EUKARYOTIC CELL, Oct. 2011, p. 1340–1347 Vol. 10, No. 10
1535-9778/11/$12.00 doi:10.1128/EC.05181-11

RNA-Binding Protein Khd1 and Ccr4 Deadenylase Play Overlapping Roles in the Cell Wall Integrity Pathway in *Saccharomyces cerevisiae*▽

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The *Saccharomyces cerevisiae* RNA-binding protein Khd1/Hek2 associates with hundreds of potential mRNA targets preferentially, including the mRNAs encoding proteins localized to the cell wall and plasma membrane. We have previously revealed that Khd1 positively regulates expression of *MTL1* mRNA encoding a membrane sensor in the cell wall integrity (CWI) pathway. However, a *khd1*Δ mutation has no detectable phenotype on cell wall synthesis. Here we show that the *khd1*Δ mutation causes a severe cell lysis when combined with the deletion of the *CCR4* gene encoding a cytoplasmic deadenylase. We identified the *ROM2* mRNA, encoding a guanine nucleotide exchange factor (GEF) for Rho1, as a target for Khd1 and Ccr4. The *ROM2* mRNA level was decreased in the *khd1*Δ *ccr4*Δ mutant, and *ROM2* overexpression suppressed the cell lysis of the *khd1*Δ *ccr4*Δ mutant. We also found that Ccr4 negatively regulates expression of the *LRG1* mRNA encoding a GTPase-activating protein (GAP) for Rho1. The *LRG1* mRNA level was increased in the *ccr4*Δ and *khd1*Δ *ccr4*Δ mutants, and deletion of *LRG1* suppressed the cell lysis of the *khd1*Δ *ccr4*Δ mutant. Our results presented here suggest that Khd1 and Ccr4 modulate a signal from Rho1 in the CWI pathway by regulating the expression of RhoGEF and RhoGAP.

Regulation of gene expression is achieved not only at the transcriptional level but also at the posttranscriptional level. In the nucleus, the primary transcript is processed by the addition of 5′-cap structure, the removal of introns, and the addition of a polyadenosine [poly(A)] tail. Upon export to the cytoplasm, the mature mRNA is subject to quality control and occasionally localized to particular subcellular compartments for translation. Finally, mRNAs are degraded in processing bodies (P-bodies), a process that must also be tightly controlled. The integration of all these regulatory steps ensures that a given mRNA produces the proper proteins at the right time and place. RNA-binding proteins play important roles in posttranscriptional regulation of gene expression (14, 16, 19, 23, 41). Eukaryotic cells encode a large number of RNA-binding proteins, each of which has unique RNA-binding activity and protein-protein interaction characteristics.

A *Saccharomyces cerevisiae* RNA-binding protein, K homology (KH) domain protein 1 (Khd1), also known as a heterogeneous nuclear RNP K-like gene (Hek2), contains three K homology (KH) RNA-binding domains (here after we use Khd1) and is most related to mammalian heterogeneous nuclear ribonucleoprotein K (hnRNP K) and poly(C)-binding proteins (PCBP1 to -4) (5, 10, 21, 28). Khd1 is required for efficient localization of *ASH1* mRNA to the bud tip in dividing yeast by possibly acting as a repressor of translation during mRNA transport (21, 35). *ASH1* encodes a transcriptional repressor for HO endonuclease and thus prevents mating type switching in daughter cells (4, 40). Khd1 is also involved in the regulation of the telomeric position effect and telomere length (10, 11). More recently, Khd1 appears to regulate asymmetric expression of *FLO11* to determine daughter cell fate during filamentous growth (46). We have previously identified target mRNAs for Khd1 on a genome-wide level and found that Khd1 associates with hundreds of mRNAs, comprising almost 20% of the yeast's transcriptome (17). A significant fraction of the potential Khd1 mRNA targets encode proteins localized to the cell periphery, such as the cell wall and plasma membrane, and also nuclear proteins involved in transcriptional regulation. Despite these many mRNA targets, the *khd1*Δ mutant has no drastic phenotype in rich media except for the partial delocalization of *ASH1* mRNA and protein, resulting in slightly reduced expression of *HO* and the reduced expression of Mtl1, a membrane sensor in the cell wall integrity (CWI) signaling pathway (17, 21, 35). Thus, physiological functions of Khd1 in the CWI pathway and other processes still remain unclear.

The cell wall of the budding yeast *Saccharomyces cerevisiae* is required to maintain the cell shape and integrity (24). The cell must remodel this rigid structure during vegetative growth and during pheromone-induced morphogenesis. Cell wall remodeling is monitored and regulated by the CWI signaling pathway, which activates the protein kinase C (Pkc1)-activated Mpk1/Slt2 mitogen-activated protein (MAP) kinase (MAPK) cascade and a glucan synthesis (26). In the CWI signaling pathway, signals are initiated at the plasma membrane through the cell surface sensors Wsc1, Wsc2, Wsc3, Mid2, and Mtl1. Together with phosphatidylinositol-4,5-bisphosphate ($PI4,5P_2$), which recruits the Rom1/2 guanine nucleotide exchange factors (GEFs) to the plasma membrane, the sensors stimulate nucleotide exchange on a small G-protein Rho1. Rho1 activates five effectors, including the Pkc1-MAPK cascade, the β1,3-glucan synthase, the Bni1 formin protein, the exocyst component Sec3, and the Skn7 transcription factor. The MAP kinase cascade, which is comprised of Bck1, Mkk1/2, and Mpk1, is activated by Pkc1. Two transcription fac-

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▽ Published ahead of print on 26 August 2011.

TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
10B	*MATα ade2 trp1 can1 leu2 his3 ura3 GAL psi*$^{+}$ *HOp-ADE2-HO 3′UTR*	21
10BD	*MAT**a**/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3*	21
10BD-c163	*MAT**a**/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3 KHD1/khd1Δ::CgHIS3 CCR4/ccr4Δ::CgLEU2*	This study
10BD-p163	*MAT**a**/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3 KHD1/khd1Δ::CgHIS3 CAF1/caf1Δ::CgLEU2*	This study
10BD-cp163	*MAT**a**/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3 KHD1/khd1Δ::CgTRP1 CCR4/ccr4Δ::CgLEU2 CAF1/caf1Δ::CgHIS3*	This study
10BD-163-n3	*MAT**a**/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3 KHD1/khd1Δ::CgHIS3 NOT3/not3Δ::CgLEU2*	This study
10BD-163-n4	*MAT**a**/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3 KHD1/khd1Δ::CgHIS3 NOT4/not4Δ::CgLEU2*	This study
10BD-163-c40	*MAT**a**/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3 KHD1/khd1Δ::CgHIS3 CAF40/caf40Δ::CgLEU2*	This study
10BD-163-c130	*MAT**a**/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3 KHD1/khd1Δ::CgHIS3 CAF130/caf130Δ::CgLEU2*	This study
10BD-163-p2	*MAT**a**/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3 KHD1/khd1Δ::CgHIS3 PAN2/pan2Δ::CgLEU2*	This study
10BD-163-p3	*MAT**a**/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3 KHD1/khd1Δ::CgHIS3 PAN3/pan3Δ::CgLEU2*	This study
10BD-c163-r1	*MAT**a**/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3 KHD1/khd1Δ::CgTRP1 CCR4/ccr4Δ::CgLEU2 ROM1/rom1Δ::CgHIS3*	This study
10BD-c163-r2	*MAT**a**/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3 KHD1/khd1Δ::CgTRP1 CCR4/ccr4Δ::CgLEU2 ROM2/rom2Δ::CgHIS3*	This study
10BD-c163-l1	*MAT**a**/MATα ade2/ade2 trp1/trp1 can1/can1 leu2/leu2 his3/his3 ura3/ura3 KHD1/khd1Δ::CgTRP1 CCR4/ccr4Δ::CgLEU2 LRG1/lrg1Δ::CgHIS3*	This study
c1H-1A	*MATα ade2 trp1 can1 leu2 his3 ura3*	This study
c1H-1B	*MATα ade2 trp1 can1 leu2 his3 ura3 khd1Δ::CgHIS3 ccr4Δ::CgLEU2*	This study
c1H-1C	*MAT**a*** ade2 trp1 can1 leu2 his3 ura3 khd1Δ::*CgHIS3*	This study
c1H-1D	*MAT**a*** ade2 trp1 can1 leu2 his3 ura3 ccr4Δ::*CgLEU2*	This study
p1H-2A	*MATα ade2 trp1 can1 leu2 his3 ura3*	This study
p1H-2B	*MATα ade2 trp1 can1 leu2 his3 ura3 caf1Δ::CgLEU2*	This study
p1H-2C	*MAT**a*** ade2 trp1 can1 leu2 his3 ura3 khd1Δ::*CgHIS3 caf1Δ::CgLEU2*	This study
p1H-2D	*MAT**a*** ade2 trp1 can1 leu2 his3 ura3 khd1Δ::*CgHIS3*	This study
cp3-2C	*MAT**a*** ade2 trp1 can1 leu2 his3 ura3 ccr4Δ::*CgLEU2 caf1Δ::CgHIS3*	This study
cp3-5A	*MAT**a*** ade2 trp1 can1 leu2 his3 ura3 khd1Δ::*CgTRP1 ccr4Δ::CgLEU2 caf1Δ::CgHIS3*	This study
cl4-1B	*MATα ade2 trp1 can1 leu2 his3 ura3 khd1Δ::CgTRP1 ccr4Δ::CgLEU2 lrg1Δ::CgHIS3*	This study
KY803	*MAT**a*** gcn4-Δ*1 leu2 ura3 trp1*	7
KY803-36	*MAT**a*** gcn4-Δ*1 leu2 ura3 trp1 not2-1*	7
KY803-39	*MAT**a*** gcn4-Δ*1 leu2 ura3 trp1 not1-2*	7
KY803k	*MAT**a*** gcn4-Δ*1 leu2 ura3 trp1 khd1Δ::CgLEU2*	This study
KY803-36k	*MAT**a*** gcn4-Δ*1 leu2 ura3 trp1 not2-1 khd1Δ::CgLEU2*	This study
KY803-39k	*MAT**a*** gcn4-Δ*1 leu2 ura3 trp1 not1-2 khd1Δ::CgLEU2*	This study

tors, Rlm1 and the SBF complex (Swi4/Swi6), are targets of the MAP kinase. Loss of function of any protein kinase downstream of Pkc1 (or both Mkk1 and Mkk2) results in cell lysis at elevated growth temperature. The growth defects of these mutants are osmoremedial (e.g., with 1 M sorbitol), consistent with a primary defect in cell wall biogenesis. Loss of *PKC1* results in osmoremedial cell lysis at all growth temperatures. Unlike loss of Pkc1, loss of Rho1 or loss of both of the redundant catalytic subunits of glucan synthase, Fks1 and Fks2, does not result in osmoremedial cell lysis but is lethal, since cell wall synthesis is completely impaired in the *rho1*Δ and *fks1*Δ *fks2*Δ mutants.

In this study, we show that the *khd1*Δ mutation causes cell lysis when combined with deletion of the *CCR4* gene encoding a cytoplasmic deadenylase (6). While the *ccr4*Δ single mutant showed a weak cell lysis as previously reported (18), the *khd1*Δ *ccr4*Δ double mutant showed more severe cell lysis. We then searched the target mRNAs for Khd1 and Ccr4 in the CWI pathway and found the *ROM2* mRNA encoding the RhoGEF as one of the target mRNA. We also found that Ccr4 negatively regulates expression of the *LRG1* mRNA encoding a GTPase-activating protein (GAP) for Rho1. Our results indicate that Khd1 and Ccr4 modulate a signal from Rho1 in the CWI pathway by regulating the expression of RhoGEF and RhoGAP.

MATERIALS AND METHODS

Strains and general methods. *Escherichia coli* DH5a was used for DNA manipulations. Strains used in this study are described in Table 1. Standard procedures were followed for yeast manipulations (22). The media used in this study included rich medium, synthetic complete (SC) medium, and synthetic minimal (SD) medium (22). SC media lacking amino acids or other nutrients (e.g., SC-Ura corresponds to SC medium lacking uracil) were used to select transformants. Recombinant DNA procedures were carried out as described previously (37).

Plasmids. Plasmids used in this study are described in Table 2. Plasmids YCplac33-KHD1FLAG and YCplac-KHD1FLAG-L284N were used for the immunoprecipitation. Plasmid YCplac-RHO1-Q68L expressing the *RHO1*(*Q68L*)

TABLE 2. Plasmids used in this study

Plasmid	Relevant markers	Source or reference
YCplac33	*URA3 CEN-ARS*	13
YCplac33-KHD1FLAG	*URA3 CEN-ARS KHD1-FLAG*	This study
YCplac33-KHD1FLAG-L284N	*URA3 CEN-ARS KHD(L284N)-FLAG*	This study
YEplac195	*URA* 2μm	13
YEplac195-ROM2	*URA3* 2μm *ROM2*	This study
YEplac195-WSC2	*URA3* 2μm *WSC2*	This study
YCplac33-RHO1-Q68L	*URA3 CEN-ARS RHO1(Q68L)*	39
pRS316-PKC1-R398P	*URA3 CEN-ARS PKC1(R398P)*	32
pCgLEU2	*C. glabrata LEU2* carried by pUC19	36
pCgHIS3	*C. glabrata HIS3* carried by pUC19	36
pCgTRP1	*C. glabrata TRP1* carried by pUC19	36
pFA6a-13myc-kanMX6	*13myc-ADH 3′UTR-kanMX6*	27

allele was kindly provided by Y. Ohya and S. Nogami (39). YCplac33-PKC1-R398P expresses the *PKC1*(*R398P*) allele (32). Plasmids pCgLEU2, pCgHIS3, and pCgTRP1 are pUC19 carrying the *Candida glabrata LEU2*, *HIS3*, and *TRP1* genes, respectively (36).

Gene deletion and protein tagging. Deletions of *KHD1*, *CCR4*, *POP2*, *NOT3*, *NOT4*, *NOT5*, *CAF40*, *CAF130*, *PAN2*, *PAN3*, *ROM1*, *ROM2*, and *LRG1* were constructed by a PCR-based gene deletion method (2, 36, 38, 43). Primer sets were designed such that 46 bases at the 5′ ends of the primers were complementary to those at the corresponding region of the target gene and 20 bases at their 3′ ends were complementary to the pUC19 sequence outside the polylinker region in the plasmid pCgLEU2, pCgHIS3, or pCgTRP1. Primer sets for PCR were designed to delete the open reading frame (ORF) completely. The PCR products were transformed into the wild-type strain and selected for Leu^+, His^+, or Trp^+.

Determination of cell lysis. Aniline blue staining was done as previously described (39). Cell lysis was determined for aliquots of cell cultures as previously described (25) using propidium iodide staining. A minimum of 200 cells were counted for each sample.

Northern blot analysis. Total RNA was prepared from cells using ISOGEN reagent (Nippon Gene) and the RNeasy minikit (Qiagen). RNA samples were separated by 1.5% denatured agarose gel electrophoresis and transferred to a nylon membrane. Then, RNA was hybridized using a digoxigenin (DIG)-labeled antisense probe. The primer set j298 (TGACGATATGATGAGCTCCTCCTT ACGTCA) and j297 (TTAACCCCAGAAATCTAACGACG) and the primer set j259 (ATGATTCAAAATTCTGCTGGTTA) and j260 (GCCAATATTTAT GAATTCCATAAC) were used to detect transcript containing *ROM2* and *LRG1*, respectively. After washing and blocking, the membrane was incubated with alkaline phosphatase-conjugated anti-DIG antibody, and the signal was detected by enhanced chemiluminescence.

Screening for multicopy suppressors of the growth defect of the *khd1*Δ *ccr4*Δ mutant. The *khd1*Δ *ccr4*Δ strain (c1H-1B) was transformed with a genomic library carried in YEp24, a multicopy vector marked with *URA3*. Transformants were plated on SC-Ura plates containing 10% sorbitol and incubated at room temperature for 4 days. The plates were replica plated to yeast extract-peptone-dextrose (YPD) plates and continuously incubated at 37°C for 3 days. Eight transformants that formed colonies at 37°C were identified. The corresponding plasmids were isolated from the transformants, and those that conferred the ability to proliferate at 37°C to the *khd1*Δ *ccr4*Δ strain were identified by retransformation. Sequencing of the insert DNAs of the eight recovered plasmids revealed that four contained the *CCR4* gene, two contained the *SSD1* gene, one contained the *ROM2* gene, and one contained the *WSC2* gene. Regional analysis of the suppression ability confirmed that the *CCR4*, *SSD1*, *ROM2*, and *WSC2* genes were responsible to ensure the growth of the *khd1*Δ *ccr4*Δ strain.

Immunoprecipitation of Khd1-FLAG and RT-PCR analysis. Cells were grown in SC-Ura at 30°C to mid-log phase and harvested by centrifugation. The cells were washed twice in XT buffer (50 mM HEPES-KOH at pH 7.3, 20 mM potassium acetate, 2 mM EDTA, 0.1% Triton X-100, 5% glycerol) and resuspended in XT buffer containing protease inhibitors, phenylmethylsulfonyl fluoride (PMSF), aprotinin, and leupeptin. Glass beads were added, and the cells were broken by rigid vortexing at 4°C (4 times at 3,500 rpm for 30 s each). The supernatant was removed and centrifuged for 10 min at 5,000 × *g*. To immunoprecipitate FLAG-tagged Khd1 (Khd1-FLAG), 200 μl of extract was incubated with anti-FLAG antibody (M2) coupled to protein G Sepharose beads (20 μl; GE Healthcare) for 2 h at 4°C. Beads were washed three times with 400 μl XT buffer, and bound material was eluted with 50 μl elution buffer (0.1 μg/μl 3× FLAG peptide in XT buffer) for 10 min at 4°C. Western blots were performed using anti-FLAG antibody (M2). Reverse transcription (RT)-PCR analyses with RNAs isolated from total extracts and from Khd1-FLAG-immunoprecipitates were performed as previously described (21). The number of amplification cycles was adjusted to avoid reaching a plateau phase during PCR.

RESULTS

The *khd1*Δ mutation displays a synthetic growth defect with the *ccr4*Δ mutation. Despite the many mRNA targets, the *khd1*Δ mutant has no drastic phenotype in rich media except for the partial delocalization of *ASH1* mRNA and protein, resulting in slightly reduced expression of *HO* and reduced expression of Mtl1 (17, 21, 35). One possibility is that Khd1 shares its redundant function(s) with some other gene products. During the analysis of *MTL1* mRNA stability control by Khd1, we found that the *khd1*Δ *ccr4*Δ mutant cells grow slower than each single mutant cell, and this phenotype is clear in the W303 background (data not shown). Ccr4 is a catalytic subunits of the major cytoplasmic deadenylase involved in mRNA poly(A) tail shortening, and is also a component of the Ccr4-Not complex (6). To clarify this result, we performed a standard genetic analysis of diploid strain 10BD-c163 that was heterozygous for *khd1*Δ and *ccr4*Δ alleles in the W303 background. Tetrad analysis revealed that the *khd1*Δ mutant cells grew similarly to wild-type cells, the *ccr4*Δ mutant cells grew slower than wild-type cells, and the *khd1*Δ *ccr4*Δ double mutant cells grew much more slowly than the *ccr4*Δ mutant cells (Fig. 1A). The *khd1*Δ *ccr4*Δ double mutant cells grew very slowly at 25°C but failed to grow at elevated temperatures of

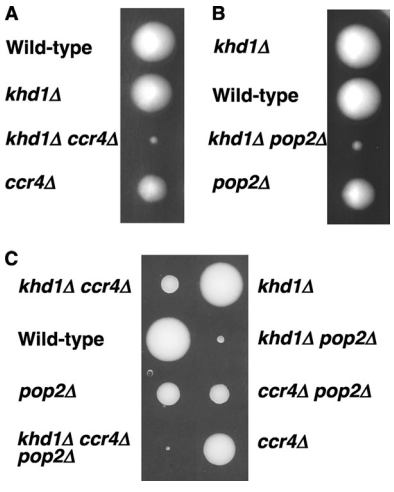

FIG. 1. Growth of the *khd1*Δ *ccr4*Δ and *khd1*Δ *pop2*Δ mutant strains. (A) Strain 10BD-c163 that was heterozygous for the *khd1*Δ and *ccr4*Δ alleles was sporulated, and the tetrad was dissected onto a YPD plate. Growth after 4 days at 25°C is shown. Genotypes are indicated on the left side. More than 50 tetrads were dissected, and representative data are shown. (B) Strain 10BD-p163 that was heterozygous for the *khd1*Δ and *pop2*Δ alleles was sporulated, and the tetrad was dissected onto a YPD plate. Growth after 4 days at 25°C is shown. Genotypes are indicated on the left side. More than 50 tetrads were dissected, and representative data are shown. (C) Strain 10BD-cp163 that was heterozygous for the *khd1*Δ, *ccr4*Δ, and *pop2*Δ alleles was sporulated, and the tetrad was dissected onto YPD containing 10% sorbitol. Growth after 6 days at 25°C is shown. Genotypes are indicated on the both sides. More than 30 tetrads were dissected, and representative data are shown.

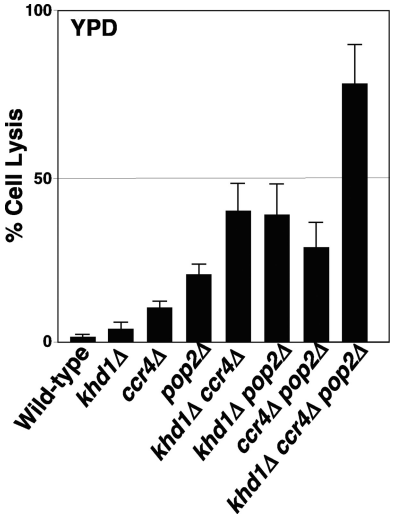

FIG. 2. The *khd1*Δ *ccr4*Δ double mutant cells show severe cell lysis. Cell lysis assay in YPD medium. Wild-type (c1H-1A), *khd1*Δ (c1H-1C), *ccr4*Δ (c1H-1D), *khd1*Δ *ccr4*Δ (c1H-1B), *pop2*Δ (p1H-2B), *khd1*Δ *ccr4*Δ (p1H-2C), *ccr4*Δ *pop2*Δ (cp3-2C), and *khd1*Δ *ccr4*Δ *pop2*Δ (cp3-5A) cells were grown in YPD medium to mid-log phase at 25°C and shifted to 37°C for 4 h. Cell lysis was monitored by propidium iodide staining. The graph represents averages from three independent cultures for each strain. Error bars depict the standard deviations. At least 200 cells were counted per each strain.

37°C (data not shown). Thus, the *khd1*Δ mutation showed a synthetic growth defect with the *ccr4*Δ mutation. We then confirmed the synthetic growth defect of the *khd1*Δ *ccr4*Δ double mutant using the *KHD1* and *CCR4* genes expressed on the plasmid. The *KHD1* and *CCR4* genes expressed on the plasmid complemented the slow growth of the *khd1*Δ *ccr4*Δ double mutant (data not shown).

The *khd1*Δ *ccr4*Δ mutant shows severe cell lysis. Previous studies demonstrated that the *ccr4*Δ mutant showed a temperature-sensitive cell lysis (18). Then, we next examined whether the *khd1*Δ *ccr4*Δ double mutants showed more severe cell lysis than the *ccr4*Δ single mutant. Microscopy analysis revealed that the *khd1*Δ *ccr4*Δ double mutant cells showed larger cell sizes and aberrant morphology, and some of the cells were lysed ghost cells (data not shown). Such aberrant morphology and lysed ghost cells were not observed in wild-type and *khd1*Δ single mutant cells. The *ccr4*Δ single mutant cells were a little larger than wild-type cells as previously reported (30), and few *ccr4*Δ cells showed lysed ghost, but the population was less than that of the *khd1*Δ *ccr4*Δ double mutant. The quantitative analysis of cell lysis using propidium iodide (see Materials and Methods) revealed that the *khd1*Δ *ccr4*Δ double mutants indeed showed severe cell lysis (Fig. 2). Aniline blue staining with 1,3-β-glucan also revealed that the *khd1*Δ *ccr4*Δ double mutant cells had abnormal deposition of the staining (data not shown). These results suggest that Khd1 together with Ccr4 has a role in the CWI signaling pathway.

The *khd1*Δ mutant displays a synthetic growth defect, with mutation in *POP2* as well as *CCR4*, but not other components of the Ccr4-Not complex. Ccr4 is one of the components in the Ccr4-Not complex involved in various cellular processes such as transcriptional control and mRNA degradation (6). Therefore, we then examined whether the *khd1*Δ mutation also showed a synthetic growth defect with mutations in other components of the Ccr4-Not complex, *POP2*, *NOT1*/*CDC39*, *NOT2*/*CDC36*, *NOT3*, *NOT4*, *CAF40*, and *CAF130*. Tetrad analysis revealed that the *khd1*Δ mutation also showed a synthetic growth defect with a deletion mutation in *POP2* that encodes another catalytic subunit of cytoplasmic deadenylase (Fig. 1B). Similar to the *khd1*Δ *ccr4*Δ double mutant, the *khd1*Δ *pop2*Δ double mutants also showed a more marked cell lysis than the *pop2*Δ single mutant (Fig. 2). It should be noted that the *khd1*Δ *pop2*Δ double mutant grew slower than the *khd1*Δ *ccr4*Δ double mutant. The *khd1*Δ *ccr4*Δ *pop2*Δ triple mutants indeed showed slower growth and more severe cell lysis (Fig. 1C and 2), suggesting that Ccr4 and Pop2 may act independently. The *khd1*Δ mutation did not show a synthetic growth defect with a deletion mutation in the other components of the Ccr4-Not complex, *NOT3*, *NOT4*, *CAF40*, and *CAF130* (data not shown). Since deletions of *NOT1*/*CDC39*, *NOT2*/*CDC36*, and *NOT5* were lethal in our strain background, we examined the synthetic growth defect of the *khd1*Δ mutation with conditional *not1-2* and *not2-1* alleles (7). A conditional allele of *NOT5* was not available in our hand. The cell growth of the *khd1*Δ *not1-2* and *khd1*Δ *not2-1* double mutant cells was similar to that of the *not1-2* and *not2-1* single mutants (data not shown), respectively, indicating that the *khd1*Δ mutation did not show a synthetic growth defect with these alleles. These results suggest that Khd1 shares its function on cell wall synthesis with Ccr4 and Pop2 but not other components of the Ccr4-Not complex.

Ccr4 and Pop2 are catalytic subunits of the major cytoplasmic deadenylase involved in mRNA degradation (8). Therefore, we next examined whether the *khd1*Δ mutation showed a synthetic growth defect with deletion mutations in *PAN2* and *PAN3*, encoding other cytoplasmic deadenylases. The *pan2*Δ and *pan3*Δ single mutant cells showed normal growth, and the *khd1*Δ mutation did not affect their growth (data not shown). These results suggest that Khd1 shares its function in the CWI pathway with Ccr4 and Pop2 but not with Pan2 and Pan3.

Isolation of multicopy suppressors of the *khd1*Δ *ccr4*Δ double mutant. Khd1 regulates the expression of Mtl1, one of the membrane sensors in the CWI pathway (17), implicating the possibility that the *khd1*Δ mutation enhances the cell lysis of the *ccr4*Δ mutant by reducing the upstream signal from Mtl1p. Then, we examined the growth of the *mtl1*Δ *ccr4*Δ double mutant and found that the *mtl1*Δ *ccr4*Δ double mutant grew similarly to the *ccr4*Δ single mutant (data not shown). Thus, only the reduced expression of Mtl1 by the *khd1*Δ mutation is not responsible for the severe cell lysis phenotype of the *khd1*Δ *ccr4*Δ mutant.

Khd1 and Ccr4 carry an RNA-binding protein and a cytoplasmic deadenylase, respectively, suggesting that Khd1 and Ccr4 function in the CWI pathway through regulating mRNA levels of some components that are required for the maintenance of the CWI pathway. To identify a possible target for Khd1 and Ccr4, we screened a genomic library for genes that, when overexpressed, suppressed the growth defect of the *khd1*Δ *ccr4*Δ double mutant. We identified *ROM2* and *WSC2* as multicopy suppressors (for details of the screening, see Materials and Methods). We also identified *SSD1* in this screening, but we did not study this gene further here since the W303

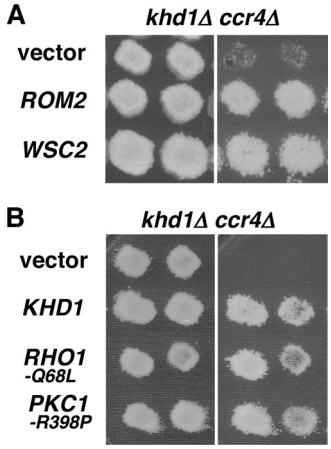

FIG. 3. Overexpression of *ROM2* and *WSC2* suppresses the growth defect of *khd1*Δ *ccr4*Δ double mutants. (A) Multicopy suppressors of *khd1*Δ *ccr4*Δ. Transformants of the *khd1*Δ *ccr4*Δ strain (c1H-1B) carrying the indicated plasmids were streaked onto YPD medium and incubated at 25°C (left) or 37°C (right). Each patch represents an independent transformant. Plasmids used were YEplac195 (vector), YEplac195-ROM2 (*ROM2*), and YEplac195-WSC2 (*WSC2*). (B) Activated Rho1 and Pkc1 alleles in *khd1*Δ *ccr4*Δ. Transformants of the *khd1*Δ *ccr4*Δ strain (c1H-1B) carrying the indicated plasmids were streaked onto YPD medium and incubated at 25°C (left) or 37°C (right). Each patch represents an independent transformant. Plasmids used were YCplac33 (vector), YCplac-RHO1-Q68L, and YCplac33-PKC1-R398P.

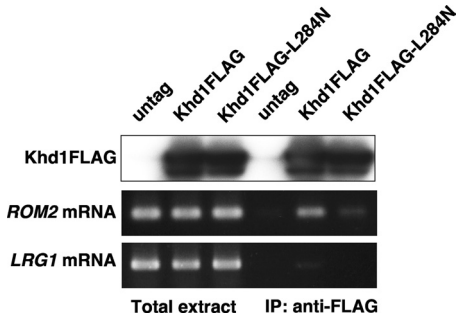

FIG. 4. Khd1 associates with the *ROM2* mRNA. Total extracts were prepared from the cells harboring the empty vector, the FLAG-tagged Khd1 plasmid, and the FLAG-tagged Khd1(L284N) plasmid. Khd1-FLAG and Khd1(L284N)-FLAG were immunoprecipitated (IP) with anti-FLAG antibodies. Samples were analyzed by immunoblot analysis with anti-FLAG to test for the presence of Khd1-FLAG proteins. The RNAs were isolated from the total extracts and from the immunoprecipitates. *ROM2* and *LRG1* mRNAs were detected by RT-PCR.

strains carry a defective *SSD1* allele. *ROM2* encodes a GEF for Rho1 in the CWI pathway (33). *WSC2* encodes a membrane sensor (26). As shown in Fig. 3A, overexpression of *ROM2* and *WSC2* suppressed the growth defect of the *khd1*Δ *ccr4*Δ double mutants. Since Rom2 and Wsc2 are upstream activators for Rho1 and Pkc1 in the CWI signaling pathway, we next examined whether the activated forms of *RHO1* and *PKC1* suppressed the growth defect of the *khd1*Δ *ccr4*Δ double mutant. Indeed, a constitutively active *RHO1* allele, *RHO1*(*Q68L*), and a constitutively active *PKC1* allele, *PKC1*(*R398P*), were able to suppress the growth defect of the *khd1*Δ *ccr4*Δ double mutant (Fig. 3B). These results, together with the observation that *ROM2* and *WSC2* act as the multicopy suppressors, suggest that the signaling upstream of Rho1 is impaired in the *khd1*Δ *ccr4*Δ double mutants.

ROM2 is a candidate target transcript linking Khd1 and Ccr4 in the CWI signaling pathway. Suppression of the *khd1*Δ *ccr4*Δ mutants by *ROM2* or *WSC2* overexpression suggests the following two possibilities. One possibility is that these genes are the direct targets for Khd1 and Ccr4, and their expression is regulated by Khd1 and Ccr4. The other possibility is that these genes are not the direct targets for Khd1 and Ccr4, but *ROM2* or *WSC2* overexpression suppressed the cell lysis by activating Rho1 and Pkc1. Our previous genome-wide analysis of Khd1-associated mRNAs revealed that both *ROM2* and *WSC2* are potential target mRNAs for Khd1 (17), and we have confirmed that the *WSC2* mRNA is associated with Khd1 *in vivo* (17). To ensure that Khd1 associates with the *ROM2* mRNA, we then performed immunoprecipitation and RT-PCR analyses with the immunoprecipitates (Fig. 4). For this purpose, we utilized plasmids expressing FLAG-tagged Khd1 (Khd1-FLAG) and Khd1(L284N) mutant protein [Khd1(L284N)-FLAG] under the control of the endogenous promoter. A replacement of Leu-284 to Asn in the third KH domain of Khd1 reduced its RNA-binding activity (17). Khd1-FLAG and Khd1(L284N)-FLAG were immunoprecipitated with anti-FLAG antibodies, and the coprecipitated mRNAs were analyzed with RT-PCR. The *ROM2* mRNA was clearly detected in RNA samples from the immunoprecipitated Khd1-FLAG, less detected in those from Khd1(L284N)-FLAG, but not detectable in untagged isolates (Fig. 4). This result suggests that Khd1 associates with the *ROM2* mRNA and that the third KH domain is required for the efficient binding of Khd1 to the RNA.

To determine whether Khd1 and Ccr4 affect the expression of *ROM2* and *WSC2*, we next examined the expression levels of *ROM2* and *WSC2* mRNAs in wild-type, *khd1*Δ, *ccr4*Δ, and *khd1*Δ *ccr4*Δ mutant cells (Fig. 5A). The *ROM2* mRNA level was not altered in the *khd1*Δ mutant compared to that in wild-type cells. In contrast, the *ROM2* mRNA level was decreased in the *ccr4*Δ mutant and more decreased in *khd1*Δ *ccr4*Δ double mutant cells. In contrast, the *WSC2* mRNA levels were not affected by the *khd1*Δ, *ccr4*Δ, or *khd1*Δ *ccr4*Δ mutations (data not shown). Together with the observations that *ROM2* overexpression suppressed the growth defect of the *khd1*Δ *ccr4*Δ mutant and that the *ROM2* mRNA is associated with Khd1, these results suggest that *ROM2* mRNA is a target mRNA for Khd1 and Ccr4.

Rom2 and Rom1 comprise a redundant pair of GEFs for Rho1 (33). Loss of *ROM2* function results in temperature-sensitive growth, whereas loss of both *ROM2* and *ROM1* is lethal. We found that the *ROM1* mRNA level was not affected by the *khd1*Δ, *ccr4*Δ, or *khd1*Δ *ccr4*Δ mutations (data not shown). Therefore, if the *ROM2* function were impaired in the *khd1*Δ *ccr4*Δ mutant, the *khd1*Δ *ccr4*Δ *rom1*Δ triple mutant would show a more severe growth defect than the *khd1*Δ *ccr4*Δ, *khd1*Δ *rom1*Δ, and *ccr4*Δ *rom1*Δ double mutants. Indeed, the *khd1*Δ *ccr4*Δ *rom1*Δ triple mutant cells showed much slower growth than the double mutant cells (Fig. 6A).

We then examined the cell growth of the *khd1*Δ *ccr4*Δ

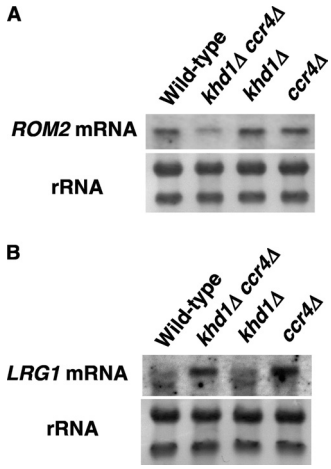

FIG. 5. Khd1 and Ccr4 regulate the *ROM2* mRNA level. (A) The *ROM2* mRNA levels in wild-type, *khd1*Δ, *ccr4*Δ, and *khd1*Δ *ccr4*Δ cells. Wild-type (c1H-1A), *khd1*Δ (c1H-1C), *ccr4*Δ (c1H-1D), and *khd1*Δ *ccr4*Δ (c1H-1B) cells were cultured to mid-logarithmic phase in YPD medium at room temperature and collected, and total RNA was prepared. The *ROM2* transcripts were quantified by Northern blotting, as described in Materials and Methods. rRNA was included as a quantity control. (B) The *LRG1* mRNA levels in wild-type, *khd1*Δ, *ccr4*Δ, and *khd1*Δ *ccr4*Δ cells. Wild-type (c1H-1A), *khd1*Δ (c1H-1C), *ccr4*Δ (c1H-1D), and *khd1*Δ *ccr4*Δ (c1H-1B) cells were cultured to mid-logarithmic phase in YPD medium at room temperature and collected, and total RNA was prepared. The *LRG1* transcripts were quantified by Northern blotting, as described in Materials and Methods. rRNA was included as a quantity control.

*rom2*Δ triple mutant cells. If *ROM2* were a sole target mRNA for Khd1 and Ccr4, the *khd1*Δ *ccr4*Δ *rom2*Δ triple mutant cells would grow similarly to that of the *rom2*Δ single or *khd1*Δ *ccr4*Δ double mutant. Surprising, the *khd1*Δ *ccr4*Δ *rom2*Δ triple mutant cells were inviable (Fig. 6B). Unlike the *rom1*Δ mutant cells that grew similarly to wild-type cells, the *rom2*Δ mutant cells grew slower than the wild-type cells, and the *ccr4*Δ *rom2*Δ double mutant cells grew more slowly than each single mutant cell (Fig. 6B). The *khd1*Δ *rom2*Δ double mutant cells grew similarly to the *rom2*Δ single mutant cells. These additive effects of the *khd1*Δ, *ccr4*Δ, and *rom2*Δ mutations suggest that Khd1 and Ccr4 have additional target mRNAs that regulate the CWI signaling pathway.

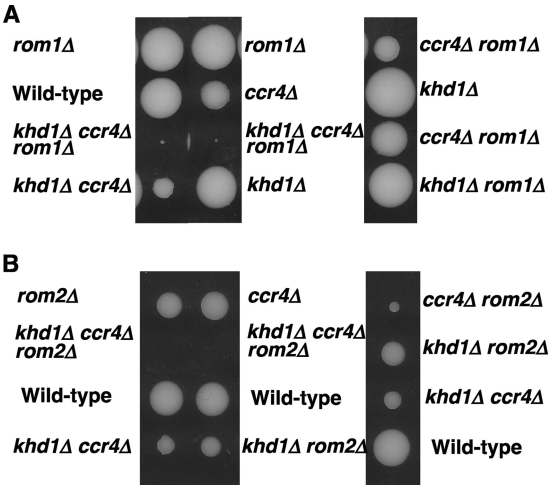

FIG. 6. Growth of the *khd1*Δ *ccr4*Δ, *khd1*Δ *ccr4*Δ *rom1*Δ, and *khd1*Δ *ccr4*Δ *rom2*Δ mutant strains. (A) Strain 10BD-c163r1 that was heterozygous for the *khd1*Δ, *ccr4*Δ, and *rom1*Δ alleles was sporulated, and the tetrad was dissected onto YPD containing 10% sorbitol. Growth after 6 days at 25°C is shown. Genotypes are indicated on both sides. More than 20 tetrads were dissected, and representative data are shown. (B) Strain 10BD-c163r2 that was heterozygous for the *khd1*Δ, *ccr4*Δ, and *rom2*Δ alleles was sporulated, and the tetrad was dissected onto YPD containing 10% sorbitol. Growth after 4 days at 25°C is shown. Genotypes are indicated on both sides. More than 20 tetrads were dissected, and representative data are shown.

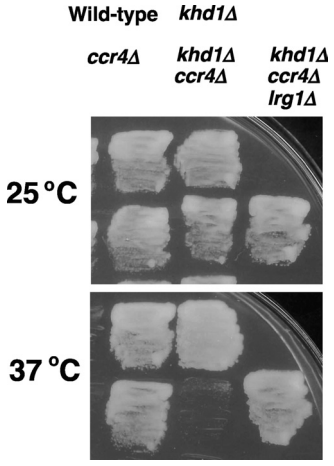

FIG. 7. Loss of *LRG1* suppresses the cell lysis of the *khd1*Δ *ccr4*Δ mutant. Wild-type (c1H-1A), *khd1*Δ (c1H-1C), *ccr4*Δ (c1H-1D), *khd1*Δ *ccr4*Δ (c1H-1B), and *khd1*Δ *ccr4*Δ *lrg1*Δ (cl4-1B) cells were plated on YPD medium plates and grown at either 25°C or 37°C for 3 days.

LRG1 is another target transcript linking Ccr4 to the CWI pathway. The activation of Rho1 is regulated by both GEFs and GAPs acting in opposition. Therefore, we next examined the possibility that Khd1 and Ccr4 regulated the levels of the mRNAs encoding RhoGAPs. Among the 11 RhoGAPs identified in *S. cerevisiae*, four GAPs, Bem2, Sac7, Bag7, and Lrg1, have been shown to act on Rho1 both *in vitro* and *in vivo* (26). We then examined the expression of RhoGAPs in wild-type, *khd1*Δ, *ccr4*Δ, and *khd1*Δ *ccr4*Δ mutant cells. We found that the *LRG1* mRNA level in the *ccr4*Δ and *khd1*Δ *ccr4*Δ mutants was increased compared to that in the wild-type cells (Fig. 5B). On the other hand, the expression level of *BEM2*, *SAC7*, or *BAG7* mRNAs was not altered significantly in the *ccr4*Δ and *khd1*Δ *ccr4*Δ mutants (data not shown). Consistent with the fact that *LRG1* was not a potential target mRNA for Khd1 in our previous analysis (17), the *LRG1* mRNA was not enriched in the RNA samples from the Khd1-FLAG immunoprecipitates (Fig. 4). Thus, Khd1 is unlikely to be involved in *LRG1* expression.

If the increased Lrg1 protein level had contributed to the cell lysis in the *khd1*Δ *ccr4*Δ mutant, a deletion of *LRG1* should suppress the growth defect of the *khd1*Δ *ccr4*Δ mutants. To test this possibility, we constructed the *khd1*Δ *ccr4*Δ *lrg1*Δ triple mutant by a standard genetic manipulation. As shown in Fig. 7, a deletion of *LRG1* efficiently suppressed the growth defect of the *khd1*Δ *ccr4*Δ mutant. These results suggest that the *LRG1* mRNA is a target mRNA for Ccr4 and that the elevated level of *LRG1* contributes to the cell lysis of the *khd1*Δ *ccr4*Δ mu-

tant. Alternatively, the elevated level of *LRG1* in the *khd1*Δ *ccr4*Δ mutant may be a consequence of the decreased *ROM2* expression caused by the *khd1*Δ *ccr4*Δ double mutation. The *lrg1*Δ mutation that conferred to an increase of Rho1-GTP could compensate for the decreased CWI signaling caused by the *khd1*Δ *ccr4*Δ double mutation.

DISCUSSION

In this study, we first showed the role of Khd1 in the CWI signaling pathway. Whereas the *khd1*Δ single mutant cells showed no detectable phenotype on cell wall synthesis, the *khd1*Δ mutation showed a severe cell wall defect when combined with the *ccr4*Δ or *pop2*Δ mutation that is known to confer a weak cell lysis (18). Although it has been reported that mutations in several genes, such as *RAD53* and *SPT15*, show a synthetic lethal or synthetic growth defect with the *ccr4*Δ and *pop2*Δ mutation (3, 34, 44), this is a first report showing that *CCR4* and *POP2* genetically interact with *KHD1*. The *khd1*Δ mutation did not show a synthetic growth defect with mutation in the other components of the Ccr4-Not complex. Genome-wide analyses of the roles of each subunit of the Ccr4-Not complex have revealed that the *ccr4*Δ and *pop2*Δ mutants show similar gene expression patterns that are different from those of other *not* deletions (1, 9). Thus, Khd1 shares a role in the expression of particular genes with Ccr4 and Pop2.

The CWI pathway consists of several membrane sensors, Wsc1, Wsc2, Wsc3, Mid2, and Mtl1 (26). Among these sensors, Wsc1 and Mid2 are reported to be the most important in the CWI signaling, and the *wsc1*Δ *mid2*Δ double mutant shows the cell lysis phenotype. Mtl1 has a minor role, and the *mtl1*Δ mutant has a normal response for the CWI signaling. Only the reduced expression of Mtl1 by the *khd1*Δ mutation is not responsible for the severe cell lysis phenotype of the *khd1*Δ *ccr4*Δ mutant, since the *mtl1*Δ *ccr4*Δ double mutant grew similarly to the *ccr4*Δ single mutant. On the other hand, overexpression of *MTL1* as well as *WSC2* suppressed the cell lysis of the *khd1*Δ *ccr4*Δ double mutant (data not shown), suggesting that overexpression of the sensors can activate the CWI signaling and overcome the cell lysis of the *khd1*Δ *ccr4*Δ double mutant presumably by Rho1 activation. The *WSC1*, *WSC2*, *WSC3*, *MID2*, and *MTL1* mRNAs are all bound to Khd1 (17). However, only the levels of *MTL1* mRNA and Mtl1 were regulated by Khd1. *MTL1* mRNA has a *cis*-acting region containing CNN repeats that direct Khd1p binding, and the *cis*-acting region is responsible for Khd1-mediated control (31). The *cis*-acting region of *MTL1* mRNA translates into an amino acid sequence containing stretches of serine residues. Although *MID2* and *WSC* mRNAs also have CNN repeats and Khd1 indeed bound to the repeats, the *khd1*Δ mutation does not affect their expression (17). The synthetic growth defect between the *khd1*Δ and *ccr4*Δ mutations implicates the possibility that the levels of Mid2 and Wsc1 to -3 besides Mtl1 are regulated by Ccr4 or by both Ccr4 and Khd1. However, it is not the case, the Mid2 or Wsc2 protein level was not affected by the *khd1*Δ *ccr4*Δ double mutation (data not shown). Physiological relevance of the binding of Khd1 to *MID2* or *WSC* mRNAs remains to be elucidated.

We show the *ROM2* mRNA encoding RhoGEF, as one of the targets for Khd1 and Ccr4 in the CWI signaling pathway. *ROM2* has been shown to be a potential target mRNA for Khd1 and contains three CNN repeats in the coding sequence (17). We then confirmed that Khd1 associates with the *ROM2* mRNA in a manner dependent on the RNA-binding activity of Khd1. We also show that *ROM2* overexpression suppresses the cell lysis of the *khd1*Δ *ccr4*Δ mutant. The *ROM2* mRNA level was slightly decreased in the *ccr4*Δ mutant and further decreased in the *khd1*Δ *ccr4*Δ double mutant. Consistent with the decrease in *ROM2* expression, the *khd1*Δ *ccr4*Δ double mutant cells showed a severe cell lysis defect probably due to the decreased Rho1 activity. Since Ccr4 is a deadenylase, previously known target mRNAs for Ccr4p such as *WHI5* and *CRT1* are negatively regulated by Ccr4 through the poly(A) shorting (30, 47). How Khd1 and Ccr4 positively regulate the expression of *ROM2* should be elucidated. In case of the regulation of *MTL1* mRNA stability by Khd1, *MTL1* mRNA itself bears the multiple CNN repeats involved in destabilization by the decapping enzyme Dcp1/2 and the 5′-3′ exonuclease Xrn1, and Khd1 stabilizes *MTL1* mRNA through binding to this element (31). Similarly, Khd1 may stabilize *ROM2* mRNA through binding to the CNN repeats within the coding sequence of the *ROM2* mRNA.

The *ccr4*Δ mutant shows pleiotropic phenotypes, including a weak cell lysis, a defect in checkpoint control, a defect in cell cycle progression, and abnormal morphology (18, 30, 44, 47). Previous studies have revealed the target mRNAs for Ccr4 that account for these phenotypes of the *ccr4*Δ mutant. For checkpoint control, the *CRT1* mRNA was shown to be negatively regulated by Ccr4 (47). Deletion of *CRT1* suppresses the checkpoint defect of the *ccr4*Δ mutant. For cell cycle progression, the *WHI5* mRNA was shown to be negatively regulated by Ccr4. Loss of *WHI5* suppresses the cell cycle defect of the *ccr4*Δ mutant (30). However, it has remained unknown how cell lysis occurs in the *ccr4*Δ cells and what the target for Ccr4 is. In the present study, we identified the target for Ccr4, the *LRG1* mRNA. Similarly to *CRT1* and *WHI5* mRNAs, the *LRG1* mRNA level is negatively regulated by Ccr4. Loss of *LRG1* suppressed the cell lysis of the *khd1*Δ *ccr4*Δ double mutant. It should be noted that loss of *CRT1* did not suppress the cell lysis of the *khd1*Δ *ccr4*Δ double mutant (data not shown), suggesting that each mRNA target is responsible for each of the several phenotypes of the *ccr4*Δ mutant. Consistent with the fact that the *LRG1* mRNA was not a target mRNA for Khd1, the *LRG1* mRNA levels in the *ccr4*Δ single mutant and the *khd1*Δ *ccr4*Δ double mutant are similar. Thus, Khd1 does not seem to be involved in the *LRG1* expression. Stewart et al. (42) have indicated that another RNA-binding protein Mpt5/Puf5 negatively regulates the *LRG1* mRNA level and that the *lrg1*Δ mutation suppresses the growth defect of the *mpt5*Δ mutation. Since Mpt5 directly binds to the 3′ untranslated region (3′UTR) of the *LRG1* mRNA (12) and physically interacts with Ccr4 and Pop2 (15, 20), Ccr4 may regulate *LRG1* mRNA in a 3′UTR-dependent manner together with Mpt5. On the other hand, since the *ccr4*Δ *mpt5*Δ double mutant grew slower than each single mutant and the *ccr4*Δ *mpt5*Δ *khd1*Δ triple mutant was lethal (our unpublished data), Mpt5 and Ccr4 may act independently in the CWI pathway.

In this paper, we found that Khd1 and Ccr4 function in the

CWI signaling pathway through regulation of the *ROM2* and *LRG1* mRNAs. What is the relevance of the regulation? Khd1 has been shown to bind to bud-localized mRNAs such as *ASH1* and *MTL1* (17, 21, 35). Rho1 is known to be spatially and temporally regulated, and the regulators for the Rho1, Rom2, and Lrg1 proteins are also localized at the bud tip and bud neck (29, 45). The regulation of *ROM2* and *LRG1* mRNAs by Khd1 and Ccr4 may be involved in such spatial and temporal expressions of the encoded proteins, presumably affecting the spatial and temporal activation of Rho1.

ACKNOWLEDGMENTS

We thank Yoshikazu Ohya and Satoru Nogami (University of Tokyo) for providing the plasmid YCplac-RHO1-Q68L.

T.M. and K.I. are supported by grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports, Culture, and Technology, Japan (2009 to 2011). T.M. is supported by the Nakajima Science Foundation. X.L. is supported by the JSPS Postdoctoral Fellowship for Foreign Researchers.

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