Proteomic and Functional Consequences of Hexokinase Deficiency in Glucose-Repressible *Kluyveromyces lactis*

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Running Title
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Abbreviations
AAR, average abundance ratio; DIGE, difference gel electrophoresis; DML, post-lysis dimethyl labeling; KlHxk1, hexokinase isoenzyme 1 of *Kluyveromyces lactis*; *K. lactis*, *Kluyveromyces lactis*; PMF, peptide mass fingerprint; *S. cerevisiae*, *Saccharomyces cerevisiae*.

Keywords
Difference gel electrophoresis, dimethyl labeling; hexokinase, KlHxk1, *Kluyveromyces lactis* proteome.
Summary

The analysis of glucose signaling in the Crabtree-positive eukaryotic model organism *Saccharomyces cerevisiae* has disclosed a dual role of its hexokinase ScHxk2 which acts as a glycolytic enzyme and key signal transducer adapting central metabolism to glucose availability. In order to identify evolutionarily conserved characteristics of hexokinase structure and function, the cellular response of the Crabtree-negative yeast *Kluyveromyces lactis* to *rag5* null mutation and concomitant deficiency of its unique hexokinase KlHxk1 was analyzed by difference gel electrophoresis (DIGE). In total 2,851 fluorescent spots containing different protein species were detected in the master gel representing the entirety of *K. lactis* proteins that were solubilized from glucose-grown KlHxk1 wild-type and mutant cells. Mass spectrometric peptide analysis identified 45 individual hexokinase-dependent proteins related to carbohydrate, short-chain fatty acid and tricarboxylic acid metabolism, amino acid and protein turnover, but also to general stress response and chromatin remodeling which occurred as a consequence of KlHxk1 deficiency at minimum threefold enhanced or reduced level in the mutant proteome. In addition, three proteins exhibiting homology to 2-methylcitrate cycle enzymes of *S. cerevisiae* were detected at increased concentrations, suggesting a stimulation of pyruvate formation from amino acids and/or fatty acids. Experimental validation of the DIGE approach by post-lysis dimethyl labeling largely confirmed the abundance changes detected in the mutant proteome by the former method. Taking into consideration the high proportion of identified hexokinase-dependent proteins exhibiting increased proteomic levels, KlHxk1 is likely to exert a repressive function in a multitude of metabolic pathways. The entirety of proteomic alterations detected in the mutant classifies KlHxk1 as an enzyme with multiple functions and supports the view of an evolutionary conserva-
tion of dual-role hexokinases even in organisms that in comparison to S. cerevisiae are less specialized on glucose utilization.

**Introduction**

Glucose represents the preferred substrate for ATP regeneration, formation of metabolic precursors and maintenance of a reductive potential in eukaryotes from yeast to man [1;2]. In accordance with its pivotal significance to central metabolism, the utilization of glucose is strictly controlled by the sugar itself [2]. Several lines of experimental evidence classified hexokinases as dual-role enzymes that, in addition to their involvement in the uptake and phosphorylation of glucose, contribute to glucose sensing and signaling in yeasts, plants and men [3-5]. The exploration of glucose-dependent signal transduction in turn is of outstanding importance for the understanding of metabolic disorders such as diabetes [6] and the switch from respiration to aerobic glycolysis occurring in early tumor development [7]. Substantial insights into the molecular mechanisms of glucose sensing and signaling and, in particular, the role of hexokinases were obtained from studies using the traditional eukaryotic model organism S. cerevisiae. The phenomenon of glucose repression in this yeast is mediated through effects on transcription, RNA stability and protein degradation [8]. Most interestingly, glucose abundance is accompanied by translocation of hexokinase isoenzyme ScHxk2 and transcription factor ScMig1 into the nucleus, where both proteins act as constituents of a heterooligomeric repressor complex preventing the expression of glucose repressible genes while glucose exhaustion results in the re-translocation of both proteins into the cytosol [3]. Taking into consideration the existence of genes predicted to encode Mig1 homologs in a
variety of yeasts [8], an evolutionary conservation of transcriptional regulation including
dual-role hexokinases and Mig1-type transcriptional repressors seems conceivable.

The use of *S. cerevisiae* as a model organism for higher eukaryotes is limited by its
Crabtree-positive phenotype which is reflected by a predominance of fermentation over
respiration despite the presence of oxygen [9]. In addition, the genetic redundancy re-
sulting from a whole-genome duplication event in the evolutionary history of the genus
[10] complicates the functional analysis of its genes. With respect to glucose phosphory-
lation and signaling, the genome duplication event is associated with the expression of
two hexokinases (ScHxk1, ScHxk2), one glucokinase (ScGlk1) and one glucokinase pa-
ralog (ScEmi2). In comparison, the genome of the Crabtree-negative model organism
*K. lactis*, in which no genome duplication has occurred [10] encodes a single hexokinase
(KlHxk1) [11] and a single glucokinase (KlGlk1) [12]. Prominent physiological features of
*K. lactis* are its growth on lactose as the sole carbon source [13], the limited exploitation
of its glucose uptake capacity during aerobic growth and the low extent of aerobic etha-
nol accumulation [14]. The reduced sensitivity to glucose repression represents another
functional peculiarity of *K. lactis* which is highly strain-dependent and seems to be medi-
ated by multiple genes [15-17]. Contrary to the situation in *S. cerevisiae* where compo-
nents of the respiratory system are subject to glucose repression [18], respiration in
*K. lactis* remains essentially unaffected when glucose is abundantly available [19]. Re-
pression by glucose is more pronounced in strain JA6 than in other strains of the same
genus and appears to be related to the existence of two tandemly arranged glucose
transporter genes (*KHT1, KHT2*) at the *RAG1* locus [17] whereas the genes encoding
the unique low-affinity glucose permease Rag1 [15-17;20-22] and the high-affinity glu-
cose transporter KlHgt1 present in sequenced reference strain CBS2359/152 are both lacking in the JA6 genome [23]. KHT1 and RAG1 encode proteins that only differ at their C-termini and are similarly regulated at the transcriptional level [21]. Deletion of KHT1 still allows fermentative growth when respiration is blocked by antimycin A (resistance against antimycin A on glucose = Rag⁺ phenotype) since KHT2 expression apparently provides sufficient glucose uptake capacity. The influence of rag5 mutations on high-affinity and low-affinity glucose transport [11;20;24] and their coincidence with a relief from glucose repression [16;25] identified RAG5 (KLLA0D11352g) and its gene product KlHxk1 as further components of the glucose signaling cascade in K. lactis.

The present paper is focused on the functional significance of the unique hexokinase KlHxk1 expressed in glucose repressible strain JA6 of K. lactis [25]. Structural investigations revealed prominent molecular similarities of KlHxk1 and hexokinase isoenzyme ScHxk2 of S. cerevisiae [26] suggesting similar functions. The two kinases exhibit 73% identity on the protein level and carry similar amino acids at additional 61 positions. KlHxk1 and ScHxk2 establish dynamic monomer-homodimer equilibria [25;27] which, according to the Ostwald dilution law, depend on the enzyme concentration in a range that is likely to meet physiological conditions (K. Kettner, unpublished data). Homodimer stability is drastically reduced as a consequence of phosphorylation of conserved amino acid serine-15 present in the highly similar N-termini of both enzymes [28;29]. In case of KlHxk1, the crystal structure of a symmetrical ring-shaped homodimer of the phosphorylated enzyme displays two structurally equivalent phosphoserine-15 residues in the intersubunit interface at positions that are critical for dimer stability [29]. The regulatory significance of serine-15 phosphorylation became apparent by the finding that in
S. cerevisiae this modification promotes the association of nuclear ScHxk2 with the ScXpo1 exportin and, thereby, facilitates translocation of the enzyme into the cytosol when glucose availability is low [30;31]. Phosphoserine-15 ScHxk2 is dephosphorylated by the ScGlc7/ScReg1 phosphoprotein phosphatase complex [3;32] to allow its nuclear import and contribution to glucose repression when the sugar is abundantly available again. In contrast, the subcellular distribution of KlHxk1 and its dependence on glucose availability are still unknown. Serine-15, however, is part of a conserved N-terminal sequence motif (K7-M16), which in S. cerevisiae is essentially involved in the shuttle of ScHxk2 between cytosol and nucleus and, in particular, in glucose signaling at the level of the ScMig1 repressor complex [3;32]. Recently, protein kinases ScTda1/ScYmr291w [33] and ScSnf1 [3] were reported to be implicated in ScHxk2 phosphorylation at serine-15 in response to glucose limitation [30]. In comparison, neither the condition triggering KlHxk1 phosphorylation at the equivalent site nor the corresponding protein kinase of K. lactis is known. The identification of a K. lactis gene encoding a hypothetical protein (KLLA0A09713p) of still unknown function which is homologous to ScTda1/ScYmr291w, however, provokes the hypothesis that KlHxk1 and the upstream KlHxk1-S15 kinase might fulfill key roles in glucose-dependent signal transduction in K. lactis.

In order to verify the expectation of KlHxk1 to represent a dual-function enzyme which in addition to its catalytic function in glycolysis contributes to the transcriptional control of central metabolic pathways in K. lactis, the proteomic consequences of RAG5 disruption and concomitant KlHxk1 deficiency were studied by difference gel electrophoresis [34]. The DIGE approach compared RAG5 wild-type strain JA6 (subsequently referred to as “wild-type”) serving as reference and the congenic rag5 null mutant strain JA6Δrag5R
(subsequently referred to as “mutant”) lacking both KlHxk1 protein and hexokinase catalytic activity. Keeping in mind the involvement of KlHxk1 in glucose repression [16], the proteomic response of K. lactis to the rag5 null mutation was analyzed in detail upon growth in high-glucose medium and was compared to the proteomic changes taking place in media containing galactose or glycerol as the sole carbon source. The overall experimental outcome not only confirms and extends current knowledge on KlHxk1 involvement in glucose sensing and signaling [16;20;24], but also indicates novel functions of the RAG5 gene and its gene product KlHxk1 in chromatin remodeling and general stress response in addition to a role in tricarboxylic acid, short-chain fatty acid and amino acid metabolism and in protein turnover.

Experimental procedures

Strains

The strains used in the present work are JA6 (MATα, ade1-600 adeT-600 trp1-11 ura3-12; [35]) and its derivatives JA6△rag5 (rag5::ScURA3; Wésolowski-Louvel laboratory strain collection, University of Lyon, France), JA6△rag5R (rag5::Scura3; Breunig laboratory strain collection, University of Halle-Wittenberg, Germany) and JA6△rag5R/pTSRAG5. JA6△rag5R was obtained from JA6△rag5 by 5-fluoroorotic acid selection. The hypothetical existence of an N-terminal KlHxk1 fragment consisting of 316 amino acids encoded by the RAG5 gene and 12 amino acids encoded by the URA3 disruption cassette (DNA sequencing data not shown) in mutant strain JA6△rag5R was excluded by multiple experiments (see “Results”).

Cell Culture for DIGE analysis
Strains were grown in YNB medium pH 5.5 consisting of 0.69% (w/v) yeast nitrogen base w/o amino acids supplemented with adenine, uracil and amino acids according to [36] at 30°C and 200 rpm. Uracil was omitted when plasmid selection was required. Glucose, galactose or glycerol was added at an initial concentration of 2% (w/v). Yeast cells were grown stepwise in YNB medium in pre-cultures of 5 ml for 16 hours (1), 25 ml for 8 hours (2) and 100 ml for 16 hours (3) in the presence of the indicated carbon source. The initial OD (600 nm/1 cm) of pre-culture 3 was 0.02, while cultivation of the main cell culture (500 ml) used for protein extraction started at an OD (600 nm/1 cm) of 0.3. For preparing the latter culture, cells from pre-culture 3 were washed twice using YNB medium w/o carbon source and re-suspended in YNB medium supplemented with the indicated carbon source. The cells were finally incubated until an OD (600 nm/1 cm) of 1 was reached, collected by centrifugation (3,500 x g, 8 min, 4°C), washed twice with ice-cold double-distilled water, frozen and stored at -80°C until further use. Due to an impaired growth of strain JA6△rag5R on glucose, pre-cultures 1-3 of the mutant strain were supplemented with 2% (w/v) galactose as the sole carbon source and pre-cultures 2 and 3 scaled up to give 50 ml and 200 ml, respectively, to allow sufficient biomass accumulation. Cells from latter pre-culture 3 were washed with and re-suspended in YNB medium containing 2% (w/v) glucose to give another pre-culture (4) (500 ml) with a starting OD (600 nm/1 cm) of 1. Pre-culture 4 was cultivated for 16 hours under the above conditions. Glucose-grown mutant strain main cell cultures were finally prepared from pre-culture 4 using YNB medium supplemented with 2% (w/v) glucose and propagated from a starting OD (600 nm/1 cm) of 1 for 6 hours. Cells were harvested, washed and frozen as specified.
Protein Extraction

The frozen yeast cells were thawed and re-suspended on ice in 3 ml DIGE lysis buffer (30 mM Tris/HCl, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 10 mM dithiothreitol (DTT), 1 x protease inhibitor mix, pH 9.1) per gram wet cell mass. Cells were disrupted by repeated use of a French® Pressure Cell Press (SLM Aminco, Rochester, NY, USA) equipped with a mini cell at 20,000 psi (138 MPa) followed by nine cycles of 10 s sonication and 10 s cooling on ice, respectively, using a UP100H-type ultrasonic processor (Dr. Hielscher, Teltow, Germany) at 20% output corresponding to 28 µm amplitude. Samples were re-collected by centrifugation (5,000 x g, 30 s, 4°C) after the 3rd and 6th cycle. Insoluble material was removed after the last cycle of sonication and cooling by centrifugation at 16,000 x g, 15 min and 4°C. The resulting supernatants containing the solubilized proteins (subsequently referred to as “proteomes”) were subjected to protein determination employing the RC DC™ Protein Assay (Bio-Rad, Hercules, CA, USA), frozen and stored at -80°C until further use.

Difference Gel Electrophoresis

Equal amounts of protein extracted from 10 cultures of wild-type and mutant cells grown in media containing either 2% (w/v) glucose, 2% (w/v) galactose or 2% (w/v) glycerol (in total 60 samples) were mixed to set up an internal standard for multiplex matching of DIGE images, spot normalization and calculation of abundance changes. Internal standard, wild-type and mutant proteomes were differentially labeled with N-hydroxysuccinimidyl ester derivatives of the cyanine dyes Cy2, Cy3 and Cy5 following the manufacturer’s instructions for minimal labeling (GE Healthcare, Munich, Germany). For analytical gels, 50 µg of protein were labeled with 200 pmol of fluorescent dye deriv-
ative in a total volume of 25 µl using DIGE lysis buffer as the solvent. Cy2 was exclusively used for labeling the internal standard that was run on each gel in parallel with two wild-type and/or mutant proteomes. To avoid any labeling bias, Cy3 and Cy5 were randomly utilized to label the wild-type and mutant proteomes. The labeling reaction was stopped by the addition of 1 µl 10 mM L-lysine. Three differentially labeled proteomes were mixed, reduced by the addition of 3.75 µl 2 M DTT corresponding to a final concentration of 50 mM of the reductant, supplemented with 0.75 µl immobilized pH gradient (IPG) buffer pH 3-10 (GE Healthcare, Munich, Germany), and completed using sample rehydration solution (7 M urea, 2 M thiourea, 4% (w/v) CHAPS) to obtain a final volume of 150 µl. Isoelectric focusing (IEF) was performed using an Ettan IPGphor 3 unit (GE Healthcare, Munich, Germany). The samples were cup-loaded at the anodic side of IPG strips (24 cm, pH 3-10, linear) previously treated with 450 µl strip rehydration buffer (0.5% (v/v) IPG buffer, pH 3-10, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1.2% (v/v) DeStreak reagent) and separated at 20°C. The following conditions were applied which correspond to a total of 114,575 Vh: 150 V/3 h, linear gradient; 300 V/3 h, linear gradient; 1,000 V/3 h, linear gradient; 10,000 V/3 h, linear gradient; 10,000 V/95,000 Vh, step. The strips were treated with equilibration buffer (50 mM Tris/HCl, 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 130 mM DTT, pH 8.8) for 20 min, followed by equilibration for another 20 min with a modified buffer lacking DTT but containing 135 mM iodoacetamide for sulfhydryl group alkylation. Subsequent SDS polyacrylamide gel electrophoresis using 12.5% (w/v) gels (20.5 x 25.5 cm) was carried out at 25°C and 5 mA per gel for the first hour and 20 W per gel until completion in an Ettan Daltsix system (GE Healthcare, Munich, Germany).
Image Acquisition and Data Analysis

Digital DIGE images of wild-type, mutant and internal standard proteomes were generated by scanning the 2-D gels (in total 30) with a Typhoon 9410 Variable Mode Imager (GE Healthcare, Munich, Germany) using the excitation/emission wavelengths of 488 nm/520 nm for Cy2, 532 nm/580 nm for Cy3 and 633 nm/670 nm for Cy5. Images (in total 90) were matched and normalized and equivalent protein spots in different gels identified by employing the fully automated computer-assisted alignment module (batch processor) of the DeCyder 2-D Differential Analysis Software Version 7.0 (GE Healthcare, Munich, Germany). Matches were manually revised using the biological variation analysis module. Statistical data treatment employed the extended data analysis (EDA) component of DeCyder 2-D Differential Analysis Software Version 7.0 (GE Healthcare, Munich, Germany). The significance of proteomic differences between wild-type and mutant strain grown on the same carbon source, respectively, was analyzed by using a t-test for independent samples. For type-I error adjustment, the false discovery rate method of Benjamini and Hochberg [37] integrated in the DeCyder software was applied using an FDR of 5% which corresponds to a critical p-value of $1.7 \times 10^{-3}$ that indicates rejection of the null-hypotheses for spots with lower p-values. In order to limit the extent of false negative decisions, normalized spots exhibiting at least threefold abundance changes (AAR ≥ 3 / ≤ -3) were considered in the analysis of the proteomic consequences of KlHxk1 deficiency. In the context of the present paper, the average abundance ratio (AAR) indicates the ratio of the average amount of a protein in the mutant proteome and in the reference proteome for proteins occurring at increased level or the negative inverse ratio for proteins occurring at decreased level. Proteins fulfilling the
above criteria are subsequently referred to as “hexokinase-dependent proteins” (for statistical details see Discussion section).

Protein Identification

Gel discs containing hexokinase-dependent proteins were excised from preparative 2-D gels loaded with 150-1,000 µg protein and stained using colloidal Coomassie Brilliant Blue G-250 [38]. Prior to excision, spot patterns were manually aligned with the fluorescent image of the master gel to allow for correct spot localization. In case of low-abundance proteins, the alignment was facilitated by the addition of Cy2-labeled internal standard (50 µg per gel) to the unlabeled proteome. The excised gel discs were washed twice with deionized water followed by one wash using 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate solution, shrunk by dehydration in 100% (v/v) acetonitrile and dried in a vacuum centrifuge (240 x g, 30 min, 25°C). The dried material was incubated overnight with 20 µl of mass spectrometry-grade trypsin (Promega, Mannheim, Germany) solution (2.5 µg/ml, 50 mM ammonium bicarbonate used as the solvent) at pH 7 and 37°C. For peptide extraction, the proteolyzed samples were mixed with 20 µl of 0.5% (v/v) trifluoroacetic acid (TFA) in acetonitrile, sonicated for 5 min in an ultrasonic bath sonicator and finally incubated for another 5 min at 20°C in a shaker at 800 rpm. After centrifugation, supernatant removal and repetition of the extraction procedure using 20 µl 100% (v/v) acetonitrile per sample, the pooled supernatants were vacuum-dried. The extracted peptides were dissolved in 5 µl 0.1% (v/v) TFA in water, sonicated for 5 min as above and incubated at 20°C for another 5 min. Aliquots (0.5-1.0 µl) of each sample were pipetted on an AnchorChip™ target plate (Bruker Daltonics, Leipzig, Germany), allowed to sit for 3 min at 20-25°C and subsequently mixed on the plate with the same
volume of α-cyano-4-hydroxycinnamic acid. Peptide analysis was performed at an Ultraflex™ Automated High-performance MALDI-TOF/TOF Mass Spectrometry System (Bruker Daltonics, Bremen, Germany) in reflectron mode. Peak lists were generated using the Bruker Daltonics flexAnalysis Software Version 2.2. Peptide mass fingerprint (PMF) analysis employed the MASCOT Application Software Version 2.2 (Matrix Science, London, UK) using the following criteria for searching against the NCBInr 20121118 (21,581,546 sequences; 7,400,919,093 residues) database: taxonomy - fungi; mass accuracy - 50-90 ppm; fixed modifications - carbamidomethylation of cysteine; variable modifications - methionine oxidation and deamidation of asparagine and/or glutamine; mass values - monoisotopic; maximum of missed cleavage sites - one. Known peptide mass values of contaminating tryptic keratin fragments and autodigestion products of trypsin were excluded from database search. PMF scores higher than 74 (p < 0.05) were considered to indicate significant protein identification. In case of insignificant PMF scores, MALDI-TOF/TOF sequencing of selected tryptic peptides was performed applying a mass tolerance setting for fragment ions of ± 0.8 Da. Extended data base search included the UniProtKB/Swiss-Prot 2012_10 (538,259 sequences; 191,113,170 residues) and UniProtKB/TrEMBL 2012_10 (27,122,814 sequences; 8,765,290,755 residues) databases.

In order to reliably identify low-abundance and verify low-score hexokinase-dependent proteins that were not adequately characterized by the MALDI-TOF/TOF approach, the extracted tryptic peptides were purified on C18 StageTips [39] and separated on a Proxeon easy nLC nano HPLC system (Proxeon Biosystems, Odense, Denmark) fitted with an in-house packed 15 cm analytical reverse phase column of 75 µm inner diame-
ter containing Reprosil-AQ Pur 3 µm C18 reverse phase beads (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Peptide separation was accomplished by applying a 65 min gradient from 5-60% (v/v) acetonitrile in 0.1% formic acid. The effluent was directly electrosprayed into a Velos-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ion source (Proxeon Biosystems, Odense, Denmark). The recorded spectra were analyzed using the MaxQuant Software Package Version 1.2.2.5 [40] by matching the data to the UniProt K. lactis database version 2012 with a false discovery rate of 1% (peptides and proteins). The fixed and variable modifications were set to carbamidomethylation of cysteines and methionine oxidation, respectively. For further data analysis, the R statistical software package was used (www.r-project.org).

Experimental Determination of pI and M_r Values

Prior to DIGE separation, marker proteins for 2-D electrophoresis (Sigma-Aldrich, Taufkirchen, Germany) were labeled with Cy3 and added to the Cy2-labeled internal standard. The DIGE coordinates of these marker proteins were used to determine experimental pI values (pI(exp)) by employing an algorithm integrated in the DeCyder Software Version 7.0 (GE Healthcare, Munich, Germany). Determination of experimental M_r values (M_r(exp)) is based on manual determination of migration distances of the above marker proteins complemented by pre-stained marker proteins for 1-D electrophoresis (PageRuler™ Prestained Protein Ladder, Fermentas, St. Leon-Rot, Germany) and application of GraphPad Prism Software Version 4.03 (GraphPad Software, La Jolla, CA, USA). M_r(exp) values of individual K. lactis proteins were calculated from manu-
ally determined migration distances using a best-fit equation provided by the GraphPad Prism software.

DIGE Analysis of KlHxk1 Phosphorylation

Spike-ins of purified serine-15-phosphorylated and unphosphorylated KlHxk1 [29] were prepared by differentially labeling 5 μg of each of the two proteins with Cy3 (green fluorescence) and Cy5 (red fluorescence), respectively, in a final volume of 25 μl. DIGE analysis performed to identify the two KlHxk1 species in the 2-D images and 2-D gels employed samples consisting of 0.2 μg of each of the two fluorescently labeled enzymes, 50 μg Cy2-labeled and 99.6 μg unlabeled internal standard to reach a final loading amount of 150 μg protein per gel. In order to improve spot separation and detection, twice the amount of each of the two labeled KlHxk1 forms, 50 μg Cy2-labeled and 99.2 μg unlabeled internal standard was analyzed on pH 4-7 IPG strips. Preparative 2-D gels covering the same pH range were loaded with 1 mg unlabeled internal standard and subjected to “Blue Silver” staining [38]. The proteins present in the discs cut out of these gels at the DIGE coordinates of the fluorescently labeled unphosphorylated and serine-15-phosphorylated KlHxk1 were subjected to phosphopeptide analysis as described below.

Phosphopeptide Analysis

Purified unphosphorylated and serine-15 phosphorylated KlHxk1 [29] was subjected to SDS–polyacrylamide gel electrophoresis in 10% (w/v) acrylamide gels followed by in-gel proteolysis according to a two-step protocol using endopeptidases LysC (Wako, Osaka, Japan) and Trypsin (Promega, Mannheim, Germany) [41]. The recovered peptides were
desalted on C18 Empore Solid Phase Extraction Disks (3M, Neuss, Germany) [39], separated on a 15 cm analytical reverse phase column of 75 µm inner diameter containing Reprosil-AQ Pur 3 µm C18 reverse phase beads (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) by using a gradient of 5-60% acetonitrile in 0.1% formic acid and ionized on a Proxeon ion source (Proxeon Biosystems, Odense, Denmark). Fragment spectra of the phosphopeptide were collected using a Velos-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in multistage activation mode. The spectra were analyzed employing the MaxQuant Software Package Version 1.2.2.5 [40] and used to develop a method for single reaction monitoring (SRM). In order to verify the SRM method and to determine the retention time during reverse phase chromatography, phosphopeptide KGS(p)MADVPNIMEQIHMVTIFSSEK corresponding to KLHxk1 amino acids 13-40 was synthesized by Fmoc solid phase synthesis on a ResPep SL Microscale Automated Synthesizer (Intavis, Cologne, Germany). The proteins present in the discs cut out of the preparative 2-D gels at the DIGE coordinates of the fluorescently labeled unphosphorylated and serine-15-phosphorylated KLHxk1 were converted to peptides according to an in-gel digestion protocol using sequencing grade trypsin (Promega, Mannheim, Germany) [42]. Recovered peptides were analyzed by applying the chromatographic setup described above, but recorded on a Q-TRAP 5500 mass spectrometer (AB Sciex, Toronto, Canada) in MRM mode. The data were quantified using the MultiQuant Software Package Version 1.2.0.6 (AB Sciex, Toronto, Canada) and further analyzed by use of the R statistical analysis language (www.R-project.org).

Homology Analysis and Assignment of Protein Functions
In spite of substantial genomic and physiological differences between *S. cerevisiae* and *K. lactis*, about 82% of the *K. lactis* metabolic genes are annotated as *S. cerevisiae* homologs [43]. Therefore, *S. cerevisiae* was taken as a primary reference for the annotation of hexokinase-dependent proteins of *K. lactis* by using the NCBI HomoloGene database system (http://www.ncbi.nlm.nih.gov/homologene) when evidence for protein existence on protein or transcript level according UniProtKB (http://www.uniprot.org/) was missing.

The annotation of *K. lactis* proteins exhibiting homology to two or more *S. cerevisiae* proteins always refers to the *S. cerevisiae* protein with the higher score as calculated with BLASTp. In three cases, homology analysis identified genes existing only in yeast species other than *S. cerevisiae*. The assignment of molecular functions and biological processes to *K. lactis* proteins or their yeast homologs employed the GO (http://www.ebi.ac.uk/QuickGO/) and KEGG (http://www.genome.jp/kegg/) databases, respectively. In two indicated cases, functional assignment was complemented by literature information.

**Immunodetection of KlHxk1**

The anti-KlHxk1 polyclonal antibody used in the present study was generated by immunizing rabbits with purified non-phosphorylated KlHxk1 [29] followed by immunoaffinity chromatography of the antiserum on a HiTrap NHS-activated HP column (GE Healthcare, Munich, Germany) reacted with purified KlGlk1 to eliminate cross-reacting immunoglobulin. Strains JA6, JA6△rag5R and JA6△rag5R/pTSRAG5 were pre-grown in YNB medium supplemented with 2% (w/v) galactose for 15 hrs. The cells were washed twice using YNB medium w/o carbon source, re-suspended in 25 ml of the
above YNB-galactose medium to give an OD (600 nm/1 cm) of 0.3, propagated until an OD (600 nm/1 cm) of 1.0 was reached and washed with ice-cold lysis buffer consisting of 50 mM imidazole/HCl pH 7.0, 50 mM NaCl, 5 mM ε-aminocaproic acid, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1x proteinase inhibitor cocktail (EDTA-free; Roche, Mannheim, Germany) and 1x phosphatase inhibitor cocktails I + II (Sigma-Aldrich, Steinheim, Germany). Protein extracts were prepared by vigorously shaking the cells suspended in 0.1 ml of lysis buffer with 0.1 g glass beads of 0.4 mm diameter (Carl Roth, Karlsruhe, Germany) in 1.5 ml plastic tubes 5 x 1 min at 25 Hz in a Mixer Mill MM 200 (Retsch, Haan, Germany) with 1-min intervals for sample cooling on ice. The supernatants obtained by centrifugation (12,000 x g, 10 min, 4°C) were subjected to SDS–polyacrylamide gel electrophoresis in 10% (w/v) acrylamide gels and the separated proteins transferred onto a PVDF membrane (Millipore, Schwalbach, Germany). The membrane was probed with the affinity-purified anti-KlHxk1 polyclonal antibody and bound IgG detected with HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Danvers, USA) using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Schwalbach, Germany).

Repression Assay

Strains JA6, JA6Δrag5R and JA6Δrag5R/pTSRAG5 were pre-grown in YNB medium supplemented with 2% (w/v) galactose, shifted into YNB medium containing 2% (w/v) glucose and cultivated for ~15 h. The cells were washed twice using YNB medium w/o carbon source, re-suspended in YNB medium containing 2% (w/v) of the indicated carbon source(s) and propagated from a starting OD (600 nm/1 cm) of 0.3 until an OD (600 nm/1 cm) of 1.0 was reached. Beta-galactosidase activity of protein extracts was
determined according to [22] using o-nitrophenyl-β-D-galactopyranoside as a substrate. One milliunit (mU) of β-galactosidase activity catalyzes the formation of 1 nanomole o-nitrophenolate per min at 30°C.

Nephelometric Growth Analysis

The utilization of different carbon sources by strains JA6, JA6Δrag5R and JA6Δrag5R/pTSRAG5 was monitored in liquid media at 30°C in a NEPHELOstar Galaxy laser-based microplate nephelometer (BMG LABTECH, Offenburg, Germany) equipped with 96-well Cellstar® suspension culture plates (Greiner BioOne, Solingen, Germany) by the detection of particulate matter via forward light scattering. Cells were grown to stationary phase in 5 ml YNB medium containing 2% (w/v) galactose at 200 rpm and 30°C and were subsequently washed twice with and re-suspended in YNB medium w/o carbon source to adjust an OD (600 nm/1 cm) of 2. This suspension was used to inoculate YNB medium containing 2% (w/v) glucose, 2% (w/v) fructose, 2% (w/v) galactose or 2% glycerol in the wells of the Cellstar® plates. Typically, 5 µl cell suspension and 200 µl medium were applied per well. Medium lacking any carbon source was used as a control. Evaporation and condensation was avoided by sealing the plates with gas-permeable plastic film (Breathe-Easy, Carl Roth, Karlsruhe, Germany). Growth data are based on three independent experiments each of which consisting of assays performed in triplicate.

Biochemicals and Materials

The following biochemicals and materials were used for DIGE: CyDyes (Cy2, Cy3, Cy5), IPG strips (pH 4-7 and 3-10, linear), IPG buffers (pH 4-7 and pH 3-10), DeStreak rea-
gent, cover fluid and protease inhibitor mix from GE Healthcare (Munich, Germany), glycine, ammonium persulfate, glycerol and Tris from Carl Roth (Karlsruhe, Germany), acrylamide/bisacrylamide solution from AppliChem (Darmstadt, Germany), DTT from Fermentas (St. Leon-Rot, Germany), urea and N,N,N′,N′-tetramethylethylenediamine (TEMED) from Bio-Rad (Munich, Germany). Coomassie Brilliant Blue G-250 used for “Blue Silver” staining was from Serva (Heidelberg, Germany), ammonium sulfate and phosphoric acid from Carl Roth (Karlsruhe, Germany) and methanol from Merck (Darmstadt, Germany). The substances used for mass spectrometry were: Trifluoroacetic acid for spectroscopy from Merck (Darmstadt, Germany), deionized water (LC-MS grade) from Fisher Scientific (Schwerte, Germany), acetonitrile from Riedel-de Haën (Seelze, Germany), ammonium bicarbonate from Sigma-Aldrich (Taufkirchen, Germany), α-cyano-4-hydroxycinnamic acid from Bruker Daltonics (Bremen, Germany). Agar, yeast nitrogen base, glucose and galactose were from ForMedium (Hunstanton, UK). HEPES was obtained from Serva (Heidelberg, Germany). Unless stated otherwise, all other reagents used were from Sigma-Aldrich (Taufkirchen, Germany).

**Results**

**KIHxk1 Deficiency of Mutant Strain JA6Δrag5R of *K. lactis***

The absence of KIHxk1 protein and catalytic activity in strain JA6Δrag5R was examined by different experimental approaches all verifying the null mutation. Firstly, severely impaired growth of the mutant on glucose (Figure 1A) and lacking growth on fructose (Figure 1B) together with normal growth on galactose (Figure 1C) and glycerol (Figure 1D) indicated the absence of a functional hexokinase. The slow growth on glucose (Figure 1A) is consistent with the expression of a recently identified glucokinase (KlGlk1) of un-
known physiological function in strain JA6Δrag5 [12]. Secondly, the rate of phosphorylation of glucose and fructose determined according to [12] with extracts of glucose- and galactose-grown strain JA6Δrag5R was less than 3% compared to JA6 control extracts (data not shown). Thirdly, Western Blot analysis using polyclonal anti-KIHxk1 primary antibodies (Figure 2A) gave the expected signal with cell extracts of strains JA6 (lane 1) and JA6Δrag5R/pTSRAG5 (lane 3), but no signal for strain JA6Δrag5R (lane 2). The additional bands in lane 3 are likely to represent degradation products of the overexpressed KIHxk1 protein. Fourthly, glucose repression of β-galactosidase as observed in strain JA6 (Figure 2B, data set 1) was essentially relieved in strain JA6Δrag5R during growth on glucose and even abolished during growth on glucose + galactose (Figure 2B, data set 2) and was fully re-established upon transformation of the mutant with multicopy plasmid pTSRAG5 (Figure 2B, data set 3). Finally, the absence of KIHxk1 in the proteome of strain JA6Δrag5R was verified by determining the AAR of the KIHxk1 protein (Table 1). The latter analysis had to take into account the existence in wild-type cells of two molecular species of KIHxk1 differing in the state of phosphorylation at serine-15 [29] and consequently exhibiting different isoelectric points and positions in the 2-D gel (Figures 3 and 4). DIGE experiments employing spike-ins of purified differentially labeled unphosphorylated and serine-15-phosphorylated KIHxk1 (Figure 3) identified spot 968 as the phosphoserine-15 enzyme. Mass spectrometric quantification applying an SRM method specific for the \textsuperscript{13}KGS(p)MADVPANIMEQIHGIETIFTVSSEK\textsuperscript{40} tryptic phosphopeptide verified the latter result by indicating a 20-fold higher signal intensity for spot protein 968 compared to spot protein 976 (Supplemental Figure S1 and Supplemental Table S1). Keeping in mind these findings, the AAR values in Table 1 essentially confirm the anticipated absence of KIHxk1 in the mutant with respect to spot protein 968.
while the data calculated for spot protein 976 require special consideration (see “Dis-
cussion”). In accordance with the entirety of above data, strain JA6△rag5R was consid-
ered appropriate to analyze the proteomic consequences of KlHxk1 deficiency in glu-
cose repressible *K. lactis*.

**Comparative DIGE Analysis of JA6 and JA6△rag5R Proteomes**

The DIGE method allowed for the resolution of 2,851 fluorescent spots in the digital im-
age of the master gel containing the entirety of *K. lactis* proteins present in the proteo-
mes of wild-type and mutant during growth in high-glucose medium (Figure 4). In com-
parison, the total number of predicted protein-coding genes in the *K. lactis* genome is
5,108 [44]. Due to differences in relative protein abundance and variation of brightness
and brilliance for image generation, the master gel image (Figure 4) which was selected
from 30 DIGE images scanned at the Cy2-specific wavelength displays a number of flu-
orescent spots that apparently deviates from the former spot number. Spot analysis and
mass spectrometric peptide determination identified 59 fluorescent spots containing 45
individual proteins which occurred in 60 different molecular forms in the mutant proteo-
me at minimum threefold enhanced or reduced level (FDR 5%) as a consequence of
KlHxk1 deficiency. The identity and function(s) of these hexokinase-dependent proteins
determined by in-gel tryptic digestion, mass spectrometric peptide analysis and data-
base search are summarized in Table S2, where proteins are ordered according to their
AAR value, while Tables 2-4 contain protein groups constituted according to assigned
functions.

**Functional Groups of Hexokinase-Dependent Proteins of *K. lactis***
Hexokinase-dependent proteins that are involved in or related to glycolysis and/or gluconeogenesis, hexose metabolism and glucosaccharide turnover are listed in Table 2 while Figure 5 gives an overview of reactions of central metabolic pathways which are catalyzed by them. Except for inositol 3-phosphate synthase (spot 927) and two molecular species of pyruvate decarboxylase (spots 828 and 858), these proteins were detected at significantly increased levels in the mutant proteome suggesting their RAG5/KIHxk1-dependent repression in wild-type cells during growth on glucose. The detection of three molecular species of glyceraldehyde-3-phosphate dehydrogenase (spots 1717, 1733, 1736) exhibiting experimentally determined Mr and pI values that only slightly deviate from those of the authentic enzyme and of two additional molecular species exhibiting clearly deviating DIGE coordinates (spots 2502 and 2533) indicates covalent modifications of still unknown nature and significance. Spot protein 470 identified as β-glucosidase precursor which in its active state is involved in glucosaccharide hydrolysis represents another high-abundance hexokinase-dependent protein that in wild-type cells apparently is subject to RAG5/KIHxk1-dependent glucose repression. Interestingly, three enzymes of the lactose-galactose regulon [45] were identified (β-galactosidase, galactokinase, galactose-1-phosphate uridylyltransferase) at throughout increased concentrations with β-galactosidase (spots 226-228) and galactokinase (spots 871 and 875) occurring in different molecular forms, respectively. In case of β-galactosidase (spot 228), the extraordinarily high AAR value reflects drastic changes at the level of transcription, translation and/or proteolysis without excluding other types of covalent modification. The detection of increased levels of the latter three enzymes is likely to reflect the adaptation of K. lactis metabolism to the utilization of alternative car-
bon sources when a drastically reduced glucose phosphorylation capacity is limiting the utilization of this sugar.

Table 3 summarizes the proteomic response of strain JA6 to KLHxk1 deficiency with regard to ethanol, acetate, propionate and tricarboxylic acid metabolism while Figure 5 gives an overview of affected reactions of central metabolic pathways. Again, the majority of listed proteins was detected at significantly increased levels in the mutant proteome with acetyl-CoA synthetase 1 displaying the most pronounced abundance change (spot 589). Particularly striking was the assignment of three hexokinase-dependent proteins to the mitochondrial 2-methylcitrate cycle which allows the conversion of propionate originating in the course of degradation of odd-chain fatty acids and certain amino acids into pyruvate, a common precursor of biosynthetic and catabolic pathways [46]. These proteins are homologs of S. cerevisiae mitochondrial citrate synthase Cit3 (spot 1213) with dual-substrate specificity for citrate and 2-methylcitrate [47], mitochondrial 2-methylisocitrate lyase (spot 837) and 2-methylcitrate dehydratase (spot 965). Their detection at increased proteomic levels not only verifies the existence of the 2-methylcitrate cycle in K. lactis but also suggests its repression by glucose in a RAG5/KLHxk1-dependent way. The listing of mitochondrial aldehyde dehydrogenase and cytosolic alcohol dehydrogenases 1 and 2 (spots 992, 1411 and 1414) in Table 3 takes into account their potential role in acetate and ethanol formation, respectively, without excluding contributions to gluconeogenesis (cf. Table 2) via the glyoxylate cycle and/or to redox state maintenance and stress response (cf. Table 4). In case of alcohol dehydrogenase isoenzyme 2, the inverse abundance changes determined for spot proteins 1411 and 1414 might reflect a post-translational regulation of this enzyme.
Hexokinase-dependent proteins exhibiting actual or predicted functional relations to chromatin remodeling, amino acid and protein metabolism, redox state maintenance and stress response are listed in Table 4. The only enzyme in Table 4 with assigned functions in central metabolic pathways (spot 1828) is also included in Figure 5. Remarkably, the highest AAR determined in the present study (AAR 37.5) corresponds to the KLLA0C16225p protein (spot 2392) which is weakly similar to the S. cerevisiae Gre1 hydrophilin of unknown function [48]. The findings that the ScGRE1 gene is induced under hypoxic conditions [49] and that the response to hypoxia in K. lactis is linked to glucose metabolism [50] might reflect a functional relation between the KLLA0C16225p protein and KIHxk1. In case of protein KLLA0D07414p (spot 615), the assignment of a molecular function is similarly unsatisfactory. This protein exhibits weak similarity to the Fmo1 thiol-specific monooxygenase of S. cerevisiae which probably is required for correct folding of disulfide-bonded proteins, however, homologs of higher similarity are found in other yeasts. The identification of hexokinase-dependent proteins involved according to functions of their yeast homologs in translation, proteolysis and amino acid metabolism (e.g. spot proteins 933, 1905, 1100) suggests stimulation of protein turnover presumably as a result of enhanced oxidative protein damage and limited glycolytic energy supply in the mutant. The latter observation is complemented by the abundance change of the K. lactis KLLA0F24838p homolog of the ISWI chromatin-remodeling complex ATPase Isw2 of S. cerevisiae (spots 471 and 568), possibly indicating the association of nuclear events taking place in the mutant at the transcriptional level with the detected proteomic alterations. In comparison, the relative abundance of two K. lactis proteins exhibiting homology to the S. cerevisiae phenylpyruvate decarboxylase Aro10
(spots 743 and 749) was found decreased in the mutant despite a role of this enzyme in amino acid degradation.

The actual or predicted molecular functions and biological processes assigned to the entirety of 45 identified different hexokinase-dependent proteins of *K. lactis* glucose repressible strain JA6 are classified in Figure 6. In line with the metabolic limitations resulting from the KlHxk1 deficiency, carbohydrate metabolism represents the most frequently met functional category which is followed by amino acid metabolism. According to expectations, genetic information processing represents another large functional category. By contrast, the impact of KlHxk1 deficiency on energy metabolism appears significantly less pronounced, suggesting the existence of compensatory metabolic pathways in the mutant.

**Discussion**

The present exploratory study addresses the significance of the single hexokinase KlHxk1 to general metabolism and, in particular, to glucose sensing and signaling in the Crabtree-negative yeast *K. lactis* favoring respiration over fermentation [9]. In comparison to the extensively studied respiro-fermentative model organism *S. cerevisiae*, glucose-dependent signal transduction is less understood and/or different in *K. lactis*. Glucose repression of invertase encoded by the *KILINV1* gene, for example, does not require the transcriptional repressor KIMig1 in *K. lactis* [51], whereas ScMig1 is indispensable for invertase (*SUC2*) repression in *S. cerevisiae*. Genetic studies of hexokinase functions in *K. lactis* using *rag5* mutants indicated the involvement of *RAG5* and its gene product KlHxk1 in the transcriptional regulation of low- and high-affinity glucose
transport [11;20;24] and in glucose repression of several enzymes [16;25] but also in glucose-induced transcription of the KlPDC gene encoding pyruvate decarboxylase [52]. Since cell morphology and functions are primarily determined by the proteome, the absence of complementary information on the proteome level initiated the systematic proteomic comparison of hexokinase wild-type strain JA6 and hexokinase null mutant strain JA6Δrag5R in different metabolic situations as presented in this paper.

The application of the DIGE method to the analysis of the most prominent proteomic changes occurring in K. lactis during growth on glucose as a consequence of RAG5 disruption and concomitant KlHxk1 deficiency resulted in the identification of 60 protein species being present at minimum threefold altered – mostly increased – concentration in the mutant proteome (Table S2). Benjamini-Hochberg correction for detected 2,851 protein spots resulted in a critical p-value of $1.7 \times 10^{-3}$ which controls the probability of false positive identifications (FDR 5%) while predefinition of at least threefold abundance changes according to [53] guarantees a statistical power of approximately 80%. The corresponding calculation took into account observed variability data expressed in terms of coefficients of variation which turned out to be lower than 0.52 for 95% of the proteins listed in Supplemental Table S2. The identified protein species correspond to 45 individual K. lactis proteins from which they are derived by covalent modifications that remain to be identified. The molecular functions assigned to these hexokinase-dependent proteins and their relative abundance in the mutant proteome (Table S2) strongly support the hypothesis of KlHxk1 to play a key role in transforming the extracellular glucose signal into a specific proteomic pattern mainly by limiting the amount of proteins that are dispensable when glucose is abundantly available. This important conclusion is impres-
sively illustrated by the greatly elevated proteomic levels of β-galactosidase and maltase (Table 2) confirming previous results showing that these enzymes are subject to KlHxk1-dependent glucose repression [16;25]. The above hypothesis is consistent with the missing detection of proteins fulfilling the AAR ≥ 3 / ≤ -3 criterion when DIGE analysis was performed following cultivation on galactose or glycerol as the sole carbon source. The latter finding likely reflects an independence of galactose and glycerol utilization of hexokinase catalytic and regulatory functions. It should be noted, however, that in total four proteins from Supplemental Table S2 were identified in cells grown on galactose and glycerol, respectively, which fulfilled the FDR 5% criterion but exhibited lower than threefold abundance changes (spot numbers 937 (AAR -1.31) and 1916 (AAR 1.56) for galactose, spot numbers 825 (AAR 1.73) and 862 (AAR 1.31) for glycerol).

The identification of three hexokinase-dependent proteins that are homologs of 2-methylcitrate cycle enzymes of S. cerevisiae (Table 3) indicates on the protein level the existence of this pathway in K. lactis and suggests its repression in a RAG5/KlHxk1-dependent way. The failing detection in the present study of elevated levels of invertase and malate dehydrogenase in the mutant does not necessarily contradict the increased catalytic activities of these enzymes found in a rag5 mutant in a condition of glucose repression [16] because enzyme activity is not stringently correlated with enzyme concentration. In comparison, the decreased level of glucose-inducible pyruvate decarboxylase (Table S2, spot 828) confirms the observation of reduced transcription of the KlPDC1 gene in a rag5 mutant [52]. The additional identification of hexokinase-dependent proteins predicted to be functionally related to chromatin remodeling, amino acid and protein metabolism, redox state maintenance and stress response (Table 4) reinforces the
idea of KlHxk1 to exert functions beyond hexose phosphorylation and glucose-dependent signal transduction. Finally, the proteomic data of the present study confirm the sensitivity to RAG5/KlHxk1-dependent glucose repression of K. lactis genes encoding proteins/enzymes which are required for the utilization of alternative carbon sources and for gluconeogenesis when glucose is limiting [16;22;25;54] while genes encoding proteins involved in respiration remain essentially unaffected [19].

The obvious consequence of KlHxk1 deficiency is the slow growth of the mutant on glucose (Figure 1A) which likely reflects a limited utilization of the sugar initiated by the KlGlk1 glucokinase [12]. This impairment might cause metabolic conditions differing from those existing in wild-type cells that could affect transcription [55] and consequently the proteome of the mutant. The lacking dependence of glucose repression in S. cerevisiae on the glucose phosphorylating capacity of the cell [56], however, alleviates this concern at least with respect to proteins involved in RAG5/KlHxk1-dependent glucose repression if evolution has preserved basic regulatory mechanisms in the two yeasts. The latter consideration may not apply to glucose induction signaling because in K. lactis the expression of the Rag1 and Kht1 glucose transporters is influenced by the glycolytic flux [21;57] and, in case of Rag1, on RAG5/KlHxk1 [20;57]. In addition to the fact that only abundance changes meeting the AAR ≥ 3 / ≤ -3 criterion were considered in the present study, the interpretation of missing Kht1 identification has to take into account that the level of an individual protein represents the complex result of transcription, translation, proteolysis and/or additional covalent protein modification(s) or combinations thereof, let alone limitations associated with incomplete protein solubilization as discussed below.
The slow growth of strain JA6Δrag5R on glucose (Figure 1A) deserves special consideration also because the correlation of instantaneous growth rate and gene expression detected in *S. cerevisiae* [58] might exist in *K. lactis* as well and interfere with the identification of hexokinase-dependent proteins. The present study did not address this phenomenon, however, comparison of the AAR values in Table S2 and the growth rate response data of *S. cerevisiae* [58] identified two classes of hexokinase-dependent proteins of *K. lactis*: Proteins whose relative abundance in the mutant qualitatively matches the growth rate-dependent change of expression of their homologs in *S. cerevisiae* and proteins that do not match this correlation (besides proteins that are not listed in [58]). This situation together with the finding that only one ribosomal protein was detected at an altered level in the mutant proteome (spot 1674; Tables 4 and S2) which is in contrast to the strong growth-rate dependence of ribosomal protein expression in *S. cerevisiae* [58] questions a general influence of growth rate on gene expression in *K. lactis*. The above considerations illustrate the complexity of gene deletion studies and their limitations that are essentially attributable to the interdependence of enzyme activity, growth rate and gene expression.

In order to recognize proteomic differences that might have been caused by the different pre-cultivation conditions applied to generate sufficient biomass of the hexokinase mutant rather than by the null mutation, the proteomes of both strains were compared after pre-cultivation on galactose and final cultivation on glucose by post-lysis dimethyl labeling (DML) and ESI-MS/MS. The DML data in Supplemental Table S3 (columns K and L) indicate the proteomic consequences of KlHxk1 deficiency to be largely similar with re-
spect to the two pre-cultivation conditions. In detail, for 27 protein species the AAR values deviate from their respective mean by not more than 20% while the AAR values of additional eight protein species indicate the same tendency of abundance change when threefold or higher abundance changes are considered as in the DIGE approach. For additional 22 protein species, less than threefold concordant abundance changes were determined. The DML data support the view that final cultivation on glucose as applied in the DIGE approach is appropriate to largely abolish proteomic differences that might have been caused by the different pre-cultivation conditions.

Difference gel electrophoresis not only separates proteins and thereby reduces sample complexity prior to proteolytic digestion and subsequent mass spectrometry, but also allows prior-to-proteolysis abundance determinations, in particular for multiple forms of proteins that may arise through covalent modifications like proteolysis or phosphorylation or combinations thereof. This singularity represents a key advantage of the DIGE method which caused its application in the present study. By contrast, methodological constraints complicate the evaluation of proteomic data obtained by the same method. First of all, the detection of in total 2,851 fluorescent spots containing different protein species vs. 5,108 predicted protein-coding genes in the *K. lactis* genome [44] certainly is related to the transcriptional state of the mutant, but likely is additionally affected by incomplete protein extraction despite the application of harsh chemical and physical conditions. Limited protein solubilization represents a general methodological problem that may be overcome by combining total cell digestion, peptide separation and quantitative mass spectrometry, however, even solubilized proteins undergoing high abundance changes may escape from detection and mass spectrometric identification when their
absolute amount is limiting. This constraint is likely to apply to regulators of gene expression and indeed, no transcription factor was identified as a hexokinase-dependent protein. In addition, the DIGE method is not appropriate to determine the individual contributions of two or more proteins being present in a particular protein spot to the overall abundance of the spot protein because spot analysis is based on total fluorescence intensities (cf. Supplemental Table S2, protein spots 471, 568, 1411 and 1905). In view of the large number of unequivocally identified hexokinase-dependent proteins (Tables 2-4), however, this uncertainty does not compromise the basic conclusions of the manuscript. Image analysis represents another challenge since unspecific background fluorescence and protein interference may affect determination of relative abundance changes especially when low-abundance proteins are analyzed. The latter limitation is likely to cause the unexpectedly low absolute AAR values of unphosphorylated KlHxk1 (spot 976; Table 1) accounting for as few as approximately 0.1% of the K. lactis total protein subjected to DIGE analysis (Figures 3 and 4). Finally, the assignment of molecular functions and biological processes to the identified hexokinase-dependent proteins represents a serious challenge which is complicated by the fact that evidence for protein existence according to the UniProtKB database still is at the “inferred from homology” and “predicted” level for the vast majority of corresponding K. lactis gene loci.

The DIGE approach was validated by post-lysis dimethyl labeling and ESI-MS/MS (Supplemental Table S3). Relative protein abundance data determined by DML were compared with the AAR values resulting from DIGE analysis (Supplemental Table S3, columns J vs. K). In detail, for 16 protein species the AAR values determined by the two proteomic methods deviate from their respective mean by not more than 20% while AAR
values indicate the same tendency of abundance change for additional 22 protein species when threefold or higher abundance changes are considered as in the DIGE approach. For additional 18 protein species, less than threefold concordant abundance changes were determined. Evaluation of these data has to consider that DML is detecting peptides from all the multiple forms of a given protein that result from co- and/or posttranslational modifications while DIGE analysis typically refers to single protein species that are separated prior to mass spectrometric analysis of their peptides. The above findings together with the broad variety of molecular functions and biological processes assigned to the large number of prominent hexokinase-dependent proteins support the view of KlHxk1 to play a dual role as a catalyst and key regulator and suggest the existence of novel hexokinase signaling networks in *K. lactis* that remain to be explored.

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Figure legends

Figure 1. Growth behavior of *K. lactis* strains JA6 (full circles), JA6Δrag5R (open triangles) and JA6Δrag5R/pTSRAG5 (open circles) on different carbon sources. Cells were grown in YNB medium supplemented with the indicated carbon sources. Growth was monitored at 30°C in a NEPHELOstar Galaxy laser-based microplate nephelometer. Cell density is given in relative nephelometric units (RNU). Data represent three independent experiments each of which performed in triplicate. For clarity, only one out of four data points is plotted.

Figure 2. KlHxk1 expression and glucose repression in *K. lactis* strains JA6, JA6Δrag5R and JA6Δrag5R/pTSRAG5. A: Immunodetection of KlHxk1 in protein extracts of strains JA6 (lane 1), JA6Δrag5R (lane 2) and JA6Δrag5R/pTSRAG5 (lane 3) using rabbit polyclonal anti-KlHxk1 immunoglobulin. The cells were grown in YNB medium supplemented with 2% galactose (w/v). 20 µg of protein were applied per lane. Primary antibody detection employed HRP-conjugated goat anti-rabbit IgG and the Immobilon™ Western Chemiluminescent HRP Substrate. B: β-galactosidase activity in strains JA6 (1), JA6Δrag5R (2) and JA6Δrag5R/pTSRAG5 (3). Cells were grown in YNB medium supplemented with 2% (w/v) galactose, shifted into YNB medium containing 2% (w/v) glucose, washed with YNB medium w/o carbon source, transferred into YNB medium containing 2% (w/v) of the indicated carbon source(s) and propagated until an OD (600 nm/1 cm) of 1 was reached. The activity of β-galactosidase in the protein extracts was determined using o-nitrophenyl-β-D-galactopyranoside as a substrate. One milliunit (mU) of β-galactosidase activity catalyzes the formation of one nanomole of o-nitrophenolate per min at 30°C.
Figure 3. Identification of unphosphorylated and serine-15-phosphorylated KlHxk1 in the 2-D image of the *K. lactis* proteome. The panels show sections of the fluorescence (A) and black/white (B) 2-D image of the Cy2-labeled internal standard proteome containing spike-ins of purified Cy3-labeled serine-15-phosphorylated (green) and Cy5-labeled unphosphorylated (red) KlHxk1. The 2-D coordinates of the two fluorescent KlHxk1 species are identical with the coordinates of spot proteins 968 (green) and 976 (red) in the 2-D image of the *K. lactis* proteome shown in Figure 4. Mass spectrometric analysis of tryptic peptides employing selective reaction monitoring verified phosphorylation of spot protein 968 at serine-15 (Figure S1 and Table S1). For further experimental details, see Materials and Methods.

Figure 4. DIGE image of the *K. lactis* proteome (black/white image of the master gel) and annotation of hexokinase-dependent proteins. The identification of *K. lactis* proteins occurring during growth on glucose (2% initial concentration) at altered concentrations in the mutant proteome as a consequence of *RAG5* disruption and concomitant hexokinase deficiency employed an AAR setting of ≥ 3 / ≤ -3 and an adjusted p ≤ 0.05 criterion according to [37]. The boundaries surround fluorescent spots containing hexokinase-dependent proteins or indicate their position in the 2-D gel when the absolute amount of the respective protein was low. The dot within each boundary marks the center of protein mass, which generally represents the optimal picking location for protein identification. The proteins are annotated in accordance with the spot numbers used in Table S2, in which where submitted or recommended names, molecular functions and biological processes are additionally indicated. The positions of serine-15-phosphorylated (spot
968) and unphosphorylated KlHxk1 (spot 976) are labeled in green and red, respectively. For further experimental details, see Materials and Methods.

Figure 5. Metabolic pathways in *K. lactis* affected by *RAG5* disruption and concomitant KlHxk1 deficiency upon growth on glucose. Hexokinase-dependent proteins catalyzing central metabolic reactions are indicated by their EC numbers. Enzymes exhibiting enhanced levels in the *rag5* null mutant proteome (AAR ≥ 3) are labeled in green while enzymes exhibiting reduced levels (AAR ≤ -3) are labeled in red. Full circles represent intermediates of the citric acid and 2-methylcitrate cycle. Open circles specifically indicate *cis*-aconitate and 2-methyl-*cis*-aconitate, respectively.

Figure 6. Assignment of hexokinase-dependent proteins of *RAG5* wild-type strain JA6 of *K. lactis* to functional categories according to KEGG pathway and Gene Ontology (GO) biological process information. The categorized proteins were detected by DIGE at altered concentrations in the proteome of glucose-grown *rag5* null mutant strain JA6Δrag5R as a consequence of *RAG5* disruption and concomitant KlHxk1 deficiency (for full list of hexokinase-dependent proteins, see Table S2). Categorization of hexokinase-dependent proteins with no explicit KEGG and/or GO annotation is based on the annotation of the closest yeast homolog. Proteins assigned more than one function are accordingly considered in more than one functional category. The sum of footprints of the categorized proteins is normalized to equal 100%.
Tables

Table 1

Analysis of apparent relative abundance of serine-15-phosphorylated and unphosphorylated KlHxk1 in the proteome of \textit{rag5} null mutant strain JA6\textDelta{rag5R} of \textit{K. lactis} grown on different carbon sources. Strain JA6 served as a reference. AAR values were calculated from fluorescence intensities of spot proteins 968 and 976 analyzed after DIGE separation of wild-type and mutant proteomes by application of DeCyder Software Version 7.0. First-dimension separation used linear pH 3-10 IPG strips. KlHxk1 phosphorylation at serine-15 was verified and quantified by SRM mass spectrometry as described in “Materials and Methods” and illustrated in Figure S1 and Table S1.

| Spot No. | Protein Name (UniProtKB) | Gene locus | AAR (2\% (w/v) glucose) | AAR (2\% (w/v) galactose) | AAR (2\% (w/v) glycerol) |
|----------|--------------------------|------------|-------------------------|--------------------------|--------------------------|
| 968      | Hexokinase KlHxk1 (S15p) (HXK_KLULA) | \textit{KLLA0D11352g (RAG5)} | -6.2                    | -7.8                     | -3.7                     |
| 976      | Hexokinase KlHxk1 (S15) (HXK_KLULA) | \textit{KLLA0D11352g (RAG5)} | -3.1                    | -2.9                     | -3.6                     |
Table 2

Hexokinase-dependent proteins of RAG5 wild-type strain JA6 of K. lactis with functions in carbohydrate metabolism. The listed proteins were detected by difference gel electrophoresis at altered concentrations in the proteome of glucose-grown rag5 null mutant strain JA6Δrag5R as a consequence of RAG5 disruption and concomitant KlHxk1 deficiency (for full list of hexokinase-dependent proteins, see Table S2). Protein identification is based on mass-spectrometric peptide analysis and data base search for homologous proteins primarily in S. cerevisiae. Homology analysis and assignment of protein functions is described in detail in “Materials and Methods”.

| Spot No. | AAR | Protein ID (NCBI) | Submitted or Recommended Name (UniProtKB) | Molecular Function (K. lactis Protein or Yeast Homolog) |
|----------|-----|-------------------|------------------------------------------|---------------------------------------------------|
| 226      | 7.9 | 50304489          | Beta-galactosidase                        | Beta-galactosidase                                 |
| 227      | 4.9 |                   |                                          |                                                   |
| 228      | 20.5|                   |                                          |                                                   |
| 470      | 13.7|                   |                                          |                                                   |
| 471      | 3.5 | 50309205          | KLLA0E14631p                             | Beta-glucosidase precursor                        |
| 568      | 3.6 |                   |                                          |                                                   |
| 730      | 8.0 | 50303199          | KLLA0D00231p                             | Maltase                                           |
| 825      | 4.0 | 50304293          | KLLA0B12694p                             | Phosphoglucomutase                                |
| 828      | -3.9| 50309353          | Pyruvate decarboxylase                   | Pyruvate decarboxylase                            |
| 858      | -3.7|                   |                                          |                                                   |
| 871      | 4.2 | 50310877          | Galactokinase                            |                                                   |
| 875      | 3.2 |                   |                                          |                                                   |
| 927      | -3.7| 50307609          | KLLA0D16412p                             | Inositol-3-phosphate synthase                     |
| 937      | -3.2| 50309857          | KLLA0E21891p                             | External NADH-ubichinone oxidoreductase 1         |
| ID  | Value | Gene ID     | Description                                                                 |
|-----|-------|-------------|-----------------------------------------------------------------------------|
| 1312| 7.8   | 50310881    | Galactose-1-phosphate uridylyltransferase                                  |
| 1717| 3.4   |             | Galactose-1-phosphate uridylyltransferase                                  |
| 1733| 4.6   | 50311981    | Glyceraldehyde-3-phosphate dehydrogenase 1                                |
| 1736| 3.7   |             | Glyceraldehyde-3-phosphate dehydrogenase 1                                |
| 1945| 3.2   | 50311553    | KLLA0F16016p Hydroxyacylglutathione hydrolase, mitochondrial (Glyoxalase II) |
| 2502| 6.4   | 50311981    | Glyceraldehyde-3-phosphate dehydrogenase 1                                |
| 2533| 6.4   |             | Glyceraldehyde-3-phosphate dehydrogenase 1                                |
| 2834| 4.2   | 50302165    | KLLA0A00418p Glycerol-3-phosphate dehydrogenase (mitochondrial)           |
Table 3

Hexokinase-dependent proteins of RAG5 wild-type strain JA6 of K. lactis with functional relations to ethanol, acetate, propionate and tricarboxylic acid metabolism. The listed proteins were detected by difference gel electrophoresis at altered concentrations in the proteome of glucose-grown rag5 null mutant strain JA6 Δrag5R as a consequence of RAG5 disruption and concomitant KIHxk1 deficiency (for full list of hexokinase-dependent proteins, see Table S2). Protein identification is based on mass-spectrometric peptide analysis and data base search for homologous proteins primarily in S. cerevisiae. Homology analysis and assignment of protein functions is described in detail in “Materials and Methods”.

| Spot No. | AAR   | Protein ID (NCBI) | Submitted or Recommended Name (UniProtKB) | Molecular Function (K. lactis Protein or Yeast Homolog) |
|----------|-------|-------------------|------------------------------------------|------------------------------------------------------|
| 490      | -3.2  | 50304787          | KLLA0C03432p                             | Probable aconitase hydratase 2                        |
| 589      | 17.4  | 50302423          | Acetyl-coenzyme A synthetase 1           | Acetyl-coenzyme A synthetase 1                        |
| 603      | 8.5   |                   |                                          |                                                      |
| 775      | 5.9   | 50302319          | KLLA0A02123p                             | Carnitine acetyl-CoA transferase (mitochondrial and peroxisomal) |
| 837      | 4.2   | 50309145          | Isocitrate lyase                         | 2-Methylisocitrate lyase (mitochondrial)             |
| 965      | 9.6   | 50309171          | KLLA0E14213p                             | Probable 2-methylcitrate dehydratase                  |
| 992      | 5.8   | 50310159          | KLLA0F00440p                             | Potassium-activated aldehyde dehydrogenase (mitochondrial) |
| 1213     | 5.4   | 50309173          | Citrate synthase                         | Dual specificity citrate/2-methylcitrate synthase (mitochondrial) |
| 1248     | 3.1   | 50311261          | Citrate synthase                         | Citrate synthase (mitochondrial)                     |
| 1300     | 3.6   | 50303483          | Isocitrate dehydrogenase [NADP]          | Isocitrate dehydrogenase [NADP] (mitochondrial)      |
|     |   3.2 | 50311983 | Alcohol dehydrogenase 1 | Alcohol dehydrogenase 1 |
|-----|-------|----------|-------------------------|-------------------------|
|     | 4.3   | 50311741 | Alcohol dehydrogenase 2 | Alcohol dehydrogenase 2 |
Table 4

Hexokinase-dependent proteins of RAG5 wild-type strain JA6 of K. lactis with functional relations to chromatin remodeling, amino acid and protein metabolism, redox state maintenance and stress response. The listed proteins were detected by difference gel electrophoresis at altered concentrations in the proteome of glucose-grown rag5 null mutant strain JA6Δrag5R as a consequence of RAG5 disruption and concomitant KlHxk1 deficiency (for full list of hexokinase-dependent proteins, see Table S2). Protein identification is based on mass-spectrometric peptide analysis and data base search for homologous proteins primarily in S. cerevisiae. Homology analysis and assignment of protein functions is described in detail in “Materials and Methods”.

| Spot No. | AAR | Protein ID (NCBI) | Submitted or Recommended Name (UniProtKB) | Molecular Function (K. lactis Protein or Yeast Homolog) |
|----------|-----|------------------|------------------------------------------|-----------------------------------------------------|
| 471      | 3.5 | 49645322         | KLLA0F24838p                             | Isw2, ISWI chromatin-remodeling complex ATPase       |
| 568      | 3.6 |                  |                                          |                                                     |
| 615      | 19.2| 50306835         | KLLA0D07414p                             | Flavin-dependent monooxygenase                       |
| 676      | -4.3| 50306557         | KLLA0D04224p                             | Ssa2, member of 70 kDa heat shock protein family     |
| 743      | -4.0| 50308135         | KLLA0E02707p                             | Aro10, Transaminated amino acid decarboxylase        |
| 749      | -8.4|                  |                                          |                                                     |
| 915      | 7.7 | 50307205         | Catalase                                 | Catalase                                            |
| 920      | 6.9 |                  |                                          |                                                     |
| 933      | -5.4| 50304099         | KLLA0B10560p                             | Eukaryotic translation initiation factor eIF-4B     |
| 1100     | 4.4 | 50311777         | KLLA0F18678p                             | 3-Hydroxyisobutyryl-CoA hydrolase (mitochondrial)   |
| 1228     | -3.5| 50304639         | KLLA0C01782p                             | S-adenosylmethionine synthase                       |
| 1446     | 4.5 | 50311207         | KLLA0F12056p                             | Glutathione S-transferase omega-like 2              |
| Protein ID   | Z-score | T-Value | Accession   | Description                                      |
|-------------|---------|---------|-------------|--------------------------------------------------|
| KLLA0B05918p| -3.2    | 1674    | 50303713    | 60S Acidic ribosomal protein P0                  |
| KLLA0C08217p| -6.9    | 1828    | 50305213    | DL-Glycerol-3-phosphatase 1                      |
| KLLA0B09372p| -3.1    | 1865    | 50303991    | Spermidine synthase                              |
| KLLA0B11836p| 5.2     | 1905    | 50304213    | Proteasome component Pre6                        |
| KLLA0D00979p| 5.2     | 2059    | 50306283    | Cerevisin (Vacuolar protease B)                  |
| KLLA0F18909p| 5.7     | 2059    | 50311795    | Small heat shock protein with chaperone activity Hsp26 |
| KLLA0F04323p| 3.1     | 2367    | 50310511    | Protoplast secreted protein 2                    |
| KLLA0C16225p| 37.5    | 2392    | 50305929    | Gre1, Hydrophilin of unknown function            |
Figures

Figure 1

(A) 2 % (w/v) glucose

(C) 2 % (w/v) galactose

(B) 2 % (w/v) fructose

(D) 2 % (w/v) glycerol
Figure 2

A

kDa

B

Beta-galactosidase activity [mU/mg]

2% glucose
2% galactose
2% glucose / galactose
Figure 3

A

Isoelectric point

Molecular weight [kDa]

B

Isoelectric point

Molecular weight [kDa]
Figure 6

- Carbohydrate Metabolism (27.3%)
- Amino Acid Metabolism (14.3%)
- Energy Metabolism (6.5%)
- Nucleotide Metabolism (1.3%)
- Cellular Processes (5.2%)
- Glycan Metabolism (1.3%)
- Metabolism of Other Amino Acids (6.5%)
- Cellular Response to Stress (9.1%)
- Function not specified (5.2%)