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On sugar signalling in *Saccharomyces cerevisiae*

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Cover: Cartoon representing the best-characterised elements of the sugar sensing and signalling network.
Backcover: Night train to Abisko (above polar circle), expedition: “Paella Boreal 2006” (photo: Antonio Caballero)

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ABSTRACT

This thesis deals with the mechanisms of sensing and signalling of glucose/fructose in baker's yeast, focusing on the Snf1 transduction pathway. In the first place, we approached the question of how a reduction in glucose transport leads to a decrease in glycolytic rate. Modelling of the experimental data indicated that phosphofructokinase (PFK) might control glycolysis in order to allow adaptation to reduced sugar availability. We hypothesises that this could be due to a mechanism involving Protein Kinase A (PKA) in the generation of the PFK activator fructose-2, 6-bisphosphate. Next we addressed the question of which transcriptional responses were triggered by sensors of extracellular glucose. In a strain where glucose transport is abolished the response to glucose pulse is limited to the expression of hexotransporters. Any other significant response requires internalisation of glucose. Snf1 is an essential protein kinase for growth on poor carbon sources. Catabolism of glucose generally leads to the downregulation of Snf1 activity. We were interested in understanding how Snf1 is turned on and off. To approach this question, we undertook an interdisciplinary approach covering physiology, molecular biology, bioinformatics and single cell microscopy. We have established that the inactivation of the SNF1 pathway after addition of glucose occurs very rapidly (in less than 1 min) and therefore is independent on transcriptional events. The detailed time-course data sets obtained were used to explore the regulation upstream of Snf1 by modelling. Simulations revealed a scenario indicating that regulation of the phosphatase complex Reg1/Glc7 is sufficient to explain the activation/deactivation profile of the pathway. Hence, this supports the hypothesis that the Snf1-activating kinases do not respond to changes in the abundance of glucose.

The response to glucose has been shown to require the phosphorylation of internal glucose into glucose-6-P. Hxk2 is the predominant hexokinase during growth on glucose. In addition, to its enzymatic role it has been suggested to play part in controlling the activity of the main glucose-dependent transcription repressor, Mig1. We present data suggesting that Hxk2 affects the Mig1 activation state in a parallel pathway to the SNF1 signalling pathway. The proposed mechanism requires Hxk2 protection of Mig1 regulatory residues from the phosphorylation mediated by Snf1.

In conclusion, this thesis dives into the different glucose signalling pathways of baker's yeast and attempts to identify what is the molecular basis of sugar sensing. A deepened knowledge of these pathways behaviour in yeast may in turn be useful in understanding the development of Diabetes type II, an increasingly widespread disease in humans, which it is believed to be influenced by the failure of cells to recognise and internalise sugar.

Keywords: Yeast, Snf1, Sugar Signalling

LIST OF PUBLICATIONS

This thesis recapitulates the work contained in the following papers:

Paper 1. **Daniel Bosch Ibáñez***, Mikael Johansson*, Cecilia Ferndahl, Carl Johan Franzén, Christer Larsson and Lena Gustafsson. (2008) "Characterisation of glucose transport mutants of *Saccharomyces cerevisiae* during a nutritional upshift reveals a correlation between metabolites levels and glycolytic flux". *FEMS Yeast Res*. **8**: 10-25.

Paper 2. **Daniel Bosch Ibáñez**, Goutham N Vemuri, Christer Larsson, Lena Gustafsson, Karin Elbing, Jens Nielsen and Stefan Hohmann. (2008) "Uptake is required for glucose-stimulated transcriptional responses in *Saccharomyces cerevisiae*". (Manuscript).

Paper 3. Tian Ye, Gemma Beltran, Raúl García-Salcedo, **Daniel Bosch Ibáñez**, Peter Dahl, Karin Elbing and Stefan Hohmann. (2008) "On the functional relationship between Hxk2 and the Snf1 pathway in yeast carbon catabolite repression". (Manuscript).

Paper 4. Gemma Beltran*, **Daniel Bosch Ibáñez ***, Raúl García-Salcedo, Simone Frey, Arne Bittig, Katja Rateitschak, Olaf Wolkenhauer, Karin Elbing and Stefan Hohmann. (2008) "Studies on activation/deactivation of SNF1 pathway, a systems biology approach". (Manuscript).

(*) Authors contributed equally

The following paper is not included in this thesis:

Christian Brackmann, Joakim Norbeck, Madeleine Åkeson, **Daniel Bosch Ibáñez**, Christer Larsson, Lena Gustafsson, Annika Enejder. (2008) "CARS microscopy of living yeast cells reveals noise in neutral storage lipids" (Submitted).

My contribution to these papers was:

Paper 1. I participated in the experimental design, cultivations, sampling, interpretation of results and writing of the paper. I determined extracellular metabolites and performed uptake capacity assays. I am joint main author together with M. Johansson and corresponding author with C. Larsson.

Paper 2. I participated in the experimental design, interpretation of results and writing of the paper. I performed cultivations, sampling and determination of extracellular metabolites and RNA extraction.

Paper 3. I performed part of the experiments and analysis.

Paper 4. I participated in the experimental design, cultivation management, sampling, analysis, interpretation of results and writing of the paper. I am joint main author together with G. Beltran.

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AIMS AND FINDINGS

The aims of this thesis can be summarised as follows:

1) To find the internal cellular events that link glucose assimilation with the triggering of glucose responses.
2) To characterise the individual contributions of sensing and signalling pathways to the initial glucose response.

These aims can be divided into specific goals:

- To investigate how a reduction in sugar transport leads to a decrease in glycolytic rate.
- To elucidate which responses depend on cell surface sensors at high external concentrations of fermentable carbon sources.
- To explain mechanistically how the presence of glucose inside of the cell triggers signalling events.

The main findings of this thesis can be summarised as:

Paper 1 focuses on the slow down in glycolysis that accompanies a reduction in glucose transport into the cells. Upper glycolytic metabolites and adenosine nucleotides of strains with progressively reduced glucose uptake were determined. Modelling analysis of the data concluded that the changes or adaptation of the glycolytic rate accompanying a reduction in glucose consumption might result from alterations in the kinetics of phosphofructokinase and phosphoglucose isomerase.

Paper 2 investigates to what extent cells are able to respond to extracellular glucose. The transcriptional analysis of a strain lacking all hexose transporters suddenly exposed to glucose revealed insignificant changes. The conclusion of the study is that cell surface sensors have a very limited capacity to trigger a glucose response.

Paper 3 considers the implications of Hxk2 in the regulation of Mig1 phosphorylation. The *hxk2* mutant displayed abnormal Snf1 and Mig1 phosphorylation states and the double mutant *reg1 hxk2* was glucose insensitive. Data is presented suggesting that Reg1 and Hxk2 may work in parallel to mediate Snf1-dependent Mig1 phosphorylation and hence glucose repression/derepression.

Paper 4 presents high quality data sets addressing the Snf1 phosphorylation state during glucose consumption. A linear increase of Snf1P is described as the concentration of glucose in the medium dropped. It also underscored the rapidness of Snf1 dephosphorylation (less than 1 min) upon a glucose pulse, which indicates that the time scale of the response does not allow for transcriptional responses. These data sets were implemented into a preliminary mathematical model, which validates the assumption that the phosphatase and not the kinases regulate Snf1 phosphorylation.

1. Baker's yeast

Yeasts are unicellular fungi, eukaryotic microorganisms that can be found in a multitude of environments (SGD, 2008; Wikipedia, 2008). Baker's yeast, *Saccharomyces cerevisiae*, is an unusual yeast, it produces daughter cells by budding instead of symmetrical division and prefers fermentation (with the release of ethanol and CO_2) rather than respiration at high external sugar concentrations. These properties have been of great advantage to humans in the production of beverages (beer, wine, spirits, etc.) and the leavening of dough. It is only recently that baker's yeast has been employed as a host organism for the synthesis of lipids and heterologous proteins. It is also present in our daily life as food additive, cosmetics, etc. (EPA, 2008). In addition, baker's yeast is considered an optimal model organism for the study of basic cellular processes (Goffeau, 2000). In many cases, research about baker's yeast has led to the discovery of cellular mechanisms conserved in multicellular organisms (Forsburg, 2005). Whilst a great deal has been learnt and additional model organisms have emerged, the interest in baker's yeast has not relented. In the future, *S. cerevisiae* looks likely to contribute to the development of new molecular, genetic, genomic and systems-level techniques, as well as to the advancement of knowledge in basic biology. This, in turn, will be of great benefit for medical and pharmacological applications, because most core molecular processes are conserved from yeast to humans. In addition, it may open completely new fields of research as is presently occurring with systems biology. In this context, yeast is a fertile ground for quantitative and modelling studies of complex cellular processes, such as of signalling networks or of the cell cycle.

2. Sugar utilisation

Baker's yeast is, like many humans, a sugar-craving organism. Its life cycle is determined by the abundance and type of carbon sources. In general, yeasts dwell in fast changing environments (e.g. fruit surfaces, flowers, vegetables and grains (EPA, 2008)), characterised by long periods of nutrient limitation, water deprivation and intense sun radiation. As a result, baker's yeast is a resilient microorganism, which bases its success on fast adaptation to the changes in environment and rapid utilisation of nutrients (Carmo-Sousa, 1989). It is also a heterotrophic and opportunistic organism feeding on the available carbon sources, most of which are generally associated to plants. Baker's yeast cannot degrade polysaccharides (e.g. starch, cellulose, chitin, etc.) and lives at the expense of certain mono-, di- and trisaccharides, which in nature is dominated by sucrose in fruits (Carmo-Sousa, 1989; Stryer, 1997). *S. cerevisiae* assimilates a rather small range of sugars compared to other yeast species (Fig. 1) (Barnett, 1997):

- Monosaccharides: glucose, fructose, mannose and galactose.

- Disaccharides: sucrose and turanose (fructose-glucose) and maltose (2x glucose).
- Trisaccharides: raffinose (fructose-melibiose), maltotriose (3x glucose), etc.

Some strains can digest melibiose (galactose-glucose) due to the presence of the corresponding α-galactosidase (Mel1) (Liljeström, 1985). However, that is not the case for most laboratory strains. Monosaccharides are transported into the cell and incorporated into metabolism by phosphorylation. Disaccharides and trisaccharides are either cleaved extracellularly (i.e. sucrose and raffinose) or intracellularly (i.e. maltose and maltotriose) (Barnett, 1997). These sugars can all be metabolised in the absence of oxygen by fermentation, that is, provided that there is a source of sterols (usually ergosterol) available (Nes, 1978).

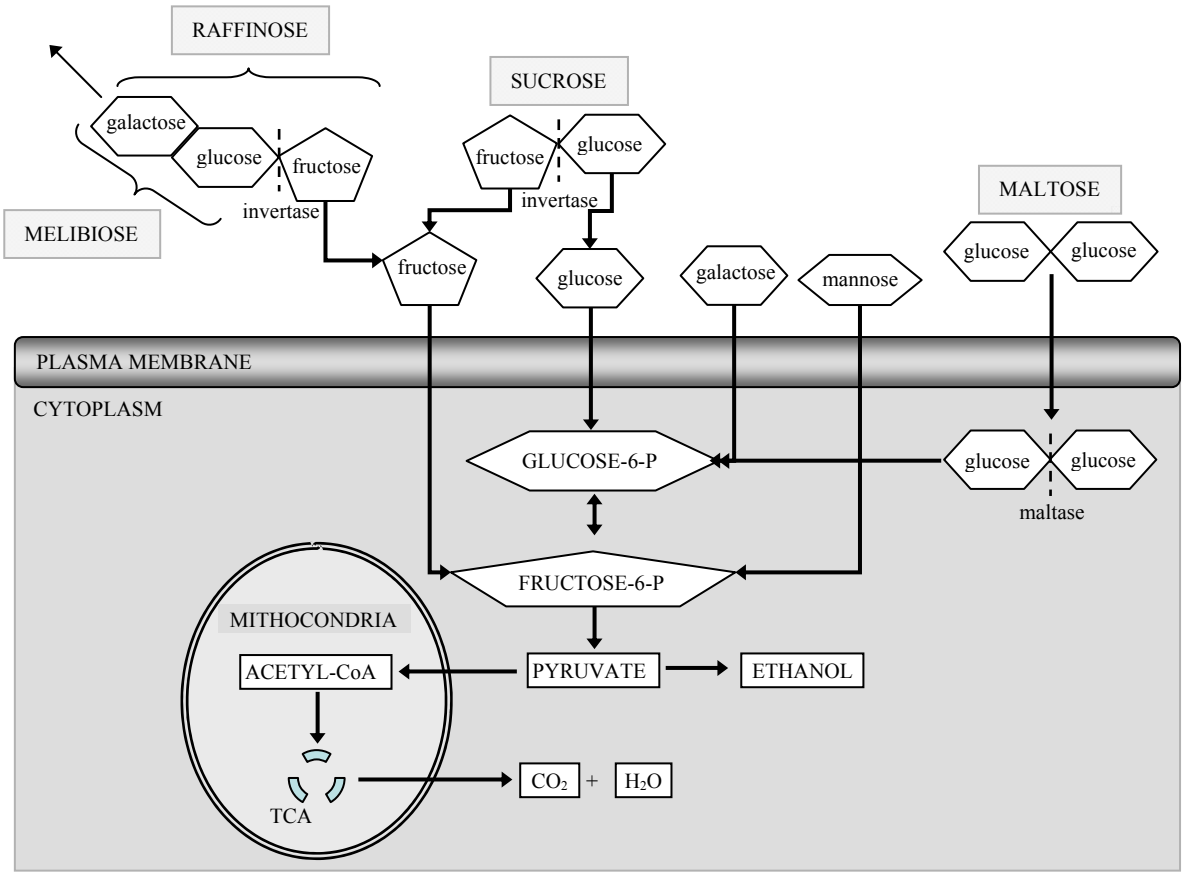

Figure 1. Assimilation of carbon sources in *S. cerevisiae*.

2.1. Extracellular sugar degradation

Sucrose is probably the favourite substrate of baker's yeast. Its degradation to fructose and glucose takes place in the periplasmic space. This reaction is mediated by **invertase**, a β–fructofuranosidase which also breaks down raffinose (Sols, 1989). Invertase was the first non-secreted enzyme to be partially purified in laboratory, as early as 1860 (Barnett, 1997). Its name was coined from the observation that it produces "invert" optical properties of sugar,

which is a 1:1 mixture of levorotatory D-fructose and dextrorotatory D-glucose (Alberto, 2004). Invertase is encoded by the *SUC* gene family (*SUC1-7*, sucrose fermentation) (Grossmann, 1979). Each of the *SUC* genes encodes a functional enzyme with sufficient activity to support growth on sucrose. Most laboratory strains bear only one gene, *SUC2* (Entian, 1997b), which encodes two distinct mRNA transcripts: a short version (1.8 kb) transcribed into a cytosolic polypeptide; and a longer transcript (1.9 kb), which gives rise to a heavily glycosylated protein found in the periplasmic space (Carlson, 1982). While the latter is the active form required for growth, the function of the cytosolic mRNA remains unknown. It has been speculated that it may take part in the degradation of intracellular sucrose (Santos, 1982). However, such a function would require the presence of a sucrose transporter, which has not yet been identified.

2.2. Sugar transport

For many years, the transport of hexoses was considered a passive process, however measurements of sugar uptake kinetics strongly indicated the involvement of protein facilitators (Bisson, 1983; Lang, 1987). All characterised sugar transporters in yeast belong to the Major Facilitator Superfamily (MFS), a conserved group of proteins that stretches from prokaryotes to eukaryotes (Boles, 2002; Pao, 1998).

The Hexose transporter family (HXT). These are a group of about 20 permeases (Hxt1-17, Gal2, Snf3 and Rgt2) identified based on their high sequence similarity (Boles, 1997; Kruckeberg, 1996) (Fig. 2). Their structure consists of twelve hydrophobic domains predicted

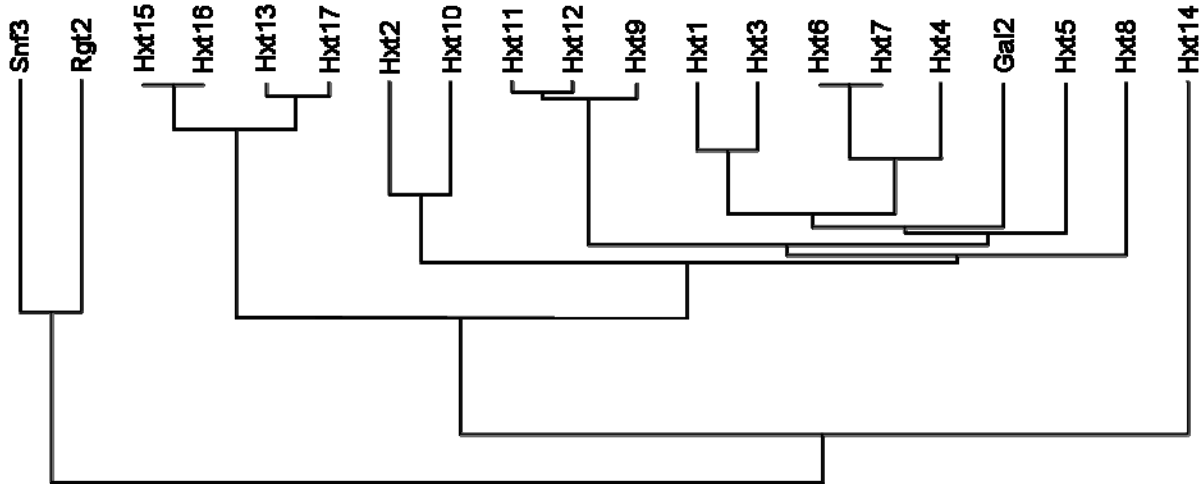

Figure 2. Dendrogram of sequence similarity among *S. cerevisiae* hexose transporters.

to span the plasma membrane and, according to the GLUT1 (mammalian glucose transporter) model, form an aqueous channel (Boles, 1997). HXT proteins have been mainly implicated in the transport of glucose, fructose, mannose and, to a lesser extent, galactose

(Reifenberger, 1997), xylose (Hamacher, 2002; Sedlak, 2004), toxic cations (i.e. arsenic trioxide (Liu, 2004)) and drugs (Nourani, 1997). (Boles, 2002) classified the HXT family based on their glucose affinity: Hxt1 and Hxt3 are low-affinity transporters (K_m = 100 mM and 60 mM, respectively), while Hxt2, Hxt4, Hxt6 and Hxt7 are high affinity transporters (K_m = 10 mM, 9 mM and 1-2 mM, respectively). Note that the values given above vary depending on the bibliography consulted, probably due to differences in the strain background and the variability of the assays performed. The remaining transporters (Hxt5 and Hxt8 to Hxt17) are poorly characterised and seem to have limited capacity to transport glucose or other compounds (Özcan, 1999). HXT proteins can mediate low affinity galactose uptake (Km = 250 mM) (Reifenberger, 1997), while high affinity uptake (K_m = 2 mM) depends on Gal2 which is part of the GAL regulon (Ciriacy, 1997; Szkutnicka, 1989). Gal2 is capable of transporting glucose, but expression of *GAL2* is strongly repressed in the presence of glucose (Ciriacy, 1997; Reifenberger, 1997).

Two members of the HXT family, Snf3 and Rgt2, are unable to support growth in a strain devoid of other transporters. Whether they retain some transport capacity is still not known. Their role in controlling gene expression of the HXT family is well established (see section 3A) (Kruckeberg, 1996).

Disaccharide transporters. The family of proton symporters, which encompasses Mal11 (Agt1), Mal21, Mal31, Mal41, Mal61, Mph2 and Mph3, is specialised in the active transport (proton symport) of maltose (Boles, 1997; Chow, 1989), but has also been reported to transport other oligosaccharides (i.e. turanose, trehalose, maltotriose, melezitose, etc.) (Han, 1995). An open question is whether there is a dedicated sucrose and trehalose transporter, as reported by (Santos, 1982), and if so, how this process is mediated and regulated (Boles, 1997) (for further details see section 2A).

HXT-resembling transporters. The following permeases could potentially be involved in sugar transport, though such a role has not been investigated. The inositol family of transporters: Itr1, Itr2 and Git1, is implicated in the uptake of inositol and inositol phosphate (Nikawa, 1995). Another proton symporter recently characterised, Stl1, is a glycerol-specific transporter (Ferreira, 2005). Both high and low affinity uptake of pentose-sugars (arabinose, xylose, ribose etc.) is well documented, however, no single transporter has been identified (Prior, 1997; van Zyl, 1993). Finally, there is a group of poorly characterised genes (*YDR387C*, *YDL199C*, *YFL040W*) identified by sequence similarity to *HXT* genes that could potentially mediate transport of hexoses (Boles, 1997).

This large diversity of sugar transporters was thought to constitute a unique feature of *S. cerevisiae*, but genome sequencing and protein characterisation of other microorganisms has revealed similar complex transport systems. Therefore, the presence of a wide range of

transporters with different, though overlapping specificity, affinity and expression profiles can constitute a strategy to secure a steady nutrient supply in an ever changing environment (Boles, 2002; Boles, 1997). A large number of transporters can be advantageous to the cell in order to fine-tune nutrient uptake. Indeed, a one-fits-all strategy, although energetically cheaper, may suffer from poor performance. This is the conclusion of a study in which strains engineered to constitutively express a single high affinity transporter grew very slow at high sugar concentrations (Boles, 2002). The large number of hexose transporter genes may have originated from genetic rearrangements, such as duplications. It has been shown that in cells subjected to long periods of carbon-limited conditions, there was an enrichment of tandem duplications of regions containing high affinity transporter genes, consequently suggesting favoured adaptation to low glucose concentrations (Brown, 1998). Interestingly, two groups of *HXT* genes occupy positions adjacent to the telomeres: *HXT1*, *HXT4* and *HXT5* on chromosome VIII and *HXT3*, *HXT6* and *HXT7* on chromosome IV (Kruckeberg, 1996). These linked triads of genes contain both high and low affinity genes that appear to be the transporters with highest Vmax. This also allows easy genetic manipulation in the laboratory.

2.3. Glycolysis

Glucose serves as a source of energy and precursors for biosynthesis. Glycolysis is the metabolic pathway that converts glucose into pyruvate with the resulting generation of ATP, NADH and an array of intermediate molecules that subsequently are substrates for other metabolic processes. The generated pyruvate may take two different paths: at low glucose concentrations, in the presence of oxygen, it is mostly converted into acetyl-CoA and transferred to the TCA cycle. This process is the most efficient in terms of ATP molecules generated per gram of glucose. However, under these conditions baker's yeast does not grow at its maximum speed (Sols, 1989). At high glucose concentrations, the bulk of pyruvate is fermented to acetaldehyde and subsequently to ethanol, without additional ATP production. This process, though generating a smaller amount of ATP per unit of glucose degraded as compared to the number gained in respiration, is very

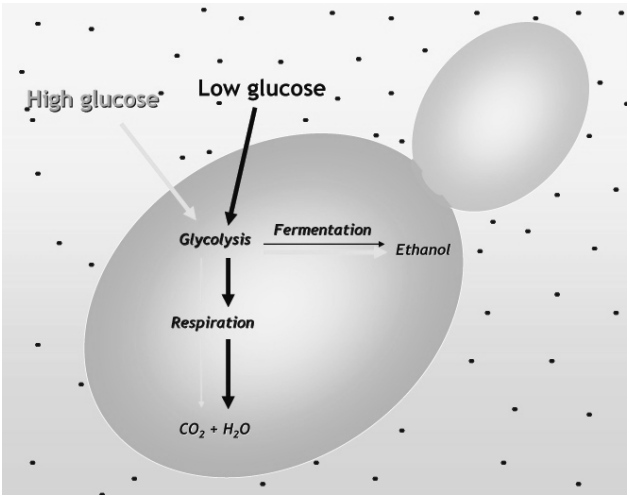

Figure 3. The balance between respiration and fermentation is dependent on the availability of glucose.

fast, allowing for rapid cell growth (Bisson, 2003). Gluconeogenesis, is the opposite process to glycolysis, in which glycolytic intermediates are generated from two- or three-carbon molecules i.e. glycerol, ethanol, acetaldehyde or TCA intermediates (e.g. acetate, succinate) (Entian, 1997a).

The fraction of glycolysis that serves respiration or fermentation during aerobic conditions is regulated by the glycolytic flux. High glycolytic fluxes are invariably accompanied by alcoholic fermentation (Pronk, 1996). Genetic manipulation aimed at increasing ethanol production by inhibiting respiration or by overproduction of several glycolytic enzymes has not resulted in increased ethanol yield (Schaaff, 1989). This points to a complex and robust control of glycolysis in which no single step determines the flux, which is shared amongst its different components (Davies, 1992; Müller, 1997). The opposite strategy, redirecting the glycolytic flux towards respiration in order to maximise biomass yield at the expense of ethanol production at high sugar concentrations, has only recently been achieved by drastically reducing sugar uptake (see section 2D).

Sugar phosphorylation

Sugars are transported into the cell by facilitated diffusion occurring via a positive gradient that is maintained by rapid sugar phosphorylation. In baker's yeast there are four hexokinases: Hxk1 and Hxk2 phosphorylate glucose, fructose and mannose; Glk1 has a preference for glucose and mannose; and Gal1 is specific for galactose (Entian, 1997a). The triple deletion *hxk1*Δ *hxk2*Δ *glk1*Δ abolishes growth on glucose and fructose (Lobo, 1977). Hxk2 is the most abundant hexokinase at high glucose concentrations, while Glk1 and Hxk1 are predominant at low glucose concentrations. Mutants with reduced Hxk2 activity show partial loss of glucose repression, indicating a putative role of Hxk2 as a sensor of glucose concentration (Entian, 1982). In addition, several observations link Hxk2 to the Snf1 signalling pathway, e.g. Hxk2 interacts with Mig1 (Moreno, 2005) and the *SUC2* promoter (Ahuatzi, 2007) and regulates the binding of Reg1 to Snf1 (Sanz, 2000a) (for more details see section 3D).

The gene *EMI2*, which shows high sequence similarity to *GLK1*, has been shown to be downregulated in a Mig1/2-dependent fashion (Lutfiyya, 1998). In principle, this would make it a good candidate as a glucose-regulated hexose kinase. However, the only noticeable phenotype of *emi2*Δ is a mild defect in glycogen accumulation (Wilson, 2002). The concurrent deletions of *hxk1*Δ, *hxk2*Δ and *glk1*Δ prevent growth on glucose, fructose or mannose (Lutfiyya, 1998), indicating that Emi2 is unable to support growth on these sugars. This could be due to poor expression of *EMI2* in the presence of glucose. To test this hypothesis, *EMI2* was overexpressed using a strong inducible promoter in a triple hexose kinase deletion mutant. Transformants failed to grow in the presence of glucose, fructose or

mannose (D. Bosch unpublished results). In conclusion, Emi2 is either a non-functional protein (sequence analysis shows that some amino acids in the catalytic domain are different from other hexose kinases (Belinchon, 2007b)) or plays a presently unidentified role. Indeed, its gene expression clusters with that of cAMP regulated genes suggesting that it could play a role in phosphorylating intermediates of biosynthetic routes.

*2.4. Alcohol-free yeast, TM6**

The TM6* strain containing a single hexose transporter has minimal ethanol production when growing at high sugar concentrations, without either releasing secondary products or a drastic reduction in growth rate (Otterstedt, 2004). The *TM6** chimera consists of a fusion between the N-terminus of *HXT7* (transmembrane domains 1-6) and the C-terminus of *HXT1* (transmembrane domains 7-12) under the control of a strong constitutive promoter. The asterisk indicates a point mutation, which is essential for the properties of the chimera. The unusually low fermentative metabolism of the TM6* strain has been suggested to be related to reduced sugar transport, such that the pyruvate produced does not exceed the capacity of PDH (Otterstedt, 2004).

There are indications that in a wild type strain the uptake step exerts little or no control over glycolysis at high glucose concentrations (Elbing, 2004a). Probably, at low glucose concentrations, transport exerts an important control of the glycolytic rate, as has been observed in strains bearing variable amounts of the high affinity Hxt7 (Ye, 1999), and in chimeric hexose transporters, such as TM6*, where glucose repression is relieved at high external glucose concentrations (Elbing, 2004a). For instance, strains expressing either Hxt1, Hxt7 or Tm6* showed a progressive decrease in glycolytic flux accompanied by an increase in the control that transport exerts on glycolysis according to metabolic control analysis (MCA) (Elbing, 2004a).

2.5. Consequences of reduced sugar transport

In paper 1, strains with reduced glucose/fructose consumption were used to study the switch between fermentation and respiration. Strains lacking all hexose transporters (Null strain) were transformed with a single artificial transporter, Hxt1, Hxt7 or Tm6*, under the control of a constitutive truncated *HXT7* promoter. The strains were grown in continuous cultures to a steady-state using ethanol as carbon source. This ensured identical growth conditions for all strains. The steady state was perturbed by addition of glucose or fructose to a final concentration known to trigger an extensive glucose response (2%). For the four strains studied, consumption of glucose/fructose correlated well with the glycolytic flux (monitored by ethanol production). Only the TM6* strain displayed a purely respiratory phenotype as

observed previously in batch cultures (Otterstedt, 2004). In contrast to a previous study where the TM6* strain produced detectable amounts of ethanol during growth on fructose (Henricsson, 2005), we were not able to observe measurable noticeable concentrations. This could be due to differences in the genetic background of the strains used (V5 for Henricsson et al. and CEN.PK in this study). (Henricsson, 2005) also showed that strains with reduced transport rate (HXT7 and TM6*) accumulated glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P), whilst showing low concentrations of fructose-1,6-bisphosphate (F16BP); that situation was reversed in strains with a high glycolytic rate (wild type and HXT1). The high concentrations of F6P and G6P together with low levels of F16BP may indicate that phosphofructokinase (PFK) activity was diminished. A well-known regulator of PFK activity is F26BP, whose synthesis and degradation is regulated by cAMP-activated protein kinases (PKA) (Heinisch, 1996). These may be impaired in strains with reduced sugar uptake. A feature associated with low PKA activity is high levels of glycogen and trehalose, which is also shown in this experiment of the mutant strains. Modelling of the data also indicates changes in the kinetics properties of PFK and PGI, these together with alterations in the sensitivity of the respiratory metabolism for NAD^+ and NADH may explain the switch from fermentative to respiratory metabolism. It would be interesting to investigate whether this regulation is dependent on PKA, because it may indicate that the PKA response is able to regulate the glycolytic rate, which in turn could influence the intracellular glucose signalling pathway Snf1/Mig1 (for more details see section 3D).

2.6. The glucose response

The metabolism of baker's yeast is heavily dependent on the availability of sugars. For instance, the transport of galactose and maltose is stimulated by the presence of the corresponding sugars and the breakdown of sucrose and raffinose requires the presence of low concentrations of glucose or fructose. However, these catabolic processes are abolished in the presence of high concentrations of glucose/fructose (Gancedo, 1998; Melcher, 1997). This indicates the existence of a mechanism prioritising the consumption of fructose and glucose ahead of other sugars. This phenomenon has been extensively studied in baker's yeast under different names such as "Crabtree effect" (Crabtree, 1929; Ibsen, 1961; Ibsen, 1962) or "catabolic or glucose repression" (Entian, 1997c). The latter term will be used in this thesis. The extent of the glucose repression response can be fully appreciated at the mRNA level. During diauxic shift there is a change in the expression of about 1700 genes, one-third of the whole genome (DeRisi, 1997). This has also been confirmed at the protein level by two-dimensional gel electrophoresis (Haurie, 2004). In general, glucose repression causes downregulation of genes implicated in mitochondrial biogenesis, the glyoxylate cycle,

gluconeogenesis and utilisation of alternative carbon sources; whilst upregulating those involved in cell proliferation and glycolysis (Santangelo, 2006). The genetic response is also dependent on the concentration of glucose, e.g. gluconeogenesis genes are repressed by small quantities of glucose (*FBP1*), whilst at the same concentrations sucrose utilisation (*SUC2*) becomes upregulated (Paper 4). Indeed, the term "glucose repression" refers to a compilation of individual gene expression responses, each of them responding to a particular glucose concentration.

3. Glucose sensing and signalling

One of the most challenging mysteries in the field of cell biology is how cells monitor changes in their external and intracellular environment. How can changes in chemical concentrations, temperature, humidity, light, rigidity or electric charge be converted into information used by the cell? Sensors are present at the plasma membrane in the form of proteins that alter their structure or binding properties when exposed to stimuli (Darnell, 1986). Figure 4 depicts the main glucose sensing and signalling pathways and their inter-

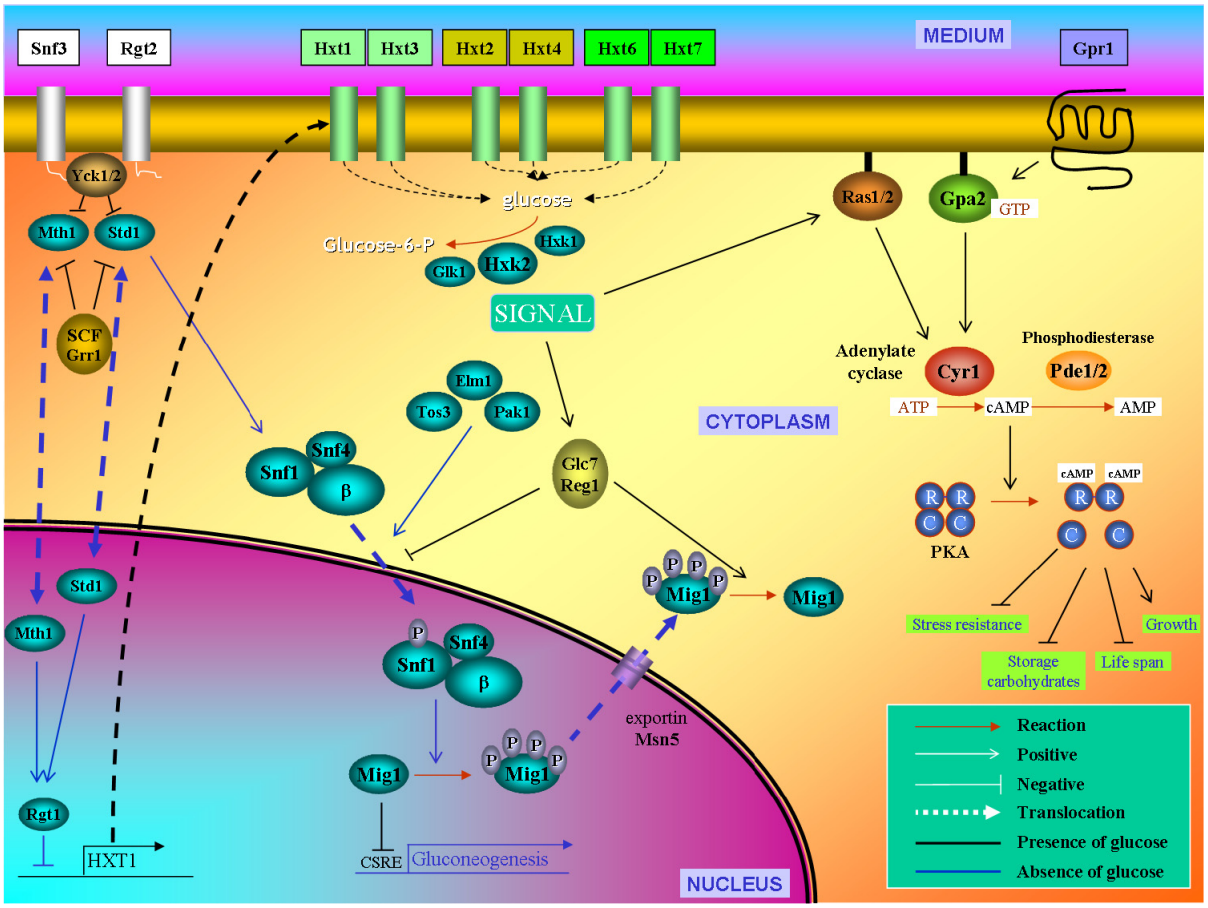

Figure 4. Sugar sensing and signalling in baker's yeast.

connexions in baker's yeast. Extracellular glucose/fructose is monitored by two different set of sensors. The first relies on a pair of modified hexose transporters, Snf3 and Rgt2, which

upon sugar binding trigger a signalling cascade leading to the expression of *HXTs*. The second depends on Gpr1, a sensor associated to a G-protein coupled receptor system (GPCR), which contributes to the activation of the PKA pathway, which in turn, is involved in cell cycle progression and stress resistance (Rolland, 2001b). Intracellular glucose sensors are more difficult to identify, since the influence of glucose affects the entire central carbon and energy metabolism. Two glucose signalling pathways have been characterised. One regulates the expression of genes whose expression is not required during fermentative growth and involves the Snf1 protein kinase and its main target the transcriptional repressor Mig1 (see review (Hedbacker, 2008)). Another corresponds to the RAS branch of the PKA pathway. In both, SNF1 and RAS pathways, glucose phosphorylation is required as the initial signal.

Since most of the research aimed at understanding sugar signalling pathways has been done using glucose as a carbon source, the terms *glucose sensing*, *glucose signalling* and *glucose signal* are commonplace in literature concerning the sugar repression response. This will also be the case in this thesis. However, it should be noted that glucose is not the only sugar able to trigger such a response; fructose is at least as effective (Gancedo, 1992).

3.1. Snf3/Rgt2 pathway

In yeast, a well-concerted regulatory pathway governs the expression of specific sugar transporters, ensuring efficient uptake at different external sugar conditions (Fig. 5).

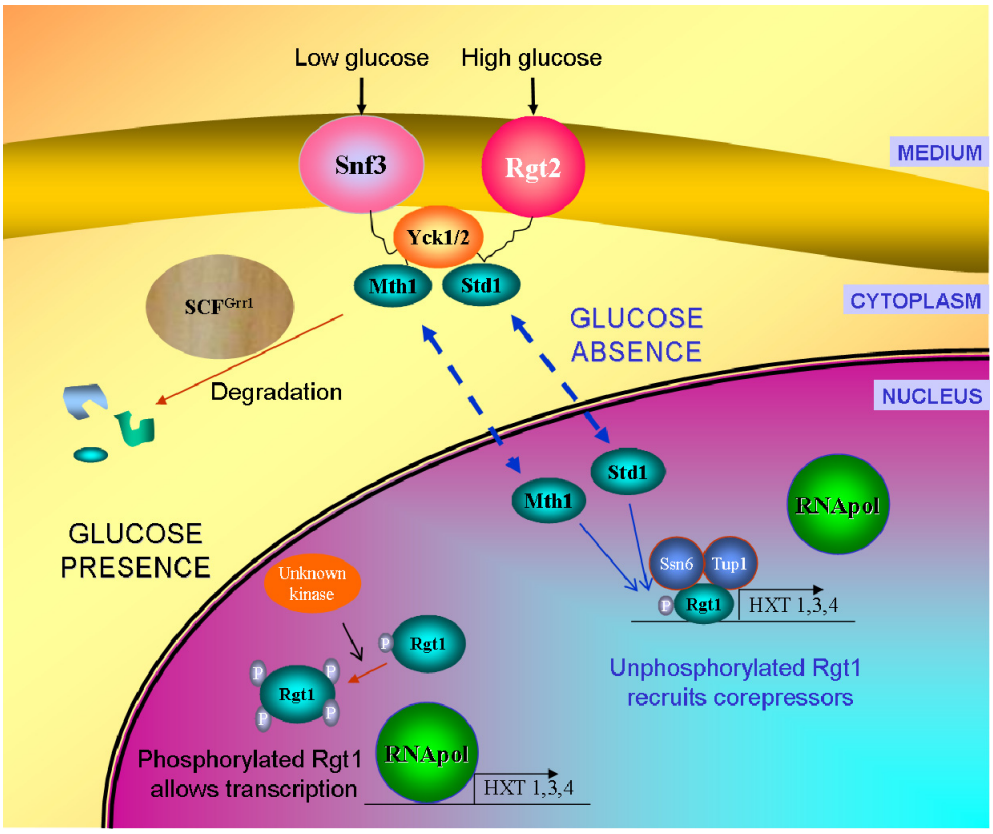

Figure 5. Snf3/Rgt2 signalling pathway.

Two cell surface sensors, Snf3/Rgt2, recognise glucose and induce expression of *HXT*. Most *HXTs* are repressed in the absence of glucose, a process mediated by the transcriptional factor Rgt1 in collaboration with Std1 and Mth1. Upon glucose addition, Snf3/Rgt2 trigger the degradation of Std1 and Mth1 and the relieve of repression by Rgt1 (see more below).

Snf3 and Rgt2

Snf3, the first component of this pathway to be identified (Neigeborn, 1984), is a high affinity sugar sensor found at the plasma membrane. Snf3 shares high amino acid similarity with the hexose transporter family (see section 2B). It contains twelve hydrophobic domains, corresponding to two groups of six membrane-spanning regions, separated by a stretch rich in charged residues. In addition, immunofluorescence studies confirmed Snf3 localisation to the plasma membrane (Celenza, 1988). Snf3 is a much larger protein than the average Hxt, with additional sequences predicted to face the cytosol at the amino (85 amino acids) and carboxy termini (303 amino acids) (Celenza, 1988). The function of the long carboxy terminus is particularly interesting constituting a key factor to understand Snf3 role as sensor. Screenings for multi-copy suppressors of the *snf3*Δ mutant in low glucose medium led to the identification of the first hexose transporters, i.e. *HXT1* (Lewis, 1991) and *HXT2* (Kruckeberg, 1990). The presence in that study (Kruckeberg, 1990) of cross-hybridising sequences in low-stringency Southern blots indicated the existence of highly related genes, that were subsequently characterised as additional hexose transporters: *HXT3*, *HXT4* (Ko, 1993; Theodoris, 1994), *HXT5-7* (Reifenberger, 1995) and *HXT8-17* (Boles, 1997).

Initially, Snf3 was thought to be also a transporter, but some of its features did not match with the identified Hxts. First, expression of *SNF3* was repressed at high glucose concentrations (Neigeborn, 1986). Second, *SNF3* expression was 500-fold weaker than that of other transporters, *HXT1-4* (Özcan, 1995). And third, the over-expression of *SNF3* in strains lacking *HXT1-7* (and hence crippled in glucose uptake) did not restore growth on glucose medium (Özcan, 1998).

The presence of *SNF3* alone does not explain the regulation of low-affinity transporters like *HXT1*, hence several authors postulated the existence of at least a second sensor (Ko, 1993; Özcan, 1995), later identified as Rgt2. This is a close relative of Snf3, sharing as much as 60% amino acid sequence identity. It also has a long carboxy-terminal stretch (218 amino acids) (Özcan, 1996a). As predicted, *rgt2*Δ strains show defects in low-affinity transport, while *snf3*Δ mutants are defective in high-affinity sugar transport, A point mutation in Rgt2 (Arg-231 to Lys), that is a conserved residue in all sugar transporters known in mammals and other organisms, makes the expression of the low-affinity transporter *HXT1* constitutive even in the absence of glucose. The same point mutation introduce into *SNF3* results in constitutive expression of the high-affinity transporter *HXT2* (Özcan, 1996a). Since Snf3 and

Rgt2 have long carboxy-terminal tails that are not present in other hexose transporters, several laboratories have studied their relevance for sugar sensing. In a *snf3*Δ mutant, the expression of the carboxy-terminal sequence of *SNF3* alone restored growth (Coons, 1997; Vagnoli, 1998) and attachment of this tail to *HXT1* or *HXT2* rendered partial *HXT1* expression (Moriya, 2004; Özcan, 1998). However, Moriya and Johnston found that overexpression of a tail-less *RGT2* could also stimulate *HXT1* expression and proposed that the carboxy-terminal domain may recruit or titrate other components of the system (Moriya, 2004) (see below).

In order to understand how Snf3 and Rgt2, two membrane proteins, regulate *HXT* expression is necessary to introduce the function of signalling components. The most decisive is probably *RGT1*, isolated in the same screening than *RGT2* (Marshall-Carlson, 1991).

Rgt1. *RGT1* encodes a putative DNA-binding protein with a predicted zinc finger motif, which is common in transcriptional regulators like *e.g.* Gal4 (Özcan, 1996b). Deletion of *RGT1* leads to the constitutive expression of both high- and low-affinity transporters (*HXT1-4*), pointing to a role as a *HXT* repressor (Özcan, 1995). Rgt1 is likely to recruit the co-repressors Cyc8 (Ssn6) and Tup1 to promoters (Smith, 2000; Tomás Cobos, 2002), since repression of *HXT* genes is constitutive in the absence of glucose in *cyc8*Δ and *tup1*Δ mutants (Özcan, 1996b). *RGT1* gene expression and protein localisation (constant nuclear accumulation) do not change at high or low glucose, indicating that is function is probably modulated by alterations in its DNA-binding capacity, which in turn has been proposed to be regulated by phosphorylation (Mosley, 2003). According to the model put forward by Mark Johnston, Rgt1 would be present in the absence of glucose in a low phosphorylation state with enhanced affinity for *HXTs* promoters. In the presence of glucose, Rgt1 would be phosphorylated at several places (i.e. serines 735, 755 and 758), thus losing the ability to act as a repressor (Kim, 2003; Mosley, 2003; Polish, 2005). Indeed, the different levels of repression observed in *HXTs* and other targets genes (*e.g. MIG2*, *MIG3*) seem to correlate well with the number of predicted Rgt1 binding sites in their promoters.

The picture that emerges from the analysis of *rgt1* mutants is the following: expression of the low-affinity transporter *HXT1* is repressed by Rgt1 in the absence of glucose; the repression is relieved by high levels of glucose, but maximal expression is dependent on a yet unknown mechanism. It has been proposed that Rgt1 would act as an activator under these conditions, but paradoxically its DNA-binding capacity is impaired (Özcan, 1996b; Polish, 2005). *HXT2* and *HXT4* are repressed by Rgt1 in the absence of glucose, and by Mig1 in the presence of high levels of glucose (deletion of genes encoding glucose repression components Hxk2, Reg1 or Mig1 leads to constitutive derepression), therefore *HXT2* and

HXT4 are only expressed at low glucose concentrations. Finally, *HXT3* expression is induced by the presence of glucose, no matter the concentration (Özcan, 1995).
Grr1, Std1 and Mth1 mediate signalling between Snf3/Rgt2 at the plasma membrane and Rgt1 in the nucleus.

Grr1. *GRR1* (Glucose Repression Resistant) was isolated in a screen for mutants defective in glucose repression of *SUC2* (Bailey, 1984). Grr1 is a F-box component of the E3 ubiquitin ligase complex (the other two components are Skp1 and a cullin protein, Cdc53) involved in ubiquitination and degradation of proteins (Li, 1997). Deletion of *GRR1* has pleiotropic effects, such as, elongated cell shape, sensitivity to osmotic stress and sporulation deficiency (Flick, 1991; Vallier, 1991). *grr1* mutants show high levels of invertase activity during growth on glucose but are unable to grow in raffinose as the sole carbon source. Sugar transport assays showed that *grr1*Δ mutants have an abnormally low uptake of glucose, therefore explaining the high *SUC2* expression levels (Özcan, 1994; Vallier, 1994). This was an early indication that glucose repression can be uncoupled from external sensing by diminishing transport. This has been shown later in strains with reduced sugar transport (*i.e.* TM6* strain) (Paper 1) (Otterstedt, 2004). The second indication of the role of Grr1 in sugar sensing was the isolation of *grr1* mutants as suppressors of the growth defects of *rgt1*Δ (Erickson, 1994). In summary, Grr1 is involved in a number of processes where it exerts regulation by targeting proteins for degradation. In sugar signalling, Grr1 antagonises the action of Rgt1, not by direct degradation of this transcription factor, but rather by targeting two other proteins that collaborate with Rgt1: Std1 and Mth1 (Flick, 2003).

Std1 and Mth1. *STD1* was identified in two different screens: first, as multi-copy suppressor of the deficiency of a TATA binding protein (Suppressor of TBP Deletion) (Ganster, 1993); and second, as multi-copy suppressor of the deficiency in *SUC2* expression of *snf1*Δ, *MSN3* (Hubbard, 1994). *MTH1* (MSN Three Homologue) was uncovered due to sequence homology to *STD1* (Hubbard, 1994), though previously its disruption was found to cause the phenotype of a *HTR1* (Hexose Transport Regulation) mutant (Özcan, 1993; Schulte, 2000). *STD1* and *MTH1* might not just be redundant. Std1 seems to have a close connection with Snf1 both physically (Hubbard, 1994), and functionally: increased dosage of *STD1* partially restores Snf1 activity in a *snf4*Δ mutant. Whereas, Mth1 does not seem to interact with Snf1 or affect its function (Hubbard, 1994). Several pieces of evidence support the specific role of Std1 in the regulation of low-affinity transport and Mth1 in the control of high affinity transport. First, *STD1* expression, like that of *RGT2*, is not dependent on glucose, while expression of *MTH1*, like that of *SNF3*, is repressed by high glucose; second, the low-affinity transporter *HXT1* is repressed in both single deletion mutants *std1*Δ or *mth1*Δ but expressed in the

double mutant. The high-affinity transporters *HXT2-4* are insensitive to *STD1* deletion, but become 400-fold derepressed if *MTH1* is knocked out (Schmidt, 1999). The mechanism of action of Std1 and Mth1 remains unclear. Several authors have shown two-hybrid interactions with the carboxy-terminal tails of both Rgt2 and Snf3 (Lafuente, 2000; Schmidt, 1999), as well as with Rgt1 (Lakshmanan, 2003). These observations together with the finding that the double mutant *mth1*Δ *std1*Δ renders Rgt1 constitutively phosphorylated irrespectively of carbon source (Lakshmanan, 2003), and that Mth1 degradation upon addition of glucose is dependent on Grr1 (Flick, 2003) suggested a new model. Mth1 and Std1 enhance the repression activity of Rgt1 in the absence of glucose and are degraded via Grr1 when glucose is present at high concentrations (Polish, 2005). Some details are still unclear due to conflictive observations. (Flick, 2003) suggests that Std1 degradation occurs independently of Grr1. However, (Kim, 2006) argue that Std1 degradation is Grr1-dependent, but this event is obscured by an increase in *STD1* expression dependent on Rgt1. Recently, degradation of both Mth1 and Std1 has been confirmed (Pasula, 2007).

Yck1 and Yck2. Proteins are often marked for degradation by phosphorylation. Screenings for kinases acting on Std1 and Mth1 led to the discovery of a new player, Casein Kinase I (CKI) (Moriya, 2004). The genes, *YCK1* and *YCK2* encode two isoforms of CKI. CKI is a membrane bound kinase that is implicated in a number of cellular processes (e.g. morphogenesis, proper septin assembly, endocytic trafficking, etc.) (Robinson, 1993). CKI also interacts with Snf3 and Rgt2 (Moriya, 2004).
A major contribution to the understanding of this signalling pathway has come from the network analysis performed by (Kaniak, 2004). All putative targets of Rgt1 were defined and their contribution to gene expression measured. The authors concluded that the Snf3/Rgt2 pathway is in close connection with the Snf1/Mig1 pathway and that crosstalk aids in fine-tuning gene expression according to glucose abundance. For instance, in the absence of glucose Rgt1 would not only repress expression of *HXT* genes, but also that of *STD1*, *MIG2* and *SUC2*. In the presence of glucose, Mig1 and Mig2 would repress expression of low-affinity transporter genes *HXT2*, *HXT4* and also *SUC2*, *SNF3*, *MTH1* and *MIG1*. However, in Paper 2, we did not observed any significant change in expression of genes participating in this network (*SUC2*, *SNF3*, *STD1*, *MTH1*, *MIG1*, *MIG2* or *MIG3*)

Current hypothesis. The simplest picture of the regulation of hexose transporters is summarised in Fig. 5. Snf3 and Rgt2 are membrane proteins with different sugar binding affinities. Snf3 transduces a signal at low glucose concentrations whilst Rgt2 does so at high concentrations. CKI, Mth1 and Std1 interact with both Snf3 and Rgt2. Probably Std1 and Mth1 shuttle between cytoplasm and nucleus and maintain a dynamic equilibrium that

favours interaction with Rgt1 in the absence of a signal. The binding of sugar to the receptors enhances the rate of phosphorylation of Mth1 and Std1 by CKI. Then, the phosphorylated forms of Std1 and Mth1 are ubiquitinated by the SCF^{grr1} complex, and presumably degraded, hence reducing the number of copies able to bind to Rgt1. Lack of Std1 and Mth1 reduces Rgt1 DNA-binding capacity or its ability to recruit the co-repressors Tup1 and Cyc8. In addition, Rgt1 is hypephosphorylated by an unknown kinase. The outcome is the release from repression of selected hexose transporter genes. In addition, there is an interesting cross talk with the glucose repression pathway, i.e. Snf1/Mig1. In the presence of high glucose, Mig1 and Mig2 seem to repress expression of *HXT* genes encoding high-affinity transporters, while in the absence of glucose Rgt1 represses *MIG2*. There is also evidence that Std1 fosters Snf1 activity under the same conditions. In summary, the Snf3/Rgt2 pathway mediates the expression of *HXT*s according to the extracellular glucose concentration.

Open questions. The model described above summarises the best-characterised components and their interactions, but it does not explain certain observations. **First**, yeast cells present a wide range of hexose affinities (see section 2B) that vary according to external glucose concentration. However, the specific sugar affinities of Snf3 and Rgt2 are unknown. How can their combined action elicit specific *HXT* expression? **Second**, the mechanisms regulating Rgt1 are poorly characterised. For instance, the kinase that mediates its phosphorylation has not yet been identified. Indeed, its suggested function as activator of *HXT1* expression at high sugar levels is insufficiently supported in the literature. **Third**, a member of this pathway uncovered by multi-copy suppression of *snf3*Δ, the kinase Sks1, has not yet been accommodated in the model (Yang, 1996).
Amongst the experiments that could shed light on the functioning of the pathway. First, (Kruckeberg, 1998) suggested to exchange the tails of Snf3 and Rgt2 to reveal whether these portions or the membrane domains retain sensing specificity. Second, measuring the sensibility of Snf3 and Rgt2 to different concentrations of sugars could be achieved by monitoring the expression of *HXT* promoters in the Null strain carrying a single sensor (Wieczorke, 1999).

Connexions to other signalling pathways. Sensing and signalling mechanisms are usually integrated to coordinate rapid responses. The regulation of *HXT1* is a case in point. (Tomás Cobos, 2004) observed that a mild osmotic stress increased *HXT1* expression even in the absence of glucose. This response was dependent on the Hog1 MAPK pathway, which monitors osmotic pressure. This indicates that Hog1 collaborates with Snf3/Rgt2 in sugar sensing. Nitrogen availability signalled via the TOR pathway also seems to play a role in

controlling *HXT1* expression, since addition of rapamycin, a TOR inhibitor, blocks *HXT1* expression (Tomás Cobos, 2005). There are two connexions between Snf1/Mig1 and Snf3/Rgt2 (Tomás Cobos, 2002). Std1 seems to be required for increased activity of Snf1 and Rgt1 regulates the expression of *MIG1* (Kaniak, 2004).

3.2. PKA pathway

Cells growing on respiratory carbon sources experience a transient increase in cAMP levels upon addition of glucose (Rolland, 2001b). cAMP functions as a second messenger initiating a phosphorylation cascade that leads to metabolic rearrangements, cell proliferation and loss of stress resistance (Geladé, 2003). In yeast, the main role of cAMP is to activate Protein Kinase A (PKA). Inactive PKA is a tetrameric complex containing two regulatory subunits, encoded by *BCY1*, and two catalytic subunits, encoded by *TPK1*, *TPK2* and *TPK3*. Bcy1 seems to sequester the catalytic subunits to prevent their activity. The binding of cAMP to Bcy1 leads to the release of Tpks, which phosphorylate specific targets. The list of downstream targets include: enzymes involved in carbon metabolism (e.g. fructose-1,6-bisphosphatase (Fbp1), 2-phosphofructokinase (Pfk26)), transcriptional regulators such as Msn2 and Msn4, protein kinases such as Rim15 and the phosphodiesterase Pde1 (Thevelein, 1999). The later is considered a feedback loop that allows the pathway to return to basal activity. A balance between synthesis, mediated by adenylate cyclase (Cyr1), and degradation catalysed by two phosphodiesterases (Pde1 and Pde2) regulates the levels of cAMP. Adenylate cyclase localisation at the cytosolic face of the plasma membrane facilitates the interaction with the two activating branches, the GPCR system, which monitors extracellular glucose, and the RAS pathway which senses glucose inside the cell (Wang, 2004).

Addition of 100 mM of glucose to a culture growing on respiratory carbon sources leads to a rapid increase in cAMP with peak-values at around 1 min after glucose addition (Rolland, 2001a). Fructose causes a less pronounced cAMP induction. Interestingly, a strain devoid of the most active transporters Hxt1-7 (hxt-Null strain) does not display a raise in cAMP levels, indicating that sugar transport is required to trigger a signal (Rolland, 2001a). Addition of small amounts of maltose (0.025%) before the glucose pulse allows for a cAMP response. Therefore, external glucose is not sufficient to trigger a cAMP response, unless is accompanied by a small intracellular glucose increase (Rolland, 2002). Glucose phosphorylation has also been shown to be required for cAMP to raise (Beullens, 1988).

The Gpr1/Gpa2 branch

The G-protein coupled receptors (GPCR) are a family of eukaryotic proteins involved in secondary messenger signalling cascades (Filmore, 2004), which are associated to G-proteins. These are generally trimeric membrane-associated proteins, where the alpha subunit is bound to GTP or GDP, and beta and gamma subunits are closely linked. The change of conformation of an active GPCR causes the G-protein to substitute GDP for GTP, resulting in a conformation, which is able to activate a membrane-bound enzyme or ion channel. The inherent GTPase activity of the alpha subunit allows the system to return to its initial state (Alberts, 2002). In *S. cerevisiae*, there are only three GPCRs, two (Ste2 and Ste3) mediate the pheromone response, and another (Gpr1), responds to the presence of sugar (Versele, 2001). In the glucose responsive system, Gpa2 is the alpha subunit, Gpb1 and Gpb2 are two beta subunits and the gamma subunit has yet to be identified (Versele, 2001). Gpa2 mediates activation of adenylate cyclase (Kraakman, 1999). The return of Gpa2 to its GDP-bound form is stimulated by Rgs2 (Rolland, 2002).

Experiments aimed at measuring the affinity of Gpr1 for different sugars have been carried out using the intracellular cAMP concentration as read out. Of all the sugars tested only glucose and sucrose at concentrations of about or higher 20-30mM were able to trigger a response. This would indicate that the system is only efficient at medium to low sugar conditions, perhaps during the shift to a respiratory metabolism (Rolland, 2001a).

The RAS branch

The small GTP-binding proteins Ras1 and Ras2 are activated by an intracellular signal, which requires phosphorylation of a fermentable sugar since it does not occur in the triple knock-out strain *hxk1*Δ *hxk2*Δ *glk1*Δ (Colombo, 2004). Although several proteins acting as regulators of Ras1/2 have been identified (i.e. Ira1/2 as negative and Cdc25/Sdc25 as positive) little is known about the initial signal that triggers their activation, except that glucose phosphorylation is required (Santangelo, 2006). The strongest effector is intracellular pH acidification, which also occurs upon glucose addition (Rolland, 2001a).

3.3. Separating intra- and extracellular responses

Work from the Gustafsson lab has shown that high extracellular glucose does not automatically lead to glucose repression. A strain (TM6*) with reduced glucose transport at high glucose levels behaves like the wild type strain at low glucose, hence indicating that glucose repression is independent of external glucose concentrations (Elbing, 2004b). In order to separate responses mediated by intra- or extra-cellular glucose, the gene expression profile of a strain with completely abolished glucose transport (Null) has been

studied (Paper 2). This strain lacks all members of the Hxt family, as well as some other putative hexose transporters (see section 2B), whilst maintaining all sensors. Therefore, *null* mutants can detect the presence of glucose, but cannot take it up. First, we investigated the gene expression profile of our wild type strain (prototrophic CEN.PK) following glucose addition to steady state ethanol-growing cells. We observed a significant change in expression for about 800 genes. This number was lower than those reported previously: 2,400 genes (Wang, 2004), 1,700 genes (DeRisi, 1997; Ronen, 2006) and 1,154 genes (Kresnowati, 2006) respectively. Strain background, cultivation methodologies, the time point after the sugar pulse and mRNA technologies might be reasons for such variations. In any case, categorisation of genes that change expression revealed a pattern common to previous studies. Following the glucose pulse there was a strong downregulation of genes implicated in mitochondrial and peroxisomal biogenesis, lipid metabolism and catabolism of alternative carbon sources. On the other hand, significantly upregulated genes encoded hexose transporters, members of RNA and DNA synthesis machinery and ribosome components.

The Null strain is completely insensitive to the presence of glucose in the environment. This indicates that the cell surface glucose sensors serve two main purposes: initiating the expression of HXTs to promote import of hexoses and to aid in the development of the glucose response in concert with intracellular signaling pathways. This double check mechanism ensures that glucose is both available in the medium and intracellularly.

3.4. Snf1/Mig1 pathway

As indicated above, yeast cells sense the external sugar concentration by means of membrane-associated proteins, but the great bulk of metabolic rearrangements that characterise the glucose repression response require sugar internalisation and phosphorylation by hexokinases (Carlson, 1999). Intracellular glucose initiates a cascade of events that lead to inactivation of the SNF1 protein kinase. How these events are connected is an enigma. SNF1 regulation is discussed in detail in the following section. For clarity, the trimeric kinase complex is denoted as SNF1 and its catalytic subunit as Snf1 (for recent reviews see (Hedbacker, 2008; Santangelo, 2006)).

In short, the pathway components are Hxk2, as the main hexose kinase during growth on glucose; Snf1 activating kinases (SAK); protein phosphatase 1 (Reg1-Glc7); SNF1 trimeric complex (Snf1, Snf4 and either Gal83, Sip1 or Sip2); Mig1 (best studied Snf1 target); *SUC2* and *FBP1* expression (read-outs of glucose repression).

SNF1 protein kinase complex

The SNF1 complex belongs to a family of well-conserved protein kinases defined by the mammalian homologue AMPK. SNF1/AMPK is implicated in the sensing and regulation of the energy state of cells and entire multi-cellular organisms (Kemp, 2003). In baker's yeast, SNF1 is mainly required for growth on poor carbon sources (Celenza, 1986; Orlova, 2006), but has also been implicated in metabolic, oxidative, high pH, high Na^+, and other stress responses. (Dubacq, 2004; Hahn, 2004; Hong, 2007; Mayordomo, 2002; Orlova, 2006; Portillo, 2005; Tian, 2007) (for review see (Sanz, 2003)). The heterotrimeric complex contains an alpha catalytic subunit (Snf1), a gamma activating module (Snf4) and a beta scaffolding component (either Gal83, Sip1 or Sip2) (Carling, 2004).

***SNF1*, alpha catalytic subunit** (Sucrose Non-Fermenting) was identified in two independent screens for mutants unable to ferment sucrose (Carlson, 1981; Zimmermann, 1977). Deletion of *SNF1* prevents growth on fermentable sugars different from glucose and fructose (sucrose, raffinose, maltose and galactose) and on non-fermentable carbon sources (glycerol, ethanol acetate). In addition, it also decreases growth rate at low glucose concentrations, an effect alleviated at high concentrations (7.5% or above). Other defects include sensitivity to heat stress, deficiency in glycogen accumulation and low sporulation success (less than 1%, probably due to the use of acetate as carbon source in sporulation media). *SNF1* was mapped and cloned in 1984 and sequenced two years later, revealing a 72 KDa protein, with similarities to serine/threonine protein kinases (Celenza, 1984a, 1984b, 1986). Snf1 was the first documented yeast protein kinase.

Until now, Snf1 gene expression was considered constitutive (Celenza, 1984b, 1986), however recent data from our laboratory indicates an increase in the abundance of *SNF1* mRNA during glucose depletion coinciding with the increase in Snf1 phosphorylation levels (R. Garcia-Salcedo, unpublished results). This effect has not been reported in glucose shift experiments, indicating that Snf1 has comparable levels during growth on high and low glucose and changes may be transient. Analysis of the *SNF1* promoter region indicates the existence of four putative Gcn4 sites. Gcn4 is a transcription factor involved in induction of genes in response to amino acid starvation, in line with evidence that Snf1 becomes more active at low nitrogen conditions (Orlova, 2006).

Snf1 is composed of a catalytic (N-terminal 1-392 aa) and a regulatory (C-terminal 393-633 aa) (Kuchin, 2003) domain (Fig. 6). The catalytic domain contains the protein kinase functions, whilst the second is required for interaction with beta and gamma subunits of the complex. The regulatory domain has been proposed to mask the catalytic domain in the presence of glucose, a phenomenon defined as auto-inhibition, thereby maintaining an open/close conformation that determines Snf1 activity (Jiang, 1996). In the N-terminal end

there is a “natural histidine tag”, thirteen consecutive His residues (18-30 aa) which seem to be dispensable for function (Celenza, 1989a). Threonine in position 210 (T210) is phosphorylated depending on growth conditions (Estruch, 1992) and determines the open or closed configuration of the protein. Changing T210 to alanine or aspartate results in a *snf* phenotype (Jiang, 1996). The conserved lysine 84 in the ATP-binding site is also essential for Snf1 activity, as point mutations abolish Snf1 kinase activity (Celenza, 1989a). Finally, replacement of the non-conserved residue 53 (glycine) with arginine bypasses the requirement for Snf4 (Celenza, 1989a; Estruch, 1992).
It has been proposed that the catalytic domain functions as a dimer (Nayak, 2006), however this is not supported by measurements of light scattering (Rudolph, 2005) indicating that it may constitute a crystallisation artefact.

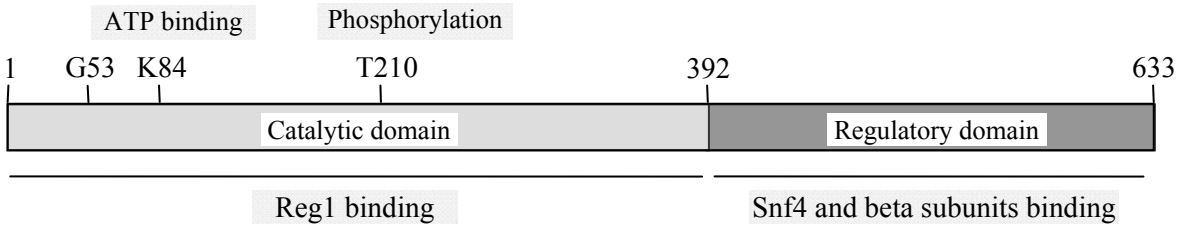

Figure 6. Simplified Snf1 structure.

***SNF4*, the gamma regulatory subunit** was isolated in a screen for mutants able to grow aerobically on glucose but not on sucrose when respiration was blocked (Neigeborn, 1984). Its deletion renders a nearly complete *snf* phenotype that can be partially complemented by overexpression of Snf1 (Neigeborn, 1984). Indeed, interaction with Snf1 was described during some of the first two-hybrid interaction analysis and has also been confirmed by co-immunoprecipitation studies (Celenza, 1989b). It has been proposed that Snf4 stabilises the open conformation of Snf1 (see more below) (Jiang, 1996). mRNA and protein levels of Snf4 do not seem to be glucose regulated (Celenza, 1989b). There is a long-standing controversy about whether Snf4 is able to bind AMP, therefore stimulating its interaction with Snf1 (see more below).

***GAL83*, *SIP1* and *SIP2*, the beta targeting subunits** were identified by their ability to bind Snf1 (Jiang, 1997; Yang, 1994). Since only one of the three can participate in the complex at any time there are *de facto* three types of SNF1. Concomitant deletion of *GAL83*, *SIP1* and *SIP2* gives rise to a *snf* phenotype (Schmidt, 2000) and deletion of two of them impairs Snf1 activity. The beta subunits are always in contact with both Snf1 and Snf4 (Jiang, 1997; Yang, 1994), therefore it has been proposed that their main role is to provide stability to the complex. Another presumed function is regulation of the subcellular localisation of the entire

complex depending on the carbon source (Vincent, 2001). In the presence of high glucose Snf1 is found mainly in the cytoplasm. Upon a shift to low glucose (generally 0.5 g/L) the complex relocates largely to the nuclear periphery and the vacuolar membrane. Gal83 has been shown to mediate nuclear accumulation of Snf1, while Sip1 and Sip2 promote Snf1 localisation to the vacuolar membrane and cytosol, respectively. Though the regulation of the β subunits remains largely uncharacterised, Snf1-Sip1 has been linked to the PKA pathway (Hedbacker, 2004). Despite the fact that the three beta subunits seem to have different targeting functions any one of them can support growth on carbon sources that require Snf1 activity, indicating that their functions are overlapping.

Upstream kinases: Elm1, Sak1 and Tos3, positive regulators of Snf1

The main regulatory switch controlling Snf1 activity rests on the phosphorylation of the T210 residue (Estruch, 1992). However, for more than ten years the nature of Snf1's upstream kinase(s) remained elusive, which then was explained by the fact that there are three such kinases with redundant functions. In 2003, two laboratories independently obtained a *snf* phenotype by concurrently knocking out three protein kinases: Elm1, Tos3 and Sak1 (previously known as Pak1) (Hong, 2003; Sutherland, 2003). All of them have functions unrelated to Snf1, although only deletion of Elm1 displays a strong phenotype (filamentous growth). Elm1 is implicated in cellular morphogenesis and forms part of the bud neck ring (Bouquin, 2000). A one-to-one correspondence between these three upstream kinases (SAKs) and the three distinct forms of SNF1 complexes has been ruled out (Hedbacker, 2004), nevertheless certain preferential interactions have been described (McCartney, 2005). *In vitro* measurements of Snf1 activity and purification studies suggest that Sak1 is the most important kinase particularly as an activator of Snf1-Gal83 (Elbing, 2006; McCartney, 2005). However, this could be a consequence of its high abundance compared to other SAKs, rather than possessing unique features (Hedbacker, 2004). Little is known about the regulation of SAKs, one possibility would be that their activity is constitutive or at least independent of the presence of glucose (Hedbacker, 2008; Rubenstein, 2007). This would indicate that the initial glucose signal is not mediated by the kinases but rather via the phosphatase Glc7-Reg1.

Protein phosphatase 1, negative regulator of Snf1

Protein Phosphatase 1 (PP1) regulates a large number of cellular processes including cell cycle control, cell wall remodelling, glycogen accumulation, sporulation, meiosis, glucose signalling, etc. (Santangelo, 2006) by dephosphorylating target proteins. Since Snf1 is

activated by phosphorylation and deactivated by dephosphorylation PP1 is a negative regulator of Snf1.

PP1 is a protein complex that consists of the catalytic subunit, Glc7, a regulatory subunit and often a scaffolding component. Glc7 is an essential protein. It is targeted to specific substrates by interaction with one of seventeen different regulatory subunits (Santangelo, 2006). Reg1 is the main regulatory subunit implicated in glucose signalling, though the paralogue Reg2 also plays a role (Jiang, 2000). The scaffolding component, Sip5, stabilises Reg1 interaction to targets such as Snf1, but it is not essential for function (Sanz, 2000b). PP1 dephosphorylates Snf1 (Ludin, 1998) and Hxk2 (Alms, 1999). Reg1 was identified in screens for mutants with constitutive *SUC2* expression, but its deletion also causes glycogen accumulation and slow growth on glucose (Niederacher, 1991). The double deletion *reg1*Δ *reg2*Δ severely impairs growth, an effect that is suppressed by deletion of *SNF1*, illustrating that hyperactive Snf1 is deleterious to the cell (Frederick, 1996). The interaction with Glc7 requires the phenylalanine 468, since a point mutation to arginine (F468R) prevents glucose repression (Alms, 1999). Two-hybrid interaction and protein purification analysis indicates that in the absence of glucose Reg1 brings Glc7 close to threonine 210 of Snf1 (Ludin, 1998; Sanz, 2000a). The capacity of Reg1 to recruit PP1 to Snf1T210 seems to correlate with the phosphorylation state of Reg1 (Sanz, 2000a). According to this model, at low glucose concentrations Snf1 phosphorylates Reg1, preventing it from recruiting Glc7 to Snf1T210. At high glucose concentrations Glc7 dephosphorylates Reg1, strengthening its interaction with Snf1. In addition, Hxk2 seems to play a role in keeping Reg1 hyper-phosphorylated during glucose-limiting conditions (Sanz, 2000a). Increasing the complexity of the situation, Reg1 regulates the phosphorylation state of Hxk2 (Alms, 1999), though it is unclear how this affects Hxk2 activity (Bisson, 2003). It appears that the system is controlled by multiple feedback and feed-forward loops, whose understanding will likely require not only detailed quantitative molecular information but also mathematical modelling.

It has been proposed that the ability of Reg1 to target Glc7 to different substrates is modulated by its association to Bmh1/2 (Dombek, 2004). The mechanism put forward relies on the capacity of Bmh1/2 plant homologues to regulate the activity of nitrate reductase when bound to AMP, therefore suggesting that Bmh1/2 could act as an energy sensor (Comparot, 2003).

Regulation of the SNF1 complex

In summary, two main factors seem to play a role in SNF1 regulation, one is the open/closed conformation of the catalytic subunit, controlled by Snf4 and T210 phosphorylation, and the second is the subcellular localisation of the complex (Santangelo, 2006).

Open/closed Snf1 conformation

- Snf4 stimulation.

Snf4 interaction with Snf1 at low/absent glucose concentrations is required to attain an open Snf1 conformation (Fig. 6) (Jiang, 1996; Sanz, 2000a). Snf4 itself does not seem to be regulated by environmental signals. This is most intriguing, because its mammalian counterpart, AMPKγ, has been shown to stimulate the activity of the alpha subunit by binding to adenosine nucleotides (Kemp, 2003). AMPKγ contains two bateman domains, which have affinity for two AMP, ADP or ATP molecules. These domains are partially conserved in Snf4 (Rudolph, 2007), however the SNF1 complex does not seem to be sensitive to activation by nucleotides, at least not *in vitro* (Hardie, 1998). Some reports indicated that a low ATP:AMP ratio correlates with elevated Snf1 activity (Wilson, 1996). Nevertheless, in Paper 1, we show that in the TM6* strain, which is constitutively de-repressed (Elbing, 2004b) the ATP:AMP ratio is considerably higher than for the corresponding wild type under the same conditions, which argues against an activating role of AMP.

To test whether SNF1 is responsive to ATP:AMP changes, our laboratory tested Sfn1 phosphorylation after a thiamine pulse. It is known that the transport and subsequent pyrophosphorylated of thiamine leads to a transient accumulation of AMP but not ADP (D. Mojžita, unpublished results). In these experiments, a transient increase in Snf1P was observed during the first seconds after the pulse. This may indicate that Snf1 indeed responds to an increase in AMP. The drop in ATP is quickly counteracted by other mechanisms, for instance by the action of adenylate kinase (Adk1). Thus, we have observed that deletion of *ADK1*, causes a constitutive activation of the Snf1 complex and partial *SUC2* de-repression (D. Mojžita, unpublished results).

So far, modifications introduced into Snf4 to express the bateman domains of AMPKγ, in order to make yeast sensitive to AMP, have failed (D. Carling, personal communication),

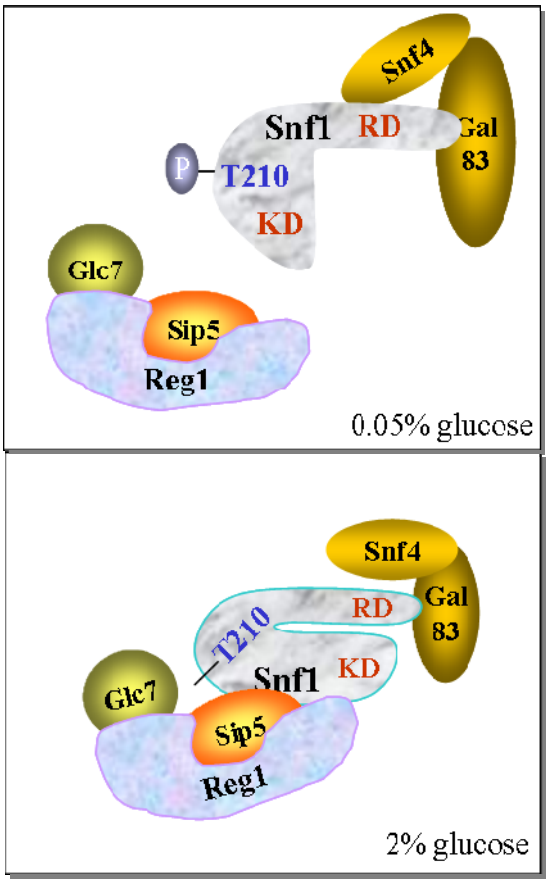

Figure 6. Open and close conformation of the SNF1 complex, redrawn according to (Sanz, 2000a).

indicating that the structure of Snf4 is very sensitive to modifications. Our laboratory has recently attained such transplantation, but strain growth was slow compare to wild type (D. Mojžita, unpublished results). It would be of great importance to develop a yeast strain with a Snf1 sensitive to AMP since it could constitute a good test system for compounds, which could be used in mammalians to fight Diabetes Type II.

- Phosphorylation of Snf1-T210.

The regulation of Snf1 conformation is dependent on phosphorylation of threonine 210, which correlates well with Snf1 activity (McCartney, 2001)(Paper 4). Time-course experiments show an inverse correlation between extracellular levels of glucose and Snf1-P (Paper 4). The isolation of Snf1 upstream kinases raised expectations for a regulatory mechanism that could explain Snf1 phosphorylation (Hong, 2003). However, SAKs purified at high and low glucose showed no differences in their kinase activity, pointing towards constitutive activity (Rubenstein, 2008). This finding, together with the modelling presented in Paper 4 redirected the interest on Snf1P from SAKs to the regulation of PP1.

There are two types of hypothesis: 1) those that consider that the glucose signal influences the conversion rate of Reg1 to Reg1P, and 2) those including another interacting or unknown protein.

1) Most likely, there is a dynamic equilibrium between the phospho and unphopohorylated forms of Reg1, governed by the strength of the glucose signal. This equilibrium could be regulated by an increase in Glc7-dependent dephosphorylation or by preventing Snf1 to access Reg1. The latter option seems the most likely since Glc7 activity is not known to be regulated. Reg1 has a constitutive cytosolic localisation (Dombek, 1999), whilst Snf1 accumulates in different compartments according to glucose abundance. This mechanism could explain Snf1 activation by escaping from its interaction with Reg1. However, this model has a major flaw, deletion of single or double beta subunits still renders an active Snf1, even though the localisation pattern is disrupted (Rubenstein, 2008).
2) Another alternative would be that another protein would protect the phosphorylation sites, preventing deactivation at high glucose concentrations. Several proteins have been suggested to play such a role, i.e. Bmh1/2, (Rubenstein, 2008) as explained above, and Hxk2. The latter has been suggested to either stimulate Snf1 dephosphoryaltion or prevent phosphorylation by directly interacting with Reg1 (Sanz, 2000a). A similar mechanism has been proposed to regulate the phosphorylation state of Mig1 (see more below).

Another interesting question concerns the proportion of total Snf1 that becomes phosphorylated at activating/deactivating conditions. We have observed that during growth at 4% glucose 10-15% of Snf1 remained phosphorylated, whilst in the absence of glucose 80% was phosphorylated (R. Garcia-Salcedo, unpublished data). In addition, we lack information on possible cell-to-cell variations in the response to glucose since all measurements are done on cell populations: when 30, 50 or 90% of Snf1 is phosphorylated does this means that there is a subpopulation of cells with 100% Snf1-P and another with none? Is Snf1-P uniformly distributed throughout the cell or does it accumulate in compartments? These questions can be addressed with current technologies. For instance, Snf1 and Snf1-P forms could be monitored by taking advantage of specific antibodies by *in situ* hybridisation (Aguilaniu, 2003) in a confocal microscope. This would help determine whether cells contain any active Snf1 and where is located. Indeed, this approach can be used to follow the distribution of several SNF1 specific complexes by simultaneously labelling beta subunits and Snf1.

SNF1 localisation

When glucose is abundant, Snf1-GFP is distributed throughout the entire cell, except the interior of the vacuole. Upon a shift to alternative carbon sources Snf1 accumulates in the nucleus, but also at the periphery of the vacuole without completely disappearing from the cytosol (Vincent, 2001). The distinct localisation is the only property of Snf1 that depends on the nature of the β subunits. However, whether it has an effect on Snf1 function is unclear, since Snf1 seems to be able to perform all its functions with a single beta subunits present (Schmidt, 2000). Since the best-characterised function of Snf1 is the phosphorylation of Mig1, and this is nuclear during glucose conditions, it can be assumed that active Snf1 should also be found in the nucleus. Indeed, Snf1-Gal83 complexes, which count for most of the activity observed *in vitro* localise to the nucleus (Vincent, 2001). However, there is another working hypothesis; that Snf1 phosphorylates Mig1 in the cytosol, thus affecting the shuttling of the protein, favouring glucose derepression.

At the same time, control of Snf1 most probably occurs in the cytosol because the SAKs and Glc7-Reg1 are largely present in this compartment. In glucose abundant conditions, Snf1 is mainly cytosolic explaining the rapid phosphorylation observed upon a shift to low glucose. However, upon glucose pulse a large portion of Snf1 becomes nuclear, How can this fraction be dephosphorylated? The answer could be a very fast continuous Snf1 shuttling. Since the nuclear importins/exportins involved in Snf1 shuttling have not been identified in genetic screens, it is likely that they constitute essential or redundant proteins. This issue could be resolved by monitoring fluorescence Snf1 shuttling in and out of the nucleus in real time. For instance, by photobleaching of the nuclear Snf1-GFP and measuring recovery time of the signal in samples treated with cycloheximide (to prevent synthesis *de novo*). It could also be

studied with a microfluidic system coupled to a microscope as well as can be done for Mig1 shuttling (Eriksson, 2007). An alternative experiment would consist of constructing a Snf1 that would be constitutively anchored to the plasma membrane (Song, 1996) and test whether this form devoid of shuttling is fully functional.

It is also noticeable that beta subunits and Snf4 can be phosphorylated at multiple places in a Snf1-dependent fashion (Yang, 1994). This post-translational modification is rather common in yeast (10-20% of the proteome is phosphorylated) and may not have biological relevance (Chi, 2007; Ficarro, 2002).

Mig1, target of Snf1

Mig1 is a DNA binding protein that mediates glucose/fructose repression of a large set of genes by binding to their promoters and recruiting the general repression complex Cyc8-Tup1 (Ronne, 1995; Treitel, 1995). The best characterised targets of Mig1 are genes implicated in gluconeogenesis, peroxisome and mitochondrial biogenesis and the utilisation of alternative carbon sources such as the MAL and GAL regulons and *SUC2* (Neigeborn, 1984; Sarokin, 1984, 1985).

Isolation and protein structure

MIG1 was first identified in a screen for mutations that suppressed an *SNF1*-disrupted strain growing in the presence of non-fermentable carbon sources (Carlson, 1984) and named *SSN1*. Later, the gene was again isolated and characterised in a screen for Multi-copy Inhibitors of a toxic gene under the control of *GAL1* promoter (Nehlin, 1990).

MIG1 encodes a protein of 55 KDa, which contains two predicted Cys_2-His_2 domains, also known as zinc fingers that bind to promoters of glucose-regulated genes (Fig. 7 in grey). The first finger extends from amino acids 34-61 (underlined in blue) and the second from 62-91 (underlined in red) (Nehlin, 1990). The consensus Mig1 DNA-binding site in target genes has been determined *in vitro* as 5'-ATAAAATGCGGGGAA-3' (Lutfiyya, 1998). A group of transcription factors with a similar DNA-binding domain includes: *MIG2*, *MIG3*, *NRG1* and *NRG2*.

Regulation

MIG1 is regulated both at a transcriptional and post-transcriptional level. For instance, *MIG1* is poorly transcribed when cells are grown for a long time in the absence of a fermentable carbon source. Upon addition of glucose/fructose *MIG1* becomes fully expressed, however it has been suggested to inhibit its own transcription via a feedback loop. Under these conditions it also seems to repress *MIG2* and *MIG3* transcription (Kaniak, 2004). Accordingly, Mig1 protein levels increase upon a shift from glycerol to high glucose (4%), and even more so if the shift is to low glucose medium (0.05%) (Kaniak, 2004).

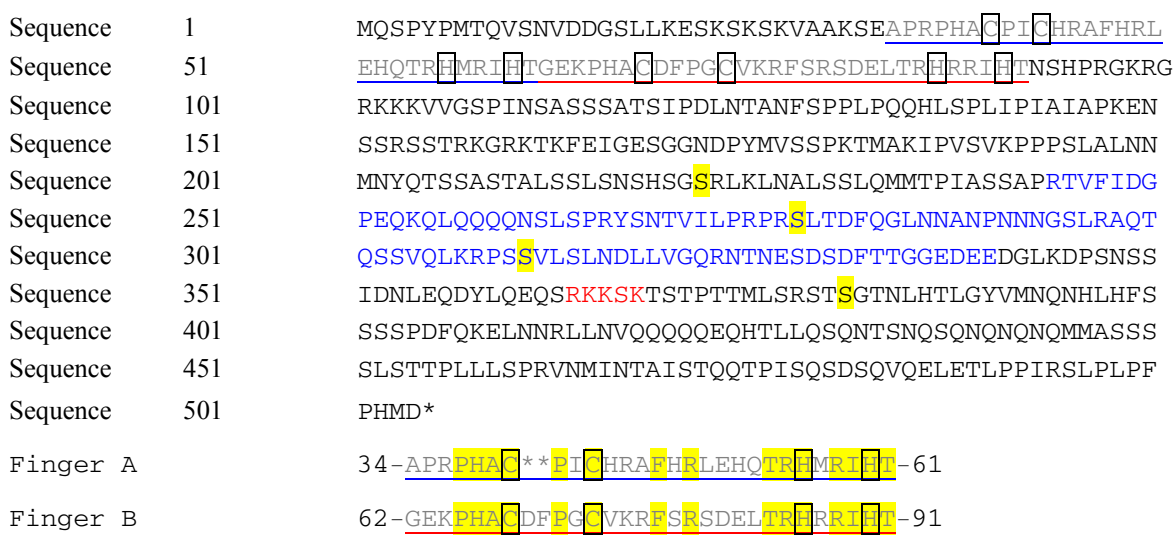

Figure 7. Mig1 predicted domains and regulatory sites: C_2H_2 zinc fingers predicted at amino acids 34-61 and 62-91, nuclear export signal (NES) mapped at 244-340 and nuclear localisation signal (NLS) amino acids 364-368. S indicates putative Snf1-phosphorylated serines *in vitro* at positions 222, 278, 311 and 381. Sequence comparison of fingers A and B, conserved amino acids highlighted (DeVit, 1999). boxes indicate C_2H_2 residues

The post-transcriptional regulation of Mig1 is based on two properties, binding capacity and localisation, both of which seem to depend on the phosphorylation state of the protein (Santangelo, 2006). Snf1 is the only kinase identified to convert Mig1 to Mig1-P, and it does so in a glucose-dependent manner (Treitel, 1998). Activation of Snf1 by other conditions (e.g. salt stress) does not lead to Mig1-P (McCartney, 2001) (T. Ye, unpublished results). Mig1 is phosphorylated at at least four biologically relevant sites (Smith, 1999), four serines placed in the stretch 222-381 (Fig. 8). Phosphorylation affects Mig1's capacity to block transcription and promotes re-localisation to the cytosol. Interestingly, one of these residues is predicted by sequence comparison to be a PKA target, however to date there is no experimental data supporting phosphorylation by PKA (Nehlin, 1990). Point mutations of the phosphorylatable residues to alanine did not result in complete loss of glucose repression, indicating that non-identified sites still exist (Treitel, 1998). It seems that phosphorylation of Mig1-P leads to loss of affinity towards Cyc8-Tup1 (Papamichos-Chronakis, 2004), and promotes recognition by the nuclear exportin Msn5 and subsequent translocation to the cytosol (DeVit, 1999). The cytosolic fate of Mig1-P is unclear. Upon a shift to glucose Glc7-Reg1 dephosphorylates Mig1, promoting its nuclear relocalisation. The only observation that implicates Glc7-Reg7 in the dephosphorylation of Mig1 is that *reg1*Δ deletion and *glc7* mutants show constitutive Mig1-P (McCartney, 2001; Treitel, 1998). Contrary to Snf1, the different phosphorylation states of Mig1 can be followed directly on western blots by monitoring band shifts (Elbing, 2004b). However, it has not been defined to which phosphorylation each band corresponds.

The Mig1 binding domain is similar to those present in transcription activators, meaning that it could have some role in activating genes required for respiratory growth (Santangelo, 2006).

In the long list of experiments that could be performed to cast light on glucose sensing, real time measurements of Mig1 shuttling in and out of the nucleus would be rather useful. This is a subject that can be studied by single cell analysis in microfluidic systems (Eriksson, 2007). If measurements were accurate, the kinetics of this process could be determined leading to a better understanding of its relation with Snf1 activity and with target genes, e.g. *SUC2*.

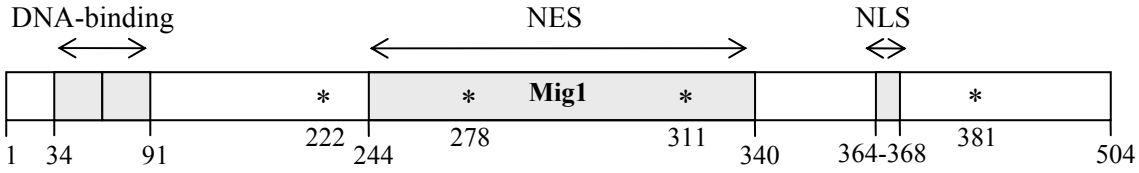

Figure 8. Two dimensional representation of Mig1 structure, * indicate putative Snf1 phosphorylation serines.

Hxk2, the intracellular glucose sensor?

In addition to its metabolic role, Hxk2 is required to trigger glucose repression (see section 2D) (Entian, 1980; Entian, 1982). (Hohmann, 1999) tried to find mutants where these two functions could be separated, that is a *hxk2* mutant retaining catalytic activity but without sensing role or a mutant unable to trigger sugar response while the metabolic activity was reduced. The isolated mutants did not offer a mechanistic explanation on how Hxk2 could transduce the signal (Bisson, 2003). A partial mechanism has been proposed after a series of observations. For instance, Hxk2 was found to localise to the nucleus (Randez-Gil, 1998) and subsequent work demonstrated association of Hxk2 to elements of a transcriptional regulatory complex (Med8), which influences *SUC2* expression (Herrero, 1998). It has also been shown to associate to Mig1, probably preventing its phosphorylation by Snf1 (Ahuatzi, 2004; Ahuatzi, 2007) (for more details see section 3D) and to Reg1, to stabilise its phosphorylated state (the inactive form) (Sanz, 2000a).

The NLS sequence of Hxk2, required for nuclear import, was identified as amino acids 8-12, however this contradicts a previous report which found dispensable the first 15 amino acids. In this deletion mutant, both hexose phosphorylation and glucose repression were similar to the wild type (Ma, 1989).

It has also been argued, that whilst both major hexokinases (Hxk1 and Hxk2) are involved in fructose repression, only Hxk2 plays a role in glucose repression. That is because Hxk1 functions to a large extend as a fructokinase (De Winde, 1996). However, it is rather intriguing that given the high sequence similarity (70%) and domain homology of Hxk1

respect to Hxk2, it would not function in a sugar signalling role. It is possible that the differences between the two hexokinases are only a consequence of their expression patterns. *HXK2* mRNA is upregulated in high glucose conditions, while *HXK1* is downregulated. Indeed, overexpression of *HXK1* has been shown to cause glucose repression to the same extent than that observed in wild type strains (Rose, 1991). In addition, Hxk1 should be able to enter the nucleus, since the first 16 amino acids of the N-terminal end, which supposedly define the nuclear localisation of Hxk2, are identical in both proteins. An experiment that would determine whether the capacity to trigger glucose repression is independent of their gene expression levels could be the exchange of promoters, using constructs such as HXK2prom-HXK1 or HXK1prom-HXK2.

Hxk2 also appears to have a complex regulation. It can be present in two forms, during glucose limiting conditions, Hxk2 is found mainly as a monomer; while upon a shift to high glucose about half of Hxk2 turns into a dimer. The transition from dimer to monomer depends on phosphorylation of serine 15 by PKA (shown *in vitro*) and dephosphorylation by Reg1-Glc7 (Bisson, 2003). An enticing hypothesis would be that PKA activation would lead to Hxk2 phosphorylation and further Snf1 inactivation, it is not clear whether dimerisation influences Hxk2 activity. For instance, a point mutation turning serine 15 to alanine produced an active form of Hxk2 that is indistinguishable from the wild type (Kriegel, 1994).

SUC2 expression as glucose response reporter

SUC2 is a classic example of “glucose repressed” gene and it has been extensively used as a reporter of the glucose repression state of the cell. The expression of *SUC2* is very low in the presence of high glucose or fructose (> 2%), this has been explained by the concerted downregulation caused by a number of repressors including Mig1, Rgt1, Sko1, Sfl1 and Gcr1 (Belinchon, 2007a; Türkel, 2003). Upon sugar exhaustion, as observed during diauxic shift, *SUC2* expression increases dramatically to high levels, which can be sustained by providing sucrose or raffinose (Carlson, 1982). When glucose is completely consumed *SUC2* mRNA levels drop to a basal state, which is still higher than during glucose repression and allows for a constant expression of invertase (our unpublished observations). Perhaps the most intriguing fact about *SUC2* is that neither activators at low glucose concentrations nor repressors in the absence of glucose have been identified.

The changes in expression observed for *SUC2* correspond to the sum of the individual expression in a cell population, assuming that these correspond to a Gaussian distribution, but is this general assumption true? To test whether every cell behaves similarly in the population or rather whether there are fractions with different behaviours or glucose thresholds we investigated *SUC2* expression across a cell population by taking advantage of flow cytometry. In order to obtain a good reporter, *SUC2* promoter was fused to a YFP

(yellow fluorescence protein) reporter tag (Sheff, 2004). We have followed *SUC2* activation by taking cells in a time-course manner as glucose was being consumed and observed a single shift in expression, therefore indicating that the entire population expresses *SUC2* uniformly (our unpublished data).

3.5. A role for Hxk2 in Mig1 regulation

Hxk2 has been shown to form a complex in the nucleus with Mig1 and to interact with Snf1 (Ahuatzi, 2004; Moreno, 2005). In a *hxk2Δ* mutant, Mig1 is partially phosphorylated at high external glucose concentrations and fully phosphorylated at low glucose. A similar phenotype can be seen in the *reg1Δ* mutant. In paper 4, we show that the double *hxk2Δ reg1Δ* mutant does not present a transient glucose repression response. We hypothesise that Hxk2 and Reg1 control glucose repression via parallel routes and not via a linear pathway. This would take into account the model proposed by (Moreno, 2005), in which Snf1 and Reg1 compete for access to the residue 311 of Mig1. Hxk2 would mask the phosphorylation site to prevent Snf1 access.

3.6. Modelling activation of SNF1 pathway

Mathematical models may offer help in order to understand complex signalling. In this way, the complexity of the pathway can be reduced to a set of differential equations. We have undertaken such an effort for the Snf1 regulatory network (Paper 4). We generated a dynamic data set with measurements of abundance of the proteins of the pathway, Snf1 kinase activity and phosphorylation state and the expression of glucose-regulated genes. This provided novel information on the pathway:

- Snf1 is constitutively active in the absence of glucose both during growth on respiratory carbon sources and in stationary phase. That is in contrast to other regulatory pathways, which are transiently activated by a stimulus (e.g. the Hog pathway shows transient Hog1 phosphorylation) (Klipp, 2005).
- Snf1 is quickly dephosphorylated by glucose addition (less than 1 min). This period probably excludes transcriptional regulation of this process.
- Snf1 phosphorylation correlates largely with Snf1 activity during glucose depletion. This was to be expected, but to our knowledge, is the first time for it to be shown in a time-course fashion.
- The classical glucose-repression marker, *SUC2* mRNA levels, does not indicate correctly the phosphorylation profile of Snf1. Instead, *FBP1* expression very closely follows Snf1 activation/deactivation.

- Determination of the components of the SNF1 complex shows a 1:1:1 equimolar relationship that it is not regulated by glucose abundance. However, the Snf1-Sip2 complex is more abundant during growth on ethanol that on glucose, as previously reported (Vincent, 2001).

The information gathered in the data sets were used to test several hypothetical scenarios of the activation of the pathway. We checked three main hypotheses regarding the regulation of the Snf1 phosphorylation state:

- It depends on changes on the Snf1-activating kinases
- On changes on the activity of the phosphatase
- On simultaneous changes on both

The best simulations obtained were those corresponding to the scenario in which the phosphatase was the only regulator of Snf1. This is in line with results from (Rubenstein, 2008) showing that the activity of the Snf1-actviating kinases was not regulated by glucose abundance. The outcome of the model suggests that the search for mechanism regulating Snf1 should be focused on understanding how the Reg1-Glc7 complex is connected to the presence of glucose in the medium.

4. Concluding remarks

This thesis may have contributed to further understanding the connexions between glucose transport and glycolysis, as well as to explore the signal causing glucose repression in yeast. The latter is still an open question that allows numerous hypotheses:

Glucose sensors

At the time the hexose transporters were discovered, they were thought to also mediate sensing, analogously to the role played by the phosphotransferase system in *E. coli* (Stülke, 1999). This hypothesis was erroneous because transport is not coupled to sensing in yeast. However, two putative hexose transporters, Snf3 and Rgt2, acting as glucose sensors, regulate the expression of hexose transporters, which in turn are essential for growth on sugars (Towle, 2005) (Paper 2). Extracellular sensors are needed for the uptake of glucose, but not for triggering glucose repression (Elbing, 2004b) (Paper 1).

cAMP

The observation that addition of glucose to respiratory cells leads to a transient increase in cAMP levels, thereby triggering the activation of PKA, has been regarded as a likely initial glucose signal (Rolland, 2002). A sharp increase in PKA activity can be interpreted as a metabolic reorganisation signal necessary to launch the rearrangements necessary for a fermentative life style. Along these lines, it has been reported that changes in mRNA abundance brought about by glucose repression can be recapitulated by a constitutively active Ras1 mutant (Wang, 2004), but also that mutants crippled in PKA activity show normal glucose repression in contradiction to previous knowledge (Mbonyi, 1990). Against an involvement of PKA in the glucose repression signal is the paucity of connections with the Snf1 pathway. Apart from converging on Msn2/4 (Estruch, 1993; Mayordomo, 2002), there is little common ground between them. Another possibility would be that PKA would influence the activity of glycolytic enzymes, therefore leading to an increase/decrease in the flux and correspondingly to the initial glucose phosphorylation step. In Paper 1, the model supports the hypothesis that glycolysis is modified in response to the transport of glucose by a change in the kinetics of PFK. This key enzymatic step could be regulated by transient changes in F26BP generation under the control of the PKA pathway.

Energy levels

The ATP:AMP ratio is a measure of energy status of the cell. As already discussed, SNF1 activation does not seem to depend on the levels of energy status as in the case of its

mammalian counterpart AMPK (Section 3D). However, ATP and AMP are substrates, products and allosteric regulators of key glycolytic regulator steps, providing support to the idea that it may constitute a good signal for glucose processing. However, it has been reported that the glycolytic rate (which is usually high at high glucose concentrations) inversely correlates with ATP levels (Larsson, 2000). Even if high glycolytic rates indicate that a larger glucose signal is generated, the next question would be, which are the components able to detect the rate and convert this into a chemical signal?

Sugar phosphorylation

No step beyond glucose phosphorylation seems to be required for glucose repression, because the glucose analogue 2-deoxyglucose, which can be phosphorylated but not further metabolised triggers repression. Consequently, 6-deoxyglucose, which can be transported but not phosphorylated, does not cause glucose repression (Bisson, 2003). Indeed, this singles out glucose phosphorylation as the upstream generator of the glucose signal. At this point, Hxk2 appears to be a plausible candidate as the sensor of glycolytic rate. Can Hxk2 "feel" that glucose is being processed and trigger downregulation of the SNF1 pathway and stimulate cAMP production? First, Hxk2 could work as a potential sensor since it binds both free glucose and ATP. Then, as a signal transducer it influences the phosphorylation state of Snf1 and we show data supporting a regulatory role in Mig1 phosphorylation too (Paper 3). No link has yet being described between Hxk2 and the PKA pathway. In conclusion, there are many indications that Hxk2 is the best candidate available as a glucose sensor but it is unknown how it could sense the rate of glucose consumption or how it might influence the activity of signalling pathways.

Tensegrity

Bisson and Kunathigan have proposed a new model to explain glucose signal transduction based on the concept of tensegrity (Bisson, 2003). That is, the capacity to generate signals through mechanical tensions. This theory has been developed in order to explain the response of cells to external stimuli (Bisson, 2003; Ingber, 2003a, 2003b). Pressures on the surface of the cell may cause a reorganisation of the actin cytoskeleton, which would trigger a response in the nucleus in the form of changes in mRNA expression. Glycolytic proteins have been observed to co-localise with components of the cytoskeleton (Walsh, 1989). Deletion of actin components temporally reduces glycolytic activity prompting the suggestion that the differences between *in vivo* and *in vitro* activities may be due to the lack of structural components in the latter (Götz, 1999). The organisation of glycolysis in a structural responsive network may explain the elusive nature of the initial glucose signal. It could also

be applied to other signalling pathways, such a Hog1, where the turgor pressure sensor has not yet been identified.

What is the initial glucose signal?

The presence of glucose in the surroundings of the cell is not sufficient to trigger a glucose response (Paper 1). Snf3/Rgt2 sensors are able to determine the carbon source abundance and regulate the expression of a small set of genes. The Gpr1/Gpa2 branch of the PKA pathway is unable to set off a response to extracellular glucose (Paper 1) (Wang, 2004), likely because its activation is insufficient to elicit a PKA response. The glucose does not depend on the external glucose, but rather of the amount of glucose processed in glycolysis. When the transport of glucose is restricted it sets the pace of glycolysis (Paper 2 and (Elbing, 2004a)). The speed of glycolysis seems to depend on PFK and PGI activities, which in turn have complex regulations. PKA may connect the regulation of these enzymes to signalling events. However, it is difficult to determine whether the glycolytic rate drives the intensity of the PKA response or the other way around.

Since glucose phosphorylation is a key step in glucose repression, it is tempting to speculate that Hxk2, the most abundant hexokinase in glucose rich medium, translates the rate of phosphorylation into an intracellular signal. Additional roles in the regulation of Reg1 and Mig1 phosphorylation (Paper 3), as well as the association to chromatin offer a compelling argument in this direction. However, it is difficult to separate the catalytic and regulatory functions of Hxk2 or connect it to the deactivation of Snf1. For instance, the fructose kinase activity of Hxk1 leads to a strong *fructose response*, however no links with signalling pathways have been described yet (Herwig, 2002). The recollection of events that lead to SNF1 activation is shrouded by complex interactions among regulators (SAKs, PP1 and Hxk2). Untangling this interplay of feedback and feedforward loops can be achieved with the help of modelling, since it offers the opportunity to explore multiple hypothetical scenarios. Such an effort, described in Paper 4, shows that SAKs may not need to be regulated and that changes in the activity of PP1 may prove sufficient to provoke activation or deactivation of SNF1.

Given the complexity of the glucose responses, most likely it does not depend on one single intracellular glucose sensor. Instead, a more probable scenario is that there is a concerted response of several signalling pathways converging into glucose responses, which are specific to every gene or group of genes.

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Paper 1

Characterization of glucose transport mutants of *Saccharomyces cerevisiae* during a nutritional upshift reveals a correlation between metabolite levels and glycolytic flux

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RESEARCH ARTICLE

Characterization of glucose transport mutants of *Saccharomyces cerevisiae* during a nutritional upshift reveals a correlation between metabolite levels and glycolytic flux

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Abstract

Saccharomyces cerevisiae shows a marked preference for glucose and fructose, revealed by the repression of genes whose products are involved in processing other carbon sources. This response seems to be driven by sugar phosphorylation in the first steps of glycolysis rather than by the external sugar concentration. To gain a further insight into the role of the internal sugar signalling mechanisms, were measured the levels of upper intracellular glycolytic metabolites and adenine nucleotides in three mutant strains, HXT1, HXT7 and TM6*, with progressively reduced uptake capacities in comparison with the wild type. Reducing the rate of sugar consumption caused an accumulation of hexose phosphates upstream of the phosphofructokinase (PFK) and a reduction of fructose-1,6-bisphosphate levels. Mathematical modelling showed that these effects may be explained by changes in the kinetics of PFK and phosphoglucose isomerase. Moreover, the model indicated a modified sensitivity of the pyruvate dehydrogenase and the trichloroacetic acid cycle enzymes towards the NAD/NADH in the TM6* strain. The activation of the SNF1 sugar signalling pathway, previously observed in the TM6* strain, does not correlate with a reduction of the ATP : AMP ratio as reported in mammals. The mechanisms that may control the glycolytic rate at reduced sugar transport rates are discussed.

Introduction

The monosaccharides glucose and fructose are the preferred carbon sources for most prokaryotic and eukaryotic cells. In nature, organisms have developed a number of mechanisms to secure a steady supply of these sugars in response to abrupt changes in their concentration. In the case of baker's yeast, *Saccharomyces cerevisiae*, glucose and fructose are incorporated by facilitated diffusion mediated by the hexose transporter family (Hxtp) (Boles & Hollenberg, 1997). This comprises at least 20 proteins with elevated sequence similarity and different sugar affinities and expression levels (Özcan & Johnston, 1999).

We have previously constructed and described a number of strains expressing different functional chimeras of the hexose transporters with a wide range of glucose uptake rates (Elbing *et al.*, 2004a). HXT1, HXT7 and TM6* were constructed by integrating a single hexose transporter gene (*HXT1*, *HXT7* and *TM6**, respectively) into the genome of the *hxt* null strain KOY.VW100P (Elbing *et al.*, 2004a, b; Otterstedt *et al.*, 2004), which lacks all known hexose transporters and is unable to take up glucose (Wieczorke *et al.*, 1999). The hexose transporters were expressed under a truncated, strong and constitutive promoter. The difference in hexose transporters resulted in strains with different sugar consumption rates.

TM6*p is a functional chimera composed of parts of the Hxt1p and Hxt7p transporters. In previous studies, it has been shown that the TM6* strain is capable of maintaining respiratory growth even in the presence of high glucose concentrations while producing negligible amounts of ethanol (Otterstedt *et al.*, 2004). This is in contrast to wild-type strains of *S. cerevisiae*, which, under similar conditions, mainly ferment glucose into ethanol and carbon dioxide

(CO_2) (Fiechter *et al.*, 1981; Verduyn *et al.*, 1984). The wild type can only attain the fully respiratory catabolism shown by the TM6* strain when it is grown in glucose-limited chemostats or fed-batch reactors at low dilution rates (Postma *et al.*, 1989).

The addition of glucose or fructose to wild-type cells growing on a respiratory carbon source leads to major changes in gene expression. A large set of genes becomes repressed, for example, those implicated in the trichloroacetic acid (TCA) cycle, glyoxylate cycle, the respiratory chain and the uptake and utilization of alternative carbon sources (Ronne, 1995; Gancedo, 1998; Carlson, 1999). After depletion of glucose or fructose, e.g. at the diauxic shift, repression is relieved (DeRisi *et al.*, 1997). Studies on the transcription pattern of the TM6* strain revealed that even at high glucose concentrations, the profile is very similar to that of the wild type in the absence of sugars (Elbing *et al.*, 2004a, b). This piece of evidence indicates that external levels of glucose do not control the metabolic switch from fermentation to respiration. Presumably, there is an intracellular signal leading to glucose repression, but its identity remains unknown. A number of potential candidates have been proposed, for instance Hxk2 (Rose *et al.*, 1991; De Winde *et al.*, 1996; Hohmann *et al.*, 1999; Rolland *et al.*, 2002), cAMP (Rolland *et al.*, 2001), the concentration of selected intracellular metabolites (Boles & Zimmermann, 1993; Boles *et al.*, 1993) and the ATP : AMP ratio (Wilson *et al.*, 1996). This signal may also control glycolytic flux by influencing the kinetics of one or several enzymes in the route; for instance, modifying the allosteric regulation of hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) (Gancedo, 1998) or the regulation of enzymes at the end of phosphorylation cascades, e.g. 6-phosphofructo-2-kinase (Pfk2) by MAPK (Dihazi *et al.*, 2004) and fructose-2, 6-biphosphatase (Fbp26) by cAMP (Boles *et al.*, 1997). Moreover, some glycolytic- and ethanol-producing enzymes have been revealed as regulators of transcriptional activity e.g. Hxk2 (de la Cera *et al.*, 2002) and Pdc1 (Mojžita, 2007) in yeast, and Hxk1 in *Arabidopsis thaliana* (Cho *et al.*, 2006).

In this study, we address how a reduction in sugar uptake leads to a slow glycolytic rate, therefore causing the shift from respiration to fermentation. We show that the intracellular levels of glycolytic metabolites and adenosine nucleotides are very different in strains with high sugar consumption rates, i.e. the wild type and HXT1 strains, in comparison with strains exhibiting low sugar consumption rates, i.e. HXT7 and TM6*, at the same external sugar concentration. Furthermore, mathematical modelling of the glycolytic flux suggests that these observations may be explained by differences in the kinetics of the PFK and phosphoglucose isomerase (PGI), and by a modified sensitivity of the pyruvate dehydrogenase (PDH) and/or the TCA cycle enzymes towards NAD^+ and NADH.

Materials and methods

Strains

All *S. cerevisiae* strains used were derived from the CEN.PK2-1C strain (van Dijken *et al.*, 2000), herein referred to as the wild type. The construction of the prototrophic strains KOY.HXT1P (HXT1), KOY.HXT7P (HXT7) and KOY.TM6*P (TM6*) has been described by Elbing *et al.* (2004a).

Growth conditions

An aerobic chemostat culture with a working volume of 2 L and a constant dilution rate of $0.1\,h^{-1}$ was prepared in a Braun Biostat A fermentor (Braun, Melsungen, Germany). In all cultivations, a two-times concentrated defined minimal medium was used (Verduyn *et al.*, 1992). An antifoaming agent (Polypropylene-2000 Fluka, Steinheim, Switzerland) was added to a final concentration of $0.1\,mL\,L^{-1}$ in the bioreactors. The temperature was maintained constant at 30 °C, and the stirring rate was 1200 r.p.m. The pH was kept at 5.0 by the automatic addition of 1 M NaOH. The airflow was controlled by a mass flow controller from Bronkhorstm, High-Tech, B.V. (Ruurlo, the Netherlands). Cells were precultured overnight in defined medium with 2% (110 mM) glucose as the sole carbon source. The preculture was subsequently used to inoculate a 2 L fermentor with 5% glucose. The feed of fresh substrate with 1% of ethanol as the sole carbon source was started following glucose depletion, as indicated by the CO_2 production rate. Once the chemostat culture achieved a constant CO_2 production for at least three volume changes, sugar was pulsed into the vessel, enough to bring the transient concentration to 2%. Simultaneously, the ethanol medium feed was shut off. Oxygen consumption and CO_2 production rate were followed online using a photoacoustic gas analyser (type 1308; Brüel and Kjær, Nærum, Denmark).

Dry weight determination

Dry weight was measured in triplicate by centrifugation of 3×10 mL of cell culture in preweighed dry glass tubes. The cells were washed twice in 5 mL of deionized water (MilliQ), dried for 24 h at 110 °C and stored in a desiccator before weighing.

Determination of extracellular substrate and products

Duplicate samples of 1 mL each were sterile filtered (0.22 μm), frozen in liquid N_2 and stored at −20 °C. The analyses of glucose, ethanol and glycerol were performed using enzymatic combination kits (Boehringer-Mannheim GmbH, Mannheim, Germany).

Consumption and production rates

The mean rates of glucose and fructose consumption and ethanol production were determined for the 2-h interval subsequent to sugar addition. The mean values of the dry weight in the specified time intervals were used in the rate calculations.

Determination of glycogen and trehalose

Duplicate samples of 5 mL were centrifuged at 4 °C and the pellets were frozen in liquid nitrogen, maintained at − 40 °C and analysed as described previously (Parrou & François, 1997). The content of storage carbohydrates was normalized to the cell dry weight of the same culture samples.

Determination of intracellular metabolites

For all metabolites, except ATP, six samples of 3 mL each were directly transferred into preweighed tubes with 10 mL of methanol maintained at − 48 °C; the final concentration of methanol (v/v) was about 75%. The extraction was performed as described previously (Gustafsson, 1979). The extraction and quantification of ATP was performed separately. Triplicate samples of 1 mL each were added to 1.2 mL of 0.51 M TCA, thereby extracting ATP as described earlier in Gustafsson (1979). The ATP concentration was analysed in triplicate on a Packard Pico-Lite luminometer (Packard Instruments, Downers Grove, IL) using ATP bioluminescence assay kits (CLSII) (Roche Diagnostics, Mannheim, Germany).

Measurement of sugar uptake capacity

Samples for determination of the sugar uptake were taken during steady-state growth on ethanol. Cells were harvested and washed twice with 0.1 M potassium phosphate buffer (pH 6.5), diluted to a cell density of 75 g L^{-1} (wet weight) and kept on ice. Uptake of [^{14}C] glucose (Amersham Life Science, Uppsala, Sweden) was assayed as described by Özcan *et al.* (1993) with the modifications developed by Walsh *et al.* (1994). Radioactivity was quantified using a liquid scintillation detector (Beckman Coulter AB, Bromma, Sweden). Total cellular protein was determined with the bichloroacetic acid kit (Bio-Rad Laboratories Inc., Hercules, CA). Three separate series at seven different sugar concentrations were performed. Data were analysed using Hanes plots.

Mathematical modelling

A kinetic model was constructed to describe respiro-fermentative growth on glucose during the 2-h transients after the glucose pulse to the four strains. The stoichiometry and kinetic rate equations of the reaction network are shown in Table 1. F16BP was not included in the model, because it is known that yeast cells adapted to grow on ethanol and subjected to a sugar pulse degrade F16BP and synthesize PFK very rapidly, making the switch instantaneous in this time scale (Regelmann *et al.*, 2003).

Polynomial approximations of the glucose uptake rate, the oxygen uptake rate and the biomass concentration were used as input to the model. Biosynthesis was described as a lumped reaction from several glycolytic intermediates. It was adapted from the aerobic case described by Sárvári Horváth *et al.* (2003). The average macromolecular composition of cells was assumed to be 50% (w/w) protein (average amino acid composition according to Albers *et al.*, 1996), 10% RNA (average nucleotide composition according to Mounolou, 1975), 30% carbohydrates, 5% lipids and 5% ash. The intracellular AMP concentration was set as the average of the measured values for each time course. The total pool of ATP and ADP was set equal to the average sum during each time course. The total NAD^+ + NADH pool was set to 3 mM, with the exception of the HXT1 strain, for which it was set to 3.4 mM, corresponding to the same ratio between the assumed total pool and the measured initial NAD^+ concentration as in the wild type. The errors between the measured and predicted intracellular concentrations of G6P, F6P, F16BP, pyruvate, ADP, ATP and NAD^+, the extracellular concentrations of ethanol and glycerol and the CO_2 evolution rates were used for parameter optimization. The maximum rates (V_{max} values) for PFK, aldolase, GapDH, Lp-PEP, PK, PDC, ADH, Lp-GDP and Lp-ATP were optimized. For Lp-PDH and complete TCA cycle reactions, V_{max} as well as K_{NAD} and $K_{I,NADH}$ were fitted.

The increase in expression during the pulse was taken into consideration by fitting an expression factor for the upper part of glycolysis, and one for PDC, such that the V_{max} values of these reactions were multiplied by a factor (1 + Expr t). ADH and GapDH were assumed to be linearly expressed from zero to full expression during the 120 min of the pulse for the wild type and HXT1 strains, while constant expression gave a better fit for the HXT7 and the TM6* strains. For the TM6* strain, V_{max} for phosphoglucose isomerase was also adjusted.

Parameters were estimated using the nonlinear least squares minimization function lsqnonlin.m and the stiff ordinary differential equation solver ode15s.m in MATLAB® 6.5. The parameter values were scaled with 10^{-5} times the inverse of the initial values, to give a uniform parameter set for improved efficiency of the minimization routine. Owing to evaporation likely leading to erroneous ethanol measurements, ethanol was weighted by a factor 0.5 in the sum of squares, while glycerol was weighted by a factor 10. Global minimum cannot be 100% guaranteed, but the optimum was the best result from several different initial guesses for the parameter values. For the HXT7 and the TM6* strains, this procedure unfortunately only produced infeasible results (negative F16BP, acetaldehyde, or ethanol

Table 1. Stoichiometry and kinetics of the reaction in the kinetic model

Rate equation	Parameter values
Glucose transport	
$$\mathrm{Glcx} \rightarrow \mathrm{Glc}$$	$V_{\mathrm{GlcTransp}}$ = measured
$$V_{\mathrm{GlcTransp}}$$	
Hexokinase	Hynne *et al*. (2001)
$$\mathrm{Glc} + \mathrm{ATP} \rightarrow \mathrm{G6P} + \mathrm{ADP}$$	$V_{3\,\mathrm{m}} = 1000$ mM min^{-1} (est)
$$V_{\mathrm{HK}} = (\mathbf{1} + \mathrm{Expr}_1 t)\frac{V_{3m}[\mathrm{Glc}][\mathrm{ATP}]}{K_{3\mathrm{DGlc}}K_{3\mathrm{ATP}} + K_{3\mathrm{Glc}}[\mathrm{ATP}] + K_{3\mathrm{ATP}}[\mathrm{Glc}] + [\mathrm{Glc}][\mathrm{ATP}]}$$	$K_{3\mathrm{ATP}} = 0.1$ mM $K_{3\mathrm{Glc}} = 0$ mM $K_{3\mathrm{DGlc}} = 0.37$ mM
Phosphoglucose isomerase	Hynne *et al*. (2001) $V_{4\,\mathrm{m}}$ = estimated
$$\mathrm{G6P} \leftrightarrow \mathrm{F6P}$$	
$$V_{\mathrm{PGI}} = (\mathbf{1} + \mathrm{Expr}_1 t)\frac{V_{4\,m}\left[[\mathrm{G6P}] - \frac{[\mathrm{F6P}]}{K_{4\mathrm{eq}}}\right]}{K_{4\mathrm{G6P}} + [\mathrm{G6P}] + \frac{K_{4\mathrm{G6P}}}{K_{4\mathrm{F6P}}}[\mathrm{F6P}]}$$	$K_{4\mathrm{G6P}} = 0.8$ mM $K_{4\mathrm{G6P}} = 0.15$ mM $K_{4\mathrm{eq}} = 0.30$ mM Expr_1 = estimated
Phosphofructokinase	Teusink *et al*. (2000) $V_{5\,\mathrm{m}}$ = estimated
$$\mathrm{F6P} + \mathrm{ATP} \rightarrow \mathrm{F16BP} + \mathrm{ADP}$$	
$$V_{\mathrm{PFK}} = (\mathbf{1} + \mathrm{Expr}_1 t)\frac{V_{5m}g_R\lambda_1\lambda_2(\mathbf{1} + \lambda_1\lambda_2 + g_R\lambda_1\lambda_2)}{(\mathbf{1} + \lambda_1\lambda_2 + g_R\lambda_1\lambda_2)^2 + L(\mathbf{1} + c_{\mathrm{ATP}}\lambda_2)^2}$$	$K_{\mathrm{ATP}} = 0.65$ mM $K_{\mathrm{AMP}} = 0.0995$ mM $K_{\mathrm{R,\,ATP}} = 0.71$ mM Expr_1 = estimated
$$\lambda_1 = \frac{[\mathrm{F6P}]}{K_{R,\,\mathrm{F6P}}};\quad \lambda_2 = \frac{[\mathrm{ATP}]}{K_{R,\,\mathrm{ATP}}}$$	$K_{\mathrm{R,\,F6P}} = 0.1$ mM $K_{\mathrm{F26BP}} = 0.000682$ mM
$$L = L_0\left[\frac{\mathbf{1} + c_{i,\,\mathrm{ATP}}[\mathrm{ATP}]/K_{\mathrm{ATP}}}{\mathbf{1} + [\mathrm{ATP}]/K_{\mathrm{ATP}}}\right]^2\left[\frac{\mathbf{1} + c_{i,\,\mathrm{AMP}}[\mathrm{AMP}]/K_{\mathrm{AMP}}}{\mathbf{1} + [\mathrm{AMP}]/K_{\mathrm{AMP}}}\right]^2\left[\frac{\mathbf{1} + c_{i,\,\mathrm{F26bP}}[\mathrm{F26bP}]/K_{\mathrm{F26bP}} + c_{i,\,\mathrm{F16bP}}[\mathrm{F16bP}]/K_{\mathrm{F16bP}}}{\mathbf{1} + [\mathrm{F26bP}]/K_{\mathrm{F26bP}} + [\mathrm{F16bP}]/K_{\mathrm{F16bP}}}\right]^2$$	$K_{\mathrm{F16BP}} = 0.111$ mM $c_{\mathrm{ATP}} = 3$ $c_{i,\,\mathrm{ATP}} = 100$ $c_{i,\,\mathrm{AMP}} = 0.0845$ $c_{i,\,F26BP} = 0.0174$ $c_{i,\,\mathrm{F16BP}} = 0.397$ $g_R = 5.12$ $L_0 = 0.66$
Aldolase	Hynne *et al*. (2001) $V_{6\,\mathrm{m}}$ = estimated
$$\mathrm{F16bP} \leftrightarrow \mathrm{DHAP} + \mathrm{GAP}$$	
$$V_{\mathrm{ALD}} = \frac{(\mathbf{1} + \mathrm{Expr}_1 t)V_{6\,\mathrm{m}}\left[[\mathrm{F16bP}] - \frac{\mathbf{5}[\mathrm{GAP}][\mathrm{DHAP}]}{K_{5\mathrm{eq}}}\right]}{\left(\begin{array}{c} K_{6\mathrm{F16bP}} + [\mathrm{F16bP}] + \frac{[\mathrm{GAP}]K_{6\mathrm{DHAP}}\mathbf{5}}{K_{6\mathrm{eq}}} + \frac{[\mathrm{DHAP}]K_{6\mathrm{GAP}}\mathbf{5}}{K_{6\mathrm{eq}}} \\ + \frac{[\mathrm{F16bP}][\mathrm{GAP}]}{K_{6\mathrm{lGAP}}} + \frac{[\mathrm{GAP}][\mathrm{DHAP}]\mathbf{5}}{K_{6\mathrm{eq}}} \end{array}\right)}$$	$K_{6\mathrm{F16BP}} = 0.3$ mM $K_{6\mathrm{DHAP}} = 2.0$ mM $K_{6\mathrm{GAP}} = 4.0$ mM $K_{6\mathrm{lGAP}} = 10.0$ mM $K_{6\mathrm{eq}} = 0.081$ mM Expr_1 = estimated

Table 1. Continued.

Rate equation	Parameter values
Triosephosphate isomerase	Hynne *et al*. (2001) $V_{7\,m} = 116.4\ \text{mM min}^{-1}$
$$\text{DHAP} \leftrightarrow \text{GAP}$$	
$$V_{TIM} = \frac{V_{7\,m}\left[[\text{DHAP}] - \frac{[\text{GAP}]}{K_{7eq}}\right]}{K_{7DHAP} + [\text{DHAP}] + \frac{K_{7DHAP}[\text{GAP}]}{K_{7GAP}}}$$	$K_{7DHAP} = 1.23\ \text{mM}$ $K_{7GAP} = 1.27\ \text{mM}$ $K_{7eq} = 0.045\ \text{mM}$
Glyceraldehyde-3-phosphate dehydrogenase	Hynne *et al*. (2001) $V_{8\,m}$ = estimated
$$\text{GAP} + \text{NAD}^+ \leftrightarrow \text{BPG} + \text{NADH} + \text{H}^+$$	
$$V_{GAPDH} = (1 + \text{Expr}_1 t)\frac{V_{8\,m}\left[[\text{GAP}][\text{NAD}]\frac{[\text{BPG}][\text{NADH}]}{K_{8eq}}\right]}{K_{8GAP}K_{8NAD}\left[1 + \frac{[\text{GAP}]}{K_{8GAP}} + \frac{[\text{BPG}]}{K_{8BPG}}\right]\left[1 + \frac{[\text{NAD}]}{K_{8NAD}} + \frac{[\text{NADH}]}{K_{8NADH}}\right]}$$	$K_{8GAP} = 0.6\ \text{mM}$ $K_{8BPG} = 0.01\ \text{mM}$ $K_{8NAD} = 0.1\ \text{mM}$ $K_{8NADH} = 0.06\ \text{mM}$ $K_{8eq} = 0.0055$ Expr_1 = estimated
Lp-PGK, Lumped reactions from BPG to PEP	Hynne *et al*. (2001) $k_{9f} = 4.44 \times 10^5\ \text{min}^{-1}$
$$\text{BPG} + \text{ADP} + \text{P}_i \leftrightarrow \text{PEP} + \text{ATP}$$	
$$V_{PEP} = k_{9f}[\text{BPG}][\text{ADP}] - k_{9r}[\text{PEP}][\text{ATP}]$$	$k_{9r} = 1530\ \text{min}^{-1}$
Pyruvate kinase	Rizzi *et al*. (1997), Johannes & Hess (1973) $V_{10\,m}$ = estimated
$$\text{PEP} + \text{ADP} + \text{P}_i \rightarrow \text{PYR} + \text{ATP}$$	
$$V_{PK} = \frac{V_{10\,m}\frac{[\text{PEP}]}{K_{PEP10}}\left[\frac{[\text{PEP}]}{K_{PEP10}} + 1\right]^{(n^{10}-1)}}{L_{0,10}\left[\frac{\frac{[\text{ATP}]}{K_{ATP10}} + 1}{\frac{[\text{F16bP}]}{K_{FBP10}} + 1}\right]^{n^{10}} + \left[1 + \frac{[\text{PEP}]}{K_{PEP10}}\right]^{n^{10}}} \frac{[\text{ADP}]}{[\text{ADP}] + K_{ADP10}}$$	$K_{PEP10} = 0.19\ \text{mM}$ $K_{ATP10} = 9.3\ \text{mM}$ $K_{FBP10} = 0.2\ \text{mM}$ $K_{ADP10} = 0.3\ \text{mM}$ $n_{10} = 4$ $L_{0,10} = 60\,000$
Lp-PDH, Lumped reactions from pyruvate to the complete TCA	Rizzi *et al*. (1997) V_{PDH} = estimated
$$\text{PYR} + 5\,\text{NAD}^+ + \text{ADP} + \text{P}_i \rightarrow 3\,\text{CO}_2 + 5\,\text{NADH} + 5\,\text{H}^+ + \text{ATP}$$	
$$V_{TCA} = \frac{V_{PDH}[\text{PYR}][\text{NAD}]}{\left(\begin{array}{c} K_{NAD13}[\text{PYR}] + K_{PYR13}[\text{NAD}] + \frac{K_{I-PYR13}K_{NAD13}[\text{NADH}]}{K_{I-NADH13}} \\ +[\text{PYR}][\text{NAD}] + \frac{K_{NAD13}[\text{PYR}][\text{NADH}]}{K_{I-NADH13}} \end{array}\right)}$$	K_{NAD13} = estimated $K_{I-NADH13}$ = estimated $K_{PYR13} = 70\ \text{mM}$ $K_{I-PYR13} = 20\ \text{mM}$
Pyruvate decarboxylase	Teusink *et al*. (2000) $V_{11\,m}$ = estimated
$$\text{PYR} \rightarrow \text{ACA} + \text{CO}_2$$	
$$V_{PDC} = (1 + \text{Expr}_2 t)\frac{V_{11m}\left[\frac{[\text{PYR}]}{K_{11}}\right]^{n^{11}}}{1 + \left[\frac{[\text{PYR}]}{K_{11}}\right]^{n^{11}}}$$	$K_{11} = 4.3\ \text{mM}$ $n_{11} = 1.9$ Expr_2 = estimated

Table 1. Continued.

Rate equation	Parameter values
Alcohol dehydrogenase (ADH1)	Ganzhorn *et al*. (1987), Teusink *et al*. (2000)
$$ACA + NADH + H^+ \leftrightarrow EtOH + NAD^+$$	$V_{12\,m}$ = estimated
$$V_{ADH} = \frac{K_t V_{12\,m}\left(\frac{[ACA][NADH]}{K_{INADH}K_{ACA}} - \frac{[EtOH\,NAD]}{K_{IEtOH}K_{NAD}}\right)}{\left(\begin{array}{l} 1 + \frac{[EtOH]}{K_{IEtOH}} + \frac{K_{EtOH}[NAD]}{K_{IEtOH}K_{NAD}} + \frac{K_{NADH}[ACA]}{K_{INADH}K_{ACA}} + \frac{[NADH]}{K_{INADH}} \\ + \frac{[EtOH][NAD]}{K_{NAD}K_{IEtOH}} + \frac{K_{NADH}[EtOH][ACA]}{K_{IEtOH}K_{INADH}K_{ACA}} + \frac{K_{EtOH}[NAD][NADH]}{K_{NAD}K_{IEtOH}K_{INADH}} \\ + \frac{[ACA][NADH]}{K_{ACA}K_{INADH}} + \frac{[EtOH][NAD][ACA]}{K_{IEtOH}K_{NAD}K_{IACA}} + \frac{[NAD][ACA][NADH]}{K_{NAD}K_{IEtOH}K_{INADH}} \end{array}\right)}$$	K_{EtOH} = 17 mM K_{IEtOH} = 90 mM K_{ACA} = 1.1 mM K_{IACA} = 1.1 mM K_{NAD} = 0.17 mM K_{INAD} = 0.92 mM K_{NADH} = 0.11 mM K_{INADH} = 0.03 mM $K_t = t$ for WT and HXT1, K_t = 1 for HXT7 and TM6*
Lp-GPD Lumped reactions from DHAP to glycerol	Hynne *et al*. (2001)
$$DHAP + NADH + H^+ \rightarrow Glyc + NAD^+$$	$V_{15\,m}$ = estimated
$$V_{lpGLYC} = \frac{K_t V_{15\,m}[DHAP]}{\left(\begin{array}{l} K_{15DAHP}\left[1 + \frac{K_{15INADH}}{[NADH]}\left(1 + \frac{[NAD]}{K_{15INAD}}\right)\right] \\ + [DHAP]\left[1 + \frac{K_{15NADH}}{[NADH]}\left(1 + \frac{[NAD]}{K_{15INAD}}\right)\right] \end{array}\right)}$$	K_{15DHAP} = 25 mM K_{15INAD} = 0.13 mM K_{15NADH} = 0.034 mM $K_{15INADH}$ = 0.13 mM $K_t = t$ for WT and HXT1, K_t = 1 for HXT7 and TM6*
Diffusion of ethanol	Hynne *et al*. (2001)
$$EtOH \leftrightarrow EtOHx$$	k_{13} = 16.72 min^{-1}
$$V_{diffEtOH} = k_{13}([EtOH] - [EtOH_e])$$	
Diffusion of glycerol	Hynne *et al*. (2001)
$$Glyc \leftrightarrow Glycx$$	k_{16} = 1.9 min^{-1}
$$V_{diffGlyc} = k_{16}([Glyc] - [Glyc_e])$$	
Oxygen consumption	$r_{O_2uptake}$ = measured
$$NADH + H^+ + ADP + P_i + 0.5O_2 \rightarrow NAD^+ + ATP + H_2O$$	
$$V_{resp} = 2r_{O_2\,uptake} - 0.002658\,vbiomass$$	
Lp-ATP (Lumped nonspecific ATP consumption)	Hynne *et al*. (2001)
$$ATP \rightarrow ADP + P_i$$	k_{23} = estimated
$$V_{cons} = k_{23}[ATP]$$	

Table 1. Continued.

Rate equation	Parameter values
Biomass formation $0.07520\,G6P + 0.02318\,BPG + 0.01551\,PEP + 0.11791\,PYR + 0.06624$ $ACA + 0.00118\,GLYC + 0.09194\,NAD^+ \rightarrow Biomass + 0.00776\,F6P + 0.00108$ $GAP + 0.09194\,(NADH + H^+) + 0.00922\,CO_2 + 0.00266\,O_2$ $V_{Biomass}$	Adapted from Sárvári Horváth *et al.* (2003) $V_{Biomass}$ = measured

concentrations, or massive accumulation of acetaldehyde or the hexose phosphates). Therefore, the parameter values for these strains were estimated by manual tuning.

The momentaneous biomass yield on ATP was calculated as the estimated growth rate [g (L cell)$^{-1}$ min^{-1}] divided by the estimated ATP consumption rate [mol (L cell)$^{-1}$ min^{-1})] at each time point.

Results

Sugar consumption rate correlated with product formation of four strains with different glycolytic flux

The four strains were cultured in chemostats with ethanol as the limiting substrate at a dilution rate of 0.1 h^{-1}. A constant CO_2 production level for at least three volume changes indicated steady-state growth (Fig. 1). The inflow was stopped, and either glucose or fructose was added to a final concentration of 110 mM (2%), turning the continuous culture into a batch cultivation. The CO_2 evolution varied for the four strains tested. In the wild type (Fig. 1a), sugar addition led to a sustained increase of CO_2 production for the 2-h span of the experiment. In contrast, the gas production rate of the HXT1 strain (Fig. 1b) reached a plateau a few minutes after the sugar pulse and thereafter remained constant. The HXT7 strain (Fig. 1c) displayed a slow increase in CO_2 over the 2 h studied. In the TM6* strain, the CO_2 profile was completely different, because it declined on sugar addition and subsequently stabilized on a reduced level (Fig. 1d).

The observed differences of the CO_2 profiles are in line with the observed sugar consumption and production rates. Figure 2a illustrates that the sugar consumption was the largest for the wild type, followed by the HXT1 and HXT7 strains, which consumed about half of the sugar added within 2 h. The TM6* strain showed a very low sugar consumption within the experimental time. These differences were further illustrated by the calculated sugar consumption rates (Table 2). The mean sugar consumption rate was reduced in all the mutant strains compared with the wild type, which showed a glucose consumption rate of 3.2 mmol glucose (g dry weight)$^{-1}$ h^{-1}. The HXT1 strain showed a 25% reduction, the HXT7 strain a 50% reduction and the TM6* strain a 90% reduction compared with the wild type.

Measurements of the sugar uptake capacity were made on steady-state cultivations fed with ethanol as the carbon source. The results were in the same range as the sugar consumption rates obtained during the pulse experiments. The V_{max} values ranged from 0.9 to 1.8 mmol glucose (g dry weight)$^{-1}$ h^{-1} and 1.7–3.9 mmol fructose (g dry weight)$^{-1}$ h^{-1}, but there were no systematic differences between the uptake capacities of the different strains.

Ethanol is the main product of yeast fermentation and there were clear differences in ethanol production for the four strains analysed (Fig. 2b). While the wild-type strain produced large quantities of ethanol, up to 70 mM during the 2 h of measurement, the production of the HXT1 strain did not exceed 30 mM. Yet, the most astonishing result was that the HXT7 strain did not produce noticeable amounts of ethanol even though the sugar consumption rate was five times higher than that of the TM6* strain, which, as expected, did not generate any detectable ethanol. Glycerol, which is a common by-product during fermentation, was also measured (Fig. 2c). The wild-type strain did not start releasing glycerol until 30 min after sugar addition. On the other hand, the HXT1 strain produced glycerol continuously and so did the HXT7 strain, albeit in smaller amounts. The glycerol production should be especially noticed because this strain did not produce any ethanol. The TM6* strain generated barely detectable amounts of glycerol.

The behaviour of the studied strains in medium containing either glucose or fructose did not show large disparities. Both sugars were consumed similarly; however, some differences in the ethanol released were apparent for wild type and HXT1 strains, the latter also showed differences in glycerol

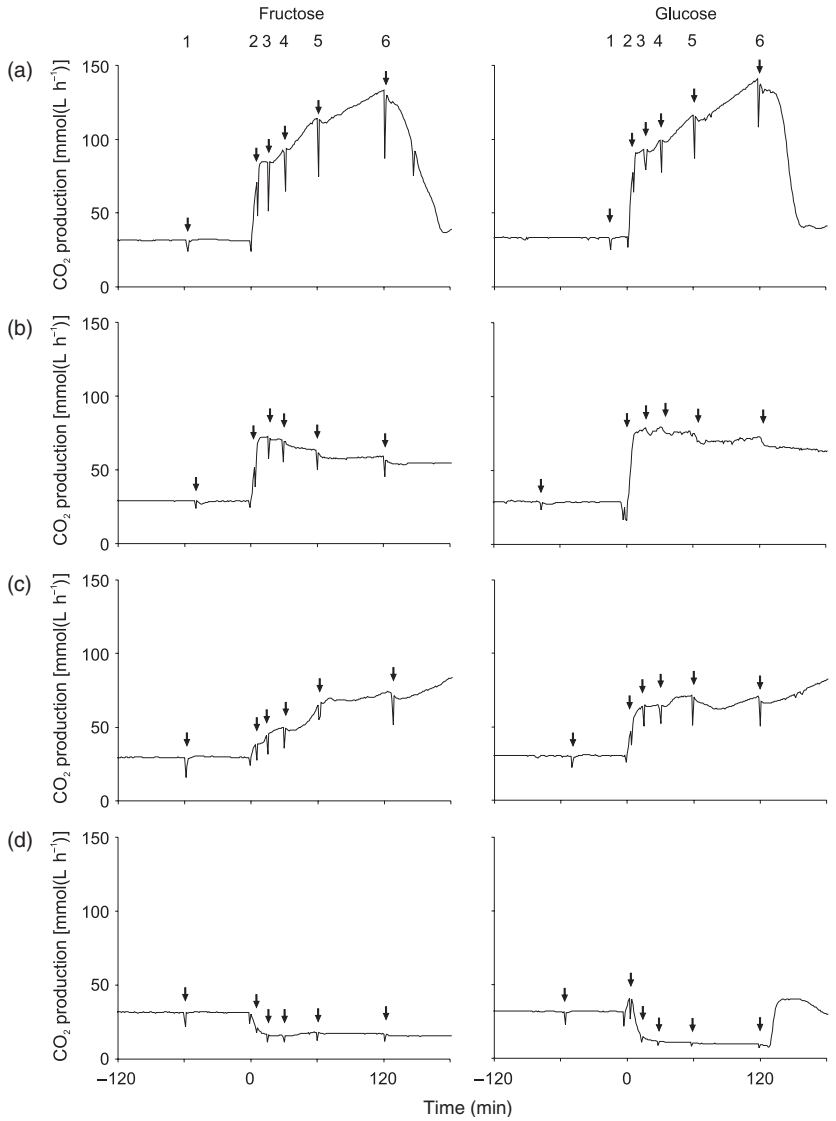

Fig. 1. Carbon dioxide production rate [mmol (L reactor)$^{-1}$ min^{-1}] during aerobic growth of the (a) wild type, (b) HXT1, (c) HXT7 and (d) TM6* strains in ethanol-limited continuous culture, with addition of sugar to a final concentration of 110 mM at time 0. The sampling points are marked with arrows and a number to facilitate identification. Arrow 1, at steady state; arrows 2–6 at 5, 15, 30, 60 and 120 min after sugar addition, respectively.

formation. This might be explained by distinct kinetics of the hexose transporters towards glucose and fructose. However, this question must be further addressed in a dedicated study (Berthels *et al*., 2004).

Intracellular levels of glycolytic intermediates and adenine nucleotides differed between strains with different rates of glycolysis

We chose to monitor the levels of the three first metabolites of glycolysis: G6P, F6P and F16BP, as well as adenine nucleotides, because some of these have been proposed to take part in the glucose repression response (Boles *et al*., 1993). The most remarkable result of these measurements was the difference between the G6P and F16BP concentration of the four strains (Fig. 3c). This correlated well with the sugar consumption rate of the different strains, F16BP being highest in the wild type, followed, in declining order, by HXT1, HXT7 and the TM6* strain. The opposite occurred for the levels of G6P; TM6* exhibited the highest G6P concentration while the wild-type level was very low after sugar addition (Fig. 3a). In HXT7, the concentration of G6P changed substantially during the measured time as well as between the two sugars. The level of F6P was very low in all four strains and showed the same pattern: a rapid decrease after addition of glucose or fructose to a level close to the detection limit (Fig. 3b). It is interesting to observe that variations in G6P concentration were particularly pronounced in the TM6* and HXT7 strains during the first 30 min after sugar addition, before stabilizing for the remaining time of the experiment. The end product of glycolysis, pyruvate, attained the highest levels in the wild type (Fig. 3d).

Large differences were observed in adenosine nucleotide levels between the TM6* and the wild-type strains. Thus, in

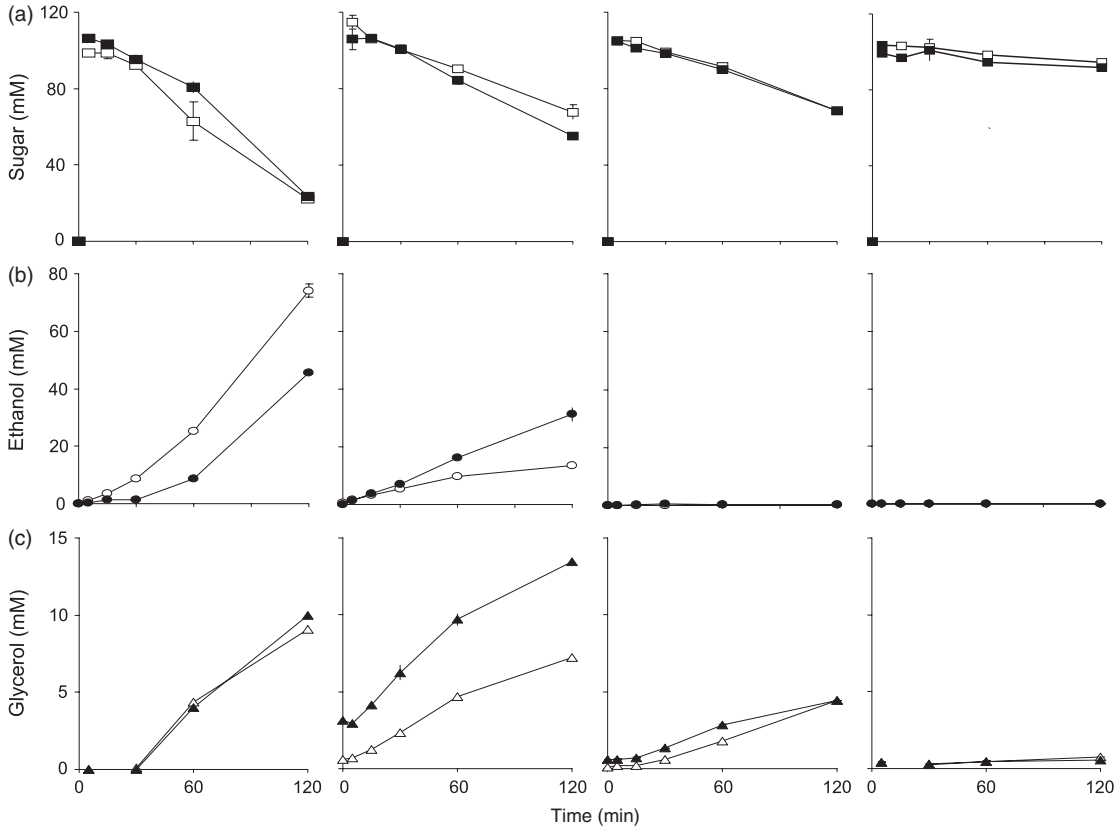

Fig. 2. Determination of extracellular (a) sugar (squares), (b) ethanol (circles) and (c) glycerol (triangles) after addition of glucose (filled symbols) or fructose (open symbols) to ethanol-limited chemostats. Error bars indicate the maximum and minimum values from duplicate samples.

Table 2. Mean sugar consumption and ethanol production rates measured over 2 h after a sugar pulse to ethanol-limited chemostats*

	Sugar consumption rate (mmol g h^{-1})		Ethanol production rate (mmol g h^{-1})	
Strains	Fructose	Glucose	Fructose	Glucose
Wild type	3.21 ± 0.07	3.21 ± 0.03	1.76 ± 0.02	3.07 ± 0.07
HXT1	2.55 ± 0.23	2.30 ± 0.25	1.51 ± 0.13	0.63 ± 0.01
HXT7	1.48 ± 0.02	1.60 ± 0.05	0.01 ± 0.00	0.01 ± 0.01
TM6*	0.33 ± 0.03	0.42 ± 0.08	0	0

*Glucose or fructose was added to a final concentration of 110 mM.

the mutant strain, ATP was higher (Fig. 4a), while ADP and AMP were lower (Fig. 4b and c) compared with the levels of the wild type. As a result of this, the ATP : ADP ratio as well as the ATP : AMP ratio were substantially higher in TM6* compared with that of the wild type, while the NAD^+ levels remained similar in both (Fig. 4d).

Storage carbohydrate accumulation varied between strains with different rates of glycolysis

The TM6*, HXT1 and HXT7 strains accumulated trehalose as well as glycogen during the ethanol-limited growth in the chemostats. The TM6* continued to accumulate these compounds also after the sugar pulse in contrast to the HXT1 and HXT7 strains, which, after the addition of sugar, consumed the storage carbohydrates produced earlier (Fig. 5). The short transient decrease in the trehalose content of the TM6* strain was correlated with the small transient increase in the CO_2 evolution rate (Fig. 1d) during the first 15 min after the glucose pulse. The wild-type strain showed accumulation neither during ethanol-limited growth nor after the pulse, except for a transient accumulation of glycogen directly after the sugar pulse (Fig. 5).

Modelling of glycolytic flux control

Yeast undergoes a tremendous and rapid reprogramming in response to a sudden glucose pulse. More than 1000 genes

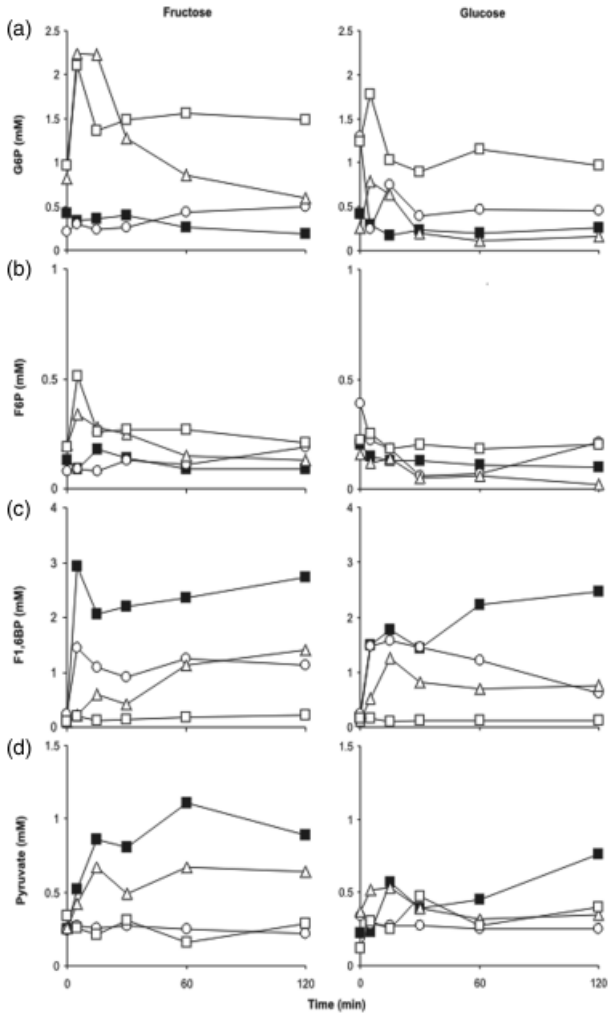

Fig. 3. Determination of intracellular (a) G6P, (b) F6P, (c) F16BP and (d) pyruvate, of the wild type (■), HXT1 (○), HXT7 (△) and TM6* (□) strains. SEM was below 15% for all measurements.

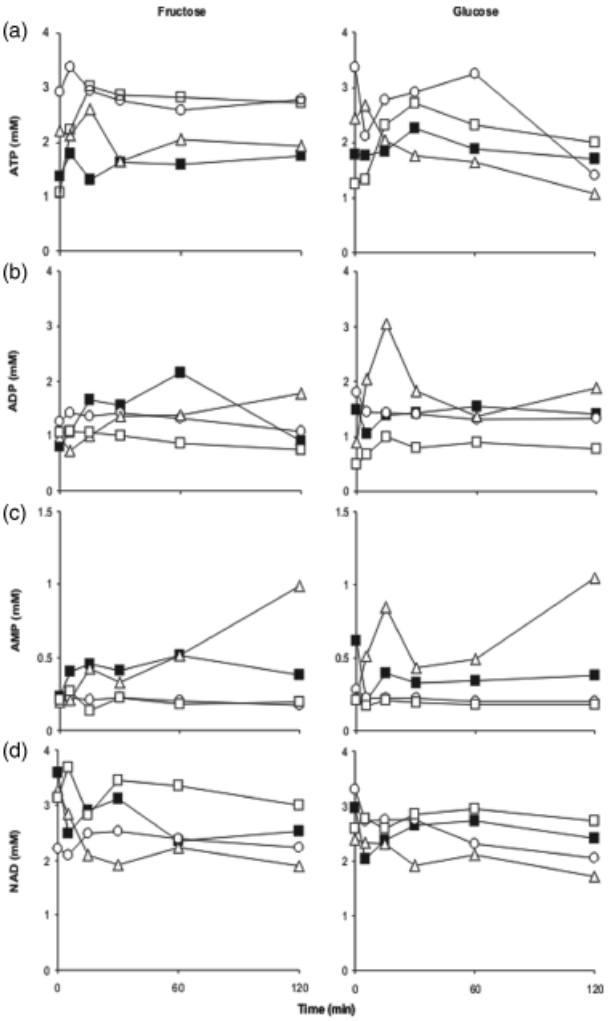

Fig. 4. Determination of intracellular (a) ATP, (b) ADP, (c) AMP and (d) NAD^+, of the wild type (■), HXT1 (○), HXT7 (△) and TM6* (□) strains. SEM was below 15% for all measurements.

were significantly affected in the first 5 min after a glucose pulse to a glucose-limited chemostat, together with significant alterations of intracellular metabolite concentrations (Kresnowati *et al.*, 2006). This initial process is still not well understood, and requires a substantially more detailed modelling approach than the one applied here. Instead, we focus on comparing the four strains during 2-h transients. During this period, metabolism is predominantly respiro-fermentative in the wild type. The model was, therefore, constructed for respiro-fermentative growth on glucose, and was not designed for detailed analysis of the short first transient from respiratory growth. The aim was to investigate whether the changes in intracellular metabolite concentrations and extracellular product profiles could be explained by shifts in enzymatic activities in the different steps of glycolysis. After parameter estimation (Table 3), the model fitted the observed trends well for most of the metabolites in all four different strains (Fig. 6). The glucose uptake rate increased throughout the transients, while the hexose phosphate concentrations, with few exceptions, remained constant or decreased slightly after the first rapid changes in the early stages. This was taken into consideration by increasing the V_{max} values of the upper part of glycolysis by a factor $(1+\mathrm{Expr}_1 t)$.

The estimated ethanol production could not be completely eliminated in the HXT7 and TM6* strains. To reach a feasible solution for these strains, a careful balance between the maximum rates of the Lp-PDH, the PDC, the ADH and the GapDH was necessary. An imbalance led to either excessive accumulation of acetaldehyde, negative acetaldehyde concentrations or erroneous glycerol formation. Therefore, parameters were fitted by manual tuning rather than by optimization for the HXT7 and the TM6* strains.

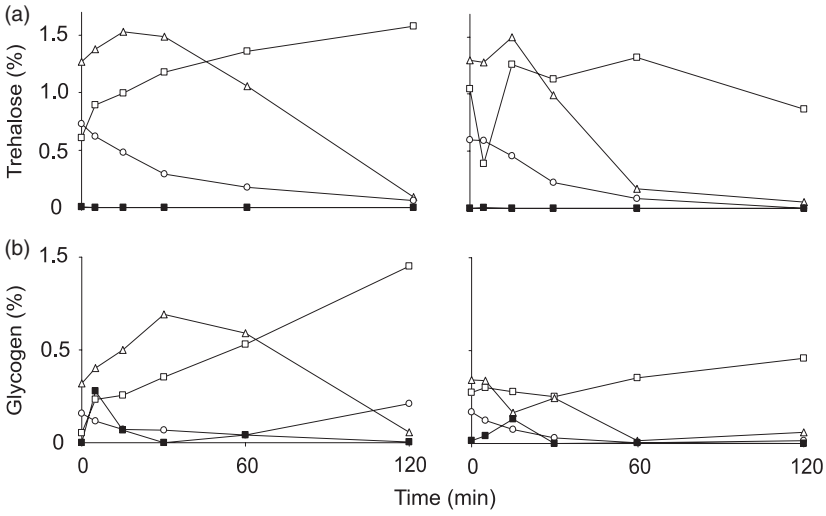

Fig. 5. Determination of (a) trehalose and (b) glycogen, of the wild type (■), HXT1 (○), HXT7 (△) and TM6* (□) strains. Concentrations are expressed as percentage [g glucose released (g dry weight)$^{-1}$].

The estimated initial V_{max} of PFK decreased for all three mutant strains – from 259 mM min^{-1} for the wild type to 9 mM min^{-1} for the TM6* strain (Table 3). The V_{max} of the PGI decreased by a factor 20 in TM6* compared with the wild type. Moreover, the rate of change of expression of the enzymes of the upper part of glycolysis was also lower in the TM6* strain. With these parameter values, the observed differences in the concentrations of G6P, F6P and F16BP could be recreated.

To obtain reasonable values of the predicted NAD^+ concentrations, it was necessary to modify the sensitivity of Lp-PDH towards NAD^+ and NADH. This was done by including the two parameters K_{NAD} and $K_{I,NADH}$ in the parameter optimization, in addition to the V_{max}. The K_{NAD} and $K_{I,NADH}$ values of the TM6* strain were *c.* 10% and 35% of the corresponding values for the wild type strain. At constant NAD^+ and NADH concentrations, a lower K_{NAD} means a higher rate, but lower sensitivity to the NAD^+ concentration, whereas a lower $K_{I,NADH}$ value means a lower rate but higher sensitivity to inhibition by NADH. In other words, the PDH and the TCA cycle reactions in the TM6* strain were, as a whole, generally closer to their maximal rates, but were also more sensitive to inhibition by NADH. In the TM6* strain, respiratory catabolism of glucose via the TCA cycle, together with a reduced growth rate, could potentially lead to an increased NADH concentration. A control structure as outlined above may, under such conditions, help to maintain the NADH levels low enough, such that any unnecessary glycerol and ethanol production is prevented.

The TM6* strain had both a reduced glycolytic rate and a reduced growth rate during the transient. However, the TM6* apparently had a greater energetic efficiency, because the rate constant for the nonspecific ATP consumption was only 7% of that of the wild type. Indeed, at the end of the 120-min transients, the biomass yield on ATP was much higher (22.6 g mol^{-1}) for TM6*, than for the wild-type strain (7.3 g mol^{-1}). The biomass yields on ATP increased steadily for all strains, indicating a continuous adaptation to the environmental conditions.

Discussion

In this study, four *S. cerevisiae* strains with different glycolytic rates were studied in order to understand the underlying mechanism driving the shift from respiration to fermentation. All strains were initially cultivated in aerobic chemostats, with ethanol as the only energy and carbon source. This guaranteed that all strains used a fully respiratory metabolism. When suddenly exposing the cells to a glucose (or fructose) pulse, the strains responded very differently. The wild type and the HXT1 turned immediately to a respiro-fermentative metabolism, while HXT7 and TM6* maintained respiratory metabolism. A reduction in sugar consumption from one strain to another, such as comparing the wild type with the TM6* strain, was accompanied by a decrease in product formation of ethanol and glycerol, accumulation of storage carbohydrates and variations in the intracellular concentrations of glycolytic metabolites and nucleotides. The intermediate consumption rates represented by the HXT1 and HXT7 strains also illustrated these trends.

In general, addition of glucose or fructose to wild-type cells triggers both glucose repression and a switch from respiratory to mainly fermentative metabolism (Rolland *et al.*, 2002). This pattern was observed for the wild-type and the HXT1 strains. Previous reports on aerobic batch cultures have shown that the ethanol production rate in the HXT7 strain is about 50% of that of the wild type (Elbing *et al.*, 2004a, b). In contrast, it was observed here that

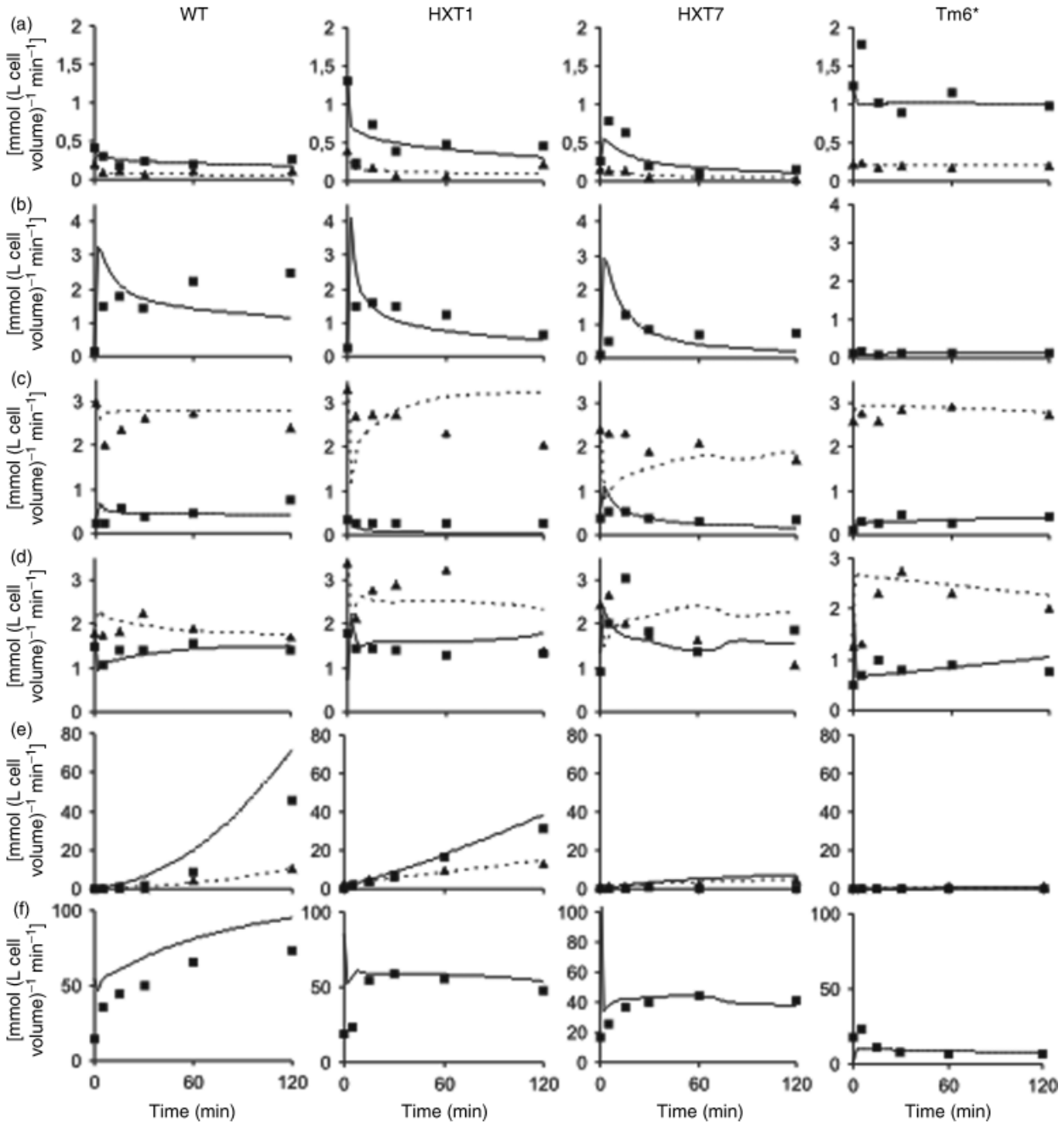

Fig. 6. Simulation of intracellular metabolite concentrations compared with experimental data. (a) G6P (squares, solid line) and F6P (triangles, dashed line), (b) F16BP, (c), pyruvate (squares, solid line) and NAD^+ (triangles, dashed line) (d) ADP (squares, solid line) and ATP (triangles, dashed line) (e) extracellular ethanol (squares, solid line) and glycerol (triangles, dashed line) and (f) CO_2 production rate [mmol (L cell volume)$^{-1}$ min^{-1}]. Symbols denote experimental data and lines indicate simulated values.

(Kresnowati *et al.*, 2006). During the 120 min duration of the experiments, the size and distribution of the AxP pool continue to change. The ATP : AMP ratio is a potential candidate for the initial glucose repression signal, because it links energy availability and sugar degradation; in addition, it is known to have regulatory functions on sensing components of mammalian cells, particularly on the AMP-activated protein kinase (AMPK) (Wilson *et al.*, 1996). However, attempts to explain glycolytic regulation in yeast according to low energetic levels have found limited support (Hardie *et al.*, 1998) particularly because Snf1, the yeast homologue of AMPK (Celenza & Carlson, 1986; Hardie

et al., 1998), does not respond to ATP or AMP *in vitro*. One hypothesis would be that the ATP : AMP ratio acts as a primary signal on glycolytic enzymes, e.g. PFK, HK and PK and the outcome affects one or several glucose responsive signalling networks such as, for example, Snf1. Nevertheless, it was found that the ATP : AMP ratio displayed in TM6* was considerably higher than that in the wild type. This could be the result of either a higher energetic efficiency (P/O ratio) or a lower nonspecific ATP consumption of the TM6* strain compared with the wild type. From the results, we cannot distinguish between these, but the effect is manifest in the lower estimated rate constant for the nonspecific ATP consumption, and in the higher biomass yield on ATP. Following up the same line of correlations as in mammals, the higher ATP : AMP ratio should result in an inactive Snf1. In contrast, it has indeed been shown that Snf1 is active in TM6* consistent with a glucose-derepressed strain (Elbing *et al*., 2004b). The conclusion is that a direct correlation between the ATP : AMP ratio and Snf1 activity does not apply, at least not in explaining the derepressed state of the TM6* strain at high external glucose concentrations.

Earlier evidence has been presented (Elbing *et al*., 2004a) that the dominating control on the glycolytic flux in TM6* was exerted by the limited uptake capacity of the hexose transporter (the Tm6* protein). This resulted in the respiratory phenotype of the TM6* strain. However, in the former study, strains were grown in batch cultures with glucose as the only carbon and energy source. In this study, on the other hand, measurements of uptake capacity of the four strains were retrieved during steady-state growth on ethanol. In this case, the uptake capacity did not show any substantial difference between the strains. Still, the cells showed large differences in response to sugar addition, both concerning the immediate response and the response registered 2 h after sugar addition. These new data complement the earlier presented hypothesis from the authors' laboratory, which pinpoints the hexose transport ability of the TM6* strain as the main controlling agent of its respiratory phenotype (Elbing *et al*., 2004a). The present study illustrates that the main controlling mechanism may differ depending on the cultivation conditions under which cells are grown. PFK has for a long time been regarded as a key enzyme in the regulation of the glycolytic rate; however, in recent years, it has been proposed that its main role would be played during carbon-source adaptation (Heinisch *et al*., 1996; Rodicio *et al*., 2000). In line with this hypothesis, we show that changed kinetics of PGI and PFK, and a modified sensitivity of PDH and the TCA cycle towards NAD^+ and NADH, may be responsible for the shift from respiro-fermentative to respiratory metabolism. The present data are not sufficient to derive the mechanisms underlying these kinetic modifications.

The initial glucose repression signal remains elusive and further work will have to be performed to identify its nature. We believe that the set of strains used in this study will contribute to disclosing the mechanism of glucose repression in yeast, for instance by studying the transcriptional response after addition of glucose. On the other hand, this study has contributed with novel information on the control of the regulation between respiro-fermentative and respiratory metabolism.

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Authors' contribution

D.B. and M.J. contributed equally to this work.

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Table 3. Estimated model parameter values for the wild type, HXT1, HXT7 and TM6* strains*

Reaction	Parameter†	Wt	HXT1	HXT7‡	TM6*‡
HK	$V_{3\,m}$ (mM min^{-1})	*1000*	*1000*	*1000*	*1000*
PGI	$V_{4\,m}$ (mM min^{-1})	*250*	*250*	*250*	13
PFK	$V_{5\,m}$ (mM min^{-1})	259	71	61	9
Ald	$V_{6\,m}$ (mM min^{-1})	88	58	149	97
TPI	$V_{7\,m}$ (mM min^{-1})	*116*	*116*	116	116
GapDH	$V_{8\,m}$ (mM min^{-1})	4.34×10^{6}	173	4.1×10^{7}	1.3×10^{5}
HKT/PGI/PFK/Ald/GapDH	$Expr_1$ (min^{-1})	0.06	0.05	0.06	0.004
PK	$V_{10\,m}$ (mM min^{-1})	*1.5×10^{5}*	*1.5×10^{5}*	*2.4×10^{8}*	*2.4×10^{8}*
PDC	$V_{11\,m}$ (mM min^{-1})	156	78	51	117
	$Expr_2$ (min^{-1})	0.20	0.09	0.29	0
ADH	$V_{12\,m}$ (mM min^{-1})	4.12×10^{7}	1.66×10^{11}	3.7×10^{9}	800
Lp-GPD	$V_{15\,m}$ (mM min^{-1})	89	60	560	63
Lp-PDH	V_m (mM min^{-1})	4.74×10^{4}	1.27×10^{5}	5.0×10^{4}	1.3×10^{3}
	K_{NAD} (mM)	2170	5440	1660	250
	$K_{I,\,NADH}$ (mM)	4.3	5.0	22	1.5
Lp-ATP	K_{23} (min^{-1})	122	60	50	8

*Parameters were estimated using nonlinear least squares minimization with Gauss–Newton algorithm in MATLAB©, except where noted. Values in italics were set as constants and were not part of the optimization. See main text for further details.

†Units refer to total cell volume, mmol (L cell volume)$^{-1}$ min^{-1}. The cellular volume was assumed to be 2 mL (g dry weight)$^{-1}$. See Table 1 for additional parameters and notation.

‡Parameters were fitted by manual tuning. Proper optimization gave infeasible solutions.

HK, hexokinase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; Ald, aldolase; TPI, triose phospate isomerase; GapDH, glyceraldehyde 3-phosphate dehydrogenase; Lp-PEP, lumped phosphoglycerate kinase, phosphoglycerate mutase, enolase; PK, pyruvate kinase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; Lp-GPD, lumped glycerol 3-phosphate dehydrogenase and glycerol 3-phosphatase; Lp-PDH, lumped pyruvate dehydrogenase and complete TCA cycle reactions; Lp-ATP, lumped, nonspecific ATP consumption.

ethanol formation was negligible in the HXT7 strain while glycerol reached a level remarkably similar to that of the wild type. TM6* showed fully respiratory metabolism on both glucose and fructose, in contrast to a previous study in which ethanol could be produced in substantial amounts during growth on fructose in batch culture (Henricsson *et al.*, 2005).

The different sugar consumption rates were also reflected in the concentrations of intracellular metabolites. G6P and F6P levels were higher for strains with a lower sugar consumption rate (i.e. HXT7 and TM6*) than for those with a higher rate (i.e. wild type and HXT1). F16BP measurements produced quite the opposite result, i.e. considerably lower levels were found in the TM6* strain compared with the wild type. A similar pattern in the enological V5.TM6*P strain has been reported previously (Henricsson *et al.*, 2005). High concentrations of F6P and G6P, together with low levels of F16BP, may indicate that PFK activity was inhibited. Elevated G6P over F6P concentrations may indicate that the PGI activity was limited, or that the equilibrium constant was affected. Furthermore, the high degree of accumulation of carbohydrates (glycogen and trehalose) may result from an increased level of their substrate (G6P). By applying the kinetic model of the central metabolic fluxes, it was found that alterations in the maximal rates of PFK and PGI could indeed explain the metabolite concentrations measured for the different strains. The simulations also reproduced the high levels of ATP in the TM6* strain. Under these conditions, ATP acts as a powerful inhibitor of PFK, without being counteracted by AMP (Reibstein *et al.*, 1986; Sols *et al.*, 1989; Larsson *et al.*, 2000). Another well-known regulator of PFK activity is F26BP, whose synthesis and degradation is regulated by cAMP-activated protein kinases (Heinisch *et al.*, 1996). Cells growing on respiratory carbon sources are known to exhibit a transient increase in cAMP levels on addition of glucose (Rolland *et al.*, 2001). F26BP was not measured, and its effect on PFK could thus only be included via an assumed constant concentration based on the literature data. Although adenylate cyclase, the enzyme responsible for synthesis of cAMP from ATP, is activated by one branch responding to external glucose concentrations (Gpr1), there is another branch that requires glucose, fructose or mannose phosphorylation (Ras). The latter could be impaired in strains with reduced sugar uptake, therefore leading to abnormally low levels of cAMP. This view is supported by the high levels of glycogen and trehalose observed in this experiment, compared with the wild type.

The total pool of adenine nucleotide as well as the individual nucleotides undergo rapid transients during the first 5 min of a glucose pulse, in part due to the massive transcriptional reprogramming and protein synthesis

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Paper 2

Uptake is required for glucose-stimulated transcriptional responses in *Saccharomyces cerevisiae*

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Uptake is required for glucose-stimulated transcriptional responses in *Saccharomyces cerevisiae*

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Running title: Response to extracellular glucose.

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Keywords: Hexose transporter, Glucose repression, Glucose signalling, Null strain.

Abstract

Cells depend on the environment for sources of nutrients and hence monitoring nutrient availability is vital to trigger adaptative responses. Nutrient sensing can be mediated by external and internal mechanisms. The model organism, *Saccharomyces cerevisiae*, relies on hexose monosaccharides as the preferred carbon source. Their presence is sensed by two modified hexose transporters, Rgt2 and Snf3, and by a G-protein coupled receptor, Gpr1. The first two regulate the expression of hexose transporters, while the latter modulates downstream Protein Kinase A (PKA) activity. So far it has been difficult to differentiate which responses depend on extracellular sensing of glucose and which to intracellular mechanisms. In the present study we sought to discriminate between these two pathways and thus characterised the transcriptional profile of a strain unable of transporting sugar. We show that all transcriptional glucose responses require glucose internalisation. This indicates that intracellular glucose or metabolites derived from it are required for transcriptional changes associated with glucose exposure.

Introduction

Glucose and fructose are the preferred sources of energy and carbon for *Saccharomyces cerevisiae*. Their presence mediates repression of systems required for the consumption of other carbohydrates and respiratory substrates by a combination of transcriptional, translational and post-translational mechanisms that are collectively known as the glucose repression response (Gancedo, 1998).

Yeast senses the presence of glucose mainly by means of three signalling pathways: Snf3/Rgt2, Gpr1/Ras/PKA and Snf1/Mig1 (Santangelo, 2006). Snf3 and Rgt2 are two hexose transporter-like

sensors of extracellular glucose, which initiate a signalling cascade aimed at maximising the sugar transport capacity by regulating the expression of the hexose transporter genes (Johnston & Kim, 2005). This is attained by controlling the activity of the transcriptional factor Rgt1. In the absence of glucose, Rgt1 represses expression of the hexose transporters genes in concert with Mth1 and Std1 (Fig. 1). The presence of glucose leads to phosphorylation of Rgt1 and its dissociation from *HXT* gene promoters as well as to degradation of Mth1 and Std1 (Flick *et al.*, 2003; Moriya & Johnston, 2004). The presence of glucose also transforms Rgt1 from a transcriptional repressor into an activator (Özcan *et al.*, 1996b). Rgt1 appears to control a very small set of genes beyond hexose transporters, of which only the Mig1-like transcriptional repressors *MIG2* and *MIG3* appear to play a role in glucose-dependent gene responses (Polish *et al.*, 2005). Although, disruptions of Snf3 or Rgt2 decrease the cell's ability to respond to glucose, this is chiefly caused by a reduction in glucose transport capacity (Özcan *et al.*, 1996a). This observation indicates that the glucose response is possibly uncoupled from the external glucose concentration.

Cells growing on respiratory carbon sources such as ethanol are known to experience a transient increase in cAMP levels upon addition of glucose (Rolland *et al.*, 2001b). cAMP functions as second messenger initiating a phosphorylation cascade that leads to changes in central carbon metabolism, cell proliferation and stress resistance (Geladé *et al.*, 2003). The role of cAMP is to mediate the activation of Protein Kinase A (PKA), thus promoting cell proliferation at the expense of stress resistance. The levels of cAMP are

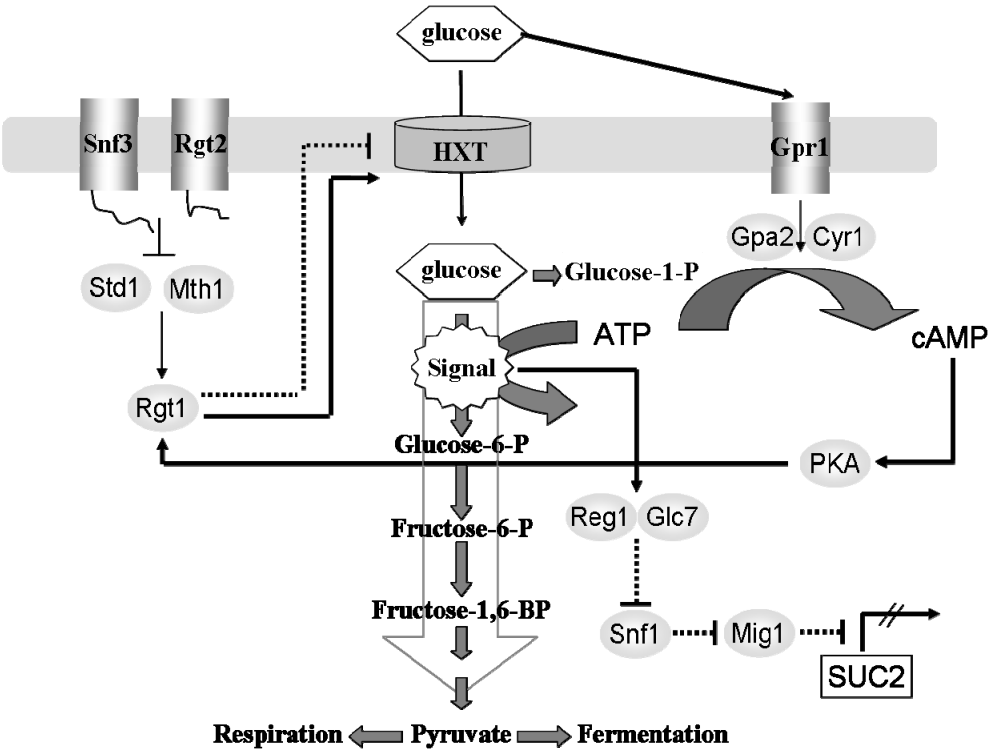

Figure 1. Sugar sensing and signalling in *S. cerevisiae*.

regulated by a balance between synthesis, mediated by adenylate cyclase (Cyr1), and degradation catalysed by two phosphodiesterases (Pde1 and Pde2) (Thevelein & de Winde, 1999). Adenylate cyclase localisation at the cytosolic face of the plasma membrane facilitates the interaction with the two activating branches, Gpr1/Gpa2, which monitor extracellular glucose, and the small GTP-binding proteins Ras1/Ras2, whose activity is stimulated by intarcelular acidification (Rolland *et al.*, 2002) (Fig. 1). Sucrose and low concentrations of extracellular glucose (20-30mM) lead to Gpr1-dependent activation of Gpa2 (Rolland *et al.*, 2001a), while the Ras1/Ras2-mediated response requires intracellular glucose phosphorylation (Colombo *et al.*, 2004).

The third glucose sensing mechanism involves Snf1, a key protein kinase, and its main target Mig1, the major transcriptional repressor of glucose-regulated genes. In the absence of glucose, Snf1 is active and stimulates the expression of glucose-repressed genes by phosphorylating and thereby inactivating Mig1 (Fig. 1). Glucose addition inactivates Snf1, leading to the conversion of Mig1 into an active repressor. Inactivation of Snf1 also requires intracellular glucose phosphorylation (Hedbacker & Carlson, 2008). More recent studies indicate considerable cross-talk between these signalling pathways, such as the Snf3/Rgt2 pathway regulating the expression of Mig1 in a glucose-dependent manner (Kaniak *et al.*, 2004). Moreover, glucose-activated PKA has been observed to affect phosphorylation of Rgt1, hence leading to the stimulated expression of hexose transporter genes (Kim & Johnston, 2006).

The three pathways appear to be activated upon the presence of glucose or its phosphorylation. Our aim is to distinguish between the cellular response arising due to the assimilation of glucose and those arising due to its mere presence. To that end we analysed the transcriptional profile of two *S. cerevisiae* strains, the wild type and a mutant unable to transport hexoses (Null). The Null strain lacks all known hexose transporters but retains the sensors Snf3/ Rgt2 and Gpr1, and it is unable to grow on sugars other than maltose.

Materials and methods

Strains

All *S. cerevisiae* strains used were derived from the wild type CEN.PK2-1C strain (*MAT***a** *leu2-3*,112 *ura3-52 trp1-289 his3*-Δ *MAL2-8*^{c} *SUC2 hxt17*Δ) (van Dijken *et al.*, 2000) and made prototrophic by integration of the corresponding markers (Otterstedt *et al.*, 2004). The phototrophic strain, called KOY.PK2-1C83P, was used as the wild type strain. The Null strain, KOY.VW101P, lacks all known hexotransporters (*gal2*Δ::*loxP* *stl1*Δ::*loxP* *agt1*Δ::*loxP* *ydl247w*Δ::*loxP* *yjr160c*Δ::*loxP* *hxt13*Δ::*loxP* *hxt15*Δ::*loxP* *hxt16*Δ::*loxP* *hxt14*Δ::*loxP* *hxt12*Δ::*loxP* *hxt9*Δ::*loxP* *hxt11*Δ::*loxP* *hxt10*Δ::*loxP* *hxt8*Δ::*loxP* *hxt514*Δ::*loxP* *hxt2*Δ::*loxP* *hxt367*Δ::*loxP)* (Wieczorke *et al.*, 1999).

Media and growth conditions

Cells were pre-cultured overnight in defined medium using 2% maltose as the sole

carbon source. The pre-culture was used to inoculate a 2 L fermentor (Braun Biostat A) with 2% maltose. A two-times concentrated defined minimal medium was used in all cultivations (Verduyn *et al.*, 1992), supplemented with 0.1 mL/L of anti-foaming agent (Polypropylene-2000 Fluka, Steinheim, Switzerland). The temperature was kept constant at 30°C with agitation at 1200 rpm. The pH was maintained at 5.0 by the automatic addition of 1 M NaOH. The airflow rate was controlled by a mass flow controller from Bronkhorstm, High-Tech, B.V. (Ruurlo, The Netherlands). The strains were grown to a steady-state in an aerobic chemostat culture with a working volume of 2 L at a dilution rate of 0.1 h^{-1} in a fermentor (Braun, Melsungen, Germany). The chemostats were initiated as batch until the maltose was depleted, after which, sterile medium consisting of 1% of ethanol was fed into the fermentor using a pre-calibrated peristaltic pump. Off-gas was monitored by a photoacoustic gas analyser (type 1308; Brüel and Kjær, Nærum, Denmark). Steady-state is defined by a constant value of CO_2 for at least 3 volume changes. Once steady-state was achieved, the ethanol feed was stopped and glucose pulsed into the vessel to a final concentration of 2%. Samples for all measurements were collected at steady-state (time = 0) and 20 min. All chemostats were performed in triplicates.

Determination of extracellular substrate and products

Duplicate samples of 1 mL each were sterile-filtered (0.22 µm), frozen in liquid N_2 and stored at -20°C. The analyses of glucose, ethanol and glycerol were performed using enzymatic combination kits (Boehringer-Mannheim GmbH, Mannheim, Germany).

Dry weight determination

Dry weight was measured in triplicates by centrifugation of 3 x 10 mL of cell culture in pre-weighed dry glass tubes. The cells were washed twice in 5 mL of deionised water (MilliQ), dried for 24 h at 110°C and stored in a desiccator before weighing.

RNA isolation and transcription analysis

Triplicate samples of 5 mL were extracted from the chemostats after the steady-state was achieved and pipetted into 10 mL ice cold water and then centrifuged at 4°C, 1 min. The pellet was washed with addition of 1 mL ice cold water and then centrifuged again. The pellet was frozen in liquid N_2 and stored at -80°C. Total RNA was extracted with the phenol/chloroform extraction method (De Winde *et al.*, 1996), purified (RNAeasy kit, Sigma) and treated with DNases (Qiagen). Global gene expression was profiled using Affymetrix Yeast Genome 2.0 arrays. All the steps involving the synthesis of cDNA from total RNA, labelling the cDNA for hybridization to the arrays, washing and straining of the arrays were performed as described in the Affymetrix geneChip® manual (www.affymetrix.com). The CEL files generated from the scanner are available at NCBI Gene Expression Omnibus (Accession number: XX). The expression data was extracted from the CEL files using dChip software (Li & Wong, 2001).

Quantification of sugar phosphates and nucleotides

Table 1. Down-regulated genes in Null strain respect to wild type strain on ethanol (16 ORFs).

ORF	Functional description	p-value
DLD3	D-lactate dehydrogenase, part of the retrograde regulon which consists of genes whose expression is stimulated by damage to mitochondria and reduced in cells grown with glutamate as the sole nitrogen source, located in the cytoplasm	3.39E-05
HXT1	Putative hexose transporter, expressed at low levels and expression is repressed by glucose	1.02E-04
HXT2	High-affinity glucose transporter of the major facilitator superfamily, expression is induced by low levels of glucose and repressed by high levels of glucose	5.43E-04
HXT3	Low affinity glucose transporter of the major facilitator superfamily, expression is induced in low or high glucose conditions	1.24E-03
HXT4	High-affinity glucose transporter of the major facilitator superfamily, expression is induced by low levels of glucose and repressed by high levels of glucose	8.52E-05
HXT5	Hexose transporter with moderate affinity for glucose, may function in accumulation of reserve carbohydrates during stress, expression induced by a decrease in growth rate, contains an extended N-terminal domain relative to other HXTs	1.19E-03
HXT7	High-affinity glucose transporter of the major facilitator superfamily, nearly identical to Hxt6p, expressed at high basal levels relative to other HXTs, expression repressed by high glucose levels /// High-affinity glucose transporter of the major facilitator superfamily, nearly identical to Hxt7p, expressed at high basal levels relative to other HXTs, repression of expression by high glucose requires *SNF3*	8.35E-04
HXT8	Protein of unknown function with similarity to hexose transporter family members, expression is induced by low levels of glucose and repressed by high levels of glucose	1.26E-04
HXT9	Putative hexose transporter that is nearly identical to Hxt11p, has similarity to major facilitator superfamily (MFS) transporters, expression of *HXT9* is regulated by transcription factors Pdr1p and Pdr3p	1.12E-02
HXT11	Possible pseudogene in strain S288C; *YIL170W*/*HXT12* and the adjacent ORF, *YIL171W*, together encode a non-functional member of the hexose transporter family /// Putative hexose transporter that is nearly identical to Hxt9p, has similarity to major facilitator superfamily (MFS) transporters and is involved in pleiotropic drug resistance	2.51E-05
RMD6	Protein required for sporulation	3.95E-06
STL1	Glycerol proton symporter of the plasma membrane, subject to glucose-induced inactivation, strongly but transiently induced when cells are subjected to osmotic shock	1.41E-03
URA3	Orotidine-5'-phosphate (OMP) decarboxylase, catalyzes the sixth enzymatic step in the de novo biosynthesis of pyrimidines, converting OMP into uridine monophosphate (UMP); converts 5-FOA into 5-fluorouracil, a toxic compound	2.06E-04
YJL216C	Protein of unknown function, similar to alpha-D-glucosidases; transcriptionally activated by both Pdr8p and Yrm1p, along with transporters and other genes involved in the pleiotropic drug resistance (PDR) phenomenon	1.1E-05
YJL217W	Cytoplasmic protein of unknown function; expression induced by calcium shortage and via the copper sensing transciption factor Mac1p during conditons of copper deficiency; mRNA is cell cycle regulated, peaking in G1 phase	1.09E-07
YJL218W	Putative protein of unknown function, similar to bacterial galactoside O-acetyltransferases; induced by oleate in an *OAF1*/*PIP2*-dependent manner; promoter contains an oleate response element consensus sequence; non-essential gene	2.89E-02

Intracellular concentration of sugar phosphates such as glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-bisphosphate; and adenine nucleotides (ATP, ADP and AMP) were measured by rapidly quenching 10 mL samples from the fermentor into chilled methanol (-60˚C). The metabolites were extracted by using TCA as previously described (Gustafsson, 1979). The sugar phosphates and nucleotides were concentrated using solid phase extracted method as described earlier (Ostergaard et al., 2001; Smits et al., 1998) and analysed using liquid chromatography. The concentration of the metabolites was determined by correlating the peak height to the concentration of the standards that have been subjected to similar sample extraction procedures, to account for losses during processing.

Results

To achieve maximal reproducibility of the results, cells were propagated in a continuous culture set-up (chemostat) under carbon limitating conditions, using ethanol as carbon and energy source. When the steady-state was established glucose was injected into the fermentor. Samples were collected during steady-state and 20 min after the glucose pulse. This time point was chosen in order to focus on primary rather than secondary responses.

1. The transcriptional profiles of the Null and wild type strains are very similar during steady state

The Null strain lacks all known glucose transporters (*HXT1-17*, *GAL2*, as well as putative transporters *AGT1*, *STL1*, *YDL247W* and *YJR160C*) (Wieczorke *et al.*, 1999). As a consequence, the strain is unable to grow on fermentable carbon sources, except maltose. Since the Null mutant has been under considerable genetic manipulation, we first compared its transcriptional profile during steady-state conditions on ethanol as sole and limiting carbon source with that of the corresponding wild type strain. No significant change of expression were observed, except for a group of 16 genes which corresponded to the deleted *HXT* genes or adjacent ORFs (Table 1). Therefore we concluded that in the absence of glucose the null strain has a similar expression profile than the wild type.

2. The glucose repression response in the wild type strain

Next, we compared the transcription profile of the wild type strain before and after the glucose pulse to estimate the reliability of the cultivation and mRNA analysis set-up. Twenty minutes after the glucose pulse 805 genes displayed significatively altered expression. Of those, the expression of 274 genes was up-regulated (Supplementary Table S1) and that of 531 genes was down-regulated (Supplementary Table S2). Both groups were further classified according to their GO annotated molecular function of their gene products (www.yeastgenome.org) (Fig. 2). Among the up-regulated genes, those encoding proteins involved in RNA, DNA metabolism, polysome and ribosomal components and glycolytic enzymes were over-represented.

Proteins participating in respiration, fatty acid oxidation, stress responses and glycogen, trehalose and energy metabolism were down-regulated.

3. The Null strain is insensitive to glucose addition

In the Null strain glucose addition resulted in no significant changes in gene expression. Only YFL068W and YFL067W were down-regulated (both about 1.5 fold) with statistical significance, but their function has not been established. Measurements of sugar phosphates in the wild type strain show a clear increase in the activity of glycolysis, however no such changes were observed in the Null strain.

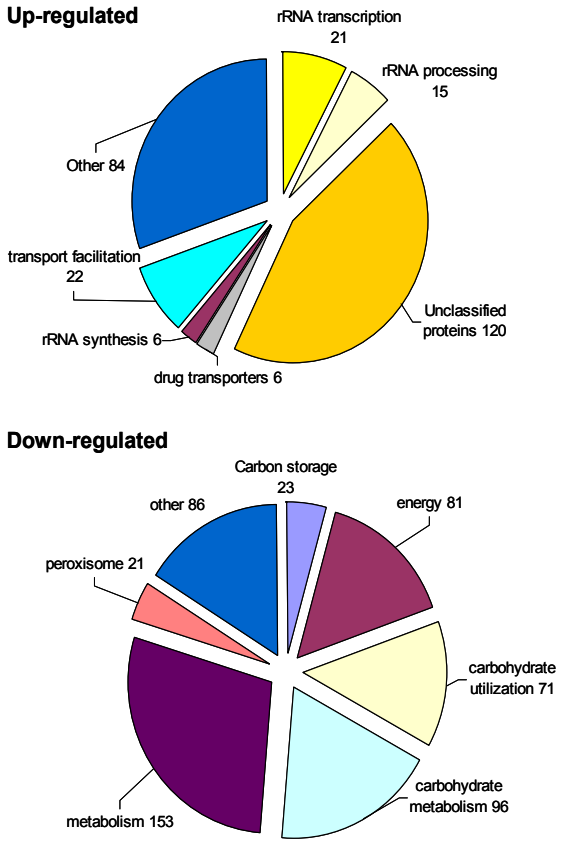

Figure 2. Functional classification of genes that changed expression significantly (2-fold) in the wild type strain before and after a 2% glucose pulse to ethanol growing cells.

Discussion

In the presence of glucose or fructose the budding yeast *S. cerevisiae* displays a broad range of responses allowing rapid adaption and growth. These changes occur at the transcriptional (Carlson, 1998; 1999; Gancedo, 1998), post-transcriptional (Kuhn *et al.*, 2001), translational (Ashe *et al.*, 2000; Kuhn *et al.*, 2001) as well as at post-translational level (Horak & Snyder, 2002; Jiang *et al.*, 2000). In this study, we address the question of whether these changes depend on the presence of extra or intracellular glucose. To that end, we used a strain that is devoid of glucose transporters.

First, Null and wild type strains were compared to ascertain that deletion of more than 20 genes did not have as a consequence a change in metabolic profile. Indeed, the number of significant changes concerned a very small group of genes whose expression was downregulated, mostly the deleted *HXT* genes.

Second, the glucose response displayed by the wild type strain was compared with previous studies. Expression of eighthundredandfive genes changed significatively in this study, which is a much smaller number than reported previously. Thus, (Wang *et al.*, 2004) found that 20 min after pulsing cells growing on glycerol with 2% glucose 41% (2343 genes) of the yeast genome displayed a 2-fold change in expression, with a similar number of up-regulated and down-regulated genes. (Kresnowati *et al.*, 2006) also showed that in cells growing on 1% ethanol subjected to

a 0.1% glucose pulse, expression of 1154 genes responded significantly. There are a number of reasons that can account for such differences between studies. For instance, the number of genes responding to glucose fluctuates with time. An early sample at 1 min presenst fewer changes than at 10 min, because to the first glucose response the one derived from growth it is also added. In addition, the number of genes responding to glucose varies with its concentration; therefore a 2% pulse is likely to trigger more changes than 1% (Yin *et al.*, 2003). Furthermore, physiological conditions such as growth system (fermentors or flasks), carbon source concentration at steady-state, dilution rate and even strain background may play a role. Another element that may have an influence is the choice of micro-arrays, normalisation procedure and the threshold above which a change is consider significant. The latter was stringent in this study to avoid false positives. On the other hand, even though the total gene number varies across studies, the functional categories of overrepresented genes remain similar. In summary, this study uncovered less genes responding to glucose than otherwise described in literature for the wild type, but the profile of the response is very similar.

Third, the Null strain did not show significant changes in gene expression, indicating that it is insensitive to the presence of glucose in the environment. This suggests that unless transported into the cell glucose is unable to trigger large transcriptional responses. This reinforces the notion that Snf3/Rgt2 only control a small group of genes, mainly HXTs (Kaniak *et al.*, 2004). Moreover, the present results suggest that Gpr1/Gpa2 system is not sufficient to trigger PKA-dependent responses, likely because the concurrent activation of Ras proteins is required, an event that is dependent on glucose internalisation (Rolland *et al.*, 2000). These results are in line with observations from previous experiments in which the transcriptional profile of strains bearing constitutive active forms of Gpa2 and Ras2 were studied in the absence of extracellular glucose (Wang *et al.*, 2004). The contributions of both branches to PKA activation were shown to be unequal. Artificially activated Gpa2 was unable to trigger a PKA response in a Ras-insensitive mutant, while Ras2 was able to cause glucose responses in the absence of Gpa2 (Rolland et al 2000). This finding underscores the importance of Ras2 and internal glucose in triggering PKA.

Since the Null strain does not respond to glucose and given that the feed of ethanol was halted upon addition of glucose it should develop a starvation response. However, no apparent starvation response could be traced in the transcriptional profile. One hypothesis would be that cells growing under carbon-limited conditions have already up-regulated stress response mechanisms, but there is clear prrof against this argument (Brauer *et al.*, 2005; Saldanha *et al.*, 2004). Therefore, we have no explanation for the lack of a response in the Null strain.

In conclusion, transcriptional and metabolic profile indicates that the Null strain is insensitive to the presence of

glucose. The role of the extracellular glucose sensors focuses on regulating the inflow of hexoses (Snf3/Rgt2 regulation of *HTX*s genes) and adaptation to fermentable carbon sources (Gpr1/Gpa2), but their activation does not lead to metabolic reprogramming. Yeast cells have developed a double check sytem to analyse the presence of extracellular glucose, but internalisation is required to develop large gene responses. Recognising the presence of a large pool of carbon source seems not to be enough guarantee for the cell to commit to a large scale transcriptional response. Only when internalisation takes place the switch to a ferementative lifestyle is developed.

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Table S1. Changes in mRNA expression in the wild type strain after a glucose pulse.

Upregulated genes in wild type after glucose pulse (274)	
ORF	**Functional description**
AAC3	Mitochondrial inner membrane ADP/ATP translocator, exchanges cytosolic ADP for mitochondrially synthesized ATP; expressed under anaerobic conditions; similar to Pet9p and Aac1p; has roles in maintenance of viability and in respiration
AAH1	Adenine deaminase (adenine aminohydrolase), involved in purine salvage and nitrogen catabolism
AFG2	ATPase of the CDC48/PAS1/SEC18 (AAA) family, forms a hexameric complex; may be involved in degradation of aberrant mRNAs
AFT2	Iron-regulated transcriptional activator, required for iron homeostasis and resistance to oxidative stress; similar to Aft1p
AIR1	RING finger protein that interacts with the arginine methyltransferase Hmt1p to regulate methylation of Npl3p, which modulates Npl3p function in mRNA processing and export; has similarity to Air2p
ALB1	Shuttling pre-60S factor; involved in the biogenesis of ribosomal large subunit; interacts directly with Arx1p; responsible for Tif6p recycling defects in absence of Rei1p.
ANB1	Translation initiation factor eIF-5A, promotes formation of the first peptide bond; similar to and functionally redundant with Hyp2p; undergoes an essential hypusination modification; expressed under anaerobic conditions
AQR1	Plasma membrane transporter of the major facilitator superfamily that confers resistance to short-chain monocarboxylic acids and quinidine
ARN2	Transporter, member of the ARN family of transporters that specifically recognize siderophore-iron chelates; responsible for uptake of iron bound to the siderophore triacetylfusarinine C
ARO7	Chorismate mutase, catalyzes the conversion of chorismate to prephenate to initiate the tyrosine/phenylalanine-specific branch of aromatic amino acid biosynthesis
ATC1	Nuclear protein, possibly involved in regulation of cation stress responses and/or in the establishment of bipolar budding pattern
ATF2	Alcohol acetyltransferase, may play a role in steroid detoxification; forms volatile esters during fermentation, which is important in brewing
ATG29	Protein specifically required for autophagy; may function in autophagosome formation at the pre-autophagosomal structure in collaboration with other autophagy proteins
AUA1	Protein required for the negative regulation by ammonia of Gap1p, which is a general amino acid permease
AYT1	Acetyltransferase; catalyzes trichothecene 3-O-acetylation, suggesting a possible role in trichothecene biosynthesis
BAP3	Amino acid permease involved in the uptake of cysteine, leucine, isoleucine and valine
BFR2	Essential protein possibly involved in secretion; multicopy suppressor of sensitivity to Brefeldin A
BSC1	Protein of unconfirmed function, similar to cell surface flocculin Muc1p; ORF exhibits genomic organization compatible with a translational readthrough-dependent mode of expression
BUD2	GTPase activating factor for Rsr1p/Bud1p required for both axial and bipolar budding patterns; mutants exhibit random budding in all cell types
BUD31	Protein involved in bud-site selection; analysis of integrated high-throughput datasets predicts an involvement in RNA splicing; diploid mutants display a random budding pattern instead of the wild-type bipolar pattern
CGR1	Protein involved in nucleolar integrity and processing of the pre-rRNA for the 60S ribosome subunit; transcript is induced in response to cytotoxic stress but not genotoxic stress
CNS1	TPR-containing co-chaperone; binds both Hsp82p (Hsp90) and Ssa1p (Hsp70) and stimulates the ATPase activity of SSA1, ts mutants reduce Hsp82p function while over expression suppresses the phenotypes of an HSP82 ts allele and a cpr7 deletion
CPD1	Cyclic nucleotide phosphodiesterase, hydrolyzes ADP-ribose 1'', 2''-cyclic phosphate to ADP-ribose 1''-phosphate; no detectable phenotype is conferred by null mutation or by overexpression
DAK2	Dihydroxyacetone kinase, required for detoxification of dihydroxyacetone (DHA); involved in stress adaptation
DAN1	Cell wall mannoprotein with similarity to Tir1p, Tir2p, Tir3p, and Tir4p; expressed under anaerobic conditions, completely repressed during aerobic growth
DBP2	Essential ATP-dependent RNA helicase of the DEAD-box protein family, involved in nonsense-mediated mRNA decay and rRNA processing
DBP7	Putative ATP-dependent RNA helicase of the DEAD-box family involved in ribosomal biogenesis
DBP8	Putative ATP-dependent RNA helicase of the DEAD-box family involved in biogenesis of the 40S ribosomal subunit
DEG1	Non-essential tRNA:pseudouridine synthase, introduces pseudouridines at position 38 or 39 in tRNA, important for maintenance of translation efficiency and normal cell growth, localizes to both the nucleus and cytoplasm
DHR2	Predominantly nucleolar DEAH-box RNA helicase, required for 18S rRNA synthesis
DIA1	Protein of unknown function, involved in invasive and pseudohyphal growth; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern

DIM1	Essential 18S rRNA dimethylase, responsible for conserved m6(2)Am6(2)A dimethylation in 3'-terminal loop of 18 S rRNA, part of 90S and 40S pre-particles in nucleolus, involved in pre-ribosomal RNA processing
DPH1	Protein required, along with Dph2p, Kti11p, Jjj3p, and Dph5p, for synthesis of diphthamide, which is a modified histidine residue of translation elongation factor 2 (Eft1p or Eft2p); may act in a complex with Dph2p and Kti11p
DRS1	Nucleolar DEAD-box protein required for ribosome assembly and function, including synthesis of 60S ribosomal subunits; constituent of 66S pre-ribosomal particles
DUS3	Dihydrouridine synthase, member of a widespread family of conserved proteins including Smm1p, Dus1p, and Dus4p; contains a consensus oleate response element (ORE) in its promoter region
DYS1	Deoxyhypusine synthase, catalyzes formation of deoxyhypusine, the first step in hypusine biosynthesis; triggers posttranslational hypusination of translation elongation factor eIF-5A and regulates its intracellular levels; tetrameric
ECM1	Protein of unknown function, localized in the nucleoplasm and the nucleolus, genetically interacts with MTR2 in 60S ribosomal protein subunit export
ECM11	Non-essential protein apparently involved in meiosis, GFP fusion protein is present in discrete clusters in the nucleus throughout mitosis; may be involved in maintaining chromatin structure
ECM12	Non-essential protein of unknown function
ECM2	Pre-mRNA splicing factor, facilitates the cooperative formation of U2/U6 helix II in association with stem II in the spliceosome, function may be regulated by Slu7p
ECM23	Non-essential protein of unconfirmed function; affects pre-rRNA processing, may act as a negative regulator of the transcription of genes involved in pseudohyphal growth; homologous to Srd1p
EMG1	Protein required for the maturation of the 18S rRNA and for 40S ribosome production; associated with spindle/microtubules; nuclear localization depends on physical interaction with Nop14p; may bind snoRNAs
ENP1	Protein associated with U3 and U14 snoRNAs, required for pre-rRNA processing and 40S ribosomal subunit synthesis; localized in the nucleus and concentrated in the nucleolus
ENT4	Protein of unknown function, contains an N-terminal epsin-like domain
ESF2	Essential nucleolar protein involved in pre-18S rRNA processing; component of the small subunit (SSU) processome; has sequence similarity to mABT1, a mouse transcription activator
FAF1	Protein required for pre-rRNA processing and 40S ribosomal subunit assembly
FAL1	Nucleolar protein required for maturation of 18S rRNA, member of the eIF4A subfamily of DEAD-box ATP-dependent RNA helicases
FCY21	Putative purine-cytosine permease, very similar to Fcy2p but cannot substitute for its function
FET4	Low-affinity Fe(II) transporter of the plasma membrane
FRM2	Protein of unknown function, involved in the integration of lipid signaling pathways with cellular homeostasis
FSH1	Serine hydrolase that localizes to both the nucleus and cytoplasm; sequence is similar to Fsh2p and Fsh3p
FYV7	Protein of unknown function, required for survival upon exposure to K1 killer toxin; involved in processing the 35S rRNA primary transcript to generate the 20S and 27SA2 pre-rRNA transcripts
GAT4	Protein containing GATA family zinc finger motifs
GCD1	Subunit of tRNA (1-methyladenosine) methyltransferase with Gcd14p, required for the modification of the adenine at position 58 in tRNAs, especially tRNAi-Met; first identified as a negative regulator of GCN4 expression
GFD2	Protein of unknown function, identified as a high-copy suppressor of a dbp5 mutation
GNP1	High-affinity glutamine permease, also transports Leu, Ser, Thr, Cys, Met and Asn; expression is fully dependent on Grr1p and modulated by the Ssy1p-Ptr3p-Ssy5p (SPS) sensor of extracellular amino acids
GRC3	Protein of unknown function, required for cell growth and possibly involved in rRNA processing; mRNA is cell cycle regulated
HAS1	ATP-dependent RNA helicase; localizes to both the nuclear periphery and nucleolus; highly enriched in nuclear pore complex fractions; constituent of 66S pre-ribosomal particles
HIM1	Protein of unknown function involved in DNA repair
HMS2	Protein with similarity to heat shock transcription factors; overexpression suppresses the pseudohyphal filamentation defect of a diploid mep1 mep2 homozygous null mutant
HMT1	Nuclear SAM-dependent mono- and asymmetric arginine dimethylating methyltransferase that modifies hnRNPs, including Npl3p and Hrp1p, thus facilitating nuclear export of these proteins; required for viability of npl3 mutants
HOR2	One of two redundant DL-glycerol-3-phosphatases (RHR2/GPP1 encodes the other) involved in glycerol biosynthesis; induced in response to hyperosmotic stress and oxidative stress, and during the diauxic transition
HXT1	Putative hexose transporter, expressed at low levels and expression is repressed by glucose
HXT13	Protein of unknown function with similarity to hexose transporter family members, expression is induced by low levels of glucose and repressed by high levels of glucose

HXT3	Low affinity glucose transporter of the major facilitator superfamily, expression is induced in low or high glucose conditions
HXT4	High-affinity glucose transporter of the major facilitator superfamily, expression is induced by low levels of glucose and repressed by high levels of glucose
IMD4	Inosine monophosphate dehydrogenase, catalyzes the first step of GMP biosynthesis, member of a four-gene family in S. cerevisiae, constitutively expressed
IMP3	Component of the SSU processome, which is required for pre-18S rRNA processing, essential protein that interacts with Mpp10p and mediates interactions of Imp4p and Mpp10p with U3 snoRNA
IMP4	Component of the SSU processome, which is required for pre-18S rRNA processing; interacts with Mpp10p; member of a superfamily of proteins that contain a sigma(70)-like motif and associate with RNAs
IPI1	Protein of unknown function, essential for viability, may be involved in rRNA processing
IPI3	Protein required for cell viability; computational analysis of large-scale protein-protein interaction data suggests a possible role in assembly of the ribosomal large subunit
ISU2	Conserved protein of the mitochondrial matrix, required for synthesis of mitochondrial and cytosolic iron-sulfur proteins, performs a scaffolding function in mitochondria during Fe/S cluster assembly; isu1 isu2 double mutant is inviable
KTR2	Mannosyltransferase involved in N-linked protein glycosylation; member of the KRE2/MNT1 mannosyltransferase family
LCP5	Essential protein involved in maturation of 18S rRNA; depletion leads to inhibited pre-rRNA processing and reduced polysome levels; localizes primarily to the nucleolus
LEU9	Alpha-isopropylmalate synthase II (2-isopropylmalate synthase), catalyzes the first step in the leucine biosynthesis pathway; the minor isozyme, responsible for the residual alpha-IPMS activity detected in a leu4 null mutant
LSG1	Putative GTPase involved in 60S ribosomal subunit biogenesis; required for the release of Nmd3p from 60S subunits in the cytoplasm
LTV1	Component of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; required for growth at low temperature
MAK5	Essential nucleolar protein, putative DEAD-box RNA helicase required for maintenance of M1 dsRNA virus; involved in biogenesis of large (60S) ribosomal subunits
MCH5	Plasma membrane riboflavin transporter; facilitates the uptake of vitamin B2; required for FAD-dependent processes; sequence similarity to mammalian monocarboxylate permeases, however mutants are not deficient in monocarboxylate transport
MEI4	Meiosis-specific protein involved in recombination; required for chromosome synapsis; required for production of viable spores
MEI5	Meiosis specific protein involved in DMC1-dependent meiotic recombination, forms heterodimer with Sae3p; proposed to be an assembly factor for Dmc1p
MET31	Zinc-finger DNA-binding protein, involved in regulating expression of the methionine biosynthetic genes, similar to Met32p
MIG2	Protein containing zinc fingers, involved in repression, along with Mig1p, of SUC2 (invertase) expression by high levels of glucose; binds to Mig1p-binding sites in SUC2 promoter
MIG3	Probable transcriptional repressor involved in response to toxic agents such as hydroxyurea that inhibit ribonucleotide reductase; phosphorylation by Snf1p or the Mec1p pathway inactivates Mig3p, allowing induction of damage response genes
MND1	Protein required for recombination and meiotic nuclear division; forms a complex with Hop2p, which is involved in chromosome pairing and repair of meiotic double-strand breaks
MRD1	Essential conserved protein that associates with 35S precursor rRNA and is required for its initial processing at the A(0)-A(2) cleavage sites, shows partial nucleolar localization, contains five consensus RNA-binding domains
MTR4	Dead-box family ATP dependent helicase required for mRNA export from the nucleus; co-factor of the exosome complex, required for 3' end formation of 5.8S rRNA
NAF1	Protein required for the assembly of box H/ACA snoRNPs and for pre-rRNA processing, forms a complex with Shq1p and interacts with H/ACA snoRNP components Nhp2p and Cbf5p; has similarity to Gar1p and other RNA-binding proteins
NAT4	N alpha-acetyl-transferase, involved in acetylation of the N-terminal residues of histones H4 and H2A
NOC2	Protein that forms a nucleolar complex with Mak21p that binds to 90S and 66S pre-ribosomes, as well as a nuclear complex with Noc3p that binds to 66S pre-ribosomes; both complexes mediate intranuclear transport of ribosomal precursors
NOC3	Protein that forms a nuclear complex with Noc2p that binds to 66S ribosomal precursors to mediate their intranuclear transport; also binds to chromatin to promote the association of DNA replication factors and replication initiation
NOC4	Nucleolar protein, forms a complex with Nop14p that mediates maturation and nuclear export of 40S ribosomal subunits
NOP13	Protein of unknown function, localizes to the nucleolus and nucleoplasm; contains an RNA recognition motif (RRM) and has similarity to Nop12p, which is required for processing of pre-18S rRNA

NOP14	Nucleolar protein, forms a complex with Noc4p that mediates maturation and nuclear export of 40S ribosomal subunits; also present in the small subunit processome complex, which is required for processing of pre-18S rRNA
NOP7	Nucleolar protein involved in rRNA processing and 60S ribosomal subunit biogenesis; constituent of several different pre-ribosomal particles; required for exit from G₀ and the initiation of cell proliferation
NOP8	Nucleolar protein required for 60S ribosomal subunit biogenesis
NOP9	Essential nucleolar protein required for 18S rRNA synthesis
OPT1	Plasma membrane transporter that transports tetra- and pentapeptides and glutathione; member of the OPT family
PDE2	High-affinity cyclic AMP phosphodiesterase, component of the cAMP-dependent protein kinase signaling system, protects the cell from extracellular cAMP, contains readthrough motif surrounding termination codon
PFK27	6-phosphofructo-2-kinase, catalyzes synthesis of fructose-2,6-bisphosphate; inhibited by phosphoenolpyruvate and sn-glycerol 3-phosphate, expression induced by glucose and sucrose, transcriptional regulation involves protein kinase A
PHO3	Constitutively expressed acid phosphatase similar to Pho5p; brought to the cell surface by transport vesicles; hydrolyzes thiamin phosphates in the periplasmic space, increasing cellular thiamin uptake; expression is repressed by thiamin
PHO84	High-affinity inorganic phosphate (Pi) transporter and low-affinity manganese transporter; regulated by Pho4p and Spt7p; mutation confers resistance to arsenate; exit from the ER during maturation requires Pho86p
PHO9	Low-affinity phosphate transporter; deletion of pho84, pho87, pho89, pho90, and pho91 causes synthetic lethality; transcription independent of Pi and Pho4p activity; overexpression results in vigorous growth
POP3	Subunit of both RNase MRP, which cleaves pre-rRNA, and nuclear RNase P, which cleaves tRNA precursors to generate mature 5' ends
POR2	Putative mitochondrial porin (voltage-dependent anion channel), related to Por1p but not required for mitochondrial membrane permeability or mitochondrial osmotic stability
PPM2	Putative carboxyl methyl transferase, has similarity to Ppm1p but biochemical activity not yet demonstrated
PPT1	Protein serine/threonine phosphatase with similarity to human phosphatase PP5; present in both the nucleus and cytoplasm; expressed during logarithmic growth
PRM1	Pheromone-regulated protein, predicted to have 5 transmembrane segments
PRM3	Pheromone-regulated protein required for karyogamy; localizes to the inner membrane of the nuclear envelope
PRM7	Pheromone-regulated protein, predicted to have one transmembrane segment; promoter contains Gcn4p binding elements
PRP43	RNA helicase in the DEAH-box family, involved in release of the lariat-intron from the spliceosome
PUS1	tRNA:pseudouridine synthase, introduces pseudouridines at positions 26-28, 34-36, 65, and 67 of tRNA; nuclear protein that appears to be involved in tRNA export; also acts on U2 snRNA
PUS7	Pseudouridine synthase, catalyzes pseudouridylation at position 35 in U2 snRNA, position 13 in cytoplasmic tRNAs, and position 35 in pre-tRNA(Tyr); Asp(256) mutation abolishes activity; conserved in archaea, some bacteria, and vertebrates
PWP1	Protein with WD-40 repeats involved in rRNA processing; associates with trans-acting ribosome biogenesis factors; similar to beta-transducin superfamily
PWP2	Conserved 90S pre-ribosomal component essential for proper endonucleolytic cleavage of the 35 S rRNA precursor at A0, A1, and A2 sites; contains eight WD-repeats; PWP2 deletion leads to defects in cell cycle and bud morphogenesis
PXR1	Essential protein involved in rRNA and snoRNA maturation; competes with TLC1 RNA for binding to Est2p, suggesting a role in regulation of telomerase; human homolog inhibits telomerase; contains a G-patch RNA interacting domain
RAD16	Protein that recognizes and binds damaged DNA in an ATP-dependent manner (with Rad7p) during nucleotide excision repair; subunit of Nucleotide Excision Repair Factor 4 (NEF4); member of the SWI/SNF family
RCK1	Protein kinase involved in the response to oxidative stress; identified as suppressor of S. pombe cell cycle checkpoint mutations
RDS1	Zinc cluster protein involved in conferring resistance to cycloheximide
REI1	Cytoplasmic pre-60S factor; required for the correct recycling of shuttling factors Alb1, Arx1 and Tif6 at the end of the ribosomal large subunit biogenesis; involved in bud growth in the mitotic signaling network
REX4	Putative RNA exonuclease possibly involved in pre-rRNA processing and ribosome assembly
RGM1	Putative transcriptional repressor with proline-rich zinc fingers; overproduction impairs cell growth
RGS2	Negative regulator of glucose-induced cAMP signaling; directly activates the GTPase activity of the heterotrimeric G protein alpha subunit Gpa2p

RIX1	Essential protein involved in the processing of the ITS2 region of the rRNA locus; required for the maturation and nuclear export of the 60S ribosomal subunit
RNT1	RNAase III; cleaves a stem-loop structure at the 3' end of U2 snRNA to ensure formation of the correct U2 3' end
ROG3	Protein that binds to Rsp5p, which is a hect-type ubiquitin ligase, via its 2 PY motifs; has similarity to Rod1p; mutation suppresses the temperature sensitivity of an mck1 rim11 double mutant
RPA12	RNA polymerase I subunit A12.2; contains two zinc binding domains, and the N terminal domain is responsible for anchoring to the RNA pol I complex
RPA43	RNA polymerase I subunit A43
RPA49	RNA polymerase I subunit A49
RPC17	RNA polymerase III subunit C17; physically interacts with C31, C11, and TFIIIB70; may be involved in the recruitment of pol III by the preinitiation complex
RPC53	RNA polymerase III subunit C53
RRN11	Protein required for rDNA transcription by RNA polymerase I, component of the core factor (CF) of rDNA transcription factor, which also contains Rrn6p and Rrn7p
RRN7	Protein involved in the transcription of 35S rRNA genes by RNA polymerase I; component of the core factor (CF) complex also composed of Rrn11p, Rrn6p and TATA-binding protein
RRP1	Essential evolutionarily conserved nucleolar protein necessary for biogenesis of 60S ribosomal subunits and processing of pre-rRNAs to mature rRNAs, associated with several distinct 66S pre-ribosomal particles
RRP8	Nucleolar protein involved in rRNA processing, pre-rRNA cleavage at site A2; also involved in telomere maintenance; mutation is synthetically lethal with a gar1 mutation
RSA4	WD-repeat protein involved in ribosome biogenesis; required for maturation and efficient intra-nuclear transport or pre-60S ribosomal subunits, localizes to the nucleolus
SDO1	Essential protein involved in RNA metabolism, one of two yeast homologs (with Yhr087wp) of the human protein SBDS responsible for autosomal recessive Shwachman-Bodian-Diamond Syndrome, also conserved in Archaea
SER2	Phosphoserine phosphatase of the phosphoglycerate pathway, involved in serine and glycine biosynthesis, expression is regulated by the available nitrogen source
SET6	Protein of unknown function; deletion heterozygote is sensitive to compounds that target ergosterol biosynthesis, may be involved in compound availability
SHQ1	Essential nuclear protein, required for accumulation of box H/ACA snoRNAs and for rRNA processing; interacts with Naf1p
SHU1	Protein of unassigned function involved in mutation suppression, important for error-free repair of spontaneous and induced DNA lesions to protect the genome from mutation; associates with Shu2p, Psy3p, and Csm2p
SIK1	Essential evolutionarily-conserved nucleolar protein component of the box C/D snoRNP complexes that direct 2'-O-methylation of pre-rRNA during its maturation; overexpression causes spindle orientation defects
SLD5	Subunit of the GINS complex (Sld5p, Psf1p, Psf2p, Psf3p), which is localized to DNA replication origins and implicated in assembly of the DNA replication machinery
SNM1	Subunit of RNase MRP, which cleaves pre-rRNA; not shared between Rnase MRP and nuclear Rnase P, in contrast to all other Rnase MRP protein subunits; binds to the NME1 RNA subunit of Rnase MRP
SPO77	Meiosis-specific protein of unknown function, required for spore wall formation during sporulation; dispensable for both nuclear divisions during meiosis
SPS18	Protein of unknown function, contains a putative zinc-binding domain; expressed during sporulation
SPT14	UDP-GlcNAc-binding and catalytic subunit of the enzyme that mediates the first step in glycosylphosphatidylinositol (GPI) biosynthesis, mutations cause defects in transcription and in biogenesis of cell wall proteins
SRO9	Cytoplasmic RNA-binding protein that associates with translating ribosomes; involved in heme regulation of Hap1p as a component of the HMC complex, also involved in the organization of actin filaments; contains a La motif
SRP4	Nucleolar, serine-rich protein with a role in preribosome assembly or transport; may function as a chaperone of small nucleolar ribonucleoprotein particles (snoRNPs); immunologically and structurally to rat Nopp140
SSF1	Constituent of 66S pre-ribosomal particles, required for ribosomal large subunit maturation; functionally redundant with Ssf2p; member of the Brix family
STD1	Protein involved in control of glucose-regulated gene expression; interacts with protein kinase Snf1p, glucose sensors Snf3p and Rgt2p, and TATA-binding protein Spt15p; acts as a regulator of the transcription factor Rgt1p
SUA5	Protein required for respiratory growth; null mutation suppresses the Cyc1p translation defect caused by the presence of an aberrant ATG codon upstream of the correct start
TIR2	Putative cell wall mannoprotein of the Srp1p/Tip1p family of serine-alanine-rich proteins; transcription is induced by cold shock and anaerobiosis

TPA1	Hypothetical protein
TPO2	Polyamine transport protein specific for spermine; localizes to the plasma membrane; transcription of TPO2 is regulated by Haa1p; member of the major facilitator superfamily
TRM11	Catalytic subunit of an adoMet-dependent tRNA methyltransferase complex (Trm11p-Trm112p), required for the methylation of the guanosine nucleotide at position 10 (m2G10) in tRNAs; contains a THUMP domain and a methyltransferase domain
TRM5	tRNA(m(1)G37)methyltransferase, methylates a tRNA base adjacent to the anticodon that has a role in prevention of frameshifting; highly conserved across Archaea, Bacteria, and Eukarya
TRM7	2'-O-ribose methyltransferase, methylates the 2'-O-ribose of nucleotides at positions 32 and 34 of the tRNA anticodon loop
TSR2	Protein with a potential role in pre-rRNA processing
UBC11	Ubiquitin-conjugating enzyme most similar in sequence to Xenopus ubiquitin-conjugating enzyme E2-C, but not a true functional homolog of this E2; unlike E2-C, not required for the degradation of mitotic cyclin Clb2
URB2	Nucleolar protein required for normal metabolism of the rRNA primary transcript, proposed to be involved in ribosome biogenesis
URK1	Uridine/cytidine kinase, component of the pyrimidine ribonucleotide salvage pathway that converts uridine into UMP and cytidine into CMP; involved in the pyrimidine deoxyribonucleotide salvage pathway, converting deoxycytidine into dCMP
UTP11	Nucleolar protein, component of the small subunit (SSU) processome containing the U3 snoRNA that is involved in processing of pre-18S rRNA
UTP15	Nucleolar protein, component of the small subunit (SSU) processome containing the U3 snoRNA that is involved in processing of pre-18S rRNA
UTP18	Possible U3 snoRNP protein involved in maturation of pre-18S rRNA, based on computational analysis of large-scale protein-protein interaction data
UTP3	Possible U3 snoRNP protein involved in maturation of pre-18S rRNA, based on computational analysis of large-scale protein-protein interaction data
UTP5	Nucleolar protein, component of the small subunit (SSU) processome containing the U3 snoRNA that is involved in processing of pre-18S rRNA
UTP6	Nucleolar protein, component of the small subunit (SSU) processome containing the U3 snoRNA that is involved in processing of pre-18S rRNA
UTP8	Nucleolar protein required for export of tRNAs from the nucleus; also copurifies with the small subunit (SSU) processome containing the U3 snoRNA that is involved in processing of pre-18S rRNA
UTP9	Nucleolar protein, component of the small subunit (SSU) processome containing the U3 snoRNA that is involved in processing of pre-18S rRNA
UTR2	Putative glycosidase, glycosylphosphatidylinositol (GPI)-anchored protein localized to the bud neck; has a role in cell wall maintenance
VID24	Peripheral membrane protein located at Vid (vacuole import and degradation) vesicles; regulates fructose-1,6-bisphosphatase (FBPase) targeting to the vacuole; involved in proteasome-dependent catabolite degradation of FBPase
YAL065C	Hypothetical protein
YAR028W	Putative integral membrane protein, member of DUP240 gene family
YAR029W	Member of DUP240 gene family but contains no transmembrane domains; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern
YAR075W	Non-functional protein with homology IMP dehydrogenase; YAR073W/IMD1 and YAR075W comprise a continuous reading frame in some strains of S. cerevisiae.
YBL028C	Hypothetical protein
YBL029W	Hypothetical protein
YBL044W	Hypothetical protein
YBL054W	Hypothetical protein
YBL071C-B	Identified by gene-trapping, microarray-based expression analysis, and genome-wide homology searching
YBL081W	Hypothetical protein
YBR141C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the nucleolus; YBR141C is not an essential gene
YBR200W-A	Putative protein of unknown function; identified by fungal homology and RT-PCR
YBR221W-A	Putative protein of unknown function; identified by expression profiling and mass spectrometry
YBR238C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and mitochondrion; YBR238C is not an essential gene; transcriptionally up-regulated by TOR and deletion increases life span
YBR271W	Putative S-adenosylmethionine-dependent methyltransferase of the seven beta-strand family; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YBR271W is not as essential gene
YCL058C	Protein of unknown function, required for survival upon exposure to K1 killer toxin; involved in ion

	homeostasis
YCR016W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the nucleolus and nucleus; YCR016W is not an essential gene
YCR087C-A	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the nucleolus; YCR087C-A is not an essential gene
YDL038C	Putative protein of unknown function; YDL038C is not an essential gene
YDL063C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and nucleus; YDL063C is not an essential gene
YDL241W	Hypothetical protein
YDR020C	Hypothetical protein
YDR034C-A	Similar to probable membrane protein YLR334C and ORF YOL106W
YDR042C	Hypothetical protein
YDR124W	Hypothetical protein
YDR412W	Protein required for cell viability
YER085C	Hypothetical protein
YER187W	Hypothetical protein
YFL012W	Putative protein of unknown function; transcribed during sporulation; null mutant exhibits increased resistance to rapamycin
YFL051C	Putative protein of unknown function; YFL051C is not an essential gene
YFR032C	Putative protein of unknown function; transcribed during sporulation; YFR032C is not an essential gene
YFR055W	Putative cystathionine beta-lyase; involved in copper ion homeostasis and sulfur metabolism; null mutant displays increased levels of spontaneous Rad52 foci
YFR057W	Hypothetical protein
YGL157W	Oxidoreductase, catalyzes NADPH-dependent reduction of the bicyclic diketone bicyclo[2.2.2]octane-2,6-dione (BCO2,6D) to the chiral ketoalcohol (1R,4S,6S)-6-hydroxybicyclo[2.2.2]octane-2-one (BCO2one6ol)
YGL188C-A	Putative protein of unknown function
YGR068C	Hypothetical protein
YGR079W	Putative protein of unknown function; YGR079W is not an essential gene
YGR121W-A	Putative protein of unknown function
YGR126W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to both the cytoplasm and the nucleus
YGR146C-A	Putative protein of unknown function
YGR204C-A	Putative protein of unknown function; identified by gene-trapping, microarray-based expression analysis, and genome-wide homology searching
YGR240C-A	Putative protein of unknown function; identified by fungal homology and RT-PCR
YGR272C	Putative protein of unknown function; deletion mutant has defects in pre-rRNA processing; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and nucleus
YGR273C	Putative protein of unknown function; deletion mutant has no readily detectable phenotype
YGR283C	Putative protein of unknown function; deletion mutant is resistant to fluconazole; green fluorescent protein (GFP)-fusion protein localizes to the nucleolus
YHL012W	Putative protein of unknown function, has some homology to Ugp1p, which encodes UDP-glucose pyrophosphorylase
YHR032W	Putative protein of unknown function
YHR048W	Putative protein of unknown function, has similarity to multidrug resistance proteins; expression of gene is up-regulated in cells exhibiting reduced susceptibility to azoles
YHR126C	Hypothetical protein
YIL046W-A	Identified by expression profiling and mass spectrometry
YIL096C	Hypothetical protein
YIL110W	Putative S-adenosylmethionine-dependent methyltransferase of the seven beta-strand family; deletion mutant exhibits a weak vacuolar protein sorting defect, enhanced resistance to caspofungin, and is synthetically lethal with MEN mutants
YIL127C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the nucleolus
YIL134C-A	Putative protein of unknown function, identified by fungal homology and RT-PCR
YJL038C	Putative protein of unknown function; expression induced during sporulation and repressed during vegetative growth by Sum1p and Hst1p; similar to adjacent open reading frame, YJL037W
YJL043W	Putative protein of unknown function; YJL043W is a non-essential gene
YJL052C-A	Putative protein of unknown function, identified based on comparison to related yeast species

YJR056C	Hypothetical protein
YJR124C	Hypothetical protein
YKL037W	Putative protein of unknown function
YKR041W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and nucleus
YLR063W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YLR063W is not an essential gene
YLR073C	Protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to endosomes; YLR073C is not an esssential gene
YLR162W	Putative protein of unknown function; overexpression confers resistance to the antimicrobial peptide MiAMP1
YLR363W-A	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the nucleus
YLR412C-A	Putative protein of unknown function
YLR413W	Putative protein of unknown function; YLR413W is not an essential gene
YLR437C	Hypothetical protein
YMR230W-A	Putative protein of unknown function
YMR244W	Hypothetical protein
YMR269W	Protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the nucleolus; identified in proteomic screens of ribosomal complexes; YMR269W is not an essential gene
YNL024C	Putative protein of unknown function with seven beta-strand methyltransferase motif; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YNL024C is not an essential gene
YNL034W	Putative protein of unknown function; YNL034W is not an essential gene
YNL067W-B	Putative protein of unknown function
YNL162W-A	Hypothetical protein identified by homology. See FEBS Letters [2000] 487:31-36.
YNL234W	Similar to globins and has a functional heme-binding domain; involved in glucose signaling or metabolism; regulated by Rgt1p
YNR004W	Hypothetical protein
YNR014W	Hypothetical protein
YNR024W	Hypothetical protein
YNR066C	Hypothetical protein
YOL013W-B	Identified by SAGE
YOL014W	Hypothetical protein
YOL015W	Hypothetical protein; null mutant displays increased levels of spontaneous Rad52 foci
YOL019W-A	Identified by gene-trapping, microarray-based expression analysis, and genome-wide homology searching
YOL047C	Protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern
YOL097W-A	Identified by expression profiling and mass spectrometry
YOR004W	Essential nucleolar protein that is a component of the SSU (small subunit) processome involved in 40S ribosomal subunit biogenesis; has homology to PINc domain protein Fcf1p, although the PINc domain of Utp23p is not required for function
YOR012W	Hypothetical protein
YOR051C	Nuclear protein that inhibits replication of Brome mosaic virus in S. cerevisiae, which is a model system for studying replication of positive-strand RNA viruses in their natural hosts
YOR072W-B	Identified by expression profiling and mass spectrometry
YOR268C	Hypothetical protein
YOR287C	Protein required for cell viability
YOR293C-A	Identified by expression profiling and mass spectrometry
YOR338W	Hypothetical protein
YOR378W	Hypothetical protein
YOR381W-A	Identified by fungal homology and RT-PCR
YPL030W	Hypothetical protein
YPL068C	Hypothetical protein
YPR078C	Hypothetical protein
YPR157W	Hypothetical protein
YVH1	Protein phosphatase involved in vegetative growth at low temperatures, sporulation, and glycogen

	accumulation; transcription induced by low temperature and nitrogen starvation; member of the dual-specificity family of protein phosphatases
ZPS1	Putative GPI-anchored protein; transcription is induced under low-zinc conditions, as mediated by the Zap1p transcription factor, and at alkaline pH

	Downregulated genes in wild type after glucose pulse (531)
ORF	**Functional description**
AAD3	Putative aryl-alcohol dehydrogenase with similarity to P. chrysosporium aryl-alcohol dehydrogenase; mutational analysis has not yet revealed a physiological role
ACH1	Acetyl-coA hydrolase, primarily localized to mitochondria; required for acetate utilization and for diploid pseudohyphal growth
ACS1	Acetyl-coA synthetase isoform, expressed during growth on nonfermentable carbon sources and under aerobic conditions
ADH2	Glucose-repressible alcohol dehydrogenase II, catalyzes the conversion of ethanol to acetaldehyde; involved in the production of certain carboxylate esters; regulated by ADR1
ADK2	Mitochondrial adenylate kinase, catalyzes the reversible synthesis of GTP and AMP from GDP and ADP; may serve as a back-up for synthesizing GTP or ADP depending on metabolic conditions; 3' sequence of ADK2 varies with strain background
ADP1	Putative ATP-dependent permease of the ABC transporter family of proteins
ADR1	Carbon source-responsive zinc-finger transcription factor, required for transcription of the glucose-repressed gene ADH2, of peroxisomal protein genes, and of genes required for ethanol, glycerol, and fatty acid utilization
ADY2	Acetate transporter required for normal sporulation
ADY3	Protein required for spore wall formation, thought to mediate assembly of a Don1p-containing structure at the leading edge of the prospore membrane via interaction with spindle pole body components; potentially phosphorylated by Cdc28p
AFR1	Alpha-factor pheromone receptor regulator, negatively regulates pheromone receptor signaling; required for normal mating projection (shmoo) formation; interacts with Cdc12p
AGP2	High affinity polyamine permease, preferentially uses spermidine over putrescine; expression is down-regulated by osmotic stress; plasma membrane carnitine transporter, also functions as a low-affinity amino acid permease
AGX1	Alanine : glyoxylate aminotransferase, catalyzes the synthesis of glycine from glyoxylate, which is one of three pathways for glycine biosynthesis in yeast; has similarity to mammalian and plant alanine : glyoxylate aminotransferases
AHP1	Thiol-specific peroxiredoxin, reduces hydroperoxides to protect against oxidative damage; function in vivo requires covalent conjugation to Urm1p
AKL1	Ser-Thr protein kinase, member (with Ark1p and Prk1p) of the Ark kinase family; involved in endocytosis and actin cytoskeleton organization
ALD2	Cytoplasmic aldehyde dehydrogenase, involved in ethanol oxidation and beta-alanine biosynthesis; uses NAD+ as the preferred coenzyme; expression is stress induced and glucose repressed; very similar to Ald3p
ALD6	Cytosolic aldehyde dehydrogenase that is activated by Mg2+ and utilizes NADP+ as the preferred coenzyme; required for the conversion of acetaldehyde to acetate; constitutively expressed
ALG13	Catalytic component of UDP-GlcNAc transferase, required for the second step of dolichyl-linked oligosaccharide synthesis; anchored to the ER membrane via interaction with Alg14p; similar to bacterial and human glycosyltransferases
ALP1	Basic amino acid transporter, involved in uptake of cationic amino acids
AMS1	Vacuolar alpha mannosidase, involved in free oligosaccharide (fOS) degradation; delivered to the vacuole in a novel pathway separate from the secretory pathway
APC9	Subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C), which is a ubiquitin-protein ligase required for degradation of anaphase inhibitors, including mitotic cyclins, during the metaphase/anaphase transition
APJ1	Putative chaperone of the HSP40 (DNAJ) family; overexpression interferes with propagation of the [Psi+] prion
AQY1	Spore-specific water channel that mediates the transport of water across cell membranes, developmentally controlled; may play a role in spore maturation, probably by allowing water outflow, may be involved in freeze tolerance
ARA1	Large subunit of NADP+ dependent arabinose dehydrogenase, involved in carbohydrate metabolism; small subunit is unidentified
ARH1	Oxidoreductase of the mitochondrial inner membrane, involved in cytoplasmic and mitochondrial iron homeostasis and required for activity of Fe-S cluster-containing enzymes; one of the few mitochondrial proteins essential for viability

ARO1	Pentafunctional arom protein, catalyzes steps 2 through 6 in the biosynthesis of chorismate, which is a precursor to aromatic amino acids
ARO9	Aromatic aminotransferase, catalyzes the first step of tryptophan, phenylalanine, and tyrosine catabolism
ASI2	Predicted membrane protein; genetic interactions suggest a role in negative regulation of amino acid uptake
AST2	Protein that may have a role in targeting of plasma membrane [H+]ATPase (Pma1p) to the plasma membrane, as suggested by analysis of genetic interactions
ATG1	Protein serine/threonine kinase, required for autophagy and for the cytoplasm-to-vacuole targeting (Cvt) pathway
ATG13	Phosphorylated protein that interacts with Vac8p, required for the cytoplasm-to-vacuole targeting (Cvt) pathway and autophagy
ATG14	Subunit of an autophagy-specific phosphatidylinositol 3-kinase complex (with Vps34p, Vps15p, and Vps30p) required for organization of a pre-autophagosomal structure; ATG14 transcription is activated by Gln3p during nitrogen starvation
ATG17	Protein that interacts with and is required for activation of Apg1p protein kinase; involved in autophagy but not in the Cvt (cytoplasm to vacuole targeting) pathway
ATG2	Protein required for transport of aminopeptidase I (Lap4p) through the cytoplasm-to-vacuole targeting pathway; binds phosphatidylinositol-3-phosphate, involved in localization of membranes to the preautophagosome, potential Cdc28p substrate
ATG2	Protein required for transport of aminopeptidase I (Lap4p) through the cytoplasm-to-vacuole targeting pathway; binds phosphatidylinositol-3-phosphate, involved in localization of membranes to the preautophagosome, potential Cdc28p substrate
ATG3	Protein involved in autophagy; E2-like enzyme that plays a role in formation of Atg8p-phosphatidylethanolamine conjugates, which are involved in membrane dynamics during autophagy
ATG4	Cysteine protease required for autophagy; cleaves Atg8p to a form required for autophagosome and Cvt vesicle generation; mediates attachment of autophagosomes to microtubules through interactions with Tub1p and Tub2p
ATG7	Autophagy-related protein and dual specificity member of the E1 family of ubiquitin-activating enzymes; mediates the conjugation of Atg12p with Atg5p and Atg8p with phosphatidylethanolamine, required steps in autophagosome formation.
ATG8	Protein required for autophagy; modified by the serial action of Atg4p, Atg7p, and Atg3p, and conjugated at the C terminus with phosphatidylethanolamine, to become the form essential for generation of autophagosomes
ATG9	Transmembrane protein involved in formation of Cvt and autophagic vesicles; cycles between the pre-autophagosomal structure and other cytosolic punctate structures, not found in autophagosomes
ATH1	Acid trehalase required for utilization of extracellular trehalose
ATO2	Putative transmembrane protein, involved in the export of ammonia, a starvation signal that promotes cell death in the center of aging colonies; member of the TC 9.B.33 YaaH family; homolog of Ady2p and Y. lipolytica Gpr1p
AVT6	Vacuolar transporter, exports aspartate and glutamate from the vacuole; member of a family of seven S. cerevisiae genes (AVT1-7) related to vesicular GABA-glycine transporters
AVT7	Putative transporter, member of a family of seven S. cerevisiae genes (AVT1-7) related to vesicular GABA-glycine transporters
AYR1	NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase found in lipid particles, ER, and mitochondrial outer membrane; involved in phosphatidic acid biosynthesis; required for spore germination; capable of metabolizing steroid hormones
AZF1	Zinc-finger transcription factor, involved in induction of CLN3 transcription in response to glucose; genetic and physical interactions indicate a possible role in mitochondrial transcription or genome maintenance
BAT2	Cytosolic branched-chain amino acid aminotransferase, homolog of murine ECA39; highly expressed during stationary phase and repressed during logarithmic phase
BBC1	Protein possibly involved in assembly of actin patches; interacts with an actin assembly factor Las17p and with the SH3 domains of Type I myosins Myo3p and Myo5p; localized predominantly to cortical actin patches
BOP2	Protein of unknown function, overproduction suppresses a pam1 slv3 double null mutation
BSC5	Protein of unknown function, ORF exhibits genomic organization compatible with a translational readthrough-dependent mode of expression
BTN2	Cytosolic coiled-coil protein that modulates arginine uptake, interacts with Rhb1p, possible role in mediating pH homeostasis between the vacuole and plasma membrane H(+)-ATPase, may have a role in intracellular protein trafficking
CAF17	Mitochondrial protein that interacts with Ccr4p in the two-hybrid system; 3'-untranslated region contains a putative mRNA localization element common to genes encoding mitochondrial proteins
CAP1	Alpha subunit of the capping protein (CP) heterodimer (Cap1p and Cap2p) which binds to the barbed ends of actin filaments preventing further polymerization; localized predominantly to cortical

	actin patches
CAT2	Carnitine acetyl-CoA transferase present in both mitochondria and peroxisomes, transfers activated acetyl groups to carnitine to form acetylcarnitine which can be shuttled across membranes
CAT5	Mitochondrial inner membrane protein directly involved in ubiquinone biosynthesis, essential for several other metabolic pathways including respiration and gluconeogenic gene activation
CAT8	Zinc cluster transcriptional activator necessary for derepression of a variety of genes under non-fermentative growth conditions, active after diauxic shift, binds carbon source responsive elements
CBP4	Mitochondrial protein required for assembly of ubiquinol cytochrome-c reductase complex (cytochrome bc1 complex); interacts with Cbp3p and function is partially redundant with that of Cbp3p
CDA2	Chitin deacetylase, together with Cda1p involved in the biosynthesis ascospore wall component, chitosan; required for proper rigidity of the ascospore wall
CDC48	ATPase in ER, nuclear membrane and cytosol with homology to mammalian p97; in a complex with Npl4p and Ufd1p participates in retrotranslocation of ubiquitinated proteins from the ER into the cytosol for degradation by the proteasome
CDC53	Cullin, structural protein of SCF complexes (which also contain Skp1p, Cdc34p, and an F-box protein) involved in ubiquitination; SCF promotes the G1-S transition by targeting G1 cyclins and the Cln-CDK inhibitor Sic1p for degradation
CIT1	Citrate synthase, catalyzes the condensation of acetyl coenzyme A and oxaloacetate to form citrate; the rate-limiting enzyme of the TCA cycle; nuclear encoded mitochondrial protein
CIT2	Citrate synthase, catalyzes the condensation of acetyl coenzyme A and oxaloacetate to form citrate, peroxisomal isozyme involved in glyoxylate cycle; expression is controlled by Rtg1p and Rtg2p transcription factors
CIT3	Citrate synthase, catalyzes the condensation of acetyl coenzyme A and oxaloacetate to form citrate, mitochondrial isozyme involved in the TCA cycle
COQ1	Hexaprenyl pyrophosphate synthetase, catalyzes the first step in ubiquinone (coenzyme Q) biosynthesis
COQ3	O-methyltransferase, catalyzes two different O-methylation steps in ubiquinone (Coenzyme Q) biosynthesis; component of a mitochondrial ubiquinone-synthesizing complex
COQ4	Protein with a role in ubiquinone (Coenzyme Q) biosynthesis, possibly functioning in stabilization of Coq7p; located on the matrix face of the mitochondrial inner membrane; component of a mitochondrial ubiquinone-synthesizing complex
COQ5	2-hexaprenyl-6-methoxy-1,4-benzoquinone methyltransferase, involved in ubiquinone (Coenzyme Q) biosynthesis; located in mitochondria
COQ9	Mitochondrial inner membrane protein required for ubiquinone (coenzyme Q) biosynthesis, which in turn is required for respiratory growth; exhibits genetic interaction with ABC1, suggesting a possible common function
COS111	Protein required for wild-type resistance to the antifungal drug ciclopirox olamine; not related to the COS family of subtelomerically-encoded proteins
COX17	Copper metallochaperone that transfers copper to Sco1p and Cox11p for eventual delivery to cytochrome c oxidase
CRC1	Mitochondrial inner membrane carnitine transporter, required for carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria during fatty acid beta-oxidation
CRF1	Transcriptional corepressor involved in the regulation of ribosomal protein gene transcription via the TOR signaling pathway and protein kinase A, phosphorylated by activated Yak1p which promotes accumulation of Crf1p in the nucleus
CSM4	Protein required for accurate chromosome segregation during meiosis
CSR2	Nuclear protein with a potential regulatory role in utilization of galactose and nonfermentable carbon sources; overproduction suppresses the lethality at high temperature of a chs5 spa2 double null mutation; potential Cdc28p substrate
CTA1	Catalase A, breaks down hydrogen peroxide in the peroxisomal matrix formed by acyl-CoA oxidase (Pox1p) during fatty acid beta-oxidation
CTT1	Cytosolic catalase T, has a role in protection from oxidative damage by hydrogen peroxide
CUE5	Protein containing a CUE domain that binds ubiquitin, which may facilitate intramolecular monoubiquitination; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern
CYB2	Cytochrome b2 (L-lactate cytochrome-c oxidoreductase), component of the mitochondrial intermembrane space, required for lactate utilization; expression is repressed by glucose and anaerobic conditions
DAP1	Heme-binding protein involved in regulation of cytochrome P450 protein Erg11p; damage response protein, related to mammalian membrane progesterone receptors; mutations lead to defects in telomeres, mitochondria, and sterol synthesis
DBP1	Putative ATP-dependent RNA helicase of the DEAD-box protein family, constituent of 66S pre-ribosomal particles; essential protein involved in ribosome biogenesis
DCI1	Peroxisomal delta(3,5)-delta(2,4)-dienoyl-CoA isomerase, involved in fatty acid metabolism, contains peroxisome targeting signals at amino and carboxy termini

DCS1	Non-essential hydrolase involved in mRNA decapping, may function in a feedback mechanism to regulate deadenylation, contains pyrophosphatase activity and a HIT (histidine triad) motif; interacts with neutral trehalase Nth1p
DCS2	Non-essential protein containing a HIT (histidine triad) motif; regulated by Msn2p, Msn4p, and the Ras-cAMP-cAPK signaling pathway, transcript accumulates under glucose limitation, similar to Dcs1p
DDI1	DNA damage-inducible v-SNARE binding protein, contains a ubiquitin-associated (UBA) domain, may act as a negative regulator of constitutive exocytosis, may play a role in S-phase checkpoint control
DDR2	Multistress response protein, expression is activated by a variety of xenobiotic agents and environmental or physiological stresses
DDR48	DNA damage-responsive protein, expression is increased in response to heat-shock stress or treatments that produce DNA lesions; contains multiple repeats of the amino acid sequence NNNDSYGS /// Hypothetical protein
DER1	Endoplasmic reticulum membrane protein, required for ER-associated protein degradation, involved in the retrograde transport of misfolded or unassembled proteins; N- and C- termini protrude into the cytoplasm, has similarity to Dfm1p
DFM1	Protein of unknown function, localizes to the ER, contains four transmembrane domains; member of the Der1p-like family
DIC1	Mitochondrial dicarboxylate carrier, integral membrane protein, catalyzes a dicarboxylate-phosphate exchange across the inner mitochondrial membrane, transports cytoplasmic dicarboxylates into the mitochondrial matrix
DNM1	Dynamin-related GTPase required for mitochondrial fission and the maintenance of mitochondrial morphology, assembles on the cytoplasmic face of mitochondrial tubules at sites at which division will occur; also participates in endocytosis
DOA1	WD repeat protein required for ubiquitin-mediated protein degradation, forms complex with Cdc48p, plays a role in controlling cellular ubiquitin concentration; also promotes efficient NHEJ in postdiauxic/stationary phase
DPP1	Diacylglycerol pyrophosphate (DGPP) phosphatase, zinc-regulated vacuolar membrane-associated lipid phosphatase, dephosphorylates DGPP to phosphatidate (PA) and Pi, then PA to diacylglycerol; involved in lipid signaling and cell metabolism
ECI1	Peroxisomal delta3,delta2-enoyl-CoA isomerase, hexameric protein that converts 3-hexenoyl-CoA to trans-2-hexenoyl-CoA, essential for the beta-oxidation of unsaturated fatty acids, oleate-induced
ECM29	Major component of the proteasome; tethers the proteasome core particle to the regulatory particle, and enhances the stability of the proteasome
ECM38	Gamma-glutamyltranspeptidase, major glutathione-degrading enzyme; expression induced mainly by nitrogen starvation
ECM4	Putative omega class glutathione transferase; not essential; similar to Ygr154cp; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm
EMI2	Non-essential protein of unknown function required for transcriptional induction of the early meiotic-specific transcription factor IME1, also required for sporulation
EMI5	Non-essential protein of unknown function required for transcriptional induction of the early meiotic-specific transcription factor IME1, also required for sporulation
EMP46	Integral membrane component of endoplasmic reticulum-derived COPII-coated vesicles, which function in ER to Golgi transport
ERP3	Protein with similarity to Emp24p and Erv25p, member of the p24 family involved in ER to Golgi transport
ERS1	Protein with similarity to human cystinosin, which is a H(+)-driven transporter involved in L-cystine export from lysosomes and implicated in the disease cystinosis; contains seven transmembrane domains
ERV29	Protein localized to COPII-coated vesicles, involved in vesicle formation and incorporation of specific secretory cargo
ETR1	2-enoyl thioester reductase, member of the medium chain dehydrogenase/reductase family; localized to in mitochondria, where it has a probable role in fatty acid synthesis
FAA2	Long chain fatty acyl-CoA synthetase; accepts a wider range of acyl chain lengths than Faa1p, preferring C9:0-C13:0; involved in the activation of endogenous pools of fatty acids
FAB1	1-phosphatidylinositol-3-phosphate 5-kinase; vacuolar membrane kinase that generates phosphatidylinositol (3,5)P2, which is involved in vacuolar sorting and homeostasis
FAR7	Protein involved in G1 cell cycle arrest in response to pheromone, in a pathway different from the Far1p-dependent pathway; interacts with Far3p, Far8p, Far9p, Far10p, and Far11p
FAR8	Protein involved in G1 cell cycle arrest in response to pheromone, in a pathway different from the Far1p-dependent pathway; interacts with Far3p, Far7p, Far9p, Far10p, and Far11p
FBP1	Fructose-1,6-bisphosphatase, key regulatory enzyme in the gluconeogenesis pathway, required for glucose metabolism
FDH1	NAD(+)-dependent formate dehydrogenase, may protect cells from exogenous formate /// NAD(+)-dependent formate dehydrogenase, may protect cells from exogenous formate; YPL275W and YPL276W comprise a continuous open reading frame in some S. cerevisiae strains but not in the

	genomic reference strain S288C
FMP12	The authentic, non-tagged protein was localized to the mitochondria
FMP16	The authentic, non-tagged protein was localized to the mitochondria
FMP21	The authentic, non-tagged protein was localized to the mitochondria
FMP27	The authentic, non-tagged protein was localized to the mitochondria
FMP37	The authentic, non-tagged protein was localized to the mitochondria
FMP4	The authentic, non-tagged protein was localized to the mitochondria.
FMP43	The authentic, non-tagged protein was localized to mitochondria
FMP45	Integral membrane protein localized to mitochondria (untagged protein) and eisosomes, immobile patches at the cortex associated with endocytosis; sporulation and sphingolipid content are altered in mutants; has homologs SUR7 and YNL194C
FOX2	Multifunctional enzyme of the peroxisomal fatty acid beta-oxidation pathway; has 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities
FYV1	Protein of unknown function, required for survival upon exposure to K1 killer toxin; involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase; contains CTLH domain
GAC1	Regulatory subunit for Glc7p type-1 protein phosphatase (PP1), tethers Glc7p to Gsy2p glycogen synthase, binds Hsf1p heat shock transcription factor, required for induction of some HSF-regulated genes under heat shock
GAL1	UDP-glucose-4-epimerase, catalyzes the interconversion of UDP-galactose and UDP-D-glucose in galactose metabolism; also catalyzes the conversion of alpha-D-glucose or alpha-D-galactose to their beta-anomers
GAL3	Transcriptional regulator involved in activation of the GAL genes in response to galactose; forms a complex with Gal80p and Gal4p to relieve inhibition by Gal80p; binds galactose and ATP but does not have galactokinase activity
GCY1	Putative NADP(+) coupled glycerol dehydrogenase, proposed to be involved in an alternative pathway for glycerol catabolism
GDB1	Glycogen debranching enzyme containing glucanotranferase and alpha-1,6-amyloglucosidase activities, required for glycogen degradation
GDH2	NAD(+)-dependent glutamate dehydrogenase, degrades glutamate to ammonia and alpha-ketoglutarate; expression sensitive to nitrogen catabolite repression and intracellular ammonia levels
GDI1	GDP dissociation inhibitor, regulates vesicle traffic in secretory pathways by regulating the dissociation of GDP from the Sec4/Ypt/rab family of GTP binding proteins
GGA1	Golgi-localized protein with homology to gamma-adaptin, interacts with and regulates Arf1p and Arf2p in a GTP-dependent manner in order to facilitate traffic through the late Golgi
GIP2	Putative regulatory subunit of the protein phosphatase Glc7p, involved in glycogen metabolism; contains a conserved motif (GVNK motif) that is also found in Gac1p, Pig1p, and Pig2p
GLC3	Glycogen branching enzyme, involved in glycogen accumulation; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern
GLG1	Self-glucosylating initiator of glycogen synthesis, also glucosylates n-dodecyl-beta-D-maltoside; similar to mammalian glycogenin
GLG2	Self-glucosylating initiator of glycogen synthesis, also glucosylates n-dodecyl-beta-D-maltoside; similar to mammalian glycogenin
GLK1	Glucokinase, catalyzes the phosphorylation of glucose at C6 in the first irreversible step of glucose metabolism; one of three glucose phosphorylating enzymes; expression regulated by non-fermentable carbon sources
GND2	6-phosphogluconate dehydrogenase (decarboxylating), catalyzes an NADPH regenerating reaction in the pentose phosphate pathway; required for growth on D-glucono-delta-lactone
GPB2	Proposed beta subunit of the heterotrimeric G protein; interacts with the receptor Grp1p, affects signaling by altering the level of phosphorylation of PKS substrates; regulates pseudohyphal growth through cAMP levels; homolog of Gpb1p
GPH1	Non-essential glycogen phosphorylase required for the mobilization of glycogen, activity is regulated by cyclic AMP-mediated phosphorylation, expression is regulated by stress-response elements and by the HOG MAP kinase pathway
GPM2	Homolog of Gpm1p phosphoglycerate mutase which converts 3-phosphoglycerate to 2-phosphoglycerate in glycolysis; may be non-functional derivative of a gene duplication event
GPT2	Glycerol-3-phosphate acyltransferase located in both lipid particles and the ER; involved in the stepwise acylation of glycerol-3-phosphate and dihydroxyacetone, which are intermediate steps in lipid biosynthesis
GPX1	Phospholipid hydroperoxide glutathione peroxidase induced by glucose starvation that protects cells from phospholipid hydroperoxides and nonphospholipid peroxides during oxidative stress
GRE1	Hydrophilin of unknown function; stress induced (osmotic, ionic, oxidative, heat shock and heavy metals); regulated by the HOG pathway
GRE3	Aldose reductase involved in methylglyoxal, d-xylose and arabinose metabolism; stress induced

	(osmotic, ionic, oxidative, heat shock, starvation and heavy metals); regulated by the HOG pathway
GRX2	Cytoplasmic glutaredoxin, thioltransferase, glutathione-dependent disulfide oxidoreductase involved in maintaining redox state of target proteins, also exhibits glutathione peroxidase activity, expression induced in response to stress
GSY1	Glycogen synthase with similarity to Gsy2p, the more highly expressed yeast homolog; expression induced by glucose limitation, nitrogen starvation, environmental stress, and entry into stationary phase
GSY2	Glycogen synthase, similar to Gsy1p; expression induced by glucose limitation, nitrogen starvation, heat shock, and stationary phase; activity regulated by cAMP-dependent, Snf1p and Pho85p kinases as well as by the Gac1p-Glc7p phosphatase
GUD1	Guanine deaminase, a catabolic enzyme of the guanine salvage pathway producing xanthine and ammonia from guanine; activity is low in exponentially-growing cultures but expression is increased in post-diauxic and stationary-phase cultures
GUT1	Glycerol kinase, converts glycerol to glycerol-3-phosphate; glucose repression of expression is mediated by Adr1p and Ino2p-Ino4p; derepression of expression on non-fermentable carbon sources is mediated by Opi1p and Rsf1p
GUT2	Mitochondrial glycerol-3-phosphate dehydrogenase; expression is repressed by both glucose and cAMP and derepressed by non-fermentable carbon sources in a Snf1p, Rsf1p, Hap2/3/4/5 complex dependent manner
GYP5	GTPase-activating protein (GAP) for yeast Rab family members, involved in ER to Golgi trafficking; exhibits GAP activity toward Ypt1p that is stimulated by Gyl1p, also acts on Sec4p; interacts with Gyl1p, Rvs161p and Rvs167p
GYP7	GTPase-activating protein for yeast Rab family members including: Ypt7p (most effective), Ypt1p, Ypt31p, and Ypt32p (in vitro); involved in vesicle mediated protein trafficking
HAL1	Cytoplasmic protein involved in halotolerance; decreases intracellular Na+ (via Ena1p) and increases intracellular K+ by decreasing efflux; expression repressed by Ssn6p-Tup1p and Sko1p and induced by NaCl, KCl, and sorbitol through Gcn4p
HBT1	Substrate of the Hub1p ubiquitin-like protein that localizes to the shmoo tip (mating projection); mutants are defective for mating projection formation, thereby implicating Hbt1p in polarized cell morphogenesis
HEF3	Translational elongation factor EF-3; paralog of YEF3 and member of the ABC superfamily; stimulates EF-1 alpha-dependent binding of aminoacyl-tRNA by the ribosome; normally expressed in zinc deficient cells
HEM15	Ferrochelatase, a mitochondrial inner membrane protein, catalyzes the insertion of ferrous iron into protoporphyrin IX, the eighth and final step in the heme biosynthetic pathway; Yfh1p mediates the use of iron by Hem15p
HFD1	Putative fatty aldehyde dehydrogenase, located in the mitochondrial outer membrane and also in lipid particles; has similarity to human fatty aldehyde dehydrogenase (FALDH) which is implicated in Sjogren-Larsson syndrome
HSP12	Plasma membrane localized protein that protects membranes from desiccation; induced by heat shock, oxidative stress, osmostress, stationary phase entry, glucose depletion, oleate and alcohol; regulated by the HOG and Ras-Pka pathways
HSP14	Heat shock protein that cooperates with Ydj1p (Hsp40) and Ssa1p (Hsp70) to refold and reactivate previously denatured, aggregated proteins; responsive to stresses including: heat, ethanol, and sodium arsenite; involved in [PSI+] propagation
HSP26	Small heat shock protein with chaperone activity that is regulated by a heat induced transition from an inactive oligomeric (24-mer) complex to an active dimer; induced by heat, upon entry into stationary phase, and during sporulation
HSP3	Hydrophobic plasma membrane localized, stress-responsive protein that negatively regulates the H(+)-ATPase Pma1p; induced by heat shock, ethanol treatment, weak organic acid, glucose limitation, and entry into stationary phase
HSP42	Small cytosolic stress-induced chaperone that forms barrel-shaped oligomers and suppresses the aggregation of non-native proteins; oligomer dissociation is not required for function; involved in cytoskeleton reorganization after heat shock
HSP78	Oligomeric mitochondrial matrix chaperone that cooperates with Ssc1p in mitochondrial thermotolerance after heat shock; prevents the aggregation of misfolded matrix proteins; component of the mitochondrial proteolysis system
HSP82	Cytoplasmic chaperone (Hsp90 family) required for pheromone signaling and negative regulation of Hsf1p; docks with the mitochondrial import receptor Tom70p for preprotein delivery; interacts with co-chaperones Cns1p, Cpr6p, Cpr7p, and Sti1p
HUA2	Cytoplasmic protein of unknown function; computational analysis of large-scale protein-protein interaction data suggests a possible role in actin patch assembly
HUL4	Protein with similarity to hect domain E3 ubiquitin-protein ligases, not essential for viability
HVG1	Protein of unknown function, has homology to Vrg4p
HXK1	Hexokinase isoenzyme 1, a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism; expression is highest during growth on non-glucose carbon sources; glucose-

	induced repression involves the hexokinase Hxk2p
HXT5	Hexose transporter with moderate affinity for glucose, may function in accumulation of reserve carbohydrates during stress, expression induced by a decrease in growth rate, contains an extended N-terminal domain relative to other HXTs
HXT7	High-affinity glucose transporter of the major facilitator superfamily, nearly identical to Hxt6p, expressed at high basal levels relative to other HXTs, expression repressed by high glucose levels /// High-affinity glucose transporter of the major facilitator superfamily, nearly identical to Hxt7p, expressed at high basal levels relative to other HXTs, repression of expression by high glucose requires SNF3
ICL1	Isocitrate lyase, catalyzes the formation of succinate and glyoxylate from isocitrate, a key reaction of the glyoxylate cycle; expression of ICL1 is induced by growth on ethanol and repressed by growth on glucose
ICL2	2-methylisocitrate lyase of the mitochondrial matrix, functions in the methylcitrate cycle to catalyze the conversion of 2-methylisocitrate to succinate and pyruvate; ICL2 transcription is repressed by glucose and induced by ethanol
IDP2	Cytosolic NADP-specific isocitrate dehydrogenase, catalyzes oxidation of isocitrate to alpha-ketoglutarate; levels are elevated during growth on non-fermentable carbon sources and reduced during growth on glucose
IDP3	Peroxisomal NADP-dependent isocitrate dehydrogenase, catalyzes oxidation of isocitrate to alpha-ketoglutarate with the formation of NADP(H+), required for growth on unsaturated fatty acids
INP54	Phosphatidylinositol 4,5-bisphosphate 5-phosphatase with a role in secretion, localizes to the endoplasmic reticulum via the C-terminal tail; lacks the Sac1 domain and proline-rich region found in the other 3 INP proteins
IPK1	Inositol 1,3,4,5,6-pentakisphosphate 2-kinase, nuclear protein required for synthesis of 1,2,3,4,5,6-hexakisphosphate (phytate), which is integral to cell function; has 2 motifs conserved in other fungi; ipk1 gle1 double mutant is inviable
ISF1	Serine-rich, hydrophilic protein with similarity to Mbr1p; overexpression suppresses growth defects of hap2, hap3, and hap4 mutants; expression is under glucose control; cotranscribed with NAM7 in a cyp1 mutant
ISN1	Inosine 5'-monophosphate (IMP)-specific 5'-nucleotidase, catalyzes the breakdown of IMP to inosine, does not show similarity to known 5'-nucleotidases from other organisms
IZH4	Membrane protein involved in zinc metabolism, member of the four-protein IZH family, expression induced by fatty acids and altered zinc levels; deletion reduces sensitivity to excess zinc; possible role in sterol metabolism
JEN1	Lactate transporter, required for uptake of lactate and pyruvate; expression is derepressed by transcriptional activator Cat8p under nonfermentative growth conditions, and repressed in the presence of glucose, fructose, and mannose
JSN1	Member of the Puf family of RNA-binding proteins, interacts with mRNAs encoding membrane-associated proteins; overexpression suppresses a tub2-150 mutation and causes increased sensitivity to benomyl in wild-type cells
KGD1	Component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes a key step in the tricarboxylic acid (TCA) cycle, the oxidative decarboxylation of alpha-ketoglutarate to form succinyl-CoA
KGD2	Dihydrolipoyl transsuccinylase, a component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes a step in the tricarboxylic acid (TCA) cycle, the oxidative decarboxylation of alpha-ketoglutarate to succinyl-CoA
KIN1	Serine/threonine protein kinase involved in regulation of exocytosis; localizes to the cytoplasmic face of the plasma membrane; closely related to Kin2p
KKQ8	Putative serine/threonine protein kinase with unknown cellular role
KNH1	Protein with similarity to Kre9p, which is involved in cell wall beta 1,6-glucan synthesis; overproduction suppresses growth defects of a kre9 null mutant
LAP3	Aminopeptidase of cysteine protease family, has a DNA binding activity and acts as bleomycin hydrolase in vitro; transcription is regulated by galactose via Gal4p
LAP4	Vacuolar aminopeptidase, often used as a marker protein in studies of autophagy and cytosol to vacuole targeting (CVT) pathway
LEE1	Zinc-finger protein of unknown function
LIP2	Lipoyl ligase, involved in the modification of mitochondrial enzymes by the attachment of lipoic acid groups
LOT6	Putative NADPH-dependent FMN reductase with strong ferricyanide reductase activity in vitro; gene expression increases in cultures shifted to a lower temperature
LSB3	Protein containing a C-terminal SH3 domain; binds Las17p, which is a homolog of human Wiskott-Aldrich Syndrome protein involved in actin patch assembly and actin polymerization
LSP1	Primary component of eisosomes, which are large immobile patch structures at the cell cortex associated with endocytosis, along with Pil1p and Sur7p ; null mutants show activation of Pkc1p/Ypk1p stress resistance pathways
MAG1	3-methyl-adenine DNA glycosylase involved in protecting DNA against alkylating agents; initiates

	base excision repair by removing damaged bases to create abasic sites that are subsequently repaired
MAL11	Maltose permease, inducible high-affinity maltose transporter (alpha-glucoside transporter); encoded in the MAL1 complex locus; member of the 12 transmembrane domain superfamily of sugar transporters
MAL32	Maltase (alpha-D-glucosidase), inducible protein involved in maltose catabolism; encoded in the MAL3 complex locus; functional in genomic reference strain S288C /// Maltase (alpha-D-glucosidase), inducible protein involved in maltose catabolism; encoded in the MAL1 complex locus
MBF1	Transcriptional coactivator that bridges the DNA-binding region of Gcn4p and TATA-binding protein Spt15p; suppressor of frameshift mutations
MBR1	Protein involved in mitochondrial functions and stress response; overexpression suppresses growth defects of hap2, hap3, and hap4 mutants
MCR1	Mitochondrial NADH-cytochrome b5 reductase, involved in ergosterol biosynthesis
MDG1	Plasma membrane protein involved in G-protein mediated pheromone signaling pathway; overproduction suppresses bem1 mutations
MDH1	Mitochondrial malate dehydrogenase, catalyzes interconversion of malate and oxaloacetate; involved in the tricarboxylic acid (TCA) cycle
MDH2	Cytoplasmic malate dehydrogenase, one of the three isozymes that catalyze interconversion of malate and oxaloacetate; involved in gluconeogenesis during growth on ethanol or acetate as carbon source; interacts with Pck1p and Fbp1p
MDH3	Cytoplasmic malate dehydrogenase, catalyzes interconversion of malate and oxaloacetate; involved in the glyoxylate cycle
MEF2	Mitochondrial elongation factor involved in translational elongation
MGS1	Protein with DNA-dependent ATPase and ssDNA annealing activities involved in maintenance of genome; interacts functionally with DNA polymerase delta; homolog of human Werner helicase interacting protein (WHIP)
MIA4	Essential protein of the mitochondrial intermembrane space, involved in import and assembly of intermembrane space proteins
MLS1	Malate synthase, enzyme of the glyoxylate cycle, involved in utilization of non-fermentable carbon sources; expression is subject to carbon catabolite repression; localizes in peroxisomes during growth in oleic acid medium
MPM1	Mitochondrial membrane protein of unknown function, contains no hydrophobic stretches
MRK1	Glycogen synthase kinase 3 (GSK-3) homolog; one of four GSK-3 homologs in S. cerevisiae that function to activate Msn2p-dependent transcription of stress responsive genes and that function in protein degradation
MRP8	Putative mitochondrial ribosomal protein, has similarity to E. coli ribosomal protein S2
MSC1	Protein of unknown function, green fluorescent protein (GFP)-fusion protein localizes to the endoplasmic reticulum; msc1 mutants are defective in directing meiotic recombination events to homologous chromatids
MSF1	Mitochondrial phenylalanyl-tRNA synthetase alpha subunit, active as a monomer, unlike the cytoplasmic subunit which is active as a dimer complexed to a beta subunit dimer; similar to the alpha subunit of E. coli phenylalanyl-tRNA synthetase
MSH6	Protein required for mismatch repair in mitosis and meiosis, forms a complex with Msh2p to repair both single-base & insertion-deletion mispairs; potentially phosphorylated by Cdc28p
MTH1	Negative regulator of the glucose-sensing signal transduction pathway, required for repression of transcription by Rgt1p; interacts with Rgt1p and the Snf3p and Rgt2p glucose sensors; phosphorylated by Yck1p, triggering Mth1p degradation
MYO3	One of two type I myosins; localizes to actin cortical patches; deletion of MYO3 has little effect on growth, but myo3 myo5 double deletion causes severe defects in growth and actin cytoskeleton organization
NCA2	Protein involved in regulation of mitochondrial expression of subunits 6 (Atp6p) and 8 (Atp8p) of the Fo-F1 ATP synthase; functions with Nca3p
NCR1	Vacuolar membrane protein that transits through the biosynthetic vacuolar protein sorting pathway, involved in sphingolipid metabolism; glycoprotein and functional orthologue of human Niemann Pick C1 (NPC1) protein
NDE2	Mitochondrial external NADH dehydrogenase, catalyzes the oxidation of cytosolic NADH; Nde1p and Nde2p are involved in providing the cytosolic NADH to the mitochondrial respiratory chain
NDI1	NADH:ubiquinone oxidoreductase, transfers electrons from NADH to ubiquinone in the respiratory chain but does not pump protons, in contrast to the higher eukaryotic multisubunit respiratory complex I; homolog of human AMID
NEM1	Protein of the nuclear envelope required for the spherical shape of the nucleus; required for normal sporulation
NGL3	Putative endonuclease, has a domain similar to a magnesium-dependent endonuclease motif in mRNA deadenylase Ccr4p; similar to Ngl1p and Ngl2p
ODC1	Mitochondrial inner membrane transporter, exports 2-oxoadipate and 2-oxoglutarate from the mitochondrial matrix to the cytosol for use in lysine and glutamate biosynthesis and in lysine

	catabolism
OM14	Integral mitochondrial outer membrane protein; abundance is decreased in cells grown in glucose relative to other carbon sources; appears to contain 3 alpha-helical transmembrane segments; ORF encodes a 97-basepair intron
OM45	Protein of unknown function, major constituent of the mitochondrial outer membrane; located on the outer (cytosolic) face of the outer membrane
OPI1	Transcriptional regulator of a variety of genes; phosphorylation by protein kinase A stimulates Opi1p function in negative regulation of phospholipid biosynthetic genes; involved in telomere maintenance
OSH2	Member of an oxysterol-binding protein family with seven members in S. cerevisiae; family members have overlapping, redundant functions in sterol metabolism and collectively perform a function essential for viability
OSH6	Member of an oxysterol-binding protein family with overlapping, redundant functions in sterol metabolism and which collectively perform a function essential for viability; GFP-fusion protein localizes to the cell periphery
OXR1	Protein of unknown function required for normal levels of resistance to oxidative damage, null mutants are sensitive to hydrogen peroxide; member of a conserved family of proteins found in eukaryotes but not in prokaryotes
PAI3	Cytoplasmic proteinase A inhibitor, dependent on Pbs2p and Hog1p protein kinases for osmotic induction; intrinsically unstructured, N-terminal half becomes ordered in the active site of proteinase A upon contact
PCD1	Peroxisomal nudix pyrophosphatase with specificity for coenzyme A and CoA derivatives, may function to remove potentially toxic oxidized CoA disulfide from peroxisomes to maintain the capacity for beta-oxidation of fatty acids
PCK1	Phosphoenolpyruvate carboxykinase, key enzyme in gluconeogenesis, catalyzes early reaction in carbohydrate biosynthesis, glucose represses transcription and accelerates mRNA degradation, regulated by Mcm1p and Cat8p, located in the cytosol
PCL6	Pho85p cyclin of the Pho80p subfamily; forms the major Glc8p kinase together with Pcl7p and Pho85p; involved in the control of glycogen storage by Pho85p; stabilized by Elongin C binding
PCL8	Cyclin, interacts with Pho85p cyclin-dependent kinase (Cdk) to phosphorylate and regulate glycogen synthase, also activates Pho85p for Glc8p phosphorylation
PDH1	Mitochondrial protein that participates in respiration, induced by diauxic shift; homologous to E. coli PrpD, may take part in the conversion of 2-methylcitrate to 2-methylisocitrate
PEP12	Target membrane receptor (t-SNARE) for vesicular intermediates traveling between the Golgi apparatus and the vacuole; controls entry of biosynthetic, endocytic, and retrograde traffic into the prevacuolar compartment; syntaxin
PEP4	Vacuolar aspartyl protease (proteinase A), required for the posttranslational precursor maturation of vacuolar proteinases; synthesized as a zymogen, self-activates
PET112	Protein required for mitochondrial translation; mutation is functionally complemented by a Bacillus subtilis ortholog
PET117	Protein required for assembly of cytochrome c oxidase
PET191	Protein required for assembly of cytochrome c oxidase
PEX17	Peroxisomal membrane protein component of the peroxisomal translocation machinery, required for peroxisome biogenesis, binds Pex14p
PEX18	Part of a two-member peroxin family (Pex18p and Pex21p) specifically required for peroxisomal targeting of the Pex7p peroxisomal signal recognition factor and PTS2 peroxisomal matrix proteins
PEX29	Peroxisomal integral membrane protein, involved in regulation of peroxisome size and number; genetic interactions suggest that Pex28p and Pex29p act at steps upstream of those mediated by Pex30p, Pex31p, and Pex32p
PEX3	Peroxisomal membrane protein (PMP) required to recruit Pex19p chaperone to peroxisomes; plays selective, essential, direct role in PMP import as a docking factor for Pex19p
PEX3	Peroxisomal membrane protein (PMP) required to recruit Pex19p chaperone to peroxisomes; plays selective, essential, direct role in PMP import as a docking factor for Pex19p
PFK26	6-phosphofructo-2-kinase, inhibited by phosphoenolpyruvate and sn-glycerol 3-phosphate, has negligible fructose-2,6-bisphosphatase activity, transcriptional regulation involves protein kinase A
PGM2	Phosphoglucomutase, catalyzes the conversion from glucose-1-phosphate to glucose-6-phosphate, which is a key step in hexose metabolism; functions as the acceptor for a Glc-phosphotransferase
PHM7	Protein of unknown function, expression is regulated by phosphate levels; green fluorescent protein (GFP)-fusion protein localizes to the cell periphery and vacuole
PHR1	DNA photolyase involved in photoreactivation, repairs pyrimidine dimers in the presence of visible light; induced by DNA damage; regulated by transcriptional repressor Rph1p
PIG2	Putative type-1 protein phosphatase targeting subunit that tethers Glc7p type-1 protein phosphatase to Gsy2p glycogen synthase
PIL1	Primary component of eisosomes, which are large immobile patch structures at the cell cortex associated with endocytosis, along with Lsp1p and Sur7p; null mutants show activation of Pkc1p/Ypk1p stress resistance pathways
PIM1	Mitochondrial ATP-dependent protease involved in intramitochondrial proteolysis; involved in

	degradation of misfolded proteins in mitochondria; required for bigenesis and maintenance of mitochondria
PKH1	Serine/threonine protein kinase involved in sphingolipid-mediated signaling pathway that controls endocytosis; activates Ypk1p and Ykr2p, components of signaling cascade required for maintenance of cell wall integrity; redundant with Pkh2p
PMC1	Vacuolar Ca2+ ATPase involved in depleting cytosol of Ca2+ ions; prevents growth inhibition by activation of calcineurin in the presence of elevated concentrations of calcium
PMT3	Protein O-mannosyltransferase, transfers mannose residues from dolichyl phosphate-D-mannose to protein serine/threonine residues; acts in a complex with Pmt5p, can instead interact with Pmt1p in some conditions; target for new antifungals
PNC1	Nicotinamidase that converts nicotinamide to nicotinic acid as part of the NAD(+) salvage pathway, required for life span extension by calorie restriction; PNC1 expression responds to all known stimuli that extend replicative life span
PNG1	Conserved peptide N-glycanase required for deglycosylation of misfolded glycoproteins during proteasome-dependent degradation, localizes to the cytoplasm and nucleus, interacts with the DNA repair protein Rad23p
POT1	3-ketoacyl-CoA thiolase with broad chain length specificity, cleaves 3-ketoacyl-CoA into acyl-CoA and acetyl-CoA during beta-oxidation of fatty acids
POX1	Fatty-acyl coenzyme A oxidase, involved in the fatty acid beta-oxidation pathway; localized to the peroxisomal matrix
PPE1	Protein with carboxyl methyl esterase activity that may have a role in demethylation of the phosphoprotein phosphatase catalytic subunit; also identified as a small subunit mitochondrial ribosomal protein
PPM1	Carboxyl methyl transferase, methylates the C terminus of the protein phosphatase 2A catalytic subunit (Pph21p or Pph22p), which is important for complex formation with regulatory subunits
PRB1	Vacuolar proteinase B (yscB), a serine protease of the subtilisin family; involved in protein degradation in the vacuole and required for full protein degradation during sporulation
PRC1	Vacuolar carboxypeptidase Y (proteinase C), involved in protein degradation in the vacuole and required for full protein degradation during sporulation
PRD1	Zinc metalloendopeptidase, found in the cytoplasm and intermembrane space of mitochondria; with Cym1p, involved in degradation of mitochondrial proteins and of presequence peptides cleaved from imported proteins
PRE1	20S proteasome beta-type subunit; localizes to the nucleus throughout the cell cycle
PRE3	20S proteasome beta-type subunit, responsible for cleavage after acidic residues in peptides
PRM4	Pheromone-regulated protein, predicted to have 1 transmembrane segment; transcriptionally regulated by Ste12p during mating and by Cat8p during the diauxic shift
PRM8	Pheromone-regulated protein with 2 predicted transmembrane segments and an FF sequence, a motif involved in COPII binding; forms a complex with Prp9p in the ER; member of DUP240 gene family
PRP12	Integral inner mitochondrial membrane protein with similarity to exonucleases; mutants exhibit an increased rate of mitochondrial DNA escape
PRR2	Protein kinase with a possible role in MAP kinase signaling in the pheromone response pathway
PRX1	Mitochondrial peroxiredoxin (1-Cys Prx) with thioredoxin peroxidase activity, has a role in reduction of hydroperoxides; induced during respiratory growth and under conditions of oxidative stress
PSK1	One of two (see also PSK2) PAS domain containing S/T protein kinases; coordinately regulates protein synthesis and carbohydrate metabolism and storage in response to a unknown metabolite that reflects nutritional status
PST2	Protein of unknown function with similarity to members of a family of flavodoxin-like proteins; induced by oxidative stress in a Yap1p dependent manner; GFP-fusion protein localizes to the cytoplasm in a punctate pattern
PTK2	Putative serine/threonine protein kinase involved in regulation of ion transport across plasma membrane; enhances spermine uptake
PUP3	Beta subunit of the 20S proteasome involved in ubiquitin-dependent catabolism; human homolog is subunit C10
PUT4	Proline permease, required for high-affinity transport of proline; also transports the toxic proline analog azetidine-2-carboxylate (AzC); PUT4 transcription is repressed in ammonia-grown cells
PXA1	Subunit of a heterodimeric peroxisomal ATP-binding cassette transporter complex (Pxa1p-Pxa2p), required for import of long-chain fatty acids into peroxisomes; similarity to human adrenoleukodystrophy transporter and ALD-related proteins
PXA2	Subunit of a heterodimeric peroxisomal ATP-binding cassette transporter complex (Pxa1p-Pxa2p), required for import of long-chain fatty acids into peroxisomes; similarity to human adrenoleukodystrophy transporter and ALD-related proteins
PYK2	Pyruvate kinase that appears to be modulated by phosphorylation; PYK2 transcription is repressed by glucose, and Pyk2p may be active under low glycolytic flux
RAV1	Subunit of the RAVE complex (Rav1p, Rav2p, Skp1p), which promotes assembly of the V-ATPase holoenzyme; required for transport between the early and late endosome/PVC and for localization of

	TGN membrane proteins; potential Cdc28p substrate
RAV2	Subunit of RAVE (Rav1p, Rav2p, Skp1p), a complex that associates with the V1 domain of the vacuolar membrane (H+)-ATPase (V-ATPase) and promotes assembly and reassembly of the holoenzyme
REG2	Regulatory subunit of the Glc7p type-1 protein phosphatase; involved with Reg1p, Glc7p, and Snf1p in regulation of glucose-repressible genes, also involved in glucose-induced proteolysis of maltose permease
RIC1	Protein involved in retrograde transport to the cis-Golgi network; forms heterodimer with Rgp1p that acts as a GTP exchange factor for Ypt6p; involved in transcription of rRNA and ribosomal protein genes
RIM11	Transcriptional repressor involved in response to pH and in cell wall construction; required for alkaline pH-stimulated haploid invasive growth and sporulation; activated by proteolytic processing; similar to A. nidulans PacC
RIM4	Putative RNA-binding protein required for the expression of early and middle sporulation genes
RMD5	Cytosolic protein required for sporulation; also required for the ubiquitination of the gluconeogenetic enzyme fructose-1,6-bisphosphatase, which is degraded rapidly after the switch from gluconeogenesis to glycolysis
RME1	Zinc finger protein involved in control of meiosis; prevents meiosis by repressing IME1 expression and promotes mitosis by activating CLN2 expression; directly repressed by a1-a2 regulator; mediates cell type control of sporulation
RNY1	RNAse; member of the T(2) family of endoribonucleases
ROD1	Membrane protein; overexpression confers resistance to the GST substrate o-dinitrobenzene as well as to zinc and calcium; contains 2 PY motifs, which are required for Rod1p interaction with Rsp5p, a hect-type ubiquitin ligase
RPN12	Subunit of the 19S regulatory particle of the 26S proteasome lid; synthetically lethal with RPT1, which is an ATPase component of the 19S regulatory particle; physically interacts with Nob1p and Rpn3p
RPN6	Essential, non-ATPase regulatory subunit of the 26S proteasome lid required for the assembly and activity of the 26S proteasome; the human homolog (S9 protein) partially rescues Rpn6p depletion
RRI2	Subunit of the COP9 signalosome (CSN) complex that cleaves the ubiquitin-like protein Nedd8 from SCF ubiquitin ligases; plays a role in the mating pheromone response
RSE1	Protein involved in pre-mRNA splicing; component of the pre-spliceosome; associates with U2 snRNA; involved in ER to Golgi transport
RTN2	Protein of unknown function; has similarity to mammalian reticulon proteins; member of the RTNLA (reticulon-like A) subfamily
RTS3	Putative component of the protein phosphatase type 2A complex
SAM5	Essential component of the Sorting and Assembly Machinery (SAM or Tob complex) of the mitochondrial outer membrane, which binds precursors of beta-barrel proteins and facilitates their outer membrane insertion; homologous to bacterial Omp85
SCO1	Copper-binding protein of the mitochondrial inner membrane, required for cytochrome c oxidase activity and respiration; may function to deliver copper to cytochrome c oxidase; has similarity to thioredoxins
SCO2	Protein anchored to the mitochondrial inner membrane, similar to Sco1p and may have a redundant function with Sco1p in delivery of copper to cytochrome c oxidase; interacts with Cox2p
SDH1	Flavoprotein subunit of succinate dehydrogenase (Sdh1p, Sdh2p, Sdh3p, Sdh4p), which couples the oxidation of succinate to the transfer of electrons to ubiquinone
SDH2	Iron-sulfur protein subunit of succinate dehydrogenase (Sdh1p, Sdh2p, Sdh3p, Sdh4p), which couples the oxidation of succinate to the transfer of electrons to ubiquinone
SDH3	Cytochrome b subunit of succinate dehydrogenase (Sdh1p, Sdh2p, Sdh3p, Sdh4p), which couples the oxidation of succinate to the transfer of electrons to ubiquinone
SDH4	Membrane anchor subunit of succinate dehydrogenase (Sdh1p, Sdh2p, Sdh3p, Sdh4p), which couples the oxidation of succinate to the transfer of electrons to ubiquinone
SDS24	One of two S. cerevisiae homologs (Sds23p and Sds24p) of the Schizosaccharomyces pombe Sds23 protein, which genetic studies have implicated in APC/cyclosome regulation; may play an indirect role in fluid-phase endocytosis
SEL1	UBX (ubiquitin regulatory X) domain-containing protein that interacts with Cdc48p, has a ubiquitin-associated (UBA) domain, interacts with ubiquitylated proteins in vivo, and is required for degradation of a ubiquitylated model substrate
SFC1	Mitochondrial succinate-fumarate transporter, transports succinate into and fumarate out of the mitochondrion; required for ethanol and acetate utilization
SHP1	UBX (ubiquitin regulatory X) domain-containing protein that regulates Glc7p phosphatase activity and interacts with Cdc48p; interacts with ubiquitylated proteins in vivo and is required for degradation of a ubiquitylated model substrate
SHY1	Mitochondrial inner membrane protein required for normal respiration, possible chaperone involved in assembly of cytochrome c oxidase; similar to SURF1 from mammals, chickens, and D. melanogaster

SIP18	Protein of unknown function whose expression is induced by osmotic stress
SIP2	One of three beta subunits of the Snf1 serine/threonine protein kinase complex involved in the response to glucose starvation; null mutants exhibit accelerated aging; N-myristoylprotein localized to the cytoplasm and the plasma membrane
SIP3	Protein that activates transcription through interaction with DNA-bound Snf1p, C-terminal region has a putative leucine zipper motif; potential Cdc28p substrate
SIP5	Protein of unknown function; interacts with both the Reg1p/Glc7p phosphatase and the Snf1p kinase
SMP2	Mg²⁺-dependent phosphatidate (PA) phosphatase, catalyzes the dephosphorylation of PA to yield diacylglycerol and P_i, responsible for de novo lipid synthesis; homologous to mammalian lipin 1
SNC1	Vesicle membrane receptor protein (v-SNARE) involved in the fusion between Golgi-derived secretory vesicles with the plasma membrane; proposed to be involved in endocytosis; member of the synaptobrevin/VAMP family of R-type v-SNARE proteins
SNF3	Plasma membrane glucose sensor that regulates glucose transport; has 12 predicted transmembrane segments; long cytoplasmic C-terminal tail is required for low glucose induction of hexose transporter genes HXT2 and HXT4
SNO4	Possible chaperone and cysteine protease with similarity to E. coli Hsp31 and S. cerevisiae Hsp31p, Hsp32p, and Hsp33p; member of the DJ-1/ThiJ/PfpI superfamily; may have a role in pyridoxine metabolism /// Possible chaperone and cysteine protease with similarity to E. coli Hsp31 and S. cerevisiae Hsp32p, Hsp33p, and Sno4p; member of the DJ-1/ThiJ/PfpI superfamily, which includes human DJ-1 involved in Parkinson's disease /// Possible chaperone and cysteine protease with similarity to E. coli Hsp31 and S. cerevisiae Hsp31p, Hsp33p, and Sno4p; member of the DJ-1/ThiJ/PfpI superfamily, which includes human DJ-1 involved in Parkinson's disease
SNX4	Sorting nexin, involved in the retrieval of late-Golgi SNAREs from the post-Golgi endosome to the trans-Golgi network and in cytoplasm to vacuole transport; contains a PX domain; forms complex with Snx41p and Atg20p
SNX41	Sorting nexin, involved in the retrieval of late-Golgi SNAREs from the post-Golgi endosome to the trans-Golgi network; forms a complex with Snx4p and Atg20p
SNZ3	Member of a stationary phase-induced gene family; transcription of SNZ2 is induced prior to diauxic shift, and also in the absence of thiamin in a Thi2p-dependent manner; forms a coregulated gene pair with SNO3 /// Member of a stationary phase-induced gene family; transcription of SNZ2 is induced prior to diauxic shift, and also in the absence of thiamin in a Thi2p-dependent manner; forms a coregulated gene pair with SNO2; interacts with Thi11p
SOD2	Manganese-containing superoxide dismutase; protects cells against oxygen toxicity
SOL4	6-phosphogluconolactonase with similarity to Sol3p
SPG1	S. cerevisiae YGR236C /GEN=YHB1 /DB_XREF=GI:6321675 /SEG=NC_001139:-962431,962820 /DEF=Protein required for survival at high temperature during stationary phase /NOTE=Ygr236cp
SPG4	Protein required for survival at high temperature during stationary phase; not required for growth on nonfermentable carbon sources
SPS19	Peroxisomal 2,4-dienoyl-CoA reductase, auxiliary enzyme of fatty acid beta-oxidation; homodimeric enzyme required for growth and sporulation on petroselineate medium; expression induced during late sporulation and in the presence of oleate
SRV2	CAP (cyclase-associated protein) subunit of adenylyl cyclase complex; N-terminus binds adenylyl cyclase and facilitates activation by RAS; C-terminus binds ADP-actin monomers, facilitating regulation of actin dynamics and cell morphogenesis
SRX1	Sulfiredoxin, contributes to oxidative stress resistance by reducing cysteine-sulfinic acid groups in the peroxiredoxins Tsa1p and Ahp1p that are formed upon exposure to oxidants; conserved in higher eukaryotes
SSE2	Member of the heat shock protein 70 (HSP70) family; may be involved in protein folding; localized to the cytoplasm; highly homologous to the heat shock protein Sse1p
STF1	Protein involved in regulation of the mitochondrial F1F0-ATP synthase; Stf1p and Stf2p act as stabilizing factors that enhance inhibitory action of the Inh1p protein
STF2	Protein involved in regulation of the mitochondrial F1F0-ATP synthase; Stf1p and Stf2p act as stabilizing factors that enhance inhibitory action of the Inh1p protein
STL1	Glycerol proton symporter of the plasma membrane, subject to glucose-induced inactivation, strongly but transiently induced when cells are subjected to osmotic shock
SUC2	Invertase, sucrose hydrolyzing enzyme; a secreted, glycosylated form is regulated by glucose repression, and an intracellular, nonglycosylated enzyme is produced constitutively
SUE1	Mitochondrial protein required for degradation of unstable forms of cytochrome c
SUL1	High affinity sulfate permease; sulfate uptake is mediated by specific sulfate transporters Sul1p and Sul2p, which control the concentration of endogenous activated sulfate intermediates
SWI4	DNA binding component of the SBF complex (Swi4p-Swi6p), a transcriptional activator that in concert with MBF (Mbp1-Swi6p) regulates late G1-specific transcription of targets including cyclins and genes required for DNA synthesis and repair

SYP1	Protein with a potential role in actin cytoskeletal organization; overexpression suppresses a pfy1 (profilin) null mutation
TCB1	Contains three calcium and lipid binding domains; green fluorescent protein (GFP)-fusion protein localizes to the cell periphery; C-terminal portion of Tcb1p, Tcb2p and Tcb3p interact
TES1	Peroxisomal acyl-CoA thioesterase likely to be involved in fatty acid oxidation rather than fatty acid synthesis; conserved protein also found in human peroxisomes; TES1 mRNA levels increase during growth on fatty acids
TFC8	One of six subunits of RNA polymerase III transcription initiation factor complex (TFIIIC); part of TFIIIC TauB domain that binds BoxB promoter sites of tRNA and other genes; linker between TauB and TauA domains; human homolog is TFIIIC-90
TFS1	Carboxypeptidase Y inhibitor, function requires acetylation by the NatB N-terminal acetyltransferase; phosphatidylethanolamine-binding protein involved in protein kinase A signaling pathway
TGL1	Steryl ester hydrolase, one of three gene products (Yeh1p, Yeh2p, Tgl1p) responsible for steryl ester hydrolase activity and involved in sterol homeostasis; localized to lipid particle membranes
THI13	Protein involved in synthesis of the thiamine precursor hydroxymethylpyrimidine (HMP); member of a subtelomeric gene family including THI5, THI11, THI12, and THI13 /// Protein involved in synthesis of the thiamine precursor hydroxymethylpyrimidine (HMP); member of a subtelomeric gene family including THI5, THI11, THI12, and THI13 /// Protein involved in synthesis of the thiamine precursor hydroxymethylpyrimidine (HMP); member of a subtelomeric gene family including THI5, THI11, THI12, and THI13 /// Protein involved in synthesis of the thiamine precursor hydroxymethylpyrimidine (HMP); member of a subtelomeric gene family including THI5, THI11, THI12, and THI13
TKL2	Transketolase, similar to Tkl1p; catalyzes conversion of xylulose-5-phosphate and ribose-5-phosphate to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate in the pentose phosphate pathway; needed for synthesis of aromatic amino acids
TLG2	Syntaxin-like t-SNARE that forms a complex with Tlg1p and Vti1p and mediates fusion of endosome-derived vesicles with the late Golgi; binds Vps45p, which prevents Tlg2p degradation and also facilitates t-SNARE complex formation
TMA1	Protein of unknown function that associates with ribosomes
TMA17	Protein of unknown function that associates with ribosomes
TPK1	Subunit of cytoplasmic cAMP-dependent protein kinase, which contains redundant catalytic subunits Tpk1p, Tpk2p, and Tpk3p and regulatory subunit Bcy1p; promotes vegetative growth in response to nutrients; inhibits filamentous growth
TPK2	Subunit of cytoplasmic cAMP-dependent protein kinase, which contains redundant catalytic subunits Tpk1p, Tpk2p, and Tpk3p and regulatory subunit Bcy1p; promotes vegetative growth in response to nutrients; activates filamentous growth
TPS1	Synthase subunit of trehalose-6-phosphate synthase/phosphatase complex, which synthesizes the storage carbohydrate trehalose; also found in a monomeric form; expression is induced by the stress response and repressed by the Ras-cAMP pathway
TPS2	Phosphatase subunit of the trehalose-6-phosphate synthase/phosphatase complex, which synthesizes the storage carbohydrate trehalose; expression is induced by stress conditions and repressed by the Ras-cAMP pathway
TPS3	Regulatory subunit of trehalose-6-phosphate synthase/phosphatase complex, which synthesizes the storage carbohydrate trehalose; expression is induced by stress conditions and repressed by the Ras-cAMP pathway
TRR2	Mitochondrial thioredoxin reductase involved in protection against oxidative stress, required with Glr1p to maintain the redox state of Trx3p, contains active-site motif (CAVC) present in prokaryotic thioredoxin reductases, binds NADPH and FAD
TSL1	Large subunit of trehalose 6-phosphate synthase (Tps1p)/phosphatase (Tps2p) complex, which converts uridine-5'-diphosphoglucose and glucose 6-phosphate to trehalose, homologous to Tps3p and may share function
TUL1	Golgi-localized RING-finger ubiquitin ligase (E3), involved in ubiquitinating and sorting membrane proteins that contain polar transmembrane domains to multivesicular bodies for delivery to the vacuole for quality control purposes
TVP15	Integral membrane protein localized to late Golgi vesicles along with the v-SNARE Tlg2p
UBA1	Ubiquitin activating enzyme, involved in ubiquitin-mediated protein degradation and essential for viability
UBC5	Ubiquitin-conjugating enzyme that mediates selective degradation of short-lived and abnormal proteins, central component of the cellular stress response; expression is heat inducible
UBC8	Ubiquitin-conjugating enzyme that negatively regulates gluconeogenesis by mediating the glucose-induced ubiquitination of fructose-1,6-bisphosphatase (FBPase); cytoplasmic enzyme that catalyzes the ubiquitination of histones in vitro
UBP15	Ubiquitin-specific protease that may play a role in ubiquitin precursor processing
UBP2	Ubiquitin-specific protease that removes ubiquitin from ubiquitinated proteins, cleaves at the C terminus of ubiquitin fusions; capable of cleaving polyubiquitin and possesses isopeptidase activity

UBX3	UBX (ubiquitin regulatory X) domain-containing protein that interacts with Cdc48p, green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern
UBX5	UBX (ubiquitin regulatory X) domain-containing protein that interacts with Cdc48p
UGA1	Gamma-aminobutyrate (GABA) transaminase (4-aminobutyrate aminotransferase) involved in the 4-aminobutyrate and glutamate degradation pathways; required for normal oxidative stress tolerance and nitrogen utilization
UGA2	Succinate semialdehyde dehydrogenase involved in the utilization of gamma-aminobutyrate (GABA) as a nitrogen source; part of the 4-aminobutyrate and glutamate degradation pathways; localized to the cytoplasm
UGO1	Protein of unknown function; outer membrane component of the mitochondrial fusion machinery; Ugo1p bind directly to Fzo1p and Mgm1p and thereby link these two GTPases during mitochondrial fusion
UGP1	UDP-glucose pyrophosphorylase (UGPase), catalyses the reversible formation of UDP-Glc from glucose 1-phosphate and UTP, involved in a wide variety of metabolic pathways, expression modulated by Pho85p through Pho4p
UGX2	Protein of unknown function, transcript accumulates in response to any combination of stress conditions
UIP4	Protein of unknown function that interacts with Ulp1p, a Ubl (ubiquitin-like protein)-specific protease for Smt3p protein conjugates
URA1	Dihydroorotate dehydrogenase, catalyzes the fourth enzymatic step in the de novo biosynthesis of pyrimidines, converting dihydroorotic acid into orotic acid
VAB2	Protein with a potential role in vacuolar function, as suggested by its ability to bind Vac8p; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern
VID28	Protein involved in proteasome-dependent catabolite degradation of fructose-1,6-bisphosphatase (FBPase); localized to the nucleus and the cytoplasm
VID3	Protein involved in proteasome-dependent catabolite degradation of fructose-1,6-bisphosphatase (FBPase); shifts the balance of nitrogen metabolism toward the production of glutamate; localized to the nucleus and the cytoplasm
VPS13	Protein of unknown function; heterooligomeric or homooligomeric complex; peripherally associated with membranes; homologous to human COH1; involved in sporulation, vacuolar protein sorting and protein-Golgi retention
VPS15	Myristoylated serine/threonine protein kinase involved in vacuolar protein sorting; functions as a membrane-associated complex with Vps34p; active form recruits Vps34p to the Golgi membrane; interacts with the GDP-bound form of Gpa1p
VPS17	Subunit of the membrane-associated retromer complex essential for endosome-to-Golgi retrograde protein transport; peripheral membrane protein that assembles onto the membrane with Vps5p to promote vesicle formation
VPS55	Late endosomal protein involved in late endosome to vacuole trafficking; functional homolog of human obesity receptor gene-related protein (OB-RGRP)
VPS68	Vacuolar membrane protein of unknown function involved in vacuolar protein sorting; also detected in the mitochondria
XBP1	Transcriptional repressor that binds to promoter sequences of the cyclin genes, CYS3, and SMF2; expression is induced by stress or starvation during mitosis, and late in meiosis; member of the Swi4p/Mbp1p family; potential Cdc28p substrate
XKS1	Xylulokinase, converts D-xylulose and ATP to xylulose 5-phosphate and ADP; rate limiting step in fermentation of xylulose; required for xylose fermentation by recombinant S. cerevisiae strains
YAK1	Serine-threonine protein kinase that is part of a glucose-sensing system involved in growth control in response to glucose availability; translocates from the cytoplasm to the nucleus and phosphorylates Pop2p in response to a glucose signal
YAL061W	putative polyol dehydrogenase
YAP181	Protein involved in clathrin cage assembly; binds Pan1p and clathrin; homologous to Yap1802p, member of the AP180 protein family
YAT1	Outer mitochondrial carnitine acetyltransferase, minor ethanol-inducible enzyme involved in transport of activated acyl groups from the cytoplasm into the mitochondrial matrix
YAT2	Carnitine acetyltransferase; has similarity to Yat1p, which is a carnitine acetyltransferase associated with the mitochondrial outer membrane
YBL039W-A	Putative protein of unknown function
YBR053C	Hypothetical protein
YBR056W	Hypothetical protein
YBR139W	Putative serine type Carboxypeptidase; green fluorescent protein (GFP)-fusion protein localizes to the vacuole; YBR139W is not an essential gene
YBR204C	Serine hydrolase; YBR204C is not an essential gene
YBR241C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the vacuolar membrane; YBR241C is not an essential gene
YBR280C	F-Box protein involved in proteasome-dependent degradation of Aah1p during entry of cells into

	quiescence; interacts with Skp1
YBR285W	Putative protein of unknown function; YBR287W is not an essential gene and deletion of the YBR287W leads to poor growth on glucose-minimal medium at 15C
YCL012C	Putative protein of unknown function; orthologs are present in S. bayanus, S. paradoxus and Ashbya gossypii; YCL012C is not an essential gene
YCL033C	Putative protein-methionine-R-oxide reductase; involved in response to oxidative stress; similar to mouse Sepx1p and fly SelRp; YCL033C is not an essential gene
YCP4	Protein of unknown function, has sequence and structural similarity to flavodoxins; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern
YDL027C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the mitochondrion; YDL027C is not an essential gene
YDL124W	NADPH-dependent alpha-keto amide reductase; reduces aromatic alpha-keto amides, aliphatic alpha-keto esters, and aromatic alpha-keto esters
YDL199C	Hypothetical protein
YDL218W	Hypothetical protein
YDR031W	Mitochondrial intermembrane space cysteine motif protein of 14 kDa
YDR107C	multispanning membrane protein
YDR379C-A	Hypothetical protein identified by homology. See FEBS Letters [2000] 487:31-36.
YER039C-A	Hypothetical protein
YER066W	Hypothetical protein
YER067W	Hypothetical protein
YER079W	Hypothetical protein
YER087W	Hypothetical protein
YFL042C	Putative protein of unknown function; YFL042C is not an essential gene
YFR017C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YFR017C is not an essential gene
YFR045W	Putative mitochondrial transport protein; null mutant is viable, exhibits decreased levels of chitin and normal resistance to calcofluor white
YGL010W	Hypothetical protein
YGL059W	Hypothetical protein
YGL146C	Hypothetical protein
YGL242C	Putative protein of unknown function; deletion mutant is viable
YGL250W	Putative protein of unknown function; deletion mutant results in reduced PIS1 expression and has growth defects on non-fermentable carbon sources and on minimal media; GFP-fusion protein localizes to both the cytoplasm and the nucleus
YGL262W	Putative protein of unknown function; null mutant displays elevated sensitivity to expression of a mutant huntingtin fragment or of alpha-synuclein; YGL262W is not an essential gene
YGR026W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cell periphery
YGR043C	Putative protein of unknown function; transcription is repressed by MOT1/YPL082C and induced by alpha-factor and during diauxic shift; green fluorescent protein (GFP)-fusion protein localizes to the nucleus
YGR053C	Hypothetical protein
YGR067C	Putative protein of unknown function; contains a zinc finger motif similar to that of Adr1p
YGR127W	Hypothetical protein
YGR130C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; specifically phosphorylated in vitro by mammalian diphosphoinositol pentakisphosphate (IP7)
YGR146C	Putative protein of unknown function; induced by iron homeostasis transcription factor Aft2p; multicopy suppressor of a temperature sensitive hsf1 mutant
YGR174W-A	Putative protein of unknown function
YGR174W-A	Putative protein of unknown function
YHR087W	Protein involved in RNA metabolism, one of two yeast homologs (with Sdo1p/Ylr022cp) of the human protein SBDS responsible for autosomal recessive Shwachman-Bodian-Diamond Syndrome, also conserved in Archaea
YHR138C	Putative protein of unknown function; has similarity to Pbi2p; double null mutant lacking Pbi2p and Yhr138p exhibits highly fragmented vacuoles
YHR140W	Putative integral membrane protein of unknown function
YHR159W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; potential Cdc28p substrate
YIG1	Protein that interacts with glycerol 3-phosphatase and plays a role in anaerobic glycerol production; localizes to the nucleus and cytosol

YIL042C	Mitochondrial kinase, phosphorylates pyruvate dehydrogenase alpha subunit Pda1p
YIL057C	Hypothetical protein
YIL077C	Hypothetical protein
YIL083C	Homolog to human PPCS
YIL087C	Hypothetical protein
YIL172C	Putative protein of unknown function with similarity to glucosidases; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm /// Protein of unknown function, expression is induced during nitrogen limitation /// Hypothetical protein
YIM1	Protein of unknown function; proposed to be involved in responding to DNA damaging agents
YIP5	Protein that interacts with Rab GTPases; computational analysis of large-scale protein-protein interaction data suggests a possible role in vesicle-mediated transport
YIR007W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YIR007W is a non-essential gene
YIR014W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the vacuole; expression directly regulated by the metabolic and meiotic transcriptional regulator Ume6p; YIR014W is a non-essential gene
YIR036C	Putative protein of unknown function; sequence similarity with short-chain dehydrogenase/reductase family members; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YIR036C is a non-essential gene
YJL045W	Minor succinate dehydrogenase isozyme; homologous to Sdh1p, the major isozyme reponsible for the oxidation of succinate and transfer of electrons to ubiquinone; induced during the diauxic shift in a Cat8p-dependent manner
YJL068C	Non-essential intracellular esterase that can function as an S-formylglutathione hydrolase; may be involved in the detoxification of formaldehyde, which can be metabolized to S-formylglutathione; similar to human esterase D
YJL070C	Putative protein of unknown function with similarity to AMP deaminases; YJL070C is a non-essential gene
YJL132W	Putative protein of unknown function; localizes to the membrane fraction; possible Zap1p-regulated target gene induced by zinc deficiency; YJL132W is a non-essential gene
YJL133C-A	S. cerevisiae YJL133C-A /GEN=LCB3 /DB_XREF=GI:33438820 /SEG=NC_001142:-159541,159765 /DEF=Yjl133c-ap /NOTE=go_component: cellular_component unknown [goid GO:0008372] [evidence ND]; go_function: molecular_function unknown [goid GO:0005554] [evidence ND]; go_process: biological_process unknown [goid GO:0000004] [evidence ND]
YJL144W	Cytoplasmic hydrophilin of unknown function, proposed to be involved in the dessication response; expression induced by osmotic stress, starvation conditions and during stationary phase; YJL144W is a non-essential gene
YJL163C	Hypothetical protein
YJL185C	Putative protein of unknown function; mRNA is weakly cell cycle regulated, peaking in G2 phase; YJL185C is a non-essential gene
YJL216C	Protein of unknown function, similar to alpha-D-glucosidases; transcriptionally activated by both Pdr8p and Yrm1p, along with transporters and other genes involved in the pleiotropic drug resistance (PDR) phenomenon
YJR008W	Putative protein of unknown function; expression repressed by inosine and choline in an Opi1p-dependent manner; expression induced by mild heat-stress on a non-fermentable carbon source.
YJR039W	Hypothetical protein
YJR096W	Putative xylose and arabinose reductase
YKL091C	Putative homolog of Sec14p, which is a phosphatidylinositol/phosphatidylcholine transfer protein involved in lipid metabolism; localizes to the nucleus
YKL096C-B	Putative protein of unknown function; identified by gene-trapping, microarray-based expression analysis, and genome-wide homology searching
YKL100C	Putative protein of unknown function with similarity to a human minor histocompatibility antigen; YKL100C is not an essential gene
YKL107W	Putative protein of unknown function
YKL133C	Hypothetical protein
YKL134C	Mitochondrial intermediate peptidase, cleaves N-terminal residues of a subset of proteins upon import, after their cleavage by mitochondrial processing peptidase (Mas1p-Mas2p); may contribute to mitochondrial iron homeostasis
YKL151C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm
YKL162C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the mitochondrion
YKL171W	Putative protein of unknown function; predicted protein kinase; implicated in proteasome function; epitope-tagged protein localizes to the cytoplasm
YKL187C	Putative protein of unknown function; detectable in highly purified mitochondria

YKL215C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm
YKR075C	Protein of unknown function; similar to YOR062Cp and Reg1p; expression regulated by glucose and Rgt1p
YKU7	Subunit of the telomeric Ku complex (Yku70p-Yku80p), involved in telomere length maintenance, structure and telomere position effect; relocates to sites of double-strand cleavage to promote nonhomologous end joining during DSB repair
YLR001C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the vacuolar membrane; also found in mitochondria; YLR001C is not an essential gene
YLR031W	Hypothetical protein
YLR053C	Hypothetical protein
YLR149C	Putative protein of unknown function; YLR149C is not an essential gene
YLR164W	Putative protein of unknown function that localizes to the mitochondrial inner membrane; similar to Tim18p and to Sdh4p
YLR211C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YLR211C is not an essential gene; ORF contains an intron
YLR218C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YLR218C is not essential; mutants exhibit glycogen storage defects and growth defects on a non-fermentable carbon source
YLR281C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to mitochondria; YLR281C is not an essential gene
YLR283W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to mitochondria; YLR283W is not an essential gene
YLR307C-A	Putative protein of unknown function
YLR312C	Hypothetical protein
YLR345W	Similar to 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase enzymes responsible for the metabolism of fructoso-2,6-bisphosphate; mRNA expression is repressed by the Rfx1p-Tup1p-Ssn6p repressor complex; YLR345W is not an essential gene
YLR346C	Putative protein of unknown function found in mitochondria; expression is regulated by transcription factors involved in pleiotropic drug resistance, Pdr1p and Yrr1p; YLR346C is not an essential gene
YLR352W	Putative protein of unknown function with similarity to F-box proteins; interacts with Skp1p and Cdc53p; YLR352W is not an essential gene
YLR408C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the endosome; YLR408C is not an essential gene
YML030W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to mitochondria; YML030W is not an essential gene
YML087C	Hypothetical protein
YML131W	Putative protein of unknown function with similarity to oxidoreductases; mRNA expression is increased in a HOG1 and SKO1-dependent manner after osmotic shock; GFP-fusion protein localizes to the cytoplasm; YML131W is not an essential gene
YMR031C	Putative protein of unknown function with similarity to YKL050C and USO1/YDL058W; found in mitochondria; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YMR031C is not an essential gene
YMR041C	Putative protein of unknown function with similarity to aldo/keto reductases; YMR041C is not an essential gene
YMR114C	Protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the nucleus and cytoplasm; YMR114C is not an essential gene
YMR118C	Protein of unknown function with similarity to succinate dehydrogenase cytochrome b subunit; YMR118C is not an essential gene
YMR160W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the membrane of the vacuole; mutant has enhanced sensitivity to overexpression of mutant huntingtin; YMR160W is not an essential gene
YMR175W-A	Putative protein of unknown function
YMR196W	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YMR196W is not an essential gene
YMR206W	Putative protein of unknown function; YMR206W is not an essential gene
YMR210W	Putative acyltransferase with similarity to Eeb1p and Eht1p, has a minor role in medium-chain fatty acid ethyl ester biosynthesis; may be involved in lipid metabolism and detoxification
YMR262W	Protein of unknown function; interacts weakly with Knr4p; YMR262W is not an essential gene
YMR31	Mitochondrial ribosomal protein of the small subunit, has similarity to human mitochondrial ribosomal protein MRP-S36
YNK1	Nucleoside diphosphate kinase, catalyzes the transfer of gamma phosphates from nucleoside triphosphates, usually ATP, to nucleoside diphosphates by a mechanism that involves formation of an autophosphorylated enzyme intermediate

YNL045W	Leucyl aminopeptidase (leukotriene A4 hydrolase) with epoxide hydrolase activity, metalloenzyme containing one zinc atom; role in vivo is not defined; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and nucleus
YNL115C	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to mitochondria; YNL115C is not an essential gene
YNL134C	Putative protein of unknown function with similarity to dehydrogenases from other model organisms; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and nucleus; YNL134C is not an essential gene
YNL144C	Putative protein of unknown function found in mitochondria; YNL144C is not an essential gene
YNL194C	Integral membrane protein localized to eisosomes, large immobile protein structures at the cell cortex associated with endocytosis; sporulation and plasma membrane sphingolipid content are altered in mutants; has homologs SUR7 and FMP45
YNL195C	Hypothetical protein
YNL200C	Hypothetical protein; similarity to human TGR-CL10C, thyroidal receptor for N-acetylglucosamine
YNL274C	Putative hydroxyisocaproate dehydrogenase
YNL305C	Hypothetical protein
YNL321W	Protein of unknown function, potential Cdc28p substrate
YNR034W-A	Hypothetical protein
YNR068C	Hypothetical protein
YOL048C	Hypothetical protein
YOL053W	Hypothetical protein
YOL083W	Hypothetical protein
YOL087C	Hypothetical protein
YOL107W	Hypothetical protein
YOL164W-A	Identified by fungal homology and RT-PCR
YOR020W-A	Identified by homology to Ashbya gossypii
YOR152C	Hypothetical protein
YOR175C	Member of the MBOAT family of putative membrane-bound O-acyltransferases
YOR186W	Hypothetical protein
YOR215C	Hypothetical protein
YOR223W	Hypothetical protein
YOR227W	Hypothetical protein
YOR228C	Protein of unknown function, localized to the mitochondrial outer membrane
YOR289W	Hypothetical protein
YPC1	Alkaline ceramidase that also has reverse (CoA-independent) ceramide synthase activity, catalyzes both breakdown and synthesis of phytoceramide; overexpression confers fumonisin B1 resistance
YPI1	Inhibitor of the type I protein phosphatase Glc7p, which is involved in regulation of a variety of metabolic processes; overproduction causes decreased cellular content of glycogen
YPK1	Serine/threonine protein kinase required for receptor-mediated endocytosis; involved in sphingolipid-mediated and cell integrity signaling pathways; localized to the bud neck, cytosol and plasma membrane; homolog of mammalian kinase SGK
YPL109C	Hypothetical protein
YPL113C	Putative dehydrogenase
YPL119C-A	Identified by expression profiling and mass spectrometry
YPL230W	Up in StarVation
YPR036W-A	S. cerevisiae YPR036W-A /GEN=VMA13 /DB_XREF=GI:23270401 /SEG=NC_001148:+645945,646148 /DEF=Ypr036w-ap /NOTE=go_component: cellular_component unknown [goid GO:0008372] [evidence ND]; go_function: molecular_function unknown [goid GO:0005554] [evidence ND]; go_process: response to drug [goid GO:0042493] [evidence IEP,ISS] [pmid 11557050]
YPR1	2-methylbutyraldehyde reductase, may be involved in isoleucine catabolism
YPR158W	Hypothetical protein
YPR172W	Protein of unknown function, transcriptionally activated by Yrm1p along with genes involved in multidrug resistance
YPS6	Putative GPI-anchored aspartic protease
YPT35	Endosomal protein of unknown function that contains a phox (PX) homology domain and binds to both phosphatidylinositol-3-phosphate (PtdIns(3)P) and proteins involved in ER-Golgi or vesicular transport
YPT53	GTPase, similar to Ypt51p and Ypt52p and to mammalian rab5; required for vacuolar protein sorting and endocytosis

YSC84	Protein involved in the organization of the actin cytoskeleton; contains SH3 domain similar to Rvs167p
YTP1	Probable type-III integral membrane protein of unknown function, has regions of similarity to mitochondrial electron transport proteins

Paper 3

On the functional relationship between Hxk2 and the Snf1 pathway in yeast carbon catabolite repression

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The functional relationship between Hxk2 and the Snf1 pathway in yeast carbon catabolite repression

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Abstract

The main glucose repression/derepression Snf1 pathway in the yeast *Saccharomyces cerevisiae* has been studied for thirty years but still fundamental questions concerning sensing and signal transmission remain unanswered. In this work, we have started a new approach to elucidate the role of hexokinase PII in glucose signalling. Deletion of *HXK2* relieves glucose repression of *SUC2* (encoding invertase), but it is not known if this effect is mediated via the Snf1 kinase. Snf1 activity is required for expression of *SUC2* at low glucose levels. Reg1 is a negative regulator of Snf1 and its deletion also causes glucose derepression of *SUC2*. Snf1 negatively controls the activity of the Mig1 repressor and also deletion of *MIG1* derepresses *SUC2* expression. Genetic evidence reported here confirms that Snf1 acts as the downstream of Reg1 and Hxk2 in the pathway controlling *SUC2* expression. However, it appears that Reg1 and Hxk2 partly mediate glucose repression independently of Mig1, perhaps via Mig2. Based on Snf1 and Mig1 phosphorylation assays it appears that the *hxk2Δ* mutant transiently responds to glucose addition but is unable to sustain glucose repression. Hence, the observed glucose repression defect of the *hxk2Δ* mutant is due to increased Snf1 activity and Mig1 phosphorylation. The Mig1 phosphorylation assay as well as *SUC2* expression data further suggest that both *hxk2Δ* and *reg1Δ* mutants still retain an ability to respond to glucose but the double mutant *hxk2Δ reg1Δ*, like the *snf1Δ* mutant, does not respond to glucose. Taken together, it appears that both Reg1 and Hxk2 control glucose repression via parallel pathways that involve Snf1. The tools used in this work, i.e. genetic analysis, time course Snf1 and Mig1 phosphorylation and gene expression assays together with monitoring of Mig1 localisation will allow a complete analysis of the signalling routes from Hxk2 to *SUC2* expression and thereby answering some of the fundamental open questions concerning yeast glucose repression.

Introduction

The yeast *Saccharomyces cerevisiae* has established versatile mechanisms to adapt to rapid changes in nutrient availability. Glucose is the preferred carbon and energy source. When glucose is abundant, expression of a large number of genes is repressed, including gluconeogenic genes, respiratory genes and genes needed for the utilisation of alternative carbon sources (reviewed in (Carlson, 1999; Gancedo, 1998; Johnston, 1999; Ronne, 1995; Santangelo, 2006)). This phenomenon is referred to as glucose repression. Components of the underlying signalling pathway include (Fig. 1): the Snf1 protein kinase complex consisting of the Snf1 kinase (alpha subunit), the Snf4 regulatory (gamma) subunit (Celenza and Carlson, 1984; Celenza and Carlson, 1986) and any of the three scaffolding (beta) subunits Gal83, Sip1 and Sip2 (Amodeo et al., 2007; Jiang and Carlson, 1997; Vincent and Carlson, 1999; Vincent et al., 2001; Yang et al., 1994), the Mig1 transcriptional repressor complex consisting of Mig1, Ssn6 and Tup1 (Carlson et al., 1984; Nehlin and Ronne, 1990), the Snf1 activating kinases Sak1, Tos3 and Elm1 (Hong et al., 2003; Nath et al., 2003; Sutherland et al., 2003) and the protein phosphatase PP1 Glc7/Reg1 (Tu and Carlson, 1994; Tu and Carlson, 1995). In addition, hexokinase PII, Hxk2, plays a role in signalling (Entian, 1980; Entian et al., 1977; Moreno and Herrero, 2002).

The Snf1 complex, the central component of the signalling pathway in glucose repression/derepression, is conserved throughout eukaryotes and homologous to the mammalian AMP-activated protein kinase (AMPK) (Aguan et al., 1994; Carling, 2004; Carling et al., 1994; Carlson, 1999). When glucose is present, Snf1 is inactive and when glucose is depleted, Snf1 becomes activated and derepresses the expression of glucose-repressed genes. Mig1 is a Cys_2His_2 zinc finger protein that binds to the promoter of glucose repressed genes in the presence of glucose (Lutfiyya et al., 1998; Lutfiyya and Johnston, 1996; Nehlin and Ronne, 1990). It is inactivated through phosphorylation by active Snf1 and then translocates from the nucleus to the cytoplasm (De Vit et al., 1997; Wu and Trumbly, 1998). This results in the release of Mig1 from the promoter of glucose repressible genes and derepression of the corresponding genes (Gancedo, 1998).

The activation of Snf1 requires phosphorylation at threonine 210 in its activation loop (Estruch et al., 1992). This phosphorylation is controlled by any of the Snf1 activating kinases Sak1, Tos3 and Elm1 (Hong et al., 2003; Nath et al., 2003; Sutherland et al.,

2003) and the protein phosphatase 1 Glc7/Reg1 (Ludin et al., 1998; Sanz et al., 2000). Snf1 activating kinases appear to constitutively activate Snf1 while the dephosphorylation step is controlled by glucose in an unknown manner (Rubenstein and Schmidt, 2007). Among the kinases, Sak1 plays a predominant role (Hedbacker et al., 2004; Hong and Carlson, 2007; Nath et al., 2003). The protein phosphatase complex comprises the regulatory subunit Reg1 and the catalytic subunit Glc7 (Frederick and Tatchell, 1996 ; Hisamoto et al., 1994 ; Ludin et al., 1998; Ramaswamy et al., 1998 ; Tachikawa et al., 2001 ; Tu et al., 1996; Wu et al., 2001). Reg1/Glc7 dephosphorylates Snf1 and Mig1 when glucose is present (Ludin et al., 1998).

Mutants lacking Hxk2 derepress expression of several glucose repressed genes (Entian, 1980; Entian et al., 1977; Ma et al., 1989; Ma and Botstein, 1986; Moreno and Herrero, 2002). Hxk2 is one of three enzymes (Hxk1, Hxk2 and Glk1) in yeast responsible for phosphorylating glucose in the first irreversible step of glucose catabolism. The predominant role of Hxk2 in glucose repression is due to the fact that expression of *HXK1* and *GLK1* is glucose-repressed. Several pieces of evidence show that Hxk1, but not Glk1, may also play a role in catabolite repression (Hohmann, 1987; Ma and Botstein, 1986; Rose et al., 1991). The mechanism by which Hxk2 confers its role in glucose repression remains still unclear. It has been reported that Hxk2 localises in both the cytoplasm and the nucleus (Ahuatzi et al., 2004; Herrero et al., 1998; Randez-Gil et al., 1998) and that nuclear localisation is regulated by the availability of glucose and by Mig1 (Ahuatzi et al., 2004).

In this study, the functional relationship between Hxk2 and Reg1 as well as Snf1 and Mig1 in response to glucose was analysed further. Here, we present direct evidence that mutants lacking Hxk2 affect the phosphorylation of Snf1 in high glucose conditions. We also show that Mig1 is partially phosphorylated in the single *hxk2Δ* and *reg1Δ* mutants whereas its phosphorylation state does not seem to be regulated anymore by glucose in the double *hxk2Δ reg1Δ* mutant. These observations indicate that Hxk2 and Reg1 may have partly parallel roles in glucose repression.

Materials and methods

Yeast strains and plasmids

The *S. cerevisiae* strains used in this study are isogenic to W303-1A (Table 1). Strains were generated either by transformation of PCR product or by crossing and tetrad analysis. The deletion of genes was confirmed by PCR using different primers. Centromeric plasmids p*MIG1-HA* and p*SNF1-HA* expressing Mig1-HA and Snf1-HA (three copies of the hemagglutinin(HA)-antigen at the C-termini) were kindly provided by Martin Schmidt (McCartney and Schmidt, 2001; Schmidt and McCartney, 2000)

Growth conditions and growth assays

Yeast cells were routinely grown in medium either containing 2% peptone and 1% yeast extract supplemented with 2% glucose as a carbon source (YPD) or containing YNB (yeast nitrogen base) supplemented with amino acid as required and with different carbon sources, 2% glucose, 2% raffinose, 2% sucrose or 2% sucrose with 200 μg/ml 2-deoxyglucose. Selection and growth of transformants carrying a plasmid was performed in YNB (Sherman et al., 1983). Plate growth assays were performed by pre-growing cells in YPD medium or uracil-deficient YNB medium. Cells were resuspended in the same medium to an optical density at 600 nm (OD_{600}) = 1.0. Five μl of a 10-fold serial dilution of this culture were spotted onto agar plates supplemented with 2% glucose or different carbon sources. Growth was monitored after 2 to 3 days at 30°C.

Gene expression analysis

For reverse transcription (RT)-PCR of *SUC2* expression, cells were cultivated in media containing 8% glucose and shifted to 0.2% glucose at an OD_{600} ~ 0.8. RNA was extracted as described previously (De Winde et al., 1996), DNase treated, and controlled on an agarose gel. Reverse transcription (Superscript II; Invitrogen) using $pd(T)_{12-18}$ as primers to produce the cDNA and quantitative RT-PCR assays using specific *SUC2* primers (Ye et al., 2006) were performed in an iCycler (Bio-Rad). PCR products were checked by agarose gel and melting curve analysis. Expression data were normalized against *ACT1*, encoding actin.

Western blot analysis

Cells (10ml) were grown to OD_{600} = 0.5-0.8 and harvested by centrifugation. Cells were then resuspended in 500µl of 2M NaOH supplemented with 7% *β*-mercaptoethanol and incubated for 2 min at room temperature. Five-hundred µl of 50% TCA (tricholoracetic acid) was added, samples were vortexed and sedimented. Samples were washed in 500µl of 1M Tris (pH=7.5) and resuspended in 120µl of 1xSDS sample buffer (62.5mM Tris-HCl pH 6.8, 3% sodium dodecyl sulfate [SDS], 10% glycerol, 5 % *β*-mercaptoethanol, 0.001% bromophenol blue) and incubated for 5 min at 100^{o}C. Forty µg of the supernatant were separated by SDS-PAGE using 7.5% polyacrylamide gels for Mig1 and analysed by immunoblotting using anti-HA antibody (Sigma) and secondary anti-mouse immunoglobulin G antibody conjugated to horseradish peroxidase. The Lumi-Light Western blotting substrate (Roche) and the FUJIFILM LAS-1000 camera were used for visualisation.

For assessing the phosphorylation state of Snf1, cells (25ml) were grown to OD_{600} 0.5-0.8. NaOH was added to the culture to final concentration of 0.1M prior to protein extraction as described above with a minor modification. Immunoprecipitation of Snf1 was described previously (McCartney and Schmidt, 2001). Forty µg of the supernatant were separated by SDS-PAGE using 7.5% polyacrylamide gels and analysed by immunoblotting using our anti-phospho-Snf1 antiserum and a secondary anti-rabbit immunoglobulin G antibody (Sigma) conjugated to horseradish peroxidase and anti-HA antibody as a loading control. The Lumi-Light Western blotting substrate (Roche) and the FUJIFILM LAS-1000 camera were used for visualisation.

Invertase assay

Cells were harvested by centrifugation 3 hours after a shift to 0.2% glucose. Protein extracts and measurements of invertase activity were performed as described previously (Goldstein and Lampen, 1975; Hohmann and Zimmermann, 1986). The protein concentration was determined by using the DC protein assay kit (Bio-Rad).

Results

Phenotypic characterization of mutants in the glucose repression pathway

To re-assess the role of several major pathway components we first analysed growth of relevant deletion mutants on YNB plate supplemented with different carbon sources (Fig. 2). As expected all strains tested could grow with glucose as sole carbon source although in some instances less well than the wild type. Growth with raffinose or sucrose, the substrates for invertase, requires Snf1 and as expected this requirement was not suppressed by deletion of *HXK2* or *REG1*. We note, however, that the triple *hxk2Δ reg1Δ snf1Δ* mutant could grow slowly on raffinose or sucrose. Growth on medium with sucrose as carbon source and the non-metabolised glucose analogue 2-deoxyglucose requires constitutive derepression of *SUC2* (Zimmermann and Scheel, 1977) as observed in *hxk2Δ*, *reg1Δ* and *mig1Δ* mutants and combinations of those deletions. The triple *hxk2Δ reg1Δ snf1Δ* mutant could grow on this medium as well, albeit slowly, indicating low constitutive expression of *SUC2*.

SUC2 expression indicates that Reg1, Hxk2 and Mig1 also have independent roles in glucose repression

In order to quantify the effects on glucose repression of some of the mutants we performed expression analysis of *SUC2* at the RNA (Fig. 3A) and protein activity level (Fig. 3B). Wild type showed clear *SUC2* repression on glucose medium and a derepression after shift to 0.2% glucose. The *hxk2Δ, reg1Δ* and *mig1Δ* single mutants displayed reduced or almost absent repression on glucose and rather poor correlation between mRNA and invertase activity levels. The double mutants with combination of *hxk2Δ, reg1Δ* and *mig1Δ* all showed further increased *SUC2* mRNA and invertase activity levels and those were not further enhanced in the triple mutant. This suggests that while Hxk2, Reg1 and Mig1 all play critical roles in glucose repression they may perform their function not (only) via a linear pathway but also (partly) independent from each other.

Deletion of HXK2 affects the phosphorylation of Snf1and Mig1

The availability of antibodies specific for the phosphorylated form of Snf1 as well as the banding pattern generated by Mig1 in Western blots dependent on its phosphorylation state allowed to revisit the role of Hxk2 in glucose repression (Fig. 4). Wild type cells grown on 4% glucose show low Snf1 phosphorylation as well as

low Mig1 phosphorylation. After shift to 0.2% glucose Snf1 and Mig1 phosphorylation is strongly increased. Also in the *hxk2Δ* mutant there is a difference in the levels of Snf1 and Mig1 phosphorylation between high and low glucose growth although levels are generally higher than in the wild type. Total Mig1 levels seem to be lower in the *hxk2Δ* mutant.

Following a shift from 0.2% to 4% glucose Snf1 and Mig1 phosphorylation levels drop within 20sec in both wild type and *hxk2Δ* mutant, although to a somewhat lower extend in the mutant. Hence, the *hxk2Δ* mutant retains an ability to respond to glucose. During later time points the Snf1 and Mig1 phosphorylation levels start to increase again and they do so faster and especially stronger in the *hxk2Δ* mutant. Hence, the glucose repression defect of the *hxk2Δ* mutants does not seem to be due to an absent initial glucose signalling but rather an ability to maintain the repressed state, consistent with previous observations (De Winde et al., 1996).

Mutants in both HXK2 and REG1 abolish glucose regulated Mig1 phosphorylation

To further characterise the relationship between Hxk2 and Reg1 in glucose repression we monitored Mig1 phosphorylation in single and double mutants (Fig. 5). As shown also in figure 4, Mig1 is poorly phosphorylated in glucose-grown cells (note that 2% glucose was used in this experiment and therefore the phosphorylation level is somewhat higher) and highly phosphorylated in cells grown at low glucose levels. In a *snf1Δ* mutant Mig1 appears to be (mainly) unphosphorylated under both conditions and no significant differences can be observed. Single *hxk2Δ* and *reg1Δ* mutants both show enhanced Mig1 phosphorylation at 2% glucose as compared to wild type but there is a clear further shift to more highly phosphorylated forms in cells grown in 0.2% glucose. In the double *hxk2Δ reg1Δ* mutant Mig1 attains an intermediate degree of phosphorylation which is not altered by the growth conditions employed in this experiment.

Discussion

The data presented here should be regarded as a first step in an analysis to revisit the role of Hxk2 (and in extension Hxk1) in glucose repression. To complete this analysis and to arrive at a more comprehensive picture will require further time course analyses using additional mutants and combinations of mutations as well as mutants

lacking the Snf1 upstream kinases. We have recently generated all combinations of upstream kinase mutations with hexokinase mutations in a large set of yeast strains. Unexpectedly, simultaneous deletion of *SAK1, ELM1, TOS3* and *HXK2* turned out to be lethal for reasons that we do not presently understand. Since the *snf1Δ hxk2Δ* mutant is viable (Ahuatzi et al., 2007) and our own data, the observed effect might be due to a presently unknown function of the upstream kinases. Since any upstream kinase is sufficient to keep the *hxk2Δ* mutant alive, this property must be shared by all three kinases.

The basic experimental approaches employed here seem to have potential to further elucidate the role of Hxk2 and other Snf1 pathway signalling components in the glucose response. Time course analysis have previously demonstrated that establishing glucose repression is (at least) a two-stage process and therefore time course analysis are critically important to understand the role of different regulators (De Winde et al., 1996). The present analysis, i.e. Snf1 and Mig1 phosphorylation as well as *SUC2* gene expression might be complemented in further experiments with Mig1 promoter binding by chromatin immunoprecipitation and/or single cell analysis using microfluidics and Mig1-GFP nuclear/cytosolic shuttling as a read-out. This setup has been established and is presently being employed for data collection.

Already at this stage data presented here point to different though overlapping roles of three critical negative regulators of the glucose response, Hxk2, Reg1 and Mig1. It should be pointed out that all three have paralogues in the yeast genome (Hxk1, Reg2 and Mig2, respectively) (Frederick and Tatchell, 1996; Jiang et al., 2000; Lutfiyya et al., 1998; Lutfiyya and Johnston, 1996; Walsh et al., 1983), which might play supplementary roles and partly replace the main regulators when those are deleted (this is certainly the case for Hxk1 and Mig1) (Klein et al., 1999; Lutfiyya et al., 1998; Ma and Botstein, 1986; Rose et al., 1991). Still we observe that deletion of any of those regulators, Hxk2, Reg1 and Mig1, does not fully prevent catabolite repression while the combination of double deletions seems to abolish catabolite repression. With the strong precaution that we have not yet performed a complete analysis of all strains using all tests available, the present data suggest that Reg1 and Hxk2 function (partly) in parallel through Snf1 in controlling *SUC2* expression. Remarkably, growth assays (that require confirmation by *SUC2* gene expression analysis and other tests) indicate that deletion of both *REG1* and *HXK2* together can

partly suppress the defect of Snf1 to express *SUC2*. These observations are largely consistent with a scenario proposed by the Moreno group (Moreno et al., 2005) where Hxk2 in the nucleus restricts access of the Snf1 kinase to Mig1 and thereby establishes glucose repression. In the *hxk2Δ reg1Δ snf1Δ* mutant other kinases might have access to Mig1 and in the lack of the cognate phosphatase allow some glucose-level-independent phosphorylation of Mig1 and *SUC2* expression. Further analyses will address the validity of this scenario.

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Table 1: Yeast strains used in this study

Strain	Genotype	Source/Reference
W303-1A	MAT a *leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2*	(Thomas and Rothstein, 1989)
PD90.4c	W303-1A *snf1Δ:: KanMX*	S Hohmann collection
YSH310	W303-1A *hxk2Δ::LEU2*	S Hohmann collection
H376	W303-1A *mig1Δ::LEU2*	(Balciunas and Ronne, 1999)
YPDahl113	W303-1A *reg1Δ::KanMX*	This study
PD118.2.6c	W303-1A *hxk2Δ::LEU2 snf1::KanMX*	This study
PD129.1.3d	W303-1A *reg1Δ::KanMX snf1Δ::KanMX*	This study
YPDahl124	W303-1A *reg1Δ::KanMX hxk2Δ::LEU2*	This study
PD127.2.4b	W303-1A *reg1Δ::KanMX mig1::LEU2*	This study
PD116.8b	W303-1A *hxk2Δ::LEU2 mig1Δ::LEU2*	This study
PD128.1.2d	W303-1A *hxk2Δ::LEU2 reg1Δ::KanMX mig1Δ::KanMX*	This study
PD129.2.2d	W303-1A *hxk2Δ::LEU2 reg1Δ::KanMX snf1Δ::KanMX*	This study

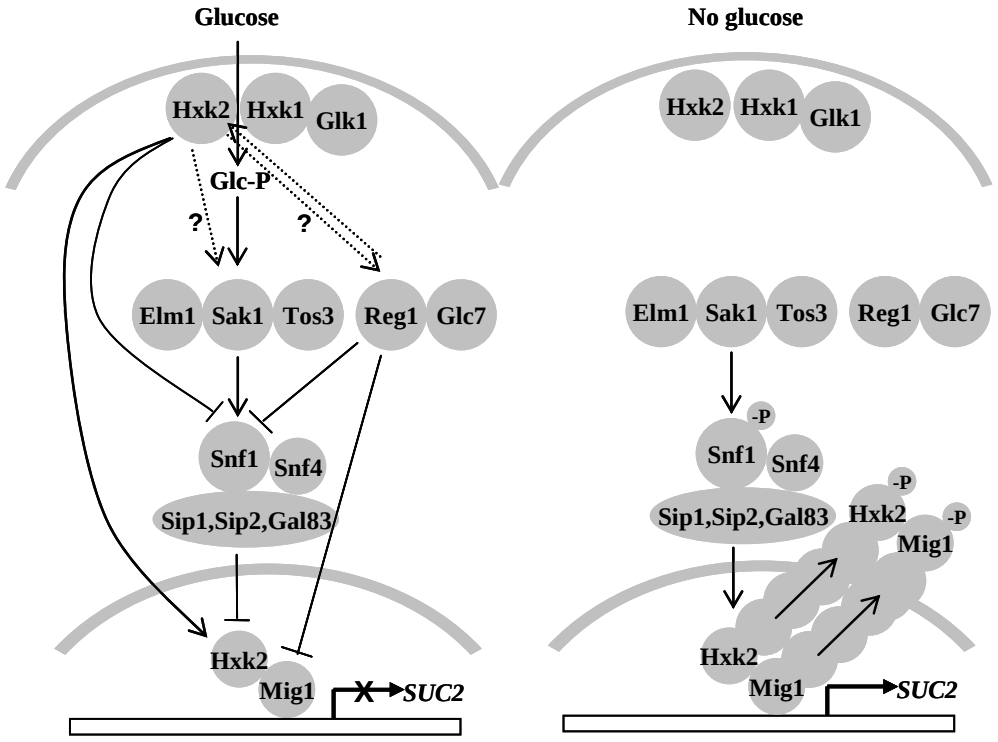

Fig. 1: Overview on the yeast Snf1 glucose repression/derepression pathway and putative roles of Hxk2 in signalling. The Snf1 heterotrimeric complex comprises the Snf1 protein kinase (alpha subunit), an activating (gamma) subunit Snf4 and any of the three scaffolding (beta) subunits Gal83, Sip1 and Sip2 (reviewed in (Carlson, 1999; Hedbacker and Carlson, 2008; Johnston, 1999)). When glucose is abundant, it is transported into the cell and rapidly phosphorylated by the hexokinases (Hxk2, Hxk1 and Glk1). Phosphorylated glucose enters glycolysis resulting in ATP generation and eventually depletion of AMP. Since AMP levels are low, Snf1 is inactive. Snf1 activity is controlled by any of the three Snf1 activating kinases Sak1, Elm1and Tos3 (Hong et al., 2003; Nath et al., 2003; Sutherland et al., 2003), the protein phosphatase PP1 Glc7/Reg1 (Tu and Carlson, 1994) and perhaps Hxk2. The three activating kinases appear to constitutively phosphorylate Snf1 (Rubenstein and Schmidt, 2007) whereas the protein phosphatase dephosphorylates Snf1 to antagonise the activating kinases (Tu and Carlson, 1994). Hxk2 appears to inhibit Snf1 activity directly or functions indirectly via Reg1 or alternative mechanisms. Furthermore, Hxk2 is partially localised in the nucleus together with Mig1 resulting in the sequestration of Snf1 and Mig1 (Moreno et al., 2005). Mig1 then can not be phosphorylated by Snf1 and consequently exerts repression of genes. When glucose is deprived, Hxk2 is phosphorylated resulting in a conformational shift (Kriegel et al., 1994) and Reg1 is no longer able to dephosphorylate Snf1. Snf1 becomes activated and phosphorylates and inactivates Mig1. Phosphorylated Mig1 translocates from the nucleus to the cytoplasm leading to derepression of expression of glucose repressed genes (De Vit et al., 1997; Ostling and Ronne, 1998; Treitel et al., 1998).

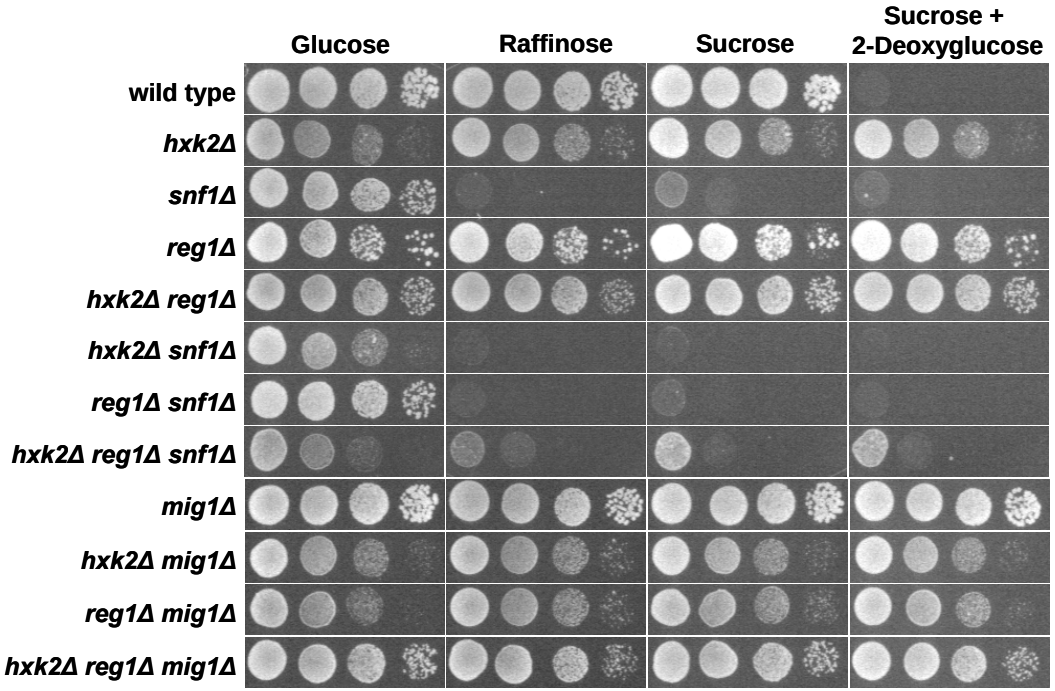

Fig. 2: Phenotypic analysis of mutants affected in the Snf1 general glucose repression/derepression pathway. Yeast strains were grown overnight on YPD plates, resuspended, adjusted to exactly an OD_{600nm} of 1.0 and 5µl were spotted in 1:10 dilution steps onto synthetic defined YNB medium containing 2% glucose (where all strains should grow), 2% raffinose, 2% sucrose (Snf1 activity is required for utilising those two sugars as carbon and energy sources) and 2% sucrose supplemented with 2-deoxyglucose (where only mutants with defective catabolite repression can grow). Plates were photographed after 2 days incubation at 30ºC.

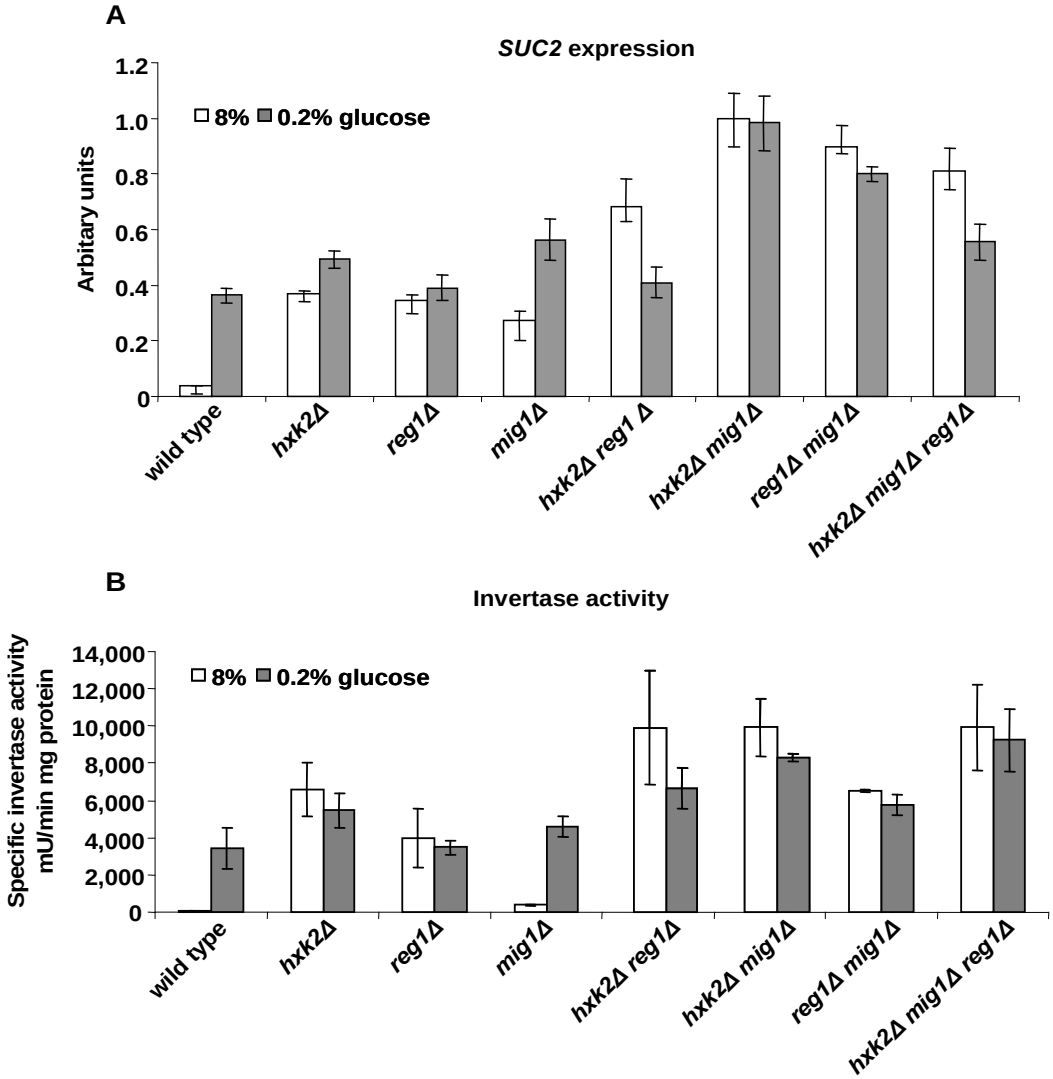

Fig. 3: Analysis of expression of invertase as a reporter for glucose repression/derepression. (A) Cells were grown in 8% glucose medium to exponential phase (approximately OD_{600} = 0.8), half of the cultures was shifted to 0.2% glucose, samples were taken after 120 min, and RNA levels of *SUC2* were monitored by RT-PCR. (B) Cells were shifted from 8% glucose to 0.2% glucose and invertase activity was measured before the shift and 3h after the shift. Data are averages of results from three independent experiments.

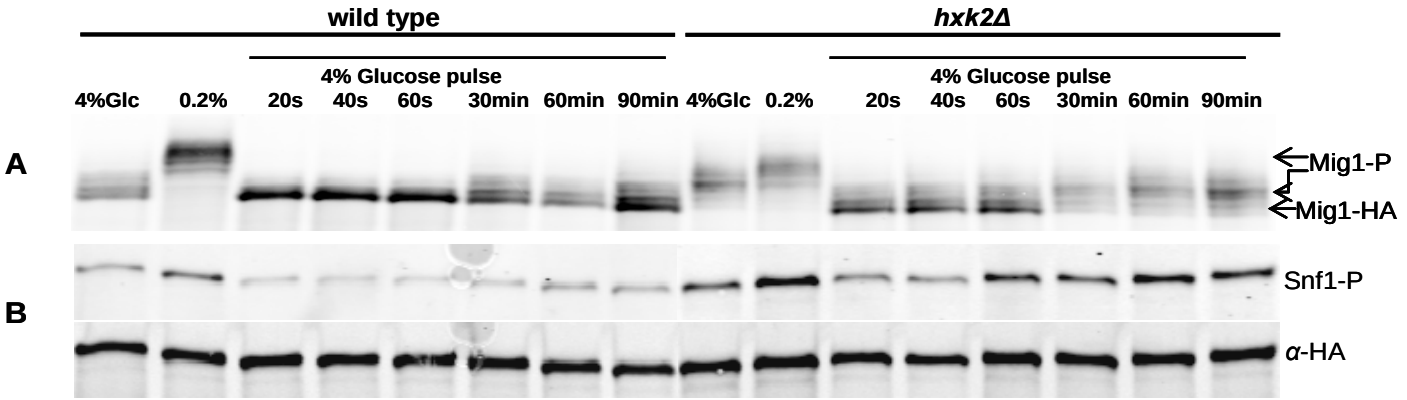

Fig. 4: The phosphorylation of Snf1 and Mig1 is affected in the *hxk2Δ* mutant. (A) Cells transformed with a plasmid expressing p*MIG1-HA* were grown on 4% glucose medium to OD_{600} = 0.8 and shifted to 0.2% glucose medium, samples were taken at the time points indicated, and the Mig1 pattern was monitored by Western blot analysis using an anti-HA antibody. (B) Cells transformed with a plasmid expressing p*SNF1-HA* were grown on 4% glucose medium to OD_{600} = 0.8 and shifted to 0.2% glucose medium, samples were taken at the time points indicated, and the phosphorylation of Snf1 was monitored by Western blotting using an anti-Snf1 phosphorylation antibody and an anti-HA antibody as a loading control.

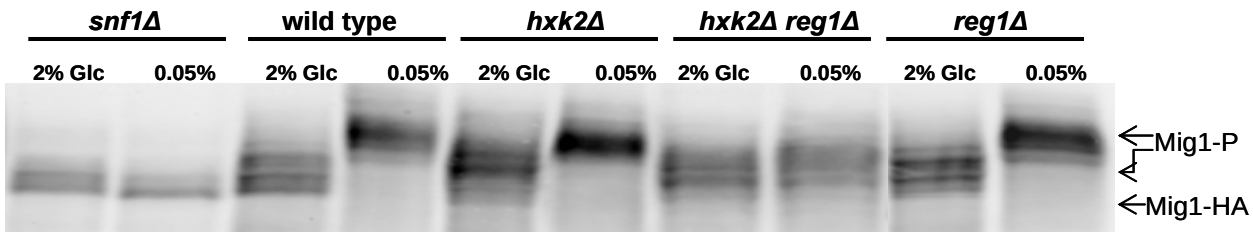

Fig. 5: Mig1 phosphorylation pattern in *hxk2Δ* and *reg1Δ* mutants. Cells transformed with a plasmid expressing p*MIG1-HA* were grown on 2% glucose medium to OD_{600} to 0.8 and were shifted to 0.2% glucose medium, samples were taken after 120 min and the phosphorylation of Mig1 was monitored by Western blot analysis using an anti-HA antibody.

Paper 4

Studies on activation/deactivation of SNF1 pathway, a systems biology approach

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Analysis of Snf1 activation and deactivation, a systems biology approach

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Running title: Studies on Snf1 activation/deactivation.

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Keywords: AMPK, Snf1, Glucose repression, Glucose signalling, Systems biology.

Abstract

The mammalian energy regulator AMP-activated kinase (AMPK) is a promising target for the design of drugs against diabetes. Its baker's yeast homologue Snf1 is required for growth on poor carbon sources. Activity of Snf1 is dependent on the phosphorylation state of a single threonine (T210). In an effort to extend our knowledge on the regulation of AMPK we have undertaken time course studies on the activation/deactivation state of Snf1. A sustained Snf1 phosphorylation (Snf1-P) is observed during growth on respiratory carbon sources or upon starvation. Snf1 is rapidly dephosphorylated upon glucose pulse (less than 1 minute after glucose pulse). For the first time, we show a detailed correlation between Snf1-P and Snf1 activity in a time-course fashion. We also identify a group of genes that can act as reporters of Snf1 phosphorylation (i.e. *ADH2*, *CAT8* and *FBP1*). Finally, protein components of the SNF1 pathway are quantified with a new technique that combines antibodies and RT-qPCR. Taken together these data and assuming that most of Snf1 regulation depends on changes on the inhibitor phosphatase, we present a model that simulates Snf1-P profile to a great extent.

1. Introduction

AMP-activated protein kinase (AMPK) is the central component of a signalling cascade that plays a key role in maintaining the energy balance within the cell and organisms (Carling, 2004). AMPK is highly conserved among eukaryotes. In mammals, AMPK regulates glucose and lipid metabolism in response to changes in energy homeostasis in cells, organs and whole body. AMPK is activated by hormones e.g. leptine and adiponectin and by stress that causes energy depletion, i.e. a rise in the AMP:ATP ratio within the cell. Once activated, AMPK mediate responses aimed at adjusting the ATP-consuming (anabolic) and ATP-generating (catabolic) pathways. AMPK activity can be modulated by drugs employed to treat metabolic disorders, such as obesity or

type II diabetes, suggesting a strong link between the development of these ailments and energy homeostasis (for reviews see (Carling, 2004; Hardie et al, 1998)).

The yeast AMPK orthologue, Snf1, is required for the adaptation to glucose limitation and growth on alternative carbon sources different from glucose or fructose (Carlson, 1999; Gancedo, 1998), nitrogen limitation (Orlova et al, 2006) and in response to certain environmental stresses (Hong & Carlson, 2007; Ye, 2007). Snf1 activity is essential for growth in the absence of glucose or fructose because it prevents the repression that these sugars cause on genes implicated in the metabolism of other carbon sources. When yeast cells are grown under conditions of limited availability of rapidly fermentable carbon sources. Snf1 is activated by a mechanism that requires phosphorylation in the protein kinase activation loop. Activation of Snf1 leads to release from glucose repression. Unlike AMPK, Snf1 does not seem to be activated *in vitro* by an increase in AMP concentration (Adams et al, 2004; Mitchelhill et al, 1994).

AMPK/Snf1 are heterotrimeric complexes. The yeast Snf1 complex consists of the catalytic alpha subunit (Snf1), an activating gamma subunit (Snf4) and one of three alternative scaffolding beta subunit (Gal83, Sip1 or Sip2) (Carlson, 1998). In the presence of glucose the SNF1 complex exists predominantly in an inactive auto-inhibited conformation, in which the regulatory domain of Snf1 masks its catalytic domain (Jiang & Carlson, 1996). Under glucose limitation conditions, the interaction of Snf4 with the regulatory domain of Snf1 leads to the release of the catalytic domain than then becomes available for phosphorylation of protein targets (Jiang & Carlson, 1996). The mechanism that controls Snf4 is not understood in yeast but is associated with AMP binding in mammalian AMPK. The beta subunit is thought to contribute to the stability of the complex and to control subcellular localisation (Vincent et al, 2001).

Activation of Snf1, in addition, requires phosphorylation of threonine 210 (T210) in the activation loop by any of three distinct upstream kinases: Sak1, Elm1 or Tos3. No evidence for a mechanism controlling the activity of these kinases has been found so far. It appears that Sak1 is the primary activator of Snf1 *in vivo.* Deletion of all three genes, *SAK1*, *TOS3* and *ELM1* results in a *snf1Δ* phenotype. Phosphorylated T210 is dephosphorylated by protein phosphatase 1 with its catalytic domain Glc7 and the regulatory/targeting domains Reg1/Reg2 (Ludin et al, 1998). Deletion of *REG1* results in constitutive Snf1 activity and hence a glucose-derepressed state even in the presence of glucose. As a result, the phosphorylation state of Snf1 is mediated by a balance between the activity of the upstream kinases and the Glc7-Reg1/2 phosphatase. The mechanisms controlling that balance and the primary signal for activation/deactivation of Snf1 remains a mystery even after 30 years of research. It is known, however, that the hexokinases Hxk1 and especially Hxk2 play a role in controlling glucose repression. Deletion of *HXK2* also leads to constitutive glucose derepression.

Snf1 controls the expression of genes encoding function required for respiration, gluconeogenesis and catabolism of carbon sources different from glucose and fructose by modifying the activity of the transcriptional activators Cat8 (Charbon et al, 2004) and Sip4 (Rahner et al, 1999) and the repressor Mig1

(Östling & Ronne, 1998)). Active Snf1 phosphorylates at least four serine residues in Mig1 (Treitel et al, 1998). Phosphorylation mediates translocation of the protein from the nucleus to the cytoplasm and relieve of glucose repression (DeVit & Johnston, 1999). This process is reversible and addition of glucose leads to Mig1 dephosphorylation, presumably mediated by Glc7/Reg1 (Alms et al, 1999) and accumulation of Mig1 in the nucleus. Recently, Hxk2 has been reported to physically interact with Mig1 and control Snf1 access to Mig1 (Ahuatzi et al, 2007). A multitude of genes in different pathways are repressed by glucose at the level of transcription (Gancedo, 1998). Classically, invertase activity has been used as a reporter of glucose repression since expression of the encoding gene, *SUC2*, is exclusively controlled by Snf1 activity (Carlson et al, 1984).

The quantitative understanding of the dynamics of complex regulatory network requires the integration of network structure and time-course data. The systems biology approach, i.e. the integration of quantitative experimentation with mathematical modelling and computer simulation, allows us to explore the regulatory properties of a pathway and elucidate systems properties such as feedback and feed forward loops, robustness against perturbation and more. Ideally, mathematical descriptions of biological system should result in models of predictive value (Klipp & Liebermeister, 2006). Mathematical models developed in baker's yeast, such as the G protein activation (Hao et al, 2003; Yi et al, 2003), the pheromone pathway (Kofahl & Klipp, 2004), the response to osmotic stress (Klipp et al, 2005) and the cell cycle (Chen et al, 2000) have illustrated the power of the approach for elucidating regulatory properties. The AMPK/Snf1 system has so far not been studied in this manner.

Here we present quantitative dynamic datasets for activation and deactivation of the Snf1 pathway, including measurements of Snf1 phosphorylation and mRNA levels of relevant reporter genes. These data will be used to generate a mathematical description of the Snf1 pathway, a first version of which is presented in this manuscript.

2. Materials and methods

2.1. Strains

Cultivations were performed with *Saccharomyces cerevisiae* W303-1A *snf1*Δ uracile auxotrophic strains, made prototrophic by transformation with either pRS316-*SNF1*-HA_3 or pRS316-*SNF1T210A*-HA_3 (McCartney & Schmidt, 2001). Quantification of proteins within the Snf1 pathway was performed in BY4741 strains with integrated TAP-tag markers (Ghaemmaghami et al, 2003).

2.2. Growth conditions

Cells were pre-cultured overnight in defined minimal medium (CBS) with 2% glucose as the sole carbon source. Subsequently, the pre-culture was used to inoculate a fermentor with 2 L of two times CBS medium containing 4% glucose (Verduyn et al, 1992). An aerobic culture with a working volume of 2 L was prepared in a Braun Biostat A fermentor (Braun, Melsungen, Germany). An anti-foaming agent (Polypropylene-2000 Fluka, Steinheim, Switzerland) was added to a final concentration of 0.1 mL/L. The temperature was kept constant at 30°C, at a stirring rate of 1000 rpm. The pH was kept at 5.0 by the automatic addition of 1 M NaOH. The airflow (0.5L/min) was regulated by a mass flow controller from Bronkhorstm, High-Tech, BV (Ruurlo, The Netherlands). Carbon

dioxide release was followed on-line (one measurement per minute) using a photoacoustic gas analyser (type 1308; Brüel and Kjær, Nærum, Denmark).

2.3. Determination of extracellular substrate and products

Duplicate samples of 1 mL were sterile-filtered (0.22 µm Ø), frozen in liquid N_2 and stored at -20°C. Glucose and ethanol were determined using enzymatic combination kits (Boehringer-Mannheim GmbH, Mannheim, Germany).

2.4. Protein extracts and western blot analysis

For phosphorylation of Snf1, a duplicate sample amounting to 25 optical density units was drawn from the fermentor, transferred to a falcon tube containing NaOH to a final concentration of 0.1 M and harvested immediately by centrifugation. The pellet was resuspended in 2 ml of 2 M NaOH containing 7% *β*-mercaptoethanol, then 2 ml of 50% TCA (trichloroacetic acid) were immediately pipetted into the mix. The resulting solution was mixed by inversion and spun down at room temperature. To neutralise the acid the pellet was resuspended in 1.5 ml of a 1:1 mixture of 1.5 M Tris (pH 7.5) and acetone and centrifuged again. The resulting pellet was resuspended in 250 µl of 1x SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 3% sodium dodecyl sulfate [SDS], 10% glycerol, 5% *β*-mercaptoethanol, 0.001% bromophenol blue) and incubated for 5 min at 100°C, then centrifuged and the supernatant stored at -20°C. Samples amounting to 400µg of total protein were separated by SDS-PAGE using 7.5% polyacrylamide gels and electroblotted to nitrocellulose membranes. The levels of total and phosphorylated Snf1 were quantified by western blot analysis using an infrared detection method (Odyssey infrared imaging system; LI-COR Biosciences). Briefly, blots were blocked overnight with solution recommended by manufacturer and incubated simultaneously with primary antibodies directed against Snf1-HA (1:2000) (Santa Cruz Biotechnology) and against the phosphorylated form of Snf1 (1:1000) (tailored antibody produced by Open Biosystems, more information (McCartney & Schmidt, 2001)). Primary antibodies were detected simultaneously with goat anti-mouse IRDye-800 (ref. number: 926-32210, LI-COR Biosciences) and anti-rabbit IRDye-680 (ref. number: 926-32221, LI-COR Biosciences) used in a 1:16000 dilution. Blots were imaged with the IR imager (Odyssey, LI-COR) in a single scan in both 700 and 800 nm channels.

Quantification of samples was performed with specific build-in software following manufacturer recommendations (Odyssey, LI-COR). The area of all boxes used in the analysis was fixed to 20.68 and included a band corresponding to both Snf1-P and Snf1-HA. Background was determined from three bordering areas at the top and bottom of every box. The analysis of the bands produced values of intensity in arbitrary units. The level of Snf1-P for every sample was calculated as the ratio of intensity between Snf1-P and Snf1-HA (Snf1-P:Snf1-HA). These values were normalized to the maximum phosphorylation intensity observed in the western blot.

2.5. Quantification of proteins.

Snf1 protein levels were measured by immuno-qPCR assay as previously reported (Lind & Norbeck, 2007). 8-well microtube strips (Robostrip, AJ Roboscreen GmbH) were incubated with 25 µL of anti-protein A antibody (1 µg/mL in carbonate buffer, pH 9.6) overnight at 4ºC. Wells were rinsed three times with washing buffer (5mM Tris-HCl, pH 7.75, 0.154

M NaCl and 0.005% Tween 20) and incubated for 1 h with 25 µL of blocking solution (phosphate buffered saline containing 5% powder milk, 0.05% Tween 20 and 4µg/mL IgY) at 37ºC. Blocking solution was removed by washing three times with washing buffer and 25 uL of protein sample diluted to 0.08 ng/µL in blocking solution were added and incubated for 1 h at room temperature. After washing six times with washing buffer the wells were incubated for 1 h with detection antibody/DNA conjugated (10000 times diluted in blocking solution) at room temperature. The wells were washed six times with washing buffer and ten times with MilliQ water and then 25 µL of BioRad SYBR Green Supermix with a final primer concentration of 0.3 µM were added. Real-time PCR was run in a BioRad iQ5 real-time PCR instrument by using the next cycling conditions: 3 min at 95ºC followed by 40 cycles of 20 s at 95ºC, 20 s at 60ºC and 20 s at 72ºC. After the run a melting curve was performed.

2.6. Snf1 kinase activity assay.

Snf1 kinase activity was used as previously described (Hedbacker et al, 2004). Cells were harvested by filtering in 45 nm pore nitrocellulose filters and broken by vortexing with glass beads in buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 % glycerol). Crude extracts were centrifuged and Snf1 was partially purified from the supernatant by DEAE-Sepharose. Elution of protein was achieved by buffer A containing 0.2 M NaCl. Protein kinase activity was determined by measuring the phosphorylation of the SAMS peptide (HMRSAMSGLHLVKRR) in the presence of [γ^{32}-P]ATP in reaction buffer (50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1mM EDTA, 0.2 mM ATP, 10 % glycerol). Kinase activity was expressed as nmol of phosphate incorporated into SAMS peptide per min and mg protein.

2.7. Determination of invertase activity.

Cells were harvested by centrifugation. Protein extracts and measurements of invertase activity were performed as described previously (Goldstein & Lampen, 1975; Hohmann & Zimmermann, 1986). The protein concentration was determined by using the DC protein assay kit (Bio-Rad).

2.8. RNA extraction and RT-PCR quantification of gene expression.

For RNA extraction samples were collected into ice-cold water. Cells were centrifuged and pellets stored -20^{o}C. RNA was extracted as previously described (De Winde et al, 1996), treated with DNAse following instructions from manufacturer (DNAse kit Qiagen). cDNA was synthesised from 40 pg·uL^{-1} of RNA using 45 ng·uL^{-1} of anchored oligo-dT primer (ABgene), 0.5 mM of dNTPs and 1 unit of SuperScript^{TM} II transcriptase (Invitrogen). The Real-Time Quantitative PCR reaction was performed by using specific primers (100 pmol uL^{-1}) for each gene and iQ SYBR Green supermix I PCR (BioRad), in an iQ5 real-time PCR detection system (BioRad). For each gene, a standard curve was constructed with 10-fold serial dilutions of yeast genomic DNA. The starting quantity of the studied gene was calculated from the standard curve by interpolation, and normalised against the quotient between the levels of the *ACT1* and *IPP1* mRNAs, as well as the maximum expression value for each gene. All samples were analysed in duplicates and the averaged expression values calculated by the analysis software (iQ5 2.0 BioRad).

2.9. Modelling.

Experimental data for extracellular glucose, cell density and Snf1-P, provided the basis for the development of a mathematical model. The structure and interpretation of the model followed the approaches by (Huang & Ferrell, 1996) and (Kholodenko, 2000) Our model describes the consumption of external glucose and the growth of the cells (measured as optical density, OD) during the linear initial phase. We use the glucose consumption rate as an approximation to the glycolytic rate:

$$\text{Glucose consumptionrate} \approx \text{Glycolytic rate} \approx \frac{V_{max}\,\text{Glcext}}{K_M + \text{Glcext}}.$$

The link from the external glucose to Snf1 is established via the glycolytic rate. Since it depends on the amount of glucose consumed and it has a regulatory effect on Snf1. Thus, the success of the model highly depends on the Km and Vmax value of the glucose consumption rate. The glycolytic rate is used as a quantitative measure for the unknown signal that regulates Snf1 phosphorylation state. It is also unknown whether this signal regulates Snf1 via the upstream kinases (UKs), the phosphatase (PP1) or both (see Fig. 1). In our model, the regulation of Snf1-P is assumed to depend exclusively on PP1. The Michaelis-Menten formalism was used to describe the rate of glycolysis, because Km and Vmax parameters were available from experiments and the substrate (external glucose) concentration is higher than the enzyme (hexotransporter) concentration. In addition, the Michaelis-Menten formalism has the advantage that only two parameters have to be estimated (instead of three when detailed enzyme kinetics were applied). The total concentrations of Snf1, UK, and PP1 were assumed to be constant.

The three conservation equations are

Snf1_total = Snf1 - Snf1PPP1 - Snf1UK - Snf1P,

PP1_tot = PP1 - Snf1PPP1, and

UK_total = UK - Snf1UK.

The model contains five differential equations:

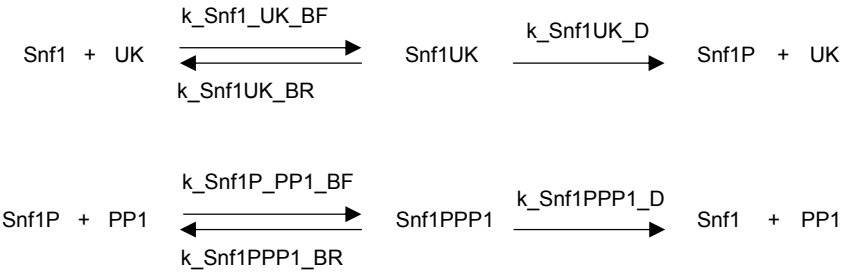
$$\frac{d}{dt}\text{OD} = \text{k\_OD}$$

$$\frac{d}{dt}\text{Glcext} = -\frac{V_{max}\,\text{Glcext}}{K_M + \text{Glcext}} \cdot \text{OD}$$

$$\frac{d}{dt}\text{Snf1P} = \text{k\_Snf1UK\_D} \cdot \text{Snf1UK} - \text{k\_Snf1P\_PP1\_BF} \cdot \text{GlycRate} \cdot \text{Snf1P} \cdot \text{PP1} + \text{k\_Snf1PPP1\_BR} \cdot \text{Snf1PPP1}$$

$$\frac{d}{dt}\text{Snf1UK} = \text{k\_Snf1\_UK\_BF} \cdot \text{Snf1} \cdot \text{UK} - (\text{k\_Snf1UK\_BR} + \text{k\_Snf1UK\_D}) \cdot \text{Snf1UK}$$

$$\frac{d}{dt}\text{Snf1PPP1} = \text{k\_Snf1P\_PP1\_BF} \cdot \text{GlycRate} \cdot \text{Snf1P} \cdot \text{PP1} - (\text{k\_Snf1PPP1\_BR} + \text{k\_Snf1PPP1\_D}) \cdot \text{Snf1PPP1}$$



In order to demonstrate regulation of Snf1 via PP1, we multiplied the glycolytic rate (denoted as GlycRate) as a regulatory factor to the rate constant k_Snf1P_PP1_BF. This means that with an increase of external glucose, there is a higher glycolytic rate, which leads to a faster formation of Snf1-P and PP1 into a complex. Thus, the dephosphorylation of Snf1-P is accelerated by an increase in glucose consumption.

We estimated Km and Vmax using the following equations:

The glycolytic rate can be calculated from the experimental data using the formula $\mathrm{GlycRate}_{\mathrm{Ref}} = \frac{\mathrm{Glcext}(\Delta t + t) - \mathrm{Glcext}(t)}{\overline{\mathrm{OD}} \cdot \Delta t}$, where $\overline{\mathrm{OD}}$ denotes the average OD in the time interval [t, t+ Δt]. The internal glucose concentration (Glcint) is assumed to be constant despite external changes. This is achieved by changes on the rate of glycolysis: large amounts of external glucose results in a fast glycolytic rate, while small amounts lead to a slower conversion of internal glucose into glucose-6-P. To that end, the Michaelis-Menten provides a description of the glycolytic rate:

$$\frac{\mathrm{d}}{\mathrm{dt}}\mathrm{Glcint} = \frac{\hat{\mathrm{V}}_{\max}\,\mathrm{Glcext}}{\mathrm{K_M} + \mathrm{Glcext}} - \mathrm{GlycRate} \cdot \mathrm{Glcint}$$

$$\frac{\hat{\mathrm{V}}_{\max}\,\mathrm{Glcext}}{\mathrm{K_M} + \mathrm{Glcext}} - \mathrm{GlycRate} \cdot \mathrm{Glcint} = 0$$

$$\Leftrightarrow \frac{\mathrm{V}_{\max}\,\mathrm{Glcext}}{\mathrm{K_M} + \mathrm{Glcext}} = \mathrm{GlycRate}, \quad \text{with } \mathrm{V}_{\max} = \frac{\hat{\mathrm{V}}_{\max}}{\mathrm{Glcint}}$$

From this knowledge we estimate Vmax and Km as follows. We define a cost function, $\mathrm{cost} = \sum \min_{Km,V\,\max} \left(\mathrm{GlycRate}_{\mathrm{Ref}} - \mathrm{GlycRate}\right)^2$ which calculates the difference between the two rate equations according to the least square method using a simplex algorithm. The parameter estimation is thus turned into a mathematical optimization problem, seeking the minimisation of the cost function, for which we here have used a genetic algorithm.

The procedure estimating the parameters for OD, Snf1, UK and PP1 was done using the SBTOOLBOX2 (www.sbtoolbox2.org) with the simplex-algorithm and the pswarm-algorithm developed by (Vaz & Vicente, 2007). Although an evaluation of the number of total concentrations of the main components is available, estimations showed that there are still too many unknown parameters that caused correlations and thus result in the non-identifiability of the parameter set. In subsequent estimation runs, we reduced the number of parameters by excluding those which were correlated (keeping them fixed). This result can therefore only be interpreted under consideration of the fixed parameters and still the model is not identifiable. However, by cancelling out correlated parameters,

we found that most parameters could be reduced to smaller intervals. For example, repeating estimations for fixed values of total Snf1P of 23000, total UK of 15300 and fixed values for k_Snf1P_PP1_BF, k_Snf1PPP1_BR, and k_Snf1UK_D, resulted in total PP1 concentrations between 23000 and 23500.

3. Results

The yeast AMPK pathway is interconnected to other signalling networks. In the first step of this study, literature was reviewed to build a descriptive model using CellDesigner™ (Funahashi et al, 2003). The resulting network was not amenable to modelling studies, given the large number of unknown parameters; hence it was reduced to the most important events leading to SNF1 complex activation and deactivation. These reactions are depicted in Fig. 1. The main assumptions in this depiction are (a) that an yet unknown signal derived from glucose controls the Glc7/Reg1 phosphatase and (b) that the upstream kinases, lumped together as one kinase, are not regulated by constitutively active. This is presently the most likely scenario given experimental evidence reported in (Rubenstein et al, 2008).

3.1. Snf1 phosphorylation persists in the absence of glucose.

Activation of the Snf1 pathway was studied during controlled batch cultivations (fermentor) with an initial glucose concentration of 4%. With the aid of on-line measurements of CO_2 to define the metabolic activity of the cells, samples were collected during the glucose consumption phase, the diauxic shift, the ethanol consumption phase and stationary phase (Fig. 2A). The activation of Snf1 was monitored via the phosphorylation state of the Snf1T210 residue (Fig. 2B). So far, Snf1 activation has only been studied by shifting cells from high to low glucose medium or vice versa, while gradual glucose consumption is probably a more natural scenario (Elbing et al, 2006; Hong & Carlson, 2007; McCartney & Schmidt, 2001). Snf1-P was quantified and

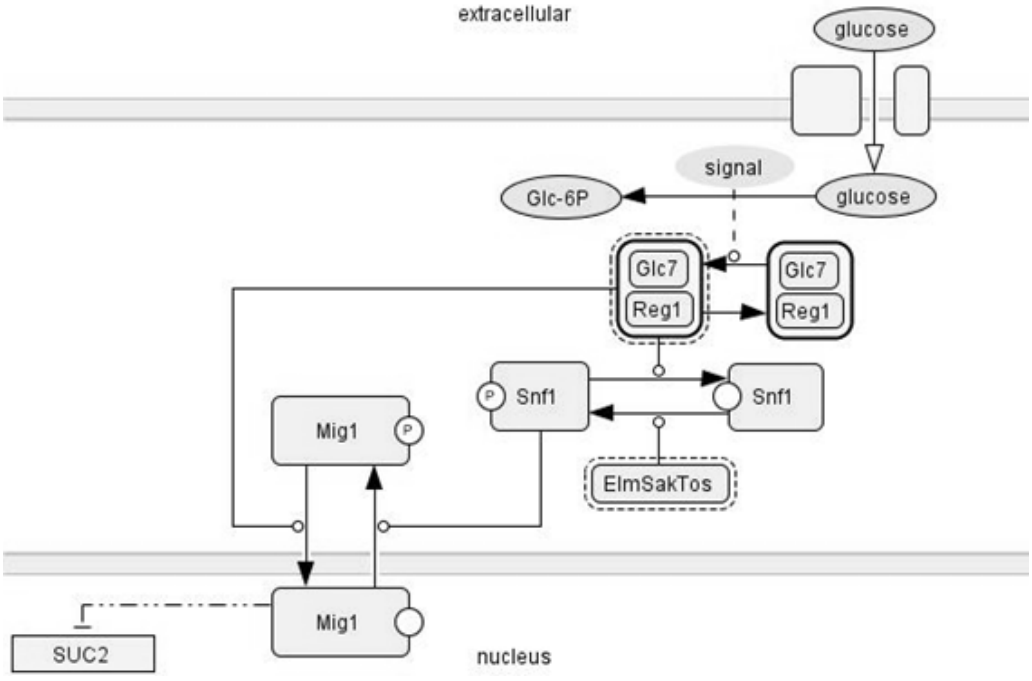


Figure 1. Schematic representation of SNF1 signalling pathway.

compared to the glucose concentration (Fig. 2C). The levels of Snf1-P increased rapidly when the external glucose concentration dropped below 0.5%. The Snf1-P level remained high during diauxic shift, growth on ethanol and carbon starvation. The response to glucose depletion was further monitored by measuring *SUC2* mRNA levels and the specific

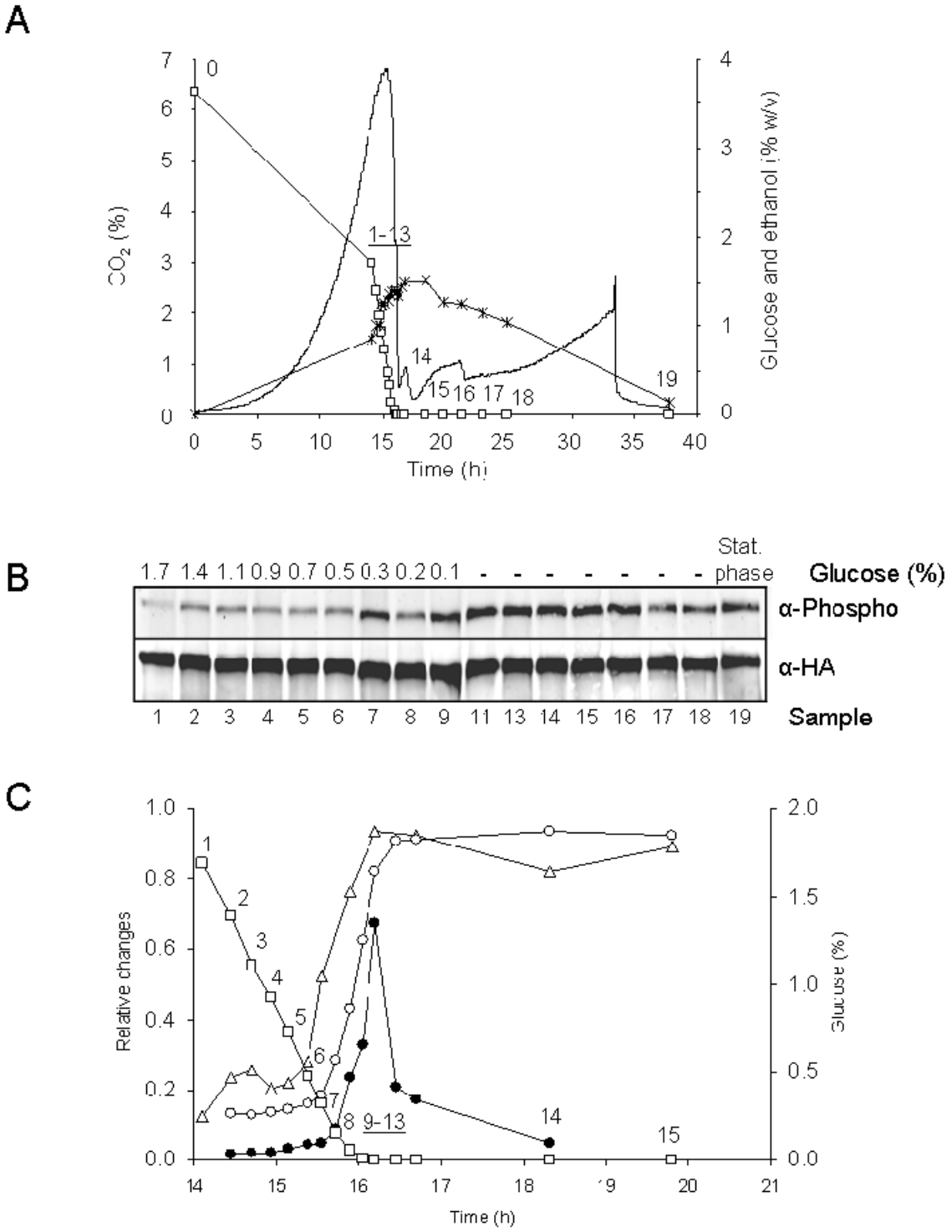


Figure 2. Time course activation of Snf1. Profile of a representative cultivation with an initial glucose concentration of 3.6%. (A) Relevant parameters: CO2 (plain line), glucose (□) and ethanol (stars) plotted respect to time. (B) Detection of Snf1-P and total Snf1 with specific antibodies. (C) Relative values of glucose (□), Snf1-P (Δ), invertase activity (○) and *SUC2* expression (●) respect to fermentation time.

activity of its gene product, invertase. The *SUC2* mRNA level rapidly increased at glucose concentrations below 0.2% (Fig. 2C). When glucose was completely exhausted *SUC2* mRNA dropped to a low expression level consistent with the observation that *SUC2* expression is stimulated by low glucose levels (Özcan et al, 1997). The specific activity of invertase correlated well with *SUC2* mRNA levels. Invertase activity remained high even after *SUC2* had been down-regulated in the absence of glucose, which is due to the high stability of the enzyme (Gancedo, 1998).

3.2. Glucose addition triggers immediate deactivation of Snf1.

To study deactivation/reactivation of the pathway in a time-course fashion we employed the same set-up described above. 2h after glucose exhaustion glucose was added again to a final concentration of 4%. Less than 1min after the pulse, CO_2 levels rose sharply (data not shown) indicating that glucose was being metabolised. At the same time, i.e. within less than 1 min, Snf1 became dephosphorylated (Fig. 3A). Interestingly, Snf1-P levels started to increase again about 1h after glucose addition, even though at this stage the glucose concentration was still around 3%.

Specific invertase activity decreased progressively over time following glucose addition, which can be attributed to dilution by growth. Invertase is an extracellular enzyme and therefore not regulated at activity level.

3.3. Transcriptional responses dependent on Snf1 activation / deactivation

Having characterised Snf1-P levels with respect

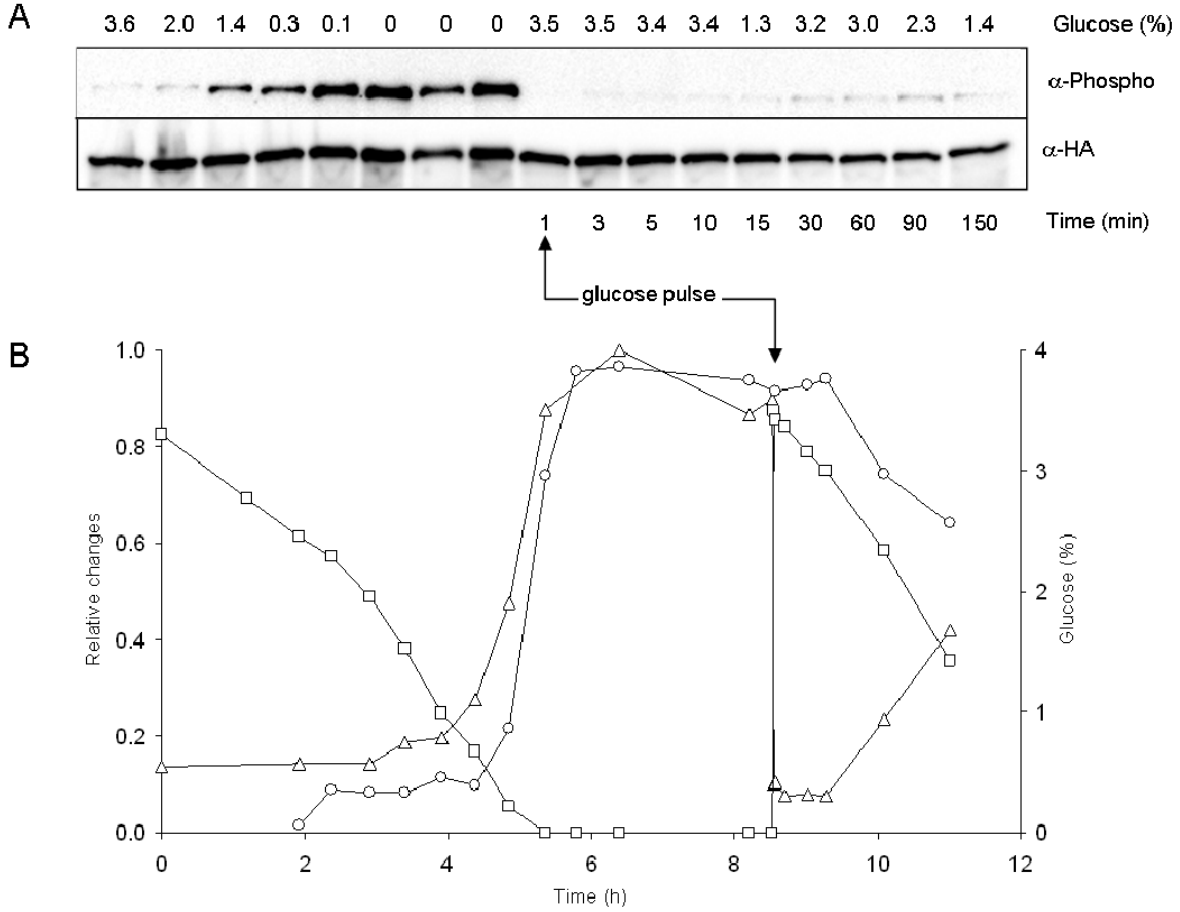


Figure 3. Time course activation and deactivation of Snf1. Profile of a representative. Approximately 2h after glucose depletion glucose was pulsed into the fermentor to a final concentration of 3.6%. (A) Detection of Snf1-P and total Snf1 with specific antibodies. (B) Summary of responses to glucose depletion: Snf1-P (Δ), invertase activity (○) and glucose concentration (□).

to glucose concentration we tried to identify a reporter gene, whose mRNA level correlated well with Snf1 activity. We tested genes whose products are implicated in alternative sugar utilisation (*SUC2*), gluconeogenesis (*FBP1* and *ADH2*) and transcriptional activation (*CAT8*) (Gancedo, 1998) using the same experimental set-up than in the previous section. Two genes, whose expression is known to be up-regulated by glucose were also included as controls, *HXT1* and *PDC1*. At low glucose concentrations (0.2%), *SUC2* and *CAT8* mRNA levels became up-regulated, while *FBP1* and *ADH2* mRNA levels only increased after glucose exhaustion (Fig. 4A and 4B) with somewhat different kinetics. At that time point, *SUC2* mRNA levels dropped (see also Fig. 2C). During growth on ethanol the mRNA levels of *CAT8* and *ADH2* continued to increase and *FBP1* mRNA levels remained high. Upon glucose addition, *CAT8*, *FBP1* and *SUC2* mRNA levels first dropped very rapidly to below 10% of the initial level and then dropped slowly to below detection. The level of *SUC2* mRNA, already low in the absence of glucose, also decreased slowly after glucose addition. In summary, *SUC2* mRNA levels correlates with those of Snf1-P at low glucose concentrations, while the *FBP1* mRNA levels follow the kinetics of Snf1-P best.

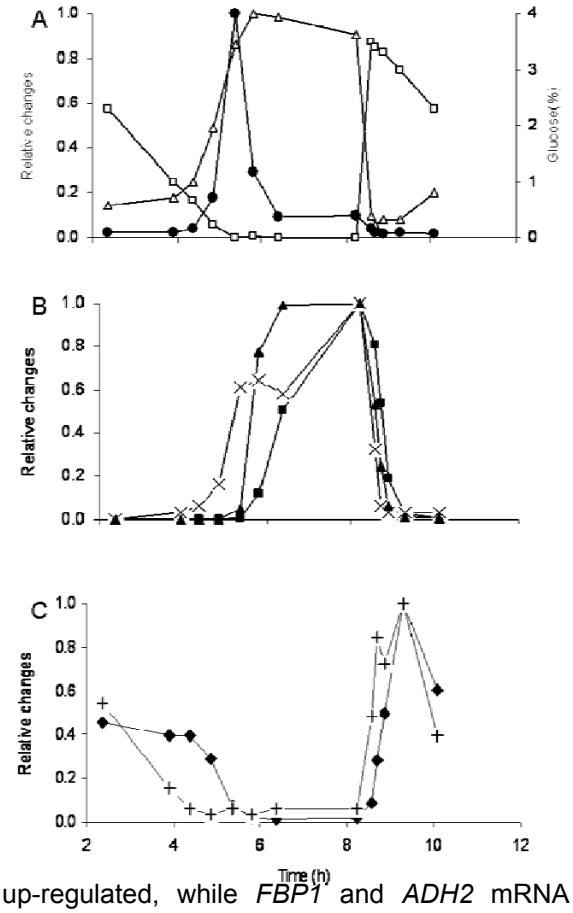


Figure 4. Relative mRNA abundance of glucose-regulated genes. (A) *SUC2* expression (●) respect to glucose concentration (□) and Snf1-P (Δ). (B) Glucose repressed genes and (C) Glucose induced genes: ADH2 (■), CAT8 (X), FBP1 (▲), PDC1 (♦) and HXT1 (+). Gene expression was normalised against the quotient between the levels of the *ACT1* and *IPP1* mRNAs, as well as the maximum expression value for each gene. The profile of Snf1-P was determined by western blot.

The expression pattern of *PDC1*, encoding pyruvate decarboxylase, and *HXT1*, encoding the low-affinity glucose transporter, followed the concentration of external glucose (Fig. 4C). Their mRNA levels decreased during the glucose consumption phase, to recover only after the glucose pulse. The *HXT1* profile followed more closely the glucose levels than *PDC1*.

In order to investigate to what extent the transcriptional profiles of glucose-regulated genes depends on Snf1 activity, we performed an analysis using a mutant expressing an unphosphorylatable form of Snf1 (T210A). The CO_2 profile was very similar to that of the wild type strain during the glucose consumption phase, but the strain was metabolically inactive upon sugar depletion (data not shown). Expression of *CAT8, ADH2* and *FBP1* was not up-regulated in the absence of glucose in this mutant (Fig. 5) This observation was consistent with the low levels of invertase activity in the Snf1 (T210A) strain (data not shown, (Estruch et al, 1992)). These data confirm previous

results showing a Snf1-dependent expression of these genes (Gancedo, 1998). The expression profile of *HXT1* and *PDC1* was similar in both mutant and wild type strains; hence they do not seem to be under the control of Snf1. However, *HXT1* expression in Snf1T210A strain was higher than in the wild type. This could be in accordance with the recently discovered role that Snf1 plays in the degradation of Mth1 and Std1, two negative *HXT1* regulators (Pasula et al, 2007).

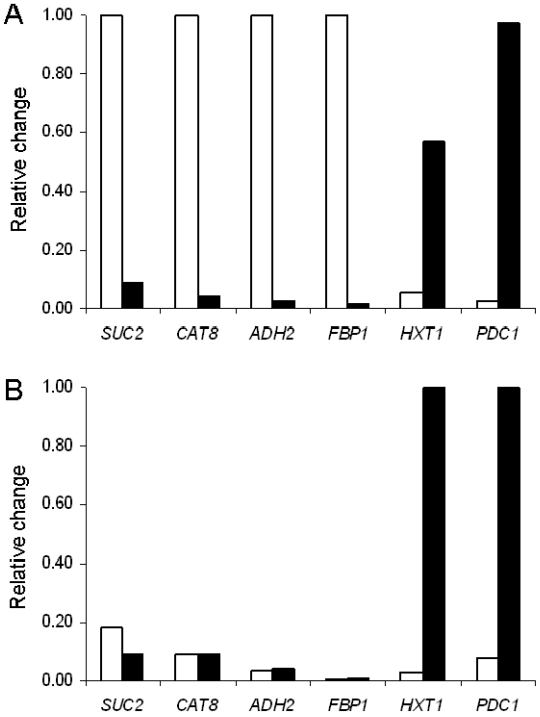


Figure 5. Snf1-dependent expression of glucose regulated genes. Changes in mRNA levels of a strain bearing an unphosphorylatable Snf1 (Snf1-T210A) and wild type were analysed in two conditions: glucose depletion and after a pulse with 4% glucose. The expression was normalised against the quotient between the levels of *ACT1* and *IPP1* mRNAs, and to the maximum expression value for each gene. Standard error was below 10% for all measurements.

3.4. Correlation between Snf1 kinase activity and Snf1-P.

It is generally assumed that the level of Snf1-P correlates with Snf1 activity (Woods et al, 1994) but this has never been thoroughly documented. Moreover, since Snf1 activity is assumed to be regulated not only by phosphorylation but also by Snf4 (and perhaps effectors controlling Snf4) it is possible that this correlation does not always hold. We studied Snf1-P and Snf1 activity in parallel during a time-course experiment, following glucose addition and subsequent consumption. Both curves are almost superimposible confirming the correlation between Snf1-P and activity (Fig. 6).

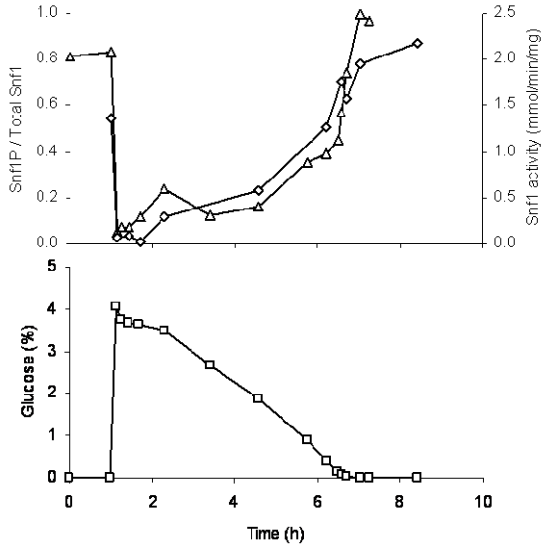


Figure 6. Levels of Snf1 activity and Snf1-P. The absolute activity of Snf1 (Δ) and the relative level of phosphorylation (◊) were quantified. A representative cultivation is shown.

3.5. Monitoring absolute protein levels in the Snf1 pathway

An essential piece of information for constraining model parameters is the quantification of the proteins involved in the pathway, i.e. the determination of absolute protein levels. This was determined on cells cultivated in flasks with or without glucose. The results are summarised in Table 1. We observe that the stoichiometry of the SNF1 complex is roughly 1:1:1 between Snf1, Snf4 and three beta subunits together, both during growth on glucose and ethanol. It is interesting to note that Gal83 levels decrease noticeably during

Protein	*Subunit role*	*Glucose*	*SD*	*Ethanol*	*SD*
Reg1	PP1 targeting	30085	5891	28419	5891
Glc7	PP1 catalytic	45919	2134	35230	2134
Sak1	Upstream kinase	5054	1032	3711	1032
Elm1	Upstream kinase	4407	765	2932	765
Snf1	α-catalytic	28256	4936	22177	4936
Snf4	γ-activating	19919	3045	14052	3045
Gal83	β	14298	1538	9386	1538
Sip1	β	5133	3330	6359	3330
Sip2	β	3819	2386	10270	2386

Table 1. Quantification of proteins involved in the Snf1 pathway. The abundance of SNF1 complex components, regulators and a target were determined by immuno-QPCR.

ethanol growth, while Sip2 increases dramatically, as previously reported (Vincent et al, 2001). We did not quantify Tos3, but assuming a concentration of the same order of magnitude as for Sak1 and Elm1, the concentration of Snf1 activating kinases probably sums up to 15000 molecules per cell. That is, half as much as Reg1, indicating a larger abundance of PP1.

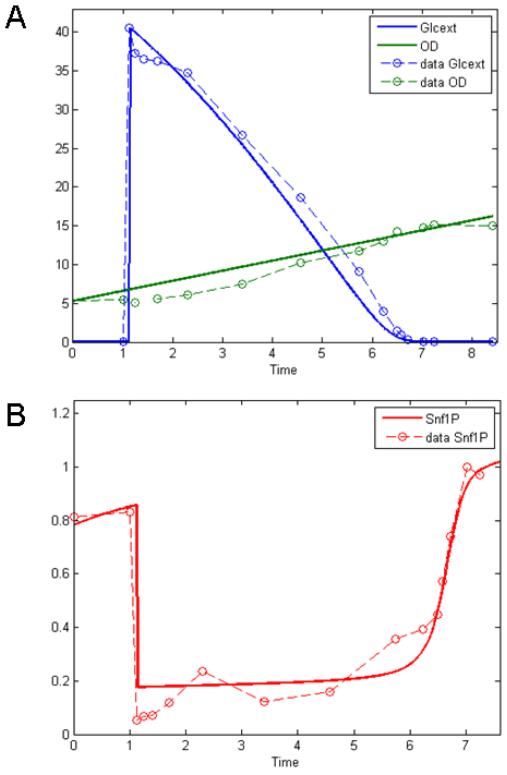


Figure 7. Simulation of a glucose pulse in a representative cultivation. The model simulates A) glucose, optical density (OD) and B) Snf1-P during Snf1 pathway deactivation/activation.

3.6. Simulation of pathway deactivation/activation.

The data sets presented above were used to build an initial dynamic model of the pathway using as input Snf1-P and the external glucose concentration. Therefore, the current model favours a scenario in which glucose consumption correlate with phosphatase activity, which in turn, is the only regulator of Snf1-P. We used as read-out of pathway activity the relative amount of Snf1-P. The present model does not incorporate mRNA expression. The model presented simulates a glucose depletion experiment with a 4% glucose pulse. The simulation reproduces glucose consumption, optical density and Snf1-P (Fig. 7). The present model succeeded in capturing Snf1-P profile.

Discussion

We have initiated a systematic analysis of the quantitative, systems-level properties of the yeast Snf1 pathway. To this end, we have performed time course analyses of Snf1 activation and deactivation as well as of absolute levels of Snf1 pathway components.

We followed Snf1 pathway activation during glucose consumption. Interestingly, Snf1 becomes phosphorylated gradually while

glucose is depleted. There does not seem to be a threshold level of glucose below which Snf1 is activated. We do not know at this stage if the gradual activation is apparent in each cell in the culture or if single cells experience "individual" thresholds for pathway activation. At the level of the cell population, we tested Snf1 phosphorylation increase from about 3.5% glucose until glucose was fully exhausted. This suggests that glucose is experienced over a range of glucose concentrations. This may have to do with the expression of different sets of glucose transporters with different sugar affinities. In any case, it is an unexpected and interesting property that raises further excitement concerning the elusive sensing mechanism of the Snf1 pathway.

We also noted that Snf1 remains phosphorylated and hence activated when glucose is exhausted. A similar observation was made upon activation by salt stress (Hong & Carlson, 2007). This is in contrast to, for instance the HOG osmostress pathway, which becomes transiently activated but then downregulated even though the stress remains present (Klipp et al, 2005). In other words, Snf1 stays activated as long as the stimulus, glucose starvation, remains present. This also means that Snf1 activity is needed to maintain the derepressed state. This can be tested now using a mutant form of Snf1 which can be blocked specifically by ATP analogues to inhibit its activity (Rubenstein et al, 2008). Inhibition should then lead to glucose repression even in the absence of glucose. Furthermore, the sustained Snf1 activation observed means that there are no feedback mechanisms down-regulating Snf1 as long as the stimulus is present. We estimated that at least 80% of Snf1 is active under such conditions (our unpublished data). Probably, not having 100% Snf1 activity does not make a difference, since in strains with reduced Snf1 activity (i.e. in which two of the three UKs have been deleted) cells are able to grow in the absence of fermentable carbon sources (Hong et al, 2003).

At the same time, we note that Snf1 can be dephosphorylated upon glucose addition extremely rapidly. The fastest time point we have been able to measure so far is 20sec after glucose addition. Snf1 and also Mig1 are then fully dephosphorylated. This means that sensing and signalling occur extremely fast and that yeast prepared to the eventual availability of glucose in the medium. It also means that the mechanisms for dephoshorylating Snf1 are in place, suggesting that the state of Snf1 phosphorylation under any condition is that of a dynamic equilibrium between phosphorylation and dephosphorylation. In other words, Snf1 becomes phosphorylated and dephosphorylated under all conditions and the rates of the forward and reverse reactions determine the actual state. It is still not fully clear if the phosphorylation, the dephoshorylation reaction or both, are controlled by glucose, but experiments indicating that the activity of UKs do not depend on glucose availability suggest that it is mainly or exclusively the dephosphorylation, phosphatase step that is controlled in an unknown way (Rubenstein et al, 2008). In any case, the time course data presented here should allow estimated rates of phosphorylation and dephosphorylation, which are important parameters for dynamic modelling.

In parallel to Snf1 phosphorylation we also monitored the mRNA level of several glucose-regulated genes, some of which are known Snf1 targets. In fact, most, if not all, Snf1-

regulated genes are controlled by additional mechanisms such as activation in complete glucose absence (gluconeogenesis), low glucose (invertase), presence of maltose (*MAL* genes) or galactose (*GAL* genes). Indeed, from the genes tested here only *FBP1* showed a mRNA level profile that closely matched Snf1 phosphorylation pattern, albeit with a certain delay following activation. The data are useful to dissect different regulatory mechanisms and they suggest that *FBP1* may be a more suitable Snf1 reporter than the classical *SUC2*, whose expression strongly drops in the complete absence of sugar.

For the first time, we monitored in parallel during glucose consumption Snf1 phosphorylation and Snf1 activity. The two curves turned out to be essentially superimposible. Hence, at least under those conditions, the phosphorylation state of Snf1 is a suitable reporter for the activity of the kinase. This is not incompatible with other regulatory mechanisms that might control the activity of the kinase, such as possible AMP binding to Snf1. It is well known that phosphorylation of T210 is essential for Snf1 kinase activity (Estruch et al, 1992), hence it is comforting that the phosphorylation state correlates well with kinase activity during pathway activation when glucose is consumed.

We also determined the absolute levels, i.e. molecule numbers per cell, of almost all components of the Snf1 pathway. This was achieved using a new method based on antibody binding to a tag and rt-PCR (Lind & Norbeck, 2007). The data are well comparable to our own Western blot analysis but differ in many instances vastly from those reported in a genome-wide analysis (Ghaemmaghami et al, 2003). Most importantly, it now appears that the number of subunits of the SNF1 complex, i.e. alpha:beta:gamma, are roughly equimolar. These data are critical to constrain the parameters for modelling of the dynamic operation the system. In this direction, we introduce a model which incorporates the main components of the network. The glucose consumption rate is assumed to be a good approximation to the glycolytic rate, and thus of the unknown signal which leads to the regulation of Snf1. Though the model is not yet complete, it supports the hypothesis that the regulation of Snf1 depends to a large extend on changes in the phosphatase and not on the UKs.

The time course data reported here together with network analysis (J Nielsen, personal communication) will be suitable to generate quantitative models of the SNF1 pathway. Important parameters and rates can be calculated or estimated from those data. These data will need to be complemented by single cell data using nuclear accumulation of Mig1 as reporter, which are presently generated. We would like to address questions concerning the route of signalling (via kinase or via phosphatase) as well as feedback regulation and robustness against perturbation with the help of computational simulations. Most importantly, simulations based on predictive models should help generating new hypotheses that can be tested by experiments. We believe that this approach has potential to better understand regulation of the yeast and mammalian Snf1/AMPK pathways and allow exploiting them for bioengineering and treatment of diseases.

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