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Understanding the mechanism of glucose-induced relief of Rgt1-mediated repression in yeast☆

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ABSTRACT

The yeast Rgt1 repressor inhibits transcription of the glucose transporter (*HXT*) genes in the absence of glucose. It does so by recruiting the general corepressor complex Ssn6-Tup1 and the *HXT* corepressor Mth1. In the presence of glucose, Rgt1 is phosphorylated by the cAMP-activated protein kinase A (PKA) and dissociates from the *HXT* promoters, resulting in expression of *HXT* genes. In this study, using Rgt1 chimeras that bind DNA constitutively, we investigate how glucose regulates Rgt1 function. Our results show that the DNA-bound Rgt1 constructs repress expression of the *HXT1* gene in conjunction with Ssn6-Tup1 and Mth1, and that this repression is lifted when they dissociate from Ssn6-Tup1 in high glucose conditions. Mth1 mediates the interaction between the Rgt1 constructs and Ssn6-Tup1, and glucose-induced downregulation of Mth1 enables PKA to phosphorylate the Rgt1 constructs. This phosphorylation induces dissociation of Ssn6-Tup1 from the DNA-bound Rgt1 constructs, resulting in derepression of *HXT* gene expression. Therefore, Rgt1 removal from DNA occurs in response to glucose but is not necessary for glucose induction of *HXT* gene expression, suggesting that glucose regulates Rgt1 function by primarily modulating the Rgt1 interaction with Ssn6-Tup1.

1. Introduction

The yeast Rgt1 repressor is a DNA-binding transcription factor that regulates expression of glucose responsive genes, including genes encoding a family of glucose transporters (HXTs) [1,2]. Rgt1 represses expression of *HXT* genes in the absence of glucose by recruiting the general corepressor Ssn6-Tup1 complex, which in turn recruits global corepressors, such as chromatin and nucleosome remodelers, or directly interacts with the RNA transcription machinery [1,3–5]. Ssn6-Tup1 also functions by masking the activation domain of a DNA-binding repressor and thereby preventing recruitment of the coactivators necessary for transcriptional activation [6]. Thus, Ssn6-Tup1 may act differently on different repressors, but an efficient recruitment of Ssn6-Tup1 by gene specific repressors may be critical for establishing repression.

Rgt1-dependent, Ssn6-Tup1-mediated repression occurs in conjunction with the paralogous proteins Mth1 and Std1. Rgt1 does not bind DNA, which thereby causes constitutive expression of *HXT* genes, in cells lacking Mth1 and Std1 [7–13]. Mth1 and Std1 directly interact with Rgt1, enabling Rgt1 to recruit Ssn6-Tup1 to the *HXT* promoters in the absence of glucose, but are degraded by the proteasome in the presence of high levels of glucose, implicating Mth1 and Std1 as Rgt1 regulators [10–14]. However, evidence also indicates that deletion of the *STD1* gene alone has little effect on the regulation of *HXT* gene expression [8,9,15]. Glucose stimulates proteasomal degradation of Std1 but also induces expression of *STD1* gene expression, suggesting attenuation of Std1 degradation by feedback regulation of Std1 expression. By contrast, glucose stimulates Mth1 degradation [14–17] but at the same time represses expression of the *MTH1* gene [9,15]. Therefore, Mth1 degradation is reinforced by glucose repression of *MTH1* gene expression, ensuring rapid removal of Mth1 from cells when glucose becomes available so as to enables prompt induction of *HXT* gene expression. Hence, glucose likely modulates Rgt1 function by mainly regulating Mth1 levels [18].

Rgt1 is phosphorylated and dissociated from the *HXT* promoters in cells grown in high glucose [3,19]. Rgt1 is a phosphoprotein; it is phosphorylated at a basal level in the absence of glucose, but hyperphosphorylated by PKA in high levels of glucose [20–23]. Rgt1 is

Abbreviations: ChIP, chromatin immunoprecipitation; ECL, enhanced chemiluminescence; IP, immunoprecipitation; PKA, protein kinase A.

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Table 1
S. cerevisiae strains used in this study.

Strain	Genotype	Source
BY4741	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ*	[37]
FM557	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 rgt1::kanMX*	[37]
YM6545	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 RGT2–1*	[15]
JKY98	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 rgt1::kanMX pHXT1::NAT*	This study
KFY35	*Matα his3Δ1 leu2Δ0 ura3Δ0 met15Δ mth1::kanMX*	[37]
KFY56	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ SSN6-TAP-HIS3MX6*	[38]

phosphorylated at four serine residues within its amino-terminal region, but this does not occur until Mth1 is degraded [24]. The PKA phosphorylation of Rgt1 inhibits its interaction with Ssn6-Tup1 and this phosphorylation is inhibited by Mth1, suggesting that Mth1 mediates the interaction between Rgt1 and Ssn6-Tup1 by inhibiting Rgt1 phosphorylation [25]. Interestingly, a recent work shows that Rgt1 bound to the *HXT1* promoter does not inhibit glucose induction of *HXT1* gene expression in cells lacking Ssn6 or Tup1, raising a possibility that glucose-induced Rgt1 removal from DNA may be not the primary cause of glucose induction of *HXT* gene expression [25]. The relief of Ssn6-Tup1-mediated repression comes about through the destruction or inactivation of the individual repressors, resulting in dissociation of the repressors from Ssn6-Tup1 and/or DNA [4]. Based on these observations, we have hypothesized that dissociation of Rgt1 from DNA occurs in response to glucose, but is not required for glucose induction of *HXT* gene expression, and that Rgt1 dissociation from Ssn6-Tup1 may be sufficient to lift Rgt1-mediated repression.

The goal of this study is to provide direct evidence to support this hypothesis. To do so, we examined glucose regulation of LexA-Rgt1 and GFP-Rgt1 fusions that bind DNA constitutively and found that the Rgt1 constructs repress *HXT1* gene expression in conjunction with Mth1 and Ssn6-Tup1 in the absence of glucose, and that this repression is lifted when they are phosphorylated and dissociated from Ssn6-Tup1 in the presence of glucose. We observed, however, that the Rgt1 constructs lacking PKA phosphorylation sites did not dissociate from Ssn6-Tup1 and thereby repress expression of the *HXT1* gene constitutively. Our results suggest that glucose induction of *HXT* gene expression results primarily from the disruption of the Rgt1-Ssn6-Tup1 interaction, rather than from Rgt1 removal from DNA.

2. Materials and methods

2.1. Yeast strains and plasmids

Yeast strains used in this study are listed in Table 1. Except where indicated, yeast strains were grown in YP (2% bacto-peptone, 1% yeast extract) and SC (synthetic yeast nitrogen base media containing 0.17% yeast nitrogen base and 0.5% ammonium sulfate) supplemented with the appropriate amino acids and carbon sources.

2.2. Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously [3]. Yeast strains were grown till mid-log phase ($O.D_{600nm}$ = 1.2–1.5) and incubated with formaldehyde (1% final concentration) at room temperature for 15 to 20 min. The cross-linking reaction was quenched by adding glycine to a final concentration of 125 mM for 5 min. The cells were disrupted by vortexing with acid-washed glass beads in ice cold ChIP lysis buffer (50 mM HEPES–KOH, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% Na-deoxycholate) containing protease and phosphatase inhibitors. The lysate was sonicated (ultrasonic cell disruptor with a microtip) five times with 10 s pulse. The genomic DNA fragments were immunoprecipitated with anti-HA, LexA, GFP or Ssn6 antibody (Santa Cruz) conjugated with agarose beads. After washing the immunoprecipitated beads, DNA was eluted from both immunoprecipitated and 1/100 input samples. The immunoprecipitated DNA was PCR-amplified using primer pairs directed against the *HXT1* promoter. As a negative control, primer sets were designed to amplify the actin gene promoter region. DNA-binding of Rgt1 was determined by running the PCR products of linear range in 1.5% agarose gel and visualizing by ethidium bromide staining.

2.3. Western blot and immunoprecipitation (IP) analysis

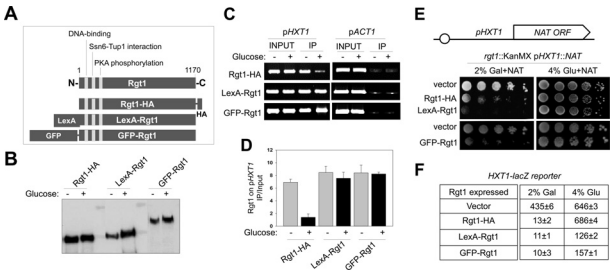
For Western blot analysis, yeast cells ($O.D_{600}$ = 1.5) were collected by centrifugation at 3000 rpm in a table-top centrifuge for 5 min. The cell pellets were resuspended in 100 μl of SDS-buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol) and boiled for 5 min. After the lysates were cleared by centrifugation at 12,000 rpm for 10 min, soluble proteins were resolved by SDS–PAGE and transferred to PVDF membrane (Millipore). The membranes were incubated with appropriate antibodies (anti-HA, anti-LexA, anti -GFP and anti-TAP antibodies, Santa Cruz) in TBST buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) and proteins were detected by the enhanced chemiluminescence (ECL) system. For IP, yeast cells were disrupted by vortexing with acid-washed glass beads in ice cold NP40 buffer (1% NP40, 150 mM NaCl, 50 mM Tris–HCl, pH 8.0) containing protease and phosphatase inhibitors. The cell lysates were incubated with appropriate antibodies at 4 °C for 3 h and further incubated with protein A/G-conjugated agarose beads at 4 °C for 1 h. The precipitated agarose beads were washed three times with ice cold NP40 buffer containing protease and phosphatase inhibitor cocktails (Sigma P8215 and Sigma P0044, respectively) and boiled in 50 μl of SDS–PAGE buffer. The resulting proteins were analyzed by Western blot using appropriate antibodies.

2.4. β-Galactosidase assay

To assay β-galactosidase activity with yeast cells expressing the *HXT1-LacZ* reporter, the yeast cells were grown to mid-log phase and the assay was performed as described previously [14]. Results were given in Miller Units [$(1000 \times O.D_{420nm})/(T \times V \times O.D_{600nm})$, where T was the incubation time in minutes, and V is the volume of cells in milliliters]. The reported enzyme activities were averages of results from triplicates of three different transformants.

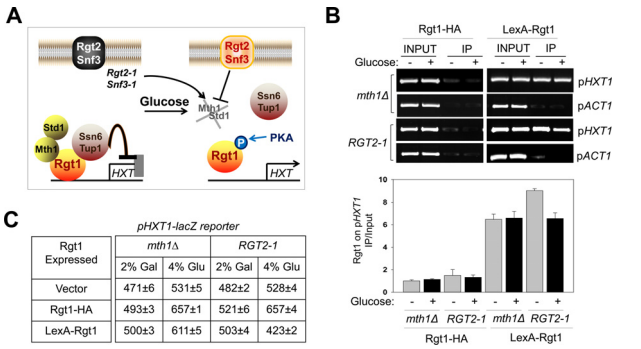
2.5. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted by RNeasy mini kit (Qiagen) following manufacturer's protocol and 2 μg of total RNA was converted to cDNA by qScript cDNA supermix (Quanta Biosciences). cDNA was analyzed by qRT-PCR using SsoFast Evagreen reagent (Bio-Rad) in CFX96 Real-time thermal cycler (Bio-Rad). *ACT1* was used as an internal control to normalize expression of *HXT1* gene. All of the shown quantification data were the averages of three independent experiments with error bars representing standard deviations (S.D.).



Rgt1 expressed	2% Gal	4% Glu
Vector	435±6	646±3
Rgt1-HA	13±2	686±4
LexA-Rgt1	11±1	126±2
GFP-Rgt1	10±3	157±1

Fig. 1. Dissociation of the Rgt1 repressor from HXT promoters is not required for glucose-induction of *HXT1* gene expression. (A) Schematic diagram of the structures of Rgt1 and its constructs. The map shows the DNA-binding domain, the Ssn6-Tup1 interaction site and the PKA phosphorylation sites. (B) Yeast cells (*rgt1Δ*) expressing Rgt1-HA (KFP60), LexA-Rgt1 (pBM3580) or GFP-Rgt1 (pBM3911) were grown in SC–2% galactose medium (−) till mid-log phase and shifted to SC–4% glucose medium (+) for 1 h. Rgt1 was subjected to Western blot analysis using anti-HA, anti-LexA or anti-GFP antibody. (C) ChIP analysis of Rgt1 binding to the *HXT1* promoter. Yeast cells (*rgt1Δ*) expressing Rgt1-HA, LexA-Rgt1 or GFP-Rgt1 were grown in SC–2% galactose medium (−) and shifted to SC–4% glucose medium (+) for 1 h, and cross-linked chromatin was precipitated using anti-HA, anti-LexA or anti-GFP antibody. Representative PCRs are shown for amplification of *HXT1* promoter. As a negative control of Rgt1 DNA binding, primer sets were designed to amplify the actin gene promoter region (p*ACT1*), which does not contain the Rgt1-binding sequence (5′-CGGANNA-3′) [3]. (D) qPCR analysis of Rgt1-binding to the *HXT1* promoter. The amount of immunoprecipitated (IP) DNA was quantified by qPCR with primer pairs directed against the *HXT1* promoter (p*HXT1*). IP/Input ratio was determined by the ratio of IP/p*HXT1* relative to the IP/p*ACT1* divided by the ratio of input/p*HXT1* relative to the INPUT/p*ACT1*. The data shown are averages of three independent experiments with error bars showing mean ± S.D. (E) The *HXT1* ORF was replaced by the *NAT* ORF by homologous recombination [26]. Empty vector, Rgt1-HA, LexA-Rgt1 and GFP-Rgt1 were expressed in the reporter strain. The reporter cells were spotted on SC-Leu or SC-Ura plate containing either 2% galactose or 4% glucose supplemented with 100 μg/ml NAT sulfate. The first spot of each row represented a count of 5×10^7 cells/ml, which is diluted 1:10 for each spot thereafter. The plates were incubated for 3 days and photographed. (F) Yeast cells (*rgt1Δ*) coexpressing p*HXT1-LacZ* reporter plasmid and Rgt1-HA, LexA-Rgt1 or GFP-Rgt1 were grown as described in (B) and assayed for β-galactosidase activity. An empty vector served as a control.



Rgt1 Expressed	mth1Δ		RGT2-1	
	2% Gal	4% Glu	2% Gal	4% Glu
Vector	471±6	531±5	482±2	528±4
Rgt1-HA	493±3	657±1	521±6	657±4
LexA-Rgt1	500±3	611±5	503±4	423±2

Fig. 2. Mth1 does not directly regulate the DNA-binding ability of Rgt1. (A) The current model of glucose-induction of *HXT* gene expression. The Rgt2 and Snf3 glucose sensors undergo a conformational change upon glucose binding and generate a signal that leads to proteasomal degradation of Mth1 and Std1. PKA phosphorylation of Rgt1, which occurs when Mth1 and Std1 are degraded, induces its dissociation from both Ssn6-Tup1 and its target promoters, leading to the expression of the *HXT* genes. *RGT2–1* and *SNF3–1* are dominant mutations that are thought to convert the proteins into the glucose-bound forms and cause glucose-independent expression of the *HXT* genes. (B) ChIP analysis of Rgt1-binding to the *HXT1* promoter. Top: Yeast cells of indicated genotypes expressing Rgt1-HA or LexA-Rgt1 were grown in SC–2% galactose medium (−) and shifted to SC–4% glucose medium (+) for 1 h and cross-linked chromatin was precipitated using anti-HA or anti-LexA antibody, and representative PCRs were shown for amplification of *HXT1* promoter. As a negative control of Rgt1 DNA-binding, primer sets were designed to amplify the actin gene promoter region (p*ACT1*). Bottom: qPCR analysis of Rgt1-binding to the *HXT1* promoter, as described in Fig. 1D. (C) Yeast cells (*mth1Δ* and *RGT2–1*) coexpressing p*HXT1-LacZ* reporter plasmid and Rgt1-HA or LexA-Rgt1 were grown as described in (B) and assayed for β-galactosidase activity. Empty vector served as a control.

3. Results

3.1. Glucose induction of HXT gene expression does not require the dissociation of Rgt1 from the HXT promoters

Rgt1 can bind DNA constitutively without inhibiting glucose induction of *HXT* gene expression in cells lacking Ssn6 or Tup1, raising the question of whether derepression of *HXT* genes does not require Rgt1 removal from the *HXT* promoters [25]. To answer this question, we tested three Rgt1 fusion proteins—Rgt1-HA (3× HA at its C-terminus, 27 aa) [19], LexA-Rgt1 (LexA at its N-terminus, 87 aa) [1], and GFP-Rgt1 (GFP at its N-terminus, 239 aa) [3]—for their ability to bind to the *HXT1* promoter (Fig. 1A and B). ChIP analysis showed that Rgt1-HA binds to the *HXT1* promoter in the absence of glucose, but is dissociated from DNA in the presence of high concentrations of glucose (Fig. 1C and D). However, LexA-Rgt1 and GFP-Rgt1 were shown to bind DNA constitutively. Thus, Rgt1-HA, like the native, untagged Rgt1, binds DNA in a glucose-regulated manner as reported previously [19,25], whereas DNA binding by LexA-Rgt1 and GFP-Rgt1 is constitutive. Thus, the addition of the LexA or GFP epitope to the N-terminus of Rgt1 seems to affect its DNA-binding property.

We next assessed the ability of the Rgt1 fusions to repress *HXT1* gene expression. The *HXT1-NAT* reporter strain expresses the *NAT* resistance gene under the control of the *HXT1* promoter. Hence, the strain is susceptible to nourseothricin in the absence of glucose (2% Gal + NAT), but exhibits resistance to the antibiotic in the presence of glucose [26]. The reporter strains expressing the Rgt1 fusions were shown to grow only in glucose-containing medium (Fig. 1E), suggesting that the Rgt1 constructs repress expression of the *HXT1* promoter in the absence of glucose but negatively regulated by glucose. We also found that expression of the *HXT1-lacZ* reporter is repressed by all the Rgt1 fusions in the absence of glucose (vector vs. Rgt1 fusions) but induced in the presence of glucose by ∼52-fold (Gal vs. Glu) in cells expressing Rgt1-HA and by ∼11- and ∼15-fold (Gal vs. Glu) in cells expressing LexA-Rgt1 and GFP-Rgt1, respectively (Figs. 1F and S1A). This indicates that expression of the *HXT1-lacZ* reporter is still ∼4–5-fold repressed by LexA-Rgt1 or GFP-Rgt1 (vector vs. LexA- or GFP-Rgt1 fusions) in the presence of glucose.

3.2. Mth1 does not directly regulate the DNA-binding ability of LexA-Rgt1

The DNA-binding activity of Rgt1 is regulated by Mth1 [8,15]. Given that LexA-Rgt1 and GFP-Rgt1 binds DNA constitutively (Fig. 1), we investigated whether the DNA-binding activity of these Rgt1 constructs is regulated by Mth1. The glucose signal that leads to *HXT* gene expression is generated by the Rgt2 and Snf3 glucose sensors at the plasma membrane [27] (Fig. 2A). Dominant mutations in the glucose sensor genes (*SNF3–1* and *RGT2–1*) cause Mth1 degradation and thereby *HXT* gene expression in a glucose-independent manner [16,28,29]. Consistent with these observations, Rgt1 does not bind DNA regardless of the presence of glucose in *mth1Δ* or *RGT2–1* strain [8]. In *mth1Δ* or *RGT2–1* strain, the Rgt1-HA fusion was shown not to bind do the *HXT1* promoter, but the DNA-binding of the LexA-Rgt1 fusion was constitutive (Fig. 2B). Thus, Mth1 is not required for the DNA-binding of LexA-Rgt1. Despite of this discrepancy, neither Rgt1-HA nor LexA-Rgt1 was able to repress expression of *HXT1-lacZ* reporter in the strain (*mth1Δ* or *RGT2–1*) (Figs. 2C and S1B). These results suggest that Mth1 may regulate the function of LexA-Rgt1 without directly affecting its DNA-binding ability.

3.3. LexA-Rgt1 function is regulated by its phosphorylation state

Our findings that LexA-Rgt1 binds DNA constitutively without significant inhibition of glucose-induction of *HXT* gene expression support the view that Rgt1 dissociation from DNA may not be required for glucose-induction of *HXT* gene expression (Fig. 1). Rgt1 function is

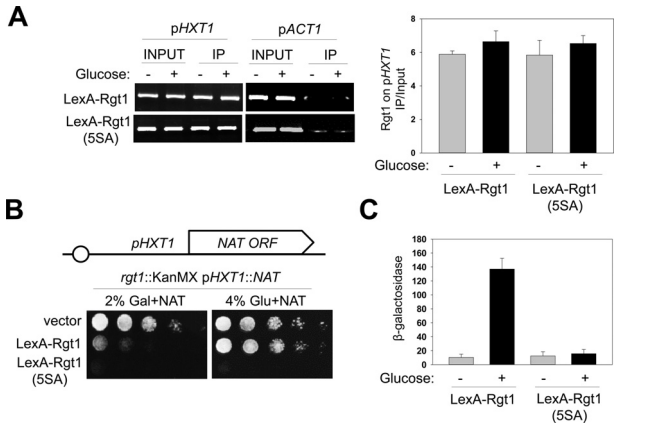


Fig. 3. Rgt1 phosphorylation at the PKA sites is required for glucose-induction of *HXT* gene expression. (A) ChIP analysis of LexA-Rgt1-binding to the *HXT1* promoter was carried out as described in Fig. 1C (left). The anti-LexA antibody was used to precipitate chromatin. Right: qPCR analysis of Rgt1-binding to the *HXT1* promoter, as described in Fig. 1D. (B) The p*HXT1-NAT* reporter strain (JKY98) expressing empty vector, LexA-Rgt1 or LexA-Rgt1 (5SA) was spotted on SC-Leu plate containing either 2% galactose or 4% glucose supplemented with 100 μg/ml NAT sulfate, as described in Fig. 1E. The plates were incubated for 3 days and photographed. (C) Yeast cells (*rgt1Δ*) coexpressing the p*HXT1-LacZ* reporter plasmid and LexA-Rgt1 or LexA-Rgt1 (5SA) were grown in SC–2% galactose medium (−) and shifted to SC–4% glucose medium (+) for 1 h and assayed for β-galactosidase activity.

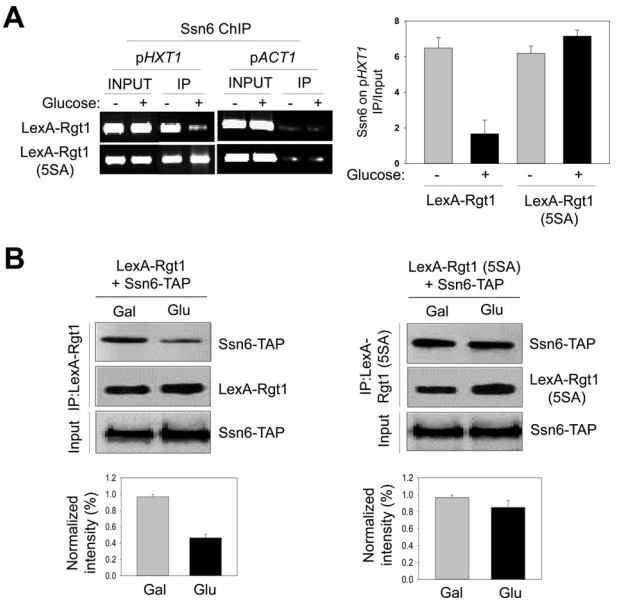


Fig. 4. The interaction of Rgt1 with Ssn6-Tup1 is critically regulated by its phosphorylation state. (A) ChIP analysis of the interaction of Ssn6 with Rgt1. Left: Yeast cells (*rgt1Δ*) expressing LexA-Rgt1 or LexA-Rgt1 (5SA) were grown in SC–2% galactose medium (−) and shifted to SC–4% glucose medium (+) for 1 h, and cross-linked chromatin was precipitated using anti-Ssn6 antibody. Representative PCRs are shown for the amplification of *HXT1* promoter. As a negative control, primer sets were designed to amplify the actin gene promoter region (p*ACT1*). Right: qPCR analysis of Rgt1-binding to the *HXT1* promoter, as described in Fig. 1D. (B) Co-IP analysis of the interaction of Rgt1 with Ssn6. Yeast cells coexpressing Ssn6-TAP [38] and LexA-Rgt1 (left) or LexA-Rgt1(5SA) (right) were grown in SC–2% galactose medium (−) till mid-log phase and shifted to SC–4% glucose medium (+) for 1 h. Cell extracts were immunoprecipitated with anti-LexA antibody (IP) and immunoblotted with either anti-LexA or anti-TAP antibody. Expression of Ssn6-TAP was analyzed by Western blot (Input). Quantification of Ssn6-TAP immunoprecipitated with LexA-Rgt1 protein is shown (bottom).

critically regulated by its phosphorylation by PKA [22–24]. Thus, we examined whether the function of the DNA-bound LexA-Rgt1 is regulated by its phosphorylation state. To this end, we explored the ability of the wild type LexA-Rgt1 and the mutant LexA-Rgt1 (5SA) lacking the PKA phosphorylation sites (S96, S146, S202, S283 and S284) to regulate the *HXT1* promoter. Both LexA-Rgt1 and LexA-Rgt1 (5SA) were shown to bind to the *HXT1* promoter constitutively, suggesting that the phosphorylation state of LexA-Rgt1 does not regulate its DNA-binding ability (Fig. 3A, top). However, the colony assay, performed as described above (Fig. 1E), demonstrated that LexA-Rgt1 (5SA), but not by LexA-Rgt1, constitutively inhibits the expression of the *NAT* resistant gene and thereby cell growth in the glucose medium (Fig. 3B). Consistently, LexA-Rgt1 (5SA) was shown to inhibit glucose-induced expression of the reporter gene (Figs. 3C and S1C). These results suggest that the function of the LexA-Rgt1 repressor, bound to the *HXT* promoters constitutively, is critically regulated by its phosphorylation state.

3.4. The phosphorylation state of LexA-Rgt1 regulates its affinity for Ssn6-Tup1

Given that glucose-induced expression of *HXT* genes requires the dissociation of Rgt1 from Ssn6-Tup1 [25], we examined the ability of LexA-Rgt1 and LexA-Rgt1 (5SA) to recruit Ssn6-Tup1 to the *HXT1* promoter by ChIP analysis using the anti-Ssn6 antibody. LexA-Rgt1 appeared to recruit Ssn6-Tup1 to the *HXT1* promoter in a glucose-dependent manner (Fig. 4A, left). Ssn6-Tup1 was associated with the *HXT1* promoter in the absence of glucose but was largely dissociated from the promoter when glucose is present; however, Ssn6-Tup1 was constitutively recruited to the *HXT1* promoter in cells expressing LexA-Rgt1 (5SA), suggesting that blocking glucose-induced PKA phosphorylation of Rgt1 enables it to recruit Ssn6-Tup1 (Fig. 4A, right).

Finally, we explored the effect of the phosphorylation defective mutation of Rgt1 (5SA) on the interaction between LexA-Rgt1 and Ssn6-Tup1. To do so, we coexpressed LexA-Rgt1 or LexA-Rgt1 (5SA) and Ssn6-TAP, and performed co-immunoprecipitation experiments with the anti-LexA antibody. The interaction of LexA-Rgt1 with Ssn6-TAP was strongly detected in galactose-grown cells, but significantly reduced in glucose-grown cells. Notably, the interaction between LexA-Rgt1 (5SA) and Ssn6-TAP occurred constitutively, reinforcing the view that the ability of LexA-Rgt1 to recruit Ssn6-Tup1 is regulated by its phosphorylation state (Fig. 4B).

4. Discussion

In this study, we provide evidence that glucose induction of *HXT* gene expression results primarily from the disruption of the Rgt1-Ssn6-Tup1 interaction, rather than from Rgt1 removal from the *HXT* promoters. It has been well established that Rgt1 binds DNA in a glucose-dependent manner. Rgt1 binds to its target promoters in the absence of glucose and dissociates from DNA in cells grown in high glucose [3,8,11,19,30,31]. An *in vitro* experiment showed that nuclear extracts from cells grown in glucose-depleted medium, but not in glucose-containing medium, can make a DNA–protein complex with a synthetic DNA sequence containing an Rgt1 recognition site ($Rgt1_{HXK2}$ probe) [23]. Here, we used three Rgt1 constructs—Rgt1-HA, LexA-Rgt1 and GFP-Rgt1 fusions—to study glucose regulation of Rgt1 function. Rgt1-HA behaves like the native, untagged Rgt1, as reported previously [11,19,25]. However, results of the ChIP experiments using anti-LexA or anti-GFP antibody show that LexA-Rgt1 and GFP-Rgt1 bind to the *HXT1* promoter constitutively (Fig. 1), and thus suggest that the N-terminal LexA or GFP moiety of the Rgt1 fusion modulates its DNA-binding property by affecting the zinc cluster DNA-binding domain at its N-terminus (aa 46–76). It should be noted that the DNA-binding of LexA-Rgt1 fusion is not detected in high glucose-grown cells by ChIP experiments using an antibody that specifically recognizes the C-terminus of Rgt1 [3,13]. Perhaps, this discrepancy is not due to the quality of these antibodies but due to

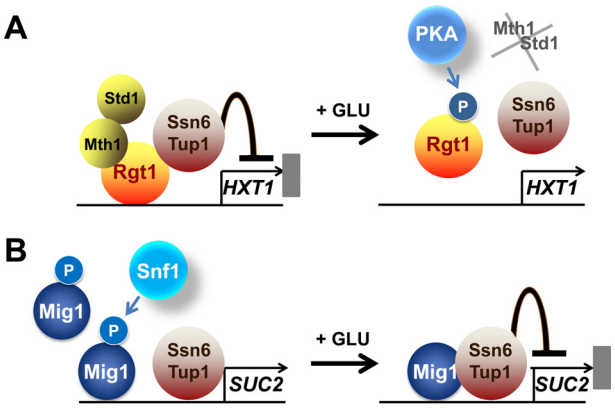


Fig. 5. Glucose regulates the function of the two major glucose responsive repressors Rgt1 and Mig1 in a similar manner. (A) A proposed model for glucose induction of *HXT* gene expression. Rgt1 recruits Ssn6-Tup1 in an Mth1-dependent manner to form a repressor complex in the absence of glucose. High glucose appears to disrupt this complex by inducing three distinct events: (1) proteasomal degradation of Mth1 via the Rgt2/Snf3 pathway; (2) repression of *MTH1* gene expression by the Snf1 (AMPK)-Mig1 pathway; and (3) Rgt1 phosphorylation by PKA (cAMP-PKA pathway). The PKA phosphorylation sites in the amino terminal region of Rgt1 become available for phosphorylation after Mth1 is degraded. Phosphorylated Rgt1 is dissociated from Ssn6-Tup1 and released from DNA, leading to expression of *HXT* genes. (B) In glucose-limited conditions, the Snf1 kinase phosphorylates and negatively regulates Mig1 by preventing the interaction between Mig1 and Ssn6-Tup1. In high glucose condition, however, Snf1 is inactive, and thereby Mig1 is dephosphorylated and recruit Ssn6-Tup1 to mediate the repression of its target genes. Therefore, the role of phosphorylation of Mig1 and Rgt1 repressors in inducing conditions is to prevent their interaction with Ssn6-Tup1 [18].

the location of the epitopes in the Rgt1 protein to which each antibody binds. Glucose induces an intramolecular interaction between the central region of Rgt1 and its N-terminal DNA-binding domain [13], suggesting the view that the C-terminus of LexA-Rgt1 may be hidden and unavailable for antibody recognition in the presence of glucose.

Glucose induces the expression of the *HXT1* gene in cells expressing Rgt1-HA, LexA-Rgt1 and GFP-Rgt1 by ~50-, ~10-, and ~15-folds, respectively. Thus, taken at face value, DNA-binding alone (by LexA-Rgt1 and GFP-Rgt1) accounts for ~4–5-fold repression (Fig. 1F). However, we argue that this repression may be associated with the ability of the Rgt1 constructs to interact with Ssn6-Tup1, rather than their ability to bind DNA. The supporting evidence is that, in response to glucose, Ssn6-Tup1 largely dissociates from Rgt1-HA but substantially associate LexA-Rgt1 (Fig. S2). The Rgt1 interaction with Ssn6-Tup1 is regulated by its phosphorylation state [25]. Hence, LexA-Rgt1 may be less efficiently phosphorylated by PKA than Rgt1-HA, enabling it to recruit Ssn6-Tup1 even in the presence of glucose. This may explain the repression of *HXT1* gene expression mediated by LexA-Rgt1 and GFP-Rgt1 in high glucose conditions. Our observations provide significant insights into the mechanism of glucose regulation of Rgt1 function: (1) Mth1 does not directly regulate the DNA-binding ability of Rgt1; rather, it mediates the Rgt1 interaction with Ssn6-Tup1 by modulating Rgt1 phosphorylation by PKA [25], (2) Rgt1 dissociation from DNA occurs in a glucose-dependent manner, but is not absolutely required for the derepression of its target genes, (3) disruption of the Rgt1-Ssn6-Tup1 interaction is necessary and sufficient to lift Rgt1-mediated repression, and (4) the interaction of Rgt1 with Ssn6-Tup1 may be regulated by its phosphorylation state.

In *Kluyveromyces lactis*, expression of the glucose transporter gene *RAG1* is repressed by the Rgt1 ortholog *kl*Rgt1 in the absence of glucose. Of note, glucose induction of *RAG1* gene expression does not require dissociation of *kl*Rgt1 from the *RAG1* promoter; *kl*Rgt1 remains bound to the *RAG1* promoter even in high glucose conditions [32]. These results reinforce the view that the primary mechanism of glucose induction of *HXT* gene expression is not Rgt1 release from *HXT* promoters but its dissociation from Ssn6-Tup1. Glucose regulates Rgt1 function in a similar manner, as it does to the glucose repressor Mig1 (Fig. 5). Mig1 recruits Ssn6-Tup1 for repression in high glucose conditions [33]; however, it dissociates from Ssn6-Tup1 upon phosphorylation by the Snf1 kinase in glucose-depleted conditions, resulting in derepression of its target genes [34,35]. Thus, Mig1 binds to its target promoters under either repressing or inducing condition, supporting the view that Snf1 controls glucose repression by modulating the Mig1-Ssn6-Tup1 interaction [36]. Likewise, PKA regulates glucose induction by controlling the Rgt1 interaction with Ssn6-Tup1.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2013.12.004.

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