repressed genes and Rph1 phosphorylation in response to DNA damage and oxidative stress. Dissociation of Rph1 from promoter regions represents a derepressed state for priming the stress-induced ESR genes for a full spectrum of activation by transcriptional activators such as Msn2/4. Alternatively, Rph1-independent repression of induced ESR genes is shown on the right (dashed line), which also requires Msn2/4 for most of the gene activation. Additional activators may cooperate with Msn2/4 to induce ESR genes.

response to stress, Rph1 is dissociated from the promoters and Msn2 is associated with them to induce gene expression.

Our present data demonstrate that the repressor Rph1 and activator Msn2 cooperate to control transcription of a subset of induced ESR genes. We propose a functional role of Rph1 as an integral component in the signaling and regulatory network to coordinate with stress-induced activators and regulate ESR gene expression. Figure 6 presents a mechanistic theme that environmental stress, such as DNA damage and oxidative stress, modulates Rph1 protein phosphorylation and relieves Rph1-mediated transcriptional repression by its dissociation from the promoters of target genes. The dissociation of Rph1 may allow for access to promoters by transcriptional activators such as Msn2/4 for subsequent gene activation.

DISCUSSION

Chromatin association at gene promoters may account for the most crucial function of Rph1-regulated transcriptional repression

The on/off switch of gene expression can be simply mediated by repressors to compete with shared DNA-binding sequences and interfere with the interaction of activators with general transcription machinery. We found that the promoters of a significant number of genes regulated by Rph1 and Msn2/4 contain the core sequence of STRE, which mediates the transcriptional activation of ESR genes in yeast. Msn2/4 likely plays a positive role, whereas Rph1 primarily functions as a repressor in gene expression, possibly by competitive binding or merely occupancy at the STRE of coregulated genes. Rph1 binds to specific promoter regions to limit the accessibility of chromatin to activators or transcription machinery in the repressive state. In the activated state, dissociation of Rph1 from chromatin is regulated by stress signals, which consequently allows for access to promoters by activators to optimize induced gene expression. Our results indicate that Rph1 and Msn2 bind to the same promoter regions of *UGX2*, *CTT1*, and *HSP26*, and oxidative stress promotes dissociation of Rph1 and association of Msn2 to activate gene expression (Figure 5, C and D). The expression of Rph1 and Msn2 is controlled by their native promoters; thus the association and dissociation with chromatin in response to oxidative stress would reflect a physiological interplay between the repressor and activator. However, whether Rph1 and Msn2 would compete with the same promoter regions when both are present simultaneously in the nucleus is unclear. Although the binding sequence of Rph1 highly resembles STRE, many Msn2/4-induced genes are unlikely Rph1 targets. Additional factors such as flanking DNA sequences and interacting proteins might contribute to the specificity of promoter association of Rph1 and Msn2/4.

In addition to restricting promoter accessibility, the repressors can also recruit chromatin-remodeling complexes, corepressors, or histone deacetylases, such as Rpd3, to establish a repressed state of the promoter regions (Gaston and Jayaraman, 2003). The histone deacetylase Rpd3L complex can be recruited by transcriptional repressors to reestablish the nucleosomal state of promoters to their pretranscriptional forms, whereas H3K36 methylation at the coding regions acts to recruit the Rpd3S complex to prevent cryptic transcription (Li *et al.*, 2007; Wagner and Carpenter, 2012). Intriguingly, Rpd3 is an important cofactor in regulating subsets of both induced and repressed ESR genes (Alejandro-Osorio *et al.*, 2009). The effects of Rph1 and Rpd3 on gene expression are highly correlated under the physiological condition ($R^2 = 0.51$; Weiner *et al.*, 2012). Coincidently, we found the expression of 58 of 158 Rph1-repressed genes was also repressed by Rpd3 (Supplemental Figure S4A), which suggests a potentially cooperative relationship between Rph1 and Rpd3 on histone modifications (methylation and deacetylation) and actions at some of the promoters of induced ESR genes. This notion is supported by our previous observation that Rph1 repressed *PHR1* expression through histone deacetylation by recruiting Rpd3 to the promoter region (Liang *et al.*, 2011), but here we did not find the histone

demethylase activity of Rph1 required for suppression of *GTT1*, *UGX2*, *CTT1*, and *HSP26* (Figure 4C).

Because of low H3K36 methylation level at the promoters under physiological conditions, we have little information regarding the relationship between H3K36 methylation and Rpd3L recruitment at the promoter regions. Of interest, ~1150 genes showed increased H3K36me3 level at the promoter regions with diamide stress (Weiner *et al*., 2012). We found that 26.6% of Rph1-repressed genes showed an increased H3K36me3 level at their promoters with diamide stress (Supplemental Figure S4B, 42 of 158, $p < 0.0001$). The low level of H3K36me3 at promoters might be due to an Rph1 association restricting accessibility of the histone methyltransferase, Set2, for active methylation or mediating demethylation at promoter regions. The recruitment of distinct Rpd3 complexes to the coding sequences (Rpd3S, high H3K36 methylation) and the promoter regions (Rpd3L, low H3K36 methylation) is likely mediated by different mechanisms. Thus the histone demethylase activity of Rph1 does not seem to be required for recruiting Rpd3 to the promoters of induced ESR genes. Most likely, Rph1 functions as a repressor for a number of induced ESR genes, by which chromatin association, instead of histone demethylase activity, of Rph1 is important for recruiting corepressors, such as Rpd3L, for transcriptional repression at the promoters.

Control of Rph1 at the protein level provides new insights into the regulation of histone demethylases

Overexpression of Rph1 leads to extremely slow growth, and *rph1*Δ is more tolerant to high-dose UV irradiation than is the WT (Liang *et al*., 2011). The protein level of Rph1 might be tightly controlled to avoid a deleterious effect on cell growth under physiological conditions and enhance cell viability under stress. The protein levels of many important transcription factors, such as p53, c-Myc, and c-Jun, are regulated by protein phosphorylation and/or proteasomal degradation crucial to maintaining cellular homeostasis (Geng *et al*., 2012). Several JmjC-containing histone demethylases are degraded by the proteasome system (Shi *et al*., 2005; Mersman *et al*., 2009; Quan *et al*., 2011; Tan *et al*., 2011; Van Rechem *et al*., 2011). Here we find that Rph1 is an unstable protein with a short half-life, and the JmjN domain is essential to maintain Rph1 stability. Protein stability may explain a critical regulatory mechanism modulating the function of members in the JmjC family. We showed that Rad53 regulates the steady-state Rph1 level but is not essential for Rph1 degradation kinetics (Figure 3). Thus, reduced Rph1 level in response to genotoxic stress (Figures 3B and 5A) might result from factors other than protein degradation. Because the transcript level of *RPH1* remained unchanged with MMS treatment, reduced protein translation of Rph1 or enhanced degradation of phosphorylated Rph1 or both may contribute to the decreased Rph1 level responding to stress. In budding yeast, the RNA-binding protein Khd1 binds to *ASH1* mRNA to hinder the recruitment of 40S ribosome and subsequently represses the initiation of translation (Paquin *et al*., 2007). Of interest, *RPH1* mRNA was identified as a target of Khd1 (Hasegawa *et al*., 2008), so the Rph1 level might also be modulated at the step of protein biogenesis.

Relief of Rph1-mediated repression might provide a cross-protection mechanism against multiple stresses

A cross-protection mechanism has been proposed to increase cell tolerance to a broad spectrum of stresses when the initial ESR is activated by individual stresses (Gasch *et al*., 2000; Berry and Gasch, 2008; Berry *et al*., 2011). Microarray analysis revealed that a considerable number of Rph1-regulated genes are involved in genotoxic stress and ESR (Table 2), which can be induced by diverse stresses, including DNA damage, oxidative stress, nutrient starvation, and heat shock. Damaged cellular components or misfolded proteins resulting from stresses are eliminated by degradation through proteasome and autophagic processes (Hochstrasser, 1996; Mizushima *et al*., 2011) or rescued by molecular chaperones (Haslbeck *et al*., 1999). We found that genes regulated by Rph1 included ESR genes, such as *UBC8*, *UIP4*, *DOA4*, *ATG7*, *14*, *16*, *17*, *23*, *34*, and *HSP26*, involved in the aforementioned cellular processes. Because Rph1 represses several dozen ESR genes under physiological conditions, it may serve as a node to regulate ESR when cells encounter stress signals and prepare the cells for the same or different stresses. Derepression of Rph1 targets may play a role in priming the gene expression for further activation induced by subsequent stress signals to achieve a full spectrum of stress responses. Therefore regulation of Rph1 function may be a cross-protection mechanism for cells to adapt to different stress conditions and survive in ever-changing environments.

MATERIALS AND METHODS

Plasmids and yeast culture

To express Rph1 fused with a 13Myc tag in an episomal plasmid under its own promoter, we constructed the following destination vectors using the Gateway recombination system (Invitrogen, Carlsbad, CA). The 800–base pair *RPH1* promoter was amplified by PCR and subcloned into pRS313-DEST-13Myc and pRS416-DEST-13Myc plasmids with *Sac*I/*Spe*1 sites. The donor clone of *RPH1* cDNA was generated as described (Liang *et al*., 2011). The mutants of Rph1, including JmjN-, H235A-, and ZF-deletion mutants, were created by site-directed mutagenesis and verified by sequencing. The yeast strains used in this study are indicated in Supplemental Table S6. All yeast manipulations followed standard methods (Sikorski and Hieter, 1989).

Microarray expression experiment and data analysis

Three biological replicates of total RNA from wild-type and *rph1*Δ strains were prepared for Affymetrix GeneChip (Yeast 2.0) microarray assays (Affymetrix, Santa Clara, CA) by the Affymetrix Gene Expression Service Lab, Academia Sinica (Taipei, Taiwan). The expression data were analyzed by use of GeneSpring GX11 (Agilent Technologies, Santa Clara, CA). Briefly, the MAS5 algorithm was used for per-chip normalization. The baseline of per-gene was transformed to the median for all samples. Genes filtered by flags (present or marginal) were selected. Only genes with FDR <0.05 were selected for fold-change analysis. Hierarchical clustering by the Cluster program (Eisen *et al*., 1998) was used to group Rph1-regulated genes by their expression profile. The microarray data were deposited in the Gene Expression Omnibus database (GSE39950).

Reverse transcription and quantitative PCR

Total RNA was extracted from yeast cells by use of a Total RNA Mini Kit (Geneaid, New Taipei City, Taiwan). RNA (1 µg) was treated with DNase I (Promega, Madison, WI), then underwent reverse transcription with use of high-performance Moloney murine leukemia virus (Epicentre Biotechnologies, Madison, WI) and an oligo-dT primer (Protech, Taipei, Taiwan). cDNA prepared from triplicate biological samples was used for PCR analysis. qPCR was performed as described (Yuen *et al*., 2002) and involved use of an Agilent MX3000P thermocycler with fluorescence detection of SYBR green (Kapa Biosystems, Woburn, MA). All primers are listed in Supplemental Table S7. Appropriate nontemplate controls were included in each

PCR, and DNA melting curves were analyzed at the end of each run to confirm the specificity of the reaction.

Stress treatments

Yeast cultures were grown in yeast extract/peptone/dextrose or synthetic-complete selection medium at 30°C until A_{600} = 0.6–0.8. For UV irradiation, cells were spread on plates and then underwent UV cross-linking treatment (Stratagene, Santa Clara, CA) for 25 mJ/cm^2. Cells were recovered for 30 min before being harvested. MMS is a DNA-alkylating agent that causes double-stranded DNA breaks. Cells were treated with 0.1% MMS for 30 min to induce the DNA-damage response. To induce oxidative stress, H_2O_2 (0.5 mM) was added to exponential cultures for 30 min, and cells were collected by centrifugation.

Trichloroacetic acid precipitation and calf intestine alkaline phosphatase treatment

Cell pellets were lysed with alkaline solution (NaOH), and total protein was precipitated with 10% trichloroacetic acid (TCA). The acetone-washed protein pellet was resuspended in 2× SDS-sample buffer for SDS–PAGE analysis as described (Knop *et al.*, 1999). Immunoblots were detected with the antibodies anti-Myc (1:5000 dilution; Roche, Indianapolis, IN) and anti-Pgk1 (1:10,000; Invitrogen). Protein intensity was quantified by use of ImageQuant (GE Healthcare Life Sciences, Piscataway, NJ). To preserve the phosphorylated peptides for phosphatase treatment, proteins were precipitated with TCA and dissolved in denaturing buffer (0.5 M Tris base, 6.5% SDS, 100 mM dithiothreitol, 12% glycerol). After heating at 65°C for 20 min, the debris was removed by centrifugation. Myc-tagged Rph1 was immunoprecipitated with anti-Myc antibody from dilution lysate with FA-lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1% sodium deoxycholate) with protease inhibitors (Sigma-Aldrich, St. Louis, MO). Dephosphorylation was catalyzed by incubation of the immunoprecipitate with 10 U of calf intestine phosphatase (New England BioLabs, Ipswich, MA) at 37°C for 30 min.

Measurement of protein half-life

Cells in log phase (A_{600} = 0.6) were treated with 5 µg/ml cycloheximide (CHX) to block protein synthesis and then harvested at the times indicated. Cell lysates were prepared by TCA precipitation and analyzed by immunoblotting. The intensity of individual protein bands was quantified by use of ImageQuant. The protein degradation was assumed to follow first-order decay kinetics and estimated as described (Belle *et al.*, 2006). Relative expression of Rph1 to Pgk1 was calculated and plotted. The protein level without CHX treatment at time zero was set to 100%.

Chromatin immunoprecipitation assay

The ChIP assay was performed as described (Kuras *et al.*, 2000; Lo *et al.*, 2005). For Rph1-Myc and Msn2-HA ChIP, samples were fixed with formaldehyde for 2 h (Robyr *et al.*, 2002). To maintain a proper size of fragmented chromatin, we optimized the sonication parameters to shear DNA to an average size of 200 base pairs, corresponding to one or two nucleosomes. Chromatin solution containing 1 mg of whole-cell extract was immunoprecipitated with anti-Myc antibody or anti-HA antibody (Roche) and purified with protein G–Sepharose (Upstate, Millipore, Billerica, CA). The precipitated DNA was analyzed by real-time qPCR. The signal for each gene primer pair in the immunoprecipitation was normalized to that of the input and then divided by the control vector to determine the fold change. Quantification of data indicated by mean ± SD was based on the number of independent biological and/or experimental replicates described in the figure legends.

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