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# Glycolysis Controls Plasma Membrane Glucose Sensors To Promote Glucose Signaling in Yeasts

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**Sensing of extracellular glucose is necessary for cells to adapt to glucose variation in their environment. In the respiratory yeast *Kluyveromyces lactis*, extracellular glucose controls the expression of major glucose permease gene *RAG1* through a cascade similar to the *Saccharomyces cerevisiae* Snf3/Rgt2/Rgt1 glucose signaling pathway. This regulation depends also on intracellular glucose metabolism since we previously showed that glucose induction of the *RAG1* gene is abolished in glycolytic mutants. Here we show that glycolysis regulates *RAG1* expression through the *K. lactis* Rgt1 (*KIRgt1*) glucose signaling pathway by targeting the localization and probably the stability of Rag4, the single Snf3/Rgt2-type glucose sensor of *K. lactis*. Additionally, the control exerted by glycolysis on glucose signaling seems to be conserved in *S. cerevisiae*. This retrocontrol might prevent yeasts from unnecessary glucose transport and intracellular glucose accumulation.**

Sensing and adaptation to environmental variations and stresses is fundamental for any cell to live and to grow properly. Among the environmental signals that cells have to consider, nutrients, and especially glucose, are of particular importance. Indeed, glucose is the principal carbon and energy source for most living organisms. Glucose signaling is a key pathway allowing cells to adapt their sugar transport system and metabolism to the quality and quantity of carbon source present in their environment.

Glucose transport and the glucose signaling network have been widely studied in the fermentative yeast model *Saccharomyces cerevisiae* (1). The yeast *Kluyveromyces lactis* is an excellent alternate and complementary model organism to investigate glucose signaling (2). Indeed, the pathway is simpler, with only two glucose permeases and little if any gene redundancy. Moreover, unlike *S. cerevisiae*, the *K. lactis* living style is preferably respiratory, making it closer to superior eukaryotes. In *K. lactis*, the fermentative growth on medium containing 5% glucose plus antimycin A (respiration inhibitor) defines the Rag-positive (Rag<sup>+</sup>) phenotype and is informative about the functionality of glucose signaling, glucose transport, and glucose metabolism.

In *K. lactis*, two glucose permeases are known: Rag1, with a low affinity for glucose, and Hgt1, having a high affinity for glucose (3, 4). Only *RAG1* expression is regulated by the extracellular glucose concentration (5). In the absence of extracellular glucose, the transcriptional repressor *K. lactis* Rgt1 (*KIRgt1*), bound to the corepressor Sms1, represses *RAG1* expression (6, 7) (see Fig. 1A for a model). Extracellular glucose is sensed by the plasma membrane glucose sensor Rag4, a glucose permease homolog unable to transport glucose (8) and exhibiting a characteristic long cytoplasmic C-terminal tail thought to have a role in glucose signaling. Glucose binding to Rag4 probably leads to a conformational change allowing interaction with the casein kinase I (CKI) Rag8 (6, 9). When activated by glucose, Rag8 is supposed to phosphorylate the corepressor Sms1, leading to Sms1 ubiquitination by the SCF<sup>Grr1</sup> complex followed by its proteasomal degradation (7). Once Sms1 is degraded, *KIRgt1* gets highly phosphorylated by a so-far-unknown kinase (6). This phosphorylation lowers *KIRgt1* affinity for target promoters and then releases the repression of *RAG1* transcription (6). In the presence of glucose, *RAG1* maximal expression also requires the action of two transcriptional ac-

tivators: Sck1, a myc-like basic helix-loop-helix activator (10), and *KISnf2*, a subunit of the *SWI/SNF* chromatin remodeling complex, required for Sck1 recruitment to its target promoters (11). In addition to *RAG1*, the *KIRgt1*-Sms1 complex acts as a direct transcriptional repressor for *SMS1* and *SCK1* genes as well as for the glycolytic enzyme genes coding for the hexokinase *KIHXX/RAG5* and for the enolase *KIENO/RAG17* (6, 7, 10). The latter observation indicates that glucose signaling controls not only glucose transport but also its metabolism via glycolysis (Fig. 1A).

The glucose signaling pathway is globally conserved between *K. lactis* and *S. cerevisiae* (Fig. 1B) (1, 12). At least 17 hexose transporter homologs (Hxts) exist in *S. cerevisiae*, with Hxt1 being the Rag1 functional homolog (13). The function of the *S. cerevisiae* Rgt1 (*ScRgt1*) main transcriptional repressor is partly comparable to that of *KIRgt1* (14, 15). Corepressors Std1 and Mth1 are homologous to Sms1 (16). Yck1 and Yck2 (Yck1/2) are casein kinase I homologs for Rag8 (17, 18). Snf3 and Rgt2 are glucose sensors similar to Rag4 (19). So far, no *HXT1* transcriptional activator has been clearly identified in *S. cerevisiae* (for a model, see reference 12).

Extracellular glucose is not the unique signal regulating *RAG1* expression and glucose transport in *K. lactis*. Mutations of the glycolytic genes encoding the hexokinase (*KIHXX*), the 3-phosphoglycerate kinase (*KIPGK*), and the enolase (*KIENO*) provoke a *RAG1* expression defect in the presence of glucose (20). Moreover, *RAG1* expression is not affected in a phosphoglucose isomerase ( $\Delta$ *Klpgi*) mutant in which the glycolytic flux can be maintained through the pentose phosphate pathway (20). All together, these

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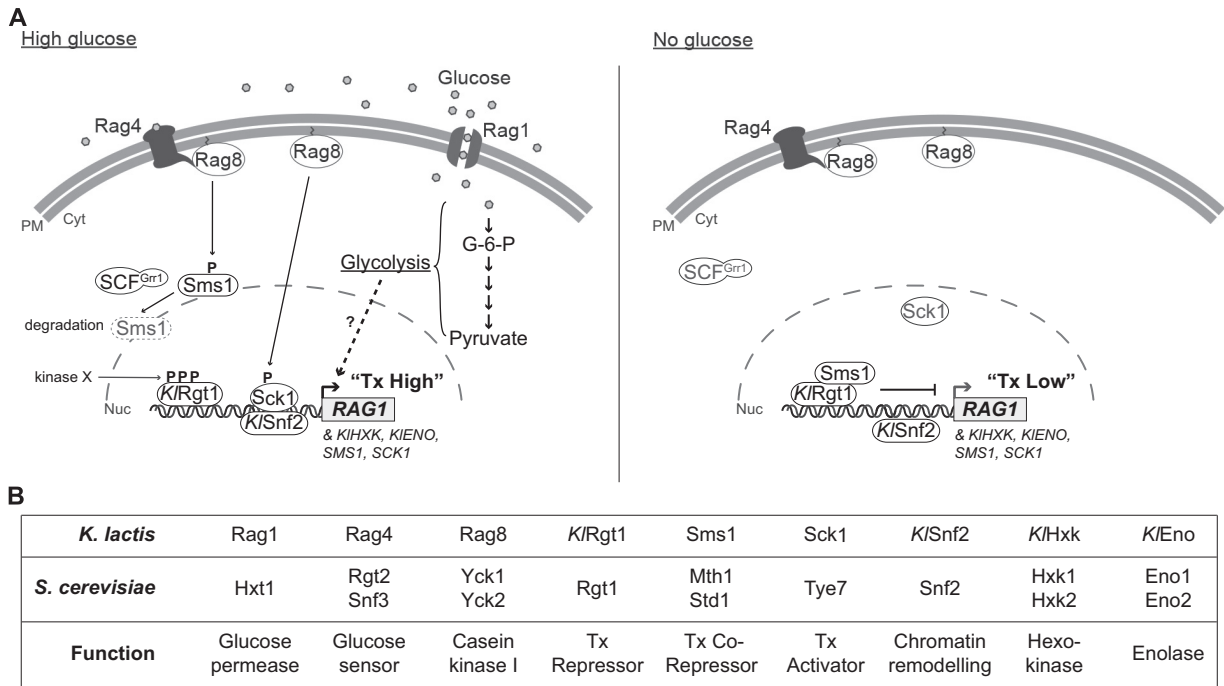
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**FIG 1** (A) A simplified model of the Rag4-K/Rgt1 glucose signaling pathway controlling *RAG1* gene expression in *Kluyveromyces lactis*. The level of *RAG1* transcription (Tx) depends on the quantity of extracellular glucose (high glucose = high transcription; no glucose = low transcription). The molecular mechanisms represented are detailed in the introduction. The dashed arrow represents the positive regulation by glycolysis on *RAG1* expression that is analyzed in this study. The glucose-induced degradation of Sms1 was positioned between the nucleus and the cytoplasm because the localization of this process is uncertain. The nuclear envelope is shown as a dashed line. Phosphorylation (P) of Sms1 and Sck1 by Rag8 and K/Rgt1 by an unknown kinase (kinase X) are presented. PM, plasma membrane; Cyt, cytosol; Nuc, nucleus; Tx, transcription; G-6-P, glucose-6-phosphate. (B) Table summarizing the functional conservation of the main factors of glucose signaling between *K. lactis* and *S. cerevisiae*. The molecular mechanisms of signal transduction of the two yeast species are similar. The function of each protein is indicated. A role for Tye7 and Snf2 in *S. cerevisiae* glucose signaling has not yet been reported. Tx, transcription.

data suggest that a functional glycolysis process controls *RAG1* expression additionally to the presence of extracellular glucose. At least two signals control *RAG1* transcription: one extracellular signal mediated by glucose via the glucose signaling pathway Rag4-Rag8-K/Rgt1 and one intracellular signal mediated by glycolysis via an unknown mechanism.

We focused on elucidating the mechanism by which glycolysis controls *RAG1* expression with special attention to a possible impact of glycolysis on glucose signaling. Here we demonstrated that glycolysis controls *RAG1* expression by promoting Rag4 stabilization at the plasma membrane which stimulates glucose signaling downstream. Moreover, this mechanism appears to be conserved in *S. cerevisiae*.

**MATERIALS AND METHODS**

**Yeast strains and growth conditions.** All the yeast strains used in this study are listed in Table 1. Yeast cells were grown at 28°C in rich medium consisting of complete yeast extract-peptone (YP) medium containing 1% Bacto yeast extract, 1% Bacto peptone (Difco) supplemented with 2% glucose (yeast extract-peptone-dextrose [YPD] medium) or minimal medium (0.7% yeast nitrogen base without amino acids [Difco] supplemented with auxotrophic requirements) supplemented with 2% glucose, 2% glycerol, 2% galactose, or 2% lactate plus 0.1% glucose as a carbon source, as mentioned. The Rag phenotype was tested on GAA medium (YP medium containing 5% glucose and 5 μM antimycin A). For G418 medium, YPD plates were supplemented with Geneticin (Life Technologies) (200 μg/ml). For Clonat medium, YPD plates were supplemented with nourseothricin (Werner Bioagents, Jena, Germany) (100 μg/ml). Glucose shift experiments were performed by addition of 2% glucose to

the glycerol- or lactate-based growth medium 30 min before cells were collected. Iodoacetate (Sigma-Aldrich) treatment was performed by addition of 0.25 to 2 mM iodoacetate to the growth medium for various times. All deletion and genomically tagged *K. lactis* strains were constructed by PCR-based gene targeting (21–23). Every epitope-tagged protein created in this study was functional since expression of the proteins in the corresponding *K. lactis* mutant restored a Rag<sup>+</sup> phenotype or correct *RAG1* transcriptional regulation by glucose. The *P<sub>ScGAL1</sub>* promoter from *S. cerevisiae* was used to constitutively express genes in *K. lactis* because this promoter is not repressed by glucose in *K. lactis* strains (our unpublished data).

*Escherichia coli* strain XL10 was used as a cloning host and for plasmid DNA propagation. *E. coli* strain BL21(DE3) was used for the production of the recombinant proteins His<sub>6</sub>-Sck1 and glutathione S-transferase-K/Rgt1<sup>338–730</sup> (GST-K/Rgt1<sup>338–730</sup>).

**Plasmid constructions.** The pACR1 plasmid is a centromeric pCXJ18 derivative plasmid (24) expressing a functional LEXA-RAG8 fusion from the *RAG8* promoter. The pACR3 plasmid expressing an inactive kinase form of LexA-Rag8 (mutation K106R) was created by one-step PCR-based mutagenesis using pACR1 as a template. The pML155 plasmid, expressing His<sub>6</sub>-SCK1 in *E. coli*, and the centromeric pHN15 plasmid, expressing a functional LEXA-SCK1, were described previously (10). The pML319 plasmid is a derivative of the pGEX-6P-3 plasmid (GE Healthcare) expressing an N-terminal GST fusion in frame with a K/Rgt1 protein internal domain (amino acids 338 to 730). Cloning details are available upon request.

**RNA extraction and RT-qPCR analysis.** Cells were grown to the mid-log phase in YPD medium before harvesting. RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) analysis were performed as described before (11). Oligonucleotide pairs were designed with Beacon

TABLE 1 Yeast strains used in this study

Strain	Genotype	Reference or source
<i>K. lactis</i>		
MW270-7B	<i>MATa leu2 metA1-1 uraA1-1</i>	4
MWK11/F1	Isogenic to MW270-7B except <i>KlhxkΔ2::ura3</i>	Laboratory collection
MWK3	Isogenic to MW270-7B except <i>KlenoΔ1::KanMX4</i>	20
MWK12	<i>MATa ade2-1 uraA1-1 rag2Δ1::URA3(ΔKlpgi)</i>	43
MWL9S1	<i>MATa leu2 lysA1-1 metA1-1 trp1 uraA1-1ΔKlnej1::loxp</i>	44
MWL1099	Isogenic to MWL9S1 except <i>RGT1-3HA::KanMX4</i>	This study
MWL1118	<i>MATa lysA1 trp1 uraA1 RGT1-3HA::KanMX4 KlhxkΔ2::ura3</i>	This study
MWL1121	<i>MATa leu2 trp1 uraA1 RGT1-3HA::KanMX4 KlenoΔ1::KanMX4</i>	This study
KLAS041	<i>MATα sms1::KanMX4 KIRGT1-3HA::KanMX4 ade2-1 uraA1-1</i>	This study
KLAS040	<i>MATa KlhxkΔ2::URA3 sms1::KanMX4 KIRGT1-3HA::KanMX4 metA1-1 trp1 uraA1-1</i>	This study
KLAS029	<i>MATα SMS1-13MYC::KanMX4 uraA1-1</i>	This study
KLAS026	<i>MATα SMS1-13MYC::KanMX4 KlhxkΔ2::URA3 uraA1-1 leu2 trp1 lysA1</i>	This study
KLAS093	Isogenic to MWL9S1 except <i>KanMX4::P<sub>ScGAL1</sub>GFP-RAG8</i>	This study
KLAS113	Isogenic to KLAS093 except <i>ΔKlhxk::NatNT2</i>	This study
MLK239	Isogenic to MWL9S1 except <i>RAG4-TAP::KanMX4</i>	This study
ACRK101	Isogenic to MLK239 except <i>ΔKlhxk::NatNT2</i>	This study
KLAS107	Isogenic to MWL9S1 except <i>KanMX4::P<sub>ScGAL1</sub>GFP-RAG4</i>	This study
KLAS109	Isogenic to KLAS107 except <i>ΔKlhxk::NatNT2</i>	This study
MLK191	Isogenic to MWL9S1 except <i>KanMX4::P<sub>ScGAL1</sub>3HA-RAG4</i>	This study
ACRK106	Isogenic to MLK191 except <i>ΔKlhxk::NatNT2</i>	This study
<i>S. cerevisiae</i>		
BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	EUROSCARF
MLY788	Isogenic to BY4742 except <i>MTH1-3HA::KanMX6</i>	This study
yAS233	Isogenic to BY4742 except <i>NatNT2::P<sub>CUP1</sub>GFP-RGT2</i>	This study
TB50a/α	<i>MATα/a leu2/leu2 ura3/ura3 rme1/rme1 trp1/trp1 his3<sup>-</sup>/his3<sup>-</sup> GAL<sup>+</sup>/GAL<sup>+</sup> HMLa/HMLa</i>	M. Hall collection
yAS223	Isogenic to TB50a/α except <i>ScRGT1/ScRGT1-3HA::KanMX4</i>	This study
W303-1A	<i>MATα leu2-3,112 ura3-1 trp1<sup>-</sup>; ade2<sup>-</sup>, his3-11,1 can1-100</i>	R. Rothstein
yAS273	Isogenic to W303-1A except <i>MTH1-9MYC::KanMX4</i>	This study
yAS275	Isogenic to W303-1A except <i>NatNT2::P<sub>CUP1</sub>GFP-RGT2</i>	This study
yAS279	Isogenic to W303-1A except <i>ScRGT1-6HA::NatNT2</i>	This study
JW02870	Isogenic to W303-1A except <i>Δhxx1::HIS3 Δhxx2::TRP1</i>	J. Winderickx
yAS276	Isogenic to JW02870 except <i>NatNT2::P<sub>CUP1</sub>GFP-RGT2</i>	This study
yAS277	Isogenic to JW02870 except <i>MTH1-9MYC::NatNT2</i>	This study
yAS280	Isogenic to JW02870 except <i>ScRGT1-6HA::NatNT2</i>	This study

Designer software (version 7.92) as follows: for *RAG1*, P548 (5'-TTTCTGGTTAGGTGTTGGT-3') and P549 (5'-CTTAAATGTTTAGGAGCGGTTT-3'); for *KIHXX*, P550 (5'-GTGCTTACTACGATGTTG-3') and P551 (5'-AGGAACCATATTCACAGT-3'); for *KIENO*, P852 (5'-CTATCGAATCCATCAAG-3') and P853 (5'-AAAGTGTCTTCAGTTTCA-3'); for *RAG4*, P854 (5'-ATGACTACTGATTCTGTTC-3') and P855 (5'-GTTACGCTCTTGCTTTC-3'); for *RAG8*, P856 (5'-CGGATTATGAAGGTTACC-3') and P857 (5'-CTATTCTATGTCTCTTACTC-3'); for *SMS1*, P858 (5'-TTAATGATATAAGGTCGTTACTC-3') and P859 (5'-GTTGATGAATGCTATGGT-3'); for *SCK1*, P860 (5'-TTCCTCCTCCGTTTATTC-3') and P861 (5'-GATTAGTAGCGTTCATTAAG-3'); and for *KIAC1*, P547 (5'-ACATCAACATCACACTTC-3') and P546 (5'-AACTGCTTCTCAATCATC-3'). The data presented are the results of at least 3 biological replicates and two technical replicates. The statistical significance of the results was assessed by performing Student's *t* test to calculate the *P* value.

**Protein extraction and Western blotting.** Total protein extraction was performed as described before (25). The primary antibodies used for Western blotting were mouse monoclonal antihemagglutinin (anti-HA) (12CA5; Roche) (1:10,000), rabbit polyclonal anti-HA (Sigma-Aldrich) (1:5,000), mouse monoclonal anti-c-Myc (9E10; Roche) (1:5,000), rabbit polyclonal anti-LexA (Millipore) (1:5,000), rabbit polyclonal anti-protein A (Sigma) (1:10,000), rabbit polyclonal anti-Pmal2 (y-300) (Santa Cruz Biotech) (1:1,000), and mouse monoclonal antiactin (mAbGEa; Novus

Biologicals) (1:10,000). Densitometric Western blot analyses were performed using Image J software.

Preparation of heavy and light membranes by ultracentrifugation was performed as follows. *K. lactis* cells were pelleted and washed before resuspension in cold lysis buffer (30 mM MOPS [morpholinepropanesulfonic acid] [pH 7], 1 mM EDTA, protease inhibitor cocktail, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Cells were broken by bead beating using a FastPrep-24 instrument (MP Biomedicals) at the maximum speed for 5 runs of 30 s each. Cell lysate was clarified from unbroken cells and debris by 5 min of centrifugation at 1,000 × *g*, and the resulting supernatant was centrifuged for 10 min at 13,000 × *g* to pellet the heavy-membrane fraction. The supernatant was centrifuged for 30 min at 100,000 × *g* to pellet the light-membrane fraction. Proteins present in the last supernatant were precipitated by the use of trichloroacetic acid (TCA) and correspond to the soluble fraction. All the different pellets were resuspended in storage buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 2 mM dithiothreitol [DTT], and 25% glycerol).

**Halo assays.** A 10-μl aliquot from an overnight culture of strain MWL9S1 grown in YPD medium was spread onto YPD or GAA plates. A sterile 6-mm-diameter filter disk (Dutscher), previously soaked with 20 μl of either serial dilutions of iodoacetate or sterile double-distilled water (dH<sub>2</sub>O), was placed onto the plates before incubation at 28°C for 48 to 72 h.



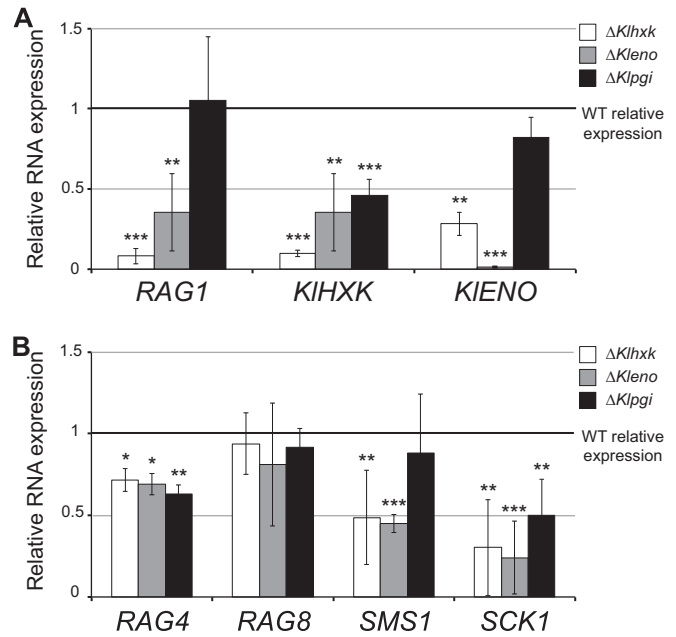
**Kinase assay.** MWL9S1 (wild-type [WT]) and MWK11/F1 ( $\Delta Klhxk$ ) strains were transformed with pACR1 and pACR3 plasmids, and kinase assays were performed as previously reported (10). Type 1 casein kinase inhibition with zinc chloride was performed as previously described (26). Densitometric analysis was achieved using Image J software.

**Fluorescence microscopy.** Cells expressing green fluorescent protein (GFP)-tagged proteins were grown to the log phase in synthetic medium containing different carbon sources. The drug treatment was achieved by adding iodoacetate to the culture. Cells were collected periodically, and protein localization was analyzed using a Zeiss AxioSkop2 fluorescence microscope set with a GFP filter. The images presented were processed by Photoshop (Adobe) and are representative of all cells observed.

**RESULTS**

**The expression of several glucose-regulated genes depends on a functional glycolysis.** Our former observation that glycolysis controls *RAG1* expression in *K. lactis* (20) prompted us to analyze the expression of other known glucose-regulated genes in three different glycolytic mutants:  $\Delta Klhxk$ ,  $\Delta Kleno$ , and  $\Delta Klpgi$ . The  $\Delta Klhxk$  and  $\Delta Kleno$  mutants are affected in the upper part and lower part of the glycolysis, respectively, and are deficient for *RAG1* gene expression (20). In the  $\Delta Klpgi$  mutant, *RAG1* gene regulation is not impaired, possibly because the glycolytic flux is maintained through the pentose phosphate pathway (20). We first analyzed by RT-qPCR the expression of *KIHXX* and *KIENO* genes in glucose-grown cells. Similarly to what was observed for *RAG1*, the *KIHXX* and *KIENO* genes were downregulated in the  $\Delta Kleno$  and  $\Delta Klhxk$  mutants, respectively, even in the presence of glucose (Fig. 2A). *SMS1* and *SCK1*, two glucose-induced genes coding for *RAG1* transcriptional regulators, were also downregulated in the  $\Delta Klhxk$  and  $\Delta Kleno$  mutants (Fig. 2B). In contrast, this transcriptional defect was absent or less pronounced in the  $\Delta Klpgi$  mutant, except for *KIHXX* and *SCK1*, for which the expression was also reduced (circa 50% of WT expression). All together, these findings demonstrate that, as observed for *RAG1*, a functional glycolysis is essential to maintain an optimal regulation of *KIHXX*, *KIENO*, *SMS1*, and *SCK1* genes by glucose. Interestingly, these five genes are known to be regulated by the *KIRgt1* transcriptional repressor (6, 7, 10). As *KIRgt1* is controlled by the *Rag4*-*Rag8* glucose signaling pathway, we investigated the expression level of the *RAG4*, *RAG8*, and *KIRGT1* genes in the glycolytic mutants. While *RAG4* expression was affected only slightly (circa 70% of WT expression), neither *RAG8* gene expression nor *KIRGT1* gene expression was significantly affected in the glycolytic mutants compared to the results seen with the WT (Fig. 2B and data not shown). This result shows that the glycolytic control of the *RAG1*, *KIHXX*, *KIENO*, *SMS1*, and *SCK1* genes is not mediated via transcriptional control of the *RAG4*, *RAG8*, and *KIRGT1* genes.

**Glycolysis controls *KIRgt1* phosphorylation and *Sms1* degradation.** Regarding the impact of *KIRgt1* posttranslational modifications on *RAG1* gene regulation, we tested whether glycolysis could influence these *KIRgt1* modifications. *KIRgt1* is hyperphosphorylated in glucose-grown WT cells, which leads to its inactivation and derepression of its targeted genes (6). Because this hyperphosphorylation is informative about *KIRgt1* activity, we analyzed the phosphorylation level of *KIRgt1* in response to glucose in the  $\Delta Klhxk$  and  $\Delta Kleno$  glycolytic mutants by a mobility shift assay as previously described (6). In the glycolytic mutants, *KIRgt1* was not phosphorylated after glucose stimulation as demonstrated by the absence of an electrophoretic-mobility shift compared to what was observed in WT cells after glucose addition (Fig. 3A). This

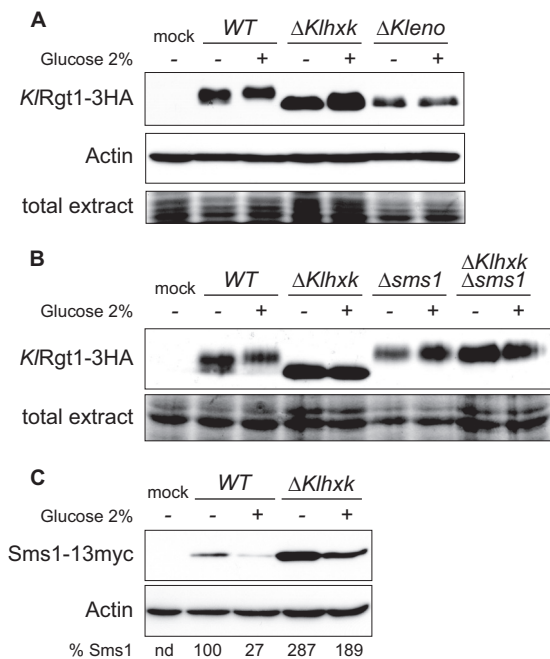


**FIG 2** Expression of glucose-regulated genes is downregulated in glycolytic mutants. The mRNA transcript levels of glucose permease gene *RAG1* and glycolysis genes *KIHXX* and *KIENO* (A) and of glucose-signaling genes *RAG4*, *RAG8*, *SMS1*, and *SCK1* (B) were determined by RT-qPCR in the wild-type strain (WT [MW270-7B]) and in  $\Delta Klhxk$  (MWK11/F1),  $\Delta Kleno$  (MWK3), and  $\Delta Klpgi$  (MWK12) mutants grown in YPD medium. Expression levels were normalized to the *KIAC1* transcript level. For each gene, the mRNA expression level in the  $\Delta Klhxk$ ,  $\Delta Kleno$ , and  $\Delta Klpgi$  mutants is presented relative to their level in the WT strain, which was set to 1 and is represented as a thick line on the graph (WT relative expression). The means of the relative-expression data from three biological replicates with standard deviation (error bars) are represented. Significance levels were determined by calculating the *P* value for each data set with Student's *t* test and are represented as asterisks: \* for *P* < 0.05, \*\* for *P* < 0.01, and \*\*\* for *P* < 0.001. It should be noted that the  $\Delta Klhxk$  mutant used in this figure (MWK11/F1) corresponds to a partial deletion of *KIHXX* coding for a nonfunctional truncated version of *KIHxk*. It is why *KIHXX* expression was still slightly observed by RT-qPCR in the  $\Delta Klhxk$  strain.

result shows that a defect in glycolysis inhibits the glucose-induced hyperphosphorylation of *KIRgt1*, which might be in a constitutive repressing form when glycolysis is not functional.

The hyperphosphorylation of *KIRgt1* in glucose-grown WT cells depends on the proteasomal degradation of its partner, *Sms1*, a process controlled by the *Rag4* glucose signaling pathway (6, 7). To test if *Sms1* mediates the effects of glycolysis on *KIRgt1* phosphorylation, we constructed a  $\Delta Klhxk \Delta sms1$  mutant and checked the phosphorylation status of *KIRgt1*  $\Delta sms1$  in response to glucose availability (Fig. 3B). *KIRgt1* was found to be hyperphosphorylated in the  $\Delta sms1$  mutant grown without glucose, confirming the protective role of *Sms1* against *KIRgt1* phosphorylation described previously (7). Interestingly, *KIRgt1* was also hyperphosphorylated in the  $\Delta Klhxk \Delta sms1$  double mutant irrespective to the carbon source. This suggests (i) that glycolysis controls *KIRgt1* phosphorylation through *Sms1* and (ii) that *KIRgt1*-phosphorylating activities are still efficient even if glycolysis is defective, at least when *Sms1* is absent.

Since *KIRgt1* was not hyperphosphorylated in a  $\Delta Klhxk$  or  $\Delta Kleno$  mutant (Fig. 3A), we investigated whether a glycolysis defect ( $\Delta Klhxk$  mutant) could prevent *Sms1* degradation in glucose-grown cells. In the absence of glucose, the *Sms1* steady-state



**FIG 3** *KIRgt1* phosphorylation and *Sms1* stability are affected in glycolytic mutants. (A) *KIRgt1* phosphorylation in glycolytic mutants. WT (MWL1099),  $\Delta Klhxk$  (MWL1118), and  $\Delta Kleno$  (MWL1121) strains expressing C-terminally 3HA-tagged *KIRgt1* (*KIRgt1*-3HA) from the genome were grown in synthetic lactate medium to the exponential phase. One half of each culture was incubated with 2% glucose (+) for 30 min and the other was not (-) before cell collection and total protein extraction. Whole-cell lysates were analyzed for *KIRgt1*-HA by Western blotting (*KIRgt1*-3HA). (B) *SMS1* is required for glycolytic control of *KIRgt1* phosphorylation. *KIRgt1*-3HA expression in the WT (MWL1099),  $\Delta Klhxk$  (MWL1118),  $\Delta sms1$  (KIAS041), and  $\Delta Klhxk \Delta sms1$  (KIAS040) strains was analyzed by Western blotting after cells were stimulated or not stimulated with 2% glucose as described for panel A. (C) *Sms1* protein level in glycolytic mutants. WT (KIAS029) and  $\Delta Klhxk$  (KIAS026) strains expressing C-terminally 13myc-tagged *Sms1* from the genome (*Sms1*-13myc) were grown and glucose stimulated as described for panels A and B. The *Sms1* protein level was analyzed by Western blotting of whole-cell lysate. The percentage of *Sms1* (% *Sms1*) was determined by densitometric analysis of the *Sms1*-13myc WB signal normalized to the actin signal (nd = not determined). The values are representative of the tendency observed in replica experiments. In all panels, a wild-type strain expressing untagged proteins (MWL9S1 or derivative) was used as a negative control (mock) and in-gel Coomassie staining of total protein extract and/or *KlAct1* (Actin) immunodetection was used as a loading control (total extract).

protein level was increased (almost 3-fold) in the hexokinase mutant cells compared to the WT cells (Fig. 3C). Furthermore, *Sms1* degradation induced by glucose was much less effective in the hexokinase mutant than in the WT cells (glucose provoked a 4-fold decrease of *Sms1* in WT cells compared to a 1.5-fold decrease in  $\Delta Klhxk$  cells) (Fig. 3C). Given that *SMS1* gene expression was downregulated in the glycolytic mutants (Fig. 2B), the high level of *Sms1* protein in the hexokinase mutant might be representative of a partial defect in its glucose-induced degradation.

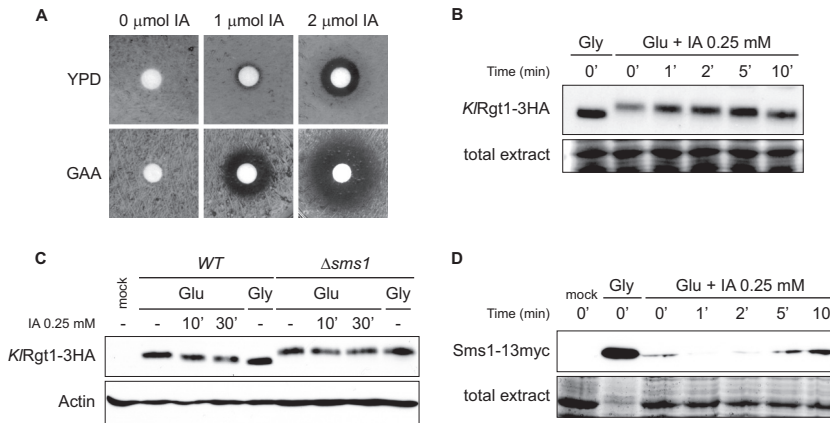
Taken together, these data demonstrate that a functional glycolysis is required to promote the optimal *Sms1* degradation in the presence of glucose, and thus subsequent *KIRgt1* hyperphosphorylation, promoting the expression of *RAG1* and other glucose-regulated genes. Furthermore, the “glycolysis signal” may act upstream of *Sms1* without affecting kinase activities regulating the *KIRgt1* repressor function.

**Glycolysis chemical inhibition affects glucose signaling.** We cannot exclude the possibility that the phenotypes observed in the glycolytic mutants (*RAG1* downregulation, *KIRgt1* hypophosphorylation, and *Sms1* stabilization) are due to cell adaptation to the mutations rather than to a defective glycolysis. In order to untangle these two possibilities, we assessed the effect of an acute chemical inhibition of glycolysis on glucose signaling by using iodoacetate, a drug already known to inhibit glycolytic GAPDH (glyceraldehyde-3-phosphate dehydrogenase) activity in *S. cerevisiae* (27, 28). We first performed a Rag phenotype assay (fermentative growth upon respiration inhibition by antimycin A) to test if addition of iodoacetate was effective for inhibition of glycolysis in the WT *K. lactis* strain. As shown in Fig. 4A, iodoacetate gave rise to a Rag<sup>-</sup> phenotype, suggesting that this drug is able to block glycolysis in *K. lactis*. We then tested the effect of iodoacetate on *KIRgt1* and *Sms1* modifications. Interestingly, treating glucose-growing WT cells with iodoacetate led to a rapid and progressive decrease in the level of the hyperphosphorylated *KIRgt1* form (Fig. 4B). However, even after a 30-min iodoacetate treatment, *KIRgt1* dephosphorylation appeared partial since its electrophoretic mobility remained lower than that of glycerol-grown WT cells (Fig. 4C) or  $\Delta Klhxk$  mutant cells (Fig. 3A and B). Importantly, in iodoacetate-treated  $\Delta sms1$  cells, the electrophoretic mobilities of the *KIRgt1* hyperphosphorylated form remained similar throughout the experimental time course, suggesting that iodoacetate treatment influences *KIRgt1* phosphorylation in a *Sms1*-dependent manner. In agreement with this hypothesis, iodoacetate addition to glucose-grown WT cells led to the partial reaccumulation of *Sms1* in the cells (Fig. 4D).

These results show that inhibiting glycolysis by an acute chemical inhibition weakens glucose signaling. However, the effects on the *KIRgt1* and *Sms1* modifications are comparable but are less pronounced than what is observed in glycolysis mutants. This might reflect the existence of other unappreciated factors that would affect glucose signaling when glycolysis is chronically altered but not when it is transiently inhibited.

**Glycolysis controls glucose sensor Rag4 but not casein kinase Rag8.** In *K. lactis*, casein kinase I (CKI) Rag8 cooperates with the Rag4 glucose sensor to initiate the signalization cascade leading to *Sms1* degradation when cells are grown on glucose (7). CKI Rag8 also phosphorylates the Sck1 transcriptional activator *in vitro* and is required for Sck1 stability *in vivo* (7). Since glucose-induced *Sms1* degradation is impaired in both  $\Delta Klhxk$  (Fig. 3C) and *rag8* (7) mutants, we wondered whether *Sms1* stabilization occurring upon glycolysis inhibition could be due to a CKI Rag8 deficiency under these conditions. Under all conditions tested, neither the *RAG8* gene expression level (Fig. 2B) nor the Rag8 steady-state protein level or localization (Fig. 5A and B) nor the Rag8 *in vivo* and *in vitro* kinase activity toward Sck1 (Fig. 5C and D) was altered in the  $\Delta Klhxk$  mutant compared to the WT strain results. Our results did not rule out the possibility that the hexokinase mutant has a reduced rate of Rag8 kinase activity because solely an endpoint kinase assay was performed here. However, taken together, these data indicate that glycolysis is probably not targeting CKI Rag8 to control glucose signaling in *K. lactis*.

The extracellular glucose signal is propagated by plasma membrane glucose sensor Rag4 via its direct interaction with CKI Rag8 (6). As Rag8 activity is not influenced by glycolysis (see above), the *Sms1* stabilization and *KIRgt1* hypophosphorylation observed in the glycolytic mutants might rather be explained by a defect in



**FIG 4** Chemical glycolysis inhibition with iodoacetate affects *KIRgt1* phosphorylation and *Sms1* stability. (A) Iodoacetate (IA) confers a Rag<sup>-</sup> phenotype. Representative examples of iodoacetate halo assays of WT cells (MWL9S1) on YPD and GAA (containing the respiratory inhibitor antimycin A) plates are shown. A 20- $\mu$ l volume of water (0  $\mu$ mol IA), 50 mM iodoacetate (1  $\mu$ mol IA), or 100 mM iodoacetate (2  $\mu$ mol IA) was dropped onto filter papers. (B) *KIRgt1* is rapidly dephosphorylated upon iodoacetate treatment. Cells expressing *KIRgt1*-3HA (MWL1099) were grown to the exponential phase in complete synthetic medium with glycerol (Gly) or glucose (Glu) as the carbon source. Glycerol-grown cells were harvested, and glucose-grown cells were incubated with 0.25 mM iodoacetate and collected after 0, 1, 2, 5, and 10 min of treatment. Whole-cell lysates were prepared and analyzed for *KIRgt1*-3HA by Western blotting. Detection of total proteins by in-gel Coomassie staining was used as a loading control (total extract). (C) *SMS1* deletion prevents iodoacetate-induced *KIRgt1* dephosphorylation. WT (MWL1099) and  $\Delta$ *sms1* (KIAS041) strains expressing *KIRgt1*-3HA were grown in glucose-containing medium (Glu) or in glycerol-containing medium (Gly) until the exponential phase. An aliquot of glucose-grown cells was treated with 0.25 mM iodoacetate for 10 and 30 min before harvest. Whole-cell lysates were prepared and analyzed for *KIRgt1*-3HA and actin (loading control) by Western blotting. The MWL9S1 wild-type strain was used as a mock treatment control. (D) Iodoacetate stabilizes *Sms1* in glucose-grown cells. *Sms1*-13myc-expressing cells (KIAS029) were grown and treated as described for panel A. Total protein extract was analyzed for *Sms1*-13myc by Western blotting. Detection of total proteins by Ponceau staining was used as a loading control (total extract). The wild-type strain MWL9S1 was used as a mock treatment control.

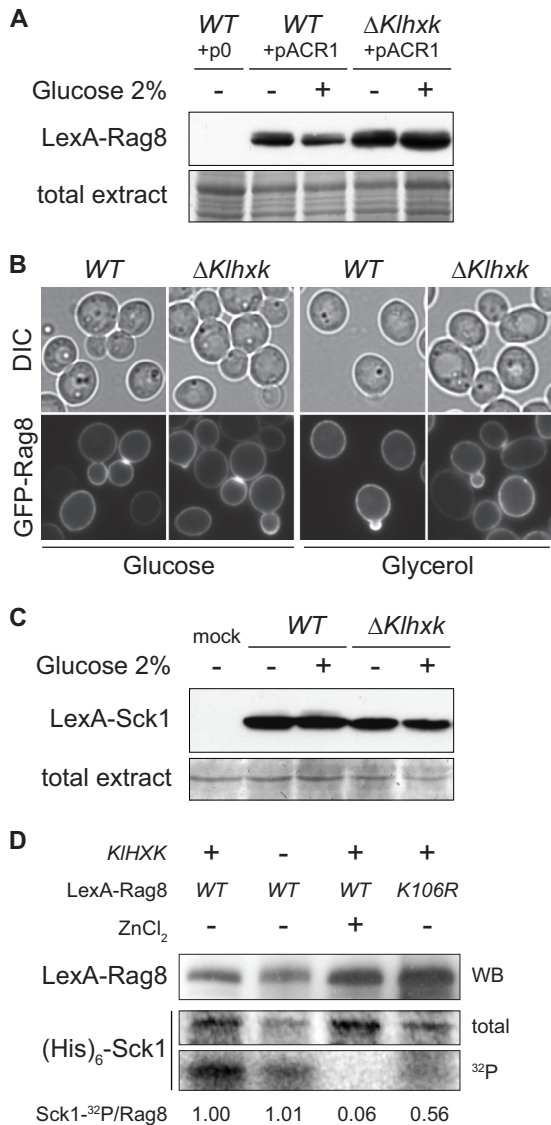
Rag4 function. Western blot analysis showed that the Rag4-TAP (where TAP is the tandem affinity purification tag) protein level (expressed from its own promoter) was decreased in the  $\Delta$ *KIHXK* mutant grown in the presence of glucose compared to the WT strain results (Fig. 6A). Furthermore, the Rag4-TAP quantity was also diminished WT grown in the absence of glucose, but in this case, deletion of *KIHXK* did not further affect the Rag4-TAP protein level (Fig. 6A). As shown in Fig. 6B, an identical effect was also observed when 3HA-Rag4 was expressed under the control of a strong nonregulated promoter (the *S. cerevisiae* *GAL1* promoter was used as it is functional and not subjected to glucose repression in *K. lactis* [our unpublished data]). All together, these data suggest that glycolysis and the nature of the carbon source control Rag4 protein stability rather than *RAG4* gene expression.

To investigate more precisely the Rag4 protein level and its localization in the  $\Delta$ *KIHXK* mutant, we performed membrane fractionation to separate heavy membranes (containing the plasma membrane, organelle membranes, and cytoskeleton) from light membranes (containing Golgi and transport vesicles) and the cytosol (29). The [H<sup>+</sup>] ATPase *KIPma1* and actin were used as controls for the membrane and cytosolic fractions, respectively (30). In glucose-grown wild-type cells, Rag4-TAP (expressed from its own promoter) was detected in both the heavy-membrane fraction and the light-membrane fraction but not in the cytosol (Fig. 6C). In glucose-grown  $\Delta$ *KIHXK* cells, the Rag4-TAP quantity in both the heavy-membrane fraction and the light-membrane fraction was severely reduced compared to the WT cell results (Fig. 6C), confirming the results obtained with total cell lysates (Fig. 6A). This reduction in Rag4-TAP protein level observed in the membrane fractions of the hexokinase mutant (Fig. 6C) suggests that the plasma membrane localization of Rag4 is probably impaired in this mutant. This hypothesis was analyzed by fluores-

cence microscopy observations using a GFP-Rag4 fusion (*P<sub>ScGAL1</sub>* promoter-driven expression). In the presence of glucose, GFP-Rag4 was localized at the plasma membrane in the WT cells whereas this plasma membrane localization almost completely vanished in the  $\Delta$ *KIHXK* mutant cells (Fig. 6D). Furthermore, in the hexokinase mutant, a strong GFP signal, probably originating from GFP-Rag4 degradation products, accumulated into the seeming vacuole (Fig. 6D). Interestingly, there was no substantial difference with respect to GFP-Rag4 localization and quantity between the WT and  $\Delta$ *KIHXK* strains when cells were grown in the presence of glycerol (Fig. 6D). Under this condition, GFP-Rag4 showed faint but persistent plasma membrane localization together with a vacuolar GFP signal in both strains. This suggests that *KIHXK* is not required for GFP-Rag4 localization in the absence of glucose. Similarly to what was observed in the hexokinase mutant (Fig. 6D), the long-term glycolysis inhibition of glucose-grown cells in the presence of iodoacetate altered the plasma membrane localization of GFP-Rag4 and favored the vacuolar accumulation of a GFP signal compared to the results seen with untreated cells (Fig. 6E, 2 h and 6 h). Short-term treatment with iodoacetate did not provoke any noticeable change in the GFP-Rag4 level or plasma membrane localization compared to the untreated-cell results (Fig. 6E, 30 min). All together, these data demonstrate that a functional glycolysis is essential to maintain Rag4 at the plasma membrane in the presence of glucose. Alteration of the glycolytic flux would lead to a mislocalization and probably vacuolar degradation of Rag4, abolishing the downstream glucose signaling.

**Glycolysis control of glucose signaling is conserved in *S. cerevisiae*.** The glucose signaling pathway is highly conserved between *K. lactis* and *S. cerevisiae* (1). Similarly to *RAG1* in *K. lactis*, the expression of the *S. cerevisiae* glucose transporter *HXT1* gene





**FIG 5** Casein kinase 1 Rag8 protein level, localization, and kinase activity in a hexokinase mutant. (A) The Rag8 protein level is stable in a glycolytic mutant. WT (MWL9S1) and  $\Delta Klhxk$  (MWK11/F1) strains transformed with the pACR1 plasmid encoding an N-terminally LexA-tagged Rag8 (*RAG8* promoter) were grown in lactate minimal media without uracil. Cells were then shifted (+) or not shifted (-) to 2% glucose and incubated for 30 min before cell collection. Whole-cell lysates were prepared and analyzed for LexA-Rag8 by Western blotting. MWL9S1 transformed with an empty plasmid was used as a mock treatment control. For a loading control, total proteins were analyzed by in-gel Coomassie staining (total extract). (B) GFP-Rag8 localization is not affected by the carbon source or by a glycolysis defect. WT (KIAS093) and  $\Delta Klhxk$  (KIAS113) strains expressing N-terminally GFP-tagged Rag8 (*P<sub>ScGAL1</sub>* promoter) from the genome were grown in glucose-based (Glucose) or glycerol-based (Glycerol) minimal medium to the log phase and then observed with a fluorescence microscope. Cells were imaged using a GFP filter (GFP-Rag8) or Nomarski optics (differential interference contrast [DIC]). The acquisition times were equal for all GFP images. (C) Sck1 protein is stable in the hexokinase mutant. WT (MWL9S1) and  $\Delta Klhxk$  (MWL1118) cells transformed with the pHN15 plasmid encoding LexA-Sck1 were grown in glycerol synthetic medium without uracil. Glucose was then added (+) or not added (-) to the cells for 30 min before being collected. The LexA-Sck1 protein level was then assessed by Western blotting of total protein extract. The mock treatment control corresponds to WT (MWL9S1) cells transformed with an empty plasmid. Total extracts were analyzed by Ponceau staining for a loading control (total extract). (D) Rag8 *in vitro* kinase activity is not affected in a glycolytic

was previously shown to be impaired in a hexokinase mutant grown in the presence of glucose (31). To assess whether glycolysis controls glucose signaling upstream of *HXT1* in *S. cerevisiae*, we analyzed the functionality of glucose signaling in a  $\Delta hxx1 \Delta hxx2$  hexokinase mutant. As shown in Fig. 7A, the glucose-induced phosphorylation of ScRgt1-6HA was decreased in the hexokinase mutant (Fig. 7A). This effect is similar to but less pronounced than the one observed in *K. lactis* (Fig. 3A). Furthermore, the glucose-dependent degradation of ScRgt1 corepressor Mth1 (Sms1 homolog) was much less effective in the hexokinase mutant than in the WT cells (glucose induced a 1,000-fold decrease of Mth1-9myc levels in WT cells compared to a 1.8-fold decrease in  $\Delta hxx1/2$  cells) (Fig. 7B). In this glycolytic mutant, the protein level of glucose sensor Rgt2 (Rag4 homolog) was also diminished upon growth in glucose (Fig. 7C) and its localization at the plasma membrane was almost completely abolished (Fig. 7D). The Rgt2 protein level and localization were also affected by the carbon source, but the absence of the hexokinases had no additive effect under these growth conditions (Fig. 7C and D). These results were confirmed and strengthened by our observations that, in glucose-grown cells, the acute chemical inhibition of glycolysis by iodoacetate provoked the dephosphorylation of ScRgt1 (Fig. 8A), the partial stabilization of Mth1 (Fig. 8B), and the delocalization of Rgt2 from the plasma membrane (Fig. 8C). As with *K. lactis*, the effects of chronic (mutant) and acute (iodoacetate) glycolysis inhibition on glucose signaling are not strictly identical in *S. cerevisiae*. This again suggests the existence of other unknown factors regulating glucose signaling when glycolysis is chronically reduced.

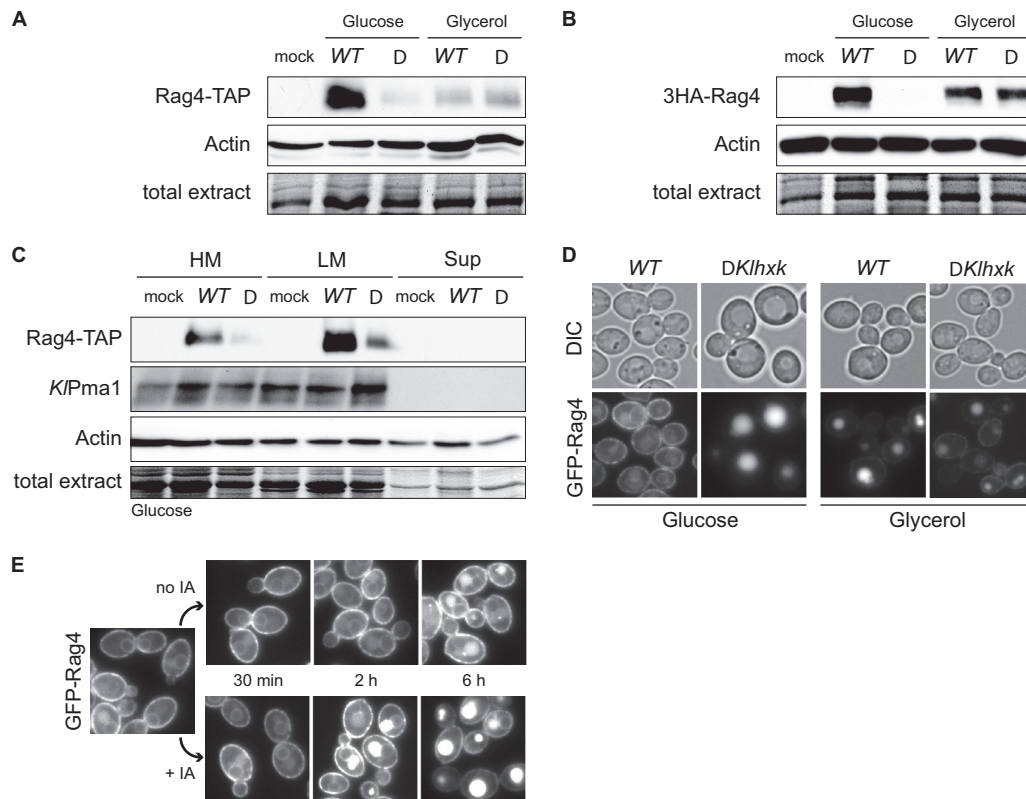
These results plus those obtained in *K. lactis* suggest that the glycolytic control exerted on glucose sensors and subsequently through glucose signaling is globally conserved between *K. lactis* and *S. cerevisiae*.

## DISCUSSION

The effect of the extracellular glucose concentration on glucose signaling has been widely studied in both *K. lactis* (11) and *S. cerevisiae* (1, 12). However, intracellular glucose and particularly its metabolism through glycolysis appear to exert regulation of glucose transport independently of the presence of extracellular hexose. Indeed, in *K. lactis*, when glucose is not correctly metabolized due to an impaired glycolysis, the expression of the main target of glucose signaling, the *RAG1* glucose permease gene, is impaired (20). In this study, we further characterized the molecular basis of *RAG1* control by glycolysis. We have demonstrated reduced not only *RAG1* expression in glycolytic mutants but also expression of various glycolysis genes and genes involved in glu-

mutant. A wild-type form (WT) and a “kinase-deficient” form (K106R) of LexA-Rag8 were immunoprecipitated from WT (MWL9S1 plus pACR1 or MWL9S1 plus pACR3) and  $\Delta Klhxk$  (MWK11/F1 plus pACR1) strains grown in synthetic glucose-containing medium without uracil (-ura). Kinase activity was assayed against 1  $\mu$ g of recombinant His<sub>6</sub>-Sck1 expressed and purified from *E. coli*. Total and phosphorylated proteins were visualized by Coomassie staining (Coomassie) and autoradiography (<sup>32</sup>P), respectively. An aliquot of immunoprecipitated LexA-Rag8 was conserved for analysis of total LexA-Rag8 by Western blotting (WB). The level of Sck1 phosphorylation relative to the Rag8 quantity was quantified by densitometric analysis and normalized to the WT strain level. The specificity of the kinase activity was controlled by pretreating purified LexA-Rag8 with 1 mM ZnCl<sub>2</sub> before performing the kinase reaction against His<sub>6</sub>-Sck1 (+ZnCl<sub>2</sub>).

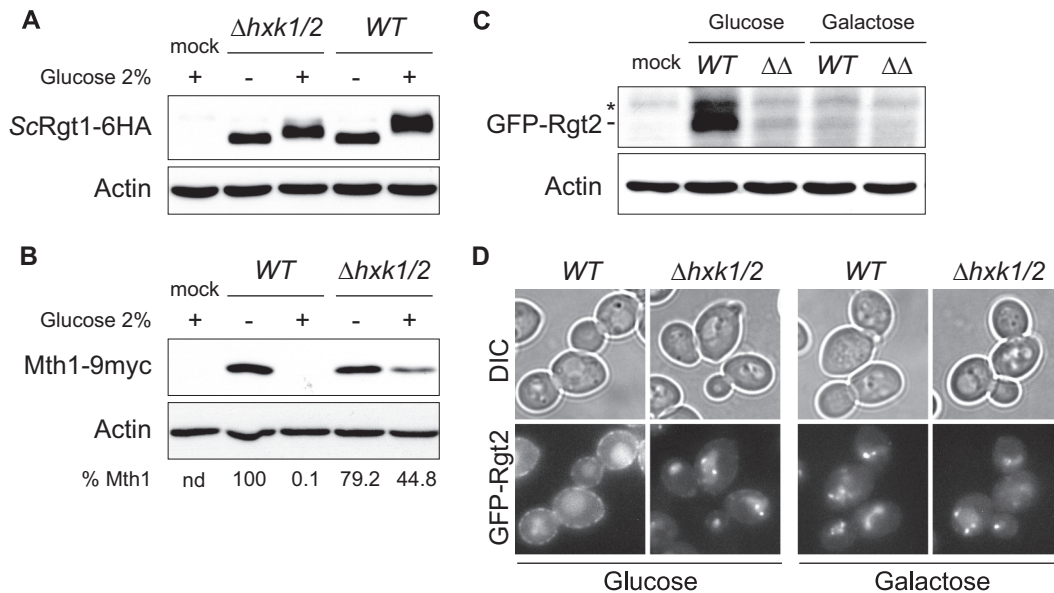




**FIG 6** The glucose sensor Rag4 is a target of glycolysis. (A and B) Rag4 protein stability is affected in a glycolysis mutant in a carbon source-dependent manner. (A) WT (MLK239) and  $\Delta Klhxk$  ( $\Delta$ ) (ACRK101) strains expressing Rag4-TAP from its own promoter were grown in synthetic medium containing glucose (Glucose) or glycerol (Glycerol) as a carbon source. Whole-cell lysates were prepared and analyzed for Rag4-TAP by Western blotting. A wild-type strain expressing untagged proteins (MWL9S1 or derivative) was used as a negative control (mock), and in-gel Coomassie staining of total protein extract (total extract) and *KIAct1* (Actin) immunodetection were used as a loading control. (B) WT (MLK191) and  $\Delta Klhxk$  (ACRK106) strains expressing N-terminally 3HA-tagged Rag4 from the  $P_{ScGAL1}$  promoter were grown and analyzed as described for panel A. (C) The Rag4 membrane distribution is impaired in a hexokinase mutant. Cell-free lysates from isogenic WT (MLK239) and  $\Delta Klhxk$  ( $\Delta$ ) (ACRK101) strains expressing Rag4-TAP and grown in synthetic glucose-containing medium (Glucose) were subjected to differential centrifugation to separate heavy membranes (HM), light membranes (LM), and cytosolic supernatant (Sup). For all fractions, equal protein quantities were examined by Western blotting to detect Rag4-TAP, *KIPma1*, and *KIAct1* (Actin). MWL9S1 (mock) was similarly processed to get a mock treatment control. Each fraction was analyzed by Ponceau staining for a loading control (total extract). (D) The *in vivo* Rag4 localization is impaired in the hexokinase mutant. WT (KIAS107) and  $\Delta Klhxk$  (KIAS109) strains expressing N-terminally GFP-tagged Rag4 ( $P_{ScGAL1}$  promoter) from the genome were grown to the exponential phase in synthetic minimal medium containing either glucose or glycerol. Cells were observed with a fluorescence microscope and imaged using a GFP filter (GFP-Rag4) or Nomarski optics (DIC). The acquisition times were equal for all GFP images. (E) Acute chemical inhibition of glycolysis by iodoacetate affects *in vivo* Rag4 localization. The KIAS107 strain used as described for panel C was grown to the log phase in YPD medium and then treated (+ IA) or not treated (no IA) with 0.25 mM iodoacetate. A fraction of the cells was collected periodically (0 min, 30 min, 2 h, and 6 h posttreatment) and observed under the microscope for the GFP signal (GFP-Rag4). The acquisition times were equal for all GFP images.

cose signaling. Strikingly, all these downregulated genes are known to be controlled by the *KIRgt1* transcriptional repressor (6, 7, 10). This suggests that *KIRgt1* might be the target of glycolytic control. Similarly to what was observed in *K. lactis*, the *S. cerevisiae* Hxk2 hexokinase was shown to be required for the optimal expression of the *HXT1* glucose permease gene in response to extracellular glucose (31). This effect seems to be mediated via the direct interaction of Hxk2, with ScRgt1 then modulating its repressor function (32). It is tempting to hypothesize that the regulation of *RAG1* expression by glycolysis in *K. lactis* is mediated via the interaction of *KIHxk* with *KIRgt1*. Despite the interaction between *KIHxk* and *KIRgt1*, which seems to be conserved in *K. lactis* (our unpublished data), here we provide several examples of evidence showing the existence of a more global effect originating from glycolysis and controlling *KIRgt1* function. First, inhibition of glycolysis at the top of the pathway ( $\Delta Klhxk$  mutant) and inhibition of glycolysis at the bottom of the pathway ( $\Delta Klno$  mutant)

similarly reduced the expression of *KIRgt1*-dependent genes in the presence of glucose. Second, the chronic (mutants) or acute (chemical) inhibition of glycolysis provokes the dephosphorylation of *KIRgt1* (repressive state) and the partial stabilization of its corepressor Sms1 in glucose-containing medium. These observations favor a model in which *KIRgt1* repressor activity is primarily regulated by the flux of glycolysis rather than through its direct interaction with the hexokinase. Moreover, in this model, glycolysis would exert its control upstream of Sms1 by tightly regulating Sms1 stability and, consequently, *KIRgt1* phosphorylation. Thus, inhibition of glycolysis would conduce to a reduced turnover of Sms1 and a more stable *KIRgt1*/Sms1 complex bound to DNA in the promoter region of *RAG1* and other target genes. This regulation seems to be conserved in *S. cerevisiae*, as (chronic or acute) glycolysis inhibition of glucose-grown cells also led to ScRgt1 dephosphorylation and Mth1 stabilization. The latter observation implies that, in *S. cerevisiae*, glycolysis controls *HXT1* gene expres-



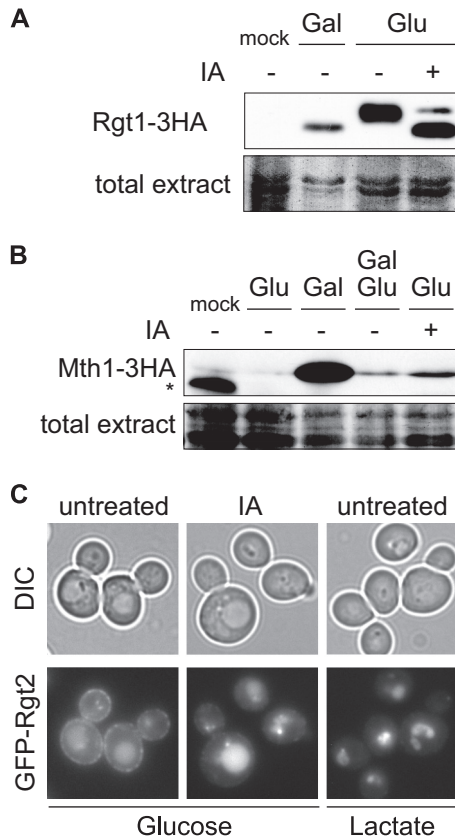
**FIG 7** The *S. cerevisiae* glucose signaling pathway is inhibited in a hexokinase mutant. (A) The glucose-dependent phosphorylation of ScRgt1 is affected in a hexokinase mutant. WT (yAS279) and  $\Delta hxxk1/2$  (yAS280) *S. cerevisiae* strains expressing C-terminally 6HA-tagged ScRgt1 (ScRgt1-6HA) from the genome were grown in synthetic galactose medium to the exponential phase. One half of each culture was incubated with 2% glucose (+) for 30 min and the other was not (-) before cell collection and total protein extraction. Whole-cell lysates were analyzed for ScRgt1-HA (ScRgt1-6HA) and actin (loading control) by Western blotting. (B) Mth1 protein level in a hexokinase mutant. WT (yAS273) and  $\Delta hxxk1/2$  (yAS280) *S. cerevisiae* strains expressing C-terminally 9myc-tagged Mth1 from the genome (Mth1-9myc) were grown and treated as described for panel A. Whole-cell lysates were prepared and analyzed for Mth1-9myc and actin (loading control) by Western blotting. The percentage of Mth1-9myc in each lane (% Mth1) was determined by densitometric analysis of the myc signal normalized to the actin signal (nd, not determined). The values are representative of the tendency observed in replica experiments. (C) The Rgt2 protein level is impaired in a hexokinase mutant in a carbon source-dependent manner. WT (yAS275) and  $\Delta hxxk1/2$  (yAS276) *S. cerevisiae* strains expressing GFP-Rgt2 ( $P_{CUP1}$  promoter) from the genome were grown to the exponential phase in synthetic medium containing 0.02 mM  $\text{CuSO}_4$  (promoter induction) and glucose or galactose as a carbon source. Whole-cell lysates were prepared and analyzed for GFP-Rgt2 and actin (loading control) by Western blotting. A wild-type strain expressing untagged proteins (W303-1A) was used as a negative control (mock). A single asterisk corresponds to an aspecific band. (D) Rgt2 *in vivo* plasma membrane localization is impaired in the hexokinase mutant. *S. cerevisiae* strains and growth conditions were as described for panel C. Living cells were then observed with a fluorescence microscope and imaged using a GFP filter (GFP) or Nomarski optics (DIC). The acquisition times were equal for all GFP images.

sion not only via the direct interaction between the hexokinase Hxk2 and ScRgt1 (32) but also by regulating glucose signaling upstream of ScRgt1. It should be noted that the effects of acute glycolysis inhibition by iodoacetate on glucose signaling in *K. lactis* and *S. cerevisiae* are similar but less pronounced than those observed in the glycolytic mutants, where glycolysis is chronically inhibited. At least two possibilities might explain these differences: either acute glycolysis inhibition and chronic glycolysis inhibition affect glucose signaling through different molecular mechanisms or the chronic inhibition of glycolysis weakens glucose signaling via additional unknown factors that are not influenced upon acute glycolysis inhibition. These two hypotheses are under investigation.

Our data strongly suggest that the glucose sensors are the target of a “glycolytic signal” controlling glucose signaling in both *K. lactis* and *S. cerevisiae*. The glucose-dependent plasma membrane localization of Rag4 in *K. lactis* and Rgt2 in *S. cerevisiae* is altered in the presence of an alternate carbon source or upon (acute or chronic) glycolysis inhibition of glucose-grown cells. However, inhibition of glycolysis did not further affect Rag4 or Rgt2 localization when cells were grown in the presence of glycerol or galactose. Our finding showing that Rag4 and Rgt2 localizations are controlled by the carbon source is in agreement with a recent report showing that the stability of Snf3 and Rgt2 glucose sensors in *S. cerevisiae* is regulated by the concentration of extracellular glucose (33). Hence, Snf3 and Rgt2 are stabilized at the plasma

membrane when the extracellular glucose concentration is in the range of their respective affinities. In the absence of glucose, Rgt2 gets rapidly internalized and degraded in the vacuole. Those authors further conclude that the *S. cerevisiae* glucose sensors are more stable in their glucose-bound conformation, favoring the transmission of the glucose signal into the cell. Our results with respect to Rag4 and Rgt2 stability fit partly with this model. Indeed, our observations that both Rag4 and Rgt2 are not stable in glucose-grown cells when the level of glycolysis is reduced suggest that the intracellular glycolytic signal may overcome the effect of extracellular glucose binding to the glucose sensors. Finally, Roy and Kim further suggest that Rgt2 stability is controlled via Rsp5-dependent ubiquitination of two lysine residues located in the Rgt2 C-terminal cytosolic extension, a region characteristic of the glucose sensors of yeasts (8, 19, 33–35). Sequence alignment and computational analysis of these C-terminal extensions revealed that the two lysines important for Rgt2 stability are not conserved in Rag4 (our unpublished data).

Interestingly, while the regulation of the stability of the glucose sensors by the carbon source in *K. lactis* is similar to that in *S. cerevisiae* (see above), the mechanisms of regulation of the type I casein kinases seem to be different in these two yeast species. Indeed, it was recently reported that the stability of *S. cerevisiae* type I casein kinases Yck1 and Yck2 is tightly regulated by the carbon source (36). These findings differ from our observations showing that Rag8 kinase activity, localization, and stability are affected



**FIG 8** Chemical glycolysis inhibition with iodoacetate affects the *S. cerevisiae* glucose signaling pathway. (A) ScRgt1 is dephosphorylated upon iodoacetate treatment. Cells expressing ScRgt1-3HA (yAS223) were grown to the exponential phase in complete synthetic medium with glucose (Glu) or galactose (Gal) as a carbon source. Galactose-grown cells were harvested, and glucose-grown cells were incubated for 30 min with drug vehicle (–) or 0.25 mM iodoacetate (+). Cells were then collected, and whole-cell lysates were prepared and analyzed for ScRgt1-3HA by Western blotting. TB50a/α was used as a mock treatment control. Total proteins were analyzed by in-gel Coomassie staining for a loading control (total extract). (B) Iodoacetate stabilizes Mth1 in glucose-grown cells. Mth1-3HA-expressing cells (MLY788) were grown to the exponential phase in complete synthetic medium with glucose (Glu) or galactose (Gal) as a carbon source. Glucose-grown cells were then treated with 0.25 mM iodoacetate (+) or drug vehicle (–) for 30 min. Glucose (2% final concentration) was added to galactose-grown cells for 30 min (Gal Glu). After cell collection, total protein extracts were prepared and analyzed for Mth1-3HA by Western blotting. Mock and loading control experiments were done as described for panel B. Asterisks represent nonspecific signal. (C) Iodoacetate treatment affects Rgt2 localization at the plasma membrane. An *S. cerevisiae* strain expressing GFP-Rgt2 (*P<sub>CUP1</sub>* promoter) from the genome (yAS233) was grown to the exponential phase in synthetic medium containing 0.1 mM CuSO<sub>4</sub> (promoter induction) and 2% glucose (Glucose) or 2% lactate plus 0.1% glucose (Lactate). Cells were then treated with 0.25 mM iodoacetate (IA) or with water (untreated) and collected after 60 min. Cells were then observed with a fluorescence microscope and imaged using a GFP filter (GFP-Rgt2) or Nomarski optics (DIC). The acquisition times were equal for all GFP images.

neither by glycolysis nor by the carbon source. Even if the glucose signaling pathway is globally well conserved between *K. lactis* and *S. cerevisiae*, the way casein kinases are regulated might be one point at which it significantly diverges.

The main function of glycolysis is to generate pyruvate from glucose through a multistep pathway that produces part of the energy necessary for cells. However, accumulating evidence demonstrates that glycolysis and, particularly, its intermediate metab-

olites have multiple regulatory functions in cells. For example, the highly cytotoxic side product of glycolysis, methylglyoxal, was previously shown to covalently modify several glycolytic enzymes and to affect glucose transport by enhancing the endocytosis of the Hxt1 glucose permease in *S. cerevisiae* (37, 38). In mammals and yeast, several metabolites such as 1,3-biphosphoglycerate, phosphoenolpyruvate, and fructose-1,6-biphosphate were shown to exert feedback control on glycolysis by affecting the activity of different glycolytic enzymes (39–41). Thus, it is reasonable to hypothesize that one of the intermediate metabolites of glycolysis might serve as a signal controlling the stability of glucose sensors in yeasts. Another possibility is that the intracellular glucose level serves as a signal controlling glucose sensors. A recent finding in *S. cerevisiae* showing that the affinity of the Snf3 glucose sensor for extracellular glucose depends on the intracellular glucose concentration supports this hypothesis (42). The identification of the glycolytic signal will be particularly important to better understand the molecular mechanism underlying the regulatory function exerted by glycolysis on glucose sensors and glucose signaling in yeast.

Our findings point out the crucial role played by glucose catabolism in control of glucose signaling in both *K. lactis* and *S. cerevisiae*. In our model, glycolysis promotes the stabilization of the glucose sensors at the plasma membrane through an intracellular signal whose molecular nature remains to be characterized. Stabilized glucose sensors can then efficiently bind extracellular glucose and propagate the signal into the cell by inducing Sms1/Mth1 degradation and then inactivating phosphorylation of the transcriptional repressor *K/Rgt1/ScRgt1*. Thus, the low-affinity glucose permease genes and other Rgt1-dependent genes get derepressed to promote glucose entry and metabolism in the cell. In the absence of glucose and/or when glycolysis is affected, the glucose sensors get internalized and degraded, which inhibits downstream signaling. This mechanism might then define a virtuous circle in which cells can signal extracellular glucose only when they can efficiently metabolize it via glycolysis.

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