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The calcineurin-responsive transcription factor Crz1 regulates the expression of *CMK2* via a sole CDRE site in its promoter in budding yeast

Linghuo Jiang[1]✉, Yiying Gu[1], Yongqiang Jiang[2], Liudan Wei[1], Lijun Meng[1] & Ni Guan[1]

As a yeast homolog of mammalian calcium/calmodulin-dependent protein kinase II (CaMKII), Cmk2 functions as a negative regulator of calcium signaling in *Saccharomyces cerevisiae*. We show here that the transcription expression of *CMK2* is controlled mainly by the transcription factor Crz1 and also by other factor(s) in response to calcium stress. There are four potential binding sites (calcium/calcineurin-dependent responsive elements; CDREs) for Crz1 in the promoter of *CMK2*. Through mutational analysis, we demonstrated that mutation of only the site (5′ G_{-177}AGGCT 3′), but not the other three sites, abolished the calcium-induction activity of the CMK2p-*lac*Z reporter. EMSA analysis indicated that the C-terminal region of Crz1, which contains its DNA-binding domains can bind to this site in vitro. ChIP analysis revealed that Crz1 binds to the promoter region containing this site in vivo. Therefore, the transcription expression of *CMK2* is controlled by Crz1 through a sole CDRE site the *CMK2* promoter.

Keywords *Saccharomyces cerevisiae*, Crz1, Cmk2, Promoter, Expression, Calcium

Abbreviations

CaMKII	Calcium/calmodulin-dependent protein kinase II
Crz1	Calcineurin-responsive zinc finger 1
CDRE	Calcium/calcineurin-dependent responsive element
PCR	Polymerase chain reaction
YPD	Yeast peptone dextron
ONPG	*O*-nitrophenyl-β-D-galactopyranoside

Mammalian calcium/calmodulin-dependent protein kinase II (CaMKII) is a central regulator of learning and memory that regulates calcium signaling in neural cells[1,2]. The calcium signaling pathway is highly conserved in eukaryotes[3,4]. The budding yeast *Saccharomyces cerevisiae* is the simplest eukaryotic model organism for studying calcium signaling[5–8]. There are two yeast homologs of mammalian CaMKII, Cmk1 and Cmk2[9]. Cmk2 works together with Rch1 to negatively regulate calcium uptake in yeast cells[10,11]. In addition, Cmk2 negatively regulates calcium signaling and the expression of *PMR1* and *PMC1* encoding calcium pumps at the membranes of the endoplasmic reticulum and the vacuole, respectively[12]. Cmk2 interacts with the β subunit Fas1 of the fatty acid synthetase in yeast cells[13]. Endoplasmic reticulum stress-induced nonapoptotic cell death is inhibited by Cmk2 in *S. cerevisiae*[14]. In another yeast *Schizosaccharomyces pombe*, Cmk2 negatively regulates translation through the Cpc2 kinase, an ortholog of mammalian RACK1, which is a constituent of the eukaryotic ribosome[15].

In *S. cerevisiae*, a previous study revealed that the transcription expression of *CMK2* is dependent on the calcineurin-responsive zinc finger 1 transcription factor Crz1 in response to calcium stress[14]. Blue light

[1]Laboratory of Yeast Biology and Ethanol Fermentation Technology, State Key Laboratory of Non-Food Biomass Energy Technology, Institute of Biological Sciences and Technology, National Engineering Research Center for Non-Food Biorefinery, Guangxi Biomass Engineering Technology Research Center, Guangxi Academy of Sciences, Nanning 530007, Guangxi, China. [2]Institute of Biology, Guangxi Academy of Sciences, Nanning 530007, Guangxi, China. ✉email: linghuojiang@hotmail.com

exposure also triggers Crz1-dependent transcription of *CMK2*[16]. Here, we demonstrated that Crz1 regulates the transcription expression of *CMK2* through the sole CDRE site (5' G_{-177}AGGCT 3') in its promoter.

Methods

Strains, media, reagents, primers and plasmids

The yeast diploid wild type strain BY4743, the haploid wild type strain BY4741 and their isogenic deletion mutants used in this study were obtained from Invitrogen Inc[17–19]. (Table 1). Yeast cells were maintained at 30 °C on YPD plates or SD plates (0.67% yeast nitrogen base without amino acids, 2% glucose, 2% agar and auxotrophic amino acids as needed). *O*-nitrophenyl-β-D-galactopyranoside (ONPG) was purchased from Sigma (Beijing, China). The plasmids and primers used in this study are listed in Table 1 and Additional file 1 Table S1, respectively.

DNA manipulation

To construct the full-length *CMK2p-lacZ* reporter, we first digested the plasmid pRS316-PMR1-lacZ DNA with *Kpn*I and *Eco*RI enzymes[8], to remove the 780-bp *PMR1*-promoter fragment and obtain the vector DNA. We then amplified the 780-bp *CMK2*-promoter fragment as the insert with two primers CMK2-Fa and CMK2-lacZ-R and cloned it into the vector DNA, yielding pRS316-CMK2p-lacZ. To construct mutations of four potential Crz1-binding sites in the *CMK2* promoter in the *CMK2p-lacZ* reporter (Fig. 1A), we amplified the 780-bp *CMK2*-promoter fragment with two primers CMK2-M1-F and CMK2-lacZ-R, and cloned it into the vector DNA, yielding pRS316-CMK2p-M1-lacZ, which carried a mutation of the first potential Crz1-binding site in the *CMK2* promoter. To construct the pRS316-CMK2p-M2-lacZ plasmid, we first amplified the upstream 190-bp DNA fragment with the primer pair CMK2-Fa/CMK2-M2-R, and the downstream 630-bp fragment with the primer pair CMK2-M2-F/CMK2-lacZ-R, and these two fragments were then fused together via fusion PCR with the primers CMK2-Fa and CMK2-lacZ-R. Similarly, the pRS316-CMK2p-M3-lacZ and the pRS316-CMK2p-M4-lacZ plasmids were generated with the primer pairs CMK2-M3-F/CMK2-M3-R and CMK2-M4-F/CMK2-M4-R, respectively.

To express His6-tagged full-length Crz1 protein in bacterial cells, we performed PCR amplification with the primers HIS6-CRZ1-F/HIS6-CRZ1-R from the genomic DNA of BY4743 cells, and the PCR product was cloned through recombination into pET28a, which generated the recombinant plasmid pET28a-ScCRZ1 expressing the recombinant His6-Crz1 protein. Similarly, the DNA fragment containing the C-terminal region (N339-S678) of Crz1 with its DNA-binding C_2H_2-type zinc finger domains was amplified with the primers HIS6-CRZ1-CF/HIS6-CRZ1-R and subsequently cloned and inserted into pET28a, which yielded pET28a-ScCrz1(N339-S678). All inserts were confirmed by DNA sequencing.

Induction and purification of His6-CRZ1 (N339-S638)

To express the Crz1 protein, we introduced pET28a-ScCRZ1 or pET28a-ScCRZ1 (N339-S638) into *Escherichia coli* BL21 cells. The recombinant His6-CRZ1 or His6-CRZ1(N339-S638) proteins were induced at 16 °C for 16 h with IPTG, and purified with magnetic nickel beads as described previously[20–22]. The protein was eluted sequentially from the nickel beads with 1× PBS buffer containing 75 mM, 100 mM, 125 mM and 150 mM imidazole. Total cell lysates with or without IPTG-induction and the eluted proteins were examined via 10% SDS-PAGE.

Electrophoretic mobility shift assay (EMSA)

The double-stranded probe EMSA_probe_Crz1/CMK2-F/R was prepared by annealing its two complimentary oligonucleotides (Additional file 1: Table S1), and labeled at the 3′ end with the DIG Gel Shift Kit (Roche) according to the manufacturer's recommendations.

Name	Genotype or description	Source
Strain		
BY4741	Mat*a leu2 his1 ura3*	Invitrogen
BY4743	Mat*a*/*α leu2*/*leu2 his1*/*his1 ura3*/*ura3*	Invitrogen
Haploid CRZ1-GFP strain	BY4741 *CRZ1-GFP::HIS3*	Invitrogen
Diploid CRZ1-GFP strain	BY4743 *crz1::kanR*/*crz1::kanR*	Invitrogen
Plasmid		
pET28a-ScCrz1	Expressing His6-CRZ1 (full-length) in pET28a vector	This study
pET28a-ScCrz1(N339-S678)	Expressing His6-CRZ1 (N339-S678)	This study
pRS316-PMR1p-lacZ	*PMR1* promoter + *lacZ* in pRS316 vector ($Amp^R URA3$)	[8]
pRS316-CMK2p-lacZ	*CMK2* promoter + *lacZ* in pRS316 vector ($Amp^R URA3$)	This study
pRS316-CMK2p-lacZ-M1	pRS316-CMK2p-lacZ with M1 mutation	This study
pRS316-CMK2p-lacZ-M2	pRS316-CMK2p-lacZ with M2 mutation	This study
pRS316-CMK2p-lacZ-M3	pRS316-CMK2p-lacZ with M3 mutation	This study
pRS316-CMK2p-lacZ-M4	pRS316-CMK2p-lacZ with M4 mutation	This study

Table 1. Strains and plasmids used in this study.

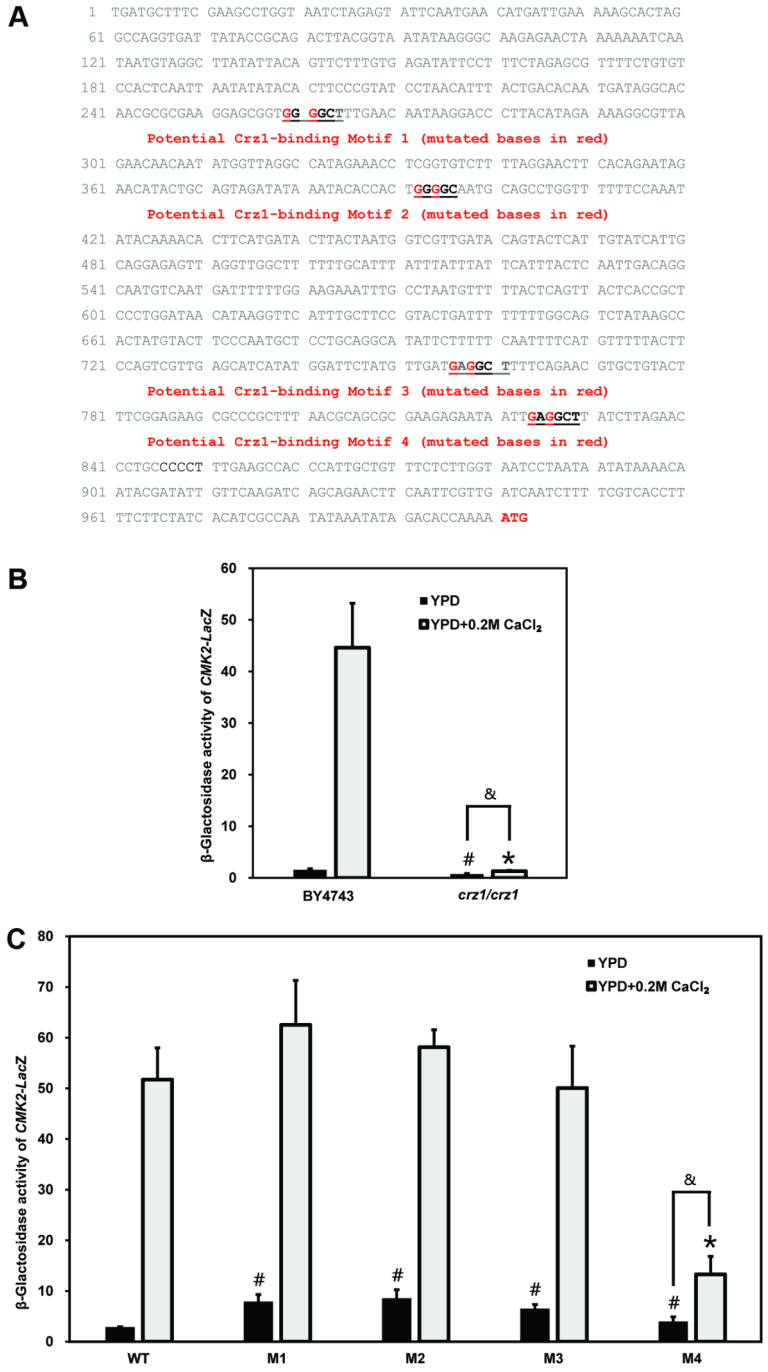

Fig. 1. The expression of *CMK2* is positively controlled by the transcription factor Crz1 through its binding to the CDRE site in the *CMK2* promoter. (**A**) Locations of four potential Crz1-binding motifs (CDRE; underlined) in the *CMK2* promoter. These motifs are predicted from the consensus motif (5' GAGGCTG 3') in the upstream regions of calcineurin/Crz1-dependent genes from a previous study[5]. Mutated nucleotides in these motifs are indicated in red. (**B**) β-Galactosidase activities of CMK2p-lacZ in the wild-type BY4743 (*CRZ1/CRZ1*) and the *crz1/crz1* mutant in the absence or presence of 0.2 M $CaCl_2$. (**C**) β-Galactosidase activities of CMK2p-lacZ (WT), CMK2p-lacZ-M1, CMK2p-lacZ-M2, CMK2p-lacZ-M3 and CMK2p-lacZ-M4 in the wild-type BY4743 in the absence or presence of 0.2 M $CaCl_2$. The data are presented as the means ± S.E.M. from six independent experiments. The cells were grown to log phase and then grown for an additional 2 h in the absence or presence of 0.2 M $CaCl_2$ before they were collected for β-galactosidase activity assay. Symbols # and * indicate statistically significant differences ($P < 0.05$) between the wild-type strain in A (or the wild-type promoter in B) and its mutant strain in A (or each of its mutant promoters in B) in the absence or presence of 0.2 M $CaCl_2$, respectively. Symbols & shows a statistically significant difference ($P < 0.05$) between CMK2p-lacZ activities of the *crz1/crz1* mutant in A (or the M4 promoter mutant in B) growing in the absence and presence of 0.2 M $CaCl_2$. The values are the means of six independent experiments.

Protein extract preparation and chromatin Immunoprecipitation (ChIP) assay

BY4741 cells expressing the nontagged wild-type Crz1 protein or the Crz1-GFP fusion protein were grown overnight in YPD and SD-HIS media, respectively. Overnight cultures were inoculated into fresh YPD media and grown to log-phase, after which they were further grown for an additional 2 h in the absence or presence of 0.2 M $CaCl_2$ before the cells were collected. The cells were treated with 1% formaldehyde, quenched with glycine and lysed with glass beads in 1 ml of lysis buffer (50 mM HEPES-KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% sodium deoxycholate; 1 mM phenylmethylsulfonyl fluoride; 1 mM benzamidine; 1 μg/ml leupeptin; and 1 μg/ml pepstatin). The resulting extracts were sonicated to obtain whole cell extracts (WCEs).

ChIP analysis was performed as previously described[8,10], except that GFP(5G4) mouse mAB magnetic beads (Cell Signaling Technology, USA) were used. The final immunoprecipitated sample was dissolved in 20 μl of TE buffer. For the PCR assays, 1 μl of the immunoprecipitated or a 1/5 dilution of WCE material was used. The primers CHIP-CMK2-F2 and CHIP-CMK2-R were designed to amplify the 530-bp fragment flanking the CDRE site in the *CMK2* promoter. The PCR fragments were separated via electrophoresis in a 2% agarose gel.

Galactosidase activity assay and statistical analysis

The galactosidase activity of the CMK2p-*lacZ* reporter was measured as described previously[8,10]. The data are from six independent transformants and are presented as the means ± SDs. Significant differences were analyzed via GraphPad Prism version 4.00 (USA). *P* values of < 0.05 were considered significant.

Results and discussion

The transcriptional expression of *CMK2* was shown to be controlled by Crz1 in response to calcium stress[14]. To determine which site(s) in the *CMK2* promoter Crz1 binds to, we constructed the CMK2p-*lacZ* reporter pRS316-CMK2p-*lacZ*. CMK2p-*lacZ* activity was increased nearly 30 fold in response to treatment with 0.2 M $CaCl_2$ for 2 h in wild type cells, but this calcium induction was dramatically reduced by the deletion of *CRZ1* (Fig. 1B). However, CMK2p-*lacZ* activity was still induced approximately two fold in response to calcium stress in BY4743 cells lacking *CRZ1* (Fig. 1B). These results are consistent with previous observations[14].

On the basis of the consensus motif (5' GAGGCTG 3') in the upstream regions of calcineurin/Crz1-dependent genes[5], we identified four potential Crz1-binding sites in the *CMK2* promoter (Fig. 1A). Mutation analysis of these four sites revealed that mutations in M1, M2 and M3 did not affect CMK2p-*lacZ* activity, but mutation of M4 dramatically reduced CMK2p-*lacZ* activity in the wild type cells in the absence or presence of 0.2 M $CaCl_2$ (Fig. 1C). However, CMK2p-*lacZ*-M4 activity was still induced approximately three fold in response to calcium stress (Fig. 1C). Taken together, these results indicate that Crz1 likely binds to the (G_{177}AGGCT) motif in the *CMK2* promoter, and that the transcription expression of *CMK2* is controlled mainly by Crz1 in response to calcium stress. Additional factor(s) are involved in the control of *CMK2* expression independent of Crz1.

To determine whether Crz1 binds in vitro to the motif (G_{177}AGGCT) in the *CMK2* promoter, we first expressed His6-tagged full-length Crz1 in bacterial cells. However, we failed to induce the expression of the full-length His6-Crz1 protein from pET28a-ScCRZ1 with IPTG (data not shown). We then constructed pET28a-ScCrz1(N339-S678) expressing the His6-tagged C-terminal region (N339-S678) of Crz1, which contains its DNA-binding domains (Fig. 2A). His6-Crz1(N339-S678) was successfully induced (Fig. 2B), and purified as a protein of approximately 40 kDa as predicted (Fig. 2C). EMSA assays demonstrated that His6-Crz1(N339-S678) bound to the DNA probe, including the motif (Fig. 3A; Lanes 2 and 4 in Fig. 3B). The binding of His6-Crz1(N339-S678) to the probe was abolished by its specific competitor, the unlabeled probe (Lanes 3 and 5 in Fig. 3B). These results indicate that Crz1 binds to this CDRE motif in the *CMK2* promoter in vitro.

To determine whether Crz1 binds to this CDRE motif in vivo, we carried out a ChIP experiment. As controls, target PCR products were amplified from whole-cell extracts (WCEs) of the untagged Crz1 BY4741 strain and the Crz1-GFP strain in the absence or presence of 0.2 M $CaCl_2$ (Lanes 1, 2, 5 and 6 in Fig. 3C) but not from the anti-GFP IPs of the untagged Crz1 BY4741 strain growing in the absence or presence of 0.2 M $CaCl_2$ (Lanes 3 and 4). The CDRE region in the *CMK2* promoter was highly enriched in the anti-GFP IPs of the Crz1-GFP strain growing in the presence of 0.2 M $CaCl_2$ (Lane 8), but was barely detected in the anti-GFP IPs of the Crz1-GFP strain growing in the absence of 0.2 M $CaCl_2$ (Lane 7). Taken together, these results demonstrate that Crz1 binds to this CDRE region in the *CMK2* promoter of yeast cells in vivo.

Previous studies have shown that a total of 41 TFs are involved in the control of *CMK2* expression under normal, acute heat shock, sudden glucose excess, and amino acid starvation conditions, but only one of them, Crz1 has been reported to regulate the expression of *CMK2* in response to calcium stress[5,19,23–27]. Together with a previous study[19], we have shown here that Crz1 is mainly responsible for, but other factor(s) also contribute to, the transcription expression of *CMK2* in response to calcium stress. In addition, through EMSA and ChIP analysis, we have demonstrated that Crz1 regulates the transcription expression of *CMK2* via a sole CDRE site [(G_{177}AGGCT)] in its promoter. Furthermore, our previous study has shown that the *cmk2 crz1* double deletion mutant is more tolerant to calcium stress than the *crz1* single deletion mutant is, suggesting that Cmk2 has an additional Crz1-independent role in promoting calcium tolerance[12]. Here, we have shown that the transcription expression of *CMK2* is still calcium-induced in yeast cells lacking *CRZ1* or in which the Crz1-binding site in the *CMK2* promoter is mutated. Taken together, these data indicate that factors other than Crz1 control *CMK2* expression, which might contribute to the calcium tolerance of yeast cells.

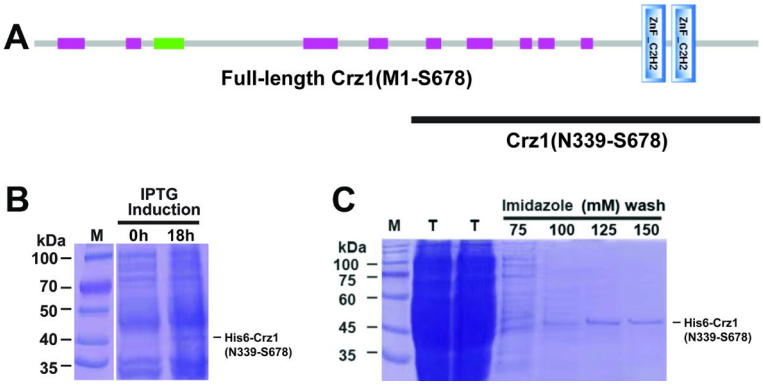

Fig. 2. Induction and purification of His6-Crz1(N339-S678). **(A)** SMART predicted full-length protein structure with two DNA-binding domains (upper panel) and the expressed C-terminal region (lower panel) of Crz1. **(B)** PAGE analysis of IPTG-induction of His6-Crz1(N339-S678). Lane M1, protein size marker (1) Lanes 0 h and 18 h, uninduced and 16-hour IPTG-induced, respectively, total cell lysate expressing His6-Crz1(N339-S678). The grouping of gels is cropped from different parts of the same gel. **(C)** PAGE analysis of His6-Crz1(N339-S678) purification. Lane M2, protein size marker (2) Lane T, sonicated total cell lysate. Lanes 75, 100, 125 and 150, elutes of nickel beads with 75 mM, 100 mM, 125 mM and 150 mM imidazole solutions after being mixed with the total cell lysate in Lane T, respectively.

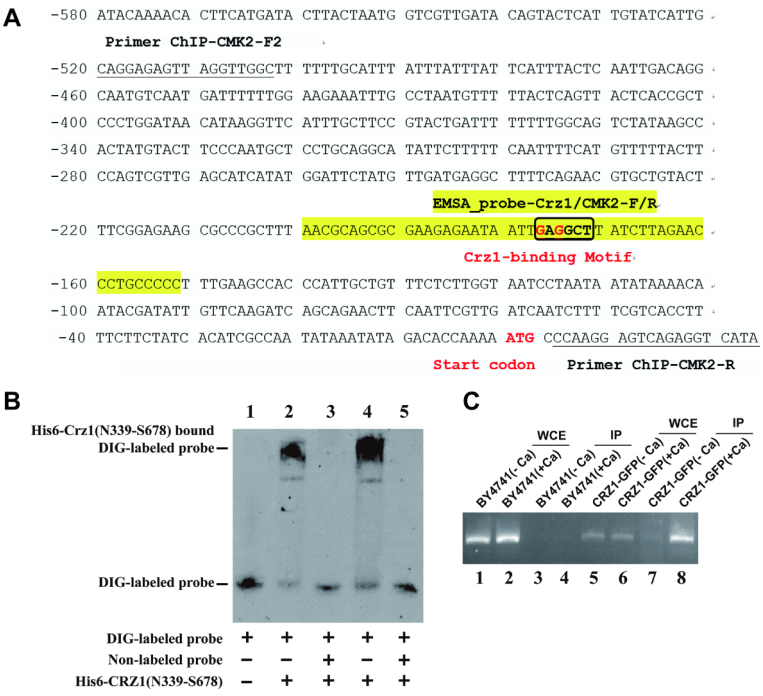

Fig. 3. The expression of *CMK2* is controlled by the transcription factor Crz1 through its binding to the sole CDRE site in the *CMK2* promoter. (**A**) Location of the sole Crz1-binding motif (CDRE; boxed) in the *CMK2* promoter that is involved in its transcription expression. The locations of the EMSA_probe-Crz1/CMK2-F/R probes are shadowed in yellow. The locations of the ChIP-PCR primer pairs [CHIP-CMK2-F and CHIP-PHR2-R] are underlined. The start codon is indicated in red. (**B**) Crz1 binds in vitro to the CDRE element (G_{177}AGGCT) in the *CMK2* promoter. An EMSA was performed with the DIG-labeled DNA probe containing the CDRE element. Lane 1, no protein control; Lanes 2 and 3, His6-CRZ1(N339-S678) (0.15 μg); Lanes 4 and 5, His6-CRZ1(N339-S678) (0.3 μg). Lanes 1–5 contain the corresponding DIG-labeled probes. Lanes 3 and 5 contain an additional specific competitor (10-fold molar excess of corresponding nonlabeled probe). (**C**) Chromatin IPs to detect Crz1-GFP binding to the CDRE motif in the *CMK2* promoter. Strains were grown in the absence (– Ca) or presence (+ Ca) of 0.2 M $CaCl_2$. DNA samples from the wild type BY4741 strain with untagged Crz1 (Lanes 1 to 4) and the Crz1-GFP strain (Lanes 5 to 8) were obtained from equal amounts of extract before (WCE; Lanes 1, 2, 5 and 6) and after anti-GFP chromatin IP (Lanes 3, 4, 7 and 8). DNA samples were used as templates to detect the 530-bp CDRE region via separate PCRs. The PCR products were separated on a 2% agarose gel.

Data availability

All data generated or analyzed during this study are included in this published article.

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Author contributions

LJ designed the study, analyzed the data and wrote the manuscript. YG, YJ, LW, LM and NG performed the experiments. All authors read and approved the final manuscript.

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Competing interests

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Additional information

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Correspondence and requests for materials should be addressed to L.J.

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