Paf1C mediates regulation of gene expression by the MAP Kinase Slt2

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Abstract
The budding yeast, *Saccharomyces cerevisiae*, has been widely used as a model organism to study the molecular mechanisms that regulate gene expression in eukaryotic cells. In the yeast Cell Wall Integrity Pathway (CWI), the protein Kinase C, Pkc1, activates the MAP Kinase Slt2, which in turn targets the transcription factors Rlm1 and SBF (Swi4-Swi6) and the transcriptional complex Paf1C, to modulate and control the expression of cell wall integrity genes. To better describe the connection between the CWI components and the transcriptional regulation of the cell integrity genes, a series of Chromatin Immunoprecipitation (ChIP) assays were performed. Our results reveal that the MAPK Slt2, associates to the promoter of several cell wall housekeeping genes like *FKS1*, *MNN1* and *GAS1*. The expression of these genes is reduced in *slt2* and *pkc1* mutant strains. However, neither the recruitment of the transcription factors Rlm1 and Swi6 to the promoter, nor the binding of the RNApol II or Paf1 to the initiation site is affected. When the association to the 3´ end of *FKS1*, *MNN1* and *GAS1* was analyzed, the RNApol II occupancy is not altered but, remarkably, the Paf1 association is importantly reduced in *slt2* and *pkc1* mutant strains. This result suggests that Slt2 is required for a stable association of Paf1C to the RNApol II along the cell wall genes and that in its absence, Paf1 dissociates from RNApol II causing a defect in RNA 3' end formation, which in turn leads to a reduced mRNA levels.

Keywords: CWI, Slt2, Paf1, transcription, yeast

Introduction

The cellular response to environmental stresses is mediated by signal transduction pathways. One of these pathways in *S. cerevisiae* is the Protein Kinase C (PKC) pathway or Cell Wall Integrity (CWI) pathway [1, 2]. The CWI pathway is activated under conditions that stress the cell wall surface, such as growth at high temperatures, hypo-osmotic shock, polarized growth, actin perturbation, or the presence of compounds or mutations that interfere with cell wall biosynthesis [3]. All these wall stresses are detected by sensor proteins that transduce the signal to the GTP bound protein Rho1, which in turn activates the kinase Pkc1. Pkc1 activates a module of protein kinases composed by the MAPKKK Bck1, 2 redundant MAPKK, Mkk1 and Mkk2 and the MAPK Slt2. Once activated, Slt2 controls the expression of the genes involved in cell wall biosynthesis through the regulation of the transcription factors Rlm1 and SBF (Swi4-Swi6), and the transcriptional complex Paf1C to maintain cell integrity [4-7].

The Paf1 complex (Paf1C) is a conserved complex in eukaryotic cells that has been related to tumorigenesis. Paf1C associates to the RNApol II throughout the entire gene regulating several transcription processes including regulation of transcriptional activators, histone modification factors, transcriptional elongation and accurate transcriptional termination [8]. The PKC pathway has been related to Paf1C since mutants in this complex showed sensitivity to different cell wall stresses, high recombination rates and defects in cell wall gene expression similar to those observed in the mutant strains *pkc1* and *slt2* [9]. More recent studies show

that Slt2 associates with Paf1C to block the premature transcription termination in the context of *FKS2*, a particular cell wall gene that is expressed only under cell wall stress conditions [7]. In this work we contribute to understand how the CWI pathway controls some of the Paf1 C functions, in the context of the transcriptional regulation of the housekeeping cell wall genes *FKS1, MNN1 and GAS1*.

Results and Discussion

***FKS1* expression is regulated by the CWI pathway.** In agreement with its function in the maintenance of the cell wall integrity, the PKC pathway regulates the expression of the *FKS1* gene, which encodes for the catalytic subunit of the (1-3)-β-glucan synthase, enzyme that catalyzes the synthesis of the cell wall polysaccharide, β-(1,3)-D-glucan [10]. We observed by northern analysis a 4-fold reduction of *FKS1* mRNA levels in the *pkc1-8* mutant strain compared with the wild type (Figure 1A). Previous studies show that the expression of *FKS1* is also reduced in the *slt2* mutant [10]. In addition, the *pkc1-8* and the *swi4* mutant strains also showed a 5-fold and 2-fold reduction, respectively, in the expression of a reporter gene driven by a fragment from -712 to -81 from the *FKS1* promoter (Figure 1B).

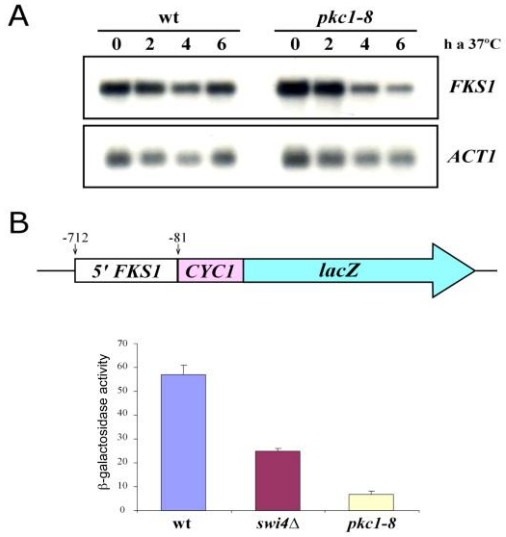


Figure 1. **Analysis of *FKS1* expression in the *pkc1-8* mutant strain.** A) Exponentially growing cultures of the wild type (W303-1a) and the *pkc1-8* (JC6-3a) mutant strain incubated at 25°C were shifted to 37°C and samples were collected at the indicated times. *FKS1* expression was analyzed by Northern blot. *ACT1* expression is shown as loading control. B) Exponentially growing cultures of the wild type (W303-1a) and the *swi4Δ* (BY604) and *pkc1-8* (JC6-3a) mutant strains transformed with a pLGL derived plasmid that contains the 5´ region of *FKS1* promoter from -712 to -81 fused to the *lacZ* coding region were incubated at 37°C for 4 hours. The β-galactosidase activity was measured as an indicator of the expression levels.

The MAP kinase Slt2 is bound to the *FKS1*atg region *in vivo*. Several MAP kinases regulate the expression of genes through its recruitment to the promoter of the target genes and the activation of the general transcriptional machinery [11, 12]. We extended the analysis to Slt2 and investigated whether the MAPK of the CWI pathway was bound to the *FKS1* upstream regulatory promoter (Figure 2B) and to the ATG region (Figure 2C). Chromatin immunoprecipitation (ChIP) assays showed that in the Slt2-HA immunoprecipitated fraction there is no specific enrichment of the *FKS1pro* fragment, but there is a specific enrichment of the *FKS1*atg fragment. This result indicated that Slt2 associates to the *FKS1* gene surrounding the ATG.

Rlm1 and Swi6 associate with the *FKS1* promoter independently of the CWI pathway. The regulation of cell wall gene expression by Slt2 is mostly mediated by the transcription factor Rlm1 [6, 13]. The ChIP analysis showed that Rlm1 was bound to its target site in the *FKS1* promoter in both the *pkc1-8* and *slt2* mutant strains (Figure 3A). This result indicated that Slt2 does not control the binding of Rlm1 to the target gene. Cell wall genes expression is also regulated by the SBF (Swi4-Swi6) transcription factor [11, 14, 15]. In fact, Slt2 interacts and phosphorylates Swi4 and Swi6 *in vitro* and *in vivo* [16, 17] and controls Swi6

subcellular localization [18]. To determine whether the PKC pathway might be controlling the SBF recruitment to the promoter of the cell wall genes we performed ChIP experiments. The ChIP results showed that Swi6 association to the *FKS1* promoter was not affected in the *slt2* mutant (Figure 3B).

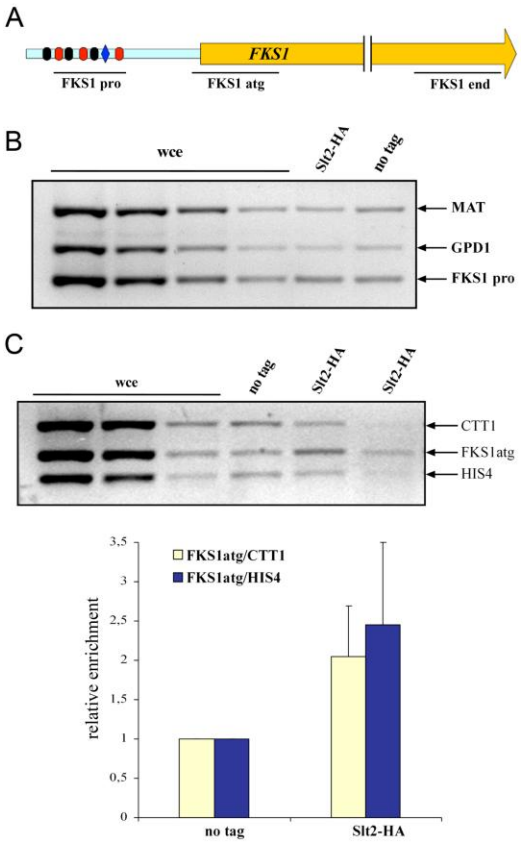


Figure 2. **Analysis of Slt2 binding to the 5' upstream region of the *FKS1* gene.** A) Diagram showing the DNA fragments amplified by PCR in the ChIP assays and the regulatory sequences in *FKS1* promoter (red: SCB; black: MCB; blue: Rlm1 bindig site). B and C) Analysis of the association of Slt2 to the promoter region of *FKS1* in wild type cells expressing the Slt2 protein tagged with HA epitope (JCY411) and exposed to heat shock at 37°C for 1h. The DNA samples were purified after crosslinking and immunoprecipitation of Slt2-HA. The association of Slt2 to the regulatory promoter region (FKS1pro fragment) or to the ATG region (FKS1atg fragment) was analyzed by ChIP using oligonucleotides that amplify the FKS1prom or FKS1atg and the control fragments *MAT* and *GDP1* or *CTT1* and *HIS4*. The lower panel shows the relative enrichment of the FKS1atg fragment in the strain that expresses the Slt2-HA protein *vs* the control strain without the tagging. All experiments were performed in triplicate. Figures show a significant experiment and graphs are the quantification of the showed experiment.

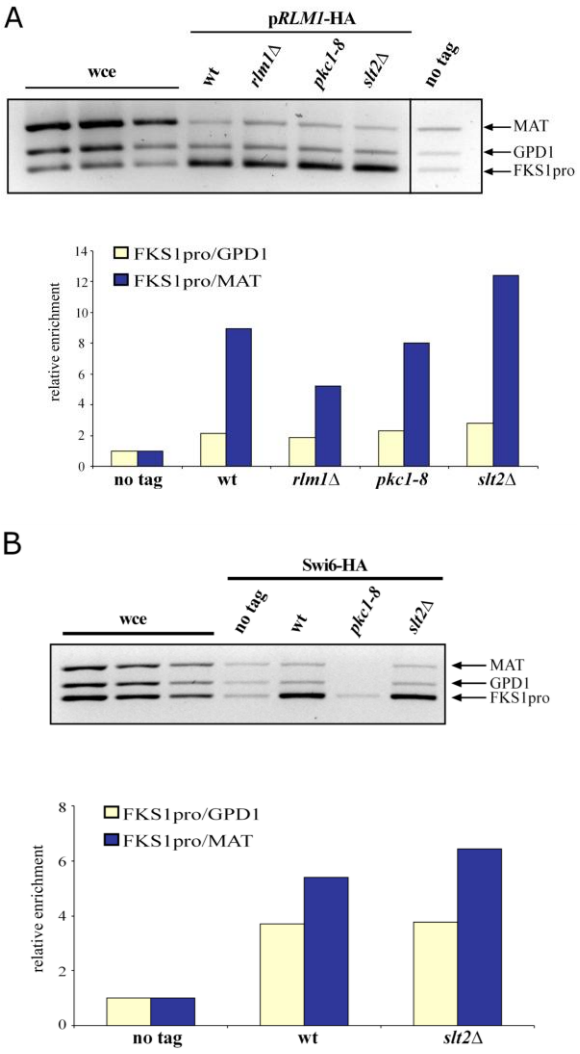
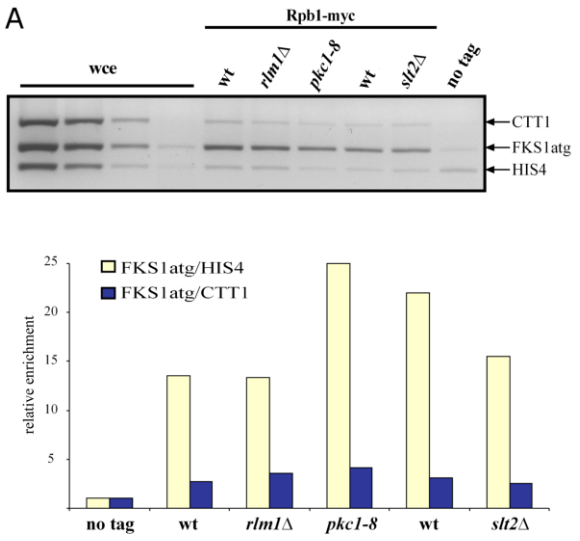


Figure 3. **Analysis of Rlm1 and Swi6 binding to *FKS1* promoter in mutants of the CWI pathway**. A) Exponentially growing cells of the wild type (W303-1a), *rlm1Δ* (JCY345), *pkc1-8* (JC63-a) and

slt2Δ (JCY464) mutants containing the pRLM1-HA plasmid were incubated at 37°C for 4 h. The association of Rlm1 to the promoter region (FKS1pro fragment) was analyzed by ChIP as described in Figure 2. B) Exponentially growing cells of the wild type (JCY114), *pkc1-8* (JCY456) and *slt2Δ* (JCY465) mutants expressing a HA-tagged version of Swi6 were incubated at 37°C for 4 h. The association of Swi6 to the promoter region (FKS1pro fragment) was analyzed by ChIP as described in Figure 2.

The Rpb1 occupancy of the ATG and the 3´end regions of *FKS1* is not regulated by the PKC pathway. In the case of the yeast MAPK Hog1, its activity is required for the binding of the RNApol II to Hog1-regulated genes [11]. Therefore, we explored whether the CWI pathway is necessary for the recruitment of the RNApol II to the cell wall genes. However, ChIP analysis revealed that the large subunit of the RNApol II, Rpb1, is normally associated to the 5´ and 3´ ends of the *FKS1* gene in the *pkc1-8*, *slt2* or *rlm1* mutant strains (Figure 4). The same result was observed in the case of other cell wall genes like *MNN1* and *GAS1* (data not shown).



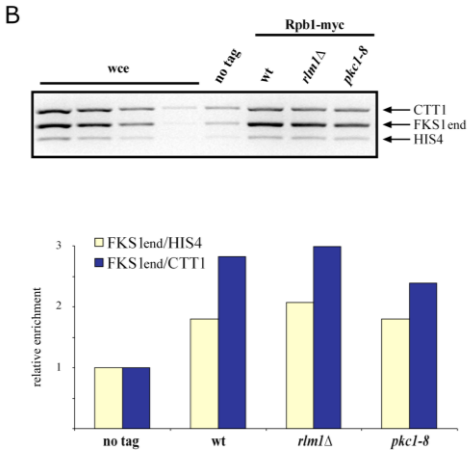


Figure 4. **Analysis of RNA pol II association to the ATG and 3' end region of *FKS1* gene in mutants of the CWI pathway.** Exponentially growing cells of the wild type strains (PAY225 and JCY651) and the *rlm1Δ* (JCY655), *pkc1-8* (JCY657) and *slt2Δ* (JCY653) mutants expressing the RNApol II subunit Rpb1 tagged with the myc epitope, were incubated at 37°C for 4 h. The association of Rpb1 to the ATG (A) or the 3' end (B) regions of the *FKS1* gene was analyzed by ChIP as described in Figure 2.

Paf1 associates to the *FKS1* ATG region independently of the PKC pathway. The *FKS1* expression in the *paf1* mutant is reduced 3-5 fold compared with the wild type strain [9]. We did not observe binding of the Paf1 protein to the upstream regulatory region of the *FKS1* promoter (data not shown). Remarkably however, Paf1 showed a strong association to the ATG region (Figure 5A). To explore the possibility whether the PKC pathway could affect the Paf1 association to the *FKS1* ATG region we carried out ChIP assay in different mutants of the PKC pathway. The results indicated that Paf1 association to the ATG region was not affected in either the *pkc1-8*, *slt2* or *rlm1* mutant cells (Figure 5A). The same result was observed in the case of other cell wall genes like *MNN1* and *GAS1* (data not shown).

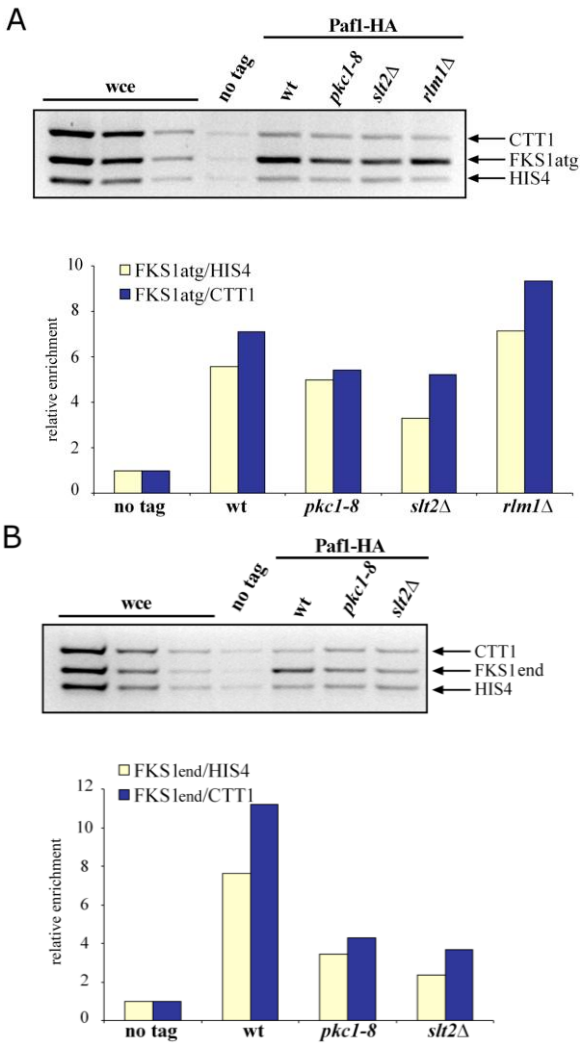


Figure 5. **Analysis of Paf1 association to the ATG and 3' end region of *FKS1* gene in mutants of the CWI pathway.** Exponentially growing cells of the wild type W303-1a (JCY870) and the *pkc1-8* (JCY893), *slt2Δ* (JCY896) and *rlm1Δ* (JCY895) mutant strains expressing the Paf1 protein tagged with the HA epitope, were incubated at 37°C for 4 h. The association of Paf1 to the ATG region (A) or to the end of the coding region (B) was analyzed by ChIP as described in Figure 2.

Stable association of Paf1 to the *FKS1* 3´ end region is dependent on the CWI pathway. Paf1 associates to the RNApol II complex in order to play different roles in transcription elongation and termination. Because of that, we also analysed Paf1 binding at the end of the *FKS1* gene. The results indicated that Paf1 is present at the 3' end of the *FKS1* gene. Remarkably, Paf1 association to the 3´ end was importantly reduced in the *slt2* and *pkc1* mutant strains (Figure 5B). This result indicates that the PKC pathway is required for a stable Paf1 association to the transcriptional machinery. Recently, it has been described that Slt2 regulates Paf1 to control transcription elongation of the *FKS2* gene by blocking the recruitment of the transcriptional attenuation complex Sen1-NrdI-Nab3 [7]. *FKS2* is a particular cell wall gene that is expressed only under cell wall stress conditions. Our results indicate that Slt2 might play a more general function in the regulation of cell wall gene expression through the control of Paf1 function. We suggest that Slt2 is required for a stable association of Paf1C to the RNApol II along the cell wall genes and that in its absence, Paf1 dissociates from RNApol II. This could cause a defect in RNA 3' end formation, which in turn could lead to a reduced mRNA level (Figure 6).

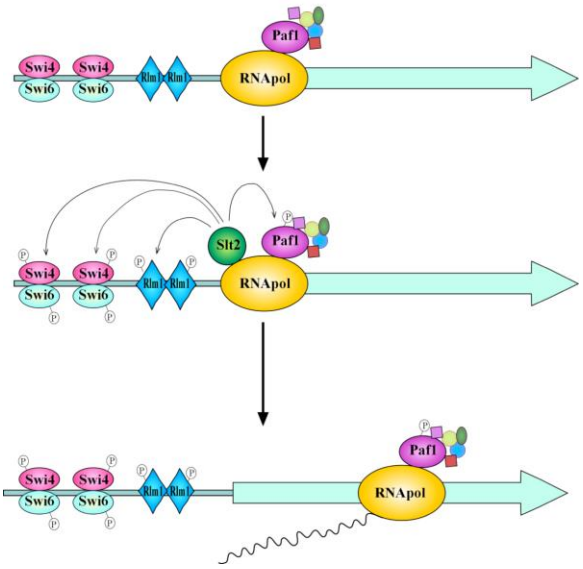


Figure 6. Model for the transcriptional regulation of *FKS1* by Slt2 (see text).

Materials and methods

Strains and growth conditions. The yeast strains used in this study are shown in Table 1. The tagging of proteins with HA or MYC epitopes was performed by integrating the PCR amplified fragments that codify for HA or MYC and the selection marker as described in Longtine *et al*., 1998 [19]. Cells were grown on standard yeast extract–peptone– dextrose or synthetic dextrose medium supplemented as required.

Plasmids. The promotor region of *FKS1* was amplify by PCR using specific oligonucleotides, and cloned in the XhoI-SmaI site at the MCS of the pLGL vector that contains the reporter gene LacZ fused to the minimum promoter region of the CYC gene. The plasmid pRLM1-HA derived from Yep181-Rlm1 (gift from Dr. Irie).

Table 1. Yeast strains

W303-1a	MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-52 can1 rad5-535	
JC6-3a	MATa ade2 trp1 leu2 his3 ura3 can1 met - pkc1-8	Our group
BY604	MATα ade2 trp1 leu2 his3 ura3 met - can1 ho::lacZ swi4::LEU2	Dr. L. Breeden
PAY225	RPB1-myc in W303-1a	Dr. P. Alepuz
SEY6211	MATa ade2 trp1 leu2 his3 ura3 suc2-Δ9	Dr. D.E. Levin
SEY6211DK	slt2::LEU2 in SEY6211	Dr. D.E. Levin
JCY114	SWI6:3HA:kanMX6 in W303-1a	Our group
JCY345	rlm1::TRP1 in W303-1a	This work
JCY411	SLT2:3HA:kanMX6 in W303-1a	Our group
JCY456	SWI6:3HA:kanMX6 in JC63a (pkc1-8)	This work
JCY464	slt2::TRP1 in W303-1a	Our group
JCY465	SWI6:3HA:kanMX6 in JCY464 (slt2::TRP1)	This work
JCY536	rlm1::LEU2 in W303-1a	This work
JCY651	RPB1:18myc:TRP1 in SEY6211 (wt)	This work
JCY653	RPB1:18myc:TRP1 in SEY6211DK (slt2::LEU2)	This work
JCY655	RPB1:18myc:TRP1 in JCY536 (rlm1::LEU2)	This work
JCY657	RPB1:18myc:TRP1 in JC63a (pkc1-8)	This work
JCY870	PAF1:3HA:kanMX6 in W303-1a	This work
JCY893	PAF1:3HA:kanMX6 in JC63a (pkc1-8)	This work
JCY895	PAF1:3HA:kanMX6 in JCY345 (rlm1::TRP1)	This work
JCY896	PAF1:3HA:kanMX6 in JCY464 (slt2::TRP1)	This work

ß-galactosidase assay. 5.10^7-10^8 cells from each sample were resuspended in 200 µL of Z Buffer (60 mM Na2HPO4, 40 mM NaH2PO4·H2O, 10 mM KCl, 1 mM MgSO4, 0.1% of β-mercaptoethanol, pH 7.0) in presence of zymoliase20T 1mg/mL. After incubation for 20 min at 30°C, and centrifugation step the extract is obtained. Equal extract quantities of each sample were adjusted with Z buffer up to a final volume of 900 µL. Next, 180 µL of ONPG (o-nitrophenil-β-D-galactopiranside (4 mg/ml) was added. Samples were incubated at 30°C and when they develop yellow color the reaction was stopped by adding 450 µL of 1M Na2CO3. The OD at 420 nm was measured. The activity is expressed as U/mg of total protein, where 1 U is defined as A420·103/min. (incubation time). The total protein concentration was quantified by Bradford method.

Chromatin Immunoprecipitation (ChIP*)* Approximately 5.10^8 cells were fixed in growth medium by adding formaldehyde to a final concentration of 1%, following by a short incubation at room temperature for 20 min and overnight incubation with agitation at 4°C. Cells were washed four times with TBS (20 mM Tris pH 7.5, 150 mM NaCl), resuspended in lysis buffer (50mM HEPES pH 7.5, 140 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1mM PMSF, complete protease inhibitors from Roche), and broken with glass beads. Lysis buffer was supplemented to a final volume of 500 µL, and samples were sonicated (four pulses of 1 min) and microfuged. Protein concentration was determined, and adjusted if necessary in order to use the same amount of total protein in each sample. An aliquot of 50 µL

was collected as a control of whole cell extract (WCE), and was supplemented with 200 µL of TE-1%SDS. Tagged proteins were immunoprecipitated by addition of anti-HA or anti-MYC (Roche) and incubation at 4°C for 7h with agitation. Samples were successively washed twice for 5 min at 4°C with 1 ml of lysis buffer, lysis buffer containing 500mM NaCl, LiCl buffer (10mM Tris pH 8.0, 250mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM EDTA) and TE. Elution of the retained proteins was done in two steps by adding 100 µL of TE-1% SDS and TE-0.67% SDS in each step and incubating at 65°C for 15 min. For the reversion of crosslinking, the samples were incubated at 65°C for 15 h. DNA for PCR reactions was purified by proteinase K digestion, phenol extraction and ethanol precipitation. The oligonucleotides used, amplify the *FKS1* regulatory region (FKS1pro) from -619 to -368, the ATG region (FKS1atg) from -11 to +291 and the 3´terminal region (FKS1end) from +5263 to +5567. Oligonucleotides were also design to amplify control fragments from *CTT1, MAD, GPD1* and *HIS4* genes. All primer sequences are available on request.

Northern blot. Assays were conducted as described by Taberner *et al*., (2012) [20].

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