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Mth1 regulates the interaction between the Rgt1 repressor and the Ssn6-Tup1 corepressor complex by modulating PKA-dependent phosphorylation of Rgt1

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ABSTRACT Glucose uptake, the first, rate-limiting step of its utilization, is facilitated by glucose transporters. Expression of several glucose transporter (*HXT*) genes in yeast is repressed by the Rgt1 repressor, which recruits the glucose-responsive transcription factor Mth1 and the general corepressor complex Ssn6-Tup1 in the absence of glucose; however, it is derepressed when Mth1 is inactivated by glucose. Here we show that Ssn6-Tup1 interferes with the DNA-binding ability of Rgt1 in the absence of Mth1 and that the Rgt1 function abrogated by Ssn6 overexpression is restored by co-overexpression of Mth1. Thus Mth1 likely regulates Rgt1 function not by modulating its DNA-binding activity directly but by functionally antagonizing Ssn6-Tup1. Mth1 does so by acting as a scaffold-like protein to recruit Ssn6-Tup1 to Rgt1. Supporting evidence shows that Mth1 blocks the protein kinase A-dependent phosphorylation of Rgt1 that impairs the ability of Rgt1 to interact with Ssn6-Tup1. Of note, Rgt1 can bind DNA in the absence of Ssn6-Tup1 but does not inhibit transcription, suggesting that dissociation of Rgt1 from Ssn6-Tup1, but not from DNA, is necessary and sufficient for the expression of its target genes. Taken together, these findings show that Mth1 is a transcriptional corepressor that facilitates the recruitment of Ssn6-Tup1 by Rgt1.

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INTRODUCTION

High-aerobic glycolysis—high propensity to ferment rather than oxidize glucose even when oxygen is abundant—is a hallmark of glucose metabolism in many types of cancer cells and the budding yeast *Saccharomyces cerevisiae* (Johnston and Kim, 2005). A key characteristic of this phenomenon is increased glucose uptake as a result of elevated expression of the glucose transporter genes. The budding yeast has at least six glucose transporter genes (hexose

transporter [*HXT*] genes *HXT1–4*, *HXT6*, and *HXT7*), whose expressions are induced by glucose but repressed when glucose is depleted (Ko *et al.*, 1993; Diderich *et al.*, 1999; Ozcan and Johnston, 1999). Repression of the *HXT* genes is largely controlled by the *HXT* repressor Rgt1, a member of the Gal4 family of transcription factors that contains the zinc binuclear cluster (Cys₆Zn₂) DNA-binding domain (Ozcan *et al.*, 1996b). Rgt1 recognizes a specific DNA sequence, 5'-CGGANNA-3', via the DNA-binding motif in its amino terminus in vitro (Kim *et al.*, 2003; Kim, 2004, 2009) and synergistically binds to multiple copies of the sequence in the upstream regions of *HXT* genes in vivo (Kim *et al.*, 2003).

Ssn6-Tup1 is a general transcription corepressor complex composed of one molecule of Ssn6 and four molecules of Tup1 (Varanasi *et al.*, 1996). The complex lacks DNA-binding ability but is instead recruited to its target promoters by sequence-specific DNA-binding repressors (Smith and Johnson, 2000b; Malave and Dent, 2006). Ssn6 and Tup1 contain the tetratricopeptide repeat (TPR) and WD domains, respectively, which serve as protein-protein interaction

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Abbreviations used: ChIP, chromatin immunoprecipitation; HA, hemagglutinin; HXT, hexose transporter; IP, immunoprecipitation; PKA, protein kinase A.

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motifs and through which they interact with different binding partners (Schultz *et al.*, 1990; Smith *et al.*, 1995; Smith and Johnson, 2000a; Jabet *et al.*, 2000; Sprague *et al.*, 2000). The mechanism of Ssn6-Tup1-mediated transcriptional repression involves the recruitment of global corepressors such as chromatin and nucleosome remodelers and the interaction with the RNA transcription machinery (Smith and Johnson, 2000b; Malave and Dent, 2006). For example, the corepressor promotes gene repression by associating with histone deacetylases, including Rpd3, Hos1, and Hos2 (Davie *et al.*, 2003). Tup1 interacts with histones H3 and H4, and its binding to hypoacetylated histones flanking repressor binding sites leads to nucleosome positioning (Edmondson *et al.*, 1996; Davie *et al.*, 2003). Ssn6-Tup1 is involved in repression of the genes regulated by diverse signaling pathways (Malave and Dent, 2006). Among them are *HXT* genes (Ozcan *et al.*, 1996b), glucose-repressible genes (Nehlin and Ronne, 1990), hypoxia-induced genes (Balasubramanian *et al.*, 1993), DNA damage-response genes (Huang *et al.*, 1998), and haploid-specific genes (Johnson and Herskowitz, 1985; Komachi *et al.*, 1994). The relief of Ssn6-Tup1-mediated repression comes about through the destruction or inactivation of the individual repressors, which leads to dissociation of the repressors from Ssn6-Tup1 and DNA (Smith and Johnson, 2000b).

Rgt1-mediated repression of the *HXT* genes occurs by a mechanism that requires the paralogous proteins Mth1 and Std1 (Hubbard *et al.*, 1994; Schmidt *et al.*, 1999). Key lines of supporting evidence are that 1) *HXT* expression is constitutive in yeast cells lacking *MTH1* and *STD1* genes (Schmidt *et al.*, 1999; Lafuente *et al.*, 2000; Lakshmanan *et al.*, 2003), 2) Mth1 and Std1 directly interact with Rgt1 (Tomas-Cobos *et al.*, 2004; Polish *et al.*, 2005), and 3) Mth1 and Std1 might form a repression complex with Rgt1 and Ssn6-Tup1 in the absence of glucose (Kim *et al.*, 2003; Lakshmanan *et al.*, 2003). Mth1 and Std1 are degraded by proteasome in the presence of high levels of glucose, resulting in disruption of the repressor complex and thereby derepression of *HXT* expression (Flick *et al.*, 2003; Moriya and Johnston, 2004; Pasula *et al.*, 2007). The glucose signal transduction pathway that leads to degradation of Mth1 and Std1 begins at the plasma membrane with the two glucose transporter-related sensor proteins Rgt2 and Snf3 (Ozcan *et al.*, 1998). There are also dominant mutations in the *MTH1* gene (*HTR1-23*, *DGT1*, or *BCP1*) that render Mth1 resistant to glucose-induced degradation (Kim *et al.*, 2006), resulting in the constitutive repression of *HXT* expression (Gamo *et al.*, 1994; Schulte *et al.*, 2000). Expression of the *MTH1* gene is also repressed by Mig1 in high-glucose conditions, reinforcing the inhibitory effect of glucose on Mth1 function (Kaniak *et al.*, 2004; Kim *et al.*, 2006). The ability of Rgt1 to bind to *HXT* promoters is correlated with its phosphorylation state: Rgt1 is phosphorylated at a basal level and binds to the promoters in the absence of glucose; it is hyperphosphorylated by protein kinase A (PKA) and dissociated from the promoters when glucose levels are high (Kim and Johnston, 2006; Palomino *et al.*, 2006). Rgt1 is also hyperphosphorylated and does not bind DNA in cells lacking Mth1 (Flick *et al.*, 2003; Mosley *et al.*, 2003), leading to the hypothesis that Mth1 and Std1 prevent the PKA-dependent phosphorylation of Rgt1 that impairs the DNA-binding ability of Rgt1.

The aim of this study is to investigate the role of Mth1 in the mechanism of Rgt1-mediated repression. We show that glucose-induced, PKA-dependent phosphorylation is a crucial step leading to dissociation of Rgt1 from Ssn6-Tup1 and consequently to derepression of *HXT* gene expression. Mth1 blocks such phosphorylation by mediating the interaction of Rgt1 with Ssn6-Tup1, thereby facilitating the formation of a functional repressor complex that inhibits transcription of *HXT* genes. We further show that Mth1 acts to

antagonize Ssn6-Tup1 inhibition of Rgt1 function; however, its expression is repressed by Ssn6-Tup1 via Mig1 in high-glucose conditions. Taken together, these results identify a functional interaction between Mth1 and Ssn6-Tup1 and provide novel insight into the mechanism of Rgt1-Ssn6-Tup1-mediated repression.

RESULTS

The DNA-binding activity of Rgt1 is antagonistically regulated by Ssn6-Tup1 and Mth1

Rgt1 forms a repressor complex with Ssn6-Tup1 on, and mediates repression of, the *HXT* promoters in the absence of glucose (Kim *et al.*, 2003), and this occurs in an Mth1/Std1-dependent manner (Schmidt *et al.*, 1999; Flick *et al.*, 2003; Mosley *et al.*, 2003). However, the underlying mechanism of this process is not understood. To understand the roles of Ssn6-Tup1, Mth1, and Std1 in the formation of the complex, we first determined whether these components regulate the ability of Rgt1 to bind to *HXT* promoters using chromatin immunoprecipitation (ChIP) analysis. The *RGT2-1* mutation causes constitutive (glucose-independent) expression of *HXT* genes (Ozcan *et al.*, 1996a), probably by inducing degradation of Mth1 and Std1 in the absence of glucose (Pasula *et al.*, 2007). ChIP analysis showed that Rgt1-binding to the *HXT1* promoter is significantly abolished in *mth1Δ std1Δ* and *RGT2-1* mutants, as reported previously (Flick *et al.*, 2003; Pasula *et al.*, 2007), but is constitutive in *ssn6Δ* and *tup1Δ* mutants (Figure 1A), without significant changes in Rgt1 protein levels (Figure 1A, top right). More important, the DNA-binding defect of Rgt1 in *mth1Δ std1Δ* and *RGT2-1* cells was restored by the removal of the *TUP1* (*mth1Δ std1Δ tup1Δ*) or *SSN6* (*RGT2-1 ssn6Δ*) gene from the mutants (Figure 2, A and B). An *mth1Δ std1Δ ssn6Δ* mutant displays an extremely slow growth phenotype compared with that of the *mth1Δ std1Δ tup1Δ* mutant, so that it could not be used in this study. These results suggest that Rgt1 by itself is able to bind to its DNA target sites, but this ability is positively and negatively regulated by Mth1/Std1 and Ssn6-Tup1, respectively.

These findings prompted us to examine whether Ssn6-Tup1 and Mth1 down-regulate each other. Western blot analysis showed that Mth1 levels are elevated in glucose-grown *ssn6Δ* and *tup1Δ* mutants compared with those of wild-type cells (Figure 1C). Elevated levels of Mth1 in *ssn6Δ* and *tup1Δ* mutants are perhaps due to derepression of *MTH1* expression (Supplemental Figure S1), consistent with our previous reports that *MTH1* expression is repressed by Mig1-Ssn6-Tup1 complex at high concentration of glucose (Kaniak *et al.*, 2004; Kim *et al.*, 2006). Mth1 was barely detectable in *RGT2-1* and *RGT2-1 ssn6Δ* mutants, suggesting that Ssn6-Tup1 is not involved in degradation of Mth1. We also confirmed that there are no appreciable changes in the levels of Ssn6 in *mth1Δ std1Δ* and *RGT2-1* mutants (Figure 1D). Taken together, these results suggest that Ssn6-Tup1 negatively regulates the DNA-binding ability of Rgt1 by repressing expression of the *MTH1* gene.

Rgt1 binds to its target promoters in the absence of Ssn6-Tup1 but does not repress transcription

Our finding that Rgt1 binds to the *HXT1* promoter in the absence of Ssn6-Tup1 (Figure 1A) raised the possibility that glucose might not regulate the DNA-binding ability of Rgt1. To test this possibility, we examined the expression of *HXT1* mRNA in the mutants tested earlier, using quantitative real-time PCR (qRT-PCR) analysis (Figure 1E). The *HXT1* mRNA is constitutively expressed in *mth1Δ std1Δ* and *RGT2-1* mutants, perhaps due to inability of Rgt1 to bind to the *HXT1* promoter in the mutants (Figure 1A), as reported previously (Mosley *et al.*, 2003; Pasula *et al.*, 2007). Of greatest note, however,

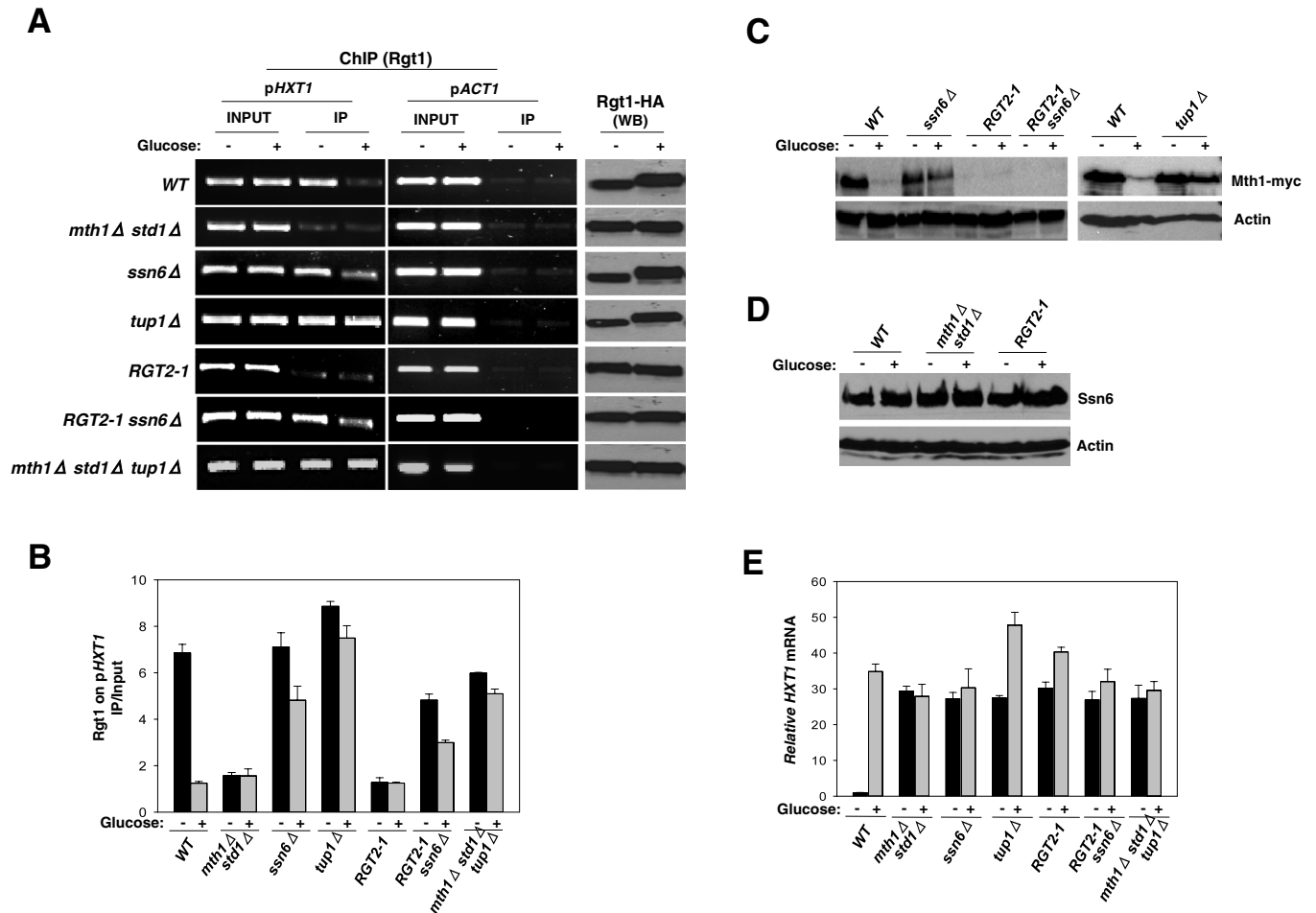


FIGURE 1: Ssn6-Tup1 negatively regulates the ability of Rgt1 to bind to its target promoters. (A) ChIP analysis of Rgt1 binding to the *HXT1* promoter in yeast cells expressing Rgt1-HA. Yeast cells of the indicated genotypes were grown in SC-2% galactose (–) and shifted to SC-4% glucose (+) for 1 h. Cross-linked chromatin was precipitated for ChIP analysis using anti-HA antibody, and representative PCRs are shown (left). As a negative control of Rgt1 DNA binding, primer sets were designed to amplify the actin gene promoter region (pACT1), which does not contain the Rgt1-binding sequence (5'CGGANNA3'; middle). Western blot analysis of Rgt1-HA expression using anti-HA antibody (right). (B) qPCR analysis of Rgt1-binding to the *HXT1* promoter in the designated yeast strains. The amount of immunoprecipitated DNA was quantified by qPCR with primer pairs directed against the *HXT1* promoter (pHXT1). The IP/Input ratio was determined by the ratio of IP/pHXT1 relative to IP/pACT1 divided by the ratio of Input/pHXT1 relative to Input/pACT1. The data are averages of three independent experiments, with error bars showing mean \pm SD. (C) Western blot analysis of expression of Mth1-myc using anti-Myc antibody. (D) Western blot analysis of expression of Ssn6 using anti-Ssn6 antibody. Actin serves as a loading control in C and D. (E) qRT-PCR analysis of *HXT1* mRNA expression. The quantification data of *HXT1* mRNA expressions are averages of three independent experiments, with error bars showing mean \pm SD.

Rgt1 was shown to be able to bind to the promoter constitutively in cells lacking *SSN6* (*ssn6Δ* and *RGT2-1 ssn6Δ* mutants) or *TUP1* (*tup1Δ* and *mth1Δ std1Δ tup1Δ* mutants; Figure 1A) but did not significantly inhibit glucose- induction of *HXT1* expression (Figure 1E). Thus our results suggest that dissociation of Ssn6-Tup1 from Rgt1 is sufficient for derepression of *HXT* gene expression regardless of the presence of both glucose and Mth1/Std1.

Mth1 acts to functionally antagonize the Ssn6-Tup1 complex

Because Rgt1 DNA-binding is oppositely regulated by Mth1/Std1 and Ssn6-Tup1 (Figure 1A), we assessed the functional interaction between Mth1 and Ssn6-Tup1 for regulating Rgt1 function in yeast cells overexpressing Ssn6 or co-overexpressing Ssn6 and Mth1. To this aim, we constructed *HXT* reporter strains that express the

nourseothricin (NAT) resistance gene under the control of the *HXT1*, *HXT2*, or *HXT3* promoter (Figure 2A). Hence the growth of the *HXT* reporter strains in a NAT-containing medium depends on the activity of the *HXT* promoters. We observed that *HXT1*-NAT and *HXT3*-NAT reporter strains grow only in high-glucose medium, whereas cells carrying the *HXT2*-NAT reporter grow in raffinose (low-glucose) medium; however, none of them grow in glycerol/ethanol medium. These results are consistent with a report that various *HXT* promoters are expressed differently in the various conditions (Ozcan and Johnston, 1995). Of interest, however, the *HXT*-NAT reporter strains—the *HXT1*-NAT reporter strain in particular—were able to grow in a galactose or glycerol/ethanol medium when Ssn6 is overexpressed from a high-copy plasmid (2 μ ; Figure 2B). The expression patterns of *HXT* mRNAs in cells overexpressing Ssn6 were similar to

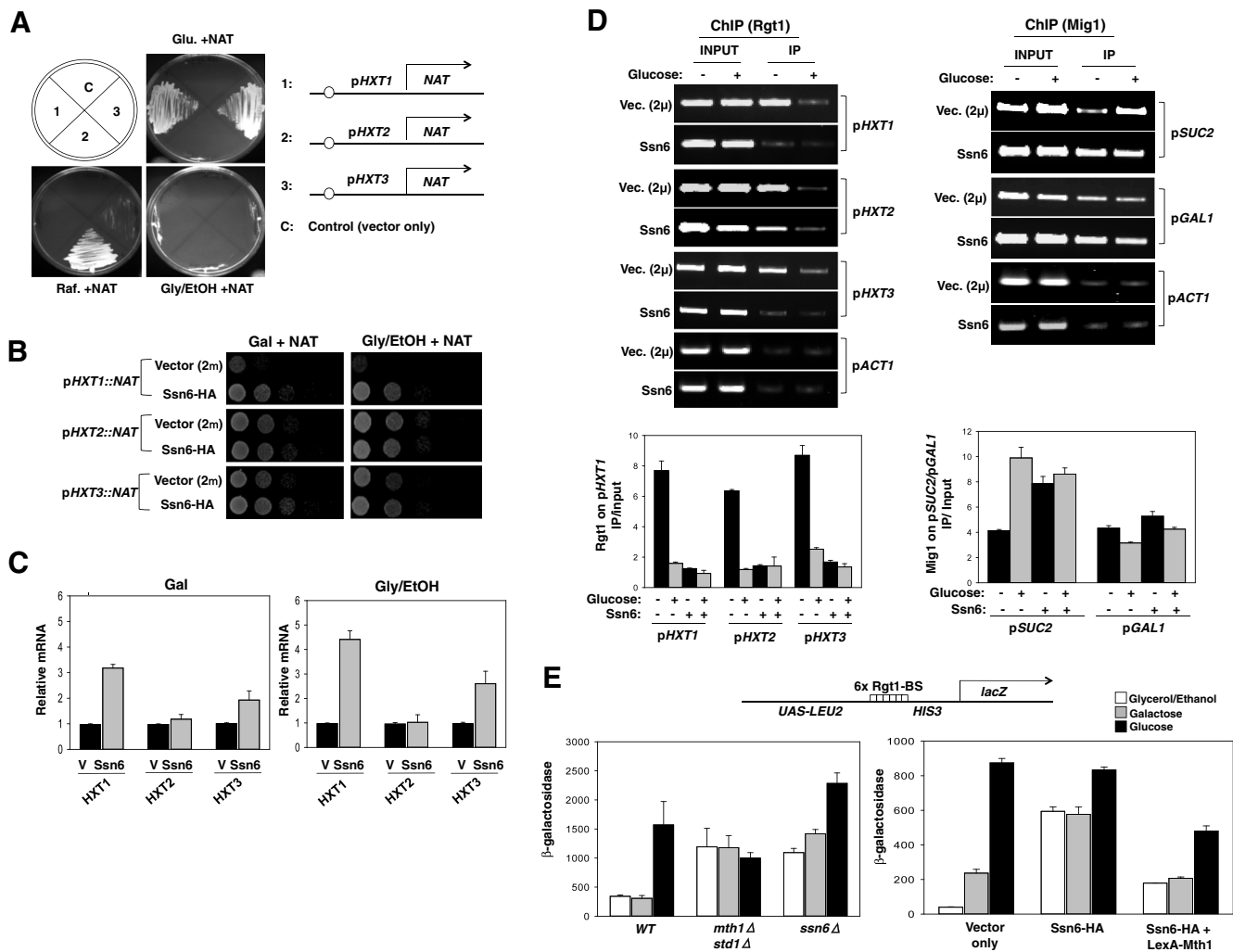


FIGURE 2: Ssn6 overexpression induces derepression of *HXT* expression in repressing conditions. (A) *HXT*-NAT reporter strains were streaked in YP plate containing 4% glucose (Glu), 5% glycerol + 2% ethanol (Gly/EtOH), or 2% raffinose (Raf) supplemented with 100 μ g/ml NAT sulfate. (B) Ssn6-HA (JKP231) was overexpressed from a high-copy 2 μ plasmid in all three NAT reporter strains. Yeast cells were spotted on YP plate containing 5% glycerol + 2% ethanol (Gly/EtOH) or 2% galactose (Gal) supplemented with 100 μ g/ml NAT sulfate. The first spot of each row represents a count of 5×10^7 cells/ml, which is diluted 1:10 for each spot thereafter. Cells were incubated for 2 d in Gal + NAT plate and 3 d in Gly/EtOH plates, respectively. (C) qRT-PCR analysis of mRNA expression of *HXT1*, *HXT2*, and *HXT3* genes. mRNA was isolated from yeast cells (BY4741) expressing either empty HA vector (V, 2 μ vector only) or Ssn6-HA (Ssn6, JKP231) grown in SC medium containing 5% glycerol + 2% ethanol (Gly/EtOH) or 2% galactose (Gal) until mid-log phase (OD_{600 nm} = 1.2–1.5). The data are averages of three independent experiments, with error bars showing mean \pm SD. (D) Yeast cells (BY4741, WT) co-overexpressing Ssn6-HA with either Rgt1-HA or Mig1-myc (pBM3076) were grown in SC-2% galactose (–) and shifted to SC-4% glucose (+) for 1 h. Cross-linked chromatin was precipitated for ChIP analysis of the indicated Rgt1 (*pHXT1*, *pHXT2*, and *pHXT3*) and Mig1 (*pSUC2* and *pGAL1*) DNA target sites, and representative PCRs are shown (top). The results of qPCR analysis of the binding of Rgt1 and Mig1 to their respective target promoters in yeast cells (bottom) are expressed as IP/Input ratio as described for Figure 1B. The data are averages of three independent experiments, with error bars showing mean \pm SD. (E) Yeast cells of indicated genotypes were transformed with a plasmid containing six copies of Rgt1-binding DNA sequence fused to the *lacZ* gene (6x Rgt1-BS-*lacZ*; JHB93) (left). BY4741 (WT) was transformed with JHB93 along with an empty HA plasmid, a plasmid expressing Ssn6-HA, or plasmids expressing Ssn6-HA and LexA-Mth1 (pBM4150; right). Transformants were grown in SC-5% glycerol + 2% ethanol medium (white bars), shifted to SC-2% galactose (gray bars) or SC-4% glucose (black bars) media for 1 h, and assayed for β -galactosidase activity.

the growth patterns of the cells (Figure 2C). ChIP analysis also showed that Rgt1 binding to the *HXT1* promoter is significantly reduced when Ssn6 is overexpressed (Figure 2D, ChIP-Rgt1, Ssn6). Next, we asked whether Ssn6 overexpression inhibits the DNA-binding ability of other Ssn6-Tup1 recruiters. The glucose repressor Mig1 is activated and mediates repression of its target genes such as

SUC2 and *GAL1* by recruiting Ssn6-Tup1 in high-glucose conditions (Treitel and Carlson, 1995). We found that Mig1 binds to its target promoters constitutively (Figure 2D, right), as reported previously (Papamichos-Chronakis *et al.*, 2004); however, the DNA-binding ability of Mig1 is not significantly affected by Ssn6 overexpression (Figure 2D and Supplemental Figure S2; ChIP-Mig1, Ssn6).

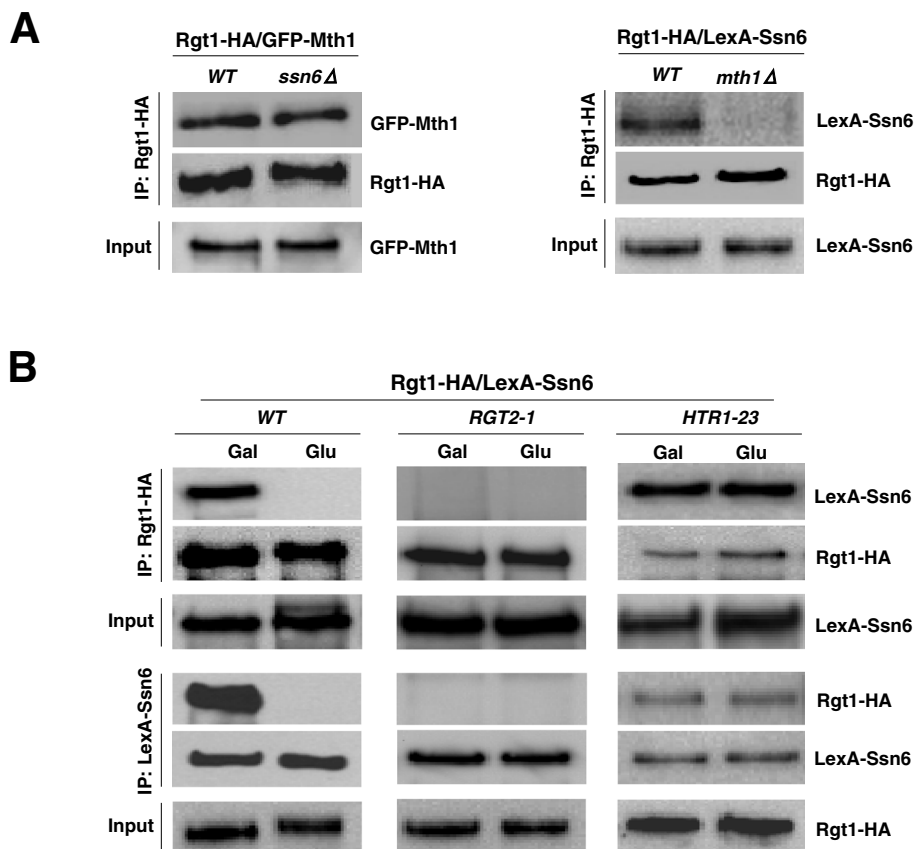


FIGURE 3: Mth1 is required for the interaction of Rgt1 with Ssn6-Tup1. (A) CoIP analysis of the interaction of Rgt1 with Mth1 or Ssn6. Yeast cells of indicated genotypes coexpressing Rgt1-HA and either green fluorescent protein (GFP)-Mth1 (pBM4748) (left) or LexA-Ssn6 (JKP173) (right) were grown in SC-2% galactose medium. Cell extracts were immunoprecipitated with anti-HA (IP) and immunoblotted with either anti-GFP or anti-LexA antibodies. Expression of GFP-Mth1 (left) or LexA-Ssn6 (right) was analyzed by Western blot using either anti-GFP or anti-LexA antibody (Input). (B) Yeast cells of indicated genotypes were cotransformed with plasmids expressing Rgt1-HA and LexA-Ssn6. Cells were first grown in SC-2% galactose medium (Gal) and shifted to SC-4% glucose medium (Glu) for 1 h. Cell extracts were subjected to IP with anti-HA or anti-LexA antibody and followed by immunoblotting with anti-HA or anti-LexA antibody. Expression of LexA-Ssn6 or Rgt1-HA was analyzed by Western blot using either anti-LexA or anti-HA antibody (Input).

Expression of *HXT* genes is regulated by not only Rgt1 but also other transcriptional factors, such as Sko1 (Tomas-Cobos *et al.*, 2004) and Mig1 (Ozcan and Johnston, 1996), raising a possibility that association of these proteins with *HXT* promoters may influence Rgt1 function. To eliminate this possibility, we used a plasmid reporter that contains six copies of the Rgt1 DNA-binding sequence (Rgt1-BS) without intervening sequences between Rgt1-BSs followed by the *lacZ* gene. Because expression of this reporter gene is solely regulated by Rgt1, this reporter system has been successfully used to measure the transcriptional repression activity of Rgt1 (Kim, 2009). We confirmed that expression of this reporter gene is negatively regulated by Mth1/Std1 and Ssn6 (Figure 2E, left). More significantly, Ssn6 overexpression (Ssn6-hemagglutinin [HA]) resulted in derepression of the *lacZ* gene in yeast cells grown under repressing conditions (galactose or glycerol plus ethanol); however, this derepression was significantly suppressed by co-overexpression of Mth1 (Ssn6-HA plus LexA-Mth1) (Figure 2E, right). These results and those in Figure 1 suggest that Ssn6-Tup1 inhibits the ability of Rgt1 to bind to its target promoters, and this inhibition is overcome by Mth1.

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Mth1 mediates the interaction of Rgt1 with Ssn6-Tup1, enabling Rgt1 to recruit Ssn6-Tup1 to *HXT* promoters

Given that Ssn6-Tup1 negatively regulates Rgt1-mediated repression (Figures 1 and 2), we examined the role of Mth1 in regulating Rgt1 function by assessing the interaction between Rgt1 and Ssn6 by coIP and Western blot analyses. Our results showed that Rgt1 interacts with both Mth1 and Ssn6 in cells grown in glucose-depleted medium, as reported previously (Polish *et al.*, 2005), and that Rgt1-Mth1 interaction is not affected by removal of the *SSN6* gene (Figure 3A, left). Surprisingly, however, Rgt1-Ssn6 interaction was abolished in the *mth1*Δ mutant (Figure 3A, right) and in the *RGT2-1* mutant (Figure 3B, *RGT2-1*), where Mth1 is constitutively degraded by proteasome (Pasula *et al.*, 2007). However, this interaction occurs constitutively in the *HTR1-23* mutant expressing a degradation-resistant Mth1 (Figure 3B, *HTR1-23*).

We previously identified domains of Rgt1 responsible for the interaction with Ssn6 (amino acids [aa] 210–250) and Mth1 (aa 310–360), respectively (Polish *et al.*, 2005). To obtain compelling evidence on whether Rgt1 interaction with Ssn6 or Mth1 affects Rgt1 function, we tested mutant Rgt1 proteins that lack the Ssn6- and Mth1-binding sites for their ability to interact with Ssn6 and Mth1, respectively. Rgt1 (Δ210-250) was able to interact with Mth1 but not with Ssn6 (Figure 4C). Furthermore, this mutant Rgt1 was able to bind to the *HXT1* promoter (Figure 4B) but did not repress transcription (Figure 4A). In contrast, Rgt1 (Δ310-360) did not strongly interact with both Mth1 (Figure 4D) and the *HXT1* promoter (Figure 4B), leading to derepression of the *HXT1* gene (Figure 4A). More important, Rgt1 (Δ310-360) was not able to interact with Ssn6 either (Figure 4D), consistent with the result that Mth1 is required for the interaction between Rgt1 and Ssn6 (Figure 3A). Taken together, these results suggest that Mth1-dependent interaction of Rgt1 with Ssn6-Tup1 enables Rgt1 to bind *HXT* promoters and thus leads to the formation of a functional Rgt1-Ssn6-Tup1 repressor complex on the promoters.

Mth1 prevents the PKA-dependent phosphorylation of Rgt1 that impairs the ability of Rgt1 to interact with Ssn6-Tup1

The ability of Rgt1 to bind to *HXT* promoters is largely correlated with the phosphorylation state of Rgt1 regulated by Mth1 (Flick *et al.*, 2003; Kim *et al.*, 2003; Mosley *et al.*, 2003). Because our findings show that the DNA-binding ability of Rgt1 is inhibited by Ssn6-Tup1 (Figure 1), we examined whether Ssn6-Tup1 modulates Rgt1 function by regulating its phosphorylation state using Western blot analysis. Rgt1 was shown to be hyperphosphorylated constitutively in the *mth1*Δ mutant (Figure 5A), as reported previously (Flick *et al.*, 2003; Mosley *et al.*, 2003). However, the phosphorylation state of Rgt1 was not significantly changed in *ssn6*Δ and *tup1*Δ mutants as compared with that of wild-type cells, regardless of the presence of

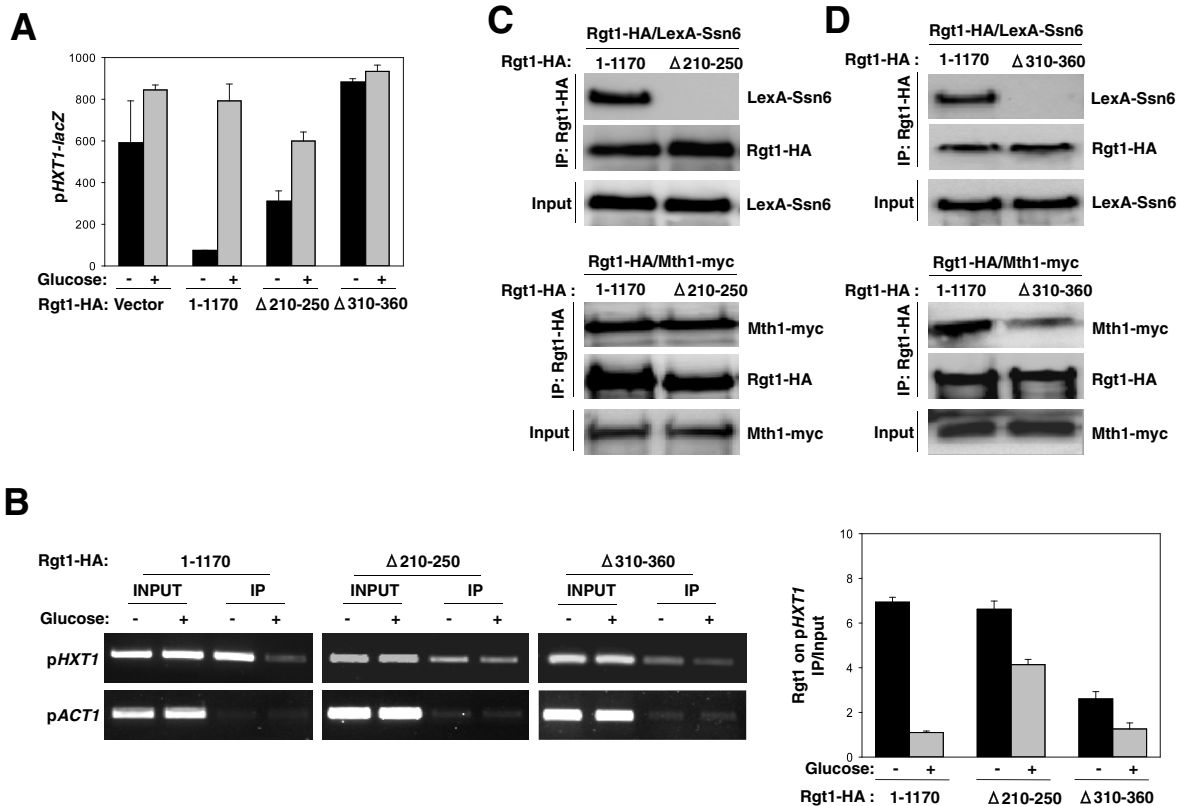


FIGURE 4: Rgt1 function is regulated positively and negatively by Mth1 and Ssn6, respectively. (A) Yeast cells (*rgt1 Δ*) were cotransformed with each of the three plasmids expressing Rgt1-HA (KFP60), Rgt1 ($\Delta 210-250$)-HA (JKP232), and Rgt1 ($\Delta 310-360$)-HA (JKP233) and the *pHXT1-lacZ* reporter plasmid (pBM2636). Transformants were first grown in SC-2% galactose medium (-) and shifted to SC-4% glucose medium (+) for 1 h and assayed for β -galactosidase activity. (B) ChIP analysis of Rgt1 binding to the *HXT1* promoter in yeast cells (*rgt1 Δ*) expressing Rgt1-HA, Rgt1 ($\Delta 210-250$)-HA, and Rgt1 ($\Delta 310-360$)-HA using anti-HA antibody. The results of qPCR analysis of Rgt1 binding to the *HXT1* promoter in yeast cells expressed as IP/Input as described in Figure 1B, using the *pACT1* promoter as a negative control of Rgt1 DNA binding (right). (C) CoIP analysis of the interaction of Rgt1 with Ssn6. Yeast cells (*rgt1 Δ*) coexpressing Rgt1-HA (1-1170 (full-length) or $\Delta 210-250$) with LexA-Ssn6 were grown in 2% galactose until mid-log phase ($OD_{600\text{ nm}} = 1.2-1.5$). Cell extracts were immunoprecipitated with anti-HA antibody and followed by immunoblotting with anti-LexA antibody. (D) CoIP analysis of the interaction of Rgt1 with Mth1. Yeast cells (*rgt1 Δ*) coexpressing Rgt1-HA (1-1170 or $\Delta 310-360$) with Mth1-myc were grown as described in C, and cell extracts were immunoprecipitated with anti-HA antibody and followed by immunoblotting with anti-Myc antibody. Expression of LexA-Ssn6 or Mth1-myc was analyzed by Western blot using either anti-LexA or anti-Myc antibody in C and D (Input).

glucose. Rgt1 was also shown to be constitutively hyperphosphorylated in *mth1 Δ std1 Δ* , *RGT2-1*, *RGT2-1 ssn6 Δ* , and *mth1 Δ std1 Δ tup1 Δ* mutants (Figure 5A).

Rgt1 is hyperphosphorylated in the presence of high levels of glucose (Flick *et al.*, 2003; Kim *et al.*, 2003; Mosley *et al.*, 2003), and this phosphorylation is catalyzed by PKA (Kim and Johnston, 2006; Palomino *et al.*, 2006). To obtain direct evidence that Mth1 regulates Rgt1 phosphorylation by PKA, we determined the phosphorylation state of Rgt1 in yeast cells lacking functional Mth1, PKA, or both. Our results showed that hyperphosphorylation of Rgt1 in the *mth1 Δ* mutant is attenuated when the mutant PKA with retarded activity (*tpk^{w1}*; Toda *et al.*, 1987) is expressed in the mutant (*mth1 Δ tpk^{w1}*; Figure 5B). We also found that Rgt1 binds to and represses the *HXT1* promoter in *mth1 Δ tpk^{w1}* mutant constitutively (Figure 5, C and D). These observations suggest that Mth1, but not Ssn6-Tup1, inhibits Rgt1 phosphorylation by PKA.

Given that Mth1 is required for the interaction between Rgt1 and Ssn6-Tup1 (Figure 3) and that Mth1 regulates Rgt1 phosphorylation (Figure 5B), we investigated whether the phosphorylation state of

Rgt1 affects its interaction with Ssn6-Tup1 using coIP and Western blot analysis. Rgt1 interaction with Ssn6 was not observed in the *mth1 Δ* mutant but was strongly detected in the *mth1 Δ tpk^{w1}* mutant (Figure 5E). Furthermore, a mutant Rgt1 that lacks PKA phosphorylation sites (Rgt1-S5A; Ser-96, Ser-146, Ser-202, Ser-283, and Ser-284; Kim and Johnston, 2006) was shown to interact with Ssn6 constitutively (Figure 5F), leading to constitutive repression of *HXT1* expression (Supplemental Figure S3). Thus these results suggest that hyperphosphorylated Rgt1 does not interact with Ssn6-Tup1 and that the role of PKA-catalyzed Rgt1 phosphorylation is to dissociate Rgt1 from Ssn6-Tup1 and consequently from *HXT* promoters.

The Ssn6-Tup1 complex is recruited to *HXT* promoters in an Rgt1-dependent manner

Although our results suggest that Rgt1 interaction with Ssn6-Tup1 is regulated by glucose (Figure 3), it is not clear whether Ssn6-Tup1 is recruited to *HXT* promoters through this interaction. To assess the recruitment of Ssn6-Tup1 to *HXT* promoters, we performed ChIP analysis of Ssn6 binding to the *HXT1* promoter using anti-Ssn6

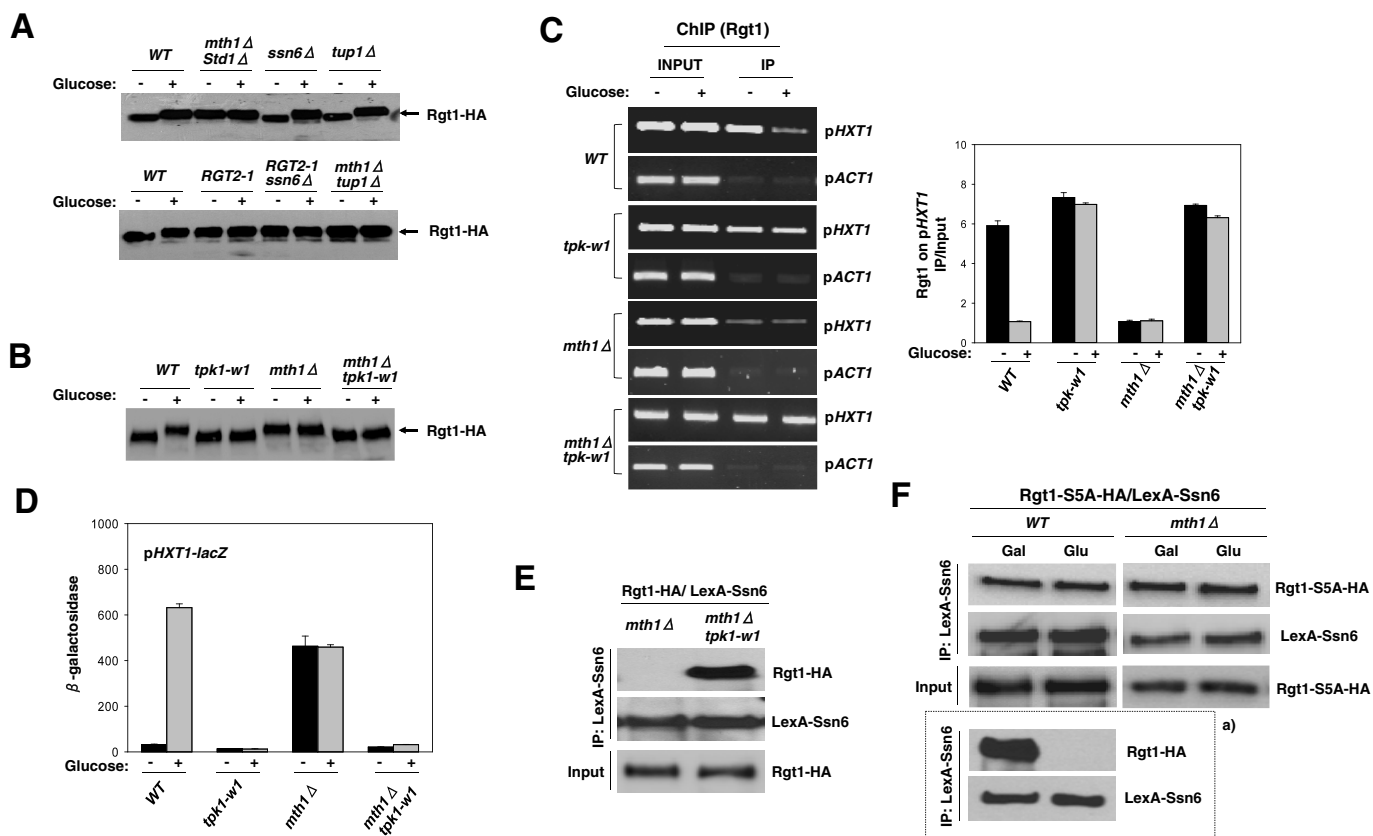


FIGURE 5: Rgt1 phosphorylation by PKA leads to the disruption of its interaction with Ssn6-Tup1. (A) Glucose-induced phosphorylation of Rgt1. Yeast cells expressing Rgt1-HA were grown in SC-2% galactose medium (-) and shifted to SC-4% glucose medium (+) for 1 h. Rgt1-HA was subjected to Western blot analysis using anti-HA antibody. (B) PKA phosphorylation of Rgt1. Western blot analysis of Rgt1, as described in A. (C) ChIP analysis of Rgt1 binding to the *HXT1* promoter. Representative PCRs are shown (left). The results of qPCR analysis of Rgt1 binding to the *HXT1* promoter expressed as IP/Input ratio as described in Figure 1B, using the *pACT1* promoter as a negative control of Rgt1 DNA-binding (right). (D) Induction of *HXT1* expression in yeast cells carrying the *pHXT1-lacZ* reporter plasmid (pBM2636). Yeast cells were grown as described in A and assayed for β -galactosidase activity. (E) CoIP analysis of Rgt1-HA and LexA-Ssn6 in *mth1* Δ and *mth1* Δ *tpk1*^{w1}. Yeast cell extracts were immunoprecipitated with anti-LexA antibody and followed by immunoblotting with anti-HA antibody. Expression of Rgt1-HA was analyzed by Western blot using anti-HA antibody (Input). (F) CoIP analysis of Rgt1-S5A-HA and LexA-Ssn6. Yeast cells (WT and *mth1* Δ) expressing Rgt1-S5A-HA (JKP234, Rgt1-S5A) and LexA-Ssn6 (JKP173) were grown as described in A. Yeast cell extracts were immunoprecipitated with anti-LexA antibody and followed by immunoblotting with anti-HA antibody. Expression of Rgt1-S5A-HA was analyzed by Western blot using anti-HA antibody (input). (a) Results of the Ssn6 interaction with wild-type Rgt1 protein depicted in Figure 3B shown for comparison.

antibody. We found that Ssn6 binds to the promoter in the absence of glucose but is removed from the promoter in the presence of high levels of glucose (Figure 6). However, this binding was not observed in the *rgt1* Δ mutant, suggesting that recruitment of Ssn6-Tup1 to *HXT* promoters occurs in an Rgt1-dependent manner. We made similar observations in the *mth1* Δ mutant, perhaps due to the inability of Rgt1 to bind to the promoter in the mutant (Figure 1A), highlighting the role of Mth1 as a mediator for the interaction of Rgt1 with Ssn6-Tup1 (Figure 3).

DISCUSSION

This study investigates the role of Mth1 in the mechanism of Rgt1-mediated repression, with the aim of understanding the glucose regulation of *HXT* expression. New findings include the following: 1) Mth1 mediates the interaction of Rgt1 with Ssn6-Tup1, leading to the formation of a functional repressor complex

on Rgt1 target genes. 2) Mutational removal or glucose inactivation of Mth1 leads to the PKA-dependent phosphorylation of Rgt1, which keeps Rgt1 from associating with Ssn6-Tup1. 3) Dissociation of Rgt1 from Ssn6-Tup1 is the most critical event for glucose induction of *HXT* genes. These findings support our previous observations that down-regulation of Mth1 level by glucose is a critical event for derepression of Rgt1 target genes (Kaniak *et al.*, 2004; Kim *et al.*, 2006). Mth1 is a common target of two glucose-signaling pathways: it is degraded in proteasome via the Rgt2/Snf3 pathway; its expression is repressed by the Snf1-Mig1 pathway. Mth1 inactivation facilitates Rgt1 phosphorylation by the cAMP-PKA pathway, leading to dissociation of Rgt1 from Ssn6-Tup1 and consequently from its DNA target sites (Jouandot *et al.*, 2011). Therefore these three different glucose-signaling pathways converge on Rgt1 to regulate expression of *HXT* genes (Figure 6C).

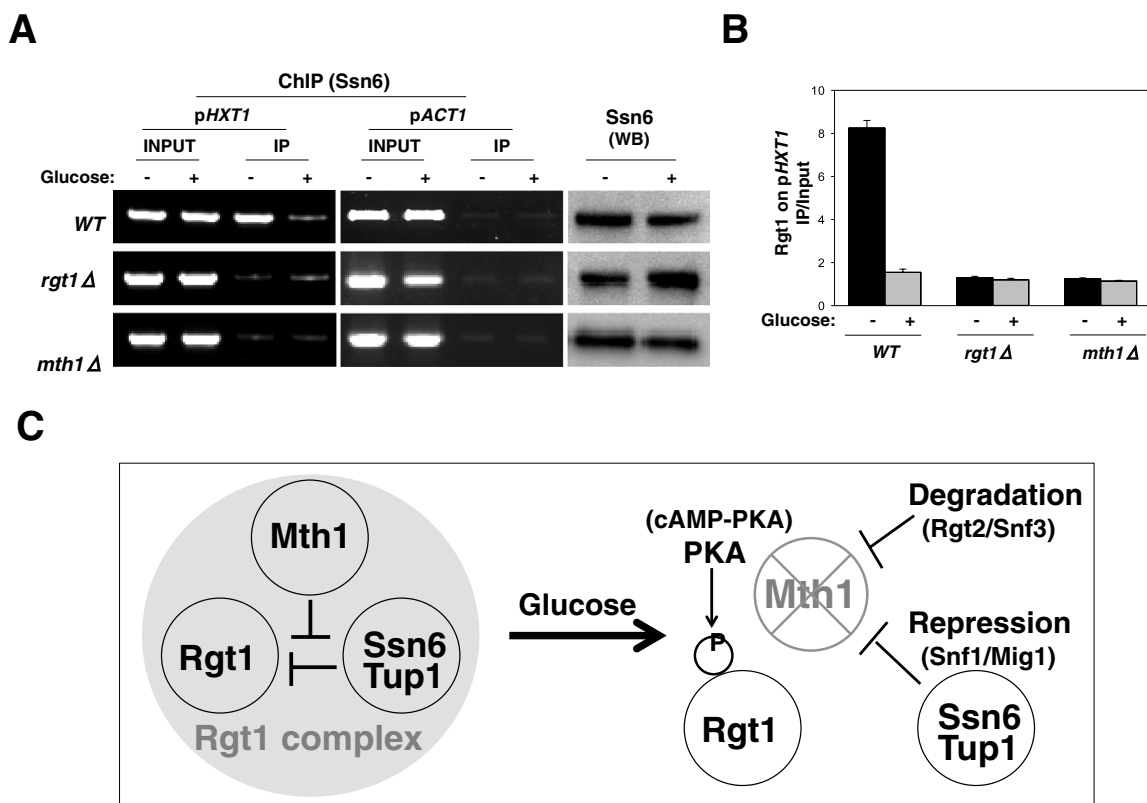


FIGURE 6: Ssn6-Tup1 is recruited to the *HXT1* promoter in an Rgt1-dependent manner. (A) Yeast cells (WT, *rgt1*Δ, and *mth1*Δ) were grown in YP-2% galactose medium (-) and shifted to YP-4% glucose medium (+) for 1 h. Cross-linked chromatin was immunoprecipitated with anti-Ssn6 antibody, and representative PCRs are shown (left and middle). Input or immunoprecipitated DNA was PCR amplified with primers specific to *pHXT1* or *pACT1* as a negative control. Western blot analysis of Ssn6 expression using anti-Ssn6 antibody (right). (B) qPCR analysis of Ssn6 binding to the *HXT1* promoter in yeast cells. The results are expressed as IP/Input ratio as described in Figure 1B, using the *pACT1* promoter as a negative control. (C) A proposed model of the role of Mth1 in Rgt1-mediated repression. In the absence of glucose, Ssn6-Tup1 interferes with Rgt1 DNA binding but is antagonized by Mth1. Mth1 mediates the interaction between Rgt1 and Ssn6-Tup1 by blocking the PKA-dependent phosphorylation of Rgt1 that impairs the ability of Rgt1 to associate with Ssn6-Tup1 and with its target DNA sites. Therefore Mth1 acts as a scaffold-like protein to recruit Ssn6-Tup1 to Rgt1. This complex is disrupted upon glucose-induced proteasomal degradation of Mth1 via the Rgt2/Snf3 pathway and followed by PKA-dependent phosphorylation of Rgt1. Expression of *MTH1* gene is also repressed by the Snf1-Mig1 pathway. Consequently, phosphorylated Rgt1 is dissociated from Ssn6-Tup1 and eventually from DNA, leading to derepression of Rgt1 target genes.

Of note, we show that Ssn6-Tup1, although required for Rgt1-mediated repression, acts to inhibit, rather than stimulate, Rgt1 function. Ssn6-Tup1 does so by inhibiting the DNA-binding ability of Rgt1 in the absence of glucose (which is antagonized by Mth1) and by repressing *MTH1* expression via the Snf1-Mig1 pathway in high-glucose conditions. This suggests that Ssn6-Tup1 can negatively regulate its recruiting DNA-binding transcription factor. As evidenced in Figure 2, Mig1, an Ssn6-Tup1 recruiter, is not negatively regulated by Ssn6-Tup1, supporting the view that Ssn6-Tup1 acts differently on different repressors in yeast cells under identical growth conditions. The biological significance of this phenomenon is not fully understood but might be related to the differential regulation of Ssn6-Tup1 target genes in response to the same stimulus. For instance, Mig1 and Rgt1 are positively and negatively regulated by glucose. Ssn6-Tup1 in high-glucose condition is recruited to Mig1 but should not be associated with Rgt1. In addition, Mig1 occupies *GAL1* continuously under either repressing or inducing conditions (Papamichos-Chronakis *et al.*, 2004). Therefore the corepressor complex in those conditions may actively inhibit its interaction with Rgt1 while associating with Mig1, thereby avoiding dysregula-

tion of genes regulated by the two glucose-responsive transcription repressors.

It is not known how Ssn6-Tup1 interferes with the DNA-binding activity of Rgt1. The purified N-terminal fragment of Rgt1 containing a DNA-binding motif is able to bind DNA in the absence of Mth1 *in vitro*, suggesting that the Rgt1 DNA-binding domain by itself can bind DNA (Kim *et al.*, 2003). Ssn6 and Tup1 contain TPR and WD40 domains, respectively (Schultz *et al.*, 1990; Jabet *et al.*, 2000; Sprague *et al.*, 2000), and appear to interact with different repressors via the domains in different manners (Smith *et al.*, 1995; Tzamarias and Struhl, 1995). The Ssn6-binding region in Rgt1 (aa 210–250) is located close to the Zn cluster DNA-binding motif (Polish *et al.*, 2005). These observations suggest that Rgt1-Ssn6-Tup1 interaction is transient but sufficient to induce a conformational change of Rgt1 and lead to dissociation of Rgt1 from *HXT* promoters. A physical interaction of Mth1 with Rgt1 prevents this from happening, enabling Rgt1 to form a functional repressor complex with Ssn6-Tup1 on *HXT* promoters.

Previous evidence showed that Ssn6-Tup1 is also actively involved in induction of gene expression (Papamichos-Chronakis

et al., 2002; Proft and Struhl, 2002; Mennella et al., 2003) and can be recruited to its target promoters in a manner independent of sequence-specific DNA-binding proteins (Papamichos-Chronakis et al., 2004; Buck and Lieb, 2006; Desimone and Laney, 2010; Hanlon et al., 2011). Recent work also shows that Ssn6-Tup1 exerts its function by masking the activation domain of a DNA-binding repressor and thereby preventing recruitment of the coactivators necessary for transcriptional activation (Wong and Struhl, 2011). Glucose induction of *HXT* expression is not inhibited by deletion of the *SSN6* or *TUP1* gene, suggesting that Ssn6-Tup1 does not act as an activator of the *HXT* genes (Ozcan et al., 1996b). Our findings in this study also indicate that Ssn6-Tup1 is recruited to *HXT* promoters by Rgt1, and this recruitment occurs in an Mth1-dependent manner in the absence of glucose. However, Ssn6-Tup1 is dissociated from Rgt1 and consequently from the *HXT* promoters upon glucose-induced down-regulation of Mth1, reinforcing the view that Mth1 plays a key role in recruitment of Ssn6-Tup1 to Rgt1.

Regulation of Rgt1 function is mechanistically similar to that of Mig1, which is phosphorylated and negatively regulated by Snf1 kinase (Treitel et al., 1998; Hedbacker and Carlson, 2008). Ssn6-Tup1 is recruited to only unphosphorylated Mig1 in the presence of high glucose and mediates repression of Mig1-target genes, including *SUC2*. Mig1-Ssn6-Tup1 interaction is disrupted when Mig1 is phosphorylated by Snf1 in low-glucose conditions, leading to derepression of these genes (Papamichos-Chronakis et al., 2004). Similarly, Rgt1-Ssn6-Tup1 interaction is disrupted when Rgt1 is phosphorylated by PKA in high levels of glucose, leading to derepression of *HXT* gene expression. Therefore it is likely that the role of phosphorylation of Mig1 and Rgt1 repressors in inducing conditions is to prevent them from associating with Ssn6-Tup1. Furthermore, Rgt1 binds to the *HXT1* promoter in the absence of Ssn6 or Tup1 in high-glucose-grown cells but does not repress the promoter (Figure 1), reinforcing the view that glucose induction of *HXT* expression is primarily due to disruption of the interaction of Rgt1 with Ssn6-Tup1 rather than dissociation of Rgt1 from *HXT* promoters.

MATERIALS AND METHODS

Yeast strains, gene deletion, 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 medium containing 0.17% yeast nitrogen base and 0.5% ammonium sulfate) supplemented with the appropriate amino acids and carbon sources. Genes were disrupted with NatMX or KanMX cassette by the homologous recombination method (Wach et al., 1994; Goldstein et al., 1999). The *HXT-NAT* reporter strains were constructed by replacing *HXT1*, *HXT2*, and *HXT3* open reading frames with the NatMX coding region by homologous recombination. JKP173 (LexA-Ssn6) and JKP231 (Ssn6-HA) were constructed by cloning the *SSN6* gene into the LexA and HA plasmids, respectively. JKP232 (Rgt1 (210-250Δ)-HA) and JKP233 (Rgt1 (310-360Δ)-HA) were constructed by gap repair.

Chromatin immunoprecipitation

ChIP was performed as described previously (Kim et al., 2003). Yeast strains were grown until mid-log phase ($OD_{600\text{ nm}} = 1.2-1.5$) and incubated with formaldehyde (1% final concentration) at room temperature for 15–20 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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-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 pulses. The genomic DNA fragments, with average size 200–500 base pairs, were immunoprecipitated with HA or Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) conjugated with agarose bead. After washing the immunoprecipitated beads with ChIP high-salt buffer (ChIP lysis buffer containing 500 mM NaCl instead of 150 mM NaCl) and then ChIP wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% NP40, 0.5% Na deoxycholate, 1 mM EDTA), we eluted DNA

Strain	Genotype	Source
BY4741	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ</i>	Kaniak et al., 2004
BY4743	<i>Mata/α his3Δ1/his3Δ1 leu2Δ0/ leu2Δ0 ura3Δ0/ ura3Δ0 met15Δ0/MET15 lys2Δ0/LYS2</i>	Kaniak et al., 2004
FM557	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 rgt1::kanMX</i>	Kaniak et al., 2004
FM645	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::URA3</i>	Toda et al., 1987
YM6266	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 mth1::kanMX</i>	Kim et al., 2006
YM6545	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 RGT2-1</i>	Kaniak et al., 2004
YM6684	<i>Mata/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/MET15 lys2Δ0/LYS2 mig1::kanMX/mig1::kanMX mig2::kanMX/mig2::kanMX mig3::kanMX/mig3::kanMX</i>	Kaniak et al., 2004
JKY 32	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 mth1::kanMX2 std1::NAT</i>	This study
JKY 66	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::URA3 mth1::NAT</i>	This study
JKY 83	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 RGT2-1 ssn6::NAT</i>	This study
JKY 87	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 mth1::kanMX std1::kanMX ssn6::NAT</i>	This study
JKY 88	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 pHXT1-NAT</i>	This study
JKY 89	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 pHXT2-NAT</i>	This study
JKY 90	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 pHXT3-NAT</i>	This study
JKY 91	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ LYS2 mth1::kanMX std1::kanMX tup1::NAT</i>	This study
JKY 93	<i>Mata ura3-52 his3-11 leu2::kanMX6 15MAL2 SUC2 GAL MET HTR1-23</i>	This study

TABLE 1: *S. cerevisiae* strains used in this study.

from both immunoprecipitated and 1/100 input samples by incubating the samples in ChIP elution buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 10 mM EDTA) at 65°C for 6–8 h. Finally, the DNAs were purified by QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The amount of immunoprecipitated DNA was quantified by real-time PCR using SsoFast Evagreen reagent (Bio-Rad, Hercules, CA) in CFX96 Real-Time Thermal Cycler (Bio-Rad) using primer pairs directed against *HXT1*, *HXT2*, *HXT3*, *SUC2*, or *GAL1* promoters. As a negative control, primer sets were designed to amplify the actin gene promoter region. DNA-binding of Rgt1 or Mig1 was determined by the ratio of IP/target promoters relative to the IP/*ACT1* promoter divided by the ratio of input (Input)/target promoters relative to the Input/*ACT1* promoter. All of the data were averages of three independent ChIP experiments, with error bars representing SDs. The sequences of the primers used for ChIP were *HXT1*, 5'-ATATAATCCCCCTCCTGAAG-3' and 5'-TGATTCTACGTTTTT-GCAAGC-3'; *HXT3*, 5'-CTTCT-CGAGATAACACCTGG-3' and 5'-CCACGAAGCTTTTCTCTGTG-3'; *SUC2*, 5'-GTAGTTCTCGCTC-CCCCAG-3' and 5'-TGGGGTCGATTAACGCTACG-3'; *GAL1*, 5'-CGAATCAAATTAACAACCATA-GGATGATA-3' and 5'-TATAGTTTT-TTCTCCTTGACGTAAAG-3'; *ACT1*, 5'-CCTGAACGAAAC-CACT-CAGAAGAA-3' and 5'-TTAAGGGTTTTGAGGATCCGATAAGG-3'.

Western blot and immunoprecipitation assays

For Western blot analysis, yeast cells ($OD_{600} = 1.5$) were collected by centrifugation at 3000 rpm in a tabletop centrifuge for 5 min. Cell pellets were resuspended in 100 μ l 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 polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membranes were incubated with appropriate antibodies (anti-HA, anti-LexA, anti-Myc, anti-Ssn6, and anti-GFP antibodies; Santa Cruz Biotechnology) in TBST buffer (10 mM Tris-HCl, pH, 7.5, 150 mM NaCl, 0.1% Tween-20), and proteins were detected by an enhanced chemiluminescence system (Pierce, Rockford, IL). 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 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 (GE Healthcare, Piscataway, NJ) at 4°C for 1 h. The precipitated agarose beads were washed three times with ice-cold NP40 buffer containing protease inhibitors and boiled in 50 μ l of SDS-PAGE buffer. The resulting proteins were subjected to Western blot analysis.

Quantitative RT-PCR

Yeast cells were grown in YP medium containing 2% galactose until mid-log phase ($OD_{600\text{ nm}} = 1.2\text{--}1.5$) and shifted to YP medium containing 4% glucose for 1 h. Total RNA was extracted by RNeasy Mini Kit (Qiagen) following the manufacturer's protocol, and 2 μ g of total RNA was converted to cDNA by qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). cDNA was analyzed by qRT-PCR using SsoFast Evagreen reagent in CFX96 Real-Time Thermal Cycler. *ACT1* was used as an internal control to normalize expression of *HXT1*, *HXT2*, or *HXT3* gene. All of the shown quantification data were the averages of three independent experiments, with error bars representing SDs.

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 (Kaniak et al., 2004). Results are given in Miller units, $(1000 \times OD_{420\text{ nm}})/(TV \times OD_{600\text{ nm}})$, where T is the incubation time in minutes and V is the volume of cells in milliliters. The reported enzyme activities are averages of results from triplicates of three different transformants.

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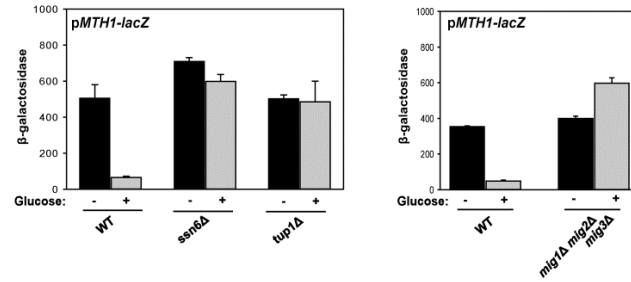


Figure S1

Figure S1. Repression of the *MTH1* gene by the Mig1-Ssn6-Tup1 complex. Yeast cells carrying the *MTH1-LacZ* reporter plasmid were first grown in SC-2% galactose medium (-) and shifted to SC-4% glucose medium (+) for 1 hr and assayed for β -galactosidase activity.

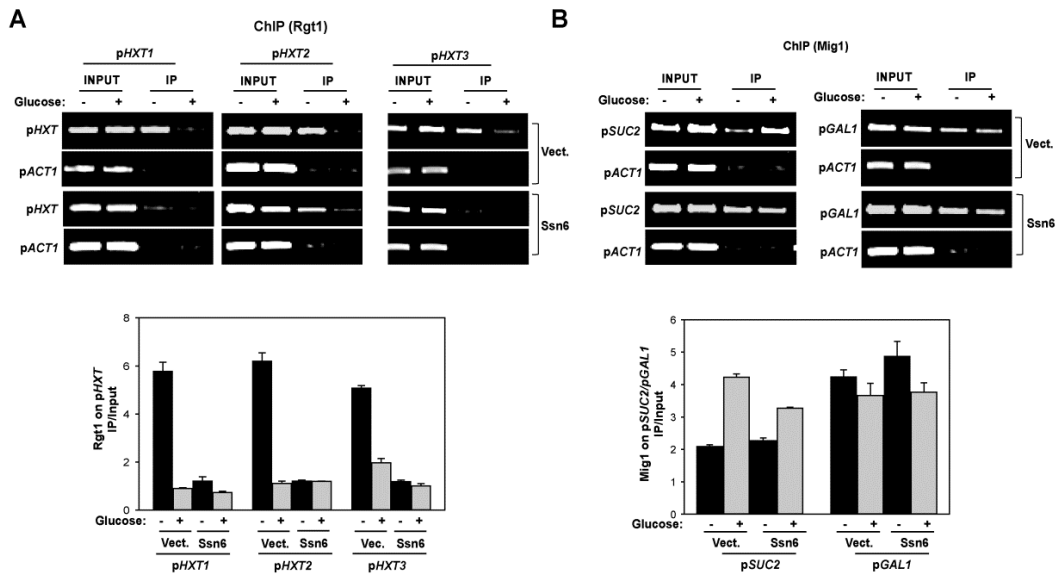


Figure S2

Figure S2. Ssn6 overexpression abrogates the DNA-binding ability of Rgt1, but not of Mig1. Yeast cells (BY4741, *WT*) cooverexpressing Ssn6-HA with Rgt1-HA were grown in SC-2% galactose (-) and shifted to SC-4% glucose (+) for 1 hr. Cross-linked chromatin was precipitated for ChIP analysis of the indicated Rgt1 (*pHXT1*, *pHXT2*, and *pHXT3*) (A) and Mig1 (*pSUC2* and *pGAL1*) (B) DNA target sites. Representative PCRs of ChIP analysis were shown (upper panels). The amount of immunoprecipitated DNA was quantified by qRT-PCR with primer pairs directed against the *HXT1*, *HXT2*, *HXT3*, *SUC2*, and *GAL1* promoters. As a control, primer sets were also used for Actin promoter (*pACT1*). qPCR analysis of the DNA-binding of Rgt1 and Mig1 was expressed as IP/Input ratio which was determined by the ratio of immunoprecipitated (IP) *pHXT* relative to the IP *pACT1* divided by the ratio of INPUT *pHXT* relative to the INPUT *pACT1* (bottom panel). The data shown were averages of three independent experiments with error bars showing mean \pm S.D.

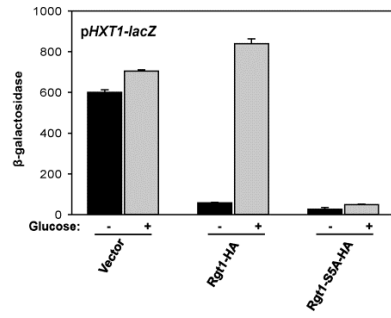


Figure S3

Figure S3. Rgt1-S5A-HA constitutively represses expression of the *HXT1* gene. Yeast cell (*rgt1Δ*) expressing Rgt1-S5A-HA was grown in SC-2% galactose medium (-) and shifted to SC-4% glucose medium (+) for 1 hr and assayed for β-galactosidase activity.