A novel role for yeast casein kinases in glucose sensing and signaling

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ABSTRACT **Yeasts have sophisticated signaling pathways for sensing glucose, their preferred carbon source, to regulate its uptake and metabolism. One of these is the sensor/receptor-repressor (SRR) pathway, which detects extracellular glucose and transmits an intracellular signal that induces expression of *HXT* genes. The yeast casein kinases (Ycks) are key players in this pathway. Our model of the SRR pathway had the Ycks functioning downstream of the glucose sensors, transmitting the signal from the sensors to the Mth1 and Std1 corepressors that are required for repression of *HXT* gene expression. However, we found that overexpression of Yck1 fails to restore glucose signaling in a glucose sensor mutant. Conversely, overexpression of a glucose sensor suppresses the signaling defect of a *yck* mutant. These results suggest that the Ycks act upstream or at the level of the glucose sensors. Indeed, we found that the glucose sensor Rgt2 is phosphorylated on Yck consensus sites in its C-terminal tail in a Yck-dependent manner and that this phosphorylation is required for corepressor binding and ultimately *HXT* expression. This leads to a revised model of the SRR pathway in which the Ycks prime a site on the cytoplasmic tails of the glucose sensors to promote binding of the corepressors.**

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INTRODUCTION

All organisms must sense nutrient availability and regulate the transport and use of nutrients to optimize growth. Glucose is the primary carbon and energy source for the yeast *Saccharomyces cerevisiae*, which uses several mechanisms to use it efficiently. One of these is the Snf3/Rgt2-Rgt1 or sensor/receptor-repressor (SRR) glucose-signaling pathway, which detects extracellular glucose and induces expression of glucose transporters that bring glucose into the cell (Özcan and Johnston, 1999). This pathway includes glucose sensors (Rgt2 and Snf3), the Yck1 and Yck2 protein kinases, and the Mth1, Std1, and Rgt1 repressors.

In the absence of glucose, the Rgt1 repressor binds to the promoters of *HXT* genes and recruits the Mth1 and Std1 corepressors to repress *HXT* gene expression (Kim *et al.*, 2003; Lakshmanan *et al.*, 2003). Glucose binds to the Rgt2 (low-affinity) and Snf3 (high-affinity) glucose sensors, and this was proposed to activate the yeast casein kinases Yck1 and Yck2 (Ycks), which are functionally redundant for glucose signaling (Moriya and Johnston, 2004). Once activated, the Ycks would be situated to phosphorylate the Mth1 and Std1 corepressors because the Ycks and the corepressors interact with the glucose sensors (Moriya and Johnston, 2004; Özcan *et al.*, 1996, 1998). The phosphorylated corepressors are recognized by the SCF^{Grr1} ubiquitin ligase and subsequently ubiquitinated, targeting them for proteasomal degradation (Flick *et al.*, 2003). Without its corepressors, Rgt1 is unable to bind the *HXT* promoters, leading to derepression of *HXT* gene expression (Polish *et al.*, 2005; Roy *et al.*, 2013).

Our working model of the SRR pathway had the binding of glucose to a glucose sensor activating the Ycks or otherwise enabling them to phosphorylate the Std1 and Mth1 corepressors. This was based on the facts that 1) Yck function is required for signaling, 2) Yck1 is able to phosphorylate the corepressors in vitro, and 3) mutation of Yck consensus phosphorylation sites in the corepressors blocks their degradation (Moriya and Johnston, 2004). However, here we report evidence that suggests that the Ycks act on the glucose sensors in the SRR signaling pathway, which leads us to a revised model of the SRR pathway in which the Ycks first phosphorylate the C-terminal tails of the glucose sensors to establish a functional site for their interaction with the Mth1 and Std1 corepressors, bringing them in the proximity of the glucose sensors to receive and transduce the glucose signal.

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Abbreviations used: SRR, Snf3/Rgt2-Rgt1 or sensor/receptor-repressor; Yck, yeast casein kinase.

RESULTS

The glucose sensors are epistatic to yeast casein kinases

Previous work suggested that the Ycks function downstream of the glucose sensors to transduce the glucose signal from the glucose sensors to the Mth1 and Std1 corepressors via their Yck-dependent phosphorylation. The Ycks are required for glucose signaling and Mth1 and Std1 phosphorylation and destruction, and Yck1 phosphorylates Mth1 and Std1 in vitro on a cluster of conserved serine residues in Yck-consensus phosphorylation sequences (Moriya and Johnston, 2004).

To test the proposed downstream function of the Ycks, we determined the epistatic relationship of *YCK2* and *RGT2*. Because inactivation of the glucose sensors and the Ycks results in the same phenotype (reduced glucose signaling), we tested the effects of overexpression of individual components of the SRR pathway in deletion mutants and assessed the effect on *HXT1* expression and Mth1 destruction (typical readouts of SRR pathway function).

If Yck2 functioned downstream of the glucose sensors, then its overexpression might rescue the glucose-sensing defect of the *rgt2snf3* mutant. However, expression of *HXT1* (measured with an *HXT1-lacZ* reporter) is not induced by glucose in an *rgt2snf3* mutant overexpressing *YCK2* (Figure 1A), nor is Mth1 degradation in response to glucose restored (Figure 1B, lane 8). This suggests that the Ycks function upstream of the glucose sensors.

If the Ycks functioned upstream of the glucose sensors, we would expect *RGT2* overexpression to suppress the glucose-signaling defect of a *yck1Δyck2*^{ts} mutant. Indeed, overexpression of *RGT2* results in full glucose induction of *HXT1* expression in the *yck1Δyck2*^{ts} mutant (Figure 2A) and restoration of glucose-induced Mth1 degradation (Figure 2B, lane 6). These results are consistent with the idea that the Ycks act upstream, or at the level of the glucose sensors.

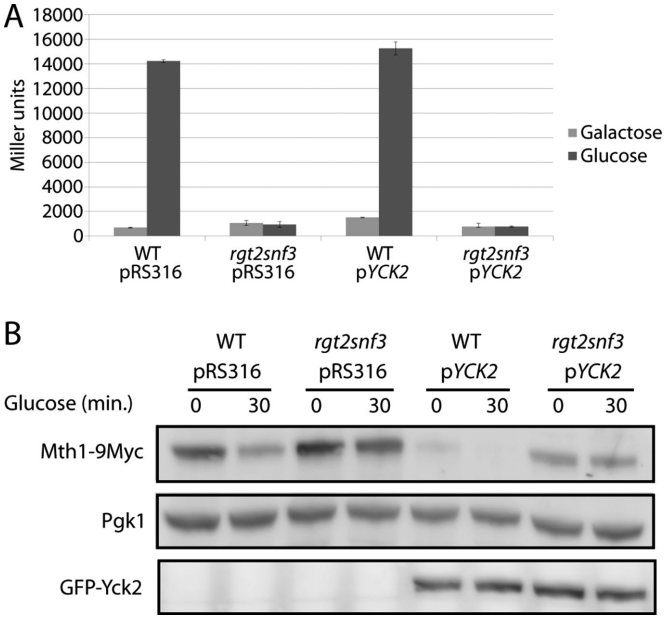

FIGURE 1: Overexpression of *YCK2* does not rescue a sensor knockout. (A) *HXT1*-lacZ expression (pBM3212) was determined as described in *Materials and Methods*. BY4742 (WT) or YM6871 (*rgt2snf3*) with pRS316 or pJB1 (p*YCK2*) was cultured at 30°C in 2% galactose (Galactose) and spiked with 4% glucose for 90 min (Glucose). (B) Mth1-9myc expressed from its own promoter on a single-copy plasmid (pBM4560) in BY4742 (WT) or YM6871 (*rgt2snf3*) with pRS316 or pJB1 (p*YCK2*) was cultured in 2% galactose (time 0) and then spiked with 4% glucose (time 30) at 30°C. The blot was probed with anti-Pgk1 as an internal standard; anti-GFP was used to confirm expression of Yck2.

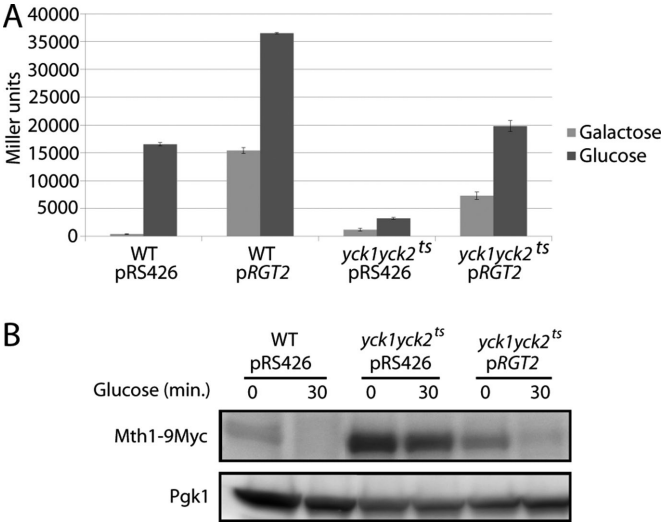

FIGURE 2: Overexpression of *RGT2* suppresses *yck1Δyck2*^{ts} mutations. (A) *HXT1*-lacZ expression (pBM3212) was determined as described in *Materials and Methods*. LRB939 (WT) or LRB1613 (*yck1Δyck2*^{ts}) with pRS426 or pBM3333 (p*RGT2*) was precultured overnight in 2% galactose at room temperature, shifted to 30°C for 4 h (Galactose), and then spiked with 4% glucose for 90 min (Glucose) before assay for β-galactosidase activity. (B) Mth1-9myc expressed from its own promoter on a single-copy plasmid (pBM4560) in LRB939 (WT) or LRB1613 (*yck1Δyck2*^{ts}) with pRS426 or pBM3333 (p*RGT2*) was precultured overnight in 2% galactose at room temperature, shifted to 30°C for 4 h (time 0), and then spiked with 4% glucose (time 30) at 30°C. The blot was probed with anti-Pgk1 as an internal standard.

The C-terminal tail of Rgt2 undergoes Yck-dependent phosphorylation

The Ycks are involved in phosphorylation of several nutrient permeases (Estrada *et al.*, 1996; Decottignies *et al.*, 1999; Marchal *et al.*, 2000; Gadura *et al.*, 2006). The glucose sensors Rgt2 and Snf3 share significant sequence similarities with glucose transporters and other nutrient permeases and could be regulated by a similar mechanism. Indeed, phosphatase treatment of Rgt2 increases its mobility in SDS–PAGE (Figure 3A, lanes 1 and 2), suggesting that Rgt2 is phosphorylated. Rgt2 from the *yck1Δyck2*^{ts} strain migrates similarly to phosphatase-treated Rgt2 from wild-type cells (Figure 3A, lanes 3 and 4), suggesting that Rgt2 phosphorylation depends on Yck function. Phosphorylation of Rgt2 does not appear to be regulated by glucose (Figure 3B).

The C-terminal tail of Rgt2 has two clusters of evolutionarily conserved potential Yck phosphorylation sites (Figure 3C). To determine whether these sites are involved in phosphorylation of Rgt2, we generated Rgt2 truncations that remove conserved sequences—box1 and box2—that are required for glucose signaling (unpublished data; Figure 3D). Potential Yck consensus sites cluster immediately downstream of box1 and box2 (Figure 3C). The SDS–PAGE migration shift of these truncated forms of Rgt2 is abolished only in the Rgt2Δbox1 mutant (Figure 3E, compare lanes 1 and 2 to lanes 3 and 4); the Rgt2Δbox2 mutant shows no change in the relative migration shift upon phosphatase treatment (Figure 3E, lanes 5 and 6). These results suggest that the potential Yck phosphorylation sites immediately downstream of box1 are in vivo targets of the Ycks.

We mutated three serines and one threonine in this cluster to alanine (Figure 3D, bottom). This Rgt2(4SA) shows no shift in

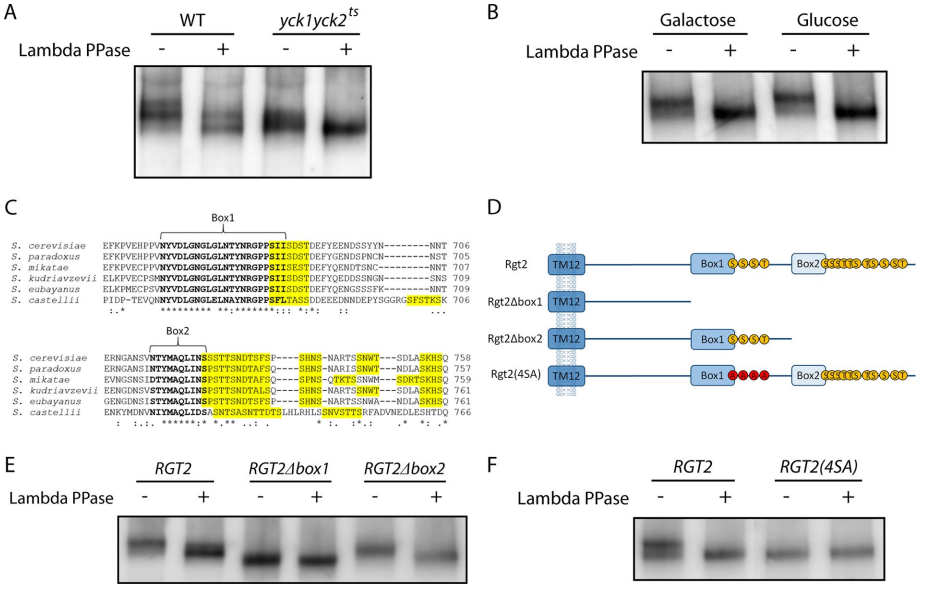

FIGURE 3: The C-terminal tail of Rgt2 is phosphorylated in a Yck-dependent manner. (A) Rgt2-13myc expressed from the *ADH1* promoter on a multicopy plasmid (pBM6232) in LRB939 (WT) or LRB1613 (*yck1Δyck2*^{ts}) was cultured in 4% glucose for 4 h and immunoprecipitated and phosphatase treated as described in *Materials and Methods*. (B) Rgt2-13myc expressed from the *ADH1* promoter on a multicopy plasmid (pBM6232) in BY4742 (WT) was cultured in 2% galactose (Galactose) or 4% glucose (Glucose) for 4 h and immunoprecipitated and phosphatase treated as described in *Materials and Methods*. (C) Multiple sequence alignment (Clustal Omega) of Rgt2 from *Saccharomyces* species shows sequence conservation in the C-terminal tail. Box1 and box2 are indicated (bold), and Yck consensus phosphorylation sites (S/T-X-X-S/T) are highlighted in yellow. (D) Diagram of three Rgt2 mutants used in this study. Transmembrane 12 of Rgt2 is indicated in the plasma membrane with the C-terminal tail shown extending into the cytoplasm. The Yck consensus phosphorylation sites are indicated with S and T for serine and threonine, respectively. The Rgt2(4SA) shows the point mutations S684A, S687A, S689A, and T690A, represented by A. (E) BY4742 with pBM6232 (*RGT2*), pBM6233 (*RGT2Δbox1*), or pBM6234 (*RGT2Δbox2*) was cultured in 4% glucose for 4 h and immunoprecipitated and phosphatase treated as described in *Materials and Methods*. (F) BY4742 with pBM6232 (*RGT2*) or pBM6235 (*RGT2(4SA)*) was cultured in 4% glucose for 4 h and immunoprecipitated and phosphatase treated as described in *Materials and Methods*.

migration pattern between phosphatase-treated and -untreated lanes in Western blot analysis (Figure 3F, lanes 3 and 4). Taken together, these results suggest that Rgt2 undergoes Yck-dependent phosphorylation on a cluster of residues immediately downstream of box1 in the C-terminal tail of the sensor.

Yck-dependent phosphorylation of Rgt2 is necessary for glucose signaling

To determine the significance of this phosphorylation of Rgt2 to SRR signaling, we analyzed the effect on glucose signaling of different mutations of Rgt2. *HXT1* expression is not induced by glucose in an *rgt2snf3* mutant, and this defect is rescued by a single copy of *RGT2* (Figure 4A). Rgt2Δbox1 does not restore glucose induction of *HXT1* expression (Figure 4A) and fails to rescue the Mth1 degradation defect of the *rgt2snf3* mutant (see later discussion of Figure 5B). Rgt2(4SA) provides no detectable glucose induction of *HXT1* expression (Figure 4A) or Mth1 degradation (Figure 4B). Rgt2Δbox2 provides a small but reproducible glucose induction of *HXT1* expression (Figure 4A) but fails to restore Mth1 degradation (Figure 4B). Thus phosphorylation of the Yck consensus sites adjacent to box1 is required for glucose signaling.

Growth on 2% glucose in the presence of antimycin A (an inhibitor of mitochondrial respiration) is another indicator of glucose sensor function. An *rgt2snf3* mutant fails to grow on glucose in the presence of antimycin A because it is unable to transport sufficient glucose to support fermentation (Schmidt *et al.*, 1999; Figure 4C). Neither Rgt2Δbox1 nor Rgt2(4SA) supports growth of an *rgt2snf3* mutant on glucose plus antimycin A, whereas the Rgt2Δbox2 mutant is capable of supporting growth (Figure 4C). These results support the conclusion that the Yck-dependent phosphorylation sites in Rgt2 are required for SRR signaling.

Stability of the glucose sensors, like other nutrient transporters that they closely resemble, is regulated. The Rgt2 high-affinity glucose sensor is stabilized by glucose and is degraded in the absence of glucose through its ubiquitination and consequent targeting to the vacuole (Roy and Kim, 2014), and Yck-dependent phosphorylation of the uracil permease Fur4 within a PEST sequence stimulates its ubiquitination and degradation (Marchal *et al.*, 2000). The C-terminal region of Rgt2 near box1, where Yck-dependent phosphorylation occurs, resembles a PEST sequence that we surmised could function to regulate the stability of Rgt2. However, the Ycks have no effect on Rgt2 stability: Rgt2 levels in glucose-grown cells are similar in wild-type and *yck1Δyck2*^{ts} cells. Rgt2 is degraded similarly after switching them to galactose (Figure 5A), and Rgt2(4SA)-13myc is degraded like the nonmutated form of Rgt2-13myc (Figure 5B). We conclude that Yck-dependent phosphorylation of Rgt2 is required for SRR signaling but does not affect Rgt2 stability.

Phosphorylation of the Rgt2 tail facilitates interaction with Mth1 and Std1

Mth1 and Std1 interact with the tails of the Rgt2 and Snf3 glucose sensors (Schmidt *et al.*, 1999; Lafuente *et al.*, 2000; Moriya and Johnston, 2004). We wondered whether Yck-dependent phosphorylation of the Rgt2 tail affects these interactions. We used the integrated modified yeast two-hybrid (iMYTH) method (Snider *et al.*, 2010) to investigate the interaction of Rgt2 with Mth1 and Std1. We constructed strains with the C-terminal half of ubiquitin (Cub) attached to the C-terminus of either *RGT2* or *RGT2(4SA)*. Genes in the SRR pathway fused to the N-terminal half of ubiquitin (Nub) were introduced into these strains. An interaction between the Cub- and Nub-tagged proteins results in expression of a *lexA-LacZ* reporter gene. This assay reveals that Mth1 and Std1 interact with Rgt2 and that this interaction is significantly reduced (by 86 and 92%, respectively) by the 4SA mutation (Figure 6). Thus the Yck-dependent phosphorylation of the Rgt2 tail potentiates Mth1 and Std1 binding.

DISCUSSION

The Ycks are required for glucose sensing and signaling via the SRR pathway and were believed to function downstream of the glucose sensors to phosphorylate Mth1 and Std1 (Moriya and Johnston, 2004). However, the results of our epistasis tests suggested that the

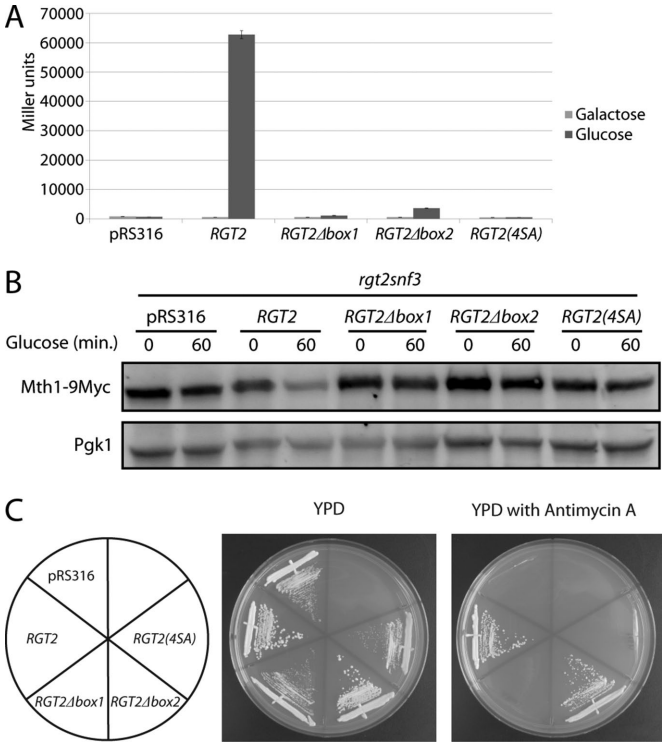

FIGURE 4: Phosphorylation of the Rgt2 tail is required for SRR signaling. (A) *HXT1*-lacZ expression (pBM3212) was determined as described in *Materials and Methods*. YM6871 (*rgt2snf3*) with pRS316, pBM6236(*RGT2*), pBM6237 (*RGT2Δbox1*), pBM6238 (*RGT2Δbox2*), or pBM6239 (*RGT2(4SA)*) was cultured at 30°C in 2% galactose (Galactose) or 4% glucose (Glucose) for 4 h. (B) Mth1-9myc expressed from its own promoter on a single-copy plasmid (pBM4560) in YM6871 (*rgt2snf3*) with pRS316, pBM6236 (*RGT2*), pBM6237 (*RGT2Δbox1*), pBM6238 (*RGT2Δbox2*), or pBM6239 (*RGT2(4SA)*) was cultured at 30°C in 2% galactose (time 0) and then spiked with 4% glucose (time 60). (C) YM6871 (*rgt2snf3*) containing pRS316, pBM6236 (*RGT2*), pBM6237 (*RGT2Δbox1*), pBM6238 (*RGT2Δbox2*), or pBM6239 (*RGT2(4SA)*) was streaked onto YPD or YPD with antimycin A and grown for 3 d at 30°C.

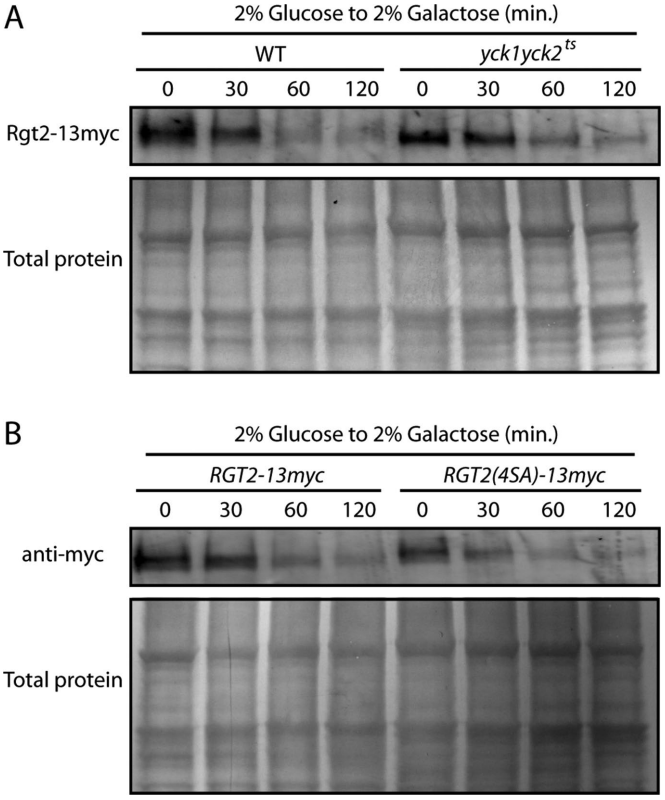

FIGURE 5: Stability of Rgt2 is not affected by the phosphorylation state of the tail. (A) *RGT2*-13myc expressed from its own promoter on a single-copy plasmid (pBM6236) in LRB939 (WT) or LRB1613 (*yck1Δyck2ts*) was cultured in 2% glucose for 4 h (time 0). Cells were washed and resuspended in 2% galactose, and samples were taken at 30, 60, and 120 min. (B) BY4742 with pBM6236 (Rgt2-13myc) or pBM6239 (Rgt2(4SA)-13myc) was cultured in 2% glucose for 4 h (time 0). Cells were washed and resuspended in 2% galactose, and samples were taken at 30, 60, and 120 min. All samples were extracted and blotted as described in *Materials and Methods*.

Ycks function upstream of or at the level of Rgt2. Indeed, we discovered that Rgt2 is phosphorylated in a Yck-dependent manner.

Knowing that the Ycks are required for stability of several membrane proteins, including transporters and permeases, and that Rgt2 and Snf3 are turned over in response to low and high glucose levels, respectively (Roy and Kim, 2014), we were surprised to find that Rgt2 is stable in a *yck1Δyck2ts* mutant and that mutation of the Yck-dependent phosphorylation sites of Rgt2 does not affect its stability (even though the phosphorylation sites on Rgt2 are in a region that resembles a PEST degradation sequence).

Conserved box1 of the Rgt2 tail is required for its interaction with Mth1 and Std1 (Moriya and Johnston, 2004), and we found that the Yck-dependent phosphorylation of a group of residues immediately downstream of box1 stimulates Mth1 and Std1 interaction with the Rgt2 tail. We imagine that this creates a scaffold for bringing the corepressors to the tail of the sensors to facilitate downstream signaling. This also suggests that the protein kinase(s) that phosphorylate the corepressors also localize to the plasma membrane and likely to the sensor tails to bring all components in the same vicinity. It is notable that deletion of conserved box2 of the Rgt2 tail causes a substantial reduction in glucose induction of *HXT1* expression without affecting the Yck-dependent phosphorylation of the tail. Thus the Yck phosphorylation sites near box1 are not the only determinants of glucose signaling in the tail.

The realization that the Ycks phosphorylate the glucose sensors requires a revision of our model of the SRR pathway. In the absence of glucose (Figure 7A), Rgt1, Mth1, and Std1 form a repressor complex that binds to the promoters of *HXT* genes to repress their transcription (Kim *et al.*, 2003; Lakshmanan *et al.*, 2003; Polish *et al.*, 2005). Yck-dependent phosphorylation of Rgt2 (and likely also Snf3)

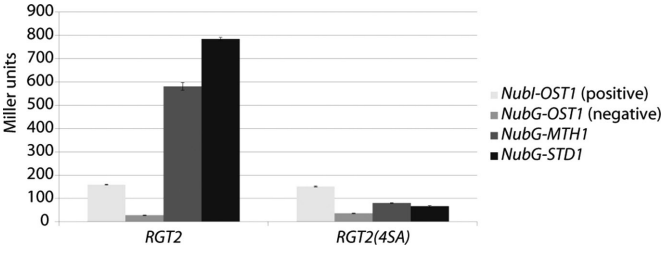

FIGURE 6: Phosphorylation of the Rgt2 tail is required for its interaction with Mth1 and Std1. THY.AP4 expressing C-terminal Cub fused to the chromosomal copy of *RGT2* (YM7807) or *RGT2(4SA)* (YM7808) and coexpressing pBM6240 (*NubI-OST1*, positive control), pBM6241 (*NubG-OST1*, negative control), pBM6242 (*NubG-MTH1*), or pBM6243 (*NubG-STD1*) was assayed for β-galactosidase activity as described in *Materials and Methods*.

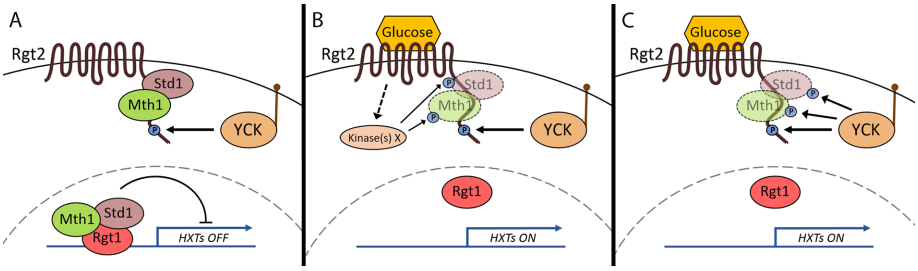

FIGURE 7: Revised model of SRR signaling. (A) In the absence of glucose, the repressor complex (Rgt1, Mth1, and Std1) is bound to the promoters of the *HXT* genes in the nucleus, blocking their transcription. The plasma membrane–bound sensor (Rgt2, and presumably also Snf3) undergoes Yck-dependent phosphorylation on its C-terminal tail. This phosphorylation of the sensor tail generates an interaction site and recruits the corepressors to the plasma membrane. This presumably brings the required signaling components together and primes the system for response to glucose. (B, C) Binding of glucose to Rgt2 activates the downstream signaling cascade. The corepressors are phosphorylated by unknown kinase(s) (B) or possibly by the Ycks (C). The phosphorylated corepressors are then ubiquitinated by the SCF^{Grr1} ubiquitin–protein ligase and targeted for degradation (shown as faded) by the proteasome. With the depletion of the cellular pool of corepressors, Rgt1 is no longer able to bind to the promoters of the *HXT* genes, relieving their repression.

facilitates its binding of the Mth1 and Std1 corepressors, effectively priming the system. Binding of glucose to the glucose sensors presumably induces a conformational change in them that activates or recruits a protein kinase(s) to phosphorylate Mth1 and Std1, targeting them for SCF^{Grr1}-dependent ubiquitination and degradation by the proteasome (Figure 7, B and C). Depletion of the corepressors robs Rgt1 of its ability to repress expression of *HXT* genes, leading to accumulation of glucose transporters in the plasma membrane.

This model leaves a potential void in the pathway: the identity of the protein kinase(s) that phosphorylate Mth1 and Std1. Our results suggest that these might not be the Ycks (Figure 7B), because they are not necessary for glucose signaling in a strain overexpressing *RGT2* (Figure 2A). However, there may be residual Yck2 protein kinase activity in the *yck1Δyck2*^{ts} mutant under our experimental conditions that is sufficient for glucose signaling when Rgt2 levels are high, and so we cannot rule out the possibility that the Ycks also act downstream of the sensors to phosphorylate the corepressors (Figure 7C). If this is the case, the residual Yck2 activity must be substantial, because overexpression of *RGT2* in the *yck1Δyck2*^{ts} mutant results in wild-type levels of glucose signaling (Figure 2A). Glucose signaling in the *yck1Δyck2*^{ts} mutant is negligible (Figure 2A), however, which suggests that Yck2 activity is very low in this mutant under these conditions. In addition, because phosphorylation of Rgt2 by the Ycks does not seem to be regulated by glucose, the glucose-induced event in the SRR pathway that initiates glucose signaling remains to be identified.

MATERIALS AND METHODS

Strains and growth conditions

All cells were cultured in synthetic complete medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate; 2% glucose or 2% galactose; 0.5% ammonium sulfate; and the amino acid supplement mixture [minus histidine, leucine, uracil, and tryptophan]). Antimycin A (A8674; Sigma-Aldrich, St. Louis, MO) was used at a concentration of 1.7 μg/ml on yeast extract/peptone/dextrose (YPD) plates. Cells for analysis of *HXT1* expression and Mth1 degradation were inoculated into synthetic medium containing 2% galactose. Cells were grown for 4 h, samples were taken for the zero time point, and glucose was added to the medium to a final concentration of 4%. Cells for immunoprecipitation, Rgt2 turnover analysis, and iMYTH analysis (Paumi *et al.*, 2007) were inoculated from overnight cultures into synthetic medium and grown for 4 h before extraction or analysis. All cultures were grown with shaking at 30°C, with the exception of those containing the *yck2* temperature-sensitive mutation, which were cultured overnight at room temperature and switched to 30°C for 4 h before sampling.

Table 1 lists strains used in this study. The Rgt2-Cub strain (YM7807) was constructed by transformation of THY.AP4 (Obrdlik *et al.*, 2004) with a PCR product generated from L2 plasmid (Paumi *et al.*, 2007; primers contained 40 base pairs of homology to *RGT2* locus upstream and downstream of the stop codon). The Rgt2(4SA)-Cub strain (YM7808) was constructed by initial disruption of *RGT2* in the box1 region of the tail with *URA3*. The *URA3* gene was removed by selection for 5-fluoroorotic acid–resistant cells transformed with an *RGT2(4SA)-CUB* fragment generated by gap repair in pCMBV4 (Snider *et al.*, 2010). Correct integration was confirmed by the PCR. Table 2 lists plasmids used in this study. All plasmids for this study were constructed by gap repair (Oldenburg *et al.*, 1997) and confirmed by PCR.

β-Galactosidase assays

The β-galactosidase kit (Pierce, Rockford, IL) was used as per the manufacturer's specifications, and reactions were stopped with 1 M sodium carbonate after 5 min or 2 h for *HXT1* or iMYTH experiments, respectively. All time points were in triplicate, and error bars represent 1 SD.

Strain	Genotype	Reference
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Laboratory collection
YM6871	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 rgt2::KanMX::NatMX snf3::KanMX*	Laboratory collection
LRB939	*MATα his3 leu2 ura3-52*	Lucy Robinson (Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, LA)
LRB1613	LRB939 *yck1::KanMX yck2-2*^{ts}	Lucy Robinson
THY.AP4	*MATa ura3 leu2 lexA::lacZ::trp1 lexA::HIS3 lexA::ADE2*	Igor Stagljar (Donnelly Centre, University of Toronto, Toronto, ON, Canada)
YM7807	THY.AP4 *RGT2-Cub::KanMX*	This study
YM7808	THY.AP4 *RGT2(4SA)-Cub::KanMX*	This study

TABLE 1: Yeast strains used in this study.

Plasmid	Description	Reference
pRS316	Yeast centromere vector with a URA3 marker and an MCS	Sikorski and Hieter (1989)
pRS426	Yeast episomal vector with a URA3 marker and an MCS	Christianson *et al.* (1992)
pJB1	*GAL1* promoter, *GFP-YCK2* in YCp50	Robinson *et al.* (1999)
pBM3212	*HXT1* promoter *lacZ* fusion, *LEU2*	Özcan *et al.* (1996)
pBM3333	*ADH1* promoter, *RGT2* in pRS426	Özcan *et al.* (1998)
pBM4560	*MTH1* promoter, *MTH1-9myc, HIS3*	Moriya and Johnston (2004)
pBM6232	pBM3333 with C-terminal 13myc tag	This study
pBM6233	pBM3333 with *RGT2deltabox1-13myc* (aa 1–664)	This study
pBM6234	pBM3333 with *RGT2deltabox2-13myc* (aa 1–714)	This study
pBM6235	pBM3333 with *RGT2(4SA)-13myc*	This study
pBM6236	*RGT2* promoter, *RGT2-13myc* in pRS316	This study
pBM6237	pBM6236 with *RGT2deltabox1-13myc* (aa 1–664)	This study
pBM6238	pBM6236 with *RGT2deltabox2-13myc* (aa 1–714)	This study
pBM6239	pBM6236 with *RGT2(4SA)-13myc*	This study
pBM6240	pPR3-N (NubI) with *OST1*	This study
pBM6241	pPR3-N (NubG) with *OST1*	This study
pBM6242	pPR3-N (NubG) with *MTH1*	This study
pBM6243	pPR3-N (NubG) with *STD1*	This study

aa, amino acids.

TABLE 2: Plasmids used in this study.

Protein extraction and Western analysis

For assaying Mth1 degradation, 1 ml of 100% trichloroacetic acid (TCA) was added to 5 ml of cell culture, followed by centrifugation at 3000 × *g* to pellet cells. The cell pellet was resuspended in 400 µl of 20% TCA and transferred to a microcentrifuge tube. Approximately 0.3 g of glass beads was added, and the samples were vortexed for 5 min. The lysate was cleared by centrifugation, and the beads were washed twice with 200 µl of 5% TCA. Samples were then centrifuged at 3000 × *g*, and the pellet was resuspended in 50 µl of nonfluorescent sample buffer (Li-Cor, Lincoln, NE) and 25 µl of 1 M Tris. Protein concentrations were determined by Coomassie elution with SDS (Marbach *et al.*, 2001).

Pelleted cells were prepared for immunoprecipitation by first resuspending them in 400 µl of lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1 mM, EDTA, 1 mM phenylmethylsulfonyl fluoride, plus Halt protease inhibitor cocktail [plus or minus phosphatase inhibitors; Thermo Scientific, Waltham, MA]), adding 0.3 g of glass beads, and vortexing for 5 min. Then NP-40 (to 1%) and deoxycholate (to 0.25%) were added, and cells were again vortexed for 5 min. Cell debris was removed by centrifugation, and the lysates were left on ice for immunoprecipitation. Analysis of Rgt2 degradation was performed as described by Roy and Kim (2014).

Protein samples were run on 10% TGS gels (Bio-Rad, Hercules, CA) and transferred to low-fluorescent polyvinylidene fluoride membranes (Bio-Rad). Membranes were probed with mouse anti-myc (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000, rabbit anti–green fluorescent protein (GFP; Sigma-Aldrich) diluted 1:1000, and mouse anti-Pgk1 (Invitrogen, Carlsbad, CA) diluted 1:10,000 in blocking buffer (Rockland, Pottstown, PA). Secondary antibodies used were anti-mouse Dylight 488 (Epitomics, Cambridge, MA) diluted 1:5000 and anti-rabbit Dylight 549 (Epitomics) diluted 1:5000 in blocking buffer. Membranes were imaged on a Bio-Rad imager.

Immunoprecipitation

Cell lysates were incubated with 25 µl of EZview Red anti–c-myc affinity gel (Sigma-Aldrich) suspended in wash buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 8.0) for 90 min at 4°C. Beads were then washed twice with wash buffer, and 50 µl of 1× λ-phosphatase buffer with $MnCl_2$ (New England Biolabs, Ipswich, MA) was added to all samples. Samples without phosphatase inhibitors were treated with 1 µl of λ-protein phosphatase (New England Biolabs) and incubated at 30°C for 20 min. Beads were washed three times with wash buffer and resuspended in 60 µl of nonfluorescent sample buffer (Li-Cor) plus 7.5% β-mercaptoethanol. For Rgt2 turnover analysis, membranes were stained with Coomassie blue after imaging to detect total protein as a loading control.

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