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Kdx1 regulates *RCK1* gene expression by interacting with Rlm1 in *Saccharomyces cerevisiae* ☆

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ABSTRACT

Kdx1 is known as a stress-responsive protein. To better understand the function of Kdx1, we performed microarray analysis in *KDX1* overexpressing cells and found that the overexpression of *KDX1* dramatically induced the expression of *RCK1*, a stress-responsive gene. This result was confirmed by northern blot analysis. Furthermore, the overexpression of *RCK1* partially rescued the growth defect caused by zymolyase stress. The expression of *RCK1* was regulated independently by Slt2 and Hog1, and Kdx1 failed to induce the expression of *RCK1* in a *HOG1* deletion strain. The transcriptional factors Smp1, Sko1, Msn2, Msn4, and Hot1, which are regulated by Hog1, did not affect *RCK1* expression, but Rlm1 did. Furthermore, the mutation of certain phosphorylation sites in *RLM1* inhibited the induction of *RCK1* expression by Kdx1. We found a conserved Rlm1 binding site in the 5′ untranslated region (UTR) of *RCK1*, and the mutation of these Rlm1 binding sites also inhibited the induction of *RCK1* expression by Kdx1. Finally, we showed that Kdx1 physically interacts with Rlm1 and that this interaction affects the ability of Rlm1 to bind to the *RCK1* 5′ UTR. Taken together, these data suggest that Kdx1 interacts with Rlm1 to activate *RCK1* gene expression in response to stress in *Saccharomyces cerevisiae*.

1. Introduction

The cell wall of the budding yeast *Saccharomyces cerevisiae* is required to maintain cell shape and integrity and to protect cells from environmental stresses [1]. The adaptive response to cell wall stress is mostly mediated by the cell wall integrity (CWI) pathway, which can be activated by treatment with cell wall-damaging agents such as Calcofluor White [2], Congo Red, and zymolyase [3–6]. In addition, other environmental stresses, including heat shock [7], osmotic stress [7,8], caffeine [9], and mating pheromone, lead to the activation of the cell wall integrity (CWI) pathway [10]. In budding yeast, the cell wall integrity pathway is activated by several membrane proteins (Mid2, Wsc1–4, and Mtl1) that act as sensors. These sensor proteins activate Pkc1, which in turn activates the MAPK module by phosphorylation. The phosphorylation of the Bck1 MAPKKK activates the Mkk1 and Mkk2 MAPKKs, which then phosphorylate Slt2 MAPK [3]. Phosphorylated Slt2 MAPK activates Rlm1 [11] and the SBF transcription factors [12].

Hog1, the homolog of the mammalian p38 MAPK, is another stress response gene that responds to extracellular osmolarity and is required for adaptation to osmotic stress [13,14]. The HOG pathway is activated by two osmosensing mechanisms. These osmosensing mechanisms involve the Sln–Ypd1–Ssk1 proteins and the transmembrane protein Sho1, which activates the MAPKKK. These proteins activate Pbs2 MAPKK by phosphorylation, and the activated Pbs2 MAPKK phosphorylates Hog1 MAPK. In response to osmotic stress, activated Hog1 induces cellular osmoadaptive responses [15–17]. Furthermore, the Hog1 pathway has been implicated in the response to other stresses, including citric acid stress [18], the presence of methylglyoxal [19], low temperature [20], zymolyase-mediated cell wall stress [21], and heavy metal stress [22,23].

In mammalian cells, many of the cellular responses involving MAPK cascades have been shown to be mediated by MAPKAPKs (MAP kinase-activated protein kinases). For example, p90/rsk (MAPKAP – K1) family members are activated by ERK and JNK MAPK by exposure to UV radiation [24]. MAPKAP kinase 2, which is a substrate for p38, activates MAPK [25]. In the budding yeast *S. cerevisiae*, two MAPKAPKs, Rck1 and Rck2, were identified as homologs of the Mkp1 and Mkp2 genes of *Schizosaccharomyces pombe* [26–28]. *S. pombe* checkpoint mutants are sensitive to hydroxyurea and radiation, and this sensitivity is suppressed by *RCK1* and *RCK2* in *S. pombe* [27]. Furthermore, *RCK1* and *RCK2* inhibit meiosis in *S. pombe* [29]. In addition, Rck2 is phosphorylated (at

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Ser519) after hyperosmotic stress in a Hog1-dependent manner. The *RCK2* protein kinase is a mediator of the osmotic stress signaling pathway, which is under the control of Hog1 [28–30]. Additionally, these kinases are phosphorylated by oxidative stress. The overexpression of *RCK2* efficiently suppresses the sensitivity of the *hog1* mutant upon exposure to oxidative stress [23]. However, very little is understood about the function of Rck1 in *S. cerevisiae*.

In this work, we demonstrated a novel role for *RCK1* in the cell wall stress response in the budding yeast *S. cerevisiae*. The overexpression of Rck1 partially suppressed the sensitive phenotype caused by cell wall stress. We identified RLM1/KDX1 as an essential element required for *RCK1* expression and showed that the RLM1/KDX1 complex is recruited to the *RCK1* promoter. *KDX1* recruitment to the *RCK1* promoter is dependent on *RLM1*. Thus, Rck1 is a MAPKAPK that acts downstream of *RLM1*. Our results demonstrated that *RCK1* is required for the adaptation to zymolyase-induced cell wall stress in yeast.

2. Materials and methods

2.1. Strains, media and growth conditions

The yeast strains used in this study were *S. cerevisiae* BY4741 (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) and its derivatives. The yeast strains were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) or synthetic defined (SD) medium (6.7 g/L yeast nitrogen base) supplemented with the required auxotrophic supplements. For the zymolyase treatment, the yeast cells were grown overnight at 30 °C in YPD. The culture was diluted to an OD of 0.2 and grown at 30 °C for 2 h 30 min. The cultures were divided into two aliquots. One aliquot was allowed to continue growing under the same conditions. The other was treated with 5 units/ml of zymolyase 100T (Seikagaku Corp.) for different periods of time.

2.2. Plasmids and two-hybrid assays

The plasmids pRS426-KDX1 and pRS425-RCK1 were constructed as follows. PCR-based gene amplification was used to construct the multi-copy plasmid containing *KDX1* from YEp13-KDX1. To construct the plasmid pRS425-RCK1, a 4.1-kb (*Xho*I/*Bam*HI) DNA fragment containing *RCK1* was prepared by PCR using 5′-CTCGAGGAACTATAGCTAAATAACCCA-3′ and 5′- GGATCCCGAGCCACAATATGCGGCAACG -3′ as the forward and reverse primers, respectively. For two-hybrid assay, *RLM1* was PCR amplified using the primers 5′-CCCGGGTATGGGTAGACGGA-3′ and 3′-GAGCTCTTATATTTTGCTTGAA-5′, and the resulting PCR product was digested with *Sac*I and *Sma*I and inserted into the pGADT7 vector. The *KDX1* fragment (1–440) was PCR amplified using the primers 5′-GGATCCGTATGGCGACTGA-3′ and 3′-CTGCAGTTAGTCACAATGTAAAAC-5′, digested with *Bam*HI and *Pst*I, and then inserted into the pGBKT7 vector.

2.3. Site-directed mutagenesis of RLM1 and RCK1

To obtain the plasmid pRS415-RLM1(S374A/S427A/T439A), a 2.9-kb (*Xho*I/*Spe*I) fragment from genomic DNA was cloned into the vector pRS415. The following forward and reverse primers were used: 5′- CTCGAGGTTGGCAAGACAATTA-3′ and 5′- ACTAGTAAAATGCAATTGAGT-3′. Site-directed mutagenesis of the wild-type *RLM1* gene was performed with the QuikChange site-directed mutagenesis kit (Stratagene). The following primers were used for the site-directed mutagenesis: 5′-CAAGTAGGTCTAGCAAAATTGCACCACTATCCGC-3′ and 3′-AATTTTGCTAGACCTACTTGTTTTGTTTTC-5′ for the Rlm1-S374A mutant, 5′-ATCCTTTCGGAAGTGGGTCTGCACCTCTTTTTTC-3′ and 3′-AGACCCACTTCCGAAAGGATAAGGGTGATT-5′ for the Rlm1-S427A mutant, and 5′-CAACACAGCCATACATTGCCGCACCCTTGCAACC-3′ and 3′-GGCAATGTATGGCTGTGTTGCAGAAAAAAG-5′ for the Rlm1-T439A mutant. The RCK1-DD (deletion of *RLM1* binding site: CTATTTTTAG) allele was produced by PCR with the primers 5′- CATAATTAAGTTTCTGGAACACGCATTGTTACGGGAGACCA-3′ and 5′- AACAATGCGTGTTCCA GAAACTTAATTATGGGAGA-3′.

2.4. Microarray and northern blot analyses

Microarray analysis was performed using two independent RNA samples of the wild-type and overexpressed *KDX1*. The yeast cultures were grown to mid-log phase in SD-ura medium and either subjected to cell wall stress (5 U/ml zymolyase, 2 h) or left untreated. Total RNAs was extracted from wild-type and overexpressed *KDX1* strains using the TRIzol reagent (Life Technologies) and performed cDNA microarray. The microarray data were analyzed using clustering program MeV4.7.1 (eBiogen Co., Seoul, Korea) and was deposited on http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45692. For the northern blot analysis, the yeast cells were grown to mid-log phase and then subjected to cell wall stress (5 U/ml zymolyase) for the indicated times.

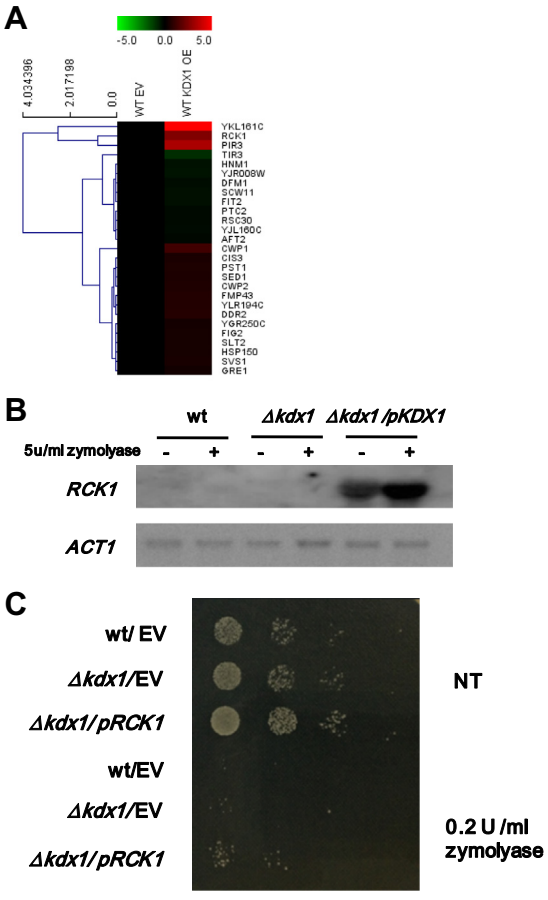

Fig. 1. Kdx1 up-regulates *RCK1* gene expression. (A) Microarray analysis was performed with wild-type and *KDX1* over-expressing cells, and the genes up-regulated by Kdx1 were identified as a heat map. EV indicates empty vector and OE indicates over-expression of *KDX1*. *CWP1* and *RCK1* were up-regulated several fold. *KDX1* served as the control. (B) The microarray data were confirmed by northern blot analysis. Total RNA was extracted from the indicated cells and separated by agarose gel electrophoresis, followed by northern blot analysis. (C) The effect of Rck1 was investigated by the plate assay. Wild-type and Kdx1 deletion mutant cells were transformed with EV or the multi-copy *RCK1* plasmid. The transformants were treated with the indicated amounts of zymolyase and spotted on SD media.

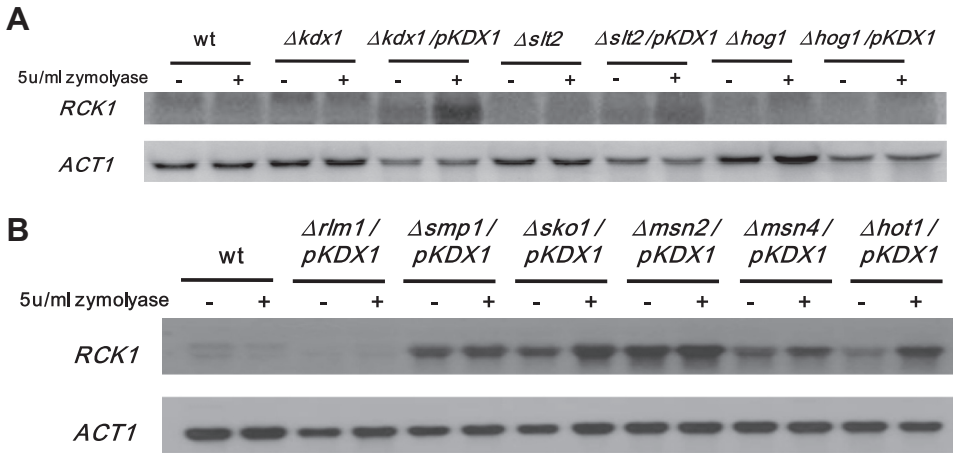

Fig. 2. The gene expression of *RCK1* is regulated by Rlm1. (A) To investigate the regulation of *RCK1*, northern blot analysis was performed in the slt2 and hog1 deletion strains. A multi-copy *KDX1* plasmid was introduced into the hog1 and slt2 deletion mutants, and total RNA was extracted from the transformed cells. A northern blot against *RCK1*was performed. (B) To further investigate the mechanism regulating *RCK1* expression, various Hog1-regulated genes were tested for the ability to modulate *RCK1* expression. A multi-copy *KDX1* plasmid was introduced into the rlm1, smp1, sko1, msn2, msn4, and hot1 deletion strains, and total RNA was extracted for subsequent *RCK1* expression analysis.

2.5. Chromatin immunoprecipitation assays

The ChIP experiments were performed as described previously [31]. The yeast cultures were grown to mid-log phase, and then the culture samples were subjected to cell wall stress (5 U/ml zymolyase; 2 h). The cells were treated with 1% formaldehyde for 20 min at room temperature to cross-link the DNA and protein. A polyclonal anti-HA antibody was used (Santa Cruz Bio). PCR was used to amplify the co-precipitated DNA sequences. The following forward and reverse primers were used: *RCK1* (promoter): 5′-ACGCATTGTTCTATTTTTAG-3′ and 5′-TCACAGTCTGAAATTCCGTG-3′, respectively. These experiments were performed to test the association of *RLM1*-HA and *KDX1*-HA with the *RCK1* promoter.

3. Results

3.1. RCK1 expression is up-regulated by Kdx1

Previously, Kdx1 was reported to be involved in cell wall stress and to be one of the members of the MAP kinase pathway that responds to external stresses in *S. cerevisiae* [5–32]. To investigate the function of Kdx1, we evaluated the global gene expression pattern caused by *KDX1* over-expression (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45692). *KDX1* was subcloned into a yeast multi-copy vector and treated with zymolyase to further induce *KDX1*. As shown in the heat map of Fig. 1A, the genes *PIR3* and *CWP1*, which encode cell wall proteins, were up-regulated several fold when *KDX1* was over-expressed. Interestingly, we found that *RCK1*, which encodes a MAPKAPK, was up-regulated by Kdx1. To confirm this change in expression, we performed northern blot analysis. As shown in Fig. 1B, the expression of *RCK1* was extremely low and difficult to detect. However, when *KDX1* was over-expressed, the expression of *RCK1* increased dramatically, and additional zymolyase treatment further increased the *RCK1* expression level. This result showed that Kdx1 regulates the expression of *RCK1*. To investigate the function of Rck1 in the zymolyase stress response, we performed a plate assay. The wild-type and Kdx1 deletion strains were transformed with a multi-copy *RCK1* plasmid, and the cells were treated with zymolyase as described in the Materials and Methods. As shown in Fig. 1C, both wild-type and Kdx1 deletion strains that were treated with zymolyase showed growth defects, but the introduction of a multi-copy *RCK1* plasmid suppressed these growth defects. This result indicates that Rck1 is involved in the response to zymolyase stress and that its gene expression is regulated by Kdx1.

3.2. RCK1 gene expression is regulated by Rlm1

Rck1 has been reported to be a member of the MAPKAPK family and to be involved in the HOG1 pathway [23–30]. However, the mechanism of its regulation has not yet been identified. To better characterize the function of Rck1 and its regulatory mechanism, we investigated the role of stress-responsive elements in the *RCK1* expression. In *S. cerevisiae*, osmotic and cell wall stresses are regulated by Hog1 and Slt2, respectively, and Rck1 has been shown to be a target of Hog1 under osmotic stress conditions [4–16]. As shown in Fig. 2A, we introduced *KDX1* expression with a multi-copy plasmid into slt2 or hog1 deletion mutants and confirmed its expression by northern blot analysis of total RNA from these cells. The gene expression of *RCK1* was increased when *KDX1* was over-expressed in the kdx1 deletion mutant. However, the gene expression of *RCK1* in the slt2 deletion mutant was only slightly increased, even when *KDX1* was over-expressed. Interestingly, no *RCK1* was detected when hog1 was deleted, even when *KDX1* was over-expressed. To confirm that the HOG1 pathway modulates *RCK1* expression, we investigated the effects of the transcription factors that are regulated by Hog1. As shown in Fig. 2B, the deletions of five transcription factors, smp1, sko1, msn2, msn4, and hot1, which are regulated by Hog1, had no effect on *RCK1* expression. However, no transcripts of *RCK1* were found in the rlm1 deletion strain, even when *KDX1* was over-expressed. These results indicate that the expression of *RCK1* is regulated by the Hog1 pathway through Rlm1.

3.3. Rlm1 phosphorylation is important for the regulation of RCK1

To further investigate the function of Rlm1 with respect to *RCK1* expression, we mutated the three identified phosphorylation sites within Rlm1 (serine 374, serine 427, and threonine 439) using site-directed mutagenesis [33]. The mutated *RLM1* was incorporated into single-copy plasmid and introduced into the rlm1 deletion strain. As shown in Fig. 3A, cells that had been transformed with the empty vector or *KDX1* overexpression plasmid showed no *RCK1* transcription, and the same results were observed in cells

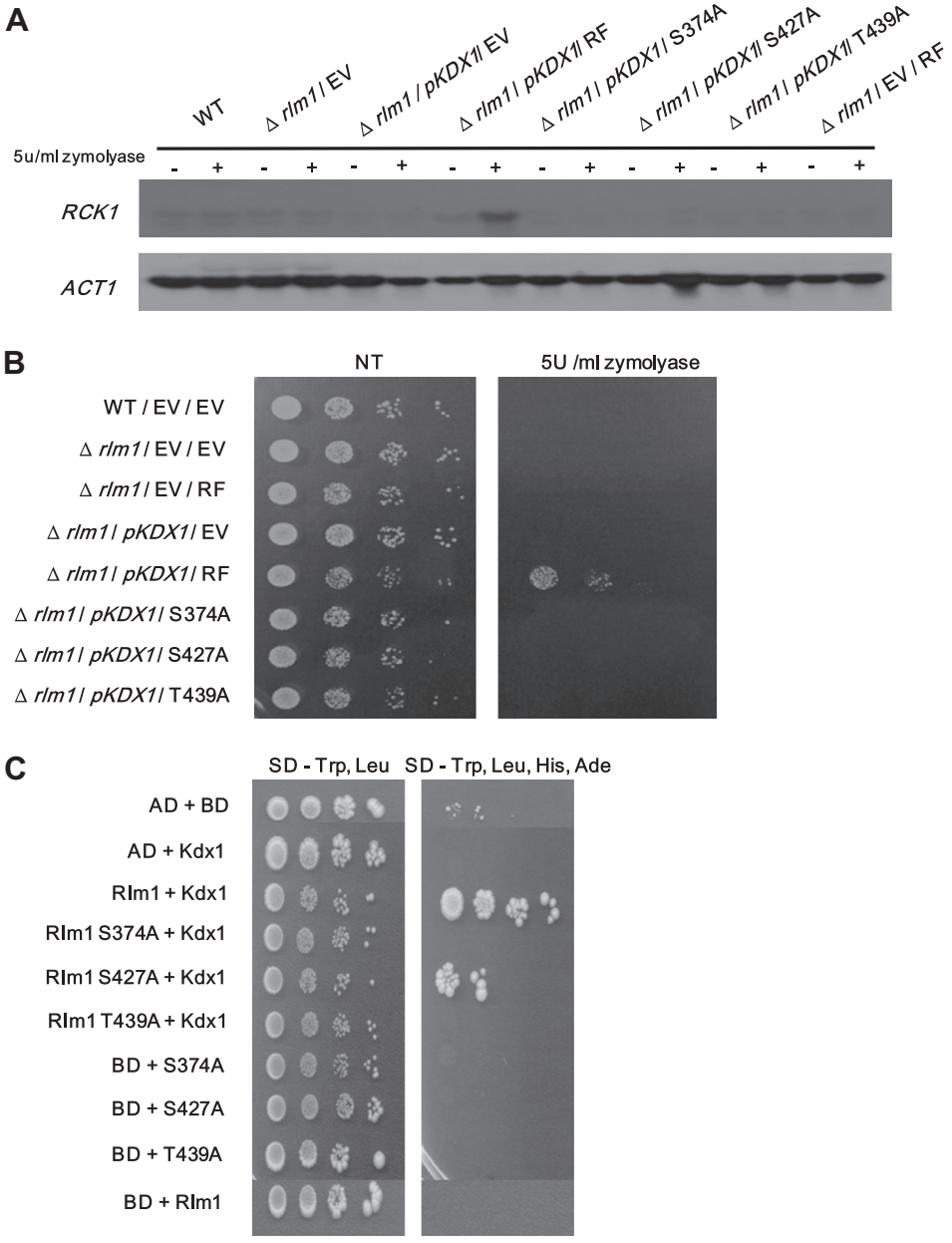

Fig. 3. The phosphorylation sites within Rlm1 are important for the regulation of *RCK1*. (A) To investigate the function of Rlm1 in *RCK1* expression, the three phosphorylation sites within Rlm1 were mutated. The multi-copy *KDX1* plasmid was introduced into the mutant strains and northern blot analysis was performed.(B) To further clarify the requirement of Rlm1 in the zymolyase stress response, the plate assay was performed. The same strains described for panel A were treated with the indicated amounts of zymolyase and spotted on SD media. (C) To test the effects of the three phosphorylation sites in Rlm1 on the interaction with Kdx1, two-hybrid analysis was performed. Mutated forms of *RLM1* and *KDX1* were co-introduced, and the plate assay was performed.

that had been transformed with the phosphorylation site mutants. However, *RCK1* gene expression was observed when intact *RLM1* was expressed. These results indicate that the phosphorylation sites of Rlm1 are important for the activation of *RCK1* expression.To determine whether the phosphorylation sites of Rlm1 is required for zymolyase stress response, we carried out a plate assay. As shown in Fig. 3B, the expression of the phosphorylation sites mutative Rlm1 in rlm1 deletion strain showed growth defects under the zymolyase stress, similar to that seen in the strains containing empty vector or*KDX1*overexpression plasmid. In contrast, expression of*RLM1* and a multi-copy *KDX1* suppressed these growth defects. To investigate the functions of the Rlm1 phosphorylation sites, we performed a two-hybrid analysis. Rlm1 has been reported to interact with Kdx1 physically [11], and we hypothesized that the Rlm1 phosphorylation sites might be important for this interaction. As shown in Fig. 3C, Rlm1 and Kdx1 interacted physically, and this interaction was abolished by the mutation of the Rlm1 phosphorylation sites. Rlm1 mutants in which serine 374 or threonine 439 had been changed to alanine failed to interact with Kdx1. These results indicate that the interaction between Rlm1 and Kdx1 is an important factor in the activation of *RCK1* gene expression. These experiments resolve several questions about the functions of Rlm1 and Kdx1 in the regulation of *RCK1* gene expression.

3.4. Rlm1 binds to the 5′ UTR of RCK1 by interacting with Kdx1

We searched for candidate Rlm1 binding sites in the RCK1 gene and found a conserved Rlm1 binding site, 5′-CTATTTTTAG-3′, in the 5′ untranslated region (UTR) [34]. As shown in Fig. 4A, this site is

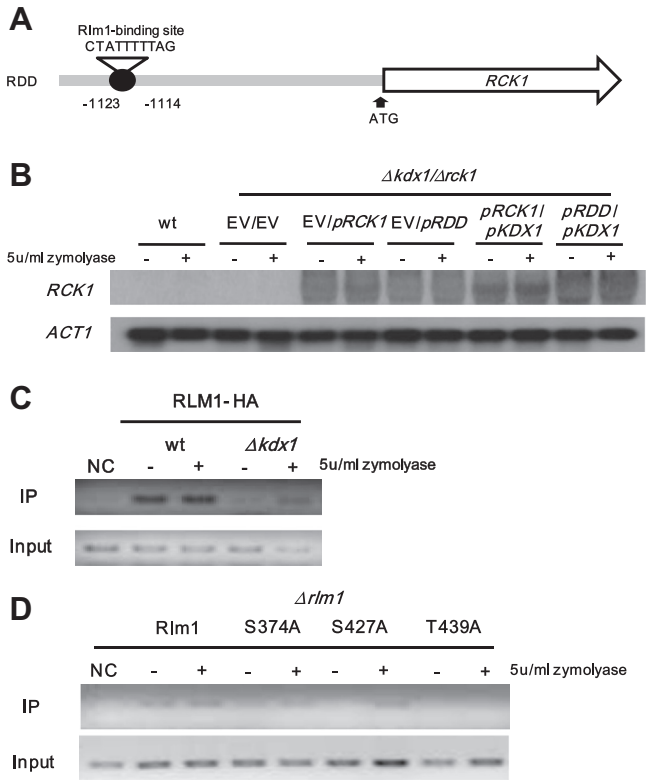

Fig. 4. Rlm1 binds to the 5′ UTR of *RCK1* by interacting with Kdx1. (A) The *RCK1* gene sequence was analyzed, and a conserved Rlm1 binding site (5′-CTATTTTTAG-3′) was found in the 5′ UTR. (B) The effect of the Rlm1 binding site on *RCK1* expression was investigated. The Rlm1 binding site was mutated by site-directed mutagenesis. The mutated form of *RCK1* (RDD) and the indicated plasmids were co-transformed into cells, and then northern blot analysis was performed. (C) A chromatin IP (ChIP) assay was performed to evaluate the binding activity of Rlm1 to the *RCK1* gene. The indicated genes were co-transformed, and ChIP was performed. (D) Rlm1 mutants (phosphorylation site mutations) were introduced into the rlm1 deletion strain, and their abilities to bind to the *RCK1* gene were tested by ChIP.

located 1123 nucleotides upstream of the translation start site. We deleted the entire Rlm1 binding site from the upstream region to investigate its function. The full-length *RCK1* and *KDX1* genes were co-transformed into a *kdx1*/*rck1* double deletion mutant, and northern blotting against *RCK1* was performed. As shown in Fig. 4B, the deletion of the Rlm1 binding site (RDD) abrogated the up-regulation of *RCK1* expression by Kdx1. We performed a chromatin IP assay to confirm that Rlm1 bound to the *RCK1* 5′ UTR. Rlm1 was tagged with an HA epitope, Rlm1-HA was pulled down with the HA antibody, and PCR was performed with *RCK1*-specific primers. The lower panel of Fig. 4C shows the input control, and the upper panel shows the pulled-down DNA fragments that were amplified by the *RCK1* primers. Interestingly, *RCK1* fragments were detected when full-length *KDX1* was introduced. When KDX1 was deleted, however, no *RCK1* fragments were detected. Furthermore, we tested the effects of the Rlm1 phosphorylation sites on the *RCK1* binding activity of Rlm1. As shown in Fig. 4D, the mutation of the phosphorylation sites decreased the binding of Rlm1, indicating that the phosphorylation of Rlm1 is an important factor in *RCK1* expression and that the binding of Rlm1 to *RCK1* requires Kdx1.

4. Discussion

Rck1 and Rck2 were identified as homologs of the Mkp1 and Mpk2 genes of *S. pombe*, and *RCK2* is known to be a protein kinase in the HOG1 pathway [26–28]. In addition, these proteins are phosphorylated in response to oxidative stress [23]. The overexpression of *RCK2* efficiently suppresses the sensitivity of the *hog1* mutant to oxidative stress. Together, these data indicate that these proteins are involved in the stress response signaling pathway. However, very little is known about the specific function and regulation of Rck1 in *S. cerevisiae*. We found that *RCK1* expression is upregulated by Kdx1. Kdx1 has been characterized as a pseudokinase and is involved in the cell wall stress response [32]. Interestingly, our microarray analysis showed that the over-expression of *KDX1* upregulated *RCK1* by several fold. To further investigate the mechanism of the regulation of *RCK1* gene expression, we analyzed the sequence of *RCK1* and identified a conserved Rlm1 binding sequence in the 5′ UTR [34]. The deletion of this Rlm1 binding site inhibited the induction of *RCK1* expression by Kdx1. Furthermore, we found that Kdx1 cannot induce the expression of *RCK1* in the absence of Rlm1. These results imply that both Kdx1 and Rlm1 are required for *RCK1* expression.

Rlm1 has been reported to interact with Kdx1 physically [11]. Therefore, we tested the effect of this interaction on *RCK1* gene expression. We tested the interaction between Rlm1 and Kdx1 using two-hybrid experiments and found that the phosphorylation sites of Rlm1 are important for its interaction with Kdx1 [33]. We found that the interaction between Rlm1 and Kdx1 was abolished by the mutation of the serine 437 and threonine 439 sites in Rlm1. We then performed a chromatin IP assay with wild-type and mutant proteins and found that neither Rlm1 nor Kdx1 alone could bind to the 5′ UTR of *RCK1*; furthermore, the mutant Rlm1 proteins also failed to bind to the 5′ non-translated region of *RCK1*, even in the presence of Kdx1. Our results suggest that Rlm1 is a transcriptional activator of *RCK1* and requires Kdx1 for its activity.

As described above, Rck1 and Rck2 are involved in stress responses and are regulated by Hog1. However, the regulatory mechanism and function of Rck1 in response to stress have not been characterized in detail. Additionally, the proteins downstream of Rck1 have not yet been identified. It will be interesting to determine whether the regulatory mechanism involves Rlm1/Kdx1 complex formation. The complex form of Rck1/Kdx1 is active, and we hypothesize that this complex may be formed when the cells become stressed.

Together, our data revealed the regulatory mechanism of *RCK1* gene expression and the function of Rck1 in the HOG pathway. These data will contribute to a better understanding of the function of Rck1.

Acknowledgments

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