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ABSTRACT **Methylglyoxal (MG) is a cytotoxic by-product of glycolysis. MG has inhibitory effect on the growth of cells ranging from microorganisms to higher eukaryotes, but its molecular targets are largely unknown. The yeast cell-surface glucose sensors Rgt2 and Snf3 function as glucose receptors that sense extracellular glucose and generate a signal for induction of expression of genes encoding glucose transporters (*HXT*s). Here we provide evidence that these glucose sensors are primary targets of MG in yeast. MG inhibits the growth of glucose-fermenting yeast cells by inducing endocytosis and degradation of the glucose sensors. However, the glucose sensors with mutations at their putative ubiquitin-acceptor lysine residues are resistant to MG-induced degradation. These results suggest that the glucose sensors are inactivated through ubiquitin-mediated endocytosis and degraded in the presence of MG. In addition, the inhibitory effect of MG on the glucose sensors is greatly enhanced in cells lacking Glo1, a key component of the MG detoxification system. Thus the stability of these glucose sensors seems to be critically regulated by intracellular MG levels. Taken together, these findings suggest that MG attenuates glycolysis by promoting degradation of the cell-surface glucose sensors and thus identify MG as a potential glycolytic inhibitor.**

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INTRODUCTION

Methylglyoxal (MG) is a reactive dicarbonyl compound formed by nonenzymatic elimination of phosphate from the triose phosphates of glycolysis, dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P), in eukaryotic cells (Richard, 1984, 1993; Phillips and Thornalley, 1993), as well as enzymatically from DHAP in a reaction catalyzed by MG synthase in bacteria (Cooper and Anderson, 1970; Hopper and Cooper, 1972). Once formed, MG irreversibly modifies amino groups of proteins, nucleic acids, and membrane lipids, forming advanced glycation end products (Thornalley, 1998), which are associated with various pathologies, including diabetes, aging-related disorders, and neurodegenerative diseases (Desai *et al.*, 2010; Rabbani and Thornalley, 2011; Beisswenger, 2014; Allaman *et al.*, 2015). Cells are protected against methylglyoxal toxicity by the glyoxalase system, which is composed of two enzymes: glyoxalase I (Glo1), which metabolizes MG to *S*-D-lactoylglutathione, and glyoxalase II (Glo2), which converts *S*-D-lactoylglutathione to D-lactate (Thornalley, 2003). MG appears to be the major physiological substrate for Glo1. Overexpression of Glo1 prevents MG accumulation (Shinohara *et al.*, 1998), whereas Glo1 inhibition increases MG accumulation, decreasing cellular viability (Kuhla *et al.*, 2006).

The budding yeast *Saccharomyces cerevisiae* prefers to ferment rather than oxidize glucose, even when oxygen is abundant—so-called aerobic glycolysis (Crabtree, 1929; Warburg, 1956;

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Abbreviations used: GFP, green fluorescence protein; HA, hemagglutinin; HXT, hexose transporter; MG, methylglyoxal.

Lagunas, 1979). Because energy generation by fermentation of glucose is inefficient, yeast cells vigorously metabolize glucose by enhancing the rate-limiting step of glucose metabolism: its transport (Johnston and Kim, 2005; Kim *et al.*, 2013; Rolland *et al.*, 2001). Yeast cells have learned how to sense the amount of glucose that is available and respond by expressing the most appropriate of its 17 glucose transporters (*HXT*s). They do this through a signal transduction pathway that begins at the cell surface with the Rgt2 and Snf3 glucose sensors and ends in the nucleus with the Rgt1 transcription factor, which regulates expression of genes encoding glucose transporters (Kim *et al.*, 2013).

Expression of *HXT* genes is repressed by the Rgt1 DNA–binding repressor in the absence of glucose (Ozcan *et al.*, 1996b). Rgt1 does this by recruiting the *HXT* corepressors Mth1 and Std1 and the general corepressor complex Ssn6-Tup1 to the *HXT* promoters (Schmidt *et al.*, 1999; Kim *et al.*, 2003, 2006; Kim, 2009; Lakshmanan *et al.*, 2003). When glucose is added to the glucose-starved cells, Mth1 and Std1 are phosphorylated by the plasma membrane–tethered casein kinases Yck1 and Yck2 (YckI), ubiquitinated by the SCF-Grr1 ubiquitin-protein ligase, and subsequently degraded by the 26S proteasome (Flick *et al.*, 2003; Moriya and Johnston, 2004). This enables protein kinase A to access and phosphorylate Rgt1, resulting in a disruption of the Rgt1 repressor complex and consequently in derepression of *HXT* genes (Kim and Johnston, 2006; Palomino *et al.*, 2006; Jouandot *et al.*, 2011; Roy *et al.*, 2013, 2014). It is unclear how an intracellular signal that induces expression of *HXT* genes is generated. However, YckI appears to be coupled to the cell-surface glucose sensors Rgt2 and Snf3, leading to the current view that glucose binding to the Rgt2 and Snf3 glucose sensors causes them to activate YckI, which phosphorylates Mth1 and Std1, which are bound to the C-terminal cytoplasmic tails of the sensors (Moriya and Johnston, 2004; Johnston and Kim, 2005).

Extracellular glucose is sensed by Rgt2 and Snf3 at the plasma membrane. These proteins are structurally similar to the Hxt glucose transporters but seem to have lost the ability to transport glucose inside the cell (Ozcan *et al.*, 1996a). They function instead as receptors that monitor extracellular glucose. A dominant mutation in the glucose sensors (R231K in Rgt2 and R229K in Snf3) leads to the constitutive expression of *HXT* genes even in the absence of glucose, possibly converting the receptors into their glucose-bound form (Ozcan *et al.*, 1996a; Pasula *et al.*, 2007). Thus it has been proposed that glucose signaling by Rgt2 and Snf3 is a receptor-mediated process, similar to hormone signaling in mammalian cells (Ozcan and Johnston, 1999; Johnston and Kim, 2005).

We recently reported that the glucose sensors are stable in cells grown in glucose but endocytosed and degraded in the vacuole when cells are starved of glucose (Roy and Kim, 2014). Of interest, it was shown that constitutively active, signaling mutants of glucose sensors are resistant to endocytosis, whereas signaling-defective sensors are constitutively targeted for degradation. Thus the stability of the glucose sensors may be correlated with their affinity for glucose. In this article, we give evidence that MG induces endocytosis and degradation of the glucose sensors to inhibit expression of the *HXT* genes and consequently attenuate glycolysis. We also discuss the possible mechanisms by which MG regulates the stability of the glucose sensors and the evaluation of MG as a glycolytic inhibitor.

RESULTS

Methylglyoxal inhibits expression of the yeast glucose transporter genes (*HXT*s)

S. cerevisiae can use glucose efficiently over a broad range of concentrations. Accordingly, yeast cells have many different glucose transporters, ~17. Several of the glucose transporters are expressed only when glucose is available and only under the appropriate conditions (Ozcan and Johnston, 1999). For example, the low-affinity glucose transporter Hxt1 is only expressed when glucose is abundant, and the high-affinity glucose transporters Hxt2 and Hxt4 are expressed only when glucose is scarce. *HXT3*, which encodes a glucose transporter of intermediate affinity, is expressed in cells exposed to both low (0.2%) and high (2%) levels of glucose (Ozcan and Johnston, 1995). MG is a cytotoxic compound produced primarily as a by-product of glycolysis (Figure 1A), but its molecular targets are largely unknown.

Raffinose is a trisaccharide consisting of fructose–glucose–galactose and for *S. cerevisiae* is equivalent to low glucose because it can cleave the fructose–glucose bond only inefficiently and thus obtains only low levels of glucose from it (Ozcan and Johnston, 1999). To investigate the effect of MG on the viability of *S. cerevisiae*, we treated the yeast cells grown in medium containing glucose or raffinose with different concentrations of MG. The results showed that MG inhibits yeast cell growth in a dose-dependent manner, suggesting the possibility of an inhibitory effect of MG on glucose utilization (Figure 1B).

Next we examined directly the effect of MG on the expression of the glucose transporter genes by measuring mRNA levels of Hxt1, Hxt2, Hxt3, and Hxt4 transporters using quantitative real-time PCR (qRT-PCR) with specific primers, as we described previously (Roy *et al.*, 2013). As mentioned, expression of *HXT1* and *HXT3* genes is induced by high glucose, whereas that of *HXT2* and *HXT4* genes is induced by low glucose (Figure 1D; see controls). However, expression of all four *HXT* genes was inhibited by MG in a dose-dependent manner, and >50% inhibition was achieved at a 10 mM concentration of MG (Figure 1D).

Methylglyoxal decreases cell-surface levels of the Rgt2 and Snf3 glucose sensors

Rgt2 and Snf3 sense different levels of extracellular glucose and function differently. Rgt2 is a low-affinity glucose sensor required for induction of *HXT1* by high glucose, whereas Snf3 is a high-affinity sensor needed for the expression of *HXT2* and *HXT4* in response to low levels of glucose (Ozcan *et al.*, 1998; Ozcan and Johnston, 1996). Thus a yeast strain lacking these genes (*rgt2*Δ*snf3*Δ) grows poorly on a glucose or raffinose (as low-glucose concentration) medium (Ozcan *et al.*, 1996a). Given that MG seems to inhibit glucose utilization by yeast, we examined whether it inactivates the Rgt2 and Snf3 glucose sensors. Expression of Rgt2 in the *rgt2snf3* double mutant restored growth in glucose medium but not in glucose medium containing 10 mM MG. Similarly, Snf3 expression complemented the growth defect of the *rgt2*Δ*snf3*Δ strain in raffinose medium but not in raffinose medium containing 10 mM MG (Figure 2A). Thus MG seems to decrease cell-surface levels of the glucose sensors.

The stability of the glucose sensors is correlated well with their respective affinities; that is, Rgt2 remains stable at the plasma membrane only in high-glucose-grown yeast cells; by contrast, this holds for Snf3 in yeast cells grown in low-glucose medium (Roy and Kim, 2014). Fluorescence microscopy and Western blot analysis confirmed that GFP-Rgt2 is stable in cells grown in high-glucose medium and endocytosed and subsequently degraded in the vacuole when cells are starved of glucose and that, by contrast, GFP-Snf3 is stable in cells in low-glucose medium and down-regulated when cells are exposed to high-glucose or glucose-free medium (Figure 2, B and C). Expression of the *SNF3* gene is repressed about fivefold by high levels of glucose, whereas the *RGT2* gene is expressed constitutively, being neither repressed nor induced by glucose

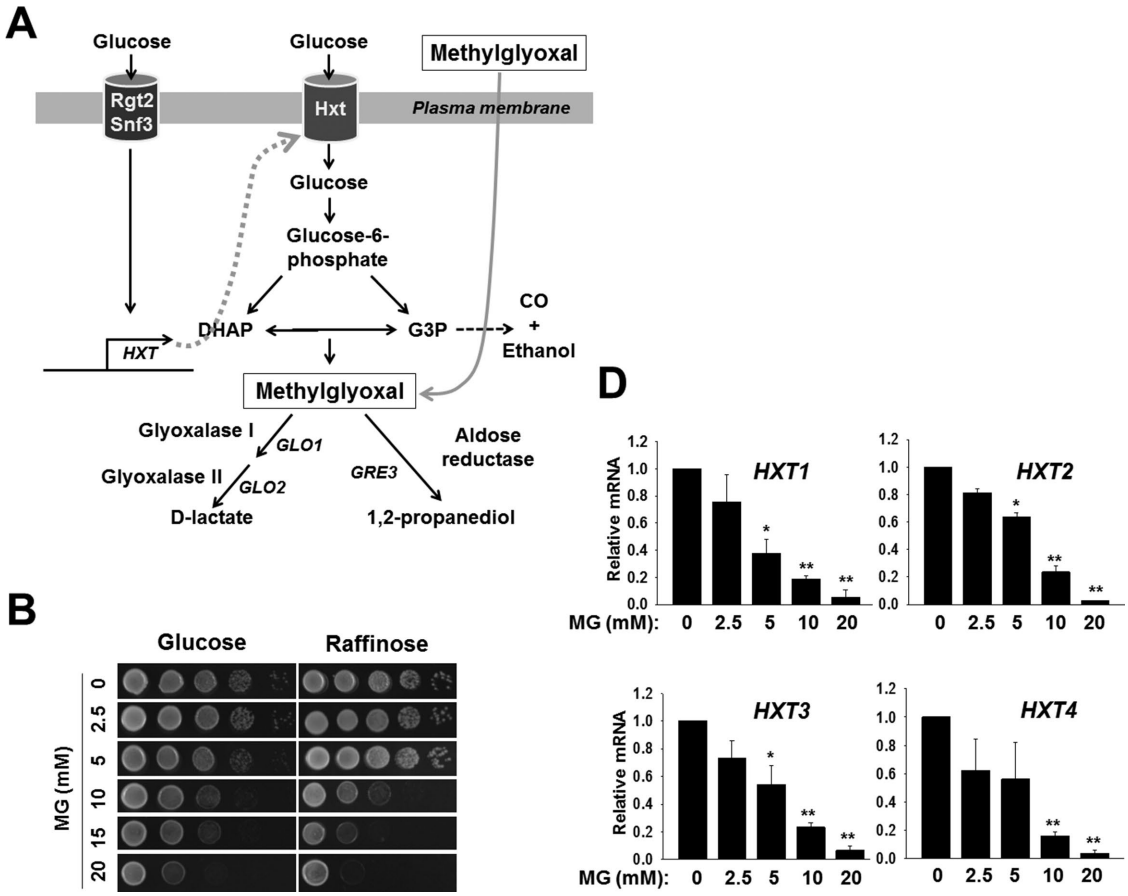

FIGURE 1: Methylglyoxal inhibits expression of the *HXT* genes. (A) Schematic representation of MG metabolism pathways. MG is produced as a by-product of glycolysis by degradation of the triose phosphates DHAP and G3P. It is then metabolized into D-lactate by glyoxalase systems (*GLO1* and *GLO2*) or 1,2-propanediol by aldolase reductase (*GRE3*). (B) Yeast cells (*WT*) were spotted on YP plates containing 2% glucose (Glucose) or 2% raffinose (Raffinose) supplemented with different concentrations of MG as shown. The first spot of each row represents a count of 5×10^7 cells/ml, which is diluted 1:10 for each spot thereafter. The plates were incubated for 2 d and photographed. (C) This figure has been removed by agreement of the authors and the journal's Editorial Board because it contained incorrect data. (D) qRT-PCR analysis of mRNA expressions of *HXT1*, *HXT2*, *HXT3*, and *HXT4* genes. mRNA was isolated from yeast cells (*WT*) grown in either 2% glucose (to quantify *HXT1* and *HXT3* gene expressions) or 2% raffinose (to quantify *HXT2* and *HXT4* gene expressions) containing the indicated amounts of MG. The data are averages of three independent experiments, with error bars showing mean ± SD. *$p < 0.05$ and **$p < 0.001$.

(Ozcan *et al.*, 1996a). We interrupted glucose regulation of expression of glucose sensor genes (*SNF3* in particular) by replacing their promoters with the promoter of *MET25*, which is not regulated by glucose (Kim *et al.*, 2006; Pasula *et al.*, 2007). Therefore changes of the GFP-Rgt2 and GFP-Snf3 levels in response to different glucose concentrations might be due not to transcriptional repression but to posttranslational regulation. We observed that GFP-Rgt2 and GFP-Snf3 are removed from the plasma membrane and degraded in the vacuole in response to MG (Figure 2D). These results suggest that MG inhibits expression of *HXT* genes by decreasing cell-surface levels of the Rgt2 and Snf3 glucose sensors.

Methylglyoxal causes degradation of the Rgt2 and Snf3 glucose sensors

To further study MG-induced inactivation of the glucose sensors, we conducted Western blot analysis of Rgt2 and Snf3 in the plasma membrane fractions of yeast cells treated with different concentrations of MG. Because there is a possibility that the green fluorescent protein (GFP) moiety of GFP-fused Rgt2 and Snf3 affects their MG-induced degradation, we expressed the glucose sensors, tagged with the 3x hemagglutinin (HA) epitope (27 amino acids), from their own promoters. The results showed that the glucose sensor proteins are degraded by MG in a concentration-dependent manner, and >50% of degradation was achieved at 10 mM MG (Figure 3A). Next we performed qRT-PCR of *RGT2* and *SNF3* genes in yeast cells treated or not with MG and found that MG does not significantly affect expression of either *RGT2* or *SNF3* (Figure 3B). Thus the disappearance of Rgt2-HA and Snf3-HA in response to MG treatment is due to degradation but not to transcriptional repression, and this was further confirmed by fluorescence microscopy of yeast cells expressing GFP-Rgt2 and GFP-Snf3 (Figure 3, C and D). We also observed that the amounts of immunodetected Rgt2-HA and Snf3-HA were reduced by ~50% within 100 min after yeast cells were treated with 10 mM MG (Figure 3E).

Methylglyoxal induces endocytosis of the Rgt2 and Snf3 glucose sensors

Given that Rgt2 and Snf3 are inactivated by endocytosis, we examined whether MG-induced inactivation of the glucose sensors is due to endocytosis, followed by degradation in the vacuole. Our results

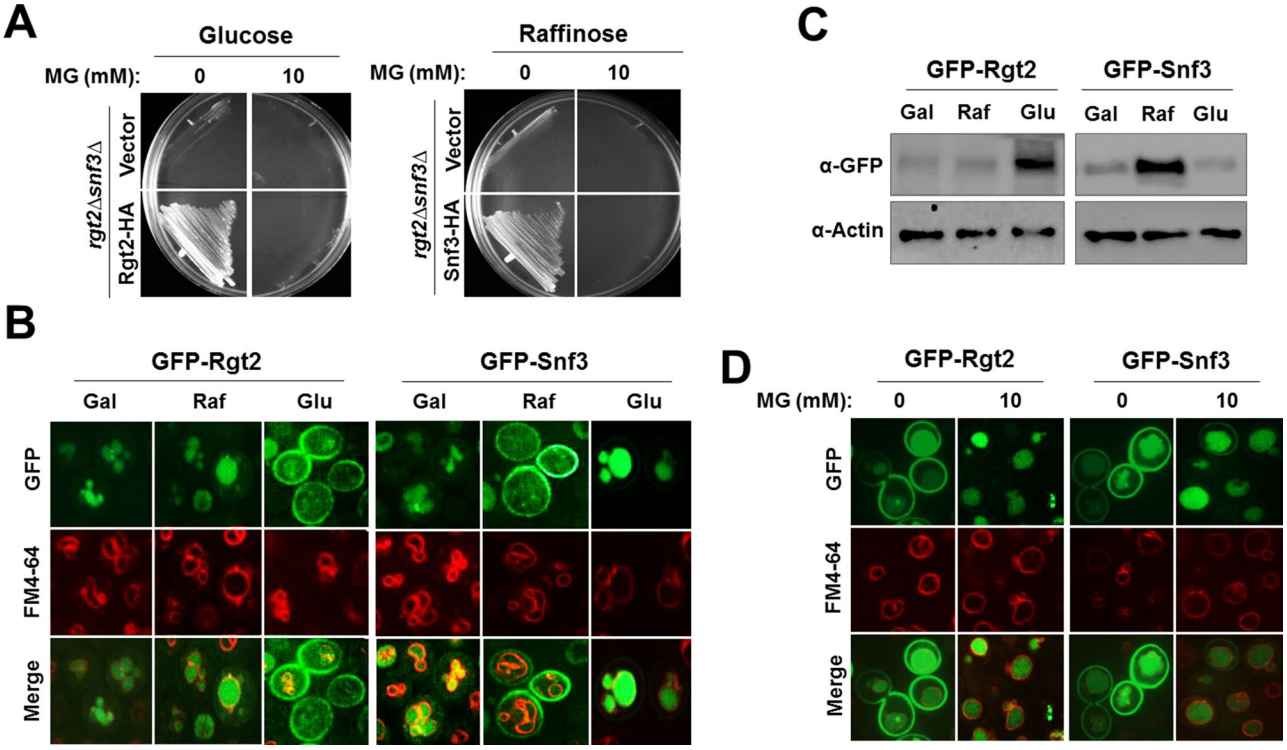

FIGURE 2: MG inactivates the yeast glucose sensors Rgt2 and Snf3. (A) Yeast cells (*rgt2Δsnf3Δ*) expressing empty vector, Rgt2-HA (left) or Snf3-HA (right panel) were streaked on SC plates containing 2% glucose (Glucose) or 2% raffinose (Raffinose), supplemented with or without 10 mM MG. The plates were incubated for 2 d and photographed. (B) Yeast cells (*WT*) expressing GFP-Rgt2 or GFP-Snf3 were grown in SC medium containing 2% galactose (Gal) until mid log phase ($A_{600\ nm}$ = 1.2–1.5), and equal amounts of cells were shifted to SC medium containing 2% raffinose (Raf) or 2% glucose (Glu). The distributions of the GFP fluorescence were analyzed by confocal microscopy. FM4-64 dye was used to visualize vacuoles. (C) Western blot analysis of GFP-Rgt2 and GFP-Snf3 levels at the plasma membrane. Yeast cells (*WT*) expressing GFP-Rgt2 or GFP-Snf3 were grown as described in B. Membrane fractions were analyzed using anti-GFP and anti-actin antibodies. (D) Yeast cells (*WT*) expressing GFP-Rgt2 or GFP-Snf3 were grown in SC medium containing 2% glucose (GFP-Rgt2) or 2% raffinose (GFP-Snf3) until mid log phase and treated with 10 mM MG for 2 h. The distributions of the GFP fluorescence were analyzed by confocal microscopy. Actin served as a loading control in C.

showed that MG treatment decreases the protein levels of Rgt2-HA and Snf3-HA in wild-type yeast cells but not in yeast cells lacking End3, a protein involved in the internalization step of endocytosis, which suggests that MG induces endocytosis of Rgt2 and Snf3 (Figure 4, A and C). This was confirmed by confocal microscopy showing that GFP-Rgt2 and GFP-Snf3 are removed from the plasma membrane and targeted to the vacuole for degradation in response to MG in wild-type yeast cells but not in the *end3Δ* mutant cells (Figure 4, B and D).

Methylglyoxal may promote Rsp5-dependent degradation of the Rgt2 and Snf3 glucose sensors

Ubiquitination is a common signal for endocytosis and subsequent degradation of plasma membrane proteins (Hicke and Dunn, 2003). A number of yeast nutrient transporters are ubiquitinated by the ubiquitin ligase Rsp5, and this process is affected by the ubiquitin isopeptidase Doa4, which is required for recycling ubiquitin from ubiquitinated substrates (Springael and Andre, 1998; Amerik *et al*., 2000; Rotin *et al*., 2000). Rsp5 is involved in degradation of the Rgt2 glucose sensor (Roy and Kim, 2014). Thus we examined the role of Rsp5 in the MG-induced endocytosis and degradation of glucose sensors. Western blot analysis showed that Rgt2-HA and Snf3-HA levels are decreased in response to MG in wild-type cells but not in *doa4Δ* or *rsp5-1*^{ts} mutant cells (Figure 5, A and B). Thus Rgt2 and Snf3 accumulated in the plasma membrane of the yeast mutants, regardless of the treatment of MG (Figure 5, C and D). We previously identified Lys-637 and Lys-657 of Rgt2 as putative ubiquitination sites (Roy and Kim, 2014). The Rgt2^{K637A,K657A} mutant is resistant to MG-induced degradation (Figure 5E), and its expression allows yeast cells to grow on MG-containing medium (Figure 5F). These results suggest that MG induces Rsp5-dependent endocytosis and vacuolar targeting of the glucose sensors.

Glo1 plays an essential role in the growth of yeast in the presence of methylglyoxal

MG, both exogenously supplied and endogenously produced via glycolysis, is detoxified by the glyoxalase system. In yeast, MG is metabolized by two pathways (Figure 1A): the glutathione-dependent glyoxalase pathway and the NADPH-dependent aldose reductase pathway (Aguilera and Prieto, 2001; Vander Jagt *et al*., 2001; Chen *et al*., 2003). The glyoxalase pathway consists of the enzymes glyoxalase I (Glo1), which catalyzes the formation of *S*-D-lactoylglutathione from methylglyoxal, and reduced glutathione (GSH) and glyoxalase II (Glo2), which recycles GSH and releases D-lactate (Martins *et al*., 2001a,b). Aldose reductase, encoded by

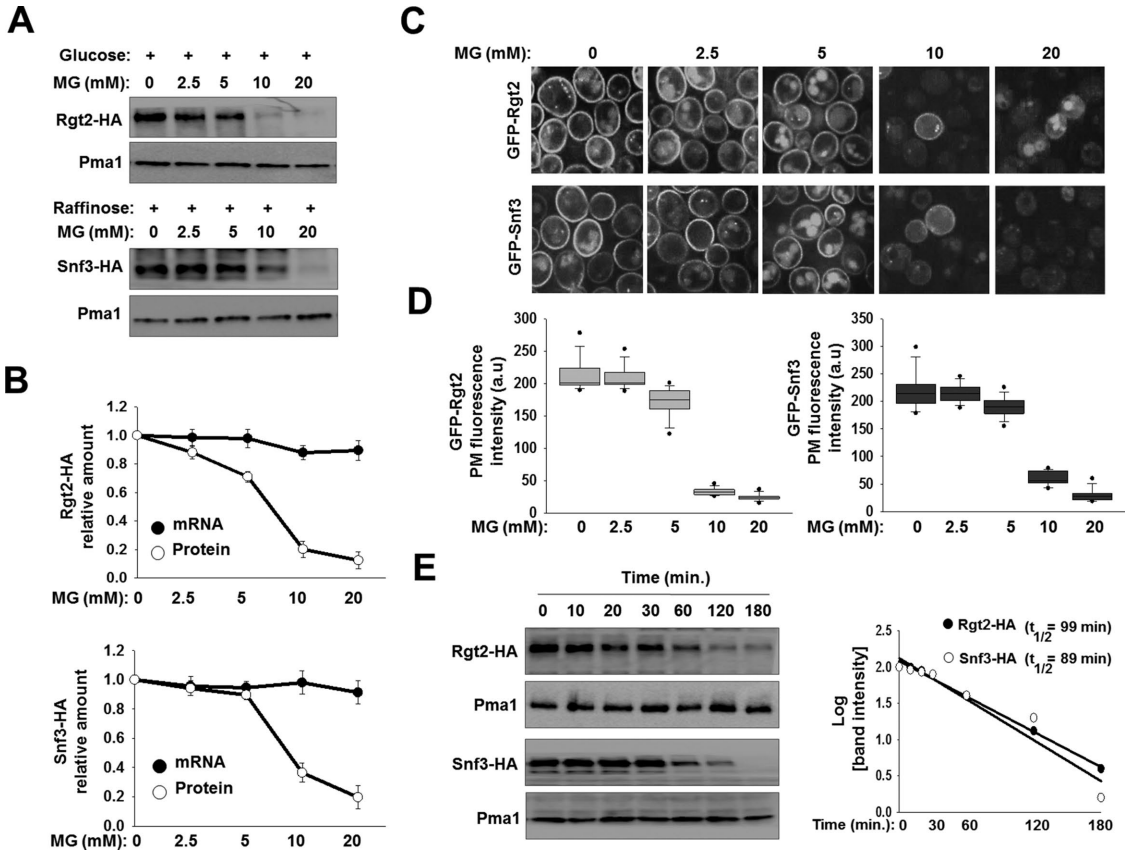

FIGURE 3: MG induces posttranslational down-regulation of Rgt2 and Snf3 in a dose-dependent manner. (A) Western blot analysis of Rgt2-HA (top) and Snf3-HA (bottom) levels at the plasma membrane. Yeast cells (*WT*) expressing Rgt2-HA or Snf3-HA were grown in SC-2% glucose or SC-2% raffinose medium, respectively, until mid log phase ($A_{600\ nm}$ = 1.2–1.5), and equal amounts of cells were shifted to SC medium containing the indicated concentrations of MG for 2 h. Membrane fractions were analyzed using anti-HA and anti-Pma1 antibodies. (B) qRT-PCR analysis of mRNA expressions (mRNA, closed circles) of *RGT2* (top) and *SNF3* (bottom) in yeast cells grown as described in A; densitometric quantification of the intensities of each of the bands on the blot in A (protein, open circles). (C) Yeast cells (*WT*) expressing GFP-Rgt2 or GFP-Snf3 were grown as described in A, and the distributions of the GFP fluorescence were analyzed by confocal microscopy. (D) Quantification of the GFP fluorescence intensities of plasma membrane (PM) from the cells depicted in C; box-and-whiskers plot (n >150). The horizontal midline represents the median; the box is bounded by the upper and lower quartiles; the whiskers denote the maximum and minimum fluorescence intensities with 5th and 95th percentiles. (E) Yeast cells (*WT*) expressing Rgt2-HA or Snf3-HA were grown in SC-2% glucose or SC-2% raffinose medium, respectively, until mid log phase ($A_{600\ nm}$ = 1.2–1.5) and shifted to SC medium containing 10 mM MG for the times as indicated. Membrane fractions were immunoblotted with anti-HA and anti-Pma1 antibodies (left), and half-lives of Rgt2-HA and Snf3-HA were calculated as described in *Materials and Methods* (right). Pma1 served as a loading control in A and E.

Gre3, catalyzes the two-step reduction of methylglyoxal to 1,2-propanediol, with acetol (hydroxyacetone) as an intermediate (Chen *et al.*, 2003). Thus the *glo1Δ* mutant was more sensitive to MG than the wild-type strain (Figure 6A), as reported previously (Maeta *et al.*, 2005). Of greater importance, the Rgt2 and Snf3 glucose sensors are more sensitive to exogenous MG in the *glo1Δ* mutant, where intracellular MG is not efficiently metabolized, than in wild-type yeast, consistent with the result that the glucose sensors are primary targets of MG (Figure 6, B and C).

DISCUSSION

MG is a cytotoxic metabolite formed as a by-product of glycolysis, but its targets are largely unknown. In this study, we found that MG inhibits expression of the *HXT* glucose transporter genes by inactivating the yeast glucose sensors Rgt2 and Snf3, which generate a signal for *HXT* expression in response to glucose. Several lines of evidence indicate that MG induces endocytosis and degradation of the glucose sensors: 1) they are removed from the plasma membrane and targeted to the vacuole for degradation in response to MG (Figures 2 and 3); 2) MG-induced degradation of these sensor proteins requires End3, Doa4, or active Rsp5 (Figures 4 and 5); and 3) glucose sensors with mutations at the putative ubiquitination sites are resistant to MG-induced endocytosis (Figure 5). The Rgt2 and Snf3 glucose sensors are essential for yeast cells to grow on glucose as sole carbon source. Our findings suggest that MG inhibits the growth of glucose-fermenting yeast cells by promoting degradation of the glucose sensors.

It is unknown how MG induces endocytosis of the glucose sensors, but our recent findings might provide a clue to the role of MG in regulation of these sensor proteins (Roy and Kim, 2014). The glucose sensors undergo a conformational change from an

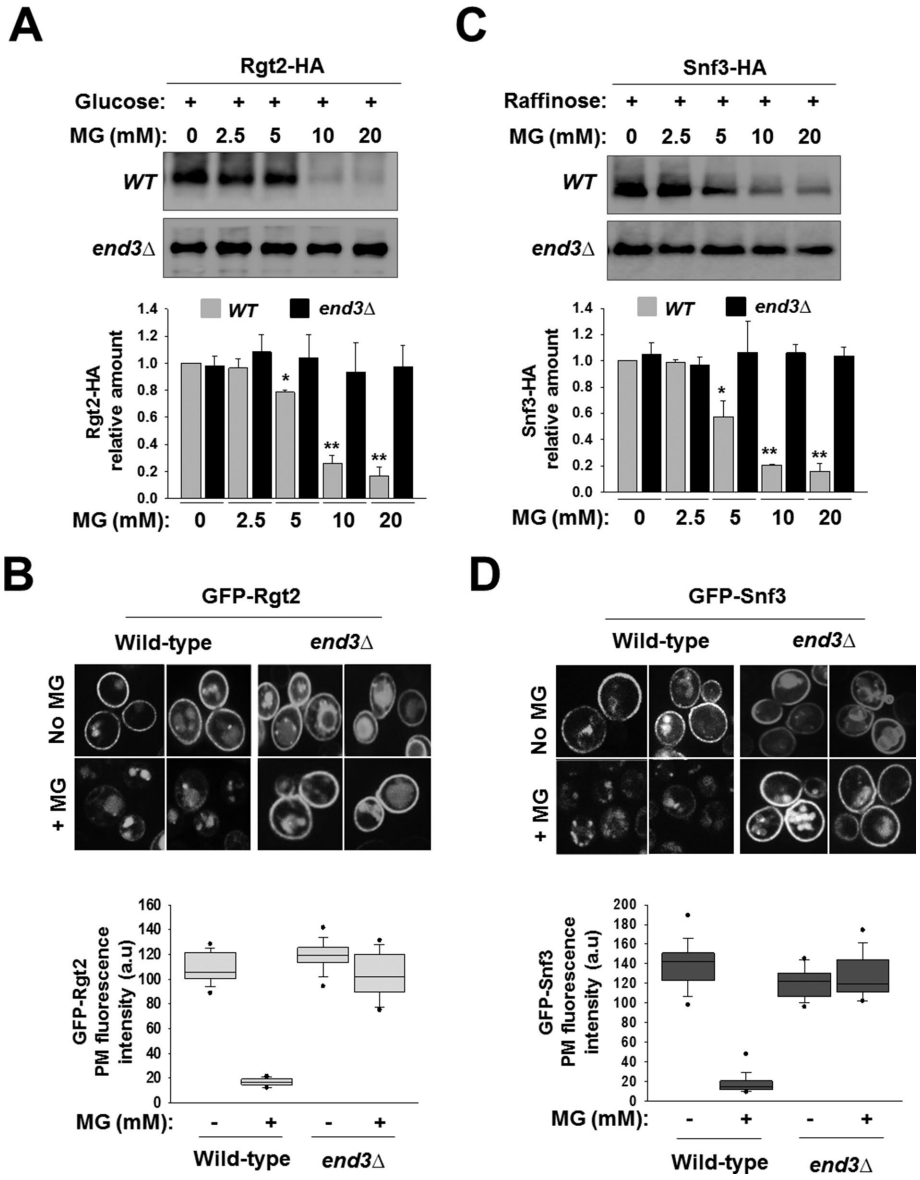

FIGURE 4: MG induces endocytosis of Rgt2 and Snf3. (A) Western blot analysis of Rgt2-HA levels at the plasma membrane. Yeast cells (*WT* and *end3Δ*) expressing Rgt2-HA were grown as described in Figure 3A. Membrane fractions were immunoblotted with anti-HA antibody (top). The intensities of each band on the blots were quantified by densitometric scanning (bottom; $*p < 0.05$ and $**p < 0.001$). (B) Yeast cells (*WT* and *end3Δ*) expressing GFP-Rgt2 were grown as described in Figure 2D. Confocal microscopy images (top) and quantifications of the GFP fluorescence intensities (bottom) as described in Figure 3D. (C) Western blot analysis of Snf3-HA levels at the plasma membrane was performed as described in A. Membrane fractions were immunoblotted with anti-HA antibody (top). The intensities of each band on the blots were quantified by densitometric scanning (bottom). (D) Confocal microscopy analysis of localization of GFP-Snf3 was carried out as described in B.

inactive to an active state upon glucose binding (Thevelein and Voordeckers, 2009). The active form (glucose bound) of these proteins localizes and functions at the plasma membrane, whereas the inactive ones (glucose unbound) are targeted to the vacuole for degradation. This notion is supported by the findings that Rgt2 and Snf3 are stabilized at the plasma membrane only when the extracellular glucose concentration is in the range of their respective affinities and that constitutively active mutants of Rgt2 and Snf3 sensors are not down-regulated even when glucose is absent (Roy and Kim, 2014). We hypothesize that MG may inhibit the glucose-induced conformational change of the glucose sensors, keeping them in the inactive state and thereby targeting them to the vacuole. Our evidence shows that endocytosis of these proteins requires the Rsp5 ubiquitin ligase. Thus it is conceivable that MG directly or indirectly modifies Rgt2 and Snf3, making them accessible to Rsp5.

In yeast, MG enters the cell via the Fps1 plasma membrane channel (Mollapour and Piper, 2007), and it is detoxified by the glyoxalase system (e.g., Glo1; Figure 1A). Our data show that the inhibitory effect of MG on the glucose sensors is greatly enhanced in the *glo1Δ* mutant, where MG accumulates (Figure 6). This suggests that degradation of Rgt2 and Snf3 is stimulated by intracellular MG. We cannot exclude the possibility that MG inhibits binding of glucose to the glucose sensors, keeping them in glucose-unbound form and vulnerable to degradation. However, this hypothesis is less tenable, in that the stability of Rgt2 and Snf3 is regulated by intracellular MG. In summary, our results suggest that MG prevents glucose-fermenting yeast cells from using glucose as sole carbon source by inhibiting expression of the *HXT* genes and thus identify MG as a glycolytic inhibitor. Intracellular MG concentrations are elevated under conditions of increased glycolytic flux, such as aerobic glycolysis associated with malignant transformation (Kawase *et al.*, 1996). These observations suggest that exogenous supply of MG in combination with pharmacological inhibitors of Glo1 may be an effective way to target cancer cells, which heavily rely on glycolysis for energy.

MATERIALS AND METHODS

Yeast strains and growth conditions

The *S. cerevisiae* strains used in this study are listed in Table 1. Cells were grown in YP (2% bactopeptone, 1% yeast extract) and SC (synthetic yeast nitrogen base medium containing 0.17% yeast nitrogen base and 0.5% ammonium sulfate) media supplemented with appropriate amino acids and carbon sources. The plasmids used in this study are listed in Table 2.

Yeast membrane preparation, Western blotting, and protein half-life measurement

Membrane-enriched fractions were essentially prepared as described previously (Galan *et al.*, 1996). Briefly, cells expressing Rgt2-HA, Snf3-HA, Rgt2$^{K637A\ K657A}$-HA, GFP-Rgt2, and GFP-Snf3 were cultured in 200-ml conical flasks containing 50 ml of SC medium to mid log phase ($A_{600\ nm}$ = 1.2–1.5), treated with different concentrations of MG (0–20 mM), and further incubated for 0–120 min, as indicated in the figures. The cells were harvested and washed with phosphate buffer, pH 7.4, containing 10 mM

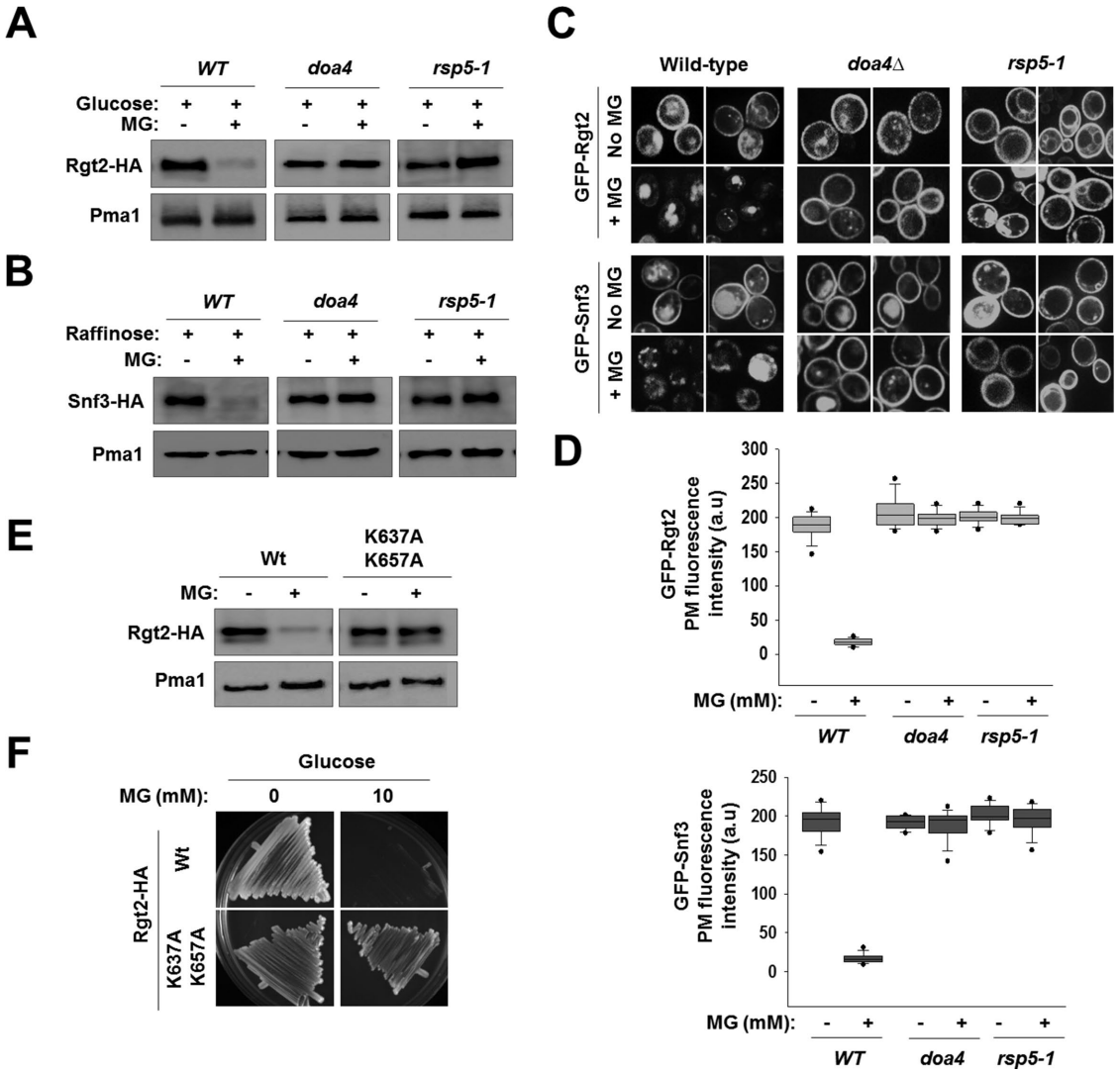

FIGURE 5: Rsp5 ubiquitin ligase is required for MG-induced vacuolar targeting and degradation of Rgt2 and Snf3. (A) Western blot analysis of Rgt2-HA levels at the plasma membrane. Yeast cells (*WT, doa4Δ* and *rsp5-1*) expressing Rgt2-HA were grown as described in Figure 2D. Membrane fractions were immunoblotted with anti-HA and anti-Pma1 antibodies. (B) Western blot analysis of Snf3-HA levels at the plasma membrane was performed as described in A. (C) Yeast cells (*WT, doa4Δ* and *rsp5-1*) expressing GFP-Rgt2 or GFP-Snf3 were grown as described in Figure 2D. The distributions of the GFP intensities were analyzed by confocal microscopy. (D) Quantification of the GFP fluorescence intensities from the cells depicted in C; box-and-whiskers plot ($n > 150$). (E) Western blot analysis of Rgt2-HA or Rgt2$^{K637A\ K657A}$-HA levels at the plasma membrane. Yeast cells (*rgt2Δsnf3Δ*) expressing Rgt2-HA or Rgt2$^{K637A\ K657A}$-HA were grown in SC-2% glucose medium until mid log phase ($A_{600\ nm}$ = 1.2–1.5) and shifted to SC medium containing 10 mM MG for 2 h. Membrane fractions were immunoblotted with anti-HA and anti-Pma1 antibodies. (F) Yeast cells (*rgt2Δsnf3Δ*) expressing Rgt2-HA or Rgt2$^{K637A\ K657A}$-HA were streaked on YP plates containing 2% glucose (Glucose), supplemented with or without 10 mM MG. The plates were incubated for 2 d and photographed. Pma1 served as a loading control in A, B, and E.

sodium azide, and the cell pellets were resuspended in ice-cold membrane isolation buffer (100 mM Tris-Cl, pH 8, 150 mM NaCl, 5 mM EDTA) containing 10 mM sodium azide and protease and phosphatase inhibitors and vortexed with acid-washed glass beads. The samples were diluted with the same buffer, and cell debris was removed by centrifugation. The membrane-enriched fractions were collected by centrifuging the samples at 12,000 rpm for 40 min at 4°C. The proteins were precipitated with 10% trichloroacetic acid, neutralized with 20 µl of 1 M Tris base, and finally dissolved in 80 µl of SDS buffer (50 mM Tris-Cl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol). The proteins were resolved by 8% SDS–PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA), and the membranes were incubated with appropriate antibodies (anti-HA, anti-GFP, anti-actin, or anti-Pma1 antibody; Santa Cruz Biotechnology, Santa Cruz, CA) in TBST buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Tween 20), and the immunoreactive bands were visualized by enhanced chemiluminescence (Pierce, Rockford, IL). The half-lives of Rgt2-HA and Snf3-HA were measured as described previously (Belle *et al.*, 2006). Briefly, the

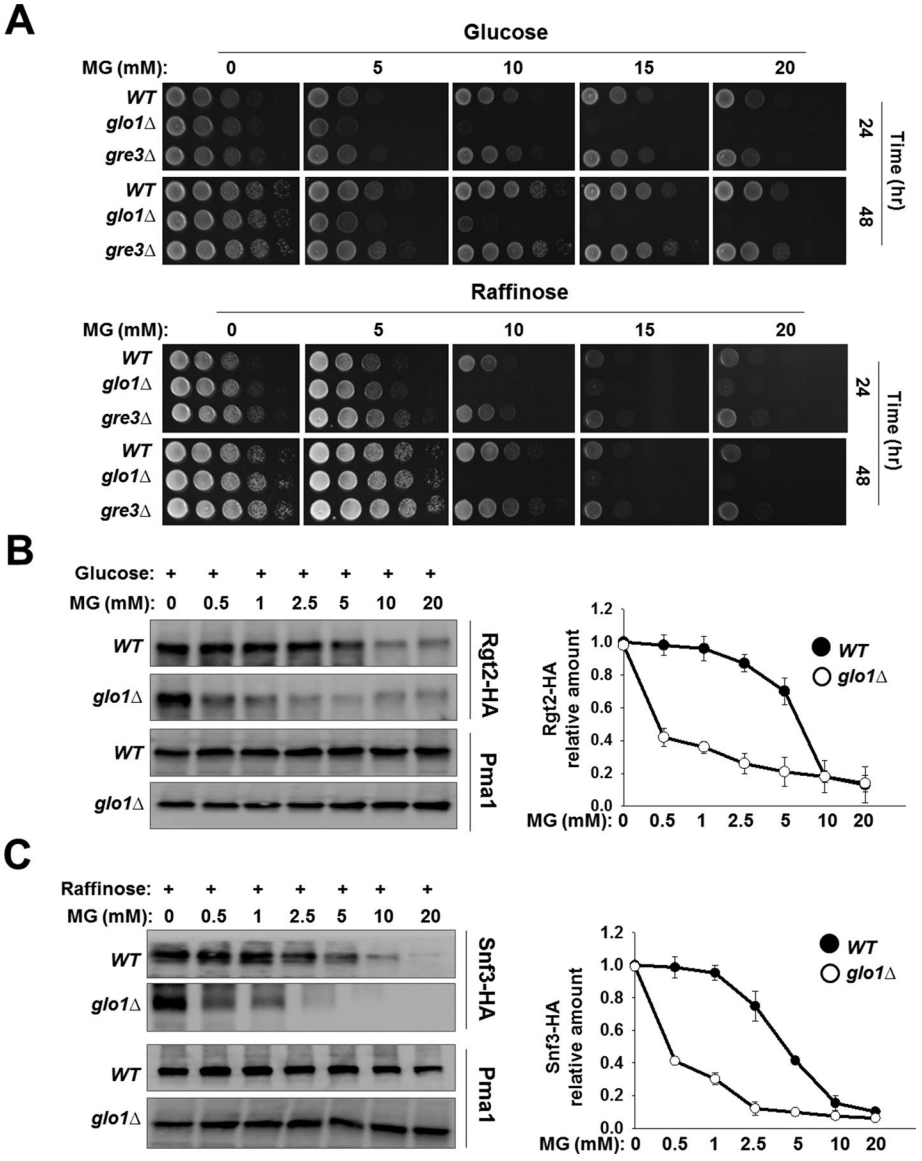

FIGURE 6: Inactivation of *GLO1* gene causes rapid down-regulation of Rgt2 and Snf3. (A) Yeast cells (*WT, glo1Δ* and *gre3Δ*) were spotted on YP plates containing 2% glucose (Glucose) or 2% raffinose (Raffinose) supplemented with the indicated concentrations of MG, as described in Figure 1B. The plates were incubated for the indicated time and photographed. (B) Western blot analysis of Rgt2-HA at the plasma membrane. Yeast cells (*WT* and *glo1Δ*) expressing Rgt2-HA were grown in SC medium containing 2% glucose until mid log phase and treated with the indicated concentrations of MG for 2 h. Membrane fractions were immunoblotted with anti-HA and anti-Pma1 antibodies (left). (C) Western blot analysis of Snf3-HA at the plasma membrane. Yeast cells (*WT* and *glo1Δ*) expressing Snf3-HA were grown in SC medium containing 2% raffinose until mid log phase and treated with the indicated concentrations of MG for 2 h. Membrane fractions were immunoblotted with anti-HA and anti-Pma1 antibodies (left). The intensities of each band on the blots in B and C were quantified by densitometric scanning (right). Pma1 served as a loading control in B and C.

band intensities were quantified by densitometry using ImageJ, version 1.4r, software (National Institutes of Health, Bethesda, MD) and normalized with the intensities of Pma1. The numbers obtained from the control samples (without MG treatment) were converted to 100, and all the values were plotted on a semilogarithmic graph against time and further fitted to an exponential line.

qRT-PCR

Total RNA was extracted by an RNeasy minikit (Qiagen, Valencia, CA) following the manufacturer's protocol, and 2 µg of total RNA was converted to cDNA by qScript cDNA supermix (Quanta Biosciences, Gaithersburg, MD). cDNA was analyzed by qRT-PCR2 using SsoFast Evagreen reagent (Bio-Rad, Hercules, CA) in a CFX96 Real-Time Thermal Cycler (Bio-Rad). *ACT1* was used as an internal control to normalize expression of *HXT1-4 RGT2*, and *SNF3* genes. All of the quantification data shown are the averages of three independent experiments, with error bars representing SD. Statistical significance was calculated by Student's *t* test using SigmaPlot software. $*p < 0.05$ and $**p < 0.001$ as compared with control.

Fluorescence microscopy and quantification

The GFP-tagged glucose sensors Rgt2 and Snf3 were imaged using a spinning-disk confocal microscope (Carl Zeiss) fitted with an Apo 150× glycerol objective (numerical aperture, 1.35). The cells were grown in SC medium supplemented with 2% galactose, 2% glucose, or 2% raffinose to mid log phase ($A_{600\ nm}$ = 1.2–1.5), and MG was added to a final concentration of 0–20 mM. The cells were further incubated for 0–120 min, as indicated in the figures, before imaging. Cells were plated onto glass-bottom dishes (Greiner Bio One, Monroe, NC) and allowed to settle for 5 min before imaging. All images from an experiment were captured with identical imaging parameters and equivalently adjusted using Adobe Photoshop software. To visualize vacuoles, cells were first incubated with 1.6 µM FM 4-64 dye for 30 min and then pelleted and resuspended in fresh medium without FM 4-64. The images were captured using a Texas red filter. The fluorescence intensities of GFP-Rgt2 and GFP-Snf3 were quantified using ImageJ, version 1.4r, software. For the quantification of fluorescence at the cell surface, the mean pixel intensities of the plasma membrane in each of at least 150 cells were measured, and the mean background pixel intensities were subtracted. The distributions of mean pixel intensities (in arbitrary units) are presented as box-and-whiskers plots generated by SigmaPlot, version 12, software. For all bar plots, each mean value represents the average for a minimum of three independent experiments. SDs were calculated and are shown as error bars. Statistical significance was calculated by Student's *t* test using SigmaPlot software. $*p < 0.05$ and $**p < 0.001$ as compared with control.

Strain	Genotype	Source
BY4741	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ*	Kaniak *et al.* (2004)
YM 6870	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 rgt2::kanMX::natMX snf3::kanMX*	Kaniak *et al.* (2004)
KFY 122	*Matα his3Δ1 leu2Δ0 ura3Δ0 doa4::KanMX*	Roy and Kim (2014)
KFY 123	*Mata his3Δ1 leu2Δ0 ura3Δ0 RSP5*	Liu *et al.* (2007)
KFY 124	*Mata his3Δ1 leu2Δ0 ura3Δ0 rsp5-1/smm1*	Liu *et al.* (2007)
KFY 127	*Matα his3Δ1 leu2Δ0 ura3Δ0 end3::KanMX*	Roy and Kim (2014)
KFY 165	*Matα his3Δ1 leu2Δ0 ura3Δ0 glo1::KanMX*	This study
KFY 166	*Matα his3Δ1 leu2Δ0 ura3Δ0 gre3::KanMX*	This study

TABLE 1: Yeast strains used in this study.

Plasmid name	Description	Source
KFP69	pAD80, 3x-HA-CYC1 terminator, Leu2, 2 μ	Kim (2009)
JKP251	pAD80-P_{RGT2}-Rgt2-3xHA	Roy and Kim (2014)
JKP293	pUG34-P_{MET25}-GFP-Rgt2, His3, CEN	Roy and Kim (2014)
JKP298	pAD80-P_{SNF3}-Snf3-3xHA	Roy and Kim (2014)
JKP308	pAD80-P_{RGT2}-Rgt2 (K637A, K657A)-3xHA	Roy and Kim (2014)
JKP309	pUG34-P_{MET25}-GFP-Snf3, His3, CEN	Roy and Kim (2014)

TABLE 2: Plasmids used in this study.

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