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Integration of Transcriptional and Posttranslational Regulation in a Glucose Signal Transduction Pathway in *Saccharomyces cerevisiae*

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Expression of the *HXT* genes encoding glucose transporters in the budding yeast *Saccharomyces cerevisiae* is regulated by two interconnected glucose-signaling pathways: the Snf3/Rgt2-Rgt1 glucose induction pathway and the Snf1-Mig1 glucose repression pathway. The Snf3 and Rgt2 glucose sensors in the membrane generate a signal in the presence of glucose that inhibits the functions of Std1 and Mth1, paralogous proteins that regulate the function of the Rgt1 transcription factor, which binds to the *HXT* promoters. It is well established that glucose induces degradation of Mth1, but the fate of its paralogue Std1 has been less clear. We present evidence that glucose-induced degradation of Std1 via the SCF<sup>Grb1</sup> ubiquitin-protein ligase and the 26S proteasome is obscured by feedback regulation of *STD1* expression. Disappearance of Std1 in response to glucose is accelerated when glucose induction of *STD1* expression due to feedback regulation by Rgt1 is prevented. The consequence of relieving feedback regulation of *STD1* expression is that reestablishment of repression of *HXT1* expression upon removal of glucose is delayed. In contrast, degradation of Mth1 is reinforced by glucose repression of *MTH1* expression: disappearance of Mth1 is slowed when glucose repression of *MTH1* expression is prevented, and this results in a delay in induction of *HXT3* expression in response to glucose. Thus, the cellular levels of Std1 and Mth1, and, as a consequence, the kinetics of induction and repression of *HXT* gene expression, are closely regulated by interwoven transcriptional and posttranslational controls mediated by two different glucose-sensing pathways.

Glucose is an important source of carbon and energy for many organisms. This is particularly apparent in the budding yeast *Saccharomyces cerevisiae*, whose sophisticated glucose-sensing and -signaling mechanisms enable it to sense a wide range of glucose concentrations and utilize glucose efficiently (2, 7, 13). One of the first responses of yeast cells to glucose is induction of expression of the *HXT* genes, encoding glucose transporters (3, 18, 21, 28, 40). This is achieved through a signal transduction pathway that begins at the cell surface with the Snf3 and Rgt2 glucose sensors and ends in the nucleus with the Rgt1 transcription factor, which binds to *HXT* gene promoters (5, 12, 14, 27, 31).

The glucose signal generated by Rgt2 and Snf3 at the cell surface alters Rgt1 function in the nucleus by stimulating degradation of Mth1 and Std1 (4, 23), paralogous proteins that bind to Rgt1 and are necessary for it to repress transcription (20, 30, 32). Mth1 and Std1 also interact with the C-terminal tails of the Rgt2 and Snf3 glucose sensors (19, 32). These places them in proximity to the Yck1 protein kinase, which is associated with the Snf3 and Rgt2 glucose sensors and is thought to catalyze phosphorylation of Mth1 and Std1 when glucose binds to the sensors (23, 37). Phosphorylated Mth1 and Std1 are targets of the SCF<sup>Grb1</sup> ubiquitin-protein ligase, which is thought to catalyze their ubiquitination, thereby targeting them for degradation by the 26S proteasome (37). In the absence of Mth1 and Std1, Rgt1 loses its ability to repress transcription, leading to derepression of *HXT* gene expression (4, 20, 24, 30, 32).

While there is ample evidence that glucose induces degradation of Mth1 via the 26S proteasome, conflicting results have been reported for the effect of glucose on Std1 (4, 23, 37). *STD1* expression is induced by glucose via the Rgt2/Snf3-Rgt1 signal transduction pathway (15), and our data suggest that Std1 degradation is dampened by this glucose induction of *STD1* expression via the Rgt2/Snf3-Rgt1 pathway. By contrast, *MTH1* expression is repressed by glucose via the Snf1-Mig1 glucose repression pathway, and our results suggest that this reinforces Mth1 degradation. Thus, opposing transcriptional regulation of *MTH1* and *STD1* expression provides for rapid induction of *HXT* gene expression in response to glucose and for prompt establishment of repression of *HXT* gene expression when the available glucose has been exhausted. Thus, the course of induction and repression of the *HXT* genes is the result of close collaboration between two different glucose-sensing pathways that helps ensure efficient utilization of this key nutrient.

**MATERIALS AND METHODS**

**Yeast strains and plasmids.** The yeast strains used in this study are listed in Table 1. Cells were grown on either YP (2% Bacto peptone, 1% yeast extract) or YNB [0.67% yeast nitrogen base plus 0.5% (NH₄)₂SO₄ lacking the appropriate amino acids] medium, supplemented with the appropriate carbon sources. Genes were disrupted by homologous recombination using HisG-URA3-HisG (1) or KanMX (39) cassettes. Sequences of the primers are available on request. To construct pBM4747 (MET25 promoter–green fluorescent protein [GFP]–*STD1*), pBM4748 (MET25 promoter–GFP–*MTH1*), and pBM4749 (MET25 promoter–GFP–Htr1-23), coding sequences of the genes were amplified by PCR and the resulting PCR products were cloned into the BamHI and SalI sites of pUG3 (pBM3642 ARSH4/CEN–HIS3–MET25 promoter–yeGFP–polylinker–CYC1 ter-

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TABLE 1. Yeast strains used in this study

| Strain          | Genotype                                      |
|-----------------|-----------------------------------------------|
| FM391           | MATa his3Δ1 leu2Δ ura3Δ met15Δ (BY4741)        |
| FM395           | MATa/MATa his3Δ1/Δ his3Δ1 leu2Δ/Δ ura3Δ/Δ met15Δ/Δ |
| FM412           | MATa trp1-901 leu2-3,112 ura3-32 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ |
| FM413           | MATa trp1-901 leu2-3,112 ura3-32 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ |
| FM439           | MATa ade2-1 his3-11,15 ura3-1 trp1-1 (CMY 18)  |
| FM452           | MATa his3-11,15 leu2-3,115 ura3-1 trp1-1 ade2-1 cdc34-2 |
| FM524           | MATa ura3 his3-11,15 leu2-3,112 can1 pre2-280 |
| FM535           | MATa cim5-1(Δ) ura3-52 his3-Δ200 leu2-Δ1  |
| YM4127          | MATa ura3-52 his3-200 ade2-101 lys2-801 leu2 trp1-903 tyr1-501 |
| YM4509          | MATa ura3-52 his3-200 ade2-101 lys2-801 leu2 trp1-903 tyr1 rgt1::HisG |
| YM6212          | MATa ura3-52 his3-200 ade2-101 lys2-801 leu2 trp1-903 leu2-3,112 tyr1-501 snf3::His3 rgt2::His3 |
| YM6244          | MATa ura3-52 his3-11,15 |
| YM6245          | MATa ura3-52 his3-11,15 MTH1-23 (HTR1-23)       |
| YM6265          | MATa his3Δ leu2Δ met15Δ ura3Δ std1::KanMX2    |
| YM6266          | MATa his3Δ leu2Δ his3-2Δ ura3Δ std1::KanMX2    |
| YM6269          | MATa ura3-52 his3-200 ade2-101 lys2-801 leu2 trp1Δ rgt1::HisG |
| YM6292          | MATa his3Δ leu2Δ met15Δ ura3Δ snl1::KanMX2 std1::KanMX2 |
| YM6328          | MATa his3Δ leu2Δ ura3Δ met1Δ snf3::HisG-URA3::HisG |
| YM6452          | MATa ade2 ura3 his3 lys2 leu2 grr1::LEU2       |

RESULTS

Mth1, Std1, and Rgt1 are required for repression of HXT gene expression. To verify the roles of Mth1, Std1, and Rgt1 in glucose signaling, we analyzed the effect of loss of these genes on expression of HXT1, which is induced by high levels of glucose (2%), and on expression of HXT3, which is induced by low levels of glucose (0.2% [a condition mimicked by 2% raffinose]), as well as by high glucose levels (28) (Table 2, genotype 1). As expected, removal of the Rgt1 repressor substantially relieved repression of the HXT genes (Table 2, genotype 2, Gal). Deletion of MTH1 relieved repression of the low-glucose-induced HXT3 gene but had little effect on expression of the high-glucose-induced HXT1 gene (Table 2, genotype 3, Gal). Further deletion of STD1 in an mth1 mutant relieved repression of HXT1 expression (Table 2, genotype 5). Deletion of STD1 alone had little effect on expression of HXT1 and HXT3 (Table 2, genotype 4). Thus, Mth1 seems to be responsible for repression of the low-glucose-induced HXT3 gene (and for that of HXT4, another low-glucose-induced gene [data not shown]), but either Std1 or Mth1 is sufficient for repression of the high-glucose-induced HXT1 gene in the absence of glucose. These results reinforce previously reported findings (4, 20, 32) and suggest that Mth1 and Std1 work together with Rgt1 to repress expression of the high-glucose-induced HXT1 gene, but that Mth1 acts alone to regulate expression of the low-glucose-induced HXT3 and HXT4 genes.

Mth1 and Std1 inhibit the ability of Rgt1 to activate transcription. Rgt1 is a transcriptional activator in cells grown on high levels of glucose but not in cells grown in the absence of glucose (on galactose) (29) (Table 3, genotype 1). Deletion of both MTH1 and STD1 causes Rgt1 to activate transcription in cells grown on galactose (Table 3, genotype 4), suggesting that Mth1 and Std1 play a role in inhibiting transcriptional activation, in addition to their roles in promoting transcriptional

TABLE 2. Roles of Rgt1, Mth1, and Std1 in regulation of HXT gene expression

| Genotype | HXT1::lacZ | HXT3::lacZ |
|----------|------------|------------|
|          | Gal | Raf | Glu | Gal | Raf | Glu |
| 1. Wild type                  | <1  | 29  | 1,220 | <1  | 20  | 629  | 744 |
| 2. rgt1 MTH1 STD1             | 27  | 57  | 326  | 225  | 632 | 559  |
| 3. RGT1 mth1 STD1             | <1  | <1  | 820  | 326  | 415 | 484  |
| 4. RGT1 MTH1 std1             | <1  | 30  | 951  | 42   | 474 | 485  |
| 5. RGT1 mth1 std1             | 127 | 607 | 632  | 148  | 893 | 760  |

a Precultures were grown to mid-log phase (OD600 = 1.5) in selective YNB medium with 2% galactose, shifted to YP medium containing the indicated sugars (all at 2%), grown for 4 h at 30°C, and then assayed for β-galactosidase activity.

b Values are means from at least four independent experiments. Standard deviations are less than 20% in all cases. Gal, galactose; Raf, raffinose; Glu, glucose.
repression in the absence of glucose. In mth1 and std1 single mutants, Rgt1 was unable to activate significant transcription in cells grown on galactose, indicating that Mth1 and Std1 are redundant inhibitors of the transcriptional activation function of Rgt1 when glucose levels are low. A form of Mth1 that is resistant to glucose-induced degradation (due to the dominant Rgt1 when glucose levels are low) is produced in the presence of high levels of glucose. Thus, in addition to promoting transcriptional repression by Rgt1 when glucose is absent (4, 30, 32), Mth1 and Std1 seem to inhibit transcriptional activation by Rgt1 when glucose levels are low. We believe that Mth1 and Std1 regulate Rgt1 function directly, because they interact with Rgt1 (20, 30, 38). In addition, Mth1 and Std1 are associated with HXT promoters (Fig. 1).

Glucose regulation of MTH1 and STD1 expression contributes to glucose signal transduction. It seems clear that Mth1 is degraded upon exposure of yeast cells to glucose, but there are conflicting reports regarding Std1 degradation in response to glucose (4, 23). Indeed, in our hands, degradation of Mth1 was reproducibly observed but degradation of Std1 in response to glucose was variable. We suspected that this was due to the different regulation of STD1 and MTH1 expression by glucose: STD1 expression is induced by glucose via the Rgt2/Snf3-Rgt1 pathway, while MTH1 expression is repressed by glucose via the Snf1-Mig1 pathway (15). Induction of STD1 expression by glucose would be expected to counteract glucose-induced deg-

### Table 3. Activation of the lexO-lacZ reporter by LexA-Rgt1

| Genotype | Active molecule(s) | β-Galactosidase activity (Miller units) |
|----------|--------------------|----------------------------------------|
|          |                    | Galactose | Glucose |
| 1. Wild type (FM391) | Mth1, Std1 | <1 | 575 |
| 2. mth1 (YM6266) | Std1 | 4 | 293 |
| 3. std1 (YM6265) | Mth1 | <1 | 325 |
| 4. mth1 std1 (YM6292) | Mth1, Std1 | 346 | 533 |
| 5. Wild type (YM6244) | Mth1, Std1 | 6 | 252 |
| 6. HTR1-23 (YM6245) | Mth1', Std1 | 3 | 2 |

* a The plasmids used are pBM1817 (lexO-lacZ reporter) and pBM3306 (LexA-Rgt1).
* b Refers to the molecules present in the cells that act on Rgt1.
* c All sugars were present at a concentration of 2% in the growth medium.
* d Mth1 constitutive repressor caused by the HTR1-23 mutation (6, 26, 33, 34).
radation of Std1 and obscure its disappearance. Conversely, repression of MTH1 expression by glucose should reinforce the glucose-induced degradation of Mth1, thereby enhancing its disappearance upon addition of glucose to cells.

We interrupted glucose regulation of STD1 and MTH1 by replacing their promoters with the promoter of MET25, which is not regulated by glucose. Expressing STD1 at the basal level of this promoter (by including methionine in the medium) makes degradation of Std1 in glucose-grown cells obvious (Fig. 2A, center panels) and significantly accelerates the rate of loss of Std1 after addition of glucose to cells (Fig. 2B). This suggests that induction of STD1 expression by glucose attenuates the glucose signal to Rgt1 by slowing the disappearance of Std1. By contrast, when repression of MTH1 expression by glucose is interrupted either by expressing MTH1 at the basal level of the MET25 promoter, by deleting MIG1 and MIG2, or by removing the Mig1/Mig2-binding sites from the MTH1 promoter, the extent (Fig. 2A) and rate (Fig. 2C) of degradation of MTH1 are reduced.

FIG. 3. Relieving transcriptional regulation of MTH1 and STD1 results in delayed induction and delayed repression, respectively, of HXT gene expression. (A) FM393 (wild type) (solid line) and YM6682 (mig1Δ mig2Δ) (dashed line) carrying HXT3::lacZ (pBM2819) were grown on 2% galactose. At time zero, 2% glucose was added to induce expression of HXT3. β-Galactosidase was assayed at the times indicated. (B) Cells (YM6292) carrying HXT7::lacZ (pBM2636) and expressing STD1 from its own promoter (pBM4540) or from the MET25 promoter (pBM4747) were grown in glucose. At time zero, the cells were pelleted, washed with water, and resuspended in 2% galactose to induce repression of HXT7 expression. Aliquots of the culture were assayed for β-galactosidase activity at the times indicated. During this time the cells approximately doubled in number.

FIG. 4. Rgt2 and Snf3 promote glucose-induced degradation of Std1 and Mth1. (A) GFP-Std1 (pBM4747) or GFP-Mth1 (pBM4748) expressed in wild-type (FM391) or rgt2 snf3 (YM6212) cells was detected by Western blotting. Control lanes (Ctl.) were loaded with extracts of cells containing the empty vector (GFP alone). (B) The dominant HTR1-23 mutation in MTH1 is resistant to degradation. GFP-Mth1 with the HTR1-23 mutation (pBM4749) was expressed in FM391 (wild type) and was detected by Western blotting.

FIG. 5. Degradation of Mth1 and Std1 requires the SCF^{Grr1} ubiquitin-protein ligase complex and the 26S proteasome. For Western blotting, cell extracts were prepared from yeast cells expressing GFP-Std1 (pBM4747) or GFP-Mth1 (pBM4748) and treated as described for Fig. 2A. Strains used were YM4127 (wild type), YM6542 (grr1Δ), FM542 (cdc34ts), and FM524 (pre2-2ts). Temperature-sensitive mutant strains were grown at 30°C overnight, then shifted to a medium containing 4% glucose, and incubated for 1 h at 30°C or 37°C. The GFP-Std1 and GFP-Mth1 proteins were then detected by Western blotting.
The effects of transcriptional regulation of \textit{MTH1} and \textit{STD1} on the rate of loss of Mth1 and Std1 are expected to be translated into effects on the rates of induction and repression of \textit{HXT} expression. We surmised that the glucose repression of \textit{MTH1} expression and the resulting acceleration of its disappearance from the cell after addition of glucose might serve to ensure speedy induction of \textit{HXT} expression. Indeed, in cells in which \textit{MTH1} expression is not repressed by glucose (due to deletion of the genes encoding the Mig1 and Mig2 glucose repressors), induction of \textit{HXT} expression by glucose is delayed relative to that in wild-type cells (Fig. 3A). Conversely, we speculated that glucose induction of \textit{STD1} expression might serve to replenish Std1 after its initial glucose-induced degradation so as to enable prompt establishment of repression of \textit{HXT} expression when glucose is exhausted in the culture. Indeed, in cells in which \textit{STD1} expression is not induced by glucose, repression of \textit{HXT} expression is established more slowly than in wild-type cells after addition of galactose (Fig. 3B). Thus, transcriptional regulation of \textit{MTH1} and \textit{STD1} significantly affects the course of induction and repression of \textit{HXT} gene expression.

Degradation of Std1 and Mth1 requires a glucose signal. Degradation of Std1 and Mth1 requires the glucose sensors Rgt2 and Snf3 (Fig. 4), as well as two components of the SCF\textsuperscript{Grr1} ubiquitin-protein ligase, Grr1 and Cdc34 (Fig. 5). Glucose addition does not cause Std1 and Mth1 to disappear in a temperature-sensitive \textit{pre2} mutant defective in a chymotrypsin-like activity of the proteasome (8–11) or in the presence of the proteasome inhibitor MG132 (Fig. 2). These results support the view that glucose binding to the Rgt2 and Snf3 glucose sensors causes them to initiate proteasome-mediated degradation of Mth1 and Std1 by targeting them for ubiquitination by the SCF\textsuperscript{Grr1} ubiquitin-protein ligase.

Potential ubiquitin attachment sites in Std1 are required for Std1 degradation. The SCF\textsuperscript{Grr1} ubiquitin-protein ligase catalyzes the covalent attachment of ubiquitin to lysine residues of the target protein (17, 22, 35, 36). Evidence has been presented that suggests that Mth1 is ubiquitinated (37), but similar evidence that Std1 is also modified in this way is lacking. Indeed,
our attempts to demonstrate this modification of Std1 have so far proven unsuccessful. We noticed 10 lysine residues in Std1 that are conserved in its orthologues in other yeasts (positions 207, 282, 287, 312, 334, 347, 354, 381, and 411). Conversion of 9 of these lysines to arginine (9KR) prevented glucose-induced degradation of Std1 (Fig. 6B) and severely reduced derepression of HXT1 expression (Fig. 6A). Changing fewer than 9 of these lysine residues (7KR and 5KR) had smaller effects on induction of HXT1 expression, suggesting that ubiquitination at only a few sites of Std1 is required to target the protein for degradation. These results provide indirect evidence supporting the idea that Std1 is targeted for degradation by ubiquitination.

**DISCUSSION**

Degradation of Std1 and Mth1 is the central event in transmission of the glucose signal to Rgt1, which results in induction of expression of the HXT genes. Glucose binding to the Snf3 and Rgt2 sensors stimulates degradation of Mth1 and Std1, probably by activating casein kinase (Yck1 and Yck2), which phosphorylates Mth1 and Std1, thereby making them substrates for the SCF<sup>Grr1</sup> ubiquitin-protein ligase and targeting them for degradation in the proteasome (23, 37). It has been difficult to demonstrate directly that Mth1 and Std1 become modified by ubiquitination when glucose is added to cells (37) (our unpublished results). Our observations that the SCF<sup>Grr1</sup> ubiquitin-protein ligase and several lysine residues in Std1 that are conserved in evolution are required for its glucose-induced degradation (Fig. 5 and 6) provide indirect evidence that ubiquitination of Std1 (and, by inference, of Mth1) plays a role in degradation (Fig. 5 and 6) provide indirect evidence that ubiquitination of Std1 (and, by inference, of Mth1) plays a role in degradation (Fig. 5 and 6) provide indirect evidence that ubiquitination of Std1 (and, by inference, of Mth1) plays a role in degradation (Fig. 5 and 6) provide indirect evidence that ubiquitination of Std1 (and, by inference, of Mth1) plays a role in degradation.

Glucose also regulates the levels of Mth1 and Std1 in cells by regulating MTH1 and STD1 transcription via feedback and feedforward regulatory mechanisms that operate through two different glucose signal transduction pathways (15). Glucose-induced disappearance of Std1 is attenuated by feedback regulation of STD1 expression via the Snf3/Rgt2-Rgt1 signal transduction pathway (Fig. 2), which causes STD1 expression to be induced by glucose, thereby replenishing Std1 soon after its degradation is initiated by addition of glucose to cells. We believe this feedback regulation evolved to provide sufficient levels of Std1 to ensure efficient reestablishment of repression of HXT expression as soon as cells exhaust the available glucose. Indeed, interruption of this regulation of STD1 expression results in slower establishment of repression of HXT expression upon removal of glucose from cells (Fig. 3B). In contrast, Mth1 degradation is reinforced by glucose repression of MTH1 expression mediated by the Snf1-Mig1 glucose-signaling pathway. Disappearance of Mth1 is slowed in cells missing Mig1 and Mig2 or lacking their binding site in the MTH1 promoter (Fig. 2). We believe the purpose of this regulation is to ensure rapid removal of Mth1 from cells when glucose becomes available so as to enable prompt induction of HXT gene expression. This idea is supported by our observation that interruption of this regulation results in delayed induction of HXT3 expression in response to glucose (Fig. 3A).

Even though Std1 and Mth1 are paralogues, they appear to have different functions in the glucose induction pathway: Mth1 collaborates with Rgt1 to repress expression of HXT1 and HXT3, whereas Std1 seems to be dedicated to regulating expression of the high-glucose-induced HXT1 gene (Table 2) (14, 32). Our results suggest that Mth1 plays a role in maintaining repression of the HXT7 genes in the absence of glucose, while Std1 may primarily be responsible for reestablishment of repression of HXT expression when the cells run out of glucose (Fig. 7). This intricate and highly evolved regulatory network ensures stringent regulation of glucose utilization.

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