The glucose metabolite methylglyoxal inhibits expression of the glucose transporter genes by inactivating the cell surface glucose sensors Rgt2 and Snf3 in yeast

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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 (HXTs). 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.

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;
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 (HXTs). 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 (Yckl), 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., 2013a, 2014). It is unclear how an intracellular signal that induces expression of HXT genes is generated. However, Yckl 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 Yckl, 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 (HXTs)

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). To test this possibility, we examined whether MG inhibits the first, rate-limiting step of glucose utilization—glucose transport. We previously constructed yeast strains that express the nourseothricin (NAT) resistance gene under the control of the HXT promoters (Roy et al., 2013b). Because the growth of these strains in a NAT-containing medium depends on the activity of the HXT promoters, the HXT1-NAT reporter strain can grow only in high-glucose medium and the HXT2-NAT reporter strain only in raffinose (low-glucose) medium. In medium containing MG, however, their growth is inhibited in a concentration-dependent manner by MG (Figure 1C).

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., 2013a). 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 (rgt2Δsnf3Δ) grows poorly on a glucose or raffinose (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 rgt2Δsnf3Δ 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 rgt2Δsnf3Δ 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 (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 post-translational 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
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 vacuolar targeting of the glucose sensors. Western blot analysis 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 (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 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 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).

**FIGURE 2:** MG inactivates the yeast glucose sensors Rgt2 and Snf3. (A) Yeast cells (rgt2Δ,snf3Δ) 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 (A600 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.
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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

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 \((A_{600 \text{ nm}} = 1.2–1.5)\), 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 \((A_{600 \text{ nm}} = 1.2–1.5)\) 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.

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 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).
MG inhibits expression of HXT genes

MG in regulation of these sensor proteins (Roy and Kim, 2014). The glucose sensors undergo a conformational change from an 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...
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 \(A_{600\text{ nm}} = 1.2–1.5\), 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 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 dilute 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.
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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 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 RNase 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-PCR 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 (A600 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

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 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.

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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.

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