Active Snf1 protein kinase inhibits expression of the *Saccharomyces cerevisiae HXT1* glucose transporter gene

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Expression of *HXT1*, a gene encoding a *Saccharomyces cerevisiae* low-affinity glucose transporter, is regulated by glucose availability, being activated in the presence of glucose and inhibited when the levels of the sugar are scarce. In this study we show that Snf1 protein kinase participates actively in the inhibition of *HXT1* expression. Activation of Snf1, either by physiological conditions (growth in low-glucose conditions) or by eliminating any of its negative regulators, such as Hxk2 or Reg1, leads to an inhibition of *HXT1* expression. We also show that Std1, another known negative regulator of *HXT1* expression, interacts physically with active Snf1 protein kinase. Std1 also interacts physically with Rgt1, a transcription factor involved in *HXT1* expression, suggesting that the transcriptional properties of Rgt1 could be modulated either directly or indirectly by Std1 and Snf1 protein kinase. Finally, we show that Rgt1 interacts physically with Ssn6, a major transcriptional repressor, to regulate negatively *HXT1* expression when glucose is depleted.

Key words: glucose induction, glucose repression, two-hybrid interaction.

INTRODUCTION

In the presence of glucose, the yeast *Saccharomyces cerevisiae* represses at the level of transcription the expression of a large number of genes, including those involved in the utilization of alternative carbon sources, gluconeogenesis and respiration (see [1–3] for reviews). Biochemical and genetic studies have identified several crucial players in this pathway. Snf1 (Cat1) is a serine/threonine protein kinase that activates transcription by inhibiting transcriptional repressors (e.g. Mig1) or by stimulating transcriptional activators (e.g. Cat8 and Sip4). The Snf1 protein kinase is found in complexes containing the activating subunit Snf4 (Cat3) and members of the Sip1/Sip2/Gal83 family [4], and its activity is negatively regulated by glucose [5]. The Reg1/Glc7 protein phosphatase complex is involved in the regulation of the activity of the Snf1 kinase complex. Reg1 (Hex2) is the regulatory subunit that targets the catalytic subunit of the PP1 phosphatase (Glc7) to substrates involved in the glucose-repression pathway [6–8]. In response to a glucose signal, Glc7 (targeted by Reg1) dephosphorylates Snf1 kinase and inactivates the complex [9,10]. In the absence of Reg1, Glc7 cannot perform its function, so the Snf1 kinase complex is constitutively in the active state, even in the presence of glucose [9,10]. Another crucial component of the glucose-repression pathway is hexokinase PII (Hxk2). This protein participates also in the regulation of the Snf1 kinase complex by regulating the phosphorylation status of Reg1 [10].

Glucose is also able to induce the expression of several genes, such as those encoding glycolytic enzymes, ribosomal proteins and some glucose transporters (see [2,3,11] for reviews). *HXT1* encodes a yeast low-affinity glucose transporter whose expression is regulated by glucose availability, being activated in the presence of glucose and inhibited when levels of the sugar are scarce (see [12] for review). In the last decade, genetic and biochemical studies have defined several components that are involved in the regulation of *HXT1* expression. Glucose availability in the surrounding medium is assessed by the sensor proteins Snf3 and Rgt2, which transmit this information to the internal cellular machinery [13,14]. This signal is transmitted through the Skp1, Cullin, F-box complex (SCF)–Grr1 ubiquitination complex [15,16], and finally modulates the activity of Rgt1, a transcription factor belonging to the Cys-6-zinc cluster protein family that may show three types of activity: (i) it is an activator of *HXT1* expression when glucose is abundant; (ii) it is a repressor when glucose is absent and (iii) it shows neutral activity when cells grow in low-glucose conditions [17]. Additional components of the glucose-induction pathway are Std1 and Mth1, two proteins that modulate *HXT1* expression negatively [12]; in the absence of Std1 and Mth1, the expression of *HXT1* becomes constitutive, not being inhibited when cells are growing in conditions of low or absent glucose [18]. Recent studies indicate that Std1 and Mth1 may interact with the C-terminal tails of the glucose sensors Rgt2 and Snf3 [18,19]. The expression of *HXT1* is also affected by mutations in *HXK2* and *REG1*, genes encoding two members of the Snf1 glucose-repression pathway (see above; [12,20]).

In this study we show that both the glucose-repression and glucose-induction pathways are interconnected in the regulation of the expression of the *HXT1* low-affinity glucose transporter gene. We observed that active Snf1 protein kinase was responsible for inhibition of *HXT1* expression when glucose was depleted.

MATERIALS AND METHODS

Strains and genetic methods

S. cerevisiae strains used in this study are listed in Table 1. Strain FY250 was a gift from Dr F. Winston (Harvard Medical School, Boston, MA, U.S.A.). To construct the double mutant *reg1*Δ *snf1*Δ, a *Bam*HI fragment from pUC-snf1Δ::KanMX4 (see below) was used to introduce the *snf1*Δ*::KanMX4* mutated allele by gene disruption [21] in a *reg1*Δ*::URA3* strain; mutants were confirmed by PCR analysis using specific oligonucleotides. Strains *hxk1*Δ*::hisG* and *hxk2*Δ*::hisG* contained respectively

Abbreviations used: GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin; SC medium, synthetic complete medium; SCF, Skp1, Cullin, F-box complex.

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Table 1 A list of the strains used in this study

Strain	Genotype	Reference
W303-1A (wild type)	*MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52*	[44]
rgt2 snf3 mth1 std1	*std1::KanMX4*, derivative of MJL30 (*rgt2::LEU2 snf3::HIS3 mth1::TRP1* [19]) in W303-1A	This study
rgt2 snf3 mth1 std1 hxk1 hxk2	*hxk1::hisG hxk2::hisG*, derivative of *rgt2 snf3 mth1 std1*	This study
FY250 (wild type)	*MATα his3Δ200 leu2Δ1 trp1Δ63 ura3-52*	From F. Winston [10]
hxk1	*hxk1::hisG*, derivative of FY250	This study
hxk2	*hxk2::TRP1*, derivative of FY250	[10]
hxk1 hxk2	*hxk1Δ::HIS3 hxk2Δ::TRP1*, derivative of FY250	[10]
hxk1 hxk2 snf1	*hxk1Δ::HIS3 hxk2Δ::TRP1 snf1Δ10*, derivative of FY250	[10]
reg1	*reg1Δ::URA3*, derivative of FY250	[10]
snf1	*snf1Δ10*, derivative of FY250	[10]
reg1 snf1	*reg1Δ::URA3 snf1Δ::KanMX4*, derivative of FY250	This study
CTY10-5d	*MATa ade2 his3 leu2 trp1 gal4 gal80 URA3::lexAop-lacZ*	From R. Sternglanz [10]

fragments from nucleotide +43 to +895 (+1ATG of *HXK1*) and from nucleotide +1 to +732 (+1ATG of *HXK2*), substituted with *Salmonella typhimurium hisG* gene [22]. Strain *std1Δ::KanMX4* contained a fragment from nucleotide +412 to +1011 (+1ATG of *STD1*) substituted with the *KanMX4* module [23]. Strain CTY10-5d was a gift from Dr R. Sternglanz (State University of New York, Stony Brook, NY, U.S.A.).

Standard methods for genetic analysis and transformation were used. Yeast cultures were grown in synthetic complete (SC) medium lacking appropriate supplements to maintain selection for plasmids [24], supplemented with different carbon sources.

Oligonucleotides

In the present study we used the following oligonucleotides. STD1-1, 5′-GCCGGATCCAGATGTTTGTTTCACCACCTC-CAGCAAC-3′ (+1 ATG is underlined); STD1-4, 5′-(+1370)-TGGGGGAATTCGTTTTTCGCTTGTTG-3′; RGT1-1, 5′-CTCCAGGATCCTTCAAATTATGAACGAGCTC-3′ (+1 ATG is underlined); RGT1-2, 5′-(+3573)GCCCTCGAGCT-GAGTCGACGGGAGAACCTGACC-3′; RGT1-GFP, 5′-(+3516)ACCTGTCGACAGCGGCCGCACTCGGAATCGT-CCAACAGC-3′; SSN6-1, 5′-CCGGGGATCCAAATGAATC-CGGGCGGTGAAC-3′ (+1 ATG is underlined), and SSN6-2, 5′-(+2929)CGCTAGTCGACTAATTTTTTGAATGCAAAC-3′. The number in parentheses corresponds to the first base of the oligonucleotide with respect to ATG at position +1.

Plasmids

To construct plasmid pACTII-Std1 (GAD-Std1) we first amplified by PCR the coding region of the *STD1* gene using oligonucleotides STD1-1/STD1-4 (see above) and genomic DNA from strain FY250 as a template. The amplified fragment was sequenced to verify that the *Taq* polymerase had not introduced any undesired mutation. It was then digested with *Bam*HI and *Eco*RI and subcloned into pACTII [25]. A *Bam*HI/*Sal*I fragment from pACTII-Std1 was introduced into plasmids pEG202 [26], pHW4 [10] and pSK93 [10] to obtain plasmids pEG202-Std1 (LexA-Std1), pGST-Std1 (GST-Std1) and pSK-Std1 (HA-Std1) respectively. Plasmid pACTII-Rgt1 (GAD-Rgt1) was constructed as above using oligonucleotides RGT1-1/RGT1-2; the amplified fragment was digested with *Bam*HI and *Xho*I and subcloned into pACTII. A *Bam*HI/*Sal*I fragment from pACTII-Rgt1 was introduced into pEG202, pWS93 [27] and pSK93 [10] to obtain pEG202-Rgt1 (LexA-Rgt1), pWS-Rgt1 (HA-Rgt1) and pSK-Rgt1 (HA-Rgt1) respectively. Plasmid pGST-Ssn6 (GST-Ssn6) was constructed as above using oligonucleotides SSN6-1/SSN6-2; the amplified fragment was digested with *Bam*HI and *Sal*I and subcloned into pHW4 [10].

Plasmid pRgt1-GFP was constructed in several steps. First, we amplified by PCR the coding region of the *RGT1* gene using oligonucleotides RGT1-1/RGT1-GFP (see above); the amplified fragment was blunt-ended, subcloned into pUC18 digested with *Sma*I and dephosphorylated to obtain pUC-Rgt1-Not1. A *Not*I fragment from pSFGP1 [28] containing the green fluorescent protein (GFP) sequence was inserted into the *Not*I site of the construct, creating a C-terminal fusion between *RGT1* and *GFP*. An *Eco*RI/*Sal*I fragment containing this fusion was subcloned into pRS424-ADH1, a pRS424 [29] derivative containing the *ADH1* gene promoter, to obtain finally pRgt1-GFP. The Rgt1-GFP fusion protein was fully functional since it restored the *HXT1* expression defect of *rgt1Δ* mutants (results not shown).

To construct the *SNF1* disruption cassette containing *KanMX4* as a selection marker, we first subcloned an *Eco*RI/*Sal*I fragment from plasmid pRJ55 (LexA-Snf1 [5]) into pUC18, obtaining pUC-Snf1. This plasmid was digested with *Nco*I, blunt-ended with Klenow DNA polymerase and dNTPs, digested with *Bgl*II and then used to subclone a *Bgl*II/*Eco*RV fragment from plasmid pFA6a-KanMX4 [23], resulting in plasmid pUC-snf1Δ::KanMX4, where an inner fragment of 537 bp of the *SNF1* gene was replaced by the *KanMX4* selection marker.

Other plasmids used in this study were pLexA-Snf1, pLexA-Snf1T210A, pLexA-Snf1KD (amino acids 1–391 of Snf1) and pLexA-Snf1RD (amino acids 392–633 of Snf1) [5], pWS-Snf1, pWS-Snf1T210A and pWS-Snf1K84R [30], pHXT1-lacZ [20] and pC-HXT1-lacZ [31].

Enzyme assays

Invertase activity was assayed in whole cells as described in [32]; 1 unit was defined as the amount of enzyme that released 1 μmol of glucose/min per 100 mg of yeast (dry weight) under the assay conditions (1 unit of absorbance at 600 nm is equivalent to 0.860 mg of cells, dry weight). β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units as in [9].

Pull-down assay

Preparation of protein extracts for pull-down assays was essentially as described previously [10]. The extraction buffer was

50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol and 10% glycerol, and contained 2 mM PMSF and complete protease inhibitor cocktail (Boehringer Mannheim). GSH–agarose (Amersham Biosciences) was used in the pull-down assays. Pelleted proteins were analysed by Western blotting using anti-haemagglutinin (HA) monoclonal antibodies.

Immunoblot analysis

Protein samples were separated by SDS/PAGE using 10% polyacrylamide gels and analysed by immunoblotting using anti-glutathione S-transferase (GST) polyclonal (Amersham Biosciences) or anti-HA monoclonal (Boehringer Mannheim) antibodies. Antibodies were detected by enhanced chemiluminescence with ECL or ECL Plus reagents (Amersham Biosciences).

Microscope observations

Exponentially growing cultures were used to visualize GFP fusion proteins. Aliquots (2 μl) of the cultures were put on microscope slides and covered with 18 mm × 18 mm coverslips. Cells were then viewed using a Zeiss Axioskop II fluorescence microscope. Images were scanned with a SPOT digital camera (Diagnostic Instruments) and processed using Adobe Photoshop 5.0 software.

RESULTS AND DISCUSSION

Activated Snf1 inhibits *HXT1* expression

As described in the Introduction, genetic studies have previously demonstrated that the expression of *HXT1* was affected in mutants lacking hexokinase PII (*hxk2* mutants; [12,20]), one of the two hexokinases present in *S. cerevisiae*. To study the involvement of Hxk2 in *HXT1* regulation, we measured the expression of *HXT1*, as a transcriptional fusion of the *HXT1* promoter to bacterial β-galactosidase encoding the *lacZ* gene, in different hexokinase mutants. As observed in Table 2, *HXK2* deletion was mainly responsible for *HXT1* inhibition, although the inhibitory effect was improved when both *HXK1* and *HXK2* genes were deleted, indicating that in the absence of Hxk2, Hxk1 could partially complement the action of Hxk2. The induction of *HXT1* by glucose was also prevented in *reg1* mutants lacking the regulatory subunit of the Reg1/Glc7 protein phosphatase complex (Table 2). Since *reg1*Δ and *hxk1hxk2*Δ are glucose-repression mutants that share in common the fact that the Snf1 protein kinase complex is abnormally active in cells growing in glucose [5,10], we checked the expression of *HXT1* in double *reg1*Δ *snf1*Δ and triple *hxk1hxk2*Δ *snf1*Δ mutants and observed a recovery in the induction of *HXT1* expression by glucose (Table 2). Therefore, the inhibition of *HXT1* expression observed in *hxk1hxk2*Δ and *reg1*Δ mutants was mainly due to the presence of an abnormally active Snf1 protein kinase.

Table 2 The activation of Snf1 inhibits *HXT1* expression

Cells containing the centromeric plasmid pC-HXT1-lacZ [31] and growing exponentially in 4% glucose were harvested. Invertase and β-galactosidase activities were measured as described in the Materials and methods section. Values for invertase are means from three different transformants (S.D. < 10% in all cases) and values for β-galactosidase are means from four–six transformants (S.D. < 15% in all cases).

	β-Galactosidase (Miller units)	Invertase (units)
Wild-type FY250	88	< 1
hxk1	87	< 1
hxk2	33	25
hxk1 hxk2	3	275
hxk1 hxk2 snf1	29	< 1
reg1	2	126
reg1 snf1	82	< 1
snf1	86	< 1

Table 3 Two-hybrid interaction between Std1 and Snf1

CTY10.5d cells expressing the corresponding LexA-Snf1 fusion proteins and Gal4-activating domain (GAD)-Std1 were grown to mid-logarithmic phase in selective SC/4% glucose medium; cells were then washed with water and shifted to SC/0.05% glucose medium for 3 h. Values are mean β-galactosidase activities from four–six transformants (S.D. < 15% in all cases). Western blotting indicated that levels of interacting proteins were similar in all cases (results not shown).

		β-Galactosidase activity (Miller units)	
LexA fusion	GAD fusion	4% Glucose	Shift to 0.05% glucose
LexA-Snf1	GAD-Std1	0.8	4.0
LexA-Snf1	GAD	0.3	0.3
LexA	GAD-Std1	0.3	0.5
LexA-Snf1T210A	GAD-Std1	0.4	0.5
LexA-Snf1KD	GAD-Std1	6.4	5.3
LexA-Snf1RD	GAD-Std1	0.4	0.4

Since in wild-type cells the expression of *HXT1* is also inhibited when the cells are growing in low glucose ([12]; see also Table 7, below), conditions in which Snf1 protein kinase is active, we suggest that the activation of Snf1, either by physiological conditions (growth in low-glucose conditions) or by eliminating negative regulators such as Hxk2 or Reg1, would lead to an inhibition of *HXT1* expression. This would define an additional function of Snf1 in transcriptional regulation, where so far only the activation of target genes has been well documented, i.e. by inhibiting repressors as Mig1, by activating activators as Cat8 or Sip4 [1] or by activating directly the transcription machinery [33,34]. Therefore, Snf1 would not only activate the expression of genes involved in the assimilation of alternative carbon sources, but it would also repress the expression of genes induced by glucose. In this way Snf1 would mimic the effect of its mammalian homologue AMP-activated protein kinase in transcriptional regulation. In mammalian hepatocytes it has been described that active AMP-activated protein kinase inhibits the expression of glucose-induced genes such as those encoding L-pyruvate kinase (L-PK), fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), spot 14 (S14) and pre-proinsulin (PPI) [35–38].

Std1 interacts with Snf1

To analyse the mechanism of action of Snf1 kinase on the regulation of *HXT1* expression, we looked for possible interactions between Snf1 and some of the components of the *HXT1* glucose-induction pathway (see Introduction). A clear candidate was Std1 (Msn3), a negative regulator of *HXT1* expression [12], since it was originally isolated as a multicopy suppressor of *snf4* deficiency (Snf4 being the activator subunit of the Snf1 protein kinase complex) [39]. It has also been described previously that the increase in gene dosage of Std1 led to an activation of the Snf1 complex, and the same authors demonstrated that Std1 interacted physically with Snf1, both by two-hybrid and co-immunoprecipitation analysis [39]. We studied further the interaction between Std1 and Snf1 and found that it was only observed when Snf1 was in its active state (cells growing in low-glucose conditions; Table 3). A mutation that inhibited Snf1 activity (T210A) [5] eliminated the interaction with Std1,

Table 4 Overexpression of Std1 inhibits *HXT1* expression through an active Snf1

FY250 *snf1*Δ cells were transformed with the centromeric pC-HXT1-lacZ plasmid, with the plasmids pWS-Snf1, pWS-Snf1K84R (kinase-dead form) and pWS-Snf1T210A (inactive Snf1) and also with the indicated plasmids. β-Galactosidase activities were measured in triple transformants growing exponentially in 4% glucose minimal medium. Values are means from four–six different transformants (S.D. < 15% in all cases). Invertase was also measured in the same cells (S.D. < 10% in all cases). Western blotting indicated that the levels of HA-Std1 were similar in the corresponding transformants (results not shown).

	pWS-Snf1		pWS-Snf1K84R		pWS-Snf1T210A	
Plasmid	Invertase (units)	pHXT1-lacZ β-galactosidase (units)	Invertase (units)	pHXT1-lacZ β-galactosidase (units)	Invertase (units)	pHXT1-lacZ β-galactosidase (units)
pSK93 (empty)	1	45	1	43	1	44
pSK-Std1	11	6	1	40	1	43

Table 5 Two-hybrid interaction between Std1 and Rgt1

CTY10.5d cells expressing LexA-Std1 and Gal4-activating domain (GAD)-Rgt1 fusion proteins were grown to mid-logarithmic phase in selective SC/4% glucose medium; cells were then washed with water and shifted to SC/0.05% glucose medium for 3 h. Values are mean β-galactosidase activities from four–six transformants (S.D. < 15% in all cases). Invertase was also measured in cells growing in 4% glucose medium (S.D. < 10% in all cases).

		β-Galactosidase (Miller units)		Invertase (units)
LexA fusion	GAD fusion	4% Glucose	Shift to 0.05% glucose	4% Glucose
LexA-Std1	GAD-Rgt1	48	45	60
LexA-Std1	GAD	< 1	< 1	60
LexA	GAD-Rgt1	< 1	< 1	< 1

confirming that Std1 only interacted with Snf1 when the kinase was in its active conformation. Table 3 also shows that Std1 interacted with the catalytic domain of Snf1 (Snf1KD) and not with its regulatory domain (Snf1RD). We also checked for possible two-hybrid interactions between Std1 and any of the Snf1 kinase regulators such as Snf4, Hxk2 or Reg1, but we did not observe any, in either high- or low-glucose conditions (results not shown).

We also studied the effects of the increase in gene dosage of Std1 on Snf1 complex activity. Table 4 shows that this increase caused a relief in *SUC2* glucose repression, as already described [39]. It also caused a dramatic decrease in *HXT1* induction (Table 4). The effects of Std1 on both *SUC2* and *HXT1* expression were dependent on the presence of an active Snf1 kinase. In the presence of inactive forms of Snf1, such as a kinase-dead Snf1K84R mutant or a form that can not be activated (Snf1T210A mutant), no effect on the expression of these genes was observed. These results were in agreement with previous observations [18].

In conclusion, the action of Std1 on *SUC2* and *HXT1* expression was mediated via the activation of Snf1, since in the absence of an active form of the kinase Std1 was unable to affect gene expression.

Std1 interacts with Rgt1

Since it has been described that Std1 could modulate negatively the expression of *HXT1* in low-glucose conditions (*std1* mutants show an increased expression of *HXT1* in low glucose [18]) and since Rgt1 is the major transcription factor involved in *HXT1* expression (see [12] for a review), we studied the possible interaction between Std1 and Rgt1. By two-hybrid analysis we observed a positive interaction between these two proteins (Table 5). This interaction did not improve when the cells were shifted from high- to low-glucose conditions, perhaps because the increase in gene dosage of Std1 had already activated the Snf1 kinase in cells growing in glucose, as demonstrated by the high levels of invertase detected in the same cells (Table 5). The physical interaction between Std1 and Rgt1 was confirmed by pull-down assays in cells expressing GST-Std1 and HA-Rgt1 as fusion proteins. Figure 1 shows that there was a specific interaction between Std1 and Rgt1.

We also assayed the two-hybrid interaction between Rgt1 and Snf1 or any of its regulators (Snf4, Hxk2 and Reg1) but we were not able to detect any interaction in either high- or low-glucose conditions (results not shown).

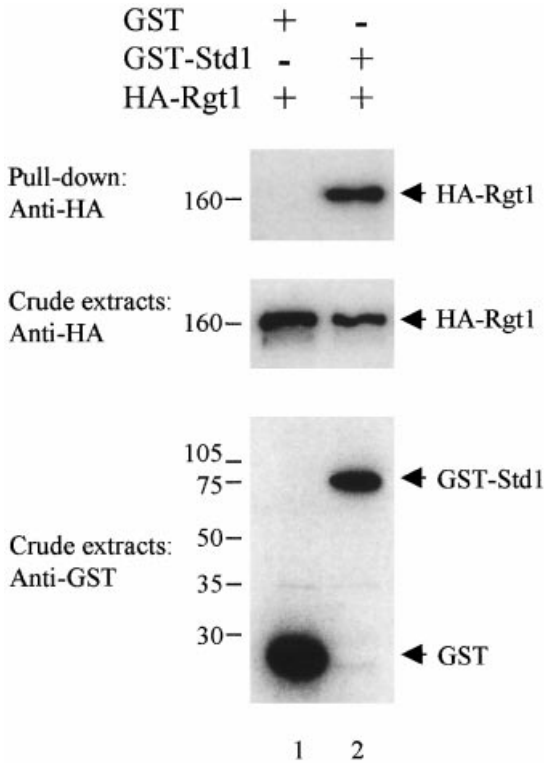

Figure 1 Pull-down assays between GST-Std1 and HA-Rgt1

Crude extracts (250 μg) were prepared from FY250 cells growing in glucose expressing GST-Std1 (plasmid pGST-Std1) and HA-Rgt1 (plasmid pSK-Rgt1) or containing the corresponding empty vectors. GST-Std1 fusion proteins were pulled down with GSH–agarose. Proteins in the pellet were analysed by SDS/PAGE and immunodetected with anti-HA monoclonal antibodies (upper panel). Proteins in the crude extracts (5 μg) were also immunodetected with either anti-HA (middle panel) or anti-GST (lower panel) antisera. Size standards are indicated in kDa.

Table 6 Overexpression of Rgt1 does not modify *HXT1* expression

FY250 cells were transformed with the centromeric pC-HXT1-lacZ plasmid and with the indicated plasmids. β-Galactosidase activities were measured in triple transformants growing exponentially in 4% glucose minimal medium. Values are means from four–six different transformants (S.D. $< 15\%$ in all cases). Invertase was also measured in the same cells (S.D. $< 10\%$ in all cases). Western blotting indicated that the corresponding transformants contained similar amounts of HA-Std1 and HA-Rgt1, respectively (results not shown).

		Invertase (units)	pHXT1-lacZ β-galactosidase (Miller units)
pSK93 (empty)	pWS93 (empty)	< 1	65
pSK-Std1	pWS93 (empty)	16	6
pSK93 (empty)	pWS-Rgt1	< 1	71
pSK-Std1	pWS-Rgt1	14	60

Since the increase in gene dosage of Std1 (by activation of Snf1 kinase) caused an inhibition of *HXT1* expression (see Table 4), we checked whether an increase in the gene dosage of Rgt1 could counteract the effects of Std1. As shown in Table 6, an increase in gene dosage of Rgt1 alone did not modify *HXT1* induction or *SUC2* repression by glucose. The combined increase in gene dosage of Rgt1 and Std1 did not show any inhibition of *HXT1* expression, either, indicating that the excess of Rgt1 could overcome the inhibitory effect of activated Snf1 kinase and still activate *HXT1* expression. This effect was specific on the induction of *HXT1* since the expression of *SUC2* was still relieved from glucose repression in these transformants, as an indication of the active state of the Snf1 kinase (Table 6).

Std1 is dispensable for the inhibitory effect of active Snf1 protein kinase on *HXT1* expression

The results presented so far indicate that under conditions in which Snf1 kinase was active, Std1 interacted with Snf1 (Table 3) and also with Rgt1 (Table 5 and Figure 1), suggesting that perhaps Std1 could act in recruiting Snf1 to Rgt1. To test this hypothesis, we analysed the expression of *HXT1* in mutant cells lacking Std1. To avoid possible interference from other components of the *HXT1* glucose-induction pathway, we used mutants lacking, in addition, Mth1 (an Std1 homologue [12]) and the two membrane glucose sensors, Snf3 and Rgt2 [12]. This quadruple mutant *rgt2*Δ *snf3*Δ *mth1*Δ *std1*Δ showed constitutive expression of *HXT1* in either high- or low-glucose conditions (Table 7), in agreement with previous results [18]. However, the additional deletion of *HXK1* and *HXK2* (sextuple *rgt2*Δ *snf3*Δ *mth1*Δ *std1*Δ *hxk1*Δ *hxk2*Δ mutant) caused constitutive activation of Snf1 kinase (measured as the presence of high levels of invertase) and prevented *HXT1* expression in both high- and low-glucose conditions (Table 7). These results indicated that activated Snf1 kinase was able to affect *HXT1* expression in the absence of Std1 and Mth1, suggesting that Std1 was mainly involved in the activation of Snf1 in wild-type cells and that if Snf1 kinase were activated by alternative mechanisms, the action of Std1 would be dispensable. The low levels of invertase detected in the quadruple mutant (*rgt2*Δ *snf3*Δ *mth1*Δ *std1*Δ) growing in low-glucose conditions would indicate that Snf1 kinase was not properly activated in these cells, and perhaps this was the reason why *HXT1* expression remained high under these conditions (Table 7). Recent data indicate that in double *std1*Δ *mth1*Δ mutants, Snf1 kinase was not properly activated in low-glucose conditions either [18], suggesting again a possible involvement of Std1 and Mth1 in the activation of Snf1 kinase.

One possibility to explain the inhibitory effect of Snf1 activation on *HXT1* expression could be that the kinase promoted the destruction of Rgt1 or changed the subcellular localization of the transcription factor, as in the case of Mig1 [40]. We ruled out the first possibility when we observed similar levels of functional HA-Rgt1 in wild-type and in *snf1* and *reg1* (where Snf1 is constitutively activated) mutants growing in glucose (results not shown). To check the second hypothesis, we constructed a functional Rgt1–GFP fusion protein (see the Materials and methods section) and studied the subcellular localization of the protein in different mutants and growth conditions. We observed that Rgt1–GFP fusion protein was always located inside the nucleus in either high- or low-glucose conditions, in either wild-type cells (Figure 2) or in *snf1*Δ or *reg1*Δ mutants (results not shown). Therefore, Snf1 activation did not modify the subcellular localization of Rgt1.

Alternatively, Snf1 could modify Rgt1 either directly or indirectly, and this could change its transcriptional properties, switching it from an activator to a repressor of *HXT1* expression. In agreement with this hypothesis, the increase in gene dosage of Rgt1 in cells having an activated Snf1 kinase (Table 6) caused regular *HXT1* induction while still having derepressed *SUC2* expression, perhaps because an excess of Rgt1 could escape Snf1 modification. To check for possible Snf1-dependent modifications of Rgt1, we analysed the electrophoretic mobility of a HA-Rgt1 fusion protein in wild-type and *snf1*Δ cells but we were unable to detect any differences in either cells growing in glucose or shifted to low-glucose conditions for 3 h (results not shown). As Rgt1 is a large protein (128 kDa, although it runs abnormally in SDS/PAGE as a protein of around 160 kDa), very rich in serine (13.2%) and threonine (7.1%) residues, which is phosphorylated in more than 30 residues [41], a more detailed analysis is required to understand the putative Snf1-dependent modifications.

Rgt1 interacts with Ssn6

It has been genetically defined that Ssn6, a general repressor of transcription in yeast [42,43], plays a major role in repressing

Table 7 Expression of *HXT1* and *SUC2* in different mutants and growth conditions

Cells containing the multicopy pHXT1-lacZ plasmid [20] and growing exponentially in 4% glucose or 2% raffinose plus 0.05% glucose were harvested. Invertase and β-galactosidase activities were measured as described in the Materials and methods section. Invertase values are means from three different transformants (S.D. $< 10\%$ in all cases). Values for β-galactosidase are means from four–six transformants (S.D. $< 15\%$ in all cases).

	pHXT1-lacZ β-galactosidase (Miller units)		*SUC2* invertase (units)	
Strain	4% Glucose	2% Raffinose	4% Glucose	2% Raffinose
Wild-type W303-1A	266	8	< 1	21
rgt2 snf3 mth1 std1	196	105	< 1	3
rgt2 snf3 mth1 std1 hxk1 hxk2	8	7	42	40

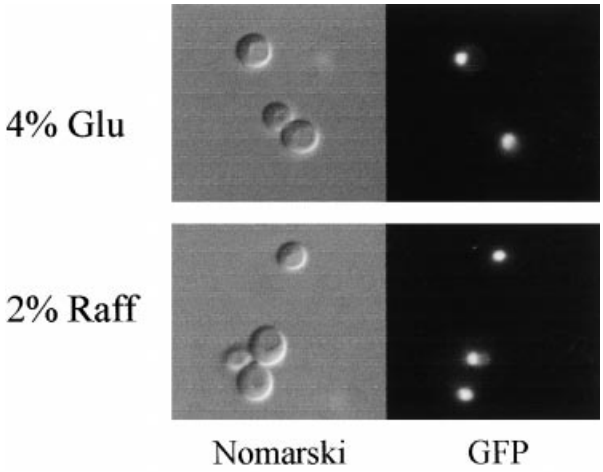

Figure 2 Subcellular localization of Rgt1–GFP

Wild-type cells expressing Rgt1–GFP fusion protein were grown in 4% glucose (Glu) or 2% raffinose (Raff) plus 0.05% glucose at 30 °C until they reached the exponential phase. Aliquots were then taken and analysed as described in the Materials and methods section. In each case, a picture of the GFP fluorescence and the Nomarski optics was taken. Similar results were observed with *snf1* and *reg1* mutant cells expressing the same fusion protein (results not shown).

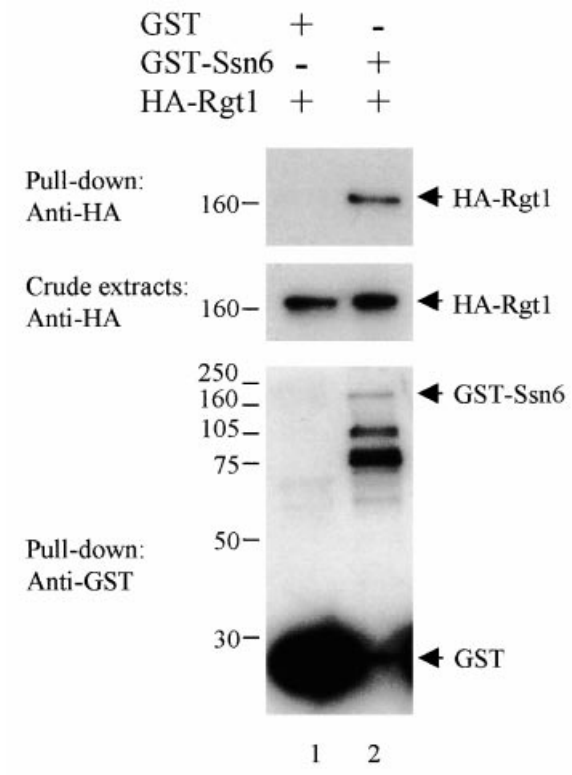

Figure 3 Pull-down assays between GST-Ssn6 and HA-Rgt1

Crude extracts (500 μg) were prepared from FY250 cells growing in glucose expressing functional GST-Ssn6 (plasmid pGST-Ssn6) and HA-Rgt1 (plasmid pSK-Rgt1) or containing the corresponding empty vectors that had been shifted to 0.05% glucose-containing medium for 30 min. GST-Ssn6 fusion proteins were pulled down with GSH–agarose. Proteins in the pellet were analysed by SDS/PAGE and immunodetected with anti-HA monoclonal (upper panel) or anti-GST polyclonal (lower panel) antibodies. Proteins in the crude extracts (5 μg) were also immunodetected with anti-HA (middle panel). Size standards are indicated in kDa.

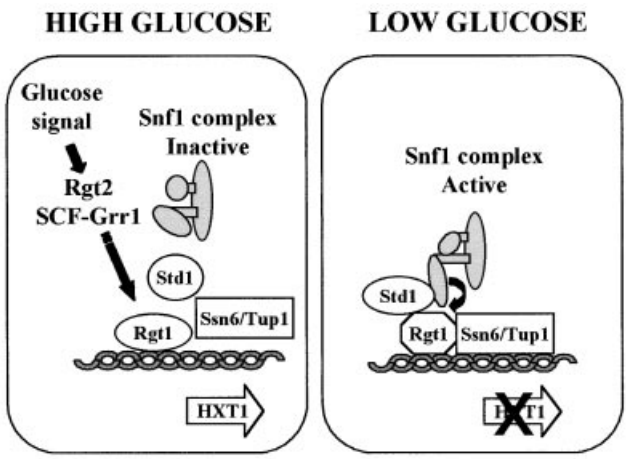

Figure 4 Model of regulation of *HXT1* expression by glucose

See text for details.

HXT1 expression; in *ssn6* mutants the expression of *HXT1* is not severely affected by the depletion of glucose [17]. It was proposed that, in low-glucose conditions, Rgt1 recruited Ssn6 to perform its repressing activity [17]. We confirmed this hypothesis by pull-down assays in cells expressing functional GST-Ssn6 and HA-Rgt1 as fusion proteins. Figure 3 shows that there was a specific interaction between Ssn6 and Rgt1.

Taking all these results together, we suggest the following model of regulation of *HXT1* expression (Figure 4). When glucose is abundant, a signal is assessed by the sensor protein Rgt2 and transmitted through the SCF–Grr1 ubiquitination complex to the Rgt1 transcription factor, activating *HXT1* expression. When the levels of glucose become depleted, Snf1 activation, caused probably by a conformational change in response to phosphorylation of its Thr-210 residue and interaction of the Snf4 activator subunit with the regulatory domain of Snf1 kinase, could modify, either directly or indirectly, Rgt1. This would allow its interaction with the Ssn6/Tup1 repressor complex that eventually would inhibit *HXT1* expression. Std1 would be involved in the activation of Snf1 in wild-type cells but if Snf1 kinase were activated by alternative mechanisms the action of Std1 would be dispensable.

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