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Glucose and regulation of cell cycle in *S. Cerevisiae*: analysis of mutants impaired in sugar uptake mechanisms

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"..better to save the mystery
than surrender to the secret…"

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Introduction

Saccharomyces cerevisiae has an unusual lifestyle

Glucose, the most abundant monosaccharide in nature is the favored carbon and energy source for most organisms. In addition to being a major nutrient, glucose can act as a "growth hormone" to regulate numerous aspects of cell growth, metabolism, and development. Thus, it is not surprising that many (all?) organisms have evolved sophisticated regulatory mechanisms for monitoring the level of the glucose in their habitat and respond quickly to changes in the sugar availability (Fig.1).

The budding yeast *S. cerevisiae* can effectively metabolize glucose over a broad range of concentration, from a few micromolar to a few molar (as it occurs in grape juice).

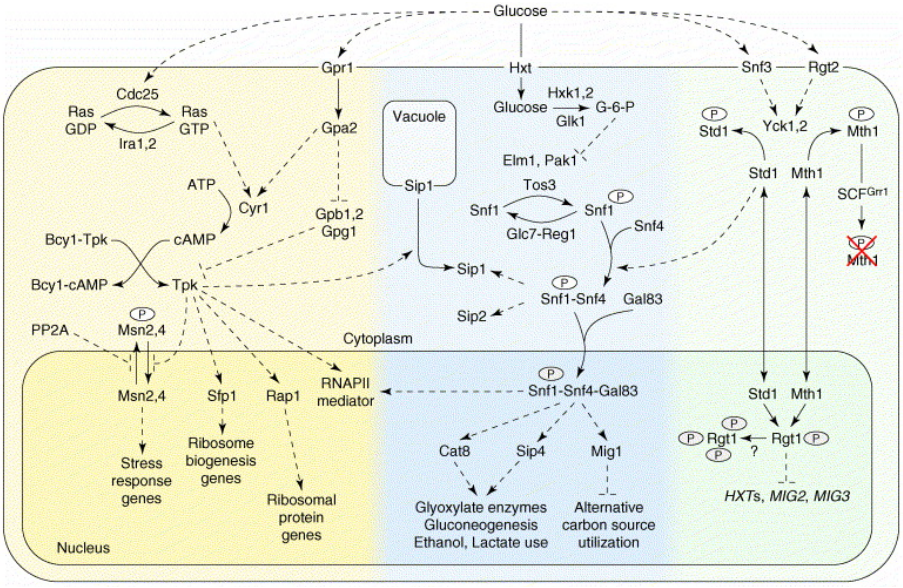


Figure 1. Diagram of yeast signaling pathways responsive to glucose.
cAMP/PKA pathway, yellow shading; Snf1 protein kinase, blue shading; Rgt2/Snf3 pathway, green shading. Solid arrows represent transformations and/or translocations, dotted lines represent regulatory or catalytic influences. (From Schneper et al., 2004)

Unlike most organisms, which under aerobic conditions fully oxidize glucose to water and carbon dioxide via the TCA cycle and resort to fermentation only when oxygen becomes limiting ("Pasteur effect"), *S. cerevisiae* prefers to metabolize glucose through the alcoholic fermentation even in presence of oxygen, despite the low energetic efficiency of this process: such a behavior is known as "Crabtree effect", named after the oncologist who originally described this phenomenon in tumor cells (Johnston & Kim, 2005).

Although, per mole of sugar, alcoholic fermentation yields fewer ATP (only 2 ATPs per molecule of glucose consumed) equivalents than respiration, it can proceed at much faster rates. Thanks to its unusual lifestyle, *S. cerevisiae* can aggressively utilize the available carbon source at the expenses of its energetically efficient but slower competitors: the rapid depletion of the sugar and the accumulation of large amounts of ethanol produced during fermentation (which is toxic for most of competing microorganisms) enable yeast cells to successfully compete for survival.

Glucose-dependent regulation of gene expression enables yeast's unique lifestyle

Several factors contribute to yeast's propensity to carry out fermentation even when oxygen is abundant.

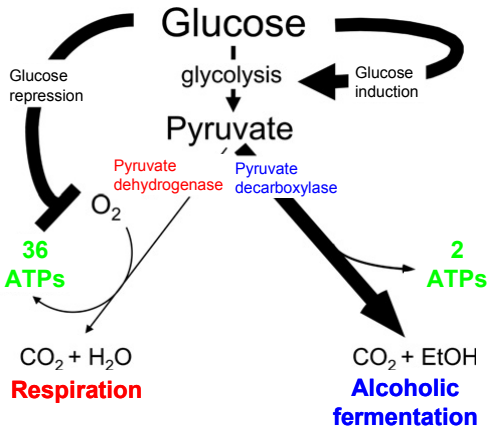


Figure 2 Simplified diagram of glucose metabolism in yeast

Aerobic ethanol production *by S. cerevisiae* is thought to depend on the relative capacities of the fermentative and respiratory pathways: in fact, pyruvate decarboxylase (Pdc), the enzyme catalyzing the first step of alcoholic fermentation has more capacity than its counterpart (pyruvate dehydrogenase, Pdh) involved in the respiratory pathway (Figure 2; Kappeli, 1986; Johnston & Kim, 2005). Consequently, the high glycolytic flux generated in presence of large amounts of glucose exceeds the capacity of the Pdh reaction and generates an overflow towards the Pdc/ethanol route (Kappeli, 1986). In contrast, at low external level of glucose *S. cerevisiae* does not produce ethanol under aerobic conditions and mainly relies on the respiratory catabolism (Kappeli, 1986; Johnston & Kim, 2005).

In addition, several key enzymes required for glucose oxidation (e.g., TCA cycle enzymes, electron transport chain proteins in the mitochondria) become repressed at the transcriptional level in presence of large amounts of glucose.

The small amount of ATP obtained via fermentation forces yeast cells to pump a large amount of sugar into glycolysis to generate sufficient energy for life: this is achieved by glucose-induced expression of many genes encoding proteins necessary for the sugar metabolism, most notably the glycolytic enzymes and the carriers that facilitate the glucose transport across the plasma membrane, the first and rate-limiting step in its metabolism (Johnston & Kim, 2005; Ozcan & Johnston, 1999).

Glucose transport in S. cerevisiae relies on a multi-component uptake system

Glucose import into the yeast cell occurs via facilitated diffusion through a group of membrane-spanning proteins, termed hexose transporters (encoded by *HXTs*). The uptake process takes place at no energy cost, with the sugar entering the cells down a concentration gradient (Boles & Hollemberg, 1997; Ozcan & Johnston, 1999).

The kinetics of glucose transport is subject to regulation in response to environmental conditions, especially the nature and concentration of the carbon source. Two uptake systems were originally described in *S. cerevisiae*: a constitutive, low affinity system (K_m 15-20mM) and a glucose-repressible, high-affinity system (K_m 1-2mM) (Bisson & Fraenkel., 1984.). It now seems clear that

these two systems represent the sum of the combined activity of multiple hexose transporters rather than being the result of the activity of individual carriers (Reifenberger et al., 1997).

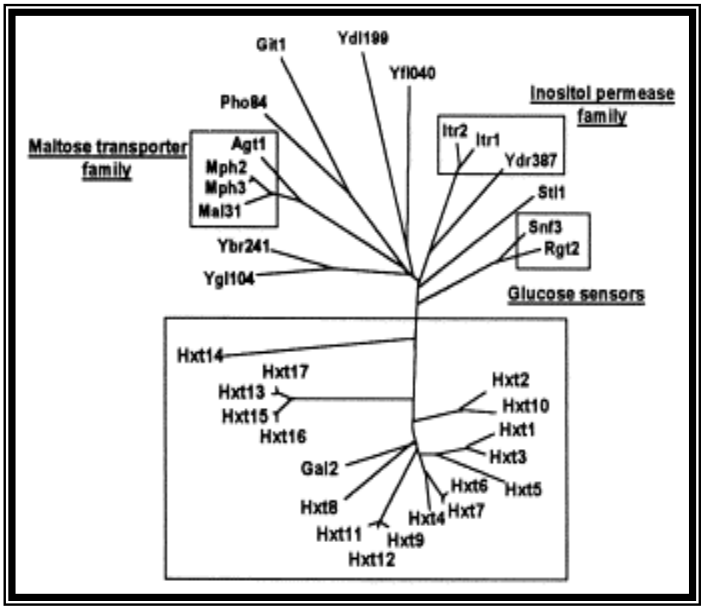


Figure 3. The Hexose Transporter family in S. cerevisiae.

S. cerevisiae possesses at least 20 glucose transporter (*HXT1* to *HXT17*, *GAL2*, *SNF3* and *RGT2*), the largest number than any known organism (Fig. 3).
Sequence alignment of the 20 hexose transporters reveals a high degree of conservation throughout the regions comprising the 12 predicted transmembrane segments (Fig 4). The amino- and carboxyl-terminal tails, both localized on the cytosolic face of the plasmatic membrane, differ considerably in length and aminoacidic composition. Little is known about the three-dimensional structure of the carrier, so the molecular mechanism of glucose transport remains an open question. However, several molecular models, developed for the mammalian Glut1p glucose transporter, which is closely related to its yeast counterparts (42-48% similarity), predict the existence of a central aqueous channel formed by the juxtaposition of at several amphipatic transmembrane helices, which would transport glucose via hydrogen binding to hydroxyl and amide-containing amino acid side chains comprising the wall of the aqueous channel (Salas-Burgos et al., 2004).
None of the transporters are essential for viability or growth on glucose (Reifenberger et al., 1997). Only the transporters encoded by *HXT1* to *HXT7* seem to be metabolically relevant, since a strain lacking these seven genes (often designed as "*hxt*-null" mutant, (MC996 background: Reifenberger et al., 1995; Reifenberger et al., 1997) is unable to grow on glucose as sole carbon source and has no detectable glycolytic flux. This apparently suggests that the remaining carriers (*HXT8-17*) do not contribute significantly to glucose import, perhaps as a consequence of their reduced expression level (at least under the most common growth conditions; Ozcan & Johnston, 1999). However, an *hxt(1-7)* deletion mutant constructed in the CEN.PK strain background still displays residual growth on glucose: such

discrepancies in experimental data may be explained with the high respiratory efficiency of the CEN.PK strain, which enables these cells to catabolize glucose even at extremely low uptake rates (Wieczorke et al. 1999). Moreover, when individually overexpressed in a *hxt*-null strain, all of the *HXTs* gene products are able to support growth on glucose, although to a variable extent (Wieczorke et al. 1999); the only exceptions are *HXT12*, a possible pseudogene, and *SNF3* and *RGT2*, which act as glucose sensors but have lost the ability to transport sugar (Wieczorke et al., 1999; Ozcan et al., 1998; see below)). Surprisingly, inactivation of *SNF3* in a strain devoid of all the known hexose carriers (*hxt(1-17) gal2*), partially restores growth on glucose, fructose, mannose and sucrose, suggesting the existence of at least one additional sugar transporter in yeast (Wieczorke et al., 1999).

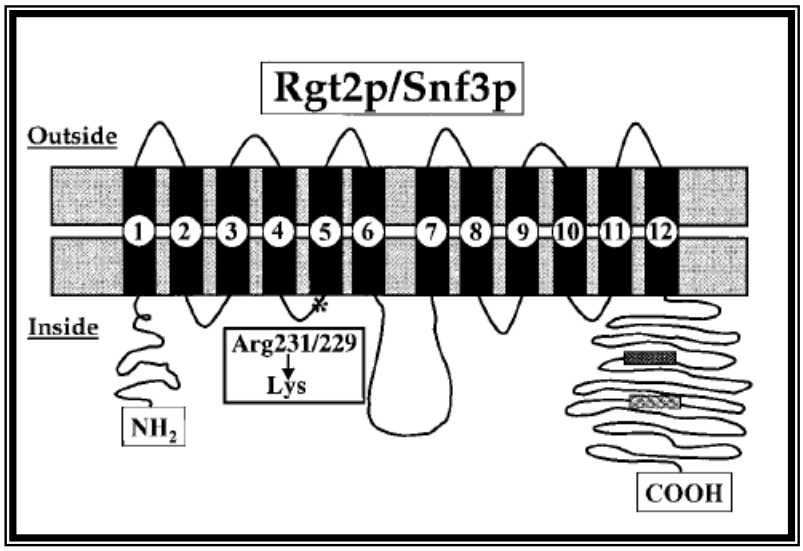


Figure 4. The predicted transmembrane topology of the Rgt2 and Snf3 glucose sensors.
The topology is based on the model for mammalian Glut1 transporter. The predicted transmembrane domains are numbered 1 to 12. The asterisk shows the position of the Arg-231 (in Rgt2) and Arg-229 (in Snf3) that is mutated to a lysine in the dominant mutants *RGT2-1* and *SNF3-1*, respectively (see below). The boxes indicate the 25-amino-acid repeat in the Snf3 and Rgt2 carboxyl-terminal tail. Snf3 has two copies and Rgt2 has only one copy of this repeat. The C-terminal segment is much shorter in the other *HXTs*. (From Ozcan & Johnston., 1999)

The presence in yeast of a multi-factorial glucose uptake system may reflect the need of this microorganism to deal with the extremely broad range of sugar concentrations occurring in its natural habitat. In fact, the diverse carriers exhibit different kinetic properties, and each of them appears particularly suited for a specific growth condition: for instance, Hxt1, a low affinity, high capacity transporter, is most useful when glucose is abundant, whereas Hxt6 and Hxt7, two high affinity carriers, are necessary when the sugar is scarce (Reifenberger et al., 1995; Reifenberger et al., 1997). Accordingly, the expression pattern of the different glucose transporters clearly correlates with their intrinsic characteristic, with low affinity carriers being maximally expressed at high external glucose concentration and high affinity transporters induced only when the sugar level declines (Ozcan et al., 1995; Ozcan et al., 1996b; Diderich et al., 1999). Thus, glucose promotes its own efficient metabolism by serving as an environmental stimulus that regulates the

quantity, types, and activity of hexose transporters, both at transcriptional and post-translational levels (Boles & Hollemberg, 1997; Ozcan & Johnston., 1999; Johnston & Kim, 2005).

Glucose dictates the expression of the most appropriate set of HXT transporters by tightly regulating their transcription

Yeast cells express only the glucose transporters most appropriate for the amount of sugar available in the environment. This is due to the combined action of different regulatory mechanisms, including transcriptional regulation of the major *HXT* genes in response to glucose (Ozcan et al., 1995; Ozcan et al., 1996b; Ozcan et al., 1998; Johnston and Kim., 2005) and inactivation of Hxt proteins under certain conditions (Korak and Wolf., 1997; Krampe et al., 1998; Ye et al., 1999; Krampe et al., 2002 van Suylekom et al., 2007). A minor contribute may also arise from the modulation of the affinity for the sugar of certain transporters (i.e. *HXT2*) (Boles & Hollemberg., 1997; Reifenberger et al., 1997; Maier et al., 2002).

The transcriptional regulation of *HXT* genes is the result of a complex interplay between at least two different pathways which constantly monitor the levels of glucose: the Snf3/Rgt2-Rgt1 and the Snf1-Mig1 pathways (see Johnston & Kim., 2005; Gancedo 2008; Santangelo 2006 for recent reviews). In addition, it has been recently pointed out that Rgt1 function, a central player in the Snf3/Rgt2 circuit, can also be modulated through phosphorylation by a third glucose-sensing pathway, the cAMP/PKA pathway (Kim and Johnston, 2006a).

Glucose induction signal: the Snf3-Rgt2 signaling transduction pathway

In *S. cerevisiae* glucose has two major effects on gene expression: it represses the transcription of genes encoding components of the respiratory pathway (e.g. cytochromes) and enzymes required for the metabolism of alternative carbon sources (e.g. galactose, sucrose and maltose, ethanol), whereas it promotes the expression of genes encoding glycolytic enzymes and glucose transporters (*HXTs*) (Zaman et al., 2008; Zaman et al., 2009).

The Snf3/Rgt2 signaling transduction pathway responsible for the induction of glucose transporter centers on the Rgt1 transcriptional repressor (Fig. 5): addition of glucose inactivates the repressor by ultimately forcing its release from the *HXT* promoters, thus enabling the expression of the hexose carriers (Johnston & Kim, 2005; Gancedo, 2008; Zaman et al., 2008; Santangelo, 2006).

Components of the Snf3/Rgt2-Rgt1 pathway

The central players in the pathway responsible for glucose induction of *HXT* gene expression are (i) Snf3 and Rgt2, two glucose sensors that reside on the cell surface and transduce the glucose signal across the membrane; ;(ii) Rgt1, a transcriptional repressor which negatively regulate the expression of *HXT* genes; and (iii) the SCF^{Grr1}ubiquitin ligase complex, that inhibits the repressor activity.

In the absence of glucose, the zinc-finger-containing repressor Rgt1 binds to the *HXT* promoters and blocks their transcription by recruiting the general repressors Ssn6 and Tup1 (Ozcan et al., 1996c). Rgt1 does this in conjunction with Mth1 and Std1 (Schmidt et al., 1999; Schulte et al., 2000; Lafuente et al., 2000), paralogous proteins that interact with Rgt1 and are essential for its activity as a repressor (Flick et al., 2003; Lakshmanan et al., 2003).

When glucose becomes available, it binds to the Snf3/Rgt2 sensors on the plasmatic membrane (Ozcan et al., 1996a; Ozcan et al., 1998) generating an intracellular signal that causes the SCF^{Grr1} complex to relieve the Rgt1-mediated repression, thereby enabling *HXT* gene transcription (Moriya and Johnston, 2004; Flick et al., 2003; Kim et al., 2006b). The key event in the entire signalling process is the degradation of Mth1 and Std1 (Flick et al., 2003; Kim et al., 2006b), which deprives Rgt1 of the proteins required for its role as transcriptional repressor and forces its release form the *HXT* promoters by inhibiting its DNA binding activity (Kim et al., 2003; Flick et al., 2003; Polish et al., 2005; Lakshmanan et al., 2003; Moriya and Johnston, 2004).

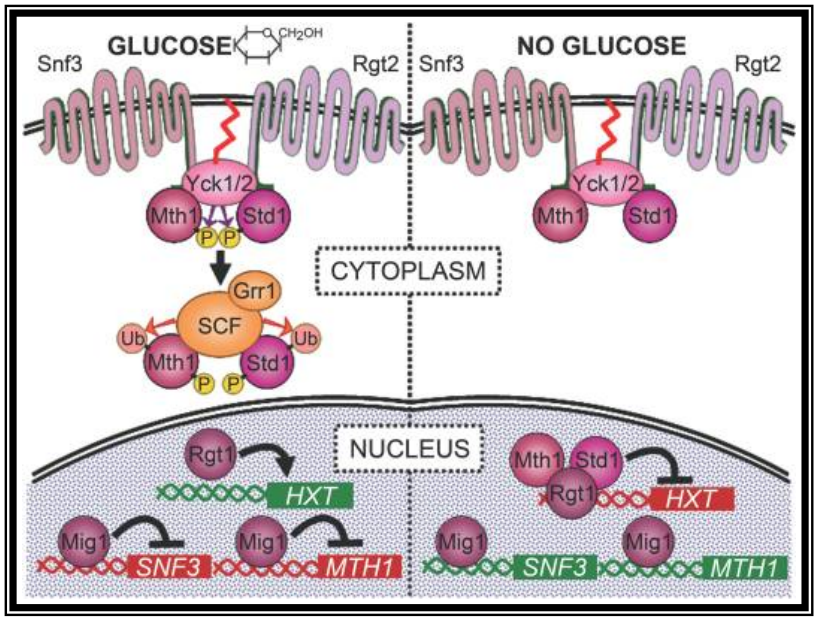


Figure 5. The Snf3/Rgt2 pathway
Transcription of hexose transporter genes (*HXT*) is repressed by Rgt1 in the absence of glucose. Glucose binding to the two membrane-spanning sensors, Snf3 and Rgt2, induces them to bind Mth1 and Std1, thereby recruiting the corepressors to the plasma membrane, where they are phosphorylated by the casein kinases Yck1 and Yck2. Once phosphorylated, the corepressors are targeted by the SCF^{Grr1} ubiquitin conjugating complex for degradation by the 26S proteasome. Elimination of the Mth1 and Std1 corepressors by proteolysis exposes Rgt1 to phosphorylation by PKA (not shown), and alleviates its repressive activity through eviction from regulated promoters. Removal of Mth1 and Std1 converts Rgt1 into a transcriptional activator that may stimulate *HXT* expression despite the absence of specific DNA binding activity. Mig1 represses transcription of its target genes in the presence of glucose and cross-regulates the Snf3/Rgt2 pathway. (From Santangelo et al., 2006).

A schematic representation of the signaling events (from glucose signal generation at the plasma membrane to signal reception by the Rgt1 DNA-bound repressor) leading to induction of the *HXT* genes in response to glucose is given in Fig. 5.

Mechanisms of glucose induction of HXT gene expression

The signaling event cascade that culminates in the Mth1 and Std1 degradation and subsequent removal of Rgt1 from the *HXT* promoters begins at the cell surface with Snf3 and Rgt2, two proteins with 12 transmembrane domains that act like glucose sensors (Fig. 4). Despite their high similarity to the Hxt glucose transporters, Snf3 and Rgt2 seem to have lost the ability to import sugars inside the cell (Ozcan et al., 1998; Wieczorke et al., 1999) and function instead as receptors that monitor the extracellular glucose*: in particular, Rgt2 seems to be a low affinity receptor required for maximal induction of *HXT1* (low affinity carrier) by high glucose, while Snf3 is a high affinity sensor needed for the transcription of *HXT2* and *HXT4* (moderately affinity carriers) genes in response to low levels of glucose (Ozcan et al., 1996a; Ozcan et al., 1998). A *snf3 rgt2* double mutant is completely defective in glucose induction of *HXT* expression and grows poorly on glucose-containing media as a consequence of its impaired sugar uptake (Ozcan et al., 1998). Whereas expression of *HXT1* from a constitutive promoter corrects the growth defect of this mutant, it does not restore glucose induced transcription of *HXT* genes (Ozcan et al., 1998). Conversely, a *hxt*-null strain has no detectable glucose uptake but exhibits normal induction of *HXT* expression in response to glucose (measured by using fusions of the *HXT* promoters to a reporter gene *(*Ozcan 2002). These observations indicate that glucose transport and metabolism are neither necessary nor sufficient for generation of the glucose induction signal. Instead, glucose signaling by Snf3 and Rgt2 is a receptor-mediated process similar to hormone signaling in mammalian cells (Ozcan et al., 1996a; Ozcan et al., 1998; Ozcan et al., 2002; Johnston & Kim, 2005). As a further confirm to this view, a dominant mutation in the Snf3 and Rgt2 sensors has been identified that leads to the constitutive expression of *HXT* genes even in complete absence of glucose (Ozcan et al., 1996a). This mutation (consisting in the replacement by a lysine of an arginine residue conserved in all glucose transporters and localized in the cytoplasmic loop preceding the 5th transmembrane domain) probably converts the receptors in their glucose-bound form, causing them to constitutively generate a signal which activates the *HXT* expression (Ozcan et al., 1996a).

Distinctive features of both Snf3 and Rgt2 are their long C-terminal tails (~200aa,), which reside in the cytoplasm and play an important role in the glucose signaling (Ozcan et al., 1998; Coons et al., 1997; Dlugai et al., 2001). The C-terminal extensions of the two receptors are quite dissimilar except for a brief sequence motif (~25aa) that occurs twice in the Snf3 tail and only once in Rgt2 and that is apparently required for the signaling function: in fact, deletion of this conserved motif, as well as of the whole C-terminal domain, impairs the ability of the sensors to induce the expression of *HXT* genes in response to glucose. Moreover, the C-terminal extensions are sufficient for glucose signaling, since attaching them to a Hxt transporter confers on it glucose signaling ability (Ozcan et al., 1998); furthermore, the expression of isolated tail domains (fused to a membrane-targeting sequence or as soluble proteins) leads to a constitutive glucose signal (Coons et al., 1997; Vagnoli et al., 1998; Dlugai et al., 2002, Moriya & Johnston, 2004).

*However, it should be noted that a direct binding of glucose to Snf3 or Rgt2 has not yet been demonstrated directly, although .several hints have already been uncovered: besides the constitutively activated versions of Rgt2 and Snf3 already described, a V404I substitution in Rgt2 virtually abolishes glucose signaling: since the corresponding residue in Hxt1 (F371) is necessary for glucose transport V404 in Rgt2 may be part of the glucose binding domain in the receptor. (Moriya & Johnston, 2004).

However, the tails are not strictly necessary for the signaling process, as shown by the fact that a tail-less version of Rgt2 can be still functional when overexpressed (Moriya & Johnston, 2004). According to the current view, the glucose signal is generated by the transmembrane domain of the glucose receptors upon binding of the sugar, while the C-terminal tails enhance signaling by facilitating the recruitment of the Mth1 and Std1 corepressors to the plasma membrane (see below; Moriya & Johnston, 2004).

Binding of glucose likely triggers a conformational change in the Rgt2/Snf3 receptors that activates Yck1 (and its paralogue Yck2), a membrane-anchored type I casein kinase involved in many cellular processes (Moriya et al., 2004; Pasula et al., 2007). Yck1 interacts with Rgt2 *in vivo* (in both the presence and absence of glucose) and its overexpression generates a constitutive glucose signal that induces *HXT1* expression. The molecular mechanism underlying activation of Yck1/2 in response to glucose remains elusive (Moriya et al., 2004; Pasula et al., 2007).

YCK1 (or *YCK2*) function is required for glucose triggered degradation of Mth1 and Std1 and for glucose induction of *HXT* transcription (Moriya et al., 2004).

Therefore, two likely substrate of the activated Yck1 are the regulators Mth1 and Std1, which are recruited in proximity of the kinase by their interaction with the long C-terminal tails of Snf3 and Rgt2 (Lafuente et al., 2000; Schmidt et al., 1999). The two corepressors bind to a common site on Rgt1 and are functionally redundant under most experimental conditions; however, Mth1 is the prominent effector of Rgt1 function since it is the more abundant of the two paralogs under conditions where both are active (i.e. in the absence of glucose) (Sabina et al., 2009)**.** Mth1 thus serves primarily to maintain repression of *HXT* in absence of glucose, whereas Std1 may play a role in the establishment of repression when glucose is exhausted (Kaniak et al., 2004; Kim et al., 2006b; Sabina et al., 2009). Mth1 and Std1 likely shuttle between the nucleus and the cell membrane, since they bind to Rgt1 inside the nucleus (Lakshmanan et al., 2003) and to the glucose sensors at the cell surface (Schmidt et al., 1999; Lafuente et al., 2000). So far, there is no evidence that the subcellular localization of Mth1 and Std1 is regulated (Johnston and Kim, 2005), although it has been proposed that the Snf1 kinase activity might favor nuclear retention of the two corepressors (Pasula et al., 2007; see below). Serine-rich sequences (consensus SXXS) have been identified both in Mth1 and Std1, which are potential targets for Yck1: removal of these aminoacids converts Mth1 and Std1 into constitutive repressors by preventing their phosphorylation and subsequent degradation in the presence of glucose (Moriya & Johnston, 2004; Pasula et al., 2007). Phosphorylation of Mth1 and Std1 triggers their recognition by Grr1, an F-box protein component of the SCF^{Grr1} ubiquitin-ligase complex (see BOX1) (Flick et al., 2003; Spielewoy et al., 2004). The ensuing ubiquitination of Mth1 and Std1 marks them for degradation by the 26S proteasome and leads to inactivation of the Rgt1 repressor (Spielewoy et al., 2004; Kim et al., 2006b). The constitutively activated Rgt2-1 sensor promotes ubiquitination (via SCF^{Grr1}) and subsequent degradation of Mth1 and Std1 regardless of the presence of glucose, thus promoting constitutive expression of the *HXT* genes (Pasula et al., 2007).

Addition of glucose inactivates Rgt1 by ultimately forcing its release from the *HXT* promoters: the key event in the entire signaling process is the degradation of the corepressors Mth1 and Std1, which exposes Rgt1 to phosphorylation by PKA and inhibits its DNA-binding activity (Kim et al., 2003; Polish et al., 2005; Flick et al., 2003; Mosley et al., 2003; Kim et al., 2006a).

Rgt1 contains a C_6-(Cys_6 Zn_2) 'zinc cluster' DNA-binding domain in its N-terminus that recognizes the consensus sequence 5'CGGANNA3 (Kim et al., 2003). Rgt1 binds synergistically to multiple sites found in the upstream regions of most of the *HXT* genes only in absence of glucose (Kim, 2009; Mosley et al., 2003; Flick et al., 2003; Kim et al., 2003).

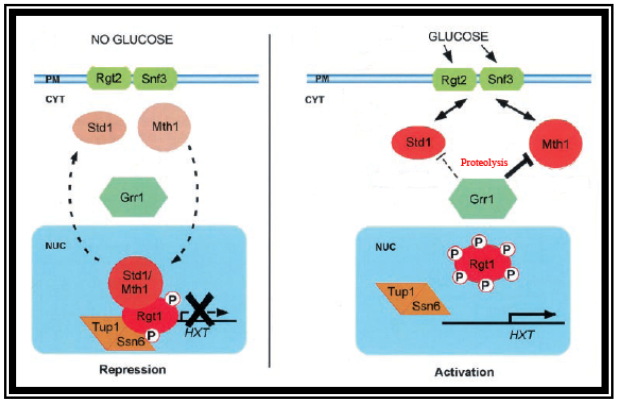


Figure 6. Rgt1 phosphorylation abolishes DNA binding activity of Rgt1 allowing transcription of HXT genes.
The corepressors Std1 and Mth1 cycle between the nucleus and the cytoplasm. In absence of glucose, the corepressors Std1 and Mth1 stabilize the interaction between the transcriptional repressor Rgt1 and the HXT gene promoters by preventing Rgt1 phosphorylation by PKA, thus repressing HXT transcription. In the presence of glucose, Mth1 and Std1 are inactivated via Grr1-dependent proteolysis, Rgt1 becomes hyper-phosphorylated and dissociates from the HXT promoters thereby activating transcription.

When glucose is available, Rgt1 becomes hyperphosphorylated and its DNA-binding activity is lost (Fig. 6;Kim et al., 2003; Mosley et al., 2003; Flick et al., 2003): consistently, a Rgt1 mutant not subjected to phosphorylation behaves as a constitutive repressor and remains associated to DNA in presence of glucose (Kim et al., 2003); Furthermore, in *snf3 rgt2* and *grr1* mutants, which are defective in glucose induction of *HXT* gene, Rgt1 is not phosphorylated (Mosley et al., 2003). The phosphorylation event allows an intramolecular interaction between the central region of Rgt1 and its zinc-finger domain, which prevents the repressor from binding DNA (Polish et al., 2005; Kim et al., 2006a) (Fig.7).
Multiple evidences support the conclusion that PKA contributes to glucose induction of *HXT* gene expression by catalyzing phosphorylation of Rgt1: i) PKA phosphorylates Rgt1 *in vitro*; ii) glucose fails to induce *HXT* genes expression in yeast cells with reduced PKA activity, whereas the transcription of *HXTs* is constitutive in strains with an hyperactive cAMP/PKA pathway; iii) several serine residues in the N-terminus of Rgt1, which are likely phosphorylated by PKA, are essential both for the intramolecular reaction of the repressor and for its release from the *HXT* promoter in response to glucose (Kim et al., 2006a).
It has been suggested that Mth1 and Std1 participate to the Rgt1-mediated repression by binding to the repressor and blocking its intramolecular interaction, likely by preventing the phosphorylation by PKA (Fig. 6-7; Polish et al, 2005): consistently, concurrent inactivation of Mth1 and Std1 leads to the hyperphosphorylation of Rgt1 and its dissociation from the *HXT* promoters even in the absence of glucose, thus

enabling constitutive expression of the hexose transporters (Lakshmanan et al., 2003; Flick et al., 2003).

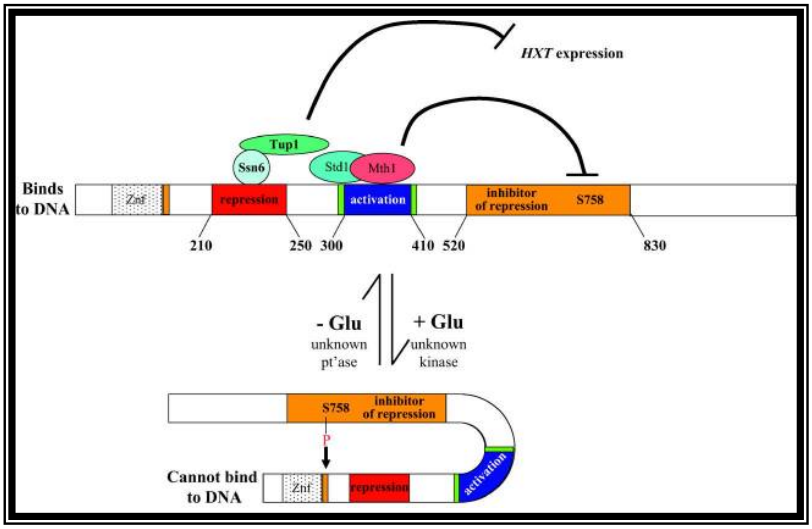


Figure 7. Regulation of Rgt1-mediated transcriptional repression by glucose.
Rgt1 function is regulated by an intramolecular interaction between its N-terminus and the middle region that inhibits function of the DNA-binding domain of Rgt1. Phosphorylation of Rgt1 by PKA in response to glucose inhibits its DNA binding activity.
In the absence of glucose (bottom), the corepressor Mth1 binds to Rgt1 and inhibits its phosphorylation (possibly at S88 and S758) and Rgt1 binds to DNA, blocking transcription by recruiting by recruiting the general repressors Ssn6 and Tup1. Addition of glucose induces degradation of Mth1, allowing phosphorylation of Rgt1 by PKA: this results in an intramolecular interaction between the central regulatory region of Rgt1 and its N-terminal DNA-binding domain (which requires amino acids 80–90), preventing Rgt1 from binding to DNA and leading to derepression of gene expression.

Therefore, two distinct glucose-induced events must occur for the removal of the Rgt1 repressor from the *HXT* promoters to take place; Mth1 and Std1 must be degraded via the Snf3-Rgt2 glucose-sensing pathway and Rgt1 must be phosphorylated via the cAMP/PKA glucose-sensing circuit (Kim et al., 2006a).
Yeast cells may take advantage of this strategy to induce different *HXT* genes in response to different levels of glucose (Kim et al., 2006a,b).. When glucose levels are low, Mth1 would be degraded, but Rgt1 would not be completely phosphorylated because PKA is not fully active under these conditions: this might result only in induction of *HXT* genes encoding high affinity glucose transporters (*e.g. HXT2*). When glucose levels are high, Mth1 would be degraded, and Rgt1 would be fully phosphorylated because PKA is fully active: this would drive to completion the intramolecular interaction of Rgt1 and result in full induction of the low affinity carriers (*i.e. HXT1* and *HXT3*) (Kim et al., 2006a,b).
As discussed in the following section, a third layer of regulation is provided by the Snf1 repression pathway, which prevents the transcription of high affinity hexose transporters when large supplies of glucose are available (Ozcan & Johnston, 1996a; Ozcan & Johnston, 1999; Johnston & Kim, 2005).

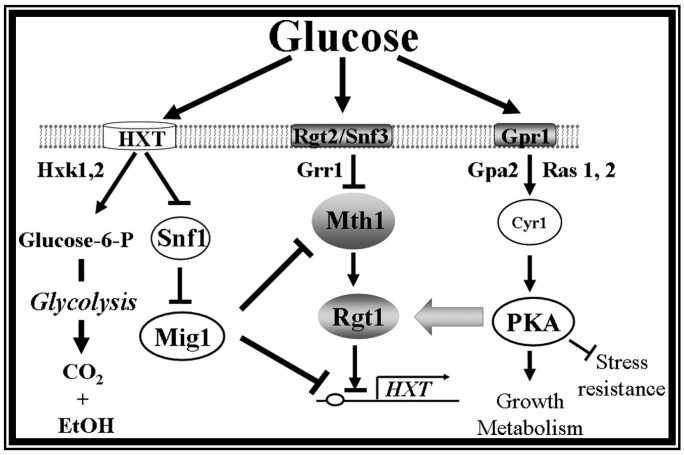


Figure 8. Multiple glucose-sensing pathways coordinately regulate the expression of HXT genes. See text for detail. (From Kim & Johnston, 2006a).

Box1. The SCF ubiquitin ligase complex

The SCF-ubiquitin ligase complex (named after its core components: Skp, Cullin and F-box protein) is a multiproteic complex catalyzing the ubiquitination of protein destined for proteosomal degradation (For a review, see Tyers & Jorgensen, 2000).
Core subunits of the complex are

- F-box protein - (Grr1, Cdc4) Contributes to the specificity of SCF by recruiting their target proteins independently of the complex and then binding to the Skp1 component, thus allowing the target to be brought into proximity with the ubiquitin-conjugating enzyme. F-box affinity for its substrates is regulated through phosphorylation of target proteins, often mediated by Cdk/cyclin,
- Skp1 - Bridging protein, forms part of the horseshoe-shaped scaffold, along with cullin. Skp1 is essential in the recognition and binding of the F-box.
- Cullin (Cdc53) forms the major structural scaffold of the SCF complex, linking the Skp1 domain with the Rbx1 domain.
- Rbx1 (Hrt1) Rbx1 contains a small zinc-binding domain called the RING Finger, to which the E2-ubiquitin conjugate binds, allowing the transfer of the ubiquitin to a cysteine residue on the target protein.
- Ubiquitin conjugating enzyme (E2: Cdc34), the catalytic subunit of the complex.

These complexes differ in the F-box-protein component. The SCF^{Cdc4} complex, for example, contains Cdc4 which is responsible for recruiting to the complex substrates such as the cyclin Clb6 or the cyclin-dependent protein kinase inhibitor Sic1, thereby causing them to be ubiquitinated and thus marked for degradation by 26S proteasome (Jackson et al., 2006; Skowyra et al., 1997; Feldman et al., 1997; Verma et al., 1997).
On the other hand, Grr1 is required for the Cdc34-dependent ubiquitination and subsequent degradation of the G1 cyclins Cln1 and Cln2, two key regulators of the cell cycle, and of Gic1 and Gic2, effectors of the GTP-binding protein Cdc42 which regulate actin polarization and bud emergence (Barral et al., 1995; Jacquenod et al., 1998; Skowyra et al., 1997).
Grr1 play a central role in coupling nutrient availability to gene expression and cell-cycle progression (Li & Johnston, 1997). Consistently, *grr1* mutant display a pleiotropic phenotype, including elongated cell morphology, increased resistance to heavy metals and sulfite, increased sensitivity to osmotic stress and nitrogen starvation, loss of aromatic amino acid transport, decreased glucose uptake, defects in glucose mediated repression of several enzymes (maltase, invertase, galactokinase) and lack of inactivation of the maltose permease in presence of glucose (Ozcan & Johnston, 1999).
The abnormal cell morphology of *grr1* mutants is likely due to hyper-accumulation of the G1 cyclins (Cln1 and Cln2) and of Gic1, which bring about anomalies in actin polarization/bud emergence ((Barral et al., 1995; Jacquenod et al., 1998; Skowyra et al., 1997).
The defect in glucose transport exhibited by grr1 cells reflect the key role of the SCFGrr1 complex in promoting the inactivation of the Rgt1 repressor via proteolytic degradation the Mth1 and Std1 corepressors (Flick et al., 2003; Kim and Johnston, 2006b; Kaniak et al., 2004; Moryiia & Johnston, 2004) . The glucose repression defect of *grr1* mutants is likely an indirect consequence of their inability to transport significant amounts of glucose (Ozcan et al., 1995; Vallier et al., 1994).

Multiple regulatory mechanisms ensure appropriate HXT gene expression

Yeast cells are able not only to detect the presence of glucose in their surroundings, but also to determine how much sugar is available and respond by expressing the most appropriate transporters (Johnston & Kim, 2005; Ozcan & Johnston, 1999).

At least three glucose-sensing pathways affect expression of the *HXT* genes (Fig. 8).

The Snf3/Rgt2-Rgt1 pathway is the main responsible for induction of *HXT* expression in presence of glucose (Ozcan et al, 1998; Ozcan et al. 1996; Johnston & Kim, 2005; Kim & Johnston, 2006a,b). Relatively few other genes have been validated as targets of this signaling circuit, suggesting that it is primarily devoted to regulating the expression of *HXT* genes (Zaman et al., 2009; Kaniak et al., 2004).

The glucose repression circuit that operates through the Snf1 protein kinase and the Mig1 transcriptional repressor prevents the expression of the high/intermediate affinity hexose carriers (encoded by *HXT2, HXT4*, *HXT6* and *HXT7)* when the sugar levels are high (Ozcan et al., 1996a; Johnston & Kim, 2005).

Finally, the cAMP/PKA glucose-sensing pathway contributes to *HXT* expression by regulating the Rgt1 function (Kim et al., 2006a).

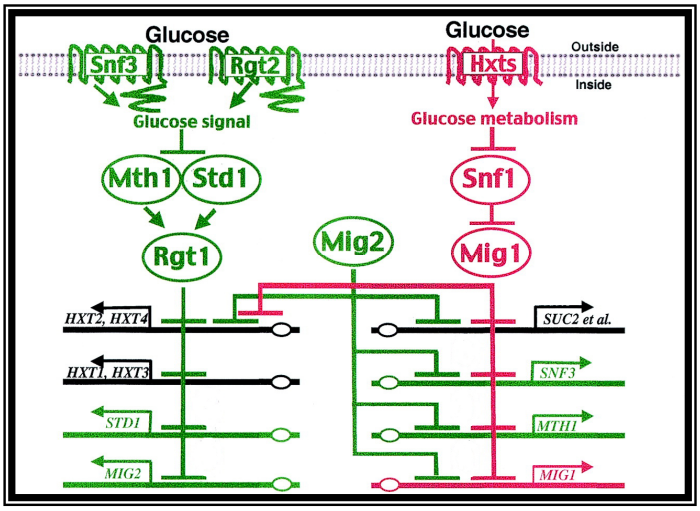


Figure 9. Multiple crosstalks between the Rgt2/Snf3 and the Snf1 glucose sensing pathways.
The components shown in green respond to the glucose signal generated by the Rgt2/Snf3 sensors, whereas components shown in red respond to the glucose signal that affects the function of the Snf1 kinase. The genes shown in black lines are the ultimate targets of these two glucose signaling pathways. Arrows denote activation, bars denote inhibition. (From Kaniak et al., 2004).

Furthermore, these three glucose sensing pathway are intertwined in a complex regulatory network with multiple feedback and feedforward regulatory loops that serves to fine-tune the cellular response to glucose availability (Fig. 9). For example,

- the expression of *STD1*, one of the regulators of Rgt1 activity, is feedback regulated: glucose inhibits Std1 function by promoting its degradation by proteasome via the Rgt2/Snf3-Rgt1 signaling pathway (Kim et al., 2006b) and concurrently induces *STD1* expression through the same pathway (Kaniak et al., 2004). Thus, *STD1* expression is stimulated at the same time that Std1 protein levels are decreasing in response to glucose: this regulation might serve to dampen glucose induction of gene expression; moreover, it may also provide a mean for the rapid re-establishment of

Rgt1-mediated repression upon glucose depletion (Kim et al., 2006b; Johnston & Kim, 2005). Std1 may also play a role in the glucose repression pathway, since it is known to interact with and regulate Snf1 (Tomas-Cobos & Sanz, 2002; Schmidt et al., 1999).

- In contrast to *STD1*, its paralogue *MTH1*, which has an overlapping function, is feedforward regulated: glucose reduces *MTH1* transcription via repression exerted by Mig1 and Mig2 while also stimulating the proteasome-mediated degradation of Mth1 (Flick et al., 2003; Spielewoy et al., 2004; Kim et al., 2006b; Schmidt et al., 1999; Johnston & Kim, 2005). Such a regulation reinforces the inhibitory effect of glucose on Mth1 function and ensures maximal glucose induction of Rgt1-repressed genes (Kaniak et al., 2004). The different modulation of the two paralogs Mth1 and Std1 justify their diverse role in assisting the Rgt1-mediated repression, with Mth1 being the primary regulator and Std1 serving to buffer the response to glucose (Kaniak et al., 2004; Kim et al., 2006b; Sabina et al., 2009; Johnston & Kim, 2005).
- The Snf3/Rgt2-Rgt1glucose induction pathway promotes the expression of the Mig2 repressor (Kaniak et al., 2004), which cooperates with Mig1 (Snf1 pathway) in the glucose-induced repression of many genes (Westholm et al., 2008; Lutfiyya et al., 1996; Lutfiyya et al., 1998; Johnston & Kim, 2005)
- *SNF3* transcription is repressed through Mig1 (Snf1 pathway) and Mig2 (Snf3/Rgt2 pathway) in presence of abundant glucose, probably reflecting the role of Snf3 as a sensor of low levels of sugar (Ozcan & Johnston, 1999; Neigeborn et al, 1986; Ozcan & Johnston, 1995).
- Besides inhibiting the expression of several components of the Snf3/Rgt2-Rgt1 circuit (i.e. *MTH1* and *SNF3*), the Snf1-Mig1 pathway can also auto-regulate its own activity by repressing the transcription of *MIG1* through a mechanism loop involving Mig1 itself (in cooperation with Mig2) (Lutfiyya et al., 1998; Kaniak et al., 2004): the effect of this auto-regulatory circuit is to mitigate the Mig1-mediated glucose repression, thus enabling a more rapid recovery from the repressed-state when the sugar is depleted.

As discussed in following sections, Snf1 represents a key player in the process of adaptation to glucose shortage, since activation of this kinase allows de-repression not only of high affinity glucose carriers, but also of many genes required for gluconeogenesis, respiration, and metabolism of alternative carbon sources: as a inevitable consequence, a Snf1-deficient strain cannot grow on low glucose (Ozcan & Johnston, 1999).

Interestingly, two well-known players in the phenomenon of the glucose repression, Mig1 and Mig2, are differentially regulated, despite their largely overlapping functions: Mig1 (which has a prominent role in the repression process) is an effector of Snf1 which responds to intracellular signals generated by glucose metabolism and regulates the subcellular localization of Mig1 (Gancedo, 20088; Santangelo, 2006; Moreno et al., 2005), whereas Mig2 (whose contribute to glucose repression is less relevant) is transcriptionally regulated by the Rgt2/Snf3-Rgt1 pathway, which monitors the extracellular glucose by a receptor-mediated mechanism (Kaniak et al., 2004).

Glucose dependent repression of several genes (i.e. *SUC2*; see next sections) is defective when the Mth1 corepressor is lost (*mth1* strain, Schmidt et al., 1999) or it cannot be degraded (Schulte et al., 2000; Kim et al., 2006b).
On the other hand, downregulation of Snf1 activity in high glucose appears to be necessary for degradation of the Mth1 and Std1 corepressors and the ensuing induction of the *HXT1* carrier (Pasula et al., 2007). Therefore, these observations imply a functional link between inactivation of Snf1 and degradation of Mth1 and Std1 (Pasula et al., 2007; Zaman et al., 2008).
Consistent with this proposal, glucose-induced degradation of Mth1/Std1 (and thus *HXT*) is prevented in strains where Snf1 is constitutively active (i.e. *reg1* and *hxk2* null strains, cells harboring a hyperactive variant of *SNF1* ($SNF1^{G53R}$) or overexpressing the *SAK1* kinase (Pasula et al., 2007; Gadura et al., 2006; Ozcan & Johnston, 1995).
It is presently unknown how Snf1 inactivation in high glucose would promote degradation of Mth1 and Std1. The current model for proteolytic removal of Mth1 and Std1 includes nuclear export of the corepressor, which must undergo phosphorylation by the membrane-tethered Yck1/2 prior to being ubiquitinated by SCF^{Grr1} (Moryiia & Johnston, 2004; Zaman et al., 2008; Gancedo, 2008; Santangelo et al., 2006). Therefore, it has been proposed that Snf1 might regulate nuclear export of Mth1 and Std1: consistently, Mth1 and Std1 are nuclear in cells harboring active Snf1 (Pasula et al., 2007).
Recent evidences have shown that Yck1 and Yck2 casein kinase might respond to glucose signals from both the Rgt2/Snf3 circuit and the Glc7/Reg1 phosphatase complex (involved in the Snf1 pathway) to induce degradation of Mth1 and Std1 with the resultant expression of the hexose transporters and *HXK2* (Gadura et al., 2006; Pasula et al., 2007). Since both glucose transport and hexokinase participate in glucose metabolism necessary for activation of Glc7/Reg1 (see below), these observations highlight a new intriguing link between the Snf3/Rgt2 pathway and the Snf1 network (Gadura et al., 2006; Pasula et al., 2007).

Furthermore, many other mechanisms apparently contribute to the regulation of *HXT* expression: for example, roles for the HOG pathway (Hirayama et al., 1995; Tomas-Cobos et al, 2004) and the TOR network (Tomas-Cobos et al., 2005) in the transcriptional regulation of *HXT1* have been proposed. Glucose phosphorylating enzymes (and in particular Hxk2) kinases also appear to influence the expression pattern of the HXT genes (Ozcan & Johnston, 1995; Petit et al., 2000; Tomas-Cobos & Sanz, 2002; Ozcan & Johnston, 1999; Belinchon & Gancedo, 2007a,b).
These issues will be discussed in detail in following sections.

The crosstalk among different glucose signaling pathways in the regulation of *HXT* transcription provide an example of how multiple regulatory circuits that respond differently to a common environmental signal can combine to provide a specific and unique pattern of gene expression.

Yeast hexose transporters in closer detail

The major hexose transporters in *S. cerevisiae*, encoded by the *HXT(1-7)* genes, cover the whole affinity range for glucose from 1 to 100mM (K_m) and have been classified as high affinity ($K_m \cong$ 1mM: Hxt7, Hxt6 and Gal2), medium affinity($K_m \cong$ 5-10mM: Hxt2 and Hxt4) and low-affinity ($K_m \cong$ 50-100mM: Hxt1 and Hxt3) glucose carriers (Table I: Maier et al.,2002). Actually, Hxt2 is quite atypical, since it exhibits biphasic uptake kinetics with a low- and high-affinity component on low glucose and an intermediate affinity on high glucose (Reifenberger et al., 1997; Maier et al., 2002).

Table I Kinetic parameters of yeast glucose transporters

Strain, % glucose	V_m **(nmol min^{-1} mg^{-1})**	K_m **(mM)**
HXT1, 2%	50.9±3.7	129±9
HXT2, 0.05%	35.3±1.3	2.8±0.1
HXT2, 2%	15.6±0.9	4.6±0.3
HXT3, 2%	18.5±2.0	34.2±3.2
HXT4, 2%	12.0±0.9	6.2±0.3
HXT6, 2%	11.4±0.5	1.4±0.1
HXT7, 2%	11.7±0.3	1.9±0.1
hxt1-7	≅0	-
hxt1-7 GAL2-ind	17.5±0.8	1.6±0.1
Wild-type, 2%	12.5±1.6	6.5±0.5
Wild-type, 0.05%	21.0±1.8	1.0±0.1

The kinetic parameters obtained from initial-uptake experiments in intact cells.
Mean values ± standard error. *GAL2*-induction was obtained by growing the *hxt(1-7)* null mutant in 2% galactose. (From Maier et al., 2002).

The expression profile of the major Hxt transporter is generally consistent with their affinity for glucose: high-affinity transporters like Hxt6 and Hxt7 are highly expressed on nonfermentable carbon-sources and repressed by high levels of glucose, whereas transporters with low affinity, such as Hxt1 and Hxt3, are induced by the presence of a high concentration of glucose. Transporters with intermediate affinity for glucose like Hxt2 and Hxt4 are induced by low levels of glucose and repressed by high levels of glucose or in absence of sugar.

At least five types of transcriptional regulation by different level of glucose have been described for the major *HXT* genes (Fig. 10): *(i)* induction by high concentrations of glucose (*HXT1*); *(ii)* induction only by low levels of glucose (*HXT2* and *HXT4*); *(iii)* induction by glucose independent of sugar concentration (*HXT3*); *(iv)* repression by high level of glucose (*HXT6* an *HXT7*); *(v)* regulation independent by glucose concentration (*HXT5*).

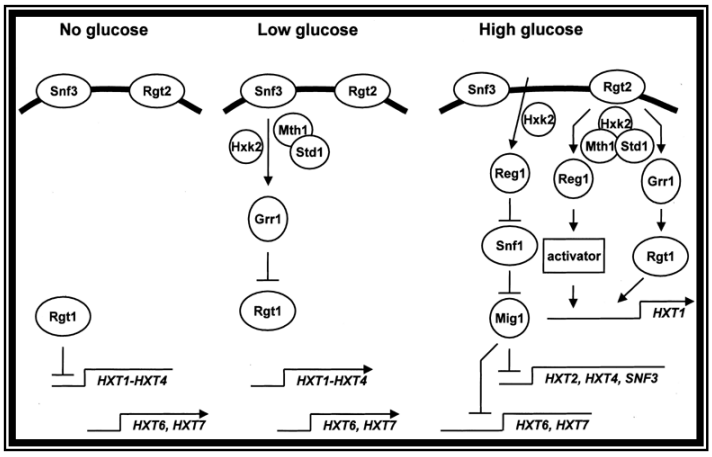


Figure 10. Glucose dependent regulation of HXT gene expression in response to glucose.
In the absence of glucose, Rgt1 represses the transcription of *HXT1-4*. Low amounts of glucose inhibit the Rgt1-repressing activity, a process triggered by Snf3 via Grr1-mediated proteolysis of the Mth1 and Std1 corepressors. At high concentrations of glucose, Rgt2 triggers *HXT1* expression through involving the conversion of Rgt1 into a transcriptional activator and another mechanism in which several components of the main glucose-repression pathway are involved. At high glucose concentrations *HXT2*, *HXT4*, *HXT6-7* and SNF3 are repressed by Mig1 via the main glucose-repression pathway. *HXT*3 expression is induced by glucose independent of sugar concentration, whereas *HXT5* regulation is glucose independent (not shown). (From Rolland et al., 2002).

I) HXT1: Induction of Transcription by High Glucose Concentrations

Originally isolated as a multicopy suppressor of the growth defect in *snf3* mutants and in *trk1 trk2* cells (Ko et al. 1993), *HXT1* encodes a 570aa transporter with an extremely low affinity for both glucose (*Km* =100mM; Table I) and fructose (*Km* =300 mM) (Reifenberger et al., 1997; Maier et al., 2002). Consistent with this property, its expression in a *hxt*-null mutant only restores growth on high concentrations of glucose (more than 1% w/v) (Reifenberger et al., 1995; Reifenberger et al., 1997).

HXT1 expression is induced about 300-fold by high concentrations of glucose (Ozcan et al., 1995; Diderich et al., 1999): this is due (i) to the relief of Rgt1-mediated repression, (ii) to the modulation of the Rgt1 activity and (iii) to another mechanism, whose molecular details have not yet been precisely defined (Ozcan et al., 1995; Mosley et al., 2003).

In the absence of the sugar, Rgt1 interacts with its corepressors Mth1 and Std1 and binds to the *HXT1* promoter, blocking its transcription by recruiting the general repressors Ssn6 and Tup1, as it does for the other *HXT* genes. In a strain lacking Rgt1, *HXT1* is about 20-fold derepressed in the absence of glucose. Besides inhibiting the transcription of *HXT* genes in absence of glucose, Rgt1 is also required for the full induction of *HXT1* when glucose is abundant (Ozcan et al., 1996c; Mosley et al., 2003). Experimental evidences suggest that Rgt1 is a bifunctional transcription factor which is converted from a repressor to an activator when large amounts of glucose become available (Ozcan et al., 1996c). In response to a high glucose signal, Rgt1 is rapidly hyperphosphorylated and dissociates from the repressor complex abandoning the *HXT1* promoter (Fig. 6). Phosphorylation also

converts Rgt1 into an activator which can indirectly promote *HXT1* transcription, possibly by stimulating the activity (expression?) of a unidentified transcription factor (Fig.11; Mosley et al., 2003): in fact, since hyper-phosphorylated Rgt1 does not bind DNA in presence of glucose, its role in the activation mechanism of *HXT1* transcription is likely indirect, (Mosley et al., 2004; Flick et al., 2003; Kim et al., 2003).

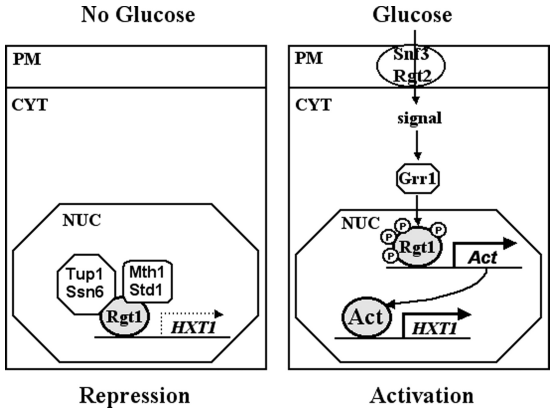


Figure 11. Regulation of HXT1 expression by Rgt1 in response to glucose.
In the absence of glucose, Rgt1 associates forms a complex with Ssn6, Tup1, Mth1, and Std1 and represses the transcription of the *HXT1* gene by direct binding to its promoter region. When glucose is abundant, Rgt1 becomes hyper-phosphorylated and dissociates from the repressor complex. Glucose-dependent phosphorylation by PKA converts Rgt1 to an activator, which then may stimulate the expression of a transcriptional activator (Act) required for maximal expression of the *HXT1* gene. (From Mosley et al., 2003).

However, even in the absence of Rgt1p, *HXT1* expression is still partially inducible by high levels of glucose: indeed, maximal transcription of *HXT1* requires an additional regulatory mechanism that shares some components (Hxk2, Reg1) with the glucose main repression pathway (Fig. 10). This induction apparently involves an putative transcriptional activator or, alternatively, a further repression mechanism that is inactivated by high glucose concentrations (Ozcan et al., 1995).
In a strain lacking Hxk2 or even all the glucose phosphorylating enzymes (*hxk2 hxk1 glk1* triple null strain), high glucose still induces up to 10-fold the expression of a reporter *HXT1–lacZ* gene, but this induction is only 20% of that reached in isogenic wild type or *HXK2*-only strains (Belinchon et al., 2007a).
Interestingly, the Snf1 protein kinase seems to actively participate in the inhibition of *HXT1* expression when glucose is depleted (Tomas-Cobos et al., 2002): activation of Snf1, either by physiological conditions (growth in low-glucose/no glucose) or by eliminating any of its negative regulators, (*hxk2* or *reg1* strains), strongly reduces *HXT1* expression (Ozcan et al., 1995; Tomas-Cobos et al., 2002, Petit et al., 2000); conversely, concurrent deletion of *SNF1* in a *hxk2* or *reg1* deficient strain partially restore the glucose inducible expression of *HXT1*. Removal of the *REG1* gene prevents degradation of the Mth1 and Std1 corepressors in the presence of high glucose, which may explain why the expression of the *HXT1* gene is defective in *reg1* cells (Gadura et al. 2006; Pasula et al., 2007). However, it is presently unknown how Snf1 prevents degradation of Mth1 and Std1 when it is not inactivated by high levels of glucose: it has been suggested that Snf1 might regulate nuclear export of Mth1 and Std1 (thus allowing their phosphorylation by Yck1,2 on

the plasma membrane and subsequent proteolysis), since Mth1 and Std1 are found inside the nucleus of the cells harboring active Snf1 (Pasula et al, 2007).
Despite these uncertainties, both the Snf1-Mig1 glucose-repression pathway and the Rgt2/Snf3-Rgt1 glucose-induction pathway apparently contribute to the transcriptional regulation of *HXT1* (Tomas-Cobos et al., 2002; Pasula et al., 2007).
Hyperactivation of the cAMP pathway leads to the constitutive expression of *HXT1* even in absence of glucose (Kim et al., 2006; Zaman et al., 2009). In contrast, loss of *GPR1* (encoding a glucose receptor required for activation of the Gpr1/Gpa2 branch of cAMP/PKA pathway (Kraakman et al. 1999; see following sections) has no clear effect on transcriptional induction of *HXT1* in high glucose (Belinchon & Gancedo, 1996a; Zaman et al., 2009).

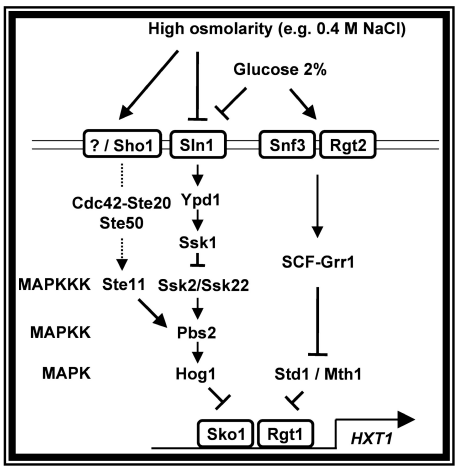


Figure 12.
The Snf3/Rgt2 and HOG pathways regulate HXT1 expression.
See text for details. (From Tomas-Cobos et al., 2004)

HXT1 expression is also inducible by hyperosmotic stress conditions (1 M NaCl or 1.5 M sorbitol) via the high osmolarity glycerol (HOG) pathway that culminates with the Sko1 transcriptional repressor (Hirayama et al., 1995; Tomas-Cobos et al, 2004) (Fig. 12). It has been proposed that increased glucose uptake under osmotic stress is necessary for the biosynthesis of glycerol, a major osmoprotectant in yeast: thus, *HOG1*-dependent increased expression of *HXT1* would provide additional substrate (namely, glucose) for the glycerol synthesis to cope with the osmostress condition (Tomas-Cobos et al., 2004; Hirayama et al., 1995). Interestingly, the induction of *HXT1* after glucose addition is completely abolished in a *hog1* strains, suggesting that the role of Hog1 in specifically regulating the *HXT1* transcription may not be limited to the osmotic stress response. Induction of *HXT1* expression in response to glucose and/or osmotic stress seems to require the coordinated activity of two independent signaling pathways: the glucose Rgt2/Snf3 pathway, which modulate the activity of the Rgt1 repressor and the HOG pathway operating through the Hog1 MAPK and the Sko1 transcriptional repressor. According to the model proposed by Tomas-Cobos and coworkers (Fig 12), in absence of glucose and osmostress both the Rgt1 and Sko1 transcription repressors would bind to the *HXT1* promoter. Glucose addition would promote the *HXT1* gene expression by directly activating the Rgt2/Snf3 pathway and thus inhibiting the Rgt1 repressor, whereas the osmotic stress caused by sugar addition would trigger the activity of the Hog1 MAPK and the ensuing inhibition the Sko1 repressor (Tomas-Cobos et al., 2004).
The release of repression by Rgt1 and Sko1, however, is not sufficient for full induction of *HXT1* in high glucose, which still requires the presence of a functional Hxk2 isoenzyme (Ozcan & Johnston, 1995; Belinchon & Gancedo, 2007a).

14-3-3 proteins Bmh1/Bmh2, positive regulators of the TOR kinase pathway, also seem to be involved in the glucose-induced transcription of *HXT1*, possibly by affecting the activity of the SCF^{Grr1} complex to which they bind (Tomas-Cobos et al., 2005; see next section).

Finally, the TOR kinase pathway also plays a role in the control of *HXT1* expression, possibly by influencing the activity of the Cdc55-PP2A protein phosphatase complex (Tomas-Cobos et al., 2005). In the absence of a TOR kinase signaling (i.e. cells growing in the presence of the drug rapamycin) the induction of *HXT1* by glucose is abolished; the Cdc55-PP2A phosphatase complex (one of the effector of TOR: see following sections) is active under these conditions and thus might interfere with the proper functioning of the Rgt2/Snf3-Rgt1 pathway: it has been proposed that the phosphatase might contrast the Yck1/2 activity by dephosphorylating Std1 and Mth1 corepressors, preventing in this way the glucose-induced transcription of *HXT1* (Tomas-Cobos et al., 2005). Consistent with this proposal, in the absence of Cdc55 (a B-subunit of the PP2A protein phosphatase complex) the transcription of *HXT1* by glucose is enhanced (Tomas-Cobos et al., 2005); furthermore, Yck1 has been demonstrated to interact with Cdc55, as well as to other subunits of the PP2A phosphatase complexes, including Pph21, Pph22 and Tpd3 (Ho et al., 2002).

Table II. Microarray analysis of the Regulation of Hexose Transporters expression.

Treatment	**Fold change (log2) in mRNA levels under the indicated treatments**								
	HXT1	***HXT2***	***HXT3***	***HXT4***	***HXT5***	***HXT6***	***HXT7***	***HXT9***	***HXT17***
Low glucose [a]	5.3	2.8	4.3	4.3	-1.3	2.7	2.7	-0.6	-0.5
High glucose [b]	6.6	-0.2	4.3	1.7	-4.7	-5.5	-5.5	-0.7	0.8
Rapamycin [c]	-2.0	-4.9	-2.3	-2.6	2.0	-2.1	-2.2	0.9	0.2
Galactose [d]	1.7	0.6	0.8	1.6	-0.4	0.6	0.8	0.2	4.0
$RAS2^{V19}$ [e]	3.7	3.7	3.5	3.0	-3.8	-0.2	-0.5	-0.6	4.2
$RAS2^{DN}$ [f]	3.8	-1.4	3.6	1.2	-1.1	-1.1	-1.1	-0.9	3.7
SCH9 OE [g]	6.1	0.1	4.9	1.3	-5.3	-5.1	-5.2	-1.3	-0.9
pka^{as} $sch9^{as}$ [h]	3.0	-3.7	2.6	-	0.1	-1.2	-1.1	0.4	-0.2
$snf1^{as}$ [i]	-0.1	-0.9	-0.2	-0.3	0.6	-0.4	-0.3	-0.3	-0.2
Glu downshift [l]	-5.6	0.9	-5.2	-0.7	4.6	6.6	6.6	0.5	3.8
$snf1^{as}$ downshift [m]	-2.1	-0.3	-2.8	3.8	4.0	6.6	6.6	0.2	3.3
gpr1 [n]	5.0	-2.5	3.8	0.8	-4.8	-5.0	-5.2	-0.8	-0.2
rgt2 [o]	6.0	-0.1	3.8	1.9	-3.6	-5.1	-5.3	-0.9	0.5
RGT2-1 [p]	4.9	2.7	3.7	5.3	-0.9	1.8	1.8	0.1	0.8

[a] 20 min after addition of 0.125 mM glucose to wild type cells growing in 2% glycerol.
[b] 20 min after addition of 100 mM glucose to wild type cells growing in 2% glycerol.
[c] 40 min after addition of 30 nM rapamycin to wild type cells growing in 2% glycerol.
[d] 20 min after addition of 100 mM galactose to *gal1* cells growing in 2% glycerol.
[e] 60 min after addition of 100 mM galactose to *gal1* P*GAL10-$RAS2^{V19}$* (constitutive active) cells growing in 2% glycerol.
[f] 20 min after addition of 100 mM glucose to *gal1* P*GAL10-$RAS2^{24N}$* (dominant negative) pregrown for 1 hr on galactose.
[g] 60 min after addition of 100 mM galactose to *gal1* P*GAL10-SCH9* cells growing in 2% glycerol.
[h] 20 min after addition of 100 mM glucose and 100 nM 1MN-PP1 to *$tpk1^{as}$ $tpk2^{as}$ $tpk3^{as}$ $sch9^{as}$* cells growing in 2% glycerol. *$tpks^{as}$* and *$sch9^{as}$* are analog-sensitive alleles encoding proteins which can be selectively inhibited by the ATP analog 1NM-PP1 (C3-1'-naphthyl-methyl PP1). *HXT2* expression in the preculture is 5 fold higher in the *$sch9^{as}$* strain than in wild type.
[i] 20 min after addition of 0.4 mM nMe-PP1 to *$snf1^{as}$* cells grown in 2% glycerol. *$snf1^{as}$* is an analog-sensitive allele which can be inhibited by the ATP analog nMe-PP1 (4-methylnapthyl-1-tert-butyl-3-phenylpyrazolo[3,4-d] pyrimidine).
[l] 2 hrs after shift from SC+2% glucose to SC+2% glycerol.
[m] 2 hrs after shift of *$snf1^{as}$* cells from SC+2% glucose to SC+2% glycerol+1 nMe-PP1.
[n] 20 min after addition of 100 mM glucose to *gpr1* cells growing in 2% glycerol. *HXT2* expression in the preculture is 6 fold higher in the *gpr1* strain than in wild type.
[o] 20 min after addition of 100 mM glucose to *rgt2* cells growing in 2% glycerol.
[p] 60 min after addition of 100 mM galactose to *gal1* P*GAL10-RGT2-1* (constitutive active) cells growing in 2% glycerol.

A recent global transcriptomic analysis has substantially confirmed the complex picture described above for the regulation of *HXT1* expression (Table II; Zaman et al., 2009). Surprisingly, the study showed that *HXT1* is induced equally by high and low glucose (Table II). The induction of *HXT1* can partially be recapitulated even in absence of glucose by activation of the cAMP/PKA pathway (via the $RAS2^{V19}$ constitutive active allele) and nearly fully recapitulated by the constitutive allele *RGT2-1* or by overexpression of *SCH9* (encoding a kinase which can suppress the lethality caused by the loss of PKA activity and that has been recently found to act as effector of the TOR network in the regulation of ribosomal biogenesis; Toda et al., 1988; Urban et al., 2007; see following sections). Expression of *HXT1* is reduced upon rapamycin treatment, which blocks TOR signalling. *HXT1* Induction by glucose is partially eliminated by concurrent inactivation of *SCH9* and *PKA* pathway. *HXT1* transcription is repressed upon glucose downshift, an event that is partially lost in the absence of Snf1 kinase activity (Zaman et al., 2009).
The properties and the expression profile of *HXT1* qualify it as an important carrier under conditions of extremely high sugar concentrations, like in grape must, one of the natural habitats of *S. cerevisiae* (Boles & Hollemberg., 1997).

II) HXT3: Induction of Transcription by Glucose Independent of Sugar Concentration

HXT3 is a 567 low-affinity hexose transporter (*Km* =60 mM) originally identified along with *HXT1* (its closest relative, with 86.4% identity) as a suppressor of the potassium import defect in *trk1 trk2* cells (Ko et al. 1993). As already seen with *HXT1,* an *hxt*-null strain expressing *HXT3* as sole hexose carrier does not grow on low-glucose medium, while it exhibits growth and sugar consumption rate similar to wild type cells at high glucose concentrations (Table I: Reifenberger et al., 1995; Reifenberger et al., 1997).
HXT3 promoter activity is induced about 10-fold in presence of glucose but is independent of sugar concentration (Ozcan et al., 1995; Diderich et al., 1999). When glucose is absent, *HXT3* is repressed by Rgt1. The modest (about 3-fold) increase in *HXT3* expression evidenced by several strains defective in glucose repression mechanisms (such as *mig1*, *ssn6*, and *tup1*) suggests that this pathway may play a minor role in regulating *HXT3* expression (Ozcan et al., 1995). Since the *HXT3* promoter contains several potential binding sites for Mig1, this transcriptional repressor may play a direct role in the process.
A recent transcriptomic study has shown that the transcriptional regulation of *HXT1* and *HXT3* is quite similar (Table II; Zaman et al., 2009; see previous *HXT1* section for details).
Together with Hxt1, Hxt3 allows glucose import when sugar availability is high. In addition, Hxt3. seems well suited to support sugar uptake after addition of large amounts of glucose to cells growing on low glucose or to stationary phase cells (Boles & Hollemberg., 1997).

III) HXT2 and HXT4: Induction of Transcription by Low Levels of Glucose

Hxt2 consists of a 541aa transporter with relatively high affinity for glucose (K_m=1.5 mM), while Hxt4 is a 576 carrier endowed with a moderate affinity for glucose (K_m= 9 mM (Table I; Reifenberger et al. 1997; Maier et al. 2002)). *HXT2* and *HXT4* were cloned as multicopy suppressors of the high-affinity glucose uptake defect in a *snf3* mutant (Kruckenberg & Bisson, 1990; Theodoris et al., 1994).

Consistent with their characteristics as high or intermediate affinity carriers, *HXT2* can restore growth both in high and low glucose media when expressed in a *hxt*-null strain, in contrast to *HXT4*, which support growth on high glucose only (Reifenberger et al., 1995). Surprisingly, the glucose uptake kinetic parameters of *HXT2*-only cells seem to depend on the growth conditions: when expressed as the sole hexose carrier, *HXT2* confers on the *hxt*-null mutant a biphasic uptake kinetics with a high- *(K_m,* = 1.5 mM) and a low-affinity component *(K_m* = 60 mM) during growth on low glucose, whereas the K_m value registered in high-glucose grown cells is about 10mM (Reifenberger et al., 1997; Maier et al., 2002).. Therefore, the affinity of the Hxt2 transporter is apparently modulated in response to the availability of the glucose. As an alternative interpretation, Hxt2 may control the expression (activity?) of one of the less characterized carriers encode by *HXT(8-17)*. Several other evidences suggest that Hxt2 may be posttranslationally regulated: for example, when expressed constitutively in a *ssn6* strain, Hxt2 contributes to high affinity glucose uptake in low but not in high-glucose media, suggesting that low levels of glucose are required to activate the Hxt2 transporter function (Wendell & Bisson., 1994).

HXT2 and *HXT4* are expressed at reduced level when glucose is absent or abundant, whereas their transcription increases approximately 10-fold on low levels of glucose (0.1%) and on raffinose (a trisaccharide consisting of fructose-glucose-galactose that is considered equivalent to low glucose, since most laboratory strains of *S. cerevisiae* can only inefficiently cleave the fructose-glucose- bond through invertase, releasing a modest amount of fructose in the process).

The transcriptional profile of *HXT2* and *HXT4* is the result of the combined activity of the Snf3-Rgt1 induction pathway and the Snf1-Mig1 repression circuit, which both respond to glucose availability. Accordingly, the *HXT2* and the *HXT4* promoters contain two Rgt1 binding sites and two Mig1 binding sites (Ozcan et al., 1996a, b).

Under complete glucose deficiency, the repressor Rgt1 binds to the *HXT2* and *HXT4* promoters preventing their transcription. At high concentrations of sugar, the expression of the two transporters is blocked by the Mig1 repressor, which is responsible for repression of many other glucose repressible genes (see below). Only at low concentrations of glucose (roughly, from 0.05 to 0.4%) both the repressors are inactive, resulting in enhanced expression of *HXT2* and *HXT4* (Ozcan and Johnston., 1996a, b),).

Deletion of *RGT1* causes *HXT2* and *HXT4* to be expressed in the absence of glucose but has no effect on Mig1-mediated repression of the two genes at high glucose concentrations. Conversely, deletion of *MIG1* makes transcription of *HXT2* and *HXT4* inducible by high levels of glucose but has no effect on the repression exerted by Rgt1 in the absence of sugar. Release from Mig1 repression requires the activity of the Snf1 kinase, which in cells growing on low glucose phosphorylates the repressor forcing its exit from the nucleus: accordingly, *HXT2* and *HXT4* induction by low glucose is completely abolished in a *snf1* strain due the constitutive activity of Mig1 (Ozcan et al., 1995; see below); conversely, the *snf1* deletion has no effect in the induced expression of the low affinity carrier Hxt1 in response to a high glucose signal (Ozcan & Johnston, 1995); Tomas-Cobos et al., 2002).

Repression exerted by Rgt1 and Mig1 requires Ssn6 and Tup1 (Ozcan & Johnston, 1995), two general repressor of transcription involved in the establishment of repressive chromatin structure through interactions with histones H3 and H4. Ssn6

and Tup1 do not bind DNA directly but are recruited to diverse promoters by several DNA binding proteins, including Rgt1 and Mig1 (Keleher et al., 1992; Williams et al., 1991). Transcription of *HXT2* and *HXT4* is constitutive (carbon source independent) in an *ssn6* mutant, since this deletion relieves repression by both Rgt1 and Mig1. (Ozcan & Johnston., 1999; Keleher et al., 1992; Treitel & Carlson, 1995; Tzamarias & Struhl., 1995; Tzamarias & Struhl., 1995; Williams et al., 1991).
Thus, the interplay of two different regulatory circuits that respond differently to the same environmental signal -glucose availability- ensures that *HXT2* and *HXT4* expression is induced only when the extracellular levels of glucose are low.
A substantial upregulation of *HXT2* has been observed in a strain expressing Hxt2 as the only functional glucose transporter (Kruckeberg et al., 1999).
Interestingly, *HXT2* (as well as *HXT7*) transcription is rapidly and potently induced after exposure to severe alkaline stress: this effect is mediated by the Snf1 kinase pathway and by calcineurin, a calcium-activated Ser/Thr protein phosphatase essential for survival under diverse stress conditions (Ruiz et al., 2008). Accordingly, the *HXT2* promoter contains a functional CDRE element (calcineurin-dependent response elements), which is recognized by the transcription factor Crz1, the prominent effector of calcineurin. *HXT2* upregulation after sudden increase of the medium pH is probably the consequence of the impaired glucose metabolism under this stress condition: in fact, besides *HXT2* and *HXT7*, a substantially large number of genes involved in carbon metabolism that are typically induced by low glucose are also upregulated upon exposure to alkaline stress (Ruiz et al., 2008).
Consistent with previous studies described above, a recent transcriptomic analysis has shown that *HXT2* transcription is induced by low (but not high) glucose and following activation of *RAS2* (but not by overespression *SCH9)*. The study also showed that the expression *HXT2* is elevated in *gpr1* or *sch9*^{as} strains during growth on glycerol, whereas this enhanced expression becomes repressed after the addition of glucose. *HXT2* Expression is also strongly repressed by rapamycin.
The transcriptional regulation of *HXT4* is quite similar to HXT2; furthermore, in the absence of Snf1 function *HXT4* is hyper-induced after a glucose-glycerol downshift (Table II; Zaman et al., 2009).
Hxt2, together with the other high affinity carriers, Hxt6, and Hxt7, plays a major role in the sugar uptake process during conditions of scarce glucose supply: consistently, its expression is maximally induced in low glucose media, the condition where the transporter is most useful (Boles & Hollemberg, 1997).
On the contrary, the transcriptional regulation of *HXT4* seems to be partially inconsistent with its characteristics as a transporter with only intermediate affinity for glucose, which is particularly suited to be used in presence of moderately high sugar concentrations (Boles & Hollemberg, 1997).

IV) HXT6 and HXT7: Repression by High Levels of Glucose

Hxt6 and Hxt7 are two highly related high affinity transporters, differing by only two amino acids over their entire 570 amino acid sequences (Reifenberger et al. 1995). Their encoding genes are arranged in tandem on the right arm of chromosome IV, immediately downstream of *HXT3*. The high similarity between these two genes extends up to 96bp upstream the start codon; further upstream, the two sequences diverge. In some strains *HXT6* and *HXT7* are fused into a single chimeric gene in which the promoter is derived from *HXT7* and the coding sequence

from the *HXT6* ORF (Liang & Gaber., 1996; Boles & Hollemberg, 1997; Ozcan & Johnston., 1999).
With a K_m value of approximately 1-2 mM, Hxt6 and Hxt7 display the highest affinity for glucose of the entire *HXT* group (Maier et al., 2002; Reifenberger et al. 1997). Consistently, both the carriers restore growth on low glucose concentration when expressed in a *hxt*-null strain and complement the sucrose fermenting defect of *snf3* cells.
Although the transcription of *HXT6* and *HXT7* is regulated similarly, the expression level of *HXT7* seems to be much higher, at least in some common laboratory strains (CEN.PK, MC996) (Diderich et al., 1999; Reifenberger et al., 1997; Reifenberger et al., 1995). However, the relative contributions of *HXT6* and *HXT7* to high-affinity glucose uptake may be strain-dependent (Liang and Gaber, 1996).
In wild-type cells, the transcription of *HXT7* (and *HXT6*) is strongly repressed by high concentrations of glucose, but increases as the sugar level declines and cells approach the diauxic shift (Diderich et al., 1999; Ye et al. 2001). In contrast to the other *HXT* genes, *HXT6/7* exhibit a high basal level of expression on non-fermentable carbon sources such as ethanol and glycerol (derepressed condition) and are only modestly induced (2-3fold) by low levels of glucose or raffinose (Diderich et al., 1999; Dlugai et al., 2001; Schulte et al., 2000; Liang & Gaber, 1996). The inhibition of *HXT6*/*7* transcription by high glucose is likely mediated by the main repression pathway operating through Mig1, while the modest induction in response to low levels of sugar involves the Snf3/Rgt1 pathway. (Ozcan & Johnston., 1999)
In addition, Snf3 and other components of the glucose induction pathway (for instance, Grr1) are required to support the high basal activity of the *HXT7* promoter during growth on ethanol (Dlugai et al., 2001). Interestingly, Snf3 seems to be necessary not only for the maximal transcription of *HXT6* on low glucose, but also to maintain the long-term repression of *HXT6* in response to a high glucose signal (Liang & Gaber, 1996): this is rather curious, since *HXT6* is the only known gene whose repression by glucose requires Snf3 (Ozcan & Johnston, 1999; Boles and Hollemberg., 1997).
Glucose repression of *HXT7* is substantially relieved in a strain expressing Hxt7 as the only glucose carrier, maybe as a consequence of the reduced transport capacity (and resulting decrease in intracellular level of glucose) observed in this mutant: accordingly, the transcription of *HXT7* is constitutive in these cells throughout the high-glucose growth phase and increases further when the sugar levels decline (Ye at al., 2001). *HXT7*-only cells can grow with a near-wild type rate on low glucose concentrations, while growth on high glucose is somewhat reduced (Reifenberger et al., 1995; Reifenberger et al., 1997; Ye et al., 1999; Ye et al., 2001). In contrast, growth of a *HXT6* only strain is strongly impaired on all glucose concentrations, probably as a consequence of the low expression level of *HXT6* in the MC996 genetic background (Reifenberger et al., 1995; Reifenberger et al., 1997).
A recent transcriptomic study has confirmed that both *HXT6* and *HXT7* are highly repressed on high glucose and modestly induced on low glucose (Table II; Zaman et al., 2009). Their transcription genes is not affected by $RAS2^{V19}$ but are strongly repressed by *SCH9* overexpression. The genes are also repressed by rapamycin treatment (Table II). Glucose mediated repression of *HXT6* and *HXT7* requires PKA activity; since it is prevented in strains expressing a dominant negative allele of *RAS2* ($RAS2^{24N}$) or analog-sensitive alleles of PKA *($tpk1^{as}$ $tpk2^{as}$ $tpk3^{as}$,* encoding variants of PKA kinase catalytic subunits which can be selectively inhibited by an

ATP analog). *HXT6* and *HXT7* transcription is strongly induced upon a glucose/glycerol downshift in a process that doesn't require Snf1 function (Table II; Zaman et al., 2009).
Because of their high affinities for glucose and the peculiarities of their regulation, Hxt6 and Hxt7 are well suited for the rapid uptake of glucose after it has been supplied to yeast growing on alternative carbon sources. Moreover, high-affinity hexose transport is required for efficient utilization of low sugar concentrations occurring in the late exponential phase which precedes the diauxic shift or during growth on sugars such as sucrose and raffinose that are hydrolyzed to monosaccharides outside the cell.

V) HXT5: regulation by growth rate (independent of extracellular glucose concentration)

HXT5 encodes a 592aa hexose transporter with moderate affinity for glucose (K_m =10mM; Maier et al., 2002; Reifenberger et al., 1997; Table I) that is maximally expressed under stress conditions that cause slow growth such as increases in temperature or osmolarity and carbon or nitrogen starvation (Diderich et al., 2001; Verwaal et al., 2002; Buziol et al., 2002; Buziol et al., 2008). *HXT5* is also highly transcribed during growth on non-fermentable carbon sources, following glucose depletion and even in stationary phase cells (Diderich et al., 1999; Diderich et al., 2001; Verwaal et al., 2002; Greatrix et al., 2006).
Given its peculiar expression profile, Hxt5 does not contribute significantly to glucose uptake under normal growth conditions and a *HXT5*-only expressing strain exhibits almost no growth on glucose medium (Reifenberger et al., 1995).
The induction of *HXT5* when glucose is exhausted correlates with the decrease in growth rate but is not dependent on the pathway involving Snf3/Rgt2 (Verwaal et al., 2002). Additionally, *HXT5* expression is not strictly subjected to the activity of the main glucose repression pathway operating through Snf1/Mig1 (Verwaal et al., 2002; Verwaal et al., 2004): in fact, deletion of *HXK2*, which is known to impair the glucose repression mechanisms, leads to the constitutive transcription of the high affinity transporters *HXT2* and *HXT7* even in presence of abundant sugar, but has no effect on the *HXT5* transcript level in high glucose (Petit et al., 2000).
HXT5 transcription is controlled by several motifs found inside its promoter (Verwaal et al., 2004): *STRE* (Stress REsponse Element, recognized by Msn2/Msn4: Martinez-Pastor et al., 1996), *HAP* (Hap2/3/4/5p) and *PDS* (post diauxic shift, bound by Gis1: Pedruzzi et al., 2000) elements. In particular, the *STRE* element most proximal to the translation initiation site seems to be involved in the induction of *HXT5* expression upon decreases in the cellular growth rate, while the *HAP* elements are required for *HXT5* transcription in glucose deprived cells or during growth on non-fermentable carbon source. The *PDS* element and a second *STRE* motif show specific involvement in regulation of *HXT5* transcription on ethanol medium (Verwaal et al., 2004). Since many *STRE* regulated genes are known targets of the cAMP/PKA signaling circuit, which represses their transcription by inhibiting the activity of the Msn2/Msn4 transcriptional factors, a possible involvement of this pathway in the regulation of *HXT5* has been proposed: accordingly, deletion of *RAS2*, which causes a low activity of the cAMP/PKA pathway, significantly derepresses *HXT5* transcription.
A recent microarray analysis has shown that *HXT5* transcription is partially repressed at low glucose concentrations and even more strongly inhibited in

presence of high glucose (Table II; Zaman et al., 2009). Glucose repression of *HXT5* requires PKA activity, since it is prevented in strains expressing the $RAS2^{24N}$ dominant negative mutant or analog-sensitive tpk^{as} alleles (Table II; Zaman et al., 2009). The transcriptional repression of *HXT5* can be fully recapitulated by $RAS2^{V19}$ or by *SCH9* overespression (Table II). *HXT5* is induced by rapamycin treatment or after a glucose-glycerol downshift though a process that doesn't require the activity of the Snf1 kinase (Zaman et al., 2009; Table II).
Consistent with its unique pattern of regulation, Hxt5 may function as a 'reserve' transporter expressed in glucose-deprived cells to ensure that they are able to utilize the sugar rapidly when it becomes available again (Diderich et al., 2001; Verwaal et al., 2002): remarkably, a shift of stationary phase cells to fresh glucose medium results in somewhat slower growth resumption in a *hxt5* deletion strain (Diderich et al., 2001).

Transcriptional Regulation of Other HXT Genes

Information regarding the physiological role and transcriptional regulation of the remaining glucose transporter encoding genes (*HXT8-17*) is still fragmentary.
All of them support growth on glucose when ectopically expressed in a hxt-null strain (Wieczorke et al. 1999) and thus they encode for functional transporters, with the exception of *HXT12*, which appears to be a pseudogene (at least in the CEN.PK genetic background). Some of these carriers may be involved in the uptake of sugars other than glucose (Ozcan & Johnston, 1999).
Apart from *HXT13*, all these *HXT* genes are expressed at very low levels (30- to 300-fold less than *HXT1* and *HXT2)*.
Transcription of *HXT10*, *HXT16* and *HXT17* is repressed to various extents (4- to 16-fold) by glucose, while *HXT8*, *HXT13*, *HXT14*, and *HXT15* are induced three- to six-fold by low levels of glucose and repressed to various degrees by high levels of glucose (Ozcan & Johnston, 1999).
The promoter region of *HXT13* was identified in a screen for targets of the transcription factor Hap2, a regulator of genes involved in mitochondrial respiration (Ozcan & Johnston, 1999). Interestingly, *HXT13* appears to be expressed at higher levels than the other member of the *HXT(8-17)* subgroup and, like all Hap2-regulated genes, is about 10-fold repressed by high concentrations of glucose. Conversely, *HXT13* (and to a lesser extent *HXT15*) transcription is up-regulated during growth on nonfermentable carbon source and it has been suggested that this transporter may assist Hxt5 in its role as "reserve" transporter during glucose starvation (Greatrix et al., 2006).
HXT9 and *HXT11* transcription is entirely independent of extracellular glucose. Rather, their expression is linked to Pdr3 (pleiotropic drug resistance), a transcription activator that regulates drug resistance (Nourani et al. 1997). Inactivation of *HXT11* or *HXT9* confers resistance to cycloheximide (protein synthesis inhibitor), sulfomethuron methyl (acetolactate synthase inhibitor) or 4-nitroquinoline-N-oxide (mutagen).
HXT17 was identified by a microarray experiment as a target of a constitutively active form of the Mac1 transcription factor (Gross et al. 2000), which regulates the expression genes required for high-affinity copper uptake genes under copper-shortage (Jungmann et al. 1993). An interesting hypothesis is that Hxt17 serves as a symporter for particular minerals (such as copper and iron) along with glucose; however, when cells are treated with a copper-specific chelator, *HXT17* is only

marginally induced. Recently, an induction of the *HXT17* promoter activity in response to a shift from pH 4.7 to 7.7 in galactose or raffinose media has also been described (Greatrix et al., 2006).

Transcriptional Regulation of GAL2

The galactose permease encoded by *GAL2* is a 574 polypeptide more than 60% identical to the other *HXT* carriers (Boles & Hollemberg, 1997; Ozcan & Johnston, 1999).

A strain lacking *GAL2* grows poorly on media containing galactose as sole carbon source (Tschopp et al., 1986.). *GAL2* is expressed only when galactose is available, because its transcription requires the galactose-activated transcription factor Gal4 and is repressed in presence of glucose by the Snf1-Mig1 main repression pathway (Ozcan & Johnston, 1999). Gal2 is also subject to glucose-induced inactivation (also known as catabolite inactivation), which consist in the internalization of Gal2 by endocytosis and its subsequent degradation in the vacuole when glucose becomes available (Horak & Wolf, 1997). When expressed from a constitutive promoter, Gal2 can also transport glucose with a high affinity kinetic (*Km* = 2 mM) (Table I; Liang and Gaber., 1996; Reifenberger et al, 1997; Maier et al., 2002).

S. cerevisiae senses glucose and galactose differently (Brown et al., 2008). In contrast to glucose induction of *HXT* gene expression, which is regulated by a receptor-mediated process (where glucose serves as a ligand for the Snf3 and Rgt2 sensors that reside on the membrane), stimulation of transcription by galactose requires the presence of intracellular galactose (Lohr et al., 1995; Ozcan & Johnston., 1999). Galactose induction of *GAL2* is mediated by the Gal3 protein, which functions as sensor and transducer of the galactose signal. Gal3 is similar to galactokinase (Gal1) but lacks any detectable phosphorylation activity (Lohr et al., 1995; Ozcan & Johnston., 1999). Gal3 seems to bind galactose (in an ATP-dependent fashion) and inhibit Gal80 (a repressor of *GAL* genes), thus enabling Gal4 to activate transcription of the *GAL* genes (Blank et al., 1997; Platt & Reece, 1998, Suzuki-Fujimoto et al., 1996; , Yano & Fukasawa, 1997; Zenke et al., 1996). Interestingly, Gal4 also activates the expression of *MTH1*, encoding one of the corepressors critical for Rgt1 activity (Brown et al., 2008).

Transcriptional Regulation of SNF3 and RGT2 Genes

Snf3 and Rgt2 are unusual members of the hexose transporter family, about 70% similar to each other but with only limited sequence homology (less than 30%) to the other *HXT* carriers (Ozcan et al., 1996b; Boles & Hollemberg, 1997; Ozcan & Johnston, 1999). As discussed in other sections, Snf3 and Rgt2 do not appear to transport glucose; instead, they serve as extracellular glucose sensors that generate an intracellular signal to regulate the expression of the major *HXT* hexose transporter. Both genes are expressed at very low levels, about 100 to 300-fold lower than the *HXT* genes (Ozcan et al., 1996b). Consistent with its role as a high-affinity glucose sensor, *SNF3* transcription is repressed at high concentrations of glucose by the Mig1 repressor (Ozcan & Johnston, 1995; Marshal-Carlson et al., 1990; , Neigeborn et al., 1986). Conversely, Rgt2 functions as a low-affinity glucose sensor, and its expression is constitutive (Ozcan & Johnston, 1999; Ozcan et al., 1996b).

Posttranslational regulation of the Hexose Transporters

In addition to the extensive regulation at the transcriptional level, the function of several sugar transporters is regulated posttranslationally.

Inactivation of glucose transport (both low- and high-affinity components) has been observed in nitrogen starved cells (Boles & Hollemberg, 1997).

In the presence of high concentrations of glucose the high-affinity transporters Hxt6 and Hxt7 are subject to glucose-induced proteolytic degradation (catabolite inactivation). Degradation occurs in the vacuole, after internalization of the transporters by endocytosis (Krampe et al, 1998; Ye at al., 2001; Krampe et al., 2002). The components of the ubiquitin machinery are required for the catabolite inactivation of the hexose carriers. However, the initial signal that triggers catabolite inactivation is not generated by the glucose sensors Snf3 and Rgt2 (Krampe et al., 1998). Hxt6 and Hxt7 are also rapidly degraded during nitrogen starvation in the presence of high concentrations of fermentable sugars: in this case, degradation is mainly due to the stimulation of general protein turnover and is not caused by a mechanism specifically triggered by glucose (Krampe et al., 1998; Krampe et al., 2002). Internalization and degradation of Hxt7 in the vacuole are blocked after truncation of its N-terminal or C-terminal domains. Remarkably, the N-terminal domain of Hxt7 contains a stretch of amino acids enriched in proline, glutamic acid, serine and threonine residues reminiscent of PEST, sequences involved in the degradation of various plasma membrane proteins, such as maltose permease (Krampe et al.,. 2002; Menditz et al., 2000).

Glucose induced internalization and vacuolar degradation has been described also for the Hxt2 (Kruckeberg et al., 1999) and Hxt5 transporters (van Suylekom et al., 2007). Interestingly, degradation of Hxt5p seems to occur in an ubiquitination-independent fashion via the endocytic pathway (van Suylekom et al., 2007).

The best characterized example of catabolite inactivation is the glucose-induced degradation of the galactose (Gal2) and maltose (Mal62) permease, which helps to ensure that yeast cells utilize these two sugars only when glucose is unavailable. Glucose-induced inactivation of Gal2 appears to be mediated by its ubiquitination, which targets it to the vacuole where it is degraded (Horak & Wolf, 1997; Ozcan & Johnston., 1999).

Sequences (in particular, a putative PEST motif) in the N-terminal cytoplasmic domain of maltose permease are required for vacuolar proteolysis but not glucose-induced internalization Gadura et al., 2006b). Vacuolar degradation of the maltose permease is also an ubiquitin dependent process that is stimulated by the activity of two glucose-signaling pathways (Menditz et al., 1998; Jiang et al., 1997; Ozcan & Johnston, 1999). One of the glucose-induced signals for maltose permease degradation is generated by the Rgt2p/Snf3p-Rgt1 pathway and thus (as discussed elsewhere in this context) does not require glucose import. Conversely, the second signal requires both glucose transport and metabolism (Jiang et al., 1997): the Glc7–Reg1 protein phosphatase seems to play a prominent role in this process by acting as an upstream activator for the Yck1,2 casein kinases (Gadura et al., 2006a).

Therefore (as already discussed elsewhere) Yck1,2 kinase appears to be the keystone of two independent regulatory pathways (involving the Snf3/Rgt2 sensors and the Glc7–Reg1 phosphatase, respectively) controlling both glucose-induced inactivation and glucose induction (Gadura et al., 2007a; Zaman et al., 2008).

Hexose kinase and glucose transport

S. cerevisiae possesses three glucose phosphorylating enzymes: two hexokinase (Hxk1 and Hxk2) and a single glucokinase (Glk1). Of the three, Hxk2 is the main phosphorylating enzyme in cells growing in abundant glucose and also plays a decisive regulatory role in the glucose repression phenomenon (for recent reviews, see Santangelo, 2006; Gancedo, 2008). Sugar kinases appear to influence glucose transport at several levels.

A triple kinase mutant (*hxk1 hxk2 glk1*) lacks high-affinity glucose uptake, which is restored by introduction of either *HXK1* or *HXK2* or *GLK1*: this suggests that glucose phosphorylation is a necessary step for high-affinity uptake (Bisson & Fraenkel, 1983a). However, high-affinity uptake of 6-deoxyglucose, a non-phosphorylable glucose analog, is also kinase-dependent, suggesting that the hexokinases may play a role in glucose uptake that is distinct from their catalytic function (Bisson & Fraenkel, 1983b; Ozcan & Johnston., 1999)

Hxk2 is required for the full induction of *HXT* expression by both low and high levels of glucose (Ozcan & Johnston, 1995; Belinchon & Gancedo, 2007a), suggesting that it might be involved in generating or transducing an intracellular glucose signal, although the exact molecular details of this mechanism remains unclear. It has been proposed that Hxk2 may be required to inactivate the kinase Snf1, since the activity of Snf1 inhibits *HXT1* expression (Tomas-Cobos & Sanz, 2002).

Albeit strongly reduced, a partial degree of induction (about ten-fold, about 20% of wild type strain)) of *HXT1* is detectable even in the absence of glucose phosphorylation (*hxk2 hxk1 glk1*) triple null strain) and is similar to that measured in a *hxk2* mutant: therefore, the release of *HXT1* promoter from repression exerted by Rgt1 (Snf3/Rgt2 pathway: Ozcan & Johnston, 1995) and Sko1(HOG pathway: Tomas-Cobos et al., 2004) does not require glucose metabolism (Belinchon & Gancedo, 2007a).

Interestingly, a *hxk2* deficient strain exhibits high-affinity hexose transport during growth on high glucose medium: this behavior is associated with an increased transcription of the *HXT2* and *HXT7* genes, encoding high-affinity transporters, and a diminished expression of *HXT1* and *HXT3*, which, encode low-affinity carriers (Petit et al., 2000).

Glucose sensing pathways: the Snf1 network

A second glucose-sensing network affecting the expression of sugar transporters is the main glucose-repression pathway (Fig. 13). In the presence of glucose, high-affinity glucose transport is repressed together with a broad range of other genes involved in the utilization of alternative carbon sources, gluconeogenesis and respiration through a process known as 'glucose repression'.

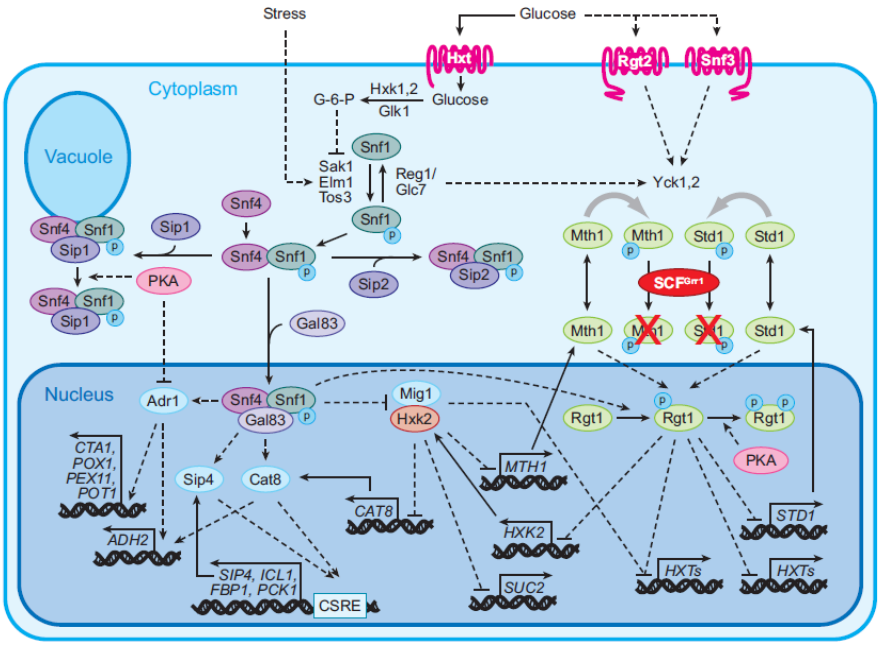


Figure 13. The interlocking Snf1 and Snf3/Rgt2 glucose sensing circuits.
Glucose regulates genes involved in carboxylic acid metabolism and fatty acid β-oxidation by inhibiting the Snf1 kinase and promotes the hexose transporter and hexokinase genes by inactivating the Rgt1 co-repressors Mth1 and Std1. These two pathways are interconnected at various points and are also influenced by PKA. (From Zaman et al., 2008)

Glucose repression: the role of the Snf1 kinase

The major downregulating effects of glucose take place at the transcriptional level Glucose represses the expression of a large number of genes, including those involved in the utilization of alternative carbon sources, gluconeogenesis and respiration through a process known as "glucose repression" (Carlson, 1999; Hedbacker & Carlson, 2008; Santangelo, 2006; Zaman et al., 2008). This mechanism involves not only the repression of transcription when glucose is available, but also the release from the glucose-repressed state when the sugar becomes limiting. A central component in the signaling pathway for the glucose repression is the Snf1 kinase (Fig. 13; Hedbacker & Carlson, 2008).

Snf1 is primarily required for the adaptation of yeast cell to glucose limitation and for growth on alternative carbon sources such as sucrose, galactose, and ethanol (Hedbacker and Carlson, 2008). Snf1 is also involved in the cellular response to various environmental stresses, including heat-shock, alkaline pH, oxidative, saline and genotoxic stress (Hong & Carlson., 2007) and has a prominent role in a number

of cellular processes, such as meiosis, pseudohyphal growth, aging and glycogen accumulation. Snf1 affects yeast regulatory processes through a variety of mechanisms, including a major remodeling of the transcriptional program and direct effects on the activity of metabolic enzymes (Hedbacker & Carlson, 2008; Santangelo, 2006; Zaman et al., 2008).

The Snf1 protein kinase complex in yeast

The Snf1 protein kinase complex (for recent reviews, see Hedbacker & Carlson, 2008; Zaman et al., 2008; Santangelo et al., 2006) is the yeast homologous to mammalian AMP-activated protein kinase (AMPK; Mitchelhill et al., 1994) and like its mammalian counterpart is a heterotrimer composed of an *α*-catalytic subunit (Snf1), a *β*-scaffold subunit (encoded in yeast by *GAL83*, *SIP1*, and *SIP2*) and a *γ*-regulatory subunit (Snf4). Snf4 binds to Snf1 and, during growth in the absence of glucose, alleviates the autoinhibition exerted on the Snf1 catalytic domain by the C-terminal regulatory domain. The Sip1, Sip2 and Gal83 proteins maintain association of Snf4 with the Snf1 kinase and confer specificity to the kinase complex, possibly through regulation of its subcellular localization. Deletion of either *SNF1* or *SNF4* impairs growth on carbon sources other than glucose, a phenotype referred to as "sucrose nonfermenting" (*snf*). The three β-scaffold-subunits are largely redundant, since only the concurrent inactivation off all their three encoding genes yields a *snf* phenotype (Hedbacker & Carlson, 2008; Zaman et al., 2008; Santangelo et al., 2006).

The uncertain nature of glucose repression signal

In accord with its central role in adaptation to glucose depletion and utilization of alternative carbon sources by S. cerevisiae, Snf1 is activated in response to glucose limitation. However, the actual signal(s) triggering its activity has not yet been identified (Hedbacker & Carlson, 2008).

Its mammalian homologue AMP-Kinase maintains cellular energy homeostasis by stimulating glucose uptake and lipid oxidation and by inhibiting ATP consuming processes when the cellular AMP:ATP ratio increases: the AMP kinase complex is stimulated by direct allosteric activation of the γ- subunit by AMP (Hedbacker & Carlson, 2008; Santangelo et al., 2006; Zaman et al., 2008).

However, yeast Snf1 is not allosterically activated by AMP, although its activity correlates remarkably well with the AMP:ATP (and ADP:ATP) ratio, which rapidly increases more than 200-fold upon glucose removal (Wilson et al., 1996; Rolland et al., 2002; Hedbacker & Carlson, 2008; Johnston, 1999). Nevertheless, it should be noted that AMP and ATP levels are quite similar during growth on glycerol and glucose media (Rolland et al., 2002).

The signal for glucose repression seems to be generated by metabolism of glucose (Hedbacker & Carlson, 2008; Santangelo et al., 2006; Zaman et al., 2008). This idea is consistent with the observation that hexokinase 2 (Hxk2), the enzyme primarily responsible for catalyzing the first step of glycolysis when glucose is abundant, plays a major –although enigmatic- role in glucose repression (de Winde at al., 1996; Gancedo, 2008; see below) Moreover, several lines of evidence suggest that Hxk2 may act as a transcriptional corepressor to inactivate the expression of genes like *SUC2* in presence of large amounts of glucose (Ahuatzi et al., 2004; Moreno et al., 20p05; Ahuatzi et al., 2007; see below)

Establishment of the glucose repression requires glucose phosphorylation but apparently no further sugar metabolism, as shown by experiments with 2-deoxyglucose (a glucose analogue that can be phosphorylated but not further metabolized), which is able to trigger repression (Rolland et al., 2002).
Glucose uptake is required to activate the repression machinery, but the diverse hexose carriers do not have a specific regulatory role in the process; instead, they are only required to provide enough sugar to sustain phosphorylation (Reifenberger et al., 1997). The level of glucose repression correlates well with the sugar-transport capacity and the glycolytic flux rate: accordingly, strains with impaired glucose uptake system show constitutive expression of many glucose-repressible genes, such as *SUC2* or *GAL1*.(Reifenberger et al., 1997; Ye et al., 1999; Elbing et al., 2004a; Otterstedt et al., 1994; Ozcan, 2002). Indeed, a strain expressing a chimeric Hxt1-Hxt7 sugar transporter as its sole hexose carrier has a strongly reduced glucose uptake capacity and exhibits a fully respiratory metabolism even in high glucose media, switching to fermentation only under anaerobic conditions (Otterstedt et al., 2004) Thus, glucose repression is fully operative only when cell posses sufficient glucose transport capacity to achieve a high glycolytic flux.

Regulation of Snf1 activity
Snf1 is active and localizes in the nucleus upon phosphorylation on threonine 210 by upstream kinases when glucose is depleted in the medium (McCartney & Schmidt, 2001). Addition of sugar converts the Snf1 kinase into an inactive, autoinhibited state by promoting the dephosphorylation of the T210 residue through the Reg1/Glc7 phosphatase complex. The subcellular localization of Snf1 is also subject to extensive regulation (Hedbacker & Carlson, 2008).
Snf1 comprises an N-terminal catalytic domain and a C-terminal regulatory region. In presence of abundant glucose, the regulatory domain binds to the catalytic domain, maintaining Snf1 in an autoinhibited conformational state. When glucose is exhausted, Snf4 counteracts autoinhibition of Snf1 by interacting with its regulatory domain and triggering a conformational change that results in activation of Snf1 (reviewed in Hedbacker & Carlson, 2008). The key event in the entire process is the phosphorylation of a conserved threonine (T210) in the activation loop of Snf1 by any one of three upstreams kinases, encoded by *SAK1, ELM1*, and *TOS3* (Nath et al., 2003; Sutherland et al., 2003; Hong et al., 2003).
Loss of this conserved threonine via a T210A mutation impairs Snf4 binding and prevents the activation of Snf1 (Jiang et al., 1996; Ludin et al., 1998); in addition, the T210A substitution alters the normal subcellular localization of Snf1, blocking the nuclear accumulation of the kinase that occurs after a nutritional shift from high to low glucose (Hedbacker et al., 2004).
The three upstream kinases are redundant in their Snf1-activating capacity, although Sak1 seems to play the prominent role in the regulatory process (Nath et al., 2003; Sutherland et al., 2003; Hong et al., 2003). Inactivation of *SAK1* leads to the largest decrease in Snf1 kinase activity and is associated with a strong reduction in glycogen accumulation, comparable with the one exhibited by *snf1* null mutants. Furthermore, in *sak1* deficient cells Snf1-Gal83 complexes maintain a cytoplasmic distribution even upon glucose depletion (Hedbacker et al., 2004).
The contributes of Tos3 and Elm1 to the activation of Snf1 are more variable depending on the carbon source availability. Tos3 plays a more active role during growth on non-fermentable carbon sources: accordingly, *tos3* mutants display

reductions in growth rate and Snf1 activity only on ethanol/glycerol media (Kim et al., 2005). Interestingly Tos3 is a direct target of the activated Snf1 (Kim et al., 2005; Hedbacker & Carlson, 2008).
Sak1 associates with Snf1 to form a stable complex, whereas the interaction of Tos3 and Elm1 with Snf1 is apparently transient (Elbing et al., 2006). The stable interaction between Snf1 and Sak1 is not regulated by glucose (Hedbacker & Carlson, 2008). Most of the Sak1 protein in the cell is apparently associated with Snf1, while only a fraction of the total Snf1 interacts with Sak1 (Elbing et al., 2006). During growth in glucose media, Sak1 resides in the cytoplasm, but exhibits some relocalization to the vacuolar membrane upon glucose exhaustion (Hedbacker et al., 2004). Tos3 distribution is always cytosolic (Kim et al., 2005), while Elm1 localizes to the bud neck (Bouquin et al., 2000), consistent with its roles in processes like cell morphology, septin organization, and cell cycle progression and cytokinesis, most of which do not depend on Snf1 (Bouquin et al., 2000, Blacketer et al., 1993; Sreenivasan et al., 1999; Sreenivasan et al., 2003).
The type 1 protein phosphatase complex, comprising the Glc7 catalytic subunit and the Reg1 regulatory subunit, counteracts the activation of Snf1 mediated by the upstream kinases (Tu & Carlson, 1995; Sanz et al., 2000; Ludin et al., 1998). When large supply of glucose becomes available, Reg1 interacts with the kinase domain of the active Snf1 complex and directs Glc7 to the activation loop of Snf1, resulting in the dephosphorylation of the Thr210 residue and the subsequent inactivation of Snf1 (McCartney&Schmidt, 2001).
In *reg1* deficient cells the activation loop of Snf1 is hyperphosphorylated and the Snf1 catalytic activity is constitutive and resistant to glucose inhibition (Hong et al., 2005). Deletion of *SAK1* suppresses many of the Snf1-dependent phenotypes observed in *reg1* null cells (Nath et al., 2003).
The critical regulatory interaction between Reg1 and the Snf1 complex was presumed to occur in the nucleus, since that is where the activated Snf1 complex is mainly located–and Glc7 is also known to be largely nuclear in growing cells. However, Reg1 is cytoplasmic and is excluded from the nuclear compartment independently of the carbon source available (Dombek et al., 1999). To resolve this discrepancy it has been suggested that active Snf1 shuttles rapidly between the nuclear and cytoplasmic compartments, allowing inactivation by Reg1/Glc7 to occur in the cytoplasm when glucose becomes available (Dombek et al., 1999).
It is not yet entirely clear whether the activities of the upstream activating kinase and/or of the Reg1/Glc7 phosphatase are regulated or not (Hedbacker & Carlson, 2008).
Reg1 expression, localization, and interaction with Glc7 do not appear to be carbon source–modulated, but the activity of the Reg1- Glc7 complex may be regulated posttranslationally: indeed, Reg1 is phosphorylated in a Snf1 dependent way in response to glucose shortage, whereas a rapid dephosphorylation of Reg1 occurs (likely mediated by Glc7) when sugar is added back to the growth medium (Sanz et al., 2000; Hedbacker & Carlson, 2008). Phosphorylation of Reg1 by Snf1 appears to stimulate both the Glc7 mediated inactivation of the Snf1 and the release of Reg1-Glc7 from its association with the Snf1 kinase complex (Sanz et al., 2000). Interestingly, the phosphorylation status of Reg1 appears to be regulated also by Hxk2, the major glucose phosphorylating enzyme which participates to the glucose repression mechanism (Sanz et al., 2000; see below).

Several lines of evidence suggest that the Snf1 upstream activating-kinases are not regulated by changes in the glucose levels (Hong et al., 2005; Hedbacker & Carlson, 2008). For instance, heterologous expression of several mammalian AMPK-activating kinases (LKB1, CaMKK alpha, or TAK1) in a strain deficient of all the endogenous upstream kinases (*sak1 tos3 elm1*) restores glucose-dependent regulation of Snf1 activity (Hong et al., 2005; Hedbacker & Carlson, 2008)... In contrast, a *sak1 tos3 elm1 reg1* quadruple null mutant strain expressing mammalian activating kinases exhibits a glucose-insensitive Snf1 activity (Hong et al., 2005). Although it is tempting to speculate that the mammalian upstream kinase homologue can sense energy status in both yeast and mammalian cells, a simpler model to reconciliate these two observations is that the upstream kinases are constitutively active, while the function of Reg1-Glc7 towards Snf1 is positively regulated by glucose signals. Indeed, a recent study has confirmed that the SAKs (upstream kinases) are active regardless of growth conditions (Rubenstein et al., 2008). In contrast, the dephosphorylation of the Snf1 activation loop is strongly stimulated in presence of high glucose levels. However, the activity of the Glc7-Reg1 phosphatase does not appear to be directly influenced by glucose, since the Glc7-Reg1 enzyme seems to be equally active in both high and low glucose: instead, under conditions of glucose limitation, the dephosphorylation of the Snf1 activation loop is apparently inhibited by an unknown factor (*X*), that limits the accessibility of the loop to the Reg1-Glc7 phosphatase (Fig. 14; Rubenstein et al., 2008).
In addition, Snf1 activity in *sak1 tos3 elm1* cells expressing mammalian CaMKK alpha responds normally to both saline and alkaline stress: this result suggest that these stress signals regulate Snf1 activity through a mechanism that bypasses the upstream kinases (Hong et al., 2005).

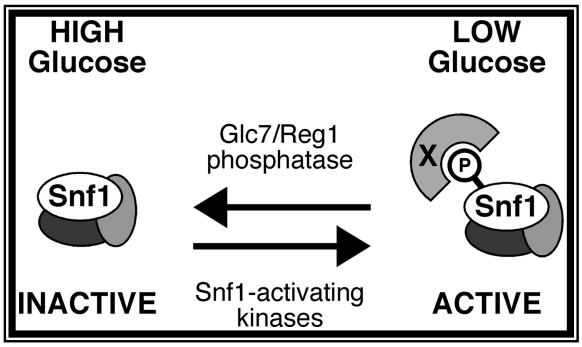


Figure 14. Model for the regulation of Snf1 kinase at the level of dephosphorylation.
In high glucose media, the Snf1 kinase is largely unphosphorylated and inactive due to the accessibility of the activation loop threonine to the Glc7-Reg1 phosphatase. Under conditions of glucose limitation, the dephosphorylation of the Snf1 activation loop is inhibited by an unknown factor (X), leading to the accumulation of phosphorylated and active Snf1 kinase.
(From Rubenstein et al., 2008).

Regulation of Snf1 subcellular localization
The activity of the Snf1 complexes is also regulated through a beta-subunit–dependent regulation of their subcellular localization (Hedbacker & Carlson, 2008).
During growth in glucose media, all the Snf1 complexes reside in the cytoplasm, regardless of the β-subunit (Vincent et al., 2001). When glucose becomes limiting, the beta subunits (and their associated complex) show unique subcellular localization patterns, dependent on their divergent N-terminal sequences: Sip2-containing complexes remains cytoplasmic, while Sip1 and Gal83 complexes relocalize to the vacuolar membrane and to the nucleus, respectively. The Snf4

subunit, which is present in excess, is both cytoplasmic and nuclear regardless of nutritional status (Vincent et al., 2001).
The three upstream activating kinases all exhibit a cytoplasmatic subcellular localization that is not affected by carbon source.
The distinct subcellular localization of the Snf1 complexes may indicate that the beta-subunits are not entirely overlapping in their function (Vincent et al., 2001).
In the absence of Gal83, Snf1 distributes relatively uniformly between nucleus and cytoplasm, regardless of the carbon source present in medium (Vincent et al., 2001).
Glucose regulates localization of Gal83-containing complexes by promoting their nuclear export. Gal83 contains a leucine-rich nuclear export signal (NES) in its N terminus, which is required for its cytoplasmic redistribution, and export depends on the Crm1 export receptor (Hedbacker & Carlson., 2006). Apparently, glucose needs to be phosphorylated in order to stimulate nuclear export of the Gal83-containing complexes: in fact, sugar addition fails to induce any Gal83-Snf1 cytoplasmatic redistribution in a *hxk1 hxk2 glk1* strain. However, further glucose metabolism is not required: addition of 2-deoxyglucose, a glucose analog that is phosphorylated but not further metabolized, suffices to localize Snf1-Gal83 complexes to the cytoplasm, whereas 6-deoxyglucose, which cannot be phosphorylated, does not induce any cytoplasmic redistribution (Hedbacker & Carlson, 2006).. Nuclear localization of Snf1-Gal83 complexes depends not only on Gal83 but also on activation of the Snf1 catalytic subunit. Nuclear accumulation of Gal83-Snf1 in the absence of glucose also requires a functional Sak1, the major upstream activating kinase for Snf1 (Hedbacker and Carlson, 2006.
In the *sip1* mutant, no vacuolar localization of Snf1 is evident (Hedbacker et al., 2004). Localization to the vacuolar membrane of the Sip1-complexes requires the N-myristoylation of consensus sequence found in Sip1. Unlike the Gal83-containing complexes, addition of 2-deoxyglucose does not trigger redistribution of the Sip1 complexes to the cytoplasm, suggesting that Sip1 may respond to a signal other than glucose phosphorylation. The localization of Sip1 to the vacuolar membrane is inhibited by protein kinase A (PKA) activity (Hedbacker et al., 2004): in a mutant lacking the three PKA catalytic subunits, Sip1 is constitutively associated to the vacuolar envelope, regardless of glucose availability, while in a mutant with high PKA activity Sip1 is always cytoplasmic, even in ethanol media (Hedbacker et al., 2004).. The physiological role of Sip1-Snf complexes is presently unknown.

To summarize, the Snf1 kinase complex in yeast does not respond directly to changes in the AMP:ATP as its mammalian counterpart. Instead, glucose modulates Snf1 activity by influencing the phosphorylation of the Snf1 activating loop. In addition, glucose exhaustion redistributes the Snf1 kinase complexes from the cytoplasm to the vacuole or the nucleus, where they can perform metabolic and transcriptional alterations. The precise mechanisms by which glucose achieve these regulations remain elusive: further studies are needed to determine if the Snf1-upstream activating kinases or the Reg1-Glc7 protein phosphatase, or both, are glucose-modulated, or instead signals to the Snf1 kinase complex affect its accessibility to the upstream kinases or phosphatase. Equally undefined are the means by which glucose promotes redistribution of Snf1 complexes between the diverse cellular compartments.

Downstream effectors of the Snf1 complex: Transcriptional control in response to glucose limitation

Snf1 regulates the expression of a large number of genes, including those involved in the metabolism of alternative carbon sources, gluconeogenesis, respiration, transport (see *HXT1* section), and meiosis. Early transcriptomic analysis showed that as many as 500 genes are modulated either directly or indirectly through a Snf1-dependent mechanisms in response to glucose depletion (Young et al., 2003; Tachibana et al., 2005), including 29 of the 40 most highly glucose regulated genes. A consistent fraction of Snf1 regulated genes is involved in transcription and signal transduction processes, reflecting the central regulatory role of this kinase (Young et al., 2003).However, only 10% of genes that show alteration of their expression profile in a *snf1* strain are direct targets of transcription factors regulated by Snf1 (Young et al., 2003; Tachibana et al., 2005). A more recent study has partially downsized the specific contribute of the Snf1 network to the overall transcriptional response to glucose: Snf1 seems to mediate only a small portion of the glucose signal and mainly does so in cooperation with other glucose-responsive pathway such as the cAMP/PKA circuit, the primary mediator of the yeast growth response (Zaman et al., 2009). However, although Snf1 regulates a limited number of genes, it specifically mediates a significant branch of the glucose repression mechanism not subject to PKA regulation (Zaman et al., 2009).

Snf1 can exert its effects on transcription through repressors, activators, chromatin remodeling, and also the transcriptional apparatus. Below is a brief summary of the downstream targets of the Snf1 pathway in the transcriptional response to carbon stress.

Snf1 affects the transcription of genes required for metabolism of alternative carbon sources (such as sucrose, galactose, and maltose) mainly by modulating the activity of the Mig1 transcription repressor (Treitel et al., 1995; Luftiyya et al., 1998; Luftiyya & Johnston, 1996; Westholm et al., 2008). Mig1 is a Cys2-His2 zinc finger protein that during growth on glucose binds to a GC-rich consensus sequence found in the promoter of its target genes and represses their transcription by recruiting the general corepressors Ssn6 and Tup1 (Treitel et al., 1995; Tzamarias et al., 1995). In the absence of glucose, Snf1 phosphorylates Mig1 (Treitel et al., 1997) inhibiting the repressor activity (likely by altering the Mig1-Ssn6-Tup1 interaction) and promoting its nuclear export through the.Msn5 importin (DeVit et al., 1997; DeVit & Johnston., 1999). When glucose becomes available, Mig1 is dephosphorylated (possibly by the Glc7 phosphatase?) and reenters the nucleus where it can repress the transcription of its target genes. However, nuclear export does not seem to be strictly necessary to inactivate Mig1, since genes like *GAL1* are normally derepressed in a *msn5* strain during growth on ethanol, despite the constitutive presence of Mig1 in the nucleus (Santangelo, 2006).

Several evidences suggest that Mig1 acts as a repressor in association with Hxk2, the major yeast hexokinase (Ahuatzi et al., 2004; Ahuatzi et al., 2007; Moreno et al., 2005). Hxk2 seems to be part of a repressor complex located on the *SUC2* promoter. A serine residue (Ser311) found on Mig1 is apparently crucial for the interaction between the repressor and the hexokinase: loss of this residue results in a nuclear localization of Mig1, regardless of the carbon source available, and in the constitutive inhibition of *SUC2* expression (Ahuatzi et al., 2004; Ahuatzi et al., 2007; Moreno et al., 2005; see below).

Two additional Snf1-regulated repressors are the poorly characterized Nrg1 and Nrg2, which contain Cys2His2 zinc fingers similar to the one found in Mig1 (Santangelo, 2006).. Both Nrg1 and Nrg2 exhibit glucose-dependent repression of a heterologous reporter gene and both interact with Snf1, although they do not appear to be phosphorylated by the kinase. Rather, Snf1 appears to modulate Nrg2 levels and is clearly required for normal Nrg1 function (Santangelo, 2006).
The Adr1 transcription factor (containing a Cys2His2-type DNA binding domain) activates expression of genes required for ethanol metabolism and β-oxidation of fatty acids (Young et al., 2003; Tachibana et al., 2005). Adr1 also affects the expression of genes involved in amino acid transport and metabolism, meiosis, and sporulation (Young et al., 2003; Tachibana et al., 2005). However, since only few genes are actually bound by Adr1 in cells grown in glucose-free media, altered regulation of most genes in *adr1* mutants may be the result of indirect regulatory or metabolic effects (Tachibana et al., 2005; Zaman et al., 2008). Adr1 is negatively regulated by PKA during growth on glucose and activated in a Snf1-dependent manner upon glucose exhaustion (Schuller, 2003; Zaman et al., 2008). The exact mechanisms of Adr1 inhibition by PKA or activation by Snf1 remain unclear. Apparently, Snf1 promotes Adr1 binding to chromatin but not transcriptional activation (Young et al., 2002). Adr1 is also under negative regulation of Reg1, since deletion of *REG1* increases the protein level of Adr1 and leads to induction of several Adr1-regulated genes, such as *ADH2* (Dombek et al., 2004). Moreover, the yeast 14-3-3 proteins, Bmh1 and Bmh2, likely act in a pathway parallel to Reg1 to inhibit expression of Adr1-regulated genes. (Dombek et al., 2004). Thus, Adr1 is sensitive to many diverse glucose-dependent inputs.
Cat8 and Sip4 (both containing aC6 zinc cluster DNA binding domain) activate expression of genes required for gluconeogenesis during growth in the absence of glucose by binding carbon source response elements (CSRE) (Hedges et al., 1995; Lesage et al., 1996; Randez-Gil et al., 1997; Vincent et al., 1998). Derepression of genes having CSRE motifs upon glucose depletion is completely abolished in *cat8 sip4* mutants (Schuller, 2003). However, the relative contributes of Cat8 and Sip4 to the transcriptional response in non-glucose media are apparently different: in fact, cat8 mutants do not grow on non-fermentable carbon source, whereas sip4 deficient cells can (Roth et al., 2004). 255 genes have been identified as putative targets of Cat8 by microarray analyses, but only 48 are actually bound by the transcription factor in vivo (Tachibana et al., 2005). Both Cat8 and Sip4 are phosphorylated in response to glucose depletion by the Snf1-Gal83 complex (Charbon et al., 2004; Lesage et al., 1996; Vincent et al., 1999; Schmidt et al., 2000). *CAT8* transcription is inhibited by Mig1 and activated by Hap2/3/4/5 whereas *SIP4* expression is upregulated by Cat8 (Zaman et al., 2008; Schuller, 2003).
Snf1 protein kinase complex also regulates certain stress responsive genes during carbon source downshift: for example, Snf1 affects the activity of the heat shock transcription factor Hsf1 in response to carbon stress (but not heat shock; Tamai et al., 1994; Hahn & Thiele, 2004).
The stress-response transcription factor Msn2 is another target of the Snf1 pathway (Mayordomo et al., 2002; De Wever et al., 2005; see following sections). Msn2 is a key player in the "general stress response system" that coordinates the induction of many stress genes through a common *STRE (Stress Responsive)* element in their promoter (Martinez-Pastor et al., 1996; Moskvina et al., 1998). Msn2 is dephosphorylated by the Glc7 phosphatase following glucose depletion and

relocalizes to the nucleus to induce expression of its target genes, such as *HSPs* (hrat-shock proteins) and *CTT1* (catalase) (De Wever et al., 2005). However, long term carbon stress induces phosphorylation of Msn2 by Snf1, thereby inhibiting its nuclear accumulation: these observations suggest that Snf1 may be involved in long-term adaptation to carbon stress by negatively regulating Msn2 transcriptional activity. Besides being regulated by Snf1, the subcellular localization and the activity of Msn2 are also affected by the cAMP/PKA and TOR pathways (see next sections; Gorner et al., 1998; Gorner et al., 2002; Santhanam et al., 2004; Beck & Hall, 1999; Sanz, 2003; Zaman et al., 2008).
Finally, Snf1 is apparently required for phosphorylation and nuclear accumulation of Gln3, a GATA transcription factor in response to glucose starvation (Bertram et al., 2002). Gln3 is also regulated by the TOR network in response to nitrogen signals (Bertram et al., 2002).
Several lines of evidence indicate that Snf1 can also affect the transcription of its target genes by stimulating chromatin remodeling or through direct effects on the transcriptional apparatus (Kuchin et al., 2000; Lin et al., 2003; Young et al., 2002; Lo et al., 2001; Shirra et al., 2005; Shirra et al., 1999; Lo et al., 2005; Liu et al., 2005; Hedbacker & Carlson, 2008; Zaman et al., 2008).
As part of its function in controlling cellular energetic status; Snf1 regulates the activity of metabolic enzymes involved in fatty acid metabolism and carbohydrate storage (Hardy et al., 1994; Wang et al., 2001; Hedbacker & Carlson, 2008).
As already discussed in previous sections, Snf1 also controls the expression of hexose transporters encoded by *HXTs* (Ozcan et al., 1996a; Tomas-Cobos & Sanz, 2002; Pasula et al., 2007) and many crosstalks between the Snf3/Rgt2 and the Snf1 pathways have been identified (Kaniak et al., 2004; Pasula et al., 2007: Gadura et al., 2006; Zaman et al., 2008).
snf1 deficient cells exhibit defects during starvation for other nutrients, including phosphate, sulfate, and nitrogen (Hedbacker & Carlson, 2008). Snf1 also plays a fundamental role in responses to many other environmental stresses besides carbon stress and also plays a fundamental role in cellular processes like sporulation and pseudohyphal growth (Hedbacker & Carlson, 2008).). However, a detailed description of the multiple role of the Snf1 in diverse cellular functions is beyond the scope of this introduction.

The enigmatic role of Hxk2 in glucose signalling

Of the three glucose phosphorylating enzyme existing in yeast (Hxk1, Hxk2 and Glk1), the hexokinase encoded by *HXK2* is the most highly expressed and has the predominant role during growth on glucose (Gancedo, 1998; Santangelo, 2006).
In addition to its canonic role in catalyzing the first step of glycolysis, Hxk2 was early identified as one of the main player involved in glucose repression, since inactivation of its encoding gene leads to derepression of genes such as *SUC2* (encoding invertase, required for sucrose metabolism) even in presence of high concentrations of glucose. Consistently, in absence of a functional Hxk2, Snf1 phosphorylates and inactivates the transcriptional repressor Mig1 even in high glucose media (Treitel et al., 1998; Ahuatzi et al., 2007).
However, if the requirement of Hxk2 in the repression signaling simply reflects the need for glucose phosphorylation or involves a separate regulatory function for the hexokinase is still controversial.

Actually, glucose repression in yeast involves two distinct mechanisms: an initial rapid response (short-term repression) is mediated through any kinase able to phosphorylate glucose (Hxk1, Hxk2, Glk1), whereas the sustained long-term repression specifically requires Hxk2 (deWinde et al., 1996). Furthermore, Hxk2 seems to be involved in a feedback loop that serves to amplify its own expression and to repress the expression of *HXK1* and *GLK1* in response to high glucose levels in the culture medium (Rodriguez et al., 2001; Palomino et al., 2005; Palomino et al., 2006).

Early reports showed a good correlation between the residual phosporylating activity associated with mutant alleles of *HXK2* and the extent of glucose repression; suggesting that Hxk2 plays a purely metabolic role (Ma et al., 1989; Rose et al., 1991). .However, more recently, mutant forms of Hxk2 with reduced catalytic activity but still functional in the establishment of the catabolite repression have been isolated, indicating that the role of Hxk2 in the glucose repression is not only to sustain the production of sugar phosphate (Hohmann et al., 1999; Kraakman et al., 1999b; Mayordomo.& Sanz, 2001). A dual function as metabolic enzyme involved in galactose phosphorylation and as transcriptional regulator has been described for the yeast galactokinase (*GAL1*): therefore, a similar situation may apply to Hxk2 (Vollenbroich et al., 1999)

Recent findings further support the idea of a more direct role for Hxk2 in signalling glucose availability to the repression machinery. Indeed, a small fraction of Hxk2 (about 15%) resides in the nucleus when glucose is present, and this nuclear localization is necessary for the repression of genes like *SUC2*, *HXK1* and *GLK1* to take place (Herrero et al., 1998a,b; Randez-Gil et al., 1998a; Rodriguez et al., 2001). Moreover, Hxk2p participates directly in DNA–protein complexes found on the promoters of these genes during their glucose-dependent repression (Rodriguez et al., 2001; Herrero et al., 1998a,b).

An interaction of Hxk2 with the transcriptional cofactor Med8 has been described (de La Cera et al., 2002). Med8 is a component of the Srb/mediator complex that enhances basal transcription and facilitates activated transcription by interacting with the C-terminal domain (CTD) of RNA polymerase II. The Hxk2-Med8 interaction may be physiologically relevant, since Med8 specifically binds to upstream activating sequences (UASs) in the *SUC2* promoter (Moreno et al., 1999; Chaves et al., 1999): it has been proposed that in the presence of high glucose levels the binding of nuclear Hxk2 to Med8 would interfere with the recruitment of the RNA polymerase II on the *SUC2* promoter, thus preventing transcription (Fig. 16; de la Cera et al., 2002).

Med8 also recognizes downstream repressing sequences (DRSs) found in *HXK2*: Med8, together with Rgt1, has been shown to be an essential factor involved in the repression of the *HXK2* gene in low glucose medium (Palomino et al., 2005; Palomino et al., 2006; see below).

Recent studies have also demonstrated that Hxk2 interacts directly with the transcriptional repressor Mig1 and that this interaction is required for Hxk2 to be retained within the nucleus in presence of abundant glucose (Ahuatzi et al., 2004; Moreno et al., 2005; Ahuatzi et al., 2007): in fact, a good correlation exists between the cellular level of Mig1 and the total amount of Hxk2 sequestered in the nuclear compartment (Ahuatzi et al., 2004). Conversely, Mig1 does not need to form a complex with Hxk2 to reside in the nucleus, since a *snf1 hxk2* double mutant strain exhibits a constitutive nuclear localization of Mig1 (Ahuatzi et al., 2004; Moreno et

al., 2005).The Mig1-Hxk2 association is detected during growth on high glucose at the DNA level (Ahuatzi et al., 2004; Moreno et al., 2005) and is mediated by the serine 311 of Mig1 and by an amino acid motif located between lysine 6 and methionine 15 of Hxk2 (Fig. 15; Ahuatzi et al., 2007). Mutation of the S311 residue confers a nuclear localization to the Mig1 repressor in both high and low glucose, resulting in the constitutive repression of the *SUC2* gene (Ahuatzi et al., 2007).

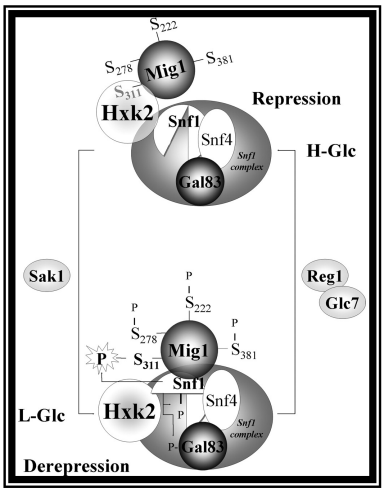


Figure 15.
Hxk2 regulates Mig1 phosphorylation by the Snf1 complex: a possible model.

In high glucose media (H-Glc), Hxk2 is present in the nucleus where it interacts with Mig1 and the Snf1 kinase. As a consequence of Hxk2 binding to Mig1, serine 311 of Mig1 is dephosphorylated and the transcriptional repression for several glucose-regulated genes is maintained.

In low glucose conditions (L-Glc), Hxk2 does not interact with Mig1 but still interacts with the Snf1 kinase. Inhibition of the Hxk2-Mig1 interaction facilitates serine 311 phosphorylation by the Snf1 kinase. Mig1 is then exported out of the nucleus together with Hxk2. The absence of the repressor complex in the nucleus activates transcription of several glucose-regulated genes.
(From Ahuatzi et al., 2007).

These findings suggest that the main regulatory role of Hxk2 in the glucose repression may be to generate together with Mig1 a repressor complex located on the promoter of glucose repressible genes (Fig. 16). At high glucose concentrations, the Hxk2-Mig1 interaction would hinder the contact between Mig1 and the Snf1 kinase, blocking the phosphorylation of the crucial serine 311 and thus preventing the inactivation of the repressor. In low glucose conditions, the Hxk2 interaction with Mig1 is abolished, while a transient increase in interaction between Snf1 and Mig1 is detected (Ahuatzi et al., 2007). This interaction could potentially stimulate Mig1 phosphorylation by Snf1, resulting in Msn5-mediated nuclear export of the repressor (and Hxk2), thereby relieving the transcriptional block (Fig. X; Ahuatzi et al., 2007).
Such a mechanism might be especially important in avoiding inappropriate cross-talks between different signaling pathways: for example, it has been observed that when cells growing on glucose are exposed to saline stress Snf1 is activated but does not phosphorylate Mig1 (Gancedo; 2008; McCartney & Schmidt, 2001).
The mechanism by which Hxk2 enters the nucleus is still unknown. Conversely, the available data suggest that the nuclear export of Hxk2 requires the Xpo1 carrier (Pelaez et al., 2009). The binding of Hxk2 and Xpo1 involve two leucine-rich nuclear export signals (NES) found in the Hxk2 protein. The phosphorylation of Hxk2 at serine 14 by an unknown kinase promotes Hxk2 export by facilitating the association between Hxk2 and Xpo1 (Pelaez et al., 2009).
As already discussed, the binding of Hxk2 and Mig1 in the nucleus suggests that the main role of Hxk2 in glucose repression is to prevent the Snf1-dependent phosphorylation of Mig1 to maintain the transcriptional repressed status (Ahuatzi et al., 2004; Moreno et al., 2005; Ahuatzi et al., 2007).

In addition, Hxk2 seems to be involved in the regulation of the phosphorylation state of Reg1, the regulatory subunit of the Glc7 protein phosphatase 1 complex (Sanz et al., 2000). Snf1-dependent phosphorylation of Reg1 is carbon source modulated and apparently promotes the inactivation of the Snf1 complex in presence of large amounts of glucose. The role of Hxk2 in this process may be to facilitate the inactivation of the Snf1 complex by the Glc7-Reg1 phosphatase, either by stimulating binding and/or phosphorylation of Reg1 or by inhibiting dephosphorylation of Reg1 by Glc7 (Sanz et al., 2000). Consistently, *REG1* overexpression partially suppresses the defects in glucose repression associated with the hxk2 mutation (Sanz et al., 2000). However, measurements of the capacity of Snf1 to phosphorylate a peptide substrate *in vitro* did not confirm an increased activity of Snf1 in extracts from *hxk2* deficient cells grown on glucose. Therefore, it remains unclear whether Hxk2 controls the intrinsic activity of Snf1 or only its capacity to phosphorylate Mig1 (Gancedo, 2008).

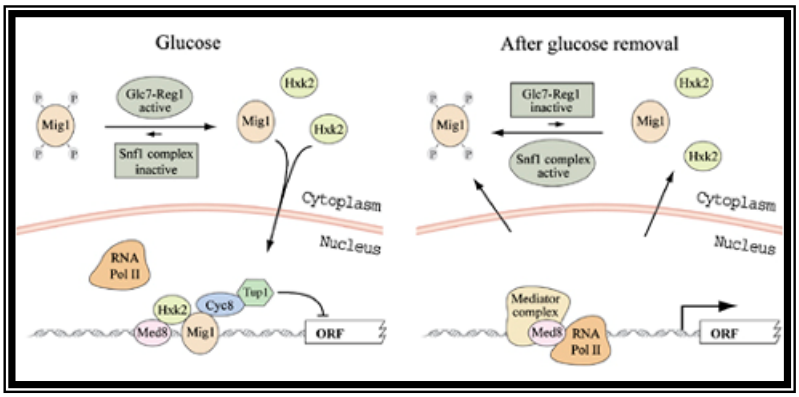


Figure. 16. Model for the role of Hxk2 in the in glucose dependent transcriptional repression.
During growth on glucose the transcriptional repressor Mig1 is mainly unphosphorylated, due to the phosphatase activity of the Glc7-Reg1 complex, coupled with a low activity of the Snf1 complex. In these conditions Mig1 exhibits a nuclear localization and Hxk2 is partially retained within the nucleus through its interaction with Mig1: this binding hinders the phosphorylation of Mig1 by any active molecule of Snf1 in the nucleus, thus reinforcing the repressing capacity of the Mig1-Cyc8-Tup1 complex. Nuclear Hxk2 also binds to Med8, a subunit of the Srb/mediator complex, and this interaction may interfere with the capacity of RNA polymerase II (RNA Pol II) to bind to the promoter of the regulated gene. When glucose is exhausted, the Snf1 complex becomes active, Mig1 is phosphorylated and abandons the nucleus accompanied by Hxk2. As Hxk2 dissociates from Med8, the mediator complex recruits RNA Pol II and transcription can proceed.
(From Gancedo, 2008).

In sum, Hxk2 is a bifunctional protein: it works as a glycolytic enzyme in the cytoplasm and as a coregulator of gene transcription in the nucleus. Hxk2 acts as a corepressor by directly interacting with components of the repression machinery (i.e. Mig1) that control the expression of several glucose-repressed genes.
Last but not least, the role of Hxk2 in glucose repression may be confined only to a subset of all the glucose-repressible genes: in fact, the presence of a functional Hxk2 is specifically required for the repression of genes such as *SUC2*, *HXK1* or the *GAL* genes (Rodrıguez et al., 2001). On the other hand, repression of genes like *ADH2* or *FOX1* is not relieved in an *hxk2* mutant (Dombek et al., 1993; Stanway et al., 1995) and relief is only partial for *CYC1*, *CYB2*, *GLK1* or *GDH2* (Rodriguez et al., 2001;

Belinchon & Gancedo, 2007b). Several genes such as *FBP1*, *PCK1* or *ICL1* are completely repressed by glucose, even in a double mutant *hxk1 hxk2* (Belinchon & Gancedo, 2007b). Furthermore, although long-term maintenance of *SUC2* repression (long-term repression) specifically requires Hxk2, *SUC2* mRNA levels show a strong, transient decrease upon addition of glucose both in a *hxk2* and in a *hxk2 hxk1* double mutant strains (short-term repression) (deWinde et al., 1996; see above).

Glucose dependent regulation of HXK2 expression: hints for a possible crosstalk among the cAMP/PKA, Rgt2/Snf2 and Snf1 pathways

Expression of *HXK2* is regulated according to glucose availability, being activated when the sugar levels are high and inhibited when they start to decline (Palomino et al., 2005; Herrero et al., 1995).

Recent evidences have demonstrated that repression of *HXK2* involves the Rgt1 repressor, a key component of the Rgt2/Snf3 pathway, together with Med8. As already discussed, Med8 is a subunit of the RNA polymerase II mediator complex which associates with core polymerase subunits to form the RNA polymerase II holoenzyme. Cofactors like Med8 mediate access to genes in chromatin and recruit the RNA polymerase II holoenzyme transcriptional machinery to specific DNA–protein regulatory complexes, thus enhancing basal transcription and facilitating activated transcription (Myers & Kornberg, 2000; Yudkovsky et al., 2000).

HXK2 transcriptional repression is dependent on the binding of Rgt1 and Med8 to their target DNA elements within the *HXK2* promoter (Palomino et al., 2005; Palomino et al., 2006). Rgt1 binds to the *RGT1* element inside the *HXK2* promoter in a carbon source-dependent manner (Palomino et al., 2005; Palomino et al., 2006), whereas Med8 binds constitutively to a DRS (downstream repressing sequence) found in the *HXK2* gene (Palomino et al. 2006; Chaves et al., 1999; Moreno et al., 1999).

Rgt1 also interacts with both Med8 and Hxk2 in a glucose dependent manner: the Rgt1-Med8 interaction occurs in low glucose but not in high glucose media, whereas Rgt1 binds to the nuclear fraction of Hxk2 only in the presence of elevated sugar concentrations (Palomino et al., 2006). Apparently, the interaction between Rgt1 and Med8 is essential for the repression of *HXK2* transcription in low glucose (Palomino et al., 2006).

Furthermore, the phosphorylation status of Rgt1 influences its interaction with both Med8 and Hxk2 (Palomino et al., 2006).

Rgt1 is hyperphosphorylated during growth in high glucose (Mosley et al., 2003; Kim et al., 2003; Mosley et al., 2003 Kim et al., 2006) and also exists in an less phosphorylated state during growth in low glucose medium (Palomino et al., 2006).

cAMP-dependent protein kinase (PKA), in particular the Tpk3 catalytic subunit, is responsible for Rgt1 hyperphosphorylation in the presence of high glucose, which determines loss of DNA binding capacity and consequent release of Rgt1 from the *HXK2* promoter (Palomino et al., 2006; Mosley et al., 2003; Kim et al., 2003). Tpk3-mediated hyperphosphorylation of Rgt1 is an essential prerequisite for DNA release, but is not necessary to induce Rgt1 interaction with nuclearHxk2 (Palomino et al. 2006).

In contrast, Snf1 protein kinase is directly or indirectly involved in activating the function of Rgt1 as a repressor in low glucose: consistently, loss of *SNF1* results in a

dephosphorylated Rgt1 protein with no DNA-binding activity that cannot repress *HXK2* transcription in low glucose medium (Palomino et al., 2006).
Therefore, the (putative) Snf1-dependent phosphorylation of Rgt1 apparently promotes DNA binding of Rgt1 to the *HXK2* promoter in low glucose, whereas Tpk3-dependent hyperphosphorylation triggers release of the repressor from the *HXK2* promoter in high glucose (Palomino et al., 2006).
A simplified scheme of how nutritional signals might converge on Rgt1 through the Snf1 and Tpk3 protein kinases to regulate *HXK2* expression is shown in Figure 17 (Palomino et al., 2006).

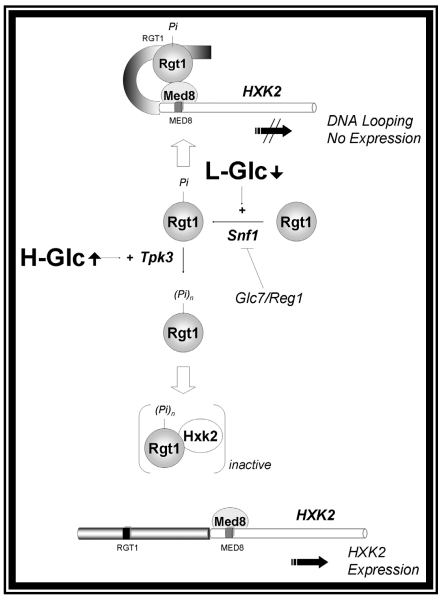


Figure 17. A model for Snf1- and Tpk3-dependent regulation of HXK2 transcription.
In low-glucose conditions (L-Glc), Rgt1 is phosphorylated by Snf1 (or by aSnf1-dependent protein kinase.) The phosphorylated Rgt1 binds to the RGT1 element of *HXK2* promoter to repress transcription by looping DNA and bringing the Rgt1 and Med8 distal binding sites into close proximity.
In high-glucose (H-Glc), Rgt1 is hyperphosphorylated by Tpk3. The hyperphosphorylated form is released from HXK2 promoter and sequestered outside by the nuclear Hxk2 protein, resulting in expression of the HXK2 gene. Arrow denotes positive expression; arrow marked with double bars denotes negative expression. Positive regulation is marked by plus. (From Palomino et al., 2006).

Apparently, *HXK2* transcription is repressed by the state of the chromatin over its promoter. In cells growing in low glucose, the *RGT1* element of the *HXK2* promoter is occupied by the phosphorylated Rgt1, which interacts with Med8. Under these conditions, the transcriptional repression is triggered by the formation of a DNA loop that includes the promoter and the coding region of the *HXK2* gene between the Rgt1 and Med8 binding sites.
In high-glucose media, Rgt1 is hyperphosphorylated in a Tpk3-dependent manner and released from *HXK2* promoter: the chromatin barrier over the *HXK2* promoter would be relieved by binding of the hyperphosphorylated Rgt1 to the nuclear Hxk2 protein (Palomino et al., 2006): Rgt1 sequestering would result in the activation of the *HXK2* expression, by rendering the promoter accessible to the RNA II polymerase complex, as well as to other mediator factors. Potentially, this model might also explain the involvement of Hxk2 in the positive-feedback loop that serves to amplify its own expression (Rodriguez et al., 2001; Palomino et al., 2006).
Furthermore, since the Hxk2 protein is necessary to generate the glucose- repression signal through the Snf1-Mig1 glucose signalling circuit, the described mechanism prefigures a new potential crosstalk among the three major glucose sensing system: the Snf1 circuit, the cAMP/PKA network and the Snf2/Rgt2 pathway (Palomino et al., 2006).

Glucose sensing pathways: the PKA signalling cascade

In their natural environment, yeast cells experience long periods of nutrient starvation, alternated with extremely short intervals of nutrient abundance. Under such conditions, fast recovery from quiescence and initiation of fermentation clearly offer a selective advantage (Thevelein & de Winde, 1999).

The addition of glucose to stationary-phase cells or to cells slowly growing on a non-fermentable carbon source cells rapidly triggers a wide variety of regulatory processes directed towards the exclusive and optimal utilization of the newly available sugar (Reviewed in Thevelein & de Winde, 1999; Zaman et al., 2008; Rolland et al., 2002; Gancedo, 2008). While glucose uptake and the glycolytic flux are stimulated, gluconeogenesis and respiration are inhibited. Genes encoding enzymes involved in the stress resistance, respiration and metabolism of alternative carbon become repressed; reserve carbohydrates are mobilized. A dramatic increase in cellular growth rate occurs which is preceded by a characteristic upshift in ribosome biogenesis and protein synthesis (Jorgensen & Tyers, 2004). Glucose elicits these dramatic changes on yeast physiology by deeply remodeling the activity of the metabolic machinery. Regulations take place at the level of gene transcription (both repression and induction), mRNA stability, translation and protein stability, whereas enzymatic activities are regulated post-transcriptionally by covalent modification or by allosteric activation or inhibition. Most of these processes are regulated by specific signaling circuits that constantly monitor glucose availability; among these, the cyclic AMP/PKA pathway plays a prominent role as central regulator of the metabolic and transcriptional status of the yeast cell.

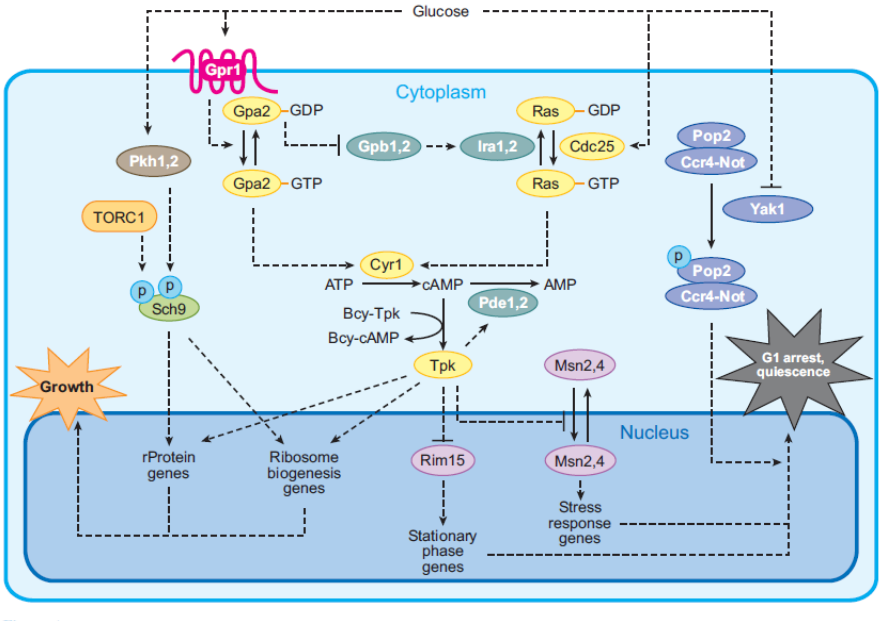


Figure 18. The cAMP/PKA signaling transduction pathway in S. cerevisiae

Glucose signaling mediated by the small G-proteins Ras and Gpa2 funnels through protein kinase A (PKA) to induce ribosome biogenesis and suppress the general stress response controlled by Msn2/Msn4 and Rim15. Dashed lines represent regulatory interactions, which may be indirect and in some cases only putative. (From Zaman et al., 2009).

The cAMP/PKA glucose sensing pathway

In yeast, a major signalling pathway activated by glucose is the cAMP/protein kinase A pathway, which regulates many aspects of cellular physiology, including growth, proliferation, metabolism, stress resistance, aging, morphogenesis and development according to nutrients availability. (Fig. 18; Zaman et al., 2008; Santangelo et al., 2006; Thevelein & deWinde, 1999; Rolland et al., 2002).

Nutrient-deprived quiescent cells or slowly-growing cells on a nonfermentable carbon source accumulate high levels of storage carbohydrates (such as trehalose and glycogen) and tolerate various stress conditions (Reviewed in Thevelein, 2004; Thevelein & de Winde, 1999; Rolland et al., 2002; Santangelo, 2006). Cells cultured on rapidly fermentable carbon source, such as glucose, show the opposite phenotype. Pseudohyphal differentiation occurs in response to nitrogen limitation in the presence of a rapidly fermentable sugar, whereas the sporulation program is triggered in the absence of both nitrogen and a carbon source. All of these characteristics are in large part determined by the activity of PKA (Thevelein & de Winde, 1999; Santangelo, 2006.

Inactivation of the PKA signaling pathway causes arrest at *START* point in G1 phase of the cell cycle, followed by entry into the stationary G0 phase (Thevelein, 2004; Santangelo, 2006; Zaman et al., 2008). Cells starved for nutrients behave in a similar way, which indicates that the cAMP–PKA pathway is involved in nutrient dependent control of growth and cell cycle progression (Thevelein & deWinde, 1999; Santangelo, 2006; Thevelein, 1994).

Mutants with reduced PKA activity exhibit several characteristics typical of stationary phase cells (enhanced stress resistance, high level of storage carbohydrates) even when supplied with a rapidly fermentable carbon source; in addition these cells are impaired in pseudohyphal growth, while their sporulation efficiency is enhanced (Thevelein & deWinde, 1999; Rolland et al., 2002; Santangelo, 2006; Thevelein, 1994; Zaman et al., 2008). Conversely, mutants with a hyperactive PKA pathway grow poorly on non fermentable carbon source, are sensitive to various stress forms and do not to arrest properly in stationary phase when deprived of nutrients; furthermore, they exhibit a vigorous filamentous growth, but fail to sporulate (; Zaman et al., 2008; Thevelein & deWinde, 1999; Rolland et al., 2002; Santangelo, 2006; Thevelein, 1994). These phenotypes partially arise from the inability to mount a stress response, but also from the lack of stored nutrients (such as glycogen or trehalose) needed to complete a round of mitotic division cell cycle upon starvation (Markwardt et al., 1995; Zaman, 2008; Thevelein, 1994; Rolland et al., 2002).

cAMP-dependent protein kinase (PKA) is a conserved serine/theonine kinase that exists in its inactive status as a heterotetrameric holoenzyme composed of two catalytic subunits (encoded in yeast by the three closely related genes: *TPK1*, *TPK2* and *TPK3*) and two regulatory subunits (encoded by *BCY1*) (Toda et al., 1987a; Toda et al., 1987b). The three catalytic subunits of PKA are largely redundant, although several specific functions have also been described for each isoforms: for example, Tpk2 is responsible for processes such as pseudohyphal growth, regulation of genes involved in trehalose degradation and iron uptake (Robertson et al., 1998; Robertson et al., 2000; Pan & Heitman, 2002). Tpk1 is required for biosynthesis of branched chain amino acid (Robertson et al., 2000), whereas Tpk3 is specifically involved in the regulation of mitochondrial enzymatic content during growth

(Chevtzoff et al., 2005). At least two different mechanisms regulate the subcellular localization of PKA: cAMP controls the localization of the Tpks catalytic subunits, whereas the carbon source determines that of the Bcy1 regulatory subunit; this regulation seems to have physiological relevance (see following section; Griffionen et al., 2000; Griffionen et al., 2001; Griffionen et al., 2002).
Only two stimuli are known to promote the activation of the PKA signaling in yeast (Thevelein & de Winde., 1999). Addition of a rapidly fermentable sugar (glucose, fructose or mannose) to derepressed yeast cells (quiescent stationary phase cells or cells slowly growing on a non-fermentable carbon source) triggers a rapid, transient increase in the cAMP level, which binds to the regulatory Bcy1 subunits and activates PKA by promoting the release of the Tpks catalytic subunits. Intracellular acidification (obtained for instance by addition of the protonophore 2,4-dinitrophenol (DNP) at low extracellular pH) results in an even more pronounced, long-lasting cAMP increase (Reviewed in Thevelein and deWinde, 1999; Rolland et al., 2002)*.
Activation of PKA elicits dramatic changes in the transcriptional program and in the activity of the biosynthetic machinery, which help the yeast cells to adapt to changes in the nutrient status. Several well known targets of PKA include glycolytic and gluconeogenetic enzymes, proteins involved in the metabolism of the storage carbohydrates, transcription factors regulating stress response, ribosomal biogenesis, and carbohydrate metabolism (Zaman et al., 2008; Gancedo et al., 2008).
The level of cAMP in yeast cell is the result of the equilibrium between its synthesis, catalyzed by the adenylate cyclase enzyme, Cyr1 (Casperson et al., 1985), and its degradation performed by the low- and high-affinity phosphodiesterases (encoded by *PDE1* and *PDE2*, respectively (Sass et al., 1986; Nikawa et al., 1987).
Adenylate cyclase activity in *S. cerevisiae* is controlled by two distinct G-protein systems: the Ras pathway and the Gpr1-Gpa2 pathway (Zaman, 2008; Santangelo, 2008; Thevelein & DeWinde, 1999; Rolland et al,, 2002).

*The physiological role of the stimulation of cAMP synthesis by intracellular acidification is rather enigmatic. One of the most accredited hypotheses is that it may help yeast cell to maintain a proper intracellular pH and ATP level during carbon starvation. Under such conditions, the intracellular pH in yeast cells drops considerably: the resulting increase in the cAMP level may activate PKA causing the mobilization of storage carbohydrates to regenerate ATP and maintain cell vitality. An increase in ATP would restore the intracellular pH by activating the H^+/ATPase on the plasma membrane (Colombo et al., 1998; Thevelein & de Winde., 1999)

The Ras-adenylate cyclase pathway
Ras1 and Ras2 are two small monomeric GTP-binding proteins capable to switch between an active GTP-bound state and an inactive GDP-bound form; when in their active conformation, Ras proteins stimulate cAMP production by direct binding to adenylate cyclase (Toda et al., 1985). The Ras-GTP/Ras-GDP ratio is controlled by the balance between the activities of the guanine nucleotide exchange factors (GEFs), Cdc25 (Camonis et al., 1986) and Sdc25 (Damak et al., 1991), which promote GTP loading on Ras, and the GTPase Activating Proteins (GAPs), Ira1 and Ira2, which stimulate GTP hydrolysis by enhancing the intrinsic Ras-GTPase activity (Tanaka et al., 1990a,b).
Ras proteins are required to maintain a basal adenylate cyclase activity and are thus essential for cell viability. Intracellular acidification enhances the GTP loading on Ras, possibly through inhibition of the Ras-GAPs Ira1 and Ira2 (Colombo et al.,

1998; see below). In addition, recent studies have demonstrated that glucose addition also causes a small but significant increase in the fraction of GTP-bound Ras (Colombo et al., 2004; Rudoni et al., 2000), thus confirming early reports which assigned to Ras a decisive role in the glucose-induced cAMP signaling (Mbonyi et al., 1988; Munder & Kuntzel, 1989; Van Aelst et al., 1990; Van Aelst et al., 1991; Bhattacharya et al., 1995). The exact mechanisms by which glucose triggers Ras activity in still uncertain: no sugar-sensing system has yet been identified that could function as an upstream activator of Cdc25 to transmit the glucose signal to the Ras proteins (Rolland et al., 2000); indeed, several evidences suggest Cdc25 may not itself be the signal receiver for glucose induced cAMP response (Goldberg et al., 1994). Recently, it has been suggested that the increase in Ras2-level in response to glucose may be mediated through inhibition of the Ira_s proteins: in fact, deletion of *IRA2* and *IRA1* or the presence of $RAS2^{V19}$ allele (encoding an hyperactive Ras2 variant insensitive to GAPs regulation) result in a constitutively high Ras GTP level that no longer increases upon glucose addition (Colombo et al., 2004).
Interestingly, a mutation in adenylate cyclase ($Cyr1^{K1876M}$) affects both glucose- and acidification-induced cAMP signalling, but not the basal cAMP level (Vanhalewyn, et al., 1999). This mutation also counteracts the hyper-activating effects of the $RAS2^{V19}$ and $GPA2^{V132}$-dominant alleles (Vanhalewyn, et al., 1999).
Consistent with a role of the Ras proteins in the glucose-induced PKA signaling, strains carrying the $ras2^{S318}$ allele exhibit normal basal levels of cAMP, whereas the glucose- induced cAMP signal is completely abolished (Jiang et al., 1998). Furthermore, cAMP production requires attachment of Ras to the plasma membrane: a mutant allele of Ras unable to be targeted to the plasma cellular envelope supports viability but not glucose-induced cAMP signalling (Bhattacharya et al., 1995).
Glucose-induced activation of the cAMP/PKA pathway is strictly controlled through a negative feedback mechanism exerted by PKA (Nikawa et al., 1987b; Ma et al., 1999). The Pde1 cAMP phosphodiesterase has been identified as a PKA target specifically involved in downregulation of agonist-induced cAMP signalling (Ma et al., 1999).
Another suggested targets for PKA mediated negative feedback loop is Cdc25: upon glucose stimulation, several residues within the long N-terminus of the GEF are phosphorylated, leading to diminished association of Cdc25 with the membrane and thus dissociation form Ras (Gross et al., 1992). Mutation of the 7 putative PKA phosphorylation sites within the N-terminus of Cdc25 substantially abolishes the attenuation of the glucose-induced cAMP response, which is responsible for signal termination (Gross et al., 1999). Furthermore, several studies have also reported that the N-terminal region of Cdc25 (as well as the C-terminal 37 amino acids), is essential for glucose induced cAMP signaling (Munder & Kuntzel, 1989; Gross et al., 1999): the transient increase in cAMP levels upon sugar addition is severely hampered by deletions within the N-terminus (Gross et al., 1999). Interestingly, the N-terminal region of Cdc25 seems to be required for carbon source modulation of cell size (Belotti et al., 2006; see next sections). It has also been proposed that the N-terminus can negatively regulate the activity of the catalytic domain of Cdc25 (located in the C-terminal region (Chen et al., 2000)). However, the actual role of the N-terminal region in the glucose signal processing is still debated, since other reports (Van Aelst et al., 1990; Paiardi et al., 2007) have shown that strains expressing Cdc25 variants lacking the amino-terminal region or even heterologous GEF domains exhibit a normal glucose-induced cAMP response: in these mutants,

the cAMP signal depends largely upon the Gpr1/Gpa2 circuit (Paiardi et al., 2007; see next sections); in contrast, the glucose-induced Ras2-GTP increase is completely abolished in all the tested N-terminus deletion mutants (Paiardi et al., 2007).

Transcriptional control by the Ras/adenylate cyclase pathway

The Ras/PKA pathway plays the leading role in the massive cellular response to glucose (Wang et al., 2004; Slattery et al., 2008; Zaman et al., 2009). Recent studies have shown that it is possible to mimic the glucose response in cells growing on nonfermentable carbon source by inducing an activated allele of Ras2 (*$RAS2^{V19}$*): the alterations occurring in the transcriptional profile are both qualitatively and quantitatively identical to the changes triggered by glucose addition. Furthermore, all of the Ras induced changes in gene expression are entirely dependent on PKA (Wang et al., 2004; Zaman et al., 2004; Zaman et al., 2008). PKA activation is both necessary and sufficient to induce the vast majority of the glucose-dependent transcriptional changes: in fact, inactivation of PKA eliminates most of the cellular transcriptional response to glucose (Zaman et al., 2009).

Activation of the PKA pathway results in induction of genes involved in ribosome biogenesis and glycolysis and repression of genes required for the stress response, respiration, gluconeogenesis, and in metabolism of storage carbohydrates (Wang et al., 2008; Zaman et al., 2009; Slattery et al., 2008). Significantly, the induction of a large fraction of genes involved in mass accumulation is mediated through the Ras/PKA route. This topic will be discussed in detail in subsequent sections.

The GPCR system

The GPCR (G-protein coupled receptor) module composed by Gpa2 and Gpr1 define a second glucose-sensing system that works in parallel with Ras to activate PKA (Fig. 18; Santangelo, 2006; Zaman et al., 2008; Rolland et al., 2002). Gpa2 is a small GTP-binding protein homologous to the mammalian Gα subunit of the heterotrimeric G proteins (Nakafuku et al., 1988). *GPR1* encodes a seven-transmembrane G protein–coupled receptor that physically interacts with Gpa2 (Xue et al., 1998; Kraakman et al., 1999a). By homology to other GPCR signaling systems, Gpa2 likely functions as the Gα subunit by coupling ligand activation of the Gpr1 receptor to an internal cellular response: it is commonly accepted that the binding of glucose to Gpr1 directs the formation of the GTP-bound, active form of Gpa2, which then stimulates adenylate cyclase to increase cAMP production (Kraakman et al., 1999a). Consistently, it has been demonstrated that adenylate cyclase physically interacts with the GTP-bound form of Gpa2, but not with GDP-Gpa2 (Peeters et al., 2006). The fact that a *gpa2* deletion (as well as *gpr1*) is synthetically lethal with *ras2* and that this phenotype is suppressed by deletion of *PDE2* is also consistent with a role for Gpa2 as stimulator of adenylate cyclase (Kubler et al., 1997; Xue et al., 1998).Moreover, the defects in pseudohyphal growth exhibited by *gpr1/gpr1* or *gpa2/gpa2* homozygous diploids strains can be suppressed by addition of exogenous cAMP (Kubler et al., 1997; Xue et al., 1998).

Unlike classic heterotrimeric G protein, Gpa2 is an atypical Gα protein for which no canonic Gβ and Gγ cognate subunits have been identified. Instead, Gpa2 interacts with Krh1 and Krh1, two kelch repeat proteins originally thought to function as substitute Gβ subunits (Harashima & Heitman, 2002; Batlle et al., 2003), and with Gpg1 which was proposed to serve as γ subunit (Zeller et al., 2007; see below). In addition, Gpa2 also binds to Rgs2, a protein that may functions as negative regulator

of the GPCR system by stimulating the intrinsic GTPase activity of Gpa2 (Versele et al., 1999): accordingly, inactivation of *RGS2* generates phenotypes consistent with a high PKA activity, while *RGS2* overespression attenuates the glucose-induced cAMP signal.
The GPCR system is specifically required for the activation of cAMP synthesis in response to high glucose (or sucrose: see below) concentrations: loss of *GPA2* or *GPR1* function completely abolishes the cAMP signal triggered by 100mM glucose, whereas the amplitude of the cAMP-responses to low (5 mM) glucose or fructose remain unaffected (Colombo et al., 1998; Kraakman et al., 1999). In contrast to the Ras circuit, the GPCR module is not required for the activation of PKA promoted by intracellular acidification and it does not seem to play an important role in the control of the basal cAMP level (Colombo et al., 1998; Kraakman et al., 1999). Consistent with this notion, both *gpr1* and *gpa2* single and double mutants are viable, unlike *ras1 ras2* or *cdc25* null strains (Kraakman et al., 1999). Furthermore, Gpa2 overproduction does suppress lethality of ras1 ras2 double null mutation (Nakafuku et al., 1988).
Deletion of *GPA2* confers to some extent the typical phenotype associated with a reduced level PKA activity (Kraakman et al., 1999a). However, the function of the GPCR system is apparently confined to the stimulation of cAMP synthesis during the transition from respirative growth on a non-fermentable carbon source to fermentative growth on glucose: in fact, inactivation of *GPA2* or *GPR1* only delays (but importantly, do not prevent) several PKA-controlled processes (such as the mobilization of the reserve carbohydrates, loss of heat resistance, repression of *STRE* responsive genes, and induction of genes encoding ribosomal proteins) that occur during the transition to growth on glucose (Kraakman et al., 1999a; Colombo et al., 1998). Furthermore, *gpa2* and *gpr1* deficient cells growing on ethanol fail to rapidly increase their size upon glucose addition (Alberghina et al., 2004; Tamaki et al., 2005; Tamaki et al., 2007; see below).

The interdependency between the GPCR system and glucose phosphorylation and the role of Ras in the glucose-induced cAMP signaling

Glucose- (or sucrose) dependent activation of cAMP signalling through the GPCR system is strictly dependent on sugar uptake and phosphorylation (Rolland et al., 2000; Rolland et al., 2001; Beullens et al., 1988): in fact, no glucose (or sucrose)-induced increase in cAMP level can be detected neither in a *hxt(1-7)* null strain, where most of the physiologically relevant hexose carrier are absent; nor in a *hxk2 hxk1 glk1* triple mutant, lacking all the three glucose phosphorylating enzymes (Rolland et al., 2001; Rolland et al., 2000; Buellens et al., 1988; Pernambuco et al., 1996). Furthermore, loss of the three sugar kinases also completely eliminates the cAMP signal induced by low (5mM instead of 100mM) glucose concentrations or by fructose (Rolland et al., 2001). Addition of sucrose to an invertase deficient strain activates cAMP synthesis only if a low level of glucose is added so that glucose phosphorylation can be sustained (Rolland et al., 2000).
No further metabolism beyond sugar phosphorylation is required to activate the cAMP signaling process, as demonstrated by a phosphogluco-isomerase deficient strain, which still respond to glucose. (Beullens et al., 1988)
The glucose transporters do not seem to have a regulatory function in the process but are only required to maintain a critical level of intracellular glucose to sustain sugar phosphorylation: as a confirm, constitutive expression of *GAL2*, encoding the

galactose permease (which can also support glucose uptake: Liang and Gaber., 1996; Reifenberger et al., 1997) suffices to restore glucose-induced cAMP signaling, making it unlikely that additional regulatory functions besides are associated with any single specific *HXT* carrier (Rolland et al., 2000). In addition, neither of the two glucose sensors Snf3 and Rgt2 has a direct role in the cAMP signaling (Rolland et al., 2001).

The constitutively active $GPA2^{V132}$ allele is able to bypass the inactivation of *GPR1* for glucose-induced activation of cAMP synthesis, but not the request for sugar uptake and phosphorylation (Rolland et al., 2000; Kraakman et al., 1999). Interestingly, the $GPA2^{V132}$allele also increases the fructose-induced cAMP signal to the same intensity as the glucose signal and enables concentrations of glucose as low as 5 mM to fully activate the cAMP/PKA pathway; this is consistent with the fact that the $GPA2^{V132}$ strain only needs to fulfill the phosphorylation request in order to activate the cAMP signaling, since the GPCR module is constitutively activated (Rolland et al., 2000; Rolland et al., 2002). Thus, $GPA2^{V132}$ can fully substitute for the requirement of high extracellular glucose, allowing ligands that are phosphorylated but not detected by the Gpr1-Gpa2 system (such as fructose (see below) and low glucose) to fully activate the cAMP circuit (Rolland et al., 2000).

In a hxt-null strain, the glucose phosphorylation requirement for cAMP signaling can be fulfilled separately from extracellular glucose detection via the GPCR system by providing a low-amount of maltose, which is transported inside the cell by a specific uptake system and converted into glucose by maltase: in this way, a pre-treatment with a low concentration of maltose before addition of glucose allows the restore the glucose-cAMP signaling in a *hxt* deficient strain (Rolland et al., 2000; Rolland et al., 2001) and the effect is entirely dependent on the presence of functional Gpr1 and Gpa2 (Rolland et al., 2000). Intracellular acidification is also able to bypass the glucose uptake requirement for cAMP signaling, but it does not suppress the absence of the hexose kinases (Rolland et al., 2001).

Hence, glucose induced cAMP signalling clearly involves two distinct processes: an extracellular glucose-sensing process that is dependent on Gpr1-Gpa2 system and an intracellular glucose-sensing process that is dependent on glucose phosphorylation (Thevelein et al., 2005; Rolland et al., 2002). It is unclear why glucose phosphorylation is required and how it is coupled to the control of cAMP synthesis (Rolland et al., 2002; Gancedo, 2008).

Neither glucose-6-phosphate nor ATP seem to act as “metabolic messengers” to trigger the cAMP production in response to glucose, since there is no strict correlation between the increase of these metabolites after glucose addition and the amplitude of the cAMP signal (Beullens et al., 1988; Rolland et al., 2001). Thus, since no further glucose metabolism is needed beyond glucose phosphorylation to activate the cAMP synthesis, a regulatory role for the sugar kinases has been proposed (Rolland et al., 2001; Beullens et al., 1988).

The interdependency between the GPCR system and the sugar phosphorylation for glucose-dependent cAMP signaling is rather puzzling: apparently, glucose, which acts as an extracellular ligand for the GPCR system, has to be transported inside the cell and phosphorylated in order to be able to stimulate its effector system (Colombo et al., 2004; Thevelein et al., 2005). Glucose phosphorylation seems to be required in some way to make adenylate cyclase responsive to activation by the GPCR system (Colombo et al., 2004; Thevelein et al., 2005).

Interestingly, the glucose-induced Ras-GTP loading is also dependent on sugar uptake and phosphorylation, while it does not require the presence of a functional GPCR system (Colombo et al., 2004). Furthermore, even low glucose levels (5mM) can trigger the increase in Ras-GTP in a *gpa2 gpr1* strain: therefore, it has been suggested that glucose phosphorylation might act through the Ras proteins to activate the cAMP signaling (Colombo et al., 2004). According to the proposed model, a glucose phosphorylation-dependent mechanism would cause inhibition of the Ira proteins, resulting in a rapid increase in Ras2-GTP levels; activated Ras would then prime adenylate cyclase for further stimulation by the GPCR system (Colombo et al., 2004).

The contribute of the GPCR system to the glucose induced transcriptional response

Recent microarray data (Wang et al., 2004; Zaman et al., 2009) have shown that induction of the $GPA2^{Q300L}$ constitutive activated allele results in the same massive reconfiguration of the transcriptional profile triggered by glucose addition: at least 90% of the transcriptional changes occurring in presence of glucose can be recapitulated by the activated Gpa2 allele and the effect is entirely mediated by PKA (Wang et al., 2004; Zaman et al., 2009). However, the magnitude of transcriptional response triggered by $Gpa2^{Q300L}$ is much weaker than those observed with an activated Ras2 allele or following glucose addition. Furthermore, inactivation of the Gpr1 receptor slightly diminishes but does not eliminate the cellular transcriptional response induced by glucose, Therefore, Ras2 seems to be the main player in mediating the glucose-induced changes in the transcriptional profile, while the role of the GPCR system in this process is more auxiliary (Wang et al., 2004; Zaman et al., 2009; Zaman et al., 2008).

The same transcriptomic analyses also revealed surprising connections between Sch9 and the Gpr1/Gpa2 module (Zaman et al., 2009). For instance, reduction of Sch9 activity or inactivation of *GPR1* promotes the transcription a common set of genes involved sterol and cell wall biosynthesis genes during growth on a non-fermentable carbon source (Zaman et al., 2009); apparently, the TORC1 network is involved in maintaining the repression of these genes under favourable growth conditions (Zaman et al., 2009). Even more interestingly, downregulation of Sch9 enhances the transcriptional repression response occurring after activation of the Gpr1/Gpa2 circuit: these observations suggest the existence of a potential crosstalk between the TOR/Sch9 and the Gpr1/Gpa2 pathways, in which attenuation of Tor signalling would increase the nutrient-induced transcriptional response mediated by cAMP/PKA pathway (Zaman et al., 2009).

This cross-talk may be particularly important under condition of nitrogen shortage for the switch to filamentous mode of growth (Zaman et al., 2009).

Gpr1: a low affinity glucose receptor to regulate the switch to fermentative metabolism?

The Gpr1-Gpa2 module is responsive to glucose and sucrose but not to structurally similar sugars such as fructose or to glucose analogues, while mannose acts as a potent antagonist of both sucrose and glucose induced cAMP signaling (Rolland et al., 2000; Lemaire et al., 2004). Although no direct binding of any sugar to Gpr1 has been reported so far, indirect evidences confirm that both sucrose and glucose interact as ligands with Gpr1: for instance, several Gpr1 mutants have been isolated,

which are specifically impaired in glucose-induced, but not in sucrose-induced, cAMP signalling, an observation which strongly supports the existence of a sugar binding site in Gpr1 (Lemaire et al., 2004).
Surprisingly, the GPCR system displays a much higher affinity for sucrose than for glucose, a finding which explains the need for a relatively high concentration of glucose to obtain maximal cAMP signaling: 20mM glucose is necessary for half-maximal activation of the cAMP signaling *in vivo*, in comparison with a measured EC50 (effector concentration for half-maximum response) value of 0.5 mM for sucrose (Lemaire et al., 2004; Rolland et al., 2000). Therefore, Gpr1 is apparently a high affinity sucrose, low affinity glucose sensor (Rolland et al., 2000; Lemaire et al., 2004). At first glance, this observation would also suggest that sucrose rather than glucose may be the relevant physiological ligand for Gpr1: detection of low amounts of this less-preferred sugar may be important for the survival of yeast cells in their natural environment, where they experience long periods of nutrient starvation, alternating with very short intervals of nutrient abundance. On the other hand, the low affinity of the GPCR system for glucose may fit with the physiological context in which this pathway is operative: in yeast cell, the complete switch from a respirative/gluconeogenetic metabolism to fermentative growth only occurs at glucose concentrations of at least 20mM (Thevelein, 1991; Rolland et al., 2000; Johnston & Carlson, 1992),a relatively high value that falls within the same range as the apparent *Ka* of 25mM estimated for the activation of cAMP synthesis by glucose. Thus, Gpr1 may have a specific role in detecting high level of glucose, in order to promote a rapid metabolic switch to fermentation only when a relatively large supply of sugar is available (Rolland et al., 2000; Lemaire et al., 2004).

Kelch proteins: a new direct route for activating PKA?
The regulatory role of the kelch proteins Krh1/Krh2 is still debated (Peeters et al., 2007; Gancedo et al., 2008; Zaman et al., 2009; Harashima et al., 2005; Harashima et al., 2006). Although they were initially proposed to mimic β-subunits, it now seems clear that they function as downstream effectors of Gpa2 to downregulate the PKA signal. Krh1/Krh2 contain seven kelch repeat domains that fold into a β propeller structure that serves to mediate protein-protein interactions and that resembles the three-dimensional configuration of the seven tandem WD-40 repeats found in canonic Gβ subunits, despite any lack of primary sequence identity (Harashima & Heitman., 2002).
However, in spite of this structural similarity, the kelch proteins do not display any of the typical functional features of the canonic Gβ subunits: they do not facilitate coupling of the GPCR Gpr1 with the α subunit Gpa2, nor do they stabilize the inactive form of Gpa2 or recruit Gpa2 to the plasma membrane (Peeters et al., 2007). *krh1* and *krh2* deficient cells exhibit the typical phenotype associated with an upregulation of the PKA pathway: increased sensitivity to heat stress, low levels of storage carbohydrates, enhanced filamentous growth and reduced sporulation efficiency (Peeters et al., 2007). Moreover, the *in vivo* phosphorylation state of PKA substrates, such as Msn2p and Sfl1p, is enhanced in strains lacking one or both of the kelch proteins (Harashima & Heitman., 2002; Peeters et al., 2006; Lu & Hirsch, 2005). The inactivation of *KRH1* results in more severe phenotypes than the *KRH2* deletion, whereas the double mutant exhibits the most pronounced effects than either of the single mutants (Peeters et al., 2007).

Taken together, these observations suggest that Krh1 and Krh2 are negative regulators of PKA signalling (Harashima & Heitman., 2002; Harashima et al., 2005; Peeters et al., 2006; Lu & Hirsch, 2005). Two different mechanisms have been proposed to explain the effect of the kelch proteins in the PKA network.

Genetic epistasis experiments indicate that the Krh proteins function downstream of both Gpa2 and adenylate cyclase and upstream of (or directly on) the PKA holoenzyme (Peeters et al., 2006). Accordingly, Krh1 and Krh2 interact with all the three Tpk catalytic subunits and in a *krh1 krh2*t mutant the interaction between Tpk1 and the regulatory subunit Bcy1 is weakened. Moreover, recent findings showed that the Gpr1-Gpa2 system is able to regulate the activity of PKA even in an adenylate cyclase null mutant (*cyr1 pde2*) where the system cannot control cAMP levels (Peeters et al., 2006) suggesting the existence of a cAMP independent mechanism for PKA activation. in fact, both the expression of a constitutively active $GPA2^{V132}$ allele or the inactivation of *KRH1/2* lower the concentration of external cAMP that must be added to the medium in order to restore growth of the *cyr1 pde2* mutant (Peeters et al., 2006). According to the proposed model, Krh1/2 might facilitate the association between the regulatory (Bcy1) and catalytic (Tpk1,2,3) subunits of PKA (Peeters et al., 2006; Peeters et al., 2007). Active Gpa2 would relieve the inhibition imposed by the kelch-repeat proteins on PKA, thereby bypassing adenylate cyclase for direct regulation of PKA; inactivation of the Krh proteins would lowers the cAMP dependency of PKA, hence triggering activation of the kinase even when the cAMP levels remain constant (Peeters et al., 2006; Peeters et al., 2007)..

The kelch repeat proteins provide one of the first examples of mechanisms for activation of PKA in the absence of an increase in the cAMP level. Interestingly, essential nutrients other than fermentable sugars, such as nitrogen sources, phosphate or sulfate, activate the PKA pathway trough an unknown, cAMP independent mechanism (see Thevelein et al., 2005). Rapid activation of the PKA pathway by these nutrients requires the presence of a rapidly fermentable sugar. A plausible model for the concerted action of the diverse nutrients is that fermentable sugars cause limited dissociation of PKA by increasing the cAMP level while other essential nutrients trigger a further increase in the activity of the free catalytic subunits by a cAMP independent mechanism (Thevelein, 1991; Peeters, et al, 2007). Intriguingly, the kelch repeat protein bypass of adenylate cyclase might provide the link to integrate different nutrient signals with the glucose-induced cAMP signaling for synergistic activation of PKA (Peeters et al., 2006; Peeters et al., 2007).

However, it should be noted that deletion of *KRH1* and *KRH2* does not suppress the lethality of a *gpa2 ras2* double deleted strain, a phenotype that may also indicate that the Krh proteins must function upstream of adenylate cyclase (Harashima et al., 2006). Krh1 and Krh2 bind to a conserved C-terminal domain of the GAPs Ira1 and Ira2 *in vitro* and stabilize them (Harashima et al., 2006). In the absence of Krh1/2 Ira1 and Ira2 are more subjected to degradation and the fraction of Ras-GTP increases, resulting in an upregulation of the cAMP signalling (Harashima et al., 2006).

Therefore, Harashima and colleagues suggested that Gpa2/Gpb/Gpg heterotrimers might regulate the activity of the PKA network in at least two ways: direct activation of adenylate cyclase promoted by Gpa2 and inhibition of Ras signaling by increasing Ira1,2 levels. These two opposing modes would function on different time scales, consistent with an initial Gpa2-dependent stimulation of the PKA

signaling followed by long-term Gpb dependent attenuation (Harashima et al., 2006; Zaman et al., 2008).
Both the models (adenylate cyclase independent (Peters et al., 2006) or adenylate cyclase dependent (Harashima et al., 2006) mechanisms) proposed to explain the role of kelch protein in PKA signaling are not mutually exclusive (Gancedo, 2008).
Recently, another WD-domain protein, Asc1, has been proposed as a substitute Gβ subunit for protein Gpa2 (Zeller et al., 2007): Asc1 binds to Gpa2 in a guanine nucleotide-dependent manner, inhibits nucleotide exchange activity on Gpa2 and diminishes glucose-induced cAMP signaling: taken together, these observations suggest that Asc1, rather than the Krh proteins, functions as a bona fide Gβ protein (Zeller et al., 2007; Peeters et al., 2007).
Based on its interaction with the Kelch protein Krh1 and Krh2, Gpg1 was initially proposed as the potential Gγ subunits in the putative heterotrimeric G-protein comprising Gpa2 and Krh1/2 as α and β subunits, respectively (Harashima & Heitman, 2002). However, recent experimental data are inconsistent with such a function for Gpg1: for instance, the effects of *GPG1* inactivation on filamentous growth are opposite to those registered in a *krh1 krh2* mutant, an unusual result since Gβ and Gγ subunits usually work as a complex. This observation would suggest for Gpg1 a role as an activator of the PKA pathway. However, it should be noted that loss of Gpg1 function does not impair the glucose-induced cAMP signalling and has only negligible effects on invasive growth, nitrogen-starvation sensitivity and glycogen accumulation (Peeters et al., 2007).

Subcellelar localization of PKA
The subcellular localization of PKA is regulated by at least two distinct mechanisms: cAMP regulates the localization of the catalytic subunit, whereas the carbon source controls that of the Bcy1 regulatory subunit (Griffionen et al., 2000; Griffionen et al., 2001; Griffionen et al., 2002); in addition, Bcy1 apparently determines the localization of the TPK subunit associated with it. Consistent with this notion, Bcy1 is almost exclusively nuclear in glucose-grown cells and the catalitically inactive PKA holoenzymes (consisting of the complexes between the regulatory subunit Bcy1 associated to the catalytic Tpks subunits) also localize to the nucleus during growth on glucose (Griffionen et al., 2000; Griffionen et al., 2001; Griffionen et al., 2002). However, glucose exhaustion leads to a pronounced relocalization of Bcy1 into the cytoplasm: in contrast to rapidly proliferating cells, Bcy1-Tpks inactive complexes are distributed between nucleus and cytoplasm in cells growing on nonfermentable carbon sources or in stationary phase cells (Griffionen et al., 2000; Griffionen et al., 2001). Deprivation of cAMP in rapidly growing yeast cells results in a pronounced nuclear localization of Tpk subunits, whereas the subsequent addition of cAMP, which activates PKA by promoting the dissociation of the Tpk-Bcy1 complexes, leads to a fast redistribution of the liberated Tpk catalytic subunit from the nucleus to the cytoplasm, where it can phosphorylate its target substrates (Griffionen et al., 2000; Griffionen et al., 2001). In contrast, the regulatory Bcy1 subunit remains in the nucleus both in the presence and absence of cAMP (Griffionen et al., 2000; Griffionen et al., 2001).
The physiological relevance of the carbon source-modulated subcellular localisation of Bcy1 is presently unknown, although several models have been proposed. For instance, the entry of Bcy1 (which inhibits Tpks activity) into the cytoplasm upon glucose exhaustion may serve to down-regulate PKA activity and consequently

control several metabolic enzymes (e.g. fructose-1,6-bisphosphatase, trehalase) localised in the cytoplasmatic compartment: this would favour the switch from a fermentative to a respiratory metabolism or the entry into quiescence and allow the accumulation of storage carbohydrates (glycogen and trehalose). Accumulation of inactive PKA holoenzyme in the cytoplasm of glucose-deprived cells may also render the kinase extremely sensitive to glucose-triggered increases in cAMP level, since this secondary messenger is produced near the plasmatic membrane: in this way, yeast cells would be able to rapidly reconfigure their metabolism when glucose is again available (Griffionen et al., 2000). Strains expressing Bcy1 versions defective in nuclear accumulation are less viable during stationary phase, sporulate with reduced efficiency (if diploids) and exhibit a delay in resumption of growth following transfer to fresh growth medium (Griffionen et al., 2000): thus, nuclear accumulation of the regulatory PKA subunit may be required for rapid recover of mitotic growth upon shift to fresh medium, whereas in cells growing on a nonfermentable carbon source it may be advantageous to increase the cytoplasmic level of the Bcy1p, in order to downregulate the cAMP signal and adjust metabolism to the gluconeogenic/respiratory mode (Griffionen et al., 2000).

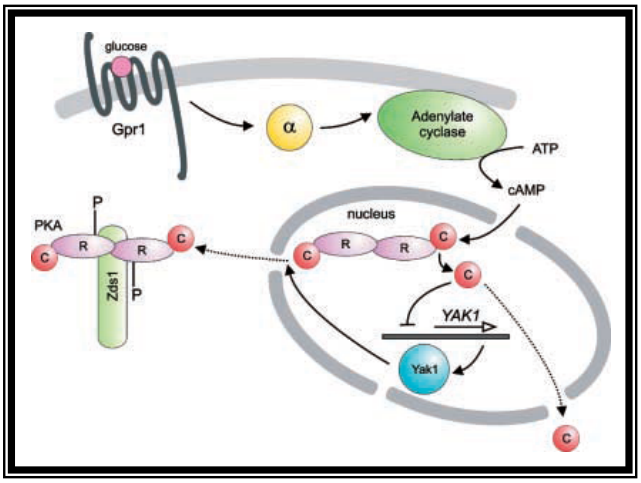


Figure 19. Model for the control of PKA subcellular localization in yeast
In glucose-growing cells, PKA inactive holoenzymes localize inside the nucleus. cAMP stimulation dissociates the catalyticYpks subunits, allowing them to enter the cytoplasm. Yak1 mediates the phosphorylation and consequently the cytoplasmic localization of the regulatory Bcy1 subunit in glucose-deprived cells. Zds1 is required for cytoplasmic sequestration of Bcy1, possibly by interacting with hyper-phosphorylated serine clusters in the N-terminus of Bcy1. Yak1 expression is negatively regulated by PKA through the transcription factors Msn2 and Msn4. (From Griffionen et al., 2002).
Note: it is not experimentally demonstrated that cAMP can enter the nucleus to liberate the catalytic Tpk subunits).

The cytoplasmic redistribution of Bcy1 in glucose-starved cells requires the phosphorylation of multiple serine residues organised in two distinct clusters (I and II) inside the N-terminal region of Bcy1 (Griffionen et al., 2001): in particular, the phosphorylation of cluster II may increase the affinity of Bcy1 for Zds1, a putative functional homologue of mammalian AKAPs (A Kinase Anchoring Proteins) that may function as an adaptor protein to sequester Bcy1 in the cytoplasmic compartment (Griffionen et al., 2001). The phosphorylation of Bcy1 in response to

carbon starvation appears to be dependent on the kinase Yak1 (Fig. 19): however, the role of Yak1 in the process may be indirect, since no evidence for a direct phosphorylation of the Bcy1 N-terminus by Yak1 has been obtained (Griffionen et al., 2001).
Interestingly, the expression of *YAK1* is regulated by the transcription factors Msn2/4, whose activity is repressed by PKA under favorable growth conditions (Smith et al. 1998): these findings apparently suggest the existence of an autoregulatory loop, in which PKA would control the subcellular localization of its own catalytic subunit via the Msn2/4 transcription factors, which would activate *YAK1* transcription upon glucose depletion (Griffionen et al., 2001; Griffionen et al., 2002). Consistent with this proposal, *yak1*, *msn2 msn4* and *msn2 msn4 yak1* null mutants all exhibit defects in cytoplasmic Bcy1 localization during growth on ethanol (Griffionen et al., 2001).
Since Bcy1 levels considerably increase as cells approach to stationary phase, the change in subcellular localization may also result from the preferential cytoplasmic accumulation of *de novo* synthesized Bcy1, besides the redistribution of the preexisting pool of Bcy1 (Griffionen et al., 2000; Griffionen et al., 2001).
Interestingly, *TPK1* expression is significantly reduced in *bcy1* cells (Schmelze et al., 2004): since the *TPK1* promoter contains several putative STRE elements (Moskvina et al., 1998) and in a *bcy1* mutant the transcription factors Msn2 and Msn4 are confined in the cytoplasm, *bcy1* cells are likely unable to activate the transcription of *TPK1* (Schmelze et al., 2004).
Finally, several evidences suggest that the TOR network controls the subcellular localization of both the PKA catalytic subunit encoded by *TPK1* and the Yak1 kinase (Schmelze et al., 2004; see following sections).

Cellular processes affected by the cAMP/PKA circuit: a brief overview
In yeast the cAMP/PKA network plays a central role in the control of metabolism, stress resistance and cell proliferation (Zaman et al., 2008; Thevelein & de Winde, 1999; Rolland et al., 2002).
When glucose is available, activation of the cAMP/PKA pathway favors rapid growth and cell proliferation by stimulating the glycolytic flux and by repressing the stress response and the expression of genes required for respiratory metabolism (Wang et al., 2008; Zaman et al., 2009; Slattery et al., 2008; Gancedo, 2008; Zaman et al., 2008). Consistent with the key role of cAMP signalling in promoting fermentation, many of the identified targets of PKA are enzymes involved in carbon and energetic metabolism (Gancedo, 2008; Zaman et al., 2008).
Glucose dependent activation of the PKA circuit promotes growth (mass accumulation) and a drastic increase in the cellular biosynthetic capacity by inducing the transcription of genes involved in ribosome biogenesis (Jorgensen et al., 2004; Klein & Struhl, 1994; Neumann et al., 1995; Zurita-Martinez & Cardenas, 2005). The PKA dependent activation domain of several genes encoding ribosomal protein maps to Rap1 binding sites (Klein & Struhl, 1994; Neumann et al., 1995). In addition, the cAMP/PKA circuit affects the activity of Sfp1, a master regulator of ribosome biogenesis (Jorgensen et al., 2004; Jorgensen & Tyers, 2004; see following sections).
Cell proliferation requires a precise coordination between growth and cell division (Jorgensen & Tyers, 2004): consistently, the cAMP pathway is involved in the control of cell cycle progression (Santangelo, 2006; Drebot et al., 1990; Anghileri et

al., 1999; Schneper et al., 2004b) and nutritional modulation of the critical cell size required for entry into the S phase (Baroni et al., 1989; Mitsuzawa, 1994; Jorgensen & Tyers. 2004). Translational control of the Cln3 cyclin synthesis by PKA has been proposed as a key link between nutrient availability and control of cell cycle progression (Hall et al., 1998; Barbet et al., 1996; Polymenis & Schmidt, 1997): interestingly, ectopic expression of the G1 cyclin *CLN3* is sufficient to suppress the lethality of mutants where the cAMP/PKA pathway is inactivated (Hall et al., 1998). These issues will be examined in more detail in following sections.

Nutrient availability, growth rate and stress response are intimately interconnected: not surprisingly, the switch to fermentative metabolism also coincides with the downregulation of the stress response (Thevelein & de Winde, 1999; Zaman et al., 2008). Two zinc-finger transcriptional factors, Msn2 and Msn4, appear to mediate the effects of the cAMP/PKA pathway on the glucose-triggered repression of stress responsive genes (Martinez-Pastor et al., 1996; Gorner et al., 1998; Smith et al., 1998; Boy-Marcotte et al., 1998; Tadi et al., 1999). Msn2 and Msn4 act as positive regulators of the general stress-response by binding to STRE motifs (stress responsive elements: consensus sequence 5'-CCCCT-3') found in the promoter of their targets genes (Martinez-Pastor et al., 1996; Moskvina et al., 1998) in response to nutrient (glucose, nitrogen) starvation and to a wide variety of stress conditions, such as heat-shock, osmotic shock, oxidative stress, low pH and high ethanol concentrations (Martinez-Pastor et al., 1996; Gash et al., 2000; Causton et al., 2001; Boy-Marcotte et al., 1998). More than 50% of the genes induced by these environmental perturbations belong to the Msn2/4 regulon, which includes genes encoding for molecular chaperones, antioxidant proteins, enzymes involved in carbohydrate metabolism and in proteolytic degradation (Gasch et al., 2000; Causton et al., 2001; Boy-Marcotte et al., 1998). Msn2 and Msn4 are functionally redundant, although increasing evidences suggest that the individual contribution of the two transcription factors may differ for specific genes and under particular stress conditions; *MSN4* transcription is itself Msn2/4 dependent and induced by stress, whereas *MSN2* expression is constitutive (Gash et al., 2000)

PKA apparently regulates processes such as growth, glycogen accumulation and stress response by suppressing the Msn2/4-mediated gene expression (Smith et al., 1998). Remarkably, PKA activity has been found to be dispensable in strains lacking Msn2 and Msn4: this observation suggests that repression of Msn2/4-dependent transcription may account for many of the pleiotropic effects of PKA signalling (Smith et al., 1998).

The main step for regulation of Msn2/4 activity takes place at the level of subcellular localization. The intracellular distribution of Msn2/4 is highly sensitive to nutrient starvation and to environmental stresses: both the transcription factors are predominantly cytoplasmic during logarithmic growth, whereas they rapidly concentrate in the nucleus in stressed cells or when nutrients such as glucose or nitrogen are depleted (Gorner et al., 1998). The accumulation of Msn2/4 in the nucleus and the subsequent activation of stress response is controlled antagonistically by stress conditions and by several nutrient sensing networks (PKA, TOR; Snf1), which affect the phosphorylation state of a nuclear localization signal (NLS) and of a less defined nuclear export signal (NES) found within the two transcription factors (Gorner et al., 1998; Gorner et al., 2002; Santhanam et al., 2004; Smith et al., 1998; Beck & Hall, 1999). In the presence of glucose, PKA-

dependent phosphorylation on al least 4 sites within the carboxy-terminal NLS inhibits the nuclear import of Msn2/4, thus repressing their transcriptional activity, whereas glucose starvation is associated with a rapid drop in PKA-dependent phosphorylation of the NLS, resulting in fast nuclear accumulation of the two transcription factors (Gorner et al., 2002; Gorner et al., 1998): therefore, Msn2 and Msn4 localize to the cytoplasm when PKA is active, whereas they reside in the nucleus when cAMP signalling is downregulated (via a Bcy1-dependent mechanism) upon glucose exhaustion (Gorner et al., 1998; Gorner et al., 2002). Nuclear import of Msn2/4 is dependent on the karyopherin Kap123 that may preferentially interact with the unphosphorylated form of the NLS within Msn2/4 (Gorner et al., unpublished data; see De Wever et al., 2005). The protein phosphatase 1 (PP1) complex (including Glc7 and an unknown regulatory subunit) appears to be the direct antagonist of PKA-dependent phosphorylation at the NLS domain within Msn2/4 and therefore it has also been suggested to be a mediator of glucose starvation signals (De weaver et al., 2005).

A recent study has shown that nuclear translocation of Msn2/4 upon glucose starvation is insufficient for their full activation, which requires an additional mechanism involving the Yak1 kinase (Lee et al., 2008). Yak1, whose activity is negatively regulated by PKA (Garret et al., 1991; Moriya et al., 2001; see below), may directly phosphorylate and activate Msn2/4 through an unidentified mechanism: since Yak1-dependent phosphorylation of Msn2/4 does not affect their DNA binding activity, the regulation of the two transcription factors by Yak1 likely occurs after the event of DNA binding (Lee et al., 2008). Interestingly, since transcription of *YAK1* is induced by Msn2/4, activation of the two transcription factor can generate a positive feedback loop by increasing the levels of Yak1: therefore, Yak1-dependent activation of Msn2/4 may provide a further layer of PKA-dependent regulation for Msn2/4 function to ensure a proper cellular response depending on the nutritional status (Lee et al., 2008). Remarkably, Yak1 seems also involved in the activation of Msn2/4 upon heat shock (Lee et al., 2008).

Apparently, the NLS localization activity responds only to glucose levels, but not to nitrogen starvation or to heat or osmotic shock: these stresses control the intracellular distribution of Msn2/4 through the PP2A phosphatase, which dephosphorylates a putative NES (nuclear export signal) inside the amino terminal region of Msn2/4 in order to prevent the export of the two transcription factors from the nuclear compartment via the Msn5 karyopherin (Santhanam et al., 2004; Gorner et al., 2002). Interestingly, the PP2A phosphatase is one of the effector of the TOR signalling pathway, which has also been found to be involved in the control of the subcellular localization of Msn2/4: inhibition of TOR upon rapamycin treatment activates PP2A, causing Msn2/4 to accumulate in the nucleus via dephosphorylation of the NES domain (Beck & Hall, 1999; Gorner et al., 2002; Santhanam et al., 2004). Remarkably, just as the NLS import signal, the nuclear export function of the amino-terminal domain of Msn2/4 also responds to glucose depletion and PKA activity, although in this case the residues target of PKA phosphorylation have yet to be determined (Gorner et al., 2002; Gorner et al., 1998). The sensitivity of the amino-terminal domain to both stress and glucose starvation suggest that it may contain a single NES which is co-regulated by glucose and stress trough a competition between PP2A and PKA activity; as an alternative, two separate sub-domains independently regulated by glucose and stress may exist within the amino-terminal region of Msn2/4 (Gorner et al., 2002).

The Snf1 kinase have also been shown to inhibit Msn2 by phosphorylating one of the four PKA targets sites in NLS during prolonged glucose starvation (S582; De Wever et al., 2005): this event may be part of an adaptive mechanism to long-term glucose deprivation (De Wever et al., 2005).
It has also been reported that Msn2 are normally sequestered in the cytoplasm by their interaction with Bmh2, a member of the 14-3-3 protein family which may function as a rapamycin sensitive cytoplasmic anchor, and that the activation of the transcription factors simply results from the dissociation of the Bmh2-Msn2/4 complex (Beck and Hall, 1999); however, a more recent study has downsized the role of 14-3-3 proteins in the regulation of Msn2/4 cellular distribution (Gorner et al., 2002).
It is still largely unknown how the different signals which affect the subcellular localization of Msn2/4 are integrated. Furthermore, nuclear translocation is not the only step for the control of Msn2/4 activity: several evidences have demonstrated that the Msn2/4 activity can also be regulated at the level of DNA binding (Hirata et al., 2003), transactivation (Boy-Marcotte et al., 2006) and protein stability (Durchschlag et al., 2004; Lallet et al., 2004; Lallet et al., 2006).
DNA binding activity of Msn2/4 is mediated by the zinc-finger domain localized in the C-terminal end and has been reported to be controlled by stress, possibly through a mechanism involving the glycogen synthase kinase-3 encoded by *GSK3* (Hirata et al., 2003). The role of Gsk3 in the process is likely indirect. (Hirata et al., 2003)
The transcriptional activation domain is located inside the amino-terminal region of Msn2/4 and its function is regulated by stress inputs, whereas it is insensitive to cAMP signalling (Boy-Marcotte et al., 2006).
Concomitantly with the activation of Msn2 and Msn4 upon stress exposure, the two transcription factors are hyperphosphorylated by the cyclin-dependent kinase Ssn3/ Srb10, a component of the Mediator complex associated with the transcriptional machinery (Garreau et al., 2000; Chi et al., 2000; Lallet et al., 2004; Lallet et al., 2006). The physiological importance of this phosphorylation event (which is inhibited by the cAMP dependent protein kinase (Garreau et al., 2000)) is not entirely known: although hyperphosphorylation is correlated with the activation of Msn2/4, it is still unclear whether it is this required for activation of Msn2 or it is rather a consequence of its activation and it may play a quite different role. Several findings suggest that hyperphosphorylation of Msn2/4 may represent a mechanism for the attenuation of the general stress response via nuclear proteolysis of the two transcription factors (Bose et al., 2005): in fact, at least a fraction of Msn2/4 is degraded inside the nucleus by the proteasome and the process requires the kinase Ssn3 (Durchschlag *et al*., 2004; Lallet *et al*., 2004; Bose et al., 2005; Lallet et al., 2006).
Therefore, induction of the STRE genes would be only transitory as a result of a dual control of Msn2/4 activity at the level of subcellular localization and nuclear degradation (Bose et al., 2005).

In absence of Msn2/4, PKA can still regulate several stress responsive genes such as *HSP12* and *HSP26* (encoding two small heat-shock proteins) by negatively modulating the activity of the transcription factor Hsf1 (Ferguson et al., 2005). Best known for its role in the heat-stress response, Hsf1 also controls the expression of a large battery of genes involved in processes such as protein folding and degradation, detoxification, energy generation, carbohydrate metabolism and cell wall

organization (Hahn et al., 2004; Eastmond & Nelson, 2006). A low level of Hsf1p activity is essential to maintain constitutive expression of genes necessary for normal cellular processes: consistent with this notion, the inactivation of *HSF1* is lethal and mutants exhibit defects in maintenance of cell wall integrity, spindle pole body duplication, protein transport and cell cycle progression (Wiederrecht et al., 1988; Smith & Yaffe, 1991; Zarzov et al., 1997; Imazu & Sakurai, 2005).

Hsf1 always localizes in the nucleus, where it binds as a homotrimeric complex to the conserved heat shock element (HSE) motifs found in the promoters of its target genes, in both constitutive and stress-inducible manners (Giardina & Lis, 1995; Jakobsen & Pelham, 1988; Hahn et al., 2004): Hsf1 binds with low affinity to many of its target promoters under physiological conditions and with high affinity upon stress exposure (Hahn et al., 2004; Hashikawa et al., 2007; Yamamoto et al., 2008).

Although heat shock is the best-studied inducing signal for Hsf1 activity, the transcription factor plays a key role in the cellular response to a wide range of stress conditions including oxidative stress, alkaline pH, heavy metals, ethanol treatment and nutrient starvation (Liu & Thiele, 1996; Tamai et al., 1994., Amoroso & Estruch, 2001; Hashikawa et al., 2007).

The exact mechanisms by which the diverse stresses activate Hsf1 are largely unknown. However, phosphorylation seems to play an important role in regulating Hsf11 activity: in unstressed cells Hsf1 is constitutively phosphorylated, but it becomes hyperphosphorylated in response to certain stresses, likely adopting an active conformation that results in the transcription of several target genes (Sorger & Pelham, 1988; Hashikawa & Sakurai, 2004; Hashikawa et al., 2006); in other studies, phosphorylation has also been proposed to serve as a regulatory mechanism to deactivate Hsf1 (Høj & Jakobsen, 1994). Interestingly, heat-shock and oxidatide stress induce distinct patterns of Hsf1 phosphorylation (Liu & Thiele, 1996): therefore, it has been suggested that Hsf1 is regulated by stress-specific differential phosphorylation events, which affect its DNA binding activity. For instance, Snf1-dependent hyper-phosphorylation upon glucose depletion enhances the DNA binding capacity of Hsf1 to low affinity target gene promoters (such as *SSA3* and *HSP30)*, whereas the activation of Hsf1 by heat-shock is not modulated through Snf1 (Hahn & Thiele, 2004).

PKA has been shown to repress Hsf1 activity by indirect inhibition of Hsf1 phosphorylation (Ferguson *et al*., 2005). The effect of PKA on Hsf1 are mediated through the Yak1 kinase, which is under the negative control of PKA and activates Hsf1 by direct phosphorylation when PKA activity decreases upon acute glucose starvation: consistently, *in vitro* phosphorylation of Hsf1 by Yak1 enhances the DNA binding activity of the transcription factor (Lee et al., 2008). However, Yak1 may not be the only effector of PKA required for Hsf1 activation, since *yak1* null cells still display a residual activation of the transcription factor (Lee et al., 2008).

Furthermore, although both Snf1 and Yak1 activate Hsf1 in response to glucose limitation, the two kinases are apparently involved in the adaptation to different physiological conditions: in particular, Yak1 (but not Snf1) seems to be primarily responsible for the activation of Hsf1 under acute glucose starvation (Lee et al., 2008). Consistent with their distinct roles, Snf1 and Yak1 phosphorylate different sites on Hsf1: the distinct patterns of phosphorylation might induce slightly different conformational changes in Hsf1, thus allowing transcription of distinct subset of genes depending on the sequence of HSE motifs (Lee et al., 2008).

In contrast to the role of PKA in the regulation of Msn2/4 dependent transcription, which affects all of STRE-containing genes, PKA activity influences only a subset of Hsf1 targets (Ferguson et al., 2005). Although limited, a partial overlap between the target genes of Hsf1 and those of Msn2/4 exists: in particular, several heat shock protein genes (*HSP12, HSP26* and *HSP104*), as well as genes involved in antioxidant and carbohydrate metabolism (Boy-Marcotte et al., 1999; Gasch et al., 2000; Causton et al., 2001), contain both heat-shock elements (HSE) and stress response elements (STRE) in their promoters and have been shown to be redundantly controlled by both the Msn2/4 and Hsf1 following heat-shock (Amoros & Estruch, 2001; Boy-Marcotte et al., 1999; Treger et al., 1998). However, recent evidences suggest that Hsf1 and Msn2/4 may play distinct roles to ensure cell survival and growth recovery upon exposure to extreme temperatures (Yamamoto et al., 2008).

One gene whose expression is abolished in a *msn2 msn4* mutant is *YAK1*, which encodes a protein kinase which acts as a negative growth regulator by antagonizing the cAMP/PKA pathway (Garret et al., 1991; Garret et al., 1989): consistent with its role, inactivation of *YAK1* restores viability in strains completely devoid of PKA kinase activity (i.e. *tpk1 tpk2 tpk3*). Sok1, a protein with unknown molecular function, appears to serve as a positive regulator in a linear pathway downstream of Yak1: in fact, restoration of growth defects by the deletion of *YAK1* in PKA-deficient mutants requires Sok1 and overexpression of *SOK1* also suppresses the lethality of a *tpk1 tpk2 tpk3* null mutant (Ward & Garrett, 1994).
Yak1 function is negatively regulated in response to glucose, supposedly via direct phosphorylation by PKA (Garrett *et al*., 1991); however, the kinase activity of Yak1 is not influenced by glucose and *in vitro* incubation with bovine PKA does not decrease Yak1 kinase activity (Moriya et al., 2001; Garrett *et al*., 1991). Instead, the glucose-dependent regulation of Yak1 takes place at the level of subcellular localization: Yak1 rapidly translocates to the nucleus upon glucose depletion, whereas addition of the sugar to glucose-starved cells leads to a redistribution of the kinase throughout the entire cell (Moriya et al., 2001); the 14-3-3 proteins Bmh1 and Bmh2, which interact with Yak1 only in the presence of glucose, likely play a role in the relocalization process (Moriya et al., 2001). Rapamycin treatment also induces a fast nuclear accumulation of Yak1, suggesting that, in addition to the cAMP/PKA pathway, the TOR network also regulates the intracellular distribution of Yak1 in response to nutritional and stress conditions (Schmelze et al., 2004; Martin et al., 2004;; Moriya et al., 2001).
The transcription of the *YAK1* gene increases in cells approaching stationary phase and correlates with cell cycle arrest (Garrett et al. 1991): as already discussed, *YAK1* expression is controlled by the transcription factors Msn2/4, whose activity is repressed by PKA and TOR under favorable growth conditions (Gorner et al., 1998; Gorner et al., 2002; Smith et al. 1998; Beck & Hall, 1999; Santhanam et al., 2004).
Yak1 inhibits growth and stimulates the stress response, possibly by downregulating PKA activity: interestingly, Bcy1, the regulatory subunit of yeast PKA, is phosphorylated and redistributes from nucleus to the cytoplasm in a Yak1-dependent manner upon glucose exhaustion (Griffionen et al., 2001). Although the physiological consequences of this regulation are not entirely clear, it has been proposed that the Yak1-mediated relocalization of Bcy1 to the cytoplasm may serve to down-regulate the PKA activity after glucose depletion, thus allowing the

adjustment of metabolism to the respiratory mode or the entry into stationary phase (Griffionen et al., 2001; Griffionen et al., 2002; see also other sections). However, the role of Yak1 in the process may be indirect, since no evidence for a direct phosphorylation of Bcy1 by Yak1 are presently available (Griffionen et al., 2001). Intriguingly, PKA appears to control the localization of its own regulatory Bcy1 subunit via negative regulation of Msn2 and Msn4: low cAMP/PKA signalling activates the two transcription factors, leading to enhanced Yak1 expression and increased cytoplasmic distribution of Bcy1 (Griffionen et al., 2001).

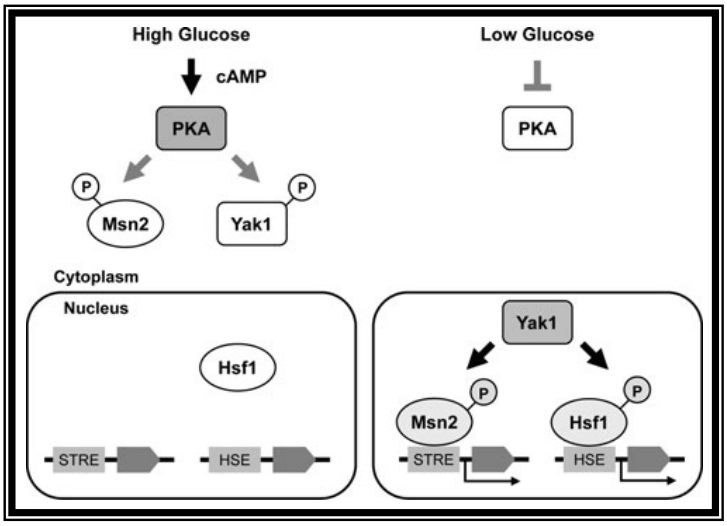


Figure 20. Yak1-dependent regulation of Hsf1 and Msn2/4.
In presence of high-glucose, PKA inhibits nuclear localization of Msn2 by direct phosphorylation. PKA also phosphorylates and inhibits Yak1, likely by preventing its nuclear accumulation. Downregulation of PKA signalling upon glucose exhaustion triggers translocation of Msn2 and Yak1 to the nucleus. Yak1 phosphorylates both Msn2/4 and Hsf1: phosphorylation of Hsf1 increases its DNA binding activity, whereas the mechanism for Yak1-dependent activation of Msn2/4 is still unknown. (From Lee et al., 2008).

Recently, Yak1 has been proposed to play a central role in coordinating the cellular response to nutrient starvation and stress by acting as a "bridge" between PKA and the stress-responsive transcription factors Hsf1 and Msn2/Msn4 (Fig. 20; Lee et al., 2008; see below). Under conditions which favour low PKA activity (for instance, acute glucose starvation), Yak1 activates Hsf1 by direct phosphorylation, increasing the DNA binding activity of the transcription factor (Lee et al., 2008). Furthermore, Yak1 is also involved in the activation of Msn2/4 (possibly by direct phosphorylation), although in this case the molecular details of the process are undefined (Lee et al., 2008); nonetheless, Yak1-dependent activation of Msn2/4 may provide a potential positive feedback loop; since transcription of *YAK1* is regulated by Msn2/4 (Lee et al., 2008). Yak1 may also be required for the activation of Msn2/4 (but not Hsf1) upon heat shock exposure (Lee et al., 2008). Apparently, Yak1-dependent activation of Hsf1 and Msn2/4 is regulated by PKA but not by the TOR network (Lee et al., 2009). The PKA-coordinated regulation of Msn2/4 and Hsf1 via Yak1 may be part of a mechanism to ensure proper balance between cell growth and stress adaptation in response to frequent changes in environmental conditions (Lee et al., 2008).

An established substrate of Yak1 is Crf1, a co-repressor of the forkhead-like transcription factor Fhl1, which regulates the transcription of genes encoding ribosomal protein (RP) (Martin *et al*., 2004). Under favourable growth conditions, TOR (and likely PKA) confines Crf1 inside the cytoplasm by repressing the Yak1 kinase activity (Martin et al., 2004). In absence of TOR signalling, Yak1 promotes the nuclear accumulation of the Crf1, which interacts with Flh1 to repress the transcription of RP genes (Martin et al., 2004; see following section).
Yak1 has also been found to stabilize or promote translation of mRNA encoding proteins involved in stress response, use of alternate carbon sources, growth inhibition and entry into stationary phase (Moriya et al., 2001). Upon glucose depletion, Yak1 directly phosphorylates Pop2, a RNase member of the Ccr4-Caf1-Not deadenylation complex that controls the stability and/or translation of a wide variety of mRNA (Moriya et al., 2001). Preventing Yak1-dependent phosphorylation of Pop2 results in a defective arrest in G1 phase upon glucose deprivation or at the end of post-diauxic growth, before entering quiescence (Moriya et al., 2001).

PKA dependent phosphorylation negatively regulates the kinase activity of Rim15, a critical regulator for entry into quiescence (Reinders et al., 1998; Swinnen et al., 2006; Pedruzzi et al., 2003). *rim15* null mutants fail to properly arrest in G0 upon nutrient exhaustion and exhibit decreased accumulation of storage carbohydrates, reduced expression of stress responsive genes and diminished thermotolerance (Reinders et al., 1998; Swinnen et al., 2006). Rim15 likely inhibits the expression of genes required for growth: consistently, inactivation of *RIM15* suppresses the lethality of *tpk1 tpk2 tpk3* triple null strain, whereas overexpression of Rim15 during exponential growth inappropriately elicits several stationary phase responses and causes a synthetic growth defect in mutants with reduced PKA activity (Reinders et al., 1998; Swinnen et al., 2006). Furthermore, nuclear/cytoplasmic distribution of Rim15 is regulated by TOR (which responds to nitrogen source), Sch9, and by phosphate-responsive signaling complex Pho80/Pho85 (Pedruzzi et al., 2003; Wanke et al., 2005; Roosen et al., 2005). Thus, at least three distinct nutrient-responsive pathways (cAMP/PKA (carbon source), Tor (nitrogen source) and Pho80/Pho85 (phosphate) converge on Rim15: how Rim15 integrates inputs from these signalling circuits to induce quiescence remains unclear. The effects of Rim15 on quiescence are due in part to the reconfiguration of the transcriptional profile, mediated through the stress response transcription factors Msn2/Msn4 and the related post diauxic shift transcription factor Gis1 (Pedruzzi et al., 2000; Cameroni et al., 2004). However, how Rim15 affects the activity of these transcription factors is not entirely understood. Rim15 also binds to the Tps1 component of the trehalose synthase complex, suggesting that part of its role in quiescence involves direct regulation of key enzymatic activities (Reinders et al., 1998; Swinnen et al., 2006).

As already discussed, a particularly relevant substrate of PKA is the transcriptional repressor Rgt1 (Kim et al., 2006a), a key component of the Snf3/Rgt2 pathway which controls the expression of the major sugar transporters encoded by the *HXT* genes (Ozcan and Johnston, 1999; see previous sections). The crosstalk between the PKA network and the Rgt2/Snf3 pathway is part of the complex regulatory circuit that allows the cell to fine tune its transcriptional program and metabolism according to the glucose availability (Zaman et al., 2008; Gancedo, 2008). Glucose triggers the phosphorylation of Rgt1 by PKA, resulting in the release of the repressor form the

promoter of the *HXT* genes and thus allowing their transcription (Kim et al., 2006a). Rgt1 also regulates the expression of *HXK2*, the main glucose phosphorylating enzyme which also plays a role in the Snf1 glucose repression pathway (see previous sections): the role of PKA in the transcriptional regulation of *HXK2* has been described in previous sections (Palomino et al., 2006).

The cAMP/PKA pathway has also been implicated in processes such as aging (Lin et al., 2002; Longo, 2003) actin polarization, bud site selection (Schneper et al., 2004b) and sporulation (Cameron et al., 1988). A key role for PKA in pseudohyphal differentiation has also been extensively described (Pan et al., 2000).
Although interesting, these topics are beyond the scope of this introduction and will not be discussed in detail.

Other nutrient sensing pathways: the TOR network in Saccharomyces cerevisiae

In addition to the cAMP/PKA signaling cascade, the other major nutrient-responsive, growth-controlling pathway in yeast is the TOR network (Martin et al., 2005; Wullschleger et al., 2006; Martin et al., 2006; De Virgilio & Loewith, 2006). Tor (Target of rapamycin) serine/threonine kinases belongs to the phosphatidylinositol- 3 kinase (PI3K) family and exert their functions in two distinct multiproteic complexes (Loewith et al., 2002; Wedaman et al., 2003): TOR Complex 1 (TORC1), which control various aspects of yeast growth and cell proliferation, and TORC2, which regulates cell polarity and organization of the actin cytoskeleton (Fig. 21). The two complexes are structurally and functionally conserved in all the eukaryotes (Wullschleger et al., 2006). In yeast, TORC1 consists of Kog1, Lst8, Tco89, and either the Tor1 or Tor2 kinase, whereas Tor2 and five other proteins (Avo1, Avo2, Avo3, Bit61, and Lst8) comprise TORC2 (Fig. 20; Loewith et al. 2002; Wedaman et al. 2003).

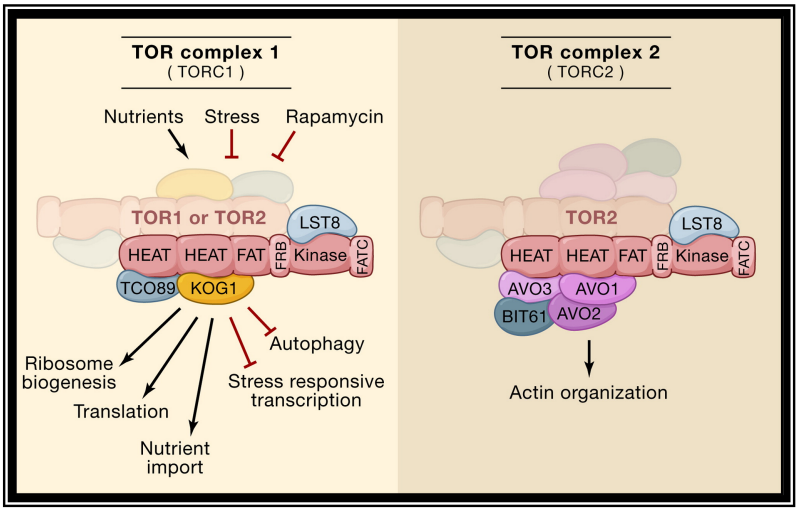


Figure 21. TOR Complex 1 (TORC1) and TOR Complex 2 (TORC2) in S. cerevisiae

TOR-associated proteins (KOG1, TCO89, LST8, AVO1-3, and BIT61) and the domains found in TOR (HEAT, FAT, FRB, Kinase, and FATC) are shown. The amino-terminal 1200 residues consist of stretches of HEAT (Huntingtin, Elongation factor 3, regulatory subunit A of PP2A, *TOR1*) repeats, which typically mediate protein-protein interactions. The 550-amino acid-long FAT (FRAP, ATM, TTRAP) domain has also been suggested to facilitate protein binding. The FAT domain is adjacent to the FKBP12-rapamycin binding site, flanked by the catalytic serine/threonine kinase domain. This kinase domain contains a conserved lipid kinase motif, making Tor1 and Tor2 members of the phosphatidylinositol-kinase-related kinase family. Finally, the carboxyl-terminal 33 residues of Tor1/2 comprise a FATC (FAT C-terminus) domain that is postulated to contribute to redox-dependent Tor protein degradation. TORC1 mediates the rapamycin-sensitive signaling branch that regulates growth (accumulation of mass). TORC2 signaling is rapamycin insensitive and is required for the organization of the actin cytoskeleton. Black arrows indicates positive regulation, whereas red bars indicate negative control. (From Wullshleger et al., 2006).

Substantial evidences suggest that TORC1 activity responds to the nutritional status, primarily the quality of the nitrogen source, and to a wide variety of stress conditions. Apparently, the TORC1 network also relays amino acid concentrations, glucose, and perhaps other nutrient signals to the cellular machinery (De Virgilio & Loewith 2006; Wullschleger et al. 2006; Dechant & Peter 2008). Its major function appears to be the regulation of translation capacity in response to environmental signals by promoting ribosome biogenesis, amino acid availability, and translation

efficiency (Wullschleger et al. 2006; Martin et al., 2005; Martin et al., 2006; Zaman et al., 2008; Inoki & Guan, 2006).
Inhibition of TORC1 by rapamycin (a macrolide drug that in complex with the prolyl-isomerase FKBP12 binds the TOR suppressing its interaction with target substrates) mimics nutrient starvation and causes G1 arrest, inhibition of protein synthesis, glycogen accumulation, induction of autophagy and entry into quiescence (Wullschleger et al. 2006). TORC1 is also intimately implicated in vesicular trafficking (Dechant & Peter 2008; Rohde et al. 2008).
The rapamycin cell cycle arrest is the consequence of downregulation of the G1-cyclins Cln1-3 and upregulation of the Cdk inhibitor Sic1 (Barbet et al., 1996; Zinzalla et al., 2007).
The subcellular localization of TOR is still matter of debate: several early studies showed TOR, its activators, and effectors often localize to intracellular membranes, which may serve as platforms for TOR signaling complexes (Wullschleger et al. 2006; Yan et al. 2006; Aronova et al. 2007; Dechant & Peter 2008; Rohde et al. 2008). More recent studies demonstrate that Tor1 is enriched on the vacuolar membrane (Sturgill et al., 2008), where TORC1 is able to phosphorylate Sch9, one of its relevant effectors (Urban et al., 2007). However, another report showed that under favorable growth conditions at least a fraction of yeast Tor1 localizes to the nucleus, but is rapidly exported to the cytoplasm after rapamycin treatment or nutrient deprivation (Li et al., 2006).

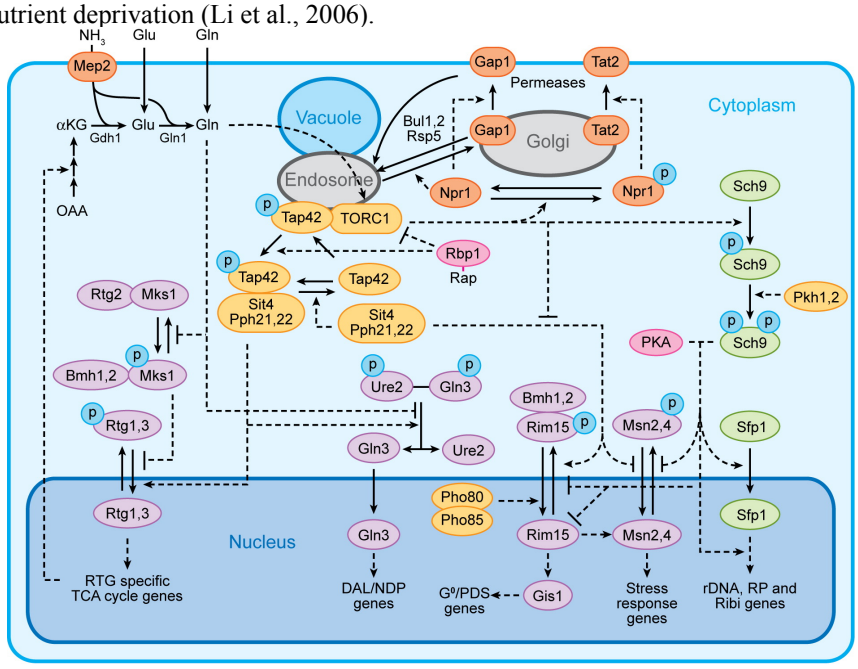


Figure 22. Nitrogen sensing and the TORC1 network in S. cerevisiae.
Nitrogen availability, detected through intracellular glutamate (Glu) and glutamine (Gln) levels, affects the activity of the Tor Complex 1 (TORC1) as well as the expression of the nitrogen discrimination pathway (DAL/NDP) and retrograde signaling (RTG) genes. TORC1 also regulates genes expression in response to nitrogen availability through modulation of Tap42-PP2A phosphatase activity. TORC1 modulates stress-responsive (STRE) and post diauxic shift (PDS) genes both through PP2A and through activation of the Sch9 kinase, which functions in parallel with PKA. The general amino acid (GAP) permease and several amino-acid-specific permeases (i.e.Tat2) are inversely regulated by TORC1 through control of vesicular trafficking by the Npr1 kinase. (From Zaman et al., 2008).

TOR governs metabolism and growth through a cohort of downstream effectors (Fig. 22): in particular, TOR exerts its control primarily at the level of gene expression, often by altering the localization of stress- and nutrient-responsive transcription factors (Duvel et al. 2003; De Virgilio & Loewith 2006).
The TOR network (Fig. 22) plays a major part in the yeast transcriptional response to nutrient availability and stresses (Slattery et al., 2008; Zaman et al., 2009).
Under nutrient-rich conditions, TORC1 inhibits the activity of transcriptional factors involved in nitrogen catabolite-repression (Gat1, Gln3; Shamji et al., 2000; Beck & Hall, 1999), retrograde response (Rtg1, Rtg3; Komeili et al., 2000; Dilova et al., 2004; Tate et al., 2002) and stress-response (Msn2, Msn4; Santhanam et al., 2004), whereas it promotes the function of transcriptional regulators involved in ribosome biogenesis (Fhl1, Spf1; Jorgensen et al., 2004; Marion et al., 2004; Martin et al., 2004). One common mechanism by which the repression of starvation-specific transcription occur is through a TORC1-mediated change in the phosphorylation state of these transcription factors, which confines them in the cytoplasm (often via binding to an anchoring protein), thus preventing their nuclear localization: these phosphorylation/dephosphorylation events are often not performed directly by the TORC1 complex but instead are carried out by downstream effectors, such as the PP2A (protein phosphatase 2A) or PP2A-like phosphatase complexes or the kinase Yak1 (Jiang & Broach, 1999; Duvel & Broach, 2004; Duvel et al., 2003; Santhanam et al., 2004; Schmelze et al., 2004; Beck & Hall, 1999).
The AGC kinase Sch9, the yeast equivalent of mammalian S6 kinase (S6K), directly mediates many of the TORC1-dependent effects on growth and mass accumulation (Urban et al. 2007; see below).
TOR activity is also linked to the cAMP-regulated protein kinase A (PKA) (Rolland et al. 2002; Thevelein and deWinde 1999). Both TOR and PKA regulate (positively or negatively) an overlapping set of genes important for control of cell growth, including genes involved in ribosome biogenesis, carbon and nitrogen metabolism and entry into stationary phase (Slattery et al., 2008; Zaman et al., 2009; Jorgensen et al. 2004; Cardenas et al. 1999; Hardwick et al. 1999; Neuman-Silverberg et al. 1995; Pedruzzi et al. 2003; Powers & Walter 1999; Shamji et al. 2000).
According to one current view, both the TOR and the PKA signaling pathways respond to nutrient signals to coordinately regulate the expression of genes required for cell growth (key metabolic enzymes, ribosomal proteins) and stress responsive genes via two parallel routes (Zurita-Martinez & Cardenas, 2005; Jorgensen et al., 2004; Zaman et al., 2009): in particular, PKA respond to carbon source availability, whereas Tor is mainly sensitive to the nitrogen source (especially glutamine) (Zaman et al., 2008; Zaman et al., 2009).
However, the exact relationship between the TOR and PKA networks is still controversial. As an alternative model, it has been proposed that TOR may act upstream of Ras to regulate PKA activity (Schmelzle et al. 2004; Martin et al., 2004): according to Hall and co-workers, the Ras/PKA circuit would represent a distinct branch of the TOR network that would regulate gene transcription (in particular RP genes) independently from the Tap42/PP2A phosphatase route (see following sections; Schmelzle et al. 2004; Martin et al., 2004). In support of this view, TOR appears to regulate the subcellular localization (and possibly the activity) of the catalytic Tpk1 subunit of PKA and of the Yak1 kinase (Schmelze et al., 2004; see elsewhere).

Despite these uncertainties, many genes require both PKA and TOR for proper nutrient regulation and the concurrent inactivation of the PKA and TOR signalling (combined with loss of glucose transport activity) is sufficient to prevent virtually the entire transcriptional response to nutrients (Slattery et al., 2008). Relevant targets of both TOR and PKA circuits in the stress response are the transcriptional factors Msn2/4 and the kinase Rim15 (Gorner et al., 2002; Santhanam et al., 2004; Pedruzzi et al., 2003). When TORC1 and Sch9 are active, both Rim15 and Msn2/4 are inhibited from entering the nucleus and are thus maintained inactive.

Nutrient-responsive localization of the zinc-finger transcription factor Sfp1, the master regulator of ribosome biogenesis (Ribi) and ribosomal protein (RP) genes, is also regulated by both TOR and PKA signaling in response to nutritional and stress inputs (Jorgensen et al. 2004; Marion et al. 2004; Fingermann et al., 2003). In actively growing cells, Sfp1 is found in the nucleus, but it rapidly translocates into the cytoplasm in response to TORC1 inactivation, oxidative stress, as well as carbon and nitrogen starvation (Jorgensen et al., 2004; Marion et al., 2004; Fingermann et al., 2003). Mrs6, a Rab escort protein involved in membrane trafficking, regulates both Sfp1 nuclear localization and TORC1 signalling (Lempiäinen et al., 2009; Singh & Tyers, 2009). In addition, TORC1 complex has recently been shown to regulate Sfp1 function by direct phosphorylation; furthermore, a feedback mechanism apparently controls the activity of Sfp1 and Sch9: in fact, Sfp1 negatively regulates TORC1 phosphorylation of Sch9 (Lempiäinen et al., 2009).

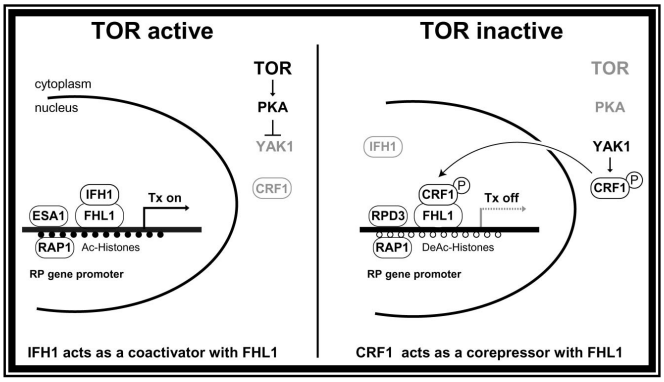


Figure 23.Model for RP gene regulation by TOR and the transcription factor Fhl1
(From Martin et al., 2004).

In addition to the above mentioned Sfp1 (Jorgensen et al. 2004; Marion et al. 2004; Fingermann et al., 2003), transcription factors that regulate RP and Ribi genes expression in response to TOR and PKA signalling include the forkhead-like protein Fhl1, together with its co-regulators Ifh1 and Crf1 (Martin et al. 2004; Schawalder et al. 2004; Wade et al. 2004; Rudra et al. 2005; Jorgensen et al., 2004).
Null mutations of *FHL1* result in a severely reduced growth rate, with cells that synthesize ribosomes at 5-10% the rate of wild-type cells and contain only 20% the normal amount of RNA (Rudra et al., 2005). Fhl1 has a dual role as an activator and a repressor in the transcription of ribosomal protein genes that is determined by its direct interactions with two specific cofactors: the coactivator Ifh1 and the corepressor Crf1 (Fig. 23; Martin et al., 2004). In growing cells, TOR maintains

Crf1 inactive in the cytoplasm by repressing the Yak1 kinase, possibly via a PKA dependent route (Martin et al., 2004). When TOR is inactive, Yak1 directly phosphorylate Crf1, thus promoting the nuclear accumulation of the corepressor: once inside the nucleus, the phosphorylated Crf1 displaces Ifh1 from Fhl1 (which is constitutively bound to RP gene promoters), thereby inhibiting transcription of RP genes (Fig. 23; Martin et al., 2004). As an additional layer of regulation, the nuclear localization both Fhl1 and Ifh1 is influenced by Sfp1: nutrient starvation or deletion of *SFP1* forces Fhl1 and Ifh1 to localize to the nucleolar regions, concomitant with reduced RP gene transcription. Surprisingly, in spite of the transcriptional repression of *RP* genes, both Ifh1 and Fhl1 maintain association with the *RP* promoters under conditions of carbon starvation (Jorgensen et al., 2004): therefore, their activity may be dictated by the nuclear environment of promoter regions (Jorgensen et al., 2004).
However, the precise functional roles and modes of action of these and other transcription factors controlled by TOR remain largely undefined.
Expression of the genes encoding the numerous constituents of ribosomes requires transcription by all three classes of nuclear RNA polymerase: TOR controls other aspects of ribosome biogenesis, such as the Pol I- and Pol III-dependent transcription of the rDNA and tRNA genes via phosphorylation of dedicated transcription factors (Mayer & Grummt 2006).
Tor1 itself may activate rDNA transcription in rich nutrient conditions by entering the nucleus and binding directly to promoters (Li et al. 2006); however, in other studies, Tor1 has been localized to internal membrane structures but not the nucleus (Wedaman et al. 2003; Aronova et al. 2007; Sturgill et al. 2008).

Effectors of TOR: the PP2A phosphatase

Many effects of the TORC1 network are mediated by the Ser/Thr protein phosphatase 2A (PP2A), which exists as an heterotrimeric complex comprising a catalytic C subunit, a scaffold A subunit and a regulatory B subunit (Duvel & Broach, 2004; Jiang & Broach, 1999; Di Como & Arndt, 1996). Three homologous genes, *PPH21, PPH22* and *PPH3*, redundantly encode for the PP2A catalytic subunit, the loss of which results in slow growth, defects in actin organization, bud morphogenesis and in progression through the mitotic cycle (Lin & Arndt, 1995; Ronne et al., 1991). The regulatory subunit is encoded by two genes, *CDC55* and *RTS1*, which likely perform distinct cellular functions, targeting the phosphatase complex to different substrates (Gentry & Hallberg, 2002; Shu et al., 1997; Wang & Burke, 1997). The scaffolding subunit is encoded by a single gene, *TPD3* (van Zyl et al., 1992). *S. cerevisiae* also possesses a PP2A-like phosphatase, consisting of the catalytic subunit, Sit4, and several regulatory subunits (Sap155, Sap190 and Sap185; Luke et al., 1996; Di Como & Arndt, 1996).
These phosphatases are normally repressed in growing cells, where TORC1 is active, but become active upon TORC1 inactivation; the activated phosphatases in turn dephosphorylate several downstream targets of the Tor signaling pathway, such as Npr1, Ure2 and Gln3 (Di Como & Jiang, 2006). In most cases, the Tor-dependent regulation of the PP2A (and PP2a-like) is mediated through the essential protein Tap42, which dynamically interacts with the phosphatases according to nutrient availability (Di Como & Arndt, 1996; Jiang & Broach, 1999). In growing cells, TORC1 directly phosphorylates Tap42 to promote its binding to the catalytic subunits of PP2A and PP2A-like phosphatase complexes (Di Como & Arndt, 1996, Jiang & Broach, 1999; Duvel & Broach, 2004), whereas nitrogen starvation or

rapamycin treatment result in dephosphorylation and subsequent dissociation of Tap42 (Jiang & Broach, 1999; Di Como & Arndt, 1996). Interestingly, Tor inactivation is accompanied by a rapid activation of the phosphatases associated with Tap42. Although there is no general consensus as to whether Tap42 stimulates or inhibits phosphatase activity, several results are consistent with a model in which Tap42p inhibits PP2A activity to promote growth and suppress the stress response (for instance, by preventing the nuclear accumulation of Msn2/4), whereas dissolution of the PP2A-Tap42 complex following rapamycin treatment releases PP2A to cause growth inhibition (Beck & Hall., 1999; Jacinto et al., 2001; Cherkasova & Hinnebusch, 2003; Schmelze et al., 2004; Santhanam et al., 2004).
However, one major limit of the above model is that Tap42 dephosphorylation occurs much more slowly than phosphatase activation (Di Como & Arndt, 1996). Furthermore, even under optimal growth conditions, Tap42 associates only with a small fraction of the phosphatase pool: for instance, only 5-10% of Sit4 and PP2A is found in association with Tap42 in actively growing cells (Di Como and Arndt, 1996).

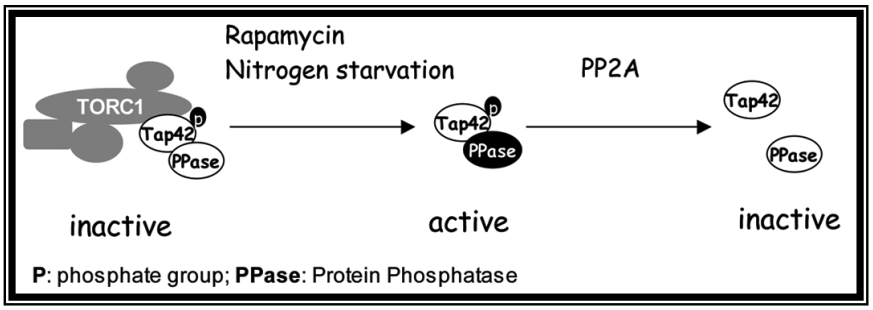


Figure 24. Model for activation of the PP2a phosphatase complex in the TOR signaling
In actively growing cells, the Tap42-phosphatase complexes are associated with TORC1 residing on membrane structures. Nitrogen starvation or rapamycin treatment disrupts the association and releases the complexes into the cytoplasm, where the Tap42-associated phosphatases become active. Once released from TORC1, Tap42 is dephosphorylated by the PP2A holoenzyme: this dephosphorylation event triggers the disassembly of the Tap42 complexes and termination of phosphatase activity.
(From Di Como & Jiang, 1996).

A recent study has established that the Tap42-phosphatase complexes exist mainly on membrane structures in association with TORC1 (Yan et al., 2006). Rapamycin (or nutrient deprivation) abrogates this association and rapidly releases the Tap42–phosphatase complexes into the cytosol. Interestingly, the Tap42-phosphatase complexes do not immediately disassembly upon release from TORC1: the dissociation occurs only later, at a much slower rate, presumably as a consequence of Tap42 dephosphorylation (Yan et al., 2006): in fact, the rate of Tap42 dephosporylation mirrors that of the disassembly of the Tap42-phosphatase complexes, suggesting that the dephosphorylation of Tap42 causes its dissociation from phosphatases (Yan et al., 2006). When the timings of the release and disassembly are compared with that of phosphatase activation (which occurs within minutes of the drug treatment or nutrient deprivation), it is clear that the release of the Tap42-phosphatase complexes from TORC1, but not the disassembly of the complexes, correlates with the activation of the Tap42-associated phosphatases (Yan et al., 2006; Di Como & Jiang, 2006). This correlation thus indicates that rapamycin (or nutrient sarvation) induces phosphatase activation by simply dissociating the Tap42-phosphatase complexes from TORC1. Therefore, the association of the

Tap42–phosphatase complexes with TORC1 represents an important mechanism by which nutrient controls Tor signaling activity; furthermore, rapamycin does not seem to act by inhibiting the kinase activity of Tor but by disrupting its interaction with downstream targets (Yan et al., 2006; Di Como & Jiang, 2006). These findings support the model depicted in Fig. 24.
According to this model Tap42 act a positive regulator of the phosphatases to which it associates, a notion in contrast the previously ascribed negative role of Tap42 in phosphatase regulation (Yan et al., 2006; Di Como & Jiang, 1996). However, substantial evidence has accumulated supporting Tap42 as a positive regulator of PP2A phosphatases (Wang et al., 2003; Duvel et al., 2003; Di Como et al., 1996; Yan et al., 2006).
Microarray analyses of*TAP42* and PP2A mutant strains have demonstrated that PP2A (and PP2a-like) phosphatases do not mediate the entire TORC1 signaling: the expression of genes encoding ribosomal proteins and components of translational apparatus is unaffected by inactivation of eitherTap42 or any of the PP2A catalytic subunits (Duvel et al., 2003). Recent results indicate that Tor control these processes via regulation of downstream kinases such as Sch9 (Urban et al., 2007).

Sch9, a prominent effector of TOR in the control of ribosome biogenesis
Sch9, a member of the AGC family of kinases, is the closest yeast homolog to the mammalian S6 kinase and prosurvival Akt/PKB. *SCH9* was originally isolated as a multicopy suppressor of the lethal phenotype of mutants in the PKA network, such as *cdc25-1, ras1 ras2, tpk1 tpk2 tpk3* (Toda et al., 1988).
The ability of Sch9 to suppress the effects of mutations in the cAMP/PKA pathway is likely due to the fact that the functions of Sch9 and PKA are partially overlapping: for instance, both the selective inhibition of Sch9 function or PKA signaling during growth in glucose media diminishes the expression of genes involved in ribosome biogenesis, whereas overexpression of *SCH9* in glycerol media promotes essentially the same reconfiguration of the transcriptional profile occurring after addition of glucose or upon artificial activation of the PKA circuit (Zaman et al, 2009). In particular, ectopic expression of *SCH9* induces the transcription of genes required for ribosome biogenesis and represses genes involved in carboxylic acid metabolism (Zaman et al., 2009).
Glucose affects Sch9 function by increasing the level of Sch9 and by inducing phosphorylation of Sch9, although the exact molecular mechanisms by which glucose availability is coupled to Sch9 phosphorylation remains largely unknown (Jorgensen et al., 2004, Urban et al., 2007).
Apparently, Sch9 acts in parallel to the cAMP/PKA pathway but has only a minor role in regulating the transcriptional response to glucose: in fact, although inactivation of Sch9 medium diminishes expression of ribosome biogenesis genes in cells growing in glucose, it does not prevent the massive reconfiguration of the transcriptional program occurring upon addition of glucose to cells growing on a non-fermentable carbon source (Zaman et al., 2009). Conversely, Sch9 plays a key role in connecting TOR-dependent nutrient sensing to ribosome biogenesis and in the coordination between growth and cell division (Jorgensen et al., 2004; Urban et al., 2007; see following sections).
Sch9 acts in both nutrient and stress-sensing circuits and is required for TORC1 network to properly regulate ribosome biogenesis, translation initiation and entry into G0 phase (but not expression of Gln3-dependent genes) (Urban et al., 2007).

TORC1 kinase directly phosphorylates multiple sites (at least six) in the carboxy-terminal domain of Sch9 and this phosphorylation event is critical for Sch9 kinase activity (Urban et al., 2007): the mutation of these residues to Ala inactivates the kinase, whereas their replacement with Asp/Glu renders Sch9 activity independent of TORC1: consistently, strains expressing one of these latter *SCH9* mutants (*SCH9*^{2D3E}) do not repress ribosome biogenesis upon rapamycin exposure (Urban et al., 2007). Furthermore, the induction of a subset of stress-responsive genes is diminished in cells expressing the TORC1 independent *SCH9* alleles, suggesting that TOR may also regulate the activity of the Msn2/4 transcription factor through Sch9 (at least partially) (Urban et al., 2007). However, Sch9 does not mediate the entire TOR signaling: strains expressing either the wild-type Sch9 or the *Sch9*^{2D3E} mutant exhibit comparable levels of Gln3-dependent and Rtg1/3-dependent gene induction in response to rapamycin (Urban et al., 2007).
TORC1 appears to prevent the entry into stationary phase via the Sch9 route (although not exclusively) (Urban et al., 2007). When TORC1 and Sch9 are active, the Rim15 kinase is confined inside the cytoplasm and thus inhibited. Following rapamycin treatment, Rim15 enters the nucleus and cells arrest with pre-synthetic DNA content while accumulating high levels of storage carbohydrates. Cells expressing an inactive Sch9 mutant display constitutive nuclear localization of Rim15 and accumulate glycogen even in the absence of rapamycin (Urban et al., 2009), whereas glycogen accumulation upon rapamycin treatment is reduced in cells expressing the *Sch9*^{2D3E} mutant compared to wild type cells (Urban et al., 2007). However, the transcriptional reprogramming associated with entry into quiescence likely requires further signals in addition to Sch9, since no significant differences are detectable between the transcriptional profiles of cells expressing the TORC1 Independent SCH9 alleles and the wild type strain (Urban et al., 2007).

TOR and PKA: a complex relationship

Several evidences suggest that the TOR network may control the subcellular localization and presumably the activity of PKA, which defines the other major nutrient-responsive, growth-controlling pathway in yeast (Schmelzle et al., 2004): similarly to cAMP depletion, inactivation of TOR in glucose growing cells by rapamycin treatment promotes the nuclear accumulation of the PKA catalytic subunit Tpk1, whereas the localization of the regulatory Bcy1 subunit is not significantly affected (i.e., Bcy1 remains nuclear); in contrast, Tpk1 remains largely cytoplasmic in rapamycin treated *bcy1* cells (Schmelzle et al., 2004), thus confirming that the nuclear accumulation of the catalytic subunit of PKA depends on the interaction with nuclear Bcy1 (Griffionen et al., 2000; Griffionen et al., 2002; Griffionen et al., 2001). Taken together, these observations apparently indicate that TOR is required to maintain PKA activity during rapid growth on glucose by preventing the accumulation of the catalytic TPK subunits in the nuclear compartment, where it resides the inhibitory Bcy1 subunit (Schmelzle et al., 2004).
Furthermore, it has been shown that constitutive activation of the Ras branch of the cAMP/PKA signaling pathway confers a marked resistance to rapamycin treatment and blocks several rapamycin-induced responses, such as the nuclear translocation of the transcription factor Msn2, induction of stress genes, accumulation of glycogen, induction of autophagy, down-regulation of ribosome biogenesis, diminished expression of the glucose transporter encoded by *HXT1* (Schmelze et al., 2004; Zurita-Martinez & Cardenas, 2005). Many of these responses seem to be

independent of the Tap42-PPA2 phosphatase complex, one of the main effectors of TOR, whereas the cellular processes controlled by TOR via the Tap42/PPA2 route remain substantially unaffected by hyperactivation of the cAMP/PKA circuit (Schmelze et al., 2004). All of these findings have been taken as evidences to support a model in which TOR signals via the PKA pathway to control several of its targets (Schmelze et al., 2004; Martin et al., 2004): PKA would thus represent a distinct effector of TOR which would act independently from the Tap42/PP2A phosphatase network to regulate gene expression (Schmelze et al., 2004; Martin et al., 2004).

However, although intriguing, the functional significance of the TOR-mediated subcellular localization of TPK1 is still uncertain; furthermore, recent studies have suggested alternative sceneries to explain the complex relationship between the TOR and PKA networks (Zurita-Martinez & Cardenas, 2005; Zaman et al., 2009; Slattery et al., 2008). For instance, a partial inactivation of the PKA signaling confers rapamycin hypersensitivity, but only modestly affects the expression of genes encoding ribosomal proteins (RP) (Zurita-Martinez & Cardenas, 2005). The complete inactivation of PKA (*tpk1 tpk2 tpk3 yak1* or *tpk1 tpk2 tpk3 msn2 msn4 strains)* impairs the RP (ribosomal proteins) genes expression and concomitantly enhances the expression of genes required for stress response and for glycogen storage; nevertheless, this altered transcriptional profile is sensitive to rapamycin treatment, and thus still subject to TOR control (Zurita-Martinez & Cardenas, 2005). Furthermore, several transcriptomic analyses have shown that both PKA and TOR promote growth under favorable conditions by contributing to a massive remodulation of the entire transcriptional profile, but they apparently do so in response to distinct nutritional inputs (Zaman et al., 2009; Slattery et al., 2008; Zaman et al., 2008).

All of these observations are more consistent with the proposal that the Tor and PKA signaling cascades coordinately (but independently) govern the expression of genes required for growth (including RP (ribosomal protein) genes) and stress response (STRE genes) by acting within two parallel, separated pathway (Zurita-Martinez & cardenas, 2005; Zaman et al., 2009; Zaman et al., 2008). This model requires that TOR and the RAS/cAMP pathway converge simultaneously on several common targets, such as the stress transcription factors Msn2 and Msn4 or Sfp1 (the master regulators of ribosome biogenesis), to regulate their activity (see following sections).

The two models described above are not mutually exclusive: crosstalks between signaling pathways are a common theme in growth control and TOR may intersect with the cAMP/PKA pathway at multiple levels. However, the exact relationship between PKA and TOR pathway remains largely to be undefined.

The transcriptional response to glucose: contributes of the diverse signaling circuits

The addition of glucose to yeast cells growing on a non-fermentable carbon source triggers a rapid and massive restructuring of the whole transcriptional profile (Wang et al., 2004; Zaman et al., 2009; Slattery et al., 2009). More than 40% of the genes in yeast genome change their expression levels by more than twofold within minutes following addition of the sugar (Wang et al., 2004; Zaman et al., 2004): genes required for glycolysis, glucose uptake and ribosome biogenesis are up-regulated, whereas the transcription of genes involved in respiratory/gluconeogenetic

metabolism, utilization of alternative carbon sources and stress response become repressed (Wang et al., 2004; Zaman et al., 2009; Slattery et al., 2008).
Several microarray analyses have recently evaluated the relative contributions of the known glucose sensing pathway to the global transcriptional response triggered by sugar addition (Zaman et al., 2008; Slattery et al., 2004; Wang et al., 2004).
The cAMP/PKA pathway appears to be the main player in the cellular response to glucose (Zaman et al., 2009; Slattery et al., 2008). Induction of an activated version of *RAS2* (*RAS2*^{V19}) in yeast cells growing on glycerol is sufficient to recapitulate both qualitatively and quantitatively ~90% of the transcriptional changes resulting from glucose addition (Zaman et al., 2009; Wang et al., 2004). A similar result can be obtained by using an activated allele of *GPA2* (*GPA2*^{Q300L}), although in this case the overall magnitude of the Gpa2-induced transcriptional changes is weaker (only half) that observed upon glucose addition (Wang et al., 2004). In contrast, induction of a dominant negative allele of *RAS2* or inactivation of PKA essentially abolishes the transcriptional response to glucose (Zaman et al., 2009).
Thus, activation of the cAMP/PKA circuit, in particular via the Ras branch, is both necessary and sufficient to induce the vast majority of glucose-induced transcriptional changes. The role of the GPCR system comprising Gpr1 and Gpa2 in this response is apparently less prominent: in fact, loss of *GPR1* reduces, but not eliminates the glucose dependent reconfiguration of the transcriptional program (Wang et al., 2004; Zaman et al., 2009).
The other signal transduction pathways appear to mediate only a small fraction of the glucose signal in yeast, often in conjunction with the PKA circuit (Zaman et al., 2009).
The Snf1 pathway mediates a significant portion of the glucose repression mechanism not subject to direct PKA control by regulating a small set of genes specialized in the metabolism of alternative carbon sources (Zaman et al., 2009); in addition, the Snf1 and the cAMP/PKA circuit cooperate in the regulation of several glucose-repressed genes (Zaman et al., 2009).
Another group of genes (most of which are targets of the heme-activated transcriptional regulators Hap1 and the Hap2/3/4/5 complex) are repressed by glucose independently both PKA and Snf1 (Zaman et al., 2009).
Finally, in the presence of glucose the Snf3/Rgt2-Rgt1 pathway induces the expression of a small set genes required for sugar uptake, such as the *HXT* genes (Zaman et al., 2009; Kaniak et al., 2004). Several of the genes subject to regulation by the Snf2/Rtg2-Rgt1 circuit also respond to PKA activation and require PKA activity for full induction by glucose (Zaman et al., 2009; Ozcan & Johnston, 1999; Kim et al., 2006).

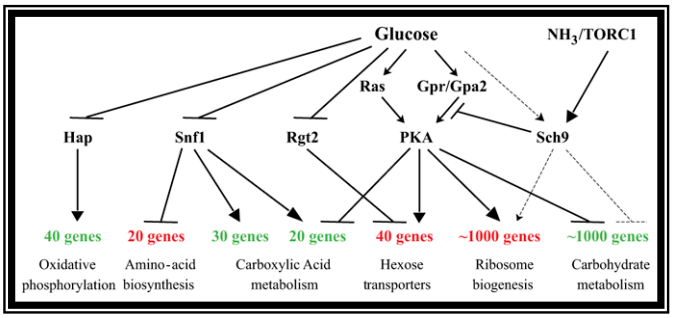


Figure 25. The glucose signaling network in Saccharomyces cerevisiae. Diagram of the regulatory wiring connecting the addition of glucose to the transcriptional responses of the yeast cell. Dotted line indicates a limited or indirect connection. (From Zaman et al., 2009).

The study by Zaman and colleagues demonstrates that PKA, Snf1, Snf3/Rgt2-Rgt1 and heme-dependent transcriptional activators are responsible for the whole glucose-induced transcriptional response (Fig. 25; Zaman et al., 2009).
In another recent paper by Slattery and colleagues, the contribute of glucose uptake to the transcriptional response has also been evaluated: it has been found that about 25% of the glucose-repressed genes and 10% of the glucose-induced genes respond to sugar addition even in the absence of PKA activity, and this regulation depends on glucose import (Slattery et al., 2008). Interestingly, many of the glucose-responsive genes whose induction depend (at least partially) on sugar uptake are involved in cell cycle progression and contain MCB and SCB regulatory motifs in their promoter (Slattery et al., 2008). In contrast, genes which require glucose transport to be repressed are often involved in the oxidative metabolism (enzymes of the TCA cycle and electron transport chain) (Slattery et al., 2008).
Although overespression of Sch9 can elicit the same transcriptional changes induced by PKA, Sch9 does not contribute significantly to the glucose response: in fact, blocking signaling through Sch9 does not affect the glucose-induced transcriptional response (Zaman et al., 2009).
Therefore, despite the fact that both Sch9 and PKA regulate a massive, nutrient-dependent reconfiguration of the transcriptional program to promote growth under favorable conditions (Zaman et al., 2009; Slattery et al., 2008), they likely do so in response to different nutritional inputs (Zaman et al., 2009; Zaman et al., 2008).
According to the model proposed by Zaman and colleagues, the "core growth related genes" (encoding ribosomal proteins and key metabolic enzymes) and stress related genes are regulated independently by the two primary nutrients: carbon and nitrogen sources (Zaman et al., 2009). Carbon source would regulate the expression of these genes through the PKA pathway, whereas nitrogen source would impinge on the expression of growth and stress related genes through the TORC1 pathway, which has been shown to directly regulate the activity of Sch9 (Urban et al., 2007).
Consistent with the proposal that the Tor and PKA signaling cascades independently coordinate the expression of genes required for growth and the stress response the inhibition of Tor signalling by rapamycin results in repression of the RP genes and induction of the STRE genes, whereas mutations that hyperactivate the PKA circuit confer resistance to rapamycin and relieve the transcriptional repression of RP genes imposed by rapamycin (Zurita-Martinez & Cardenas, 2005). By contrast, partial inactivation of the PKA signaling cascade enhances rapamycin sensitivity, but has only minor effects on RP gene expression (Zurita-Martinez & Cardenas, 2005). Complete loss of PKA function diminishes RP gene expression and concurrently up-regulates STRE gene expression; remarkably, this altered transcriptional profile is still sensitive to rapamycin and thus subject to Tor control (Zurita-Martinez & Cardenas, 2005).
How the PKA and the TOR pathway are integrated remains largely to be established (Zaman et al., 2009). Interestingly, transcriptomic analyses have revealed several intriguing connections between the TOR/Sch9 network and the Gpr1/Gpa2 branch of the cAMP/PKA signaling cascade (Zaman et al., 2009): this potential cross-talk may play a decisive role in the developmental program that yeast cells adopt under nutrient shortage.

Nutrient, transcriptional profile and growth rate

Nutrient availability influences growth rate (Zaman et al., 2008) and yeast cell adapt to nutrient status by changing their transcriptional profile. Although limitation for a certain nutrient often induces a nutrient-specific transcriptional response, many of the changes occurring in the transcriptome are substantially independent of which nutrient is limiting: in fact, a recent study has demonstrated that a surprisingly large fraction (27%) of all yeast genes are expressed in a way that is strictly correlated with the growth rate but independent of the growth-limiting nutrient (i.e. glucose, sulfate, phosphate, ammonium, aminoacids) (Brauer et al., 2008): the expression level of some genes (such as ribosome biogenesis genes) is directly proportional to the growth rate, whereas that of others is inversely proportional.

Furthermore, there is a considerable overlap between the transcriptional responses to growth limitation and a wide variety of environmental stresses: consistently, cells growing slowly are also cross protected for heat-shock (Lu et al., 2009).

In addition, metabolite concentrations can regulate gene expression, which can in turn regulate metabolic activity. Recent analyses of the metabolomic and transcriptional responses of *Saccharomyces cerevisiae* to carbon and nitrogen starvation that transcripts and metabolites show coordinated response dynamics: the extent to which functionally related transcripts and metabolites show similar patterns of concentration changes (Bradley et al., 2009). Furthermore, metabolites and gene products whose concentration profiles are alike tend to participate in related biological processes (Bradley et al., 2009).

Thus, the nutrient status appears to establish both the cellular growth rate and a corresponding highly distinctive transcriptional (and metabolomic) profile (Brauer et al., 2008; Bradley et al., 2009; Lu et al., 2009). Two recent studies have unequivocally demonstrated that the transcriptional pattern of the growth-responsive genes is regulated by nutrient sensing and not by the nutrient metabolism: in fact, activation of the PKA circuit is sufficient to mimic the glucose induced transcriptional changes even in complete absence of sugar metabolism (Slattery et al., 2008; Zaman et al., 2009).

Apparently, a yeast cell adjust its transcriptional program, metabolic machinery and growth rate solely on the basis of its perception of the nutrient status, not on the basis of metabolites actually produced from the available nutrients (Zaman et al., 2009; Slattery et al., 2008). Under most conditions, this kind of regulation is quite efficient, since the nutrients which the cell recognizes as being present in its living environment are actually available. However, a mismatch between what cell perceives and the real nutrient status (as a result of drug treatment or genetic manipulation) can have dramatic consequences: consistent with this notion, strains with an hyper-active cAMP/PKA pathway cannot grow on non-fermentable carbon sources (Thevelein & deWinde, 1999, since these cells perceives a rich nutritional environment that it does not exist (Zaman et al., 2009). In contrast, the lethality of strains lacking a functional cAMP/PKA pathway can be rescued by inactivation of *MSN2* and *MSN4*, encoding two transcriptional factors regulating the transcriptional response to unfavorable growth conditions (Smith et al., 1998).

Nutrients and cell cycle progression

An essential requisite for the survival of free living microorganism like the budding yeast *Saccharomyces cerevisiae* is the capacity to regulate growth and cell cycle progression according to the nutrient availability, so that proliferation is rapid when large supplies of nutrients are available and ceases when these becomes exhausted (Zaman et al., 2008). For instance, it would, be deleterious for a yeast cell to engage in energetically expensive cellular processes or to attempt proliferation under unfavourable conditions. Nutrients like glucose must therefore generate signals that are somehow received and elaborated by the complex machinery governing growth and cell cycle progression. Nutrients like glucose must therefore generate signals that are somehow received and elaborated by the complex machinery governing growth and cell cycle progression.

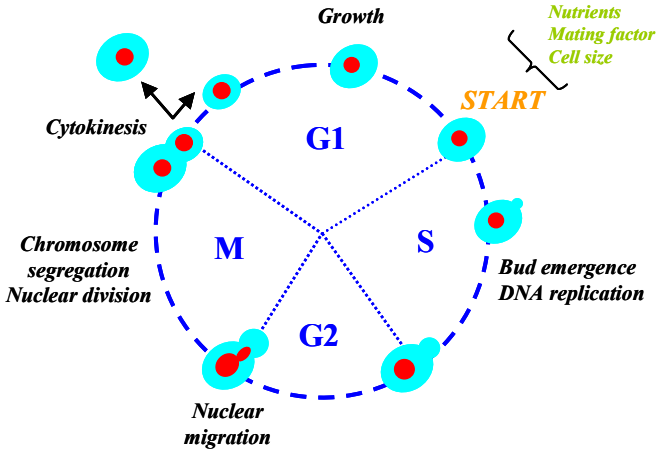


Figure 26. The cell division cycle in budding yeast

The cell cycle is a complex but orderly sequence of events that culminates in the production of two daughter cells, each containing the information and machinery necessary to repeat the process. In eukaryotes, the cycle is conventionally divided into four phases: cell growth in G1 phase, DNA replication in S phase, more growth in G2 phase, and cell division in mitosis (M phase). The system of regulators that drives transitions between the diverse phases is centered on the cyclin-dependent kinases (CDKs), enzymes that become active when regulatory proteins called cyclins bind to them. The CDK network directly or indirectly orchestrates coordinated regulation of proteins and genes involved in essentially every aspect of cell function. (Bloom & Cross, 2007)

In *Saccharomyces cerevisiae* regulation of cell cycle progression is exerted predominantly during a narrow interval in the late *G,* phase known as *START* (Fig. 26; Hartwell et al., 1974).

At *START* a yeast cell integrates environmental and internal signals (such as availability of nutrient, presence of pheromone, obtainment of a critical size, status of the metabolic machinery) and decides whether to progress toward mitosis or to undertake an alternative developmental program (sporulation, pseudohyphal differentiation, entry into stationary phase) (Hartwell et al. 1974; Jorgensen & Tyers, 2004). Execution of start irreversibly commits the cell to a new round of mitotic

division and requires the activation of Cdc28, the cyclin-dependent kinase governing the major cell cycle transitions in budding yeast. The activity of Cdc28 is regulated by its association with multiple regulatory subunits known as cyclins (see Bloom&Cross, 2007 for a recent review).
Cyclins, as their name suggest, are transcribed with cyclic periodicity during the different stages of the cell cycle, in order to restrict their presence to the specific temporal windows when they are needed. Nine different cyclins activate Cdc28 to drive the cell through the diverse phases of the cell cycle: the G1 phase cyclins Cln1, Cln2 and Cln3, are essential for the passage through Start; the S-phase Clb5 and Clb6 B-type cyclins trigger the DNA replication; the four mitotic cyclins Clb1-Clb4 regulate the G,/M transition and mitosis (Bloom & Cross, 2007).
The various cyclins are expressed in four distinct waves across the cell cycle (Fig. 29): their specificity of function combined with their timing of expression drives and orchestrates cell cycle events (Futcher, 1996; Bloom & Cross, 2007).Transcription of the *CLN3* gene is detectable throughout the cell cycle, but peaks in late M–early G1, whereas the transcription of *CLN1* and *CLN2* peaks during G1–S. Transcription of *CLB5* and *CLB6* also peak at G1–S, followed by the transcription of *CLB3* and *CLB4* and then by the transcription of *CLB1* and *CLB2* (Bloom & Cross, 2007). The transcriptional waves are autoregulatory: *CLN3* drives the expression of *CLN1* and *CLN2,* which activate Clb5 and Clb6 by removing the Sic1 inhibitor and thus indirectly promoting the expression of the mitotic B-type cyclins. Once *CLBs* are expressed, they repress CLN1 and CLN2 transcription. This feedback loop ensures the periodicity of cyclin expression (Futcher et al., 1996),

An oversimplified description of the relevant events occurring during the cell cycle progression is given in the following section (Fig. 27), whereas Fig. 28 describes the complex transcriptional network that underlies the yeast cell cycle.

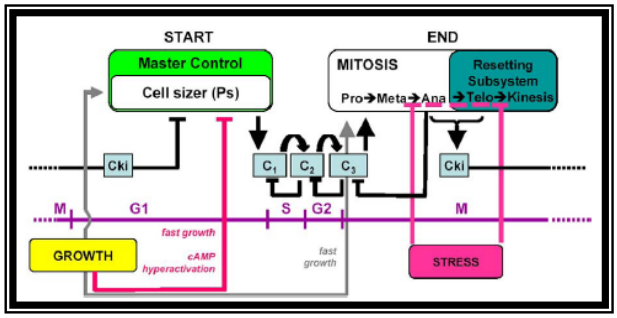


Figure 27. A block diagram of the main modules of the yeast cell cycle.
The cell cycle is driven by three functional units: a Start function that allows the G1/S transition when a critical size has been achieved; a cascade of three cyclin subsystems (C1, C2, C3) and an End function that comprises the events from mitosis to citokinesis. Insurgence of stress may delay the progression from metaphase to cell division.
Arrows indicate activation whereas bars indicate inhibition.
(From Alberghina et al., 2009).

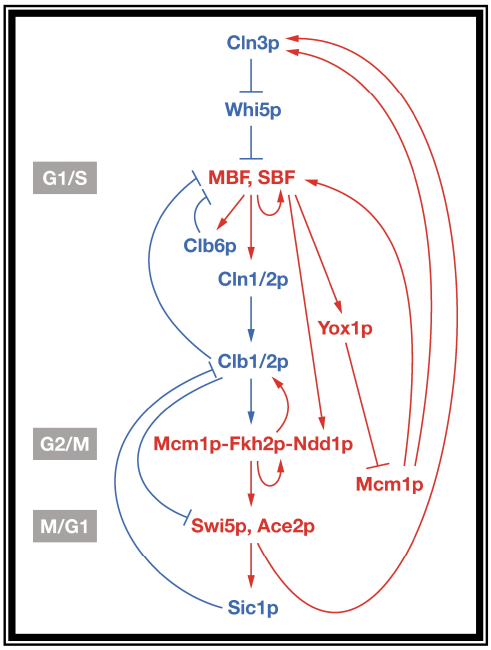


Figure 28. The main regulatory circuits that drive the gene expression program during the budding yeast cell cycle. Transcription Factors are shown in red. Red and blue lines indicate transcriptional and posttranslational regulation, respectively. Arrows: positive regulation; bars: negative regulation. The cell-cycle transitions where the main TFs act are indicated on the left side. (From Bahler, 2008)

Consecutive waves of CDK activity drive the cell cycle progression in S. cerevisiae

The budding yeast cell cycle is characterized by consecutive waves of activations and inactivations of key regulators, including cyclins, transcription factors, inhibitors and DNA replication complex components.

A single cyclin dependent kinase (a serine/threonine kinase encoded by *CDC28*) regulates the timing of different cell cycle transitions by associating with nine different cyclins which are transcribed in four distinct waves across the various stages of the cell cycle (Fig. 29); Mendenhall & Hodges, 1998; Bloom & Cross, 2007; Futcher, 1996). The diverse cyclins confer to Cdc28 stage-specific functions (Bloom & Cross, 2007). Three G1-specific cyclins (*CLN1-3*) are necessary for the G1/S transition, whereas six different B-type cyclins (*CLB1-6*) are involved in different aspects of S phase and mitosis.

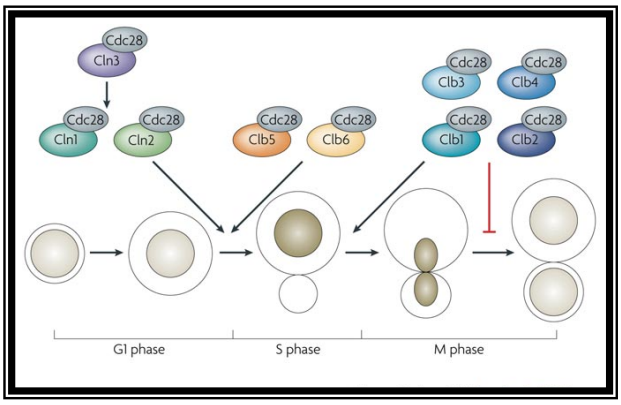
The first burst of cyclin dependent kinase (CDK) activity occurs late in G1 phase, when Cdc28 associates with the G1 cyclin Cln3 after the cell has reached the critical size required for passage through *START* (commitment to duplication and division) (Tyers et al., 1993; Jorgensen & Tyers, 2004). Cln3/Cdc28 inactivates the Whi5 transcriptional repressor, allowing the SBF (Swi4-Swi6) and MBF (Mbp1-Swi6) transcriptional factors to promote expression of hundreds of genes required for G1/S transition (the "G1/S regulon"): this gene cluster include the G1 cyclins *CLN1*, *CLN2* and the B-type cyclins *CLB5*, *CLB6* (de Bruin et al, 2004; Costanzo et al., 2004; Wittenberg & Reed, 2005).

Cln1 and Cln2 are required to promote polarized growth at the site of bud emergence, spindle pole body (SPB) duplication and inactivation of Sic1 and Cdh1 (Lew & Reed, 1993; Lew & Reed, 1995).

The Sic1 inhibitor binds and inhibits the growing pool of Clb5,6/Cdc28 cyclin complexes (Schwob et al. 1994; Knapp et al. 1996); in addition, Cdh1 promotes ubiquitination and proteolysis of mitotic Clb_s cyclins by the Anaphase Promoting

Complex/Cyclosome (APC/C) (Schwab et al. 1997; Visintin et al. 1997; Peters, 2006). Cdc28/Cln1 and Cdc28/Cln2 complexes phosphorylate both Sic1, targeting it for degradation via an ubiquitin dependent mechanism, and Cdh1, leading to its dissociation from APC (Schneider et al., 1996; Verma et al., 1997). The elimination of Sic1 triggers a wave of CDK/B-cyclin activity that drives DNA replication and entry into mitosis (Verma et al., 2001).
B-type cyclins Clb1-6 regulate Cdc28 activity during S, G2, and M phases. Cdc28 association with Clb5 and Clb6 activate DNA replication (Schwob & Nasmyth, 1993). Cdc28 association with Clb3, Clb4, and Clb5 promotes maturation and separation of spindle pole bodies and proper spindle segregation (Maekawa & Schiebel, 2004; Segal et al., 2000; Haase et al., 2001). Cdc28 association with Clb2 (and to some extent Clb1, Clb3, and Clb4) turns off the G1 SBF-transcriptional program, promotes entry into mitosis and triggers a switch in bud growth from polarized to isotropic (Fitch et al., 1992; Lew & Reed, 1995). The metaphase to anaphase transition occurs when securin (Pds1), an inhibitor of DNA segregation is destroyed by the proteasome. Mitotic CDK activity is required to target Pds1 for degradation by the Anaphase Promoting Complex (Farr & Cohen, 1999; Harper et al., 2002; Nasmyth, 2005). Cdc14, a protein phosphatase, plays a key role in mitotic exit: it is located in the nucleolus until it is liberated by the FEAR and Mitotic Exit Network during anaphase, enabling it to act on key substrates to promote a decrease in Cdc28/B-cyclin activity and mitotic exit (Saunders, 2002). Once DNA is segregated, exit from mitosis (spindle disassembly, cytokinesis and transition to the next G1) requires the turn-off of the mitotic cyclin dependent kinase activity (Surana et al., 1993).
This is accomplished by degradation of mitotic cyclins through a Cdh1/APC mediated process and inhibition of remaining mitotic activity by Sic1. Meanwhile, Whi5 is dephosphorylated by Cdc14 and reenters the nucleus, preventing a premature activation of the SBF-transcriptional program (Taberner et al., 2009; Costanzo et al., 2004). After the resetting of the cell cycle to G1 phase, .G1 cyclins can once again accumulate (Irninger, 2002; Harper et al., 2002) to promote another round of cell division once the critical size threshold has been achieved.

Figure 29. Consecutive waves of Cyclins/CDK activity drive the cell cycle progression in S. cerevisiae.



The G1-phase cyclins (Cln1, Cln2 and Cln3) promote bud emergence, spindle pole body duplication (not shown) and activation of the B-type cyclins. The S-phase cyclins (Clb5 and Clb6) advance DNA replication (shaded nucleus), and the M-phase cyclins (Clb1, Clb2, Clb3 and Clb4) promote spindle formation and the initiation of mitosis. Mitotic cyclins inhibit mitotic exit and cell division. Following cytokinesis, a mother and daughter cell are generated. (From Bloom & Cross, 2007).

Nutrients and coordination of growth and cell cycle division: the concept of critical size

A tight coordination between growth (defined as continuous accumulation of macromolecular components such as ribosome, proteins and RNA) and cell division (replication and segregation of the genetic material) is crucial to maintain cellular homeostasis over multiple generations (Fig. 30).

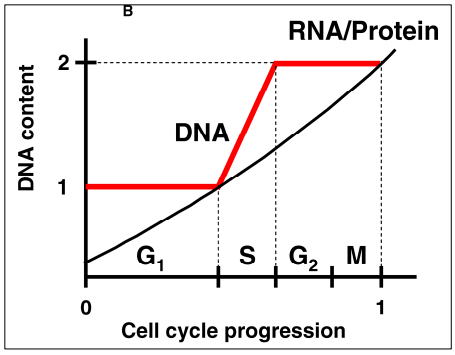


Figure 30. Coordination of growth and cell division
Cell proliferation requires a tight coordination between growth (continuous accumulation of mass (RNA, proteins), DNA replication and cell division. (Adapted from Barberis et al., 2007).

In actively proliferating cells, cell size reflects the balance between growth and division: perturbations that shift this equilibrium in favor of growth, either because of a delay in division or an increase in growth rate, result in large cell size; conversely, accelerating cell cycle progression or inhibiting growth results in small cell size (Jorgensen & Tyers, 2004; Cook & Tyers, 2007; Rudra & Warner, 2004).
Most organisms maintain cell homeostasis by rendering progress through the cell cycle dependent on growth: in particular this dependency is achieved by imposing critical cell size thresholds as a requirement for the major cell cycle transitions (i.e. G1/S transition) to occur (Jorgensen & Tyers, 2004; Cook & Tyers, 2007; Rupes, 2002). Consistently, blocking cell growth by nutrient starvation leads to a cell cycle arrest, usually in G1 phase; on the contrary, cell growth does not typically depend on cell cycle progression: in many cases, when cell cycle events are blocked by chemical treatments or genetic lesions, cell growth continues undisturbed (Pringle & Hartwell, 1981).
In *Saccharomyces cerevisiae*, the coordination between growth and cell division takes place at *START*, a short interval in late G1 phase during which the cell commits to a new round of mitotic division (Hartwell et al., 1974; see next section).
Progress through *START* is contingent on achieving a critical cell size (Johnston et al., 1977) and it also requires a minimum translation rate (Hartwell & Unger 1977; Moore, 1988), the availability of sufficient nutrients (Hartwell et al., 1974) and (for haploid cells) the absence of mating pheromone (Hartwell et al., 1974).
The existence of a critical size threshold as a prerequisite for cell cycle progression is particularly important for budding yeast, whose cytokinesis is asymmetrical and produces a mother and a daughter cell of unequal sizes (Hartwell & Unger, 1977; Porro et al., 2009). To compensate for this asymmetry, small daughter cells delay the Start transition by spending a longer time in G1 until they grow to the critical

size required for the G1/S transition to occur (Johnston et al., 1977; Hartwell & Unger, 1977); the rest of the cell cycle (the "budded phase" comprising S+G2+M phases) remains relatively constant in length in both mother and daughter cells (Fig. 31). The requirements for a critical size and a minimum translation rate also explain why slowing down growth rate increases the length of the G1 phase, whereas the time required to transit the other phases of the cell cycle is largely unaffected (Hartwell & Unger 1977). The nutritional requirement, on the other hand, forces yeast cell to check if enough resources are available to complete a new round of mitotic division.

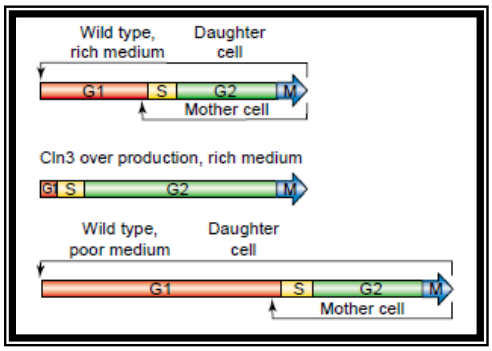


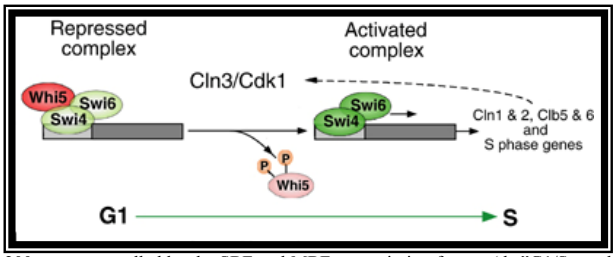
Figure 31. Nutrient status and length of cell-cycle phases in budding yeast.
Smaller daughter cells undergo a substantial growth before the G1/S transition, whereas larger mother cells rapidly enter into S phase. G1 is drastically reduced when the G1 cyclin Cln3 is overproduced. G1 becomes relatively extended under poor growth conditions, whereas S, G2 and M lengths remain largely unaffected.
(From Rupes et al., 2002)

Classic studies documented that the critical size increases with ploidy and responds dynamically to nutrients, so that yeast cells grown on poor medium are smaller than cells grown in rich medium (Johnston et al., 1977; Johnston et al., 1979; Tyers et al., 1979). Shifting cells between different nutrient conditions rapidly resets the size threshold (Johnston et al., 1977; Lorincz & McCarter, 1979; Fig.25): for example, addition of glucose to yeast culture growing on a non-fermentable carbon source transiently increases the fraction of unbudded cells (i.e. cells in G1 phase), as cells delays the G1/S transition to grow to the new threshold (Johnston et al., 1979). More recent studies using nutrient-limited chemostats reinforced these early observations by showing that the fraction of unbudded cells in cultures limited for diverse nutrients (glucose, ammonia, sulfate, or phosphate) is proportional to the doubling time of the culture (Brauer et al., 2008). Furthermore, the nutritional availability also modulates the degree of asymmetry of cell division: poor media usually yield large parent cells and very small daughters, whereas in rich media the asymmetry between parent and daughter cells is reduced (Porro et al., 2009).

The activation of the G1/S transcriptional program

Cln3 is the most upstream activator of Start, where the coordinated expression of a large family of G1-specific genes demarks the commitment to a new cell cycle (Fig. 32; Wittenberg &Reed, 2005; Breeden, 2003; Bahler, 2005; Cooper, 2006). The ~200 genes that fall into this G1-specific gene cluster govern the events associated with cell cycle initiation, including DNA replication, bud morphogenesis, and duplication of spindle pole bodies (Iyer et al., 2001; Simon et al., 2001; Bahler, 2005).

Figure 32. Regulation of the G1/S transcriptional program.



Once yeast cell has reached the critical size required for entry into S phase Cln3/Cdc28 complexes phosphorylate the Whi5 transcriptional inhibitor promoting its release from SBF and its rapid exit from the nucleus.
It is presently unknown which mechanisms repress MBF-dependent transcription in early G1 or activate MBF at Start (de Bruin et al., 2006). Inactivation of Whi5 allows the synchronous transcription of about 200 genes controlled by the SBF and MBF transcription factors (the"G1/S regulon") (de Bruin et al., 2004; Costanzo et al., 2004).The G1/S transcriptional wave, which includes the other G1 cyclins, *CLN1* and *CLN2*, drives the entry into S phase by activating the B-type cyclins, DNA replication, spindle pole body duplication, and bud emergence (Wittenberg & Reed, 2005). Later in the cell cycle, mitotic B-type cyclins inactivate SBF while Nrm1 repress MBF, thus turning off the G1/S transcriptional program (de Bruin et al., 2006; Amon et al., 1993) (From Wittenberg & Reed, 2005)

The G1 transcriptional wave is driven by a pair of heterodimeric transcription factor complexes, SBF (SCB Binding Factor) and MBF (MCB Binding Factor), each composed of unique DNA-binding components, Swi4 (SBF) or Mbp1 (MBF), and a common transcriptional coactivator, Swi6 (Wittenberg & Reed, 2005; Breeden, 2003, Bahler, 2005; Mendenhall & Hodges, 1998). The binding site for MBF is designed MCB (*Mlu*I Cell cycle Box, due to the presence of a *MluI* restriction site in the consensus sequence ACGCG), while SBF recognizes SBF (Swi4 Cell cycle Box, CRCGAAA) elements in the promoter of its target genes (Breeden, 1996; Wittenberg & Reed, 2005). In general, SBF mainly regulates transcription of genes (*CLN1/2, PCL1/2, GIN4, FKS1/2*) involved in budding, spindle pole body duplication and other growth related functions (i.e. cells wall biosynthesis), whereas genes required for DNA synthesis and repair (*CLB5/6, POL2, CDC2, RNR1*) are MBF targets (Wittenberg & Reed, 2005). However, this distinction is not absolute: each group also includes many members that do not fall neatly into these categories and a consistent fraction of genes contain both SCB and MCB elements in their promoter (Iyer et al., 2001; Simon et al., 2001; Bahler, 2005). Furthermore, Swi4 binds MCBs and Mbp1 binds SCBs in vitro (Mendenhall & Hodges, 1998): accordingly, the two transcriptional factors show a partial functional overlap, at least under some circumstances (Flick et al., 1998; Partridge et al., 1997; Bean et al., 2005). Interestingly, several SBF and/or MBF targets are transcription factors which may potentially influence the expression of thousands of genes, including those expressed in late G1 phase (Iyer et al., 2001; Simon et al., 2001; Bahler, 2005). Whereas both SBF and MBF are dependent on Cln3/Cdc28 for their activation, they are distinctly regulated and their roles in promoting periodic transcription seem to be quite different (Amon et al., 1993; Costanzo et al., 2003; de Bruin et al., 2006; Mendenhall & Hodges, 1998): in fact, while SBF is required for full transcriptional activation during G1 phase at the promoters it controls, MBF apparently restricts the

expression of its targets to the G1 phase by repressing their transcription outside of G1 (Fig.33; see below; de Bruin et al., 2006; Bean et al., 2005; Koch et al., 2003; Mendenhall & Hodge, 1989). Neither *SWI4* nor *MBP1* alone are essential for viability (Koch et al., 1993) whereas concurrent loss of both genes leads to a permanent G1 arrest and is lethal (Koch et al., 1993; Bean et al., 2005)*.

The expression of the SBF-controlled genes at *START* depends mostly upon the inactivation of transcriptional repressor Whi5 (Fig. 32) de Bruin et al., 2004; Costanzo et al., 2004). SBF complexes are pre-loaded on the promoters of their target genes in early G1 phase (Koch et al., 1993; Cosma et al., 2001), but transcription is prevented by their interaction with Whi5 (de Bruin et al., 2005; Costanzo et al., 2004). At *START*, activation of the Cln3/Cdc28 complex results in phosphorylation of Whi5: the inactivated repressor dissociates from SBF (de Bruin et al., 2004; Costanzo et al., 2004) and leaves the nuclear compartment (Costanzo et al., 2004; Di Talia et al., 2007; Skotheim et al., 2008), allowing the recruitment of the RNA polymerase II holoenzyme on the SBF promoters and the execution of the transcriptional program that triggers the G1/S transition.

At the end of mitosis, once CDK activity is quelled, a pre-existing cytoplasmic pool of Whi5 is dephosphorylated by Cdc14 and immediately enters the nucleus to ensure that transcription is not activated as soon as SBF engages its promoters in early G1 phase (Costanzo et al., 2004; Taberner et al., 2009; see following sections).This last step completes the regulatory circuit for G1 and resets the SBF-promoters for the next round of cell division (Costanzo et al., 2004; Taberner et al., 2009).

*Interestingly, the *mbp1 swi4* lethality can be rescued by constitutive expression of *CLN2*, suggesting that the phenotype arises primarily from failure to express G1 cyclins (Koch et al. 1993).

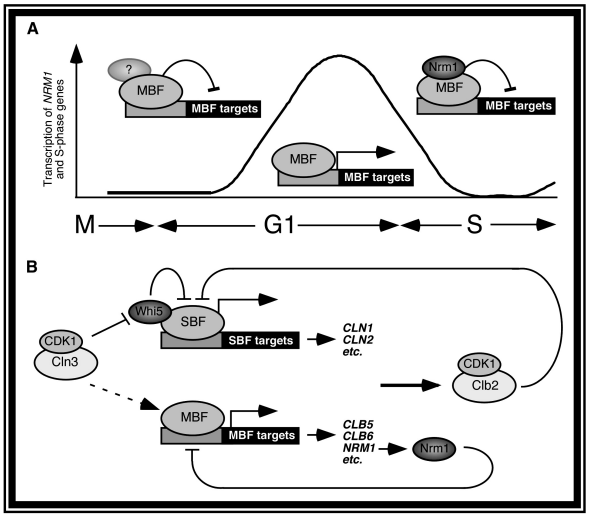
Since SBF and MBF-mediated transcription are coordinately regulated, a similar mechanism for repression of MBF targets has also been proposed (Costanzo et al. 2004). However, despite the apparent capacity of Whi5 to bind to MBF under some conditions (Costanzo et al., 2004), activation of MBF-dependent transcription seems to be largely independent of Whi5. Strikingly, in *whi5* null mutant strains, the transcription of MBF target genes remains dependent upon Cln/Cdk activity: in fact, whereas SBF dependent genes are strongly derepressed in cells lacking both *WHI5* and G1 cyclin function (de Bruin et al., 2004), MBF-dependent genes fail to be expressed (Wittenberg & Reed, 2005)); in contrast, like SBF-dependent genes, MBF-dependent genes are fully activated when Cln_s cyclins functions are retained. This suggests that Cln/Cdk1 activates MBF via a Whi5- independent mechanism.

However, it is currently unknown which mechanisms repress MBF-transcription in early G1 or activate MBF at Start (Wittenberg & Reed, 2005). So far, no Whi5 homolog has been identified that specifically binds to MBF. Stb1, a Swi6-binding protein collaborates with MBF to modulate the MBF-G1 transcriptional circuit (Costanzo et al.,2003; de Bruin et al., 2008) but the mechanism is not specific since Stb1 has also an analogous function in SBF dependent transcription (de Bruin et al., 2008). Msa1, a recently identified regulator of G1/S transcription, has also been found to interact with both SBF and MBF (Ashe et al., 2008). Moreover, recent findings have reinforced the idea that MBF acts primarily as a repressor of transcription by confining the expression of its target genes to the G1 phase (Fig. 33): consistently, inactivation of *MBP1* elevates expression levels of several MBF-dependent targets (Koch et al., 1993; Bean et al., 2005). This repressive function of

MBF depends, at least in some circumstances, upon interaction of MBF with corepressors (deBruin et al., 2006). In particular, Nrm1 (whose expression is MBF-dependent) cooperates with MBF in a negative feedback mechanism to specifically repress MBF-controlled genes in cells progressing into S phase (Fig. 33). Whether MBF acts alone or in concert with corepressors during early G1 phase, before the onset of the G1 transcriptional wave, remains to be established (de Bruin et al., 2006).

In contrast, the inactivation of SBF-mediated transcriptional program is largely accomplished by Clb_s cyclins-Cdc28 complexes which promote dissociation of Swi4 from its target promoters: in particular, Clb2 plays a central role in the transition from Clns to Clbs cyclins (Koch et al., 1996; Amon et al., 1993; Siegmund & Nasmyth, 1996; Cooper, 2006). Apparently, MBF-driven promoters are not repressed by Clb_s-Cdc28 complexes in the post-Start phases of the cell cycle (Amon et al., 1993; Mendenhall & Hodges, 1998).

Figure 33. Different control of SBF and MBF transcriptional program.



A) Model depicting the mechanism of MBF-regulated transcription. The NRM1 gene is a preferred target of MBF (SBF can also contribute to its expression), Nrm1 accumulates as cells progress into late G1 phase, binds to MBF at its target promoters and thereby represses MBF-dependent transcription as cells progress into S phase. Nrm1, like Whi5, acts as a transcriptional repressor that confines transcription to the G1 phase. However, whereas Whi5 represses G1-specific transcription during early G1 phase, Nrm1 acts at the G1/S transition. Furthermore, Whi5 binds to SBF and inhibits transcriptional activation, whereas Nrm1 binds to MBF and acts as a transcriptional corepressor.

B) The mechanisms regulating the activity of Whi5 and Nrm1 also appear to be different. Whereas Whi5 is antagonized by CDK, Nrm1 is regulated, at least in part, at the level of protein abundance. This regulation is a consequence of transcriptional regulation via a negative feedback loop involving MBF (B) and by regulated proteolysis, most likely mediated by the anaphase promoting complex (APC). (From de Bruin et al., 2006).

The molecular players at START in closer detail

CLN3, the upstream activator of the G1/S transition

The trigger of late-G1-transcriptional program depends heavily on *CLN3,* which functions as a dose-dependent upstream activator of *START* (Tyers et al., 1993; Dirick et al., 1995; Stuart & Wittenberg, 1995; Cross & Blake, 1993): transcription of the G1/S regulon is severely delayed in cells lacking *CLN3*, which also exhibit very large size; conversely, overespression of *CLN3* accelerates the passage through *START* and results in very small cells (Futcher et al., 1996; Tyers et al., 1993; Tyers et al., 1992). However, despite the alterations in the length of their G1 phase, both *cln3*-null and *CLN3*-oeverespressing strains display an overall normal growth rate due to compensation in other phases of the cell cycle (Cross, 1988; Dirick et al., 1995; Nash et al., 1988;).

Although any of the three G1 cyclins (Cln1, Cln2, or Cln3) is sufficient to promote the G1-specific transcription (Cross & Tinkelenberg, 1991; Dirick & Nasmyth, 1991;; Tyers et al., 1993), Cln3 is the primary activator under physiological conditions (Tyers et al., 1993; Dirick et al., 1995; Stuart & Wittenberg, 1995). Nevertheless, recent studies have demonstrated that the synchronous expression of the G1/S regulon is principally due to a feedback loop in which the two downstream cyclins, Cln1 and Cln2, promote their own synthesis by accelerating nuclear exclusion of Whi5 to ensure coherent entry into cell cycle (see below; Skotheim et al., 2008; Di Talia et al., 2007).

Mutants lacking all the three *CLN* genes are inviable and arrest in G1 with a phenotype resembling *cdc28* null mutants (Richardson et al., 1989).

In contrast with other cyclins, *CLN3* mRNA is transcribed at rather constant, low level throughout the cell cycle, with only a minor peak at the M/G1 boundary, which depends on Mcm1 (MacKay et al., 2001; McInerny et al., 1997). Cln3 is an extremely instable protein, with a half-life of less than 10 min in asynchronous populations (Schneider et al., 1998), and is estimated to be 50 to 100.fold less abundant than Cln1 and Cln2; in addition, the kinase activity associated with Cln3 (measured in vitro using hystone 1 as substrate) is much lower (2 to 20 fold less) than the corresponding activities for Cln1 and Cln2 (Tyers et al., 1993; Futcher, 1996; Mendenhall & Hodge). Hyperstabilization of Cln3 causes not only a dramatic decrease in the cell size required for budding and entry into S phase but also to resistance to mating pheromone-induced G1 arrest (Cross, 1988; Nash et al., 1988).

Nuclear localization of Cln3

The subcellular localization of Cln3 is predominantly nuclear (Miller and Cross, 2000; Edgington and Futcher, 2001; Miller & Cross, 2001). Cln3 contains a bipartite nuclear localization signal (NLS) at its C terminus that is sufficient for nuclear import (Edgington & Futcher, 2001; Miller & Cross, 2001). The role of Cln3 in promoting transcription is dependent on the nuclear localization of the cyclin: in fact, the introduction of a nuclear export signal into Cln3 renders the Cln3–Cdc28 complex largely non-functional (Bloom & Cross, 2007; Miller and Cross, 2000; Edgington and Futcher, 2001; Miller & Cross, 2001).

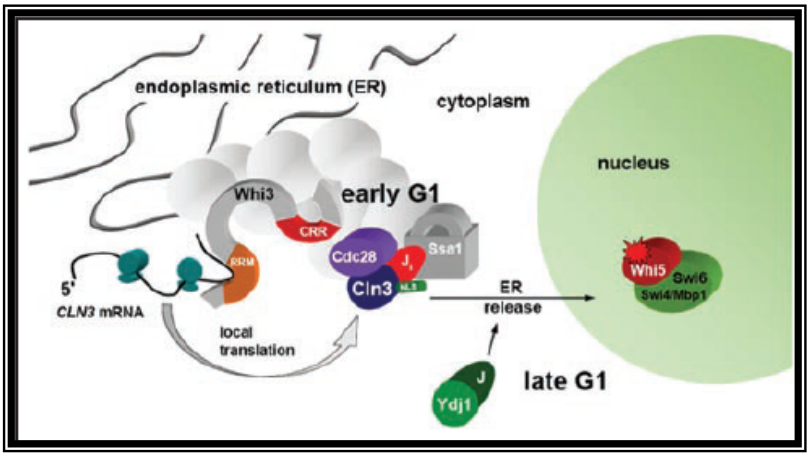


Figure 34. Cln3 is retained at the ER in early G1 and released by the Ydj1 chaperone to trigger entry into S phase.
Whi3 contains an RNA-recognition motif (RRM) that binds the *CLN3* mRNA and the Cdc28-recruitment region (CRR) to locally retain newly formed Cdc28-Cln3 complexes. By inhibiting their ATPase-dependent conformational cycle, the Ji domain of Cln3 locks the Ssa1,2 chaperones into a tightly associated ER complex with Cdc28 in early G1: in this way, nuclear import of Cln3 is prevented. In late G1 phase, once a relative surplus of Ydj1 (and most likely other folding activities) is achieved, ATPase activation by Ydj1 unlocks the Ssa1,2 complex, thus releasing Cln3 from the ER and allowing its nuclear accumulation to phosphorylate Whi5 and trigger *START*. (From Aldea et al., 2007).

Cln3 nuclear translocation is regulated by a surprisingly complex mechanism (Fig. 34). In early G1, most of Cln3 is retained on the cytoplasmic face of the endoplasmic reticulum (ER) to prevent its nuclear accumulation (Verges et al., 2007). Efficient binding of Cln3 to the ER requires Cdc28, which seems to act as an adaptor between Cln3 and ER-scaffold structures, and Whi3, a protein originally identified as involved in cell size regulation (Nash et al., 2001). Whi3 sequesters the *CLN3* mRNA to confine its translation to specific sites of the ER, where also Cdc28 is recruited via interaction with Whi3 (Verges et al., 2007; Wang et al., 2004; Garì et al., 2001). The newly formed Cdc28-Cln3 complexes are retained onto the ER by the interaction between the "Ji" domain of Cln3 and Ssa1 and Ssa2, two of the most important Hsp70 chaperones in yeast. The "j domain" is a key signature of the Hsp70-cochaperone regulators, but the one present in Cln3 is likely an inhibitory version (hence Ji), since it lacks the HPDK tetrapeptide essential to trigger the ATPase activity of Hsp70 chaperones (Verges et al., 2007): according to the proposed model, the Ji domain of Cln3 would hinder the Hsp70 chaperone cycle and effectively lock Ssa1 and Ssa2 into a tight complexes with Cdc28-Cln3 onto the ER, thus preventing unscheduled nuclear import of Cln3 in early G1. The J chaperone .1 seems to play a key and limiting role in the release of Cln3 from the ER in late G1 cells and in the subsequent nuclear accumulation of the cyclin. Ydj1 amount increase linearly during the G1 phase, eventually achieving a sufficient level to trigger the ATPase activity of Ssa1,2 and unlock the complex. The released Cln3 then freely accumulates in the nucleus to initiate the G1/S transition (Verges et al., 2007; Aldea et al., 2007).
Thus, the Cln3 retention on the ER would serve to prevent an inappropriate activation of the Start transcriptional program until the cell have reached a critical size, which would coincide with the achievement of a relative surplus of chaperone

folding activity (Verges et al., 2007; Aldea et al., 2007; see below). Later, as cells proceed into S phase, a change in the rate of protein synthesis (presumably as a direct consequence of the wave of late G1 transcription) would once more reduce chaperone availability, again resulting in Cln3 retention at the ER (Verges et al., 2007; Aldea et al., 2007). Since they have already reached enough Ydj1 levels in the previous cycle, mother cells would enter a new round of mitotic division soon after citokinesis if the growth rate remains unaltered. Therefore, in cooperation with other mechanisms that have been proposed to selectively reduce Cln3 expression in daughter cells (Laabs et al., 2003), this model may also explain one of the key features of *S. cerevisiae* cell cycle: asymmetrical division (Aldea et al., 2007).

Cln3 activity is regulated at multiple levels

Multiple signal transduction pathways that control cell cycle progression converge to regulate Cln3 transcription, translation, stability, and activity.

CLN3 transcription is maintained at rather constant level throughout the cell cycle but displays some cell cycle periodicity that depends on early cell cycle boxes (ECB) sites upstream of the *CLN3* promoter (Mai et al., 2002; McInerny et al., 1997). *CLN3* mRNA levels are high during exponential growth on glucose but dramatically drops at the diauxic shift and it further declines during stationary phase cells (Parviz et al., 1998a).

Glucose induces *CLN3* transcription (Hubler et al., 1993; Parviz et al., 1998) through a mechanism that requires glucose metabolism but that is not affected by mutations in all the known glucose sensing pathways (Parviz et al., 1998a; Newcomb et al., 2003); Azf1, a glucose-activated transcription factor, binds to repeated elements in the *CLN3* promoter (Parviz et al., 1998b; Newcomb et al., 2002).

Nitrogen starvation reduces Cln3 translation efficiency and stability (Gallego et al., 1997). Mating pheromone and osmotic-shock arrest cell cycle progression in G1 phase by decreasing the activity of the Cln3/Cdc28 kinase complex (Jeoung et al., 1998; Tyers & Futcher, 1993; Belli et al., 2001).

A leaky scanning mechanism involving a short upstream micro-ORF (Polymenis & Schmidt, 1997; Gallego et al., 1997) makes Cln3 translation extremely sensitive to the rate of protein synthesis: a 50% decrease in protein synthesis produces a 10-fold drop in Cln3 protein levels (Hall et al., 1998). This mechanism probably accounts for regulation of Cln3 translation by the Tor and protein kinase A (PKA) pathways (Hall et al., 1998; Barbet et al., 1997). Disruption of the upstream ORF permits far more efficient translation of Cln3 and leads to small cells (Polymenis & Schmidt, 1997).

CLN3 expression is apparently reduced in daughter cells through a mechanism that involves Ace2 (a daughter-specific transcription factor) and that might contribute to delay the execution of START in daughter cells (Laabs et al., 2003): although intriguing, the molecular details for this mechanism remain unclear.

Taken together, all these evidences demonstrate that Cln3 is an integrator for diverse signals that regulate cell cycle progression in *S. cerevisiae*.

Both *CLN3* mRNA and protein levels respond quickly to fluctuations in nutrient availability (especially glucose) and to changes in the cellular biosynthetic capacity (Barbet et al., 1996; Gallego et al., 1997; Polymenis & Schmidt, 1997; Newcomb et al., 2002; Parviz et al., 1998; Newcomb et al., 2003; Hall et al., 1998; Schneider et al., 2004). Because of the tight coupling between Cln3 abundance and its synthesis

rate and because transcriptional activation of the G1/S regulon is highly sensitive to Cln3 level, Cln3 is an excellent candidate for the role as a sensor of cell growth (Wittenberg & Reed, 2005). This issue will be discussed in detail in later sections.
Furthermore, Cln3 is known to be a highly unstable protein targeted for degradation by an SCF ubiquitin ligase (Tyers et al., 1992); however, the details of this regulation are unclear. Finally, *CLN3* appears to be the target of a still mysterious free-running oscillator that underlies the timing of cell cycle events (Haase & Reed, 1999).

Bck2

In absence of Cln3, the delayed onset of the G1 transcriptional wave relies on Bck2, a poorly characterized protein that is required for basal transcription of at least a portion of the G1 regulon, including the *CLN1* and *CLN2* genes (Epstein & Cross, 1994; Di Como et al., 1995; Wijnen & Futcher, 1999). The role of *BCK2* is still unclear: induction of SBF/MBF target genes by Bck2 depends partly, (but not wholly) on SBF and MBF. Unlike Cln3, Bck2 is capable of inducing its transcriptional targets even in the absence of a functional Cdc28 (Wijnen & Futcher, 1999). A recent study has shown Bck2 activates a selection of cell cycle-regulated genes from all cell cycle stages, in contrast with Cln3, which only activates G1/S phase genes. Furthermore, Bck2 activates many genes independently of Swi6, the common component of SBF and MBF; in addition to SBF and MBF, Bck2 may elicit gene expression via Ste12 and Mcm1. Thus, Bck2 apparently activates its targets by a mechanism fundamentally different from that of Cln3: thus, it may play a role as cofactor for the full expression of a subset of cell cycle-regulated genes (Ferrezuelo et al., 2009). A double bck2 cln3 knockout is lethal or very slow growing, depending upon the genetic background, and this phenotype is suppressed by ectopic expression of *CLN1* or *CLN2* (Epstein & Cross, 1994; Di Como et al., 1995; Wijnen & Futcher, 1999).Alterations of *BCK2* gene dosage have effects similar to the ones registered with *CLN3*: inactivation of *BCK2* increases cell size, whereas overexpression of the gene results in small cells (Mendenhall & Hodges, 1998).

Whi5

The lethality of the *cln3 bck2* double null mutant is also suppressed by loss of Whi5 (Costanzo et al., 2004; de Bruin et al., 2004). Consistent with its role as a dose-dependent repressor of *START* transcriptional program, inactivation of *WHI5* speeds up the G1/S transition, leading to noticeably small cells (Jorgensen et al., 2002; Zhang et al., 2002; Costanzo et al., 2004; de Bruin et al., 2004) and suppresses (although not completely) the G1 delay and large size phenotype of deficient cells (de Bruin et al., 2004; Costanzo et al., 2004). Conversely, ectopic expression of *WHI5* extends the G1 phase and significantly increases cell size in wild type cells (de Bruin et al., 2004; Costanzo et al., 2004), whereas it is lethal in a *cln3* null mutant (Costanzo et al., 2004). Furthermore, an increased *WHI5* dosage leads to a permanent G1 arrest in several strains genetically compromised for Start, such as *swi6* and *cdc28-4* strains, and severely impairs growth in a *cln1 cln2 s*train, while has little effect on *swi4* or *bck2* mutant (Costanzo et al., 2004; Jorgensen et al., 2002).
Whi5 protein associates with G1-specific promoters in an SBF-dependent manner and is released from DNA coincident with transcription of the G1 regulon (de Bruin

et al., 2004; Costanzo et al., 2004). At least two Cdc28-dependent mechanisms have been proposed to regulate the activity of Whi5: phosphorylation and nuclear export.

The role of Whi5 phosphorylation

Phosphorylation of Whi5 by Cln/Cdc28 has been proposed as a key event for the timing of SBF-dependent transcriptional activation (de Bruin et al., 2004): consistently, phosphorylation of SBF/Whi5 complexes promotes disengagement of Whi5 form the complex *in vitro* (de Bruin et al., 2004; Costanzo et al., 2004). However, the available experimental data regarding the importance of Whi5 phosphorylation *in vivo* are sometimes ambiguous. Whi5 contains twelve potential Cdk phosphorylation sites and six non-Cdc28-sites, which might be target for casein kinase I or casein kinase II: a recent analysis has confirmed that 10 of the Cdc28 sites and all of the 6 non-Cdc28 sites are phosphorylated in vivo (Wagner et al., 2009). Cells expressing a Whi5 derivative in which seven out of the 12 putative Cdk consensus sites have been eliminated exhibit a significant (~10%) increase in size and delayed transcription of the SBF-dependent gene *CLN2* (de Bruin et al., 2004). In contrast, another report shows that mutating six of the potential Cdk sites causes nuclear retention of Whi5 throughout the cell cycle, but has no effects on G1 progression (Costanzo et al., 2004)*. Even more surprisingly, overproduction of either wild type Whi5 or of a mutant form ($WHI5^{12A}$) lacking all twelve Cdk sites causes nearly indistinguishable phenotypes (at least in a wild type strain: see below): consistent with this result, in vitro studies show that Cdc28 can still promote the release of $Whi5^{12A}$ from SBF complexes (Costanzo et al., 2004). More recently, Whi5 has been found to be hypo-phosphorylated at all stages of the cell cycle, including early G1 (Wagner et al., 2009); interestingly, hypo-phosphorylation is not required for the function of Whi5 as a repressor, since cells expressing $WHI5^{18A}$ (where all potential Cdc28 and non-Cdc28 sites are mutated to alanine) exhibit normal size and overespression of this mutant is still lethal in *cln3* null cells (Wagner et al., 2009). These results also suggest that Whi5 phosphorylation is apparently not critical for the execution of the G1 transcriptional program and that other targets of Cdc28 may contribute to the timings of the *START* execution. One plausible candidate might be Swi6: in fact, the combination of Whi5 and Swi6 phosphorylation site mutants is nearly lethal, (especially when $WHI5^{18A}$ is overexpressed: Wagner et al., 2009; Costanzo et al., 2004; see below). In addition, *whi5* and *swi6* deletions are synthetically lethal (Costanzo et al., 2004).
The inconsistencies in the available data may depend on strain background differences or different experimental conditions.

*Other studies report that the redistribution form nucleus to cytoplasm of this mutant form of Whi5 is not completely abolished, although strongly reduced and delayed (Skotheim et al., 2008).

Regulation of Whi5 subcellular localization

CDK-dependent nuclear export provides a second layer of control over Whi5 activity. Whi5 enters the nucleus at the end of mitosis and remains nuclear until Start: the timing of its transit into and out of the nucleus is a consequence of the nuclear exclusion of Whi5 by all forms of cyclin-Cdc28 activity. At the end of mitosis, once CDK activity has been turned off, a preexisting cytoplasmic pool of Whi5 immediately enters the nucleus to prevent a premature transcription of the G1 regulon as soon as SBF and MBF are loaded on their promoter in early G1 (Costanzo et al., 2004).

The nuclear import depends on the beta-karyopherins of the classical import pathway Kap95 and Cse1 (Taberner et al., 2009). Whi5 contains a monopartite and a bipartite NLS localized in its N-terminal region which are functionally redundant (Taberner et al., 2009). Evidences suggest that the Whi5 nuclear import is not cell cycle-regulated. The phosphatase Cdc14 is a central player for the nuclear accumulation of Whi5 at the end of mitosis (Taberner et al., 2009). The nuclear export of Whi5 is assisted by Msn5 and requires a region of Whi5 comprised from the amino acids 51 to 167. Interestingly, this region can drive the export of a chimeric nuclear protein in a cell cycle-regulated pattern similarly to that observed for Whi5 (Wagner et al., 2009; Taberner et al., 2009). The nuclear export of Whi5 depends on the phosphorylation of specific Cdc28 consensus sites: mutants lacking several of these sites reside in the nucleus over the entire cell cycle (Costanzo et al., 2004; Taberner et al., 2009)*. However, constitutive Whi5 nuclear localization has no apparent effect on cell viability or cell cycle progression, further confirming that multiple mechanisms inactivate Whi5 to promote *START* (Costanzo et al., 2004).
In sum, the detailed mechanisms by which Cdk activity neutralizes the inhibitory function of Whi5 remains uncertain, but this do not diminish the importance of Whi5 as key negative regulator of cell cycle progression at *START* (Schaefer & Breeden, 2004).
Although elimination of *WHI5* leads to a premature transcription of the G1/S regulon, and thus premature cell cycle initiation, it does not affect the periodicity of the transcriptional program (Costanzo et al., 2004; de Bruin et al., 2004). Therefore, mechanisms that normally restrict expression of the G1 gene cluster to the G1/S window (namely Clb2-Cdc28) do not depend on Whi5 (Costanzo et al., 2004; Wittenberg & Reed, 2005; Amon et al., 1993;Koch et al., 1996; Siegmund & Nasmyth, 1996): rather, Whi5 restrains the activation of G1/S transcriptional program until the appropriate cellular size is achieved (Costanzo et al., 2004; de Bruin et al., 2004).
Interestingly, Cln3/Cdc28 can promote transcriptional activation even in the absence of Whi5: this it has been proposed that this activation might depend on the direct phosphorylation of Swi6 by Cln/Cdc28 (Wittenberg & Reed, 2005). Consistently, (as aforementioned), loss of Cdc28 phosphorylating sites on both Whi5 and Swi6 produces a dramatic growth defect (Costanzo et al., 2004; Wagner et al., 2009).

SBF and MBF as direct targets of CDK activity

Although Whi5 is clearly a critical target, Cln/Cdc28 likely phosphorylates additional substrates at SBF/MBF promoters. Both Swi6 and Swi4 are established Cdc28 targets and several studies implicate Swi6 as a critical target of Cln3/Cdc28 (Sidorova et al., 1995; Ubersax et al., 2003; Geymonat et al., 2004); however, mutation of numerous phosphorylation sites on Swi4 and Swi6 has no clear effect on the timing of the G1/S transcription (Koch et al., 1996; Sidorova et al., 1995; Wijnen et al., 2002). Nevertheless, Cdc28/Clb6 specifically phosphorylates Swi6 at Ser 160 promoting its nuclear export in early S phase (Geymonat et al., 2004); nuclear re-import of Swi6 occurs in late M phase concomitantly with its dephosphorylation, likely mediated by Cdc14, the principal effector of the mitotic exit network (Geymonat et al., 2004). Elimination of Cdc28 consensus sites impairs nuclear import of Swi6 (Sidorova et al., 1995; Geymonat et al., 2004), apparently without interfering with the timing and the extent of transcriptional activation. However, although it is not entirely known how localization of Swi6 is regulated, it is likely

that both SBF and MBF levels are influenced by the lack of availability of nuclear Swi6 outside the G1 phase (Sidorova et al., 1995; Queralt & Igual, 2003). Alternatively, it has also been proposed that nucleo-cytoplasmic cycling of Swi6 is required for transcriptional activation (Queralt & Igual, 2003). It is unclear whether limitation of nuclear Swi6 contributes to maintain the periodicity of the G1/S transcriptional program in *whi5* null cells (Wittenberg & Reed, 2005). The lack of a functional link between phosphorylation of Swi6 and transcriptional activation has led to the suggestion that Cdc28 acts indirectly via phosphorylation of another unrecognized factor (Wijnen et al., 2002). Although several protein have been shown to interact with Swi6 (i.e. Stb1; Costanzo et al., 2003; de Bruin et al., 2008) none of them appears to play a key role in the Cln/Cdc28 transcriptional program.
Swi4 is a known target of the mechanism that promotes the power-off of the SBF-mediated transcription in late G2 and the switch from Clns to Clbs cyclin: the key event in the entire process is the accumulation of the B-type cyclin Clb2, which antagonizes the binding of Swi4 to SBF-dependent promoters (Amon et al.,1993; Siegmund & Nasmyth, 1996; Wittenberg & Reed, 2005). Interestingly, a truncated version of Swi4 bypasses the need for Swi6 in transcriptional activation; when overexpressed, it causes a precocious entry in S phase and constitutive expression of SBF target genes; length of the S phase is also increased (Sidorova & Breeden, 2002).

Cln1 and Cln2.
The two *CLN1* and *CLN2* were originally identified as high-copy-number suppressors of *cdc28-4*ts mutations (Hadwiger et al., 1989). At the level of primary structure the two cyclins share a high degree of identity (~60%), whereas they are only distantly related to Cln3 (Nash et al. 1988; Hadwiger et al. 1989).
The expression of *CLN1* and *CLN2* is regulated coordinately: the two genes are periodically transcribed as part of the G1/S regulon (Tyers et al., 1993; Wittenberg & Reed, 2005) with a peak of mRNA accumulation occurring in late G1; their transcription declines during G2 (as do the Clnl and Cln2 protein levels), as a consequence of the inactivation of the G1/S transcriptional program operated by the B-type cyclins (Tyers et al., 1993; Wittenberg & Reed, 2005).
Control of *CLN1* and *CLN2* transcription plays a crucial part in the proper execution of Start. Cln1 and Cln2 hyperstable alleles accelerate the execution of Start, thus shortening G1 phase length and decreasing the minimal cell size required for budding (Cross 1988; Nash et al. 1988; Hadwiger et al. 1989). Similarly, their overexpression leads to premature cell cycle entry and reduce the mean cell size (Lew et al. 1992).
A Recent study has confirmed that Cln1 and Cln2 form a potent feedback loop to stimulate the transcription of their own encoding genes: this mechanism operates by accelerating the nuclear exclusion of Whi5 and it is essential for the synchronous transcription of the G1/S regulon (Stokheim et al., 2008; Tyers et al., 1993; Stuart & Wittenberg, 1995; Dirick & Nasmith, 1995; see later sections).
Although individual gene knockouts are not associated with dramatic phenotypes, *cln1 cln2* mutant cells grow slowly and exhibit increased size, altered morphology (Hadwiger et al., 1989) and a substantial delay in both bud emergence and DNA synthesis (Dirick & Nasmyth; Stuart & Wittenberg, 2009). Furthermore, the transcription of the G1/S regulon is desynchronized and incoherent in the *cln1 cln2*

strains: as a consequence, a significant fraction of the cell population fails to bud and accumulates permanently in G1 (Stokheim et al., 2008).
Overespression of *CLN2* restores cell cycle progression restores cell cycle progression in previously arrested *cln1 cln2* cells and also suppresses the lethality of a *mbp1 swi4* strain, which lacks functional SBF and MBF and exhibit very low expression of the G1/S regulon (Bean et al., 2005; Koch et al., 1993).
The levels of Cln1 and Cln2 proteins are modulated by nutrient status, in particular by carbon and nitrogen source: for instance, cells growing in rich media have larger amounts of G1 cyclins than cells cultivated in presence of scarce or poor quality nutrients (Schneider et al., 2004).
Although Cln1 and Cln2 are largely cytoplasmic, their function is compromised when a nuclear export signal is added, suggesting that the two cyclins have also important roles in the nucleus despite (Bloom & Cross, 2007; Miller and Cross, 2000; Edgington & Futcher, 2001; Miller & Cross, 2001).The subcellular localization of Cln2 is regulated by Cdc28- mediated phosphorylation: when the Cdc28 consensus phosphorylation sites in Cln2 are mutated, Cln2 is exclusively nuclear. Therefore, the phosphorylation event may conceal a nuclear localization signal or, alternatively, expose a nuclear export signal (Bloom & Cross, 2007; Miller and Cross, 2000; Edgington and Futcher, 2001; Miller & Cross, 2001)
Cln1 and Cln2 are pleiotropic effectors of Start with important nuclear and cytoplasmic functions (Edgington & Futcher, 2001; Miller & Cross, 2000). The two functions are apparently separable (Stokheim et al., 2008): the coherent expression of the G1/S regulon is mainly a nuclear function of Cln2, while cytoplasmic Cln2 has a role in budding (Edgington & Futcher, 2001; Miller & Cross, 2000).The mechanism by which Cln1 and Cln2 is involved in morphogenesis and bud emergence is not completely clear (Mendenhall & Hodges, 1998): the process is apparently mediated by a mechanism involving cytoplasmic Cln1 and Cln2 (Polymenis & Schmidt, 1999; Miller & Cross, 2000) and other members of the G1/S regulon, such as the cyclins Pcl1 and Pcl2 that regulate the activity of the Pho85 CDK(Moffat et al., 2004). A quadruple *cln1 cln2 pcl1 pcl2* null mutant strain fails to produce a discernable bud and arrest with 2n DNA content, thus confirming that other G1/S events such as DNA replication are not affected in this strain (Moffat et al., 2004).
The dual role of Cln1 and Cln2 (in promoting the G1/S transcriptional program and in directly driving bud emergence) provides a compact solution to ensure efficient and timely morphogenesis and synchronous expression of the G1/S regulon (Skotheim et al., 2008).
Cln1 and Cln2 indirectly participate to DNA synthesis by promoting the targeted degradation of Sic1 (Verma et al.1997; Nash et al. 2001b) and inactivating Cdh1, two negative regulators of B-type cyclins (Zachariae et al., 1998; Schwab et al., 1997; Visintin et al., 1997).
In addition to the Start functions, Cln1-Cdc28 and Cln2-Cdc28 can specifically repress pheromone inducible transcription, a function not shared with Cln3-Cdc28 or the Clb-Cdc28 complexes (Oehlen et al., 1994).
Several functional differences between Cln1 and Cln2 have also emerged: for example, overproduction of Cln2 but not Cln1 is lethal in some strain backgrounds (Richardson et al., 1989); in contrast, *CLN1* but not *CLN2* transcription is down-regulated by the cAMP-dependent protein kinase A (PKA) as part of a mechanism

that may serve to increase cell size in presence of glucose (Baroni et al, 1994; Tokiwa et al., 1994).
Degradation of Cln1 and Cln2 is performed by the 26S proteasome via an ubiquitin-dependent mechanism mediated by the SCF complex that contains the F-box protein Grr1 protein (SCF_{Grr1}) (Fig. 35; Barral et al., 1995; Skowyra et al., 1997).

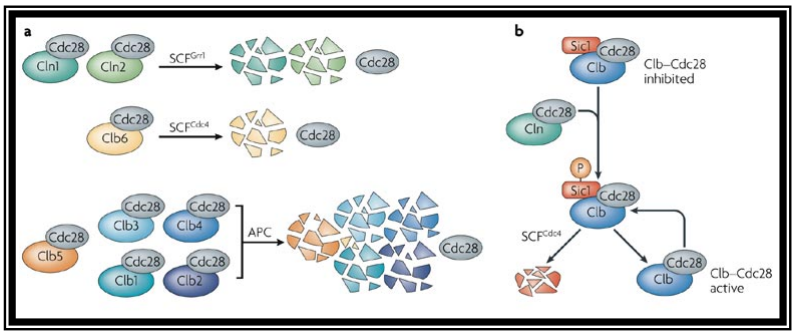


Figure 35 Degradation of cyclins and Sic1
A) Cyclins are ubiquitinated by different ubiquitin ligases and degraded by the 26S proteasome.
The G1-phase cyclins (Cln1 and Cln2) are ubiquitinated by SCF^{Grr1}, Clb6 is ubiquitinated by SCF^{Cdc4}, and the other B-type cyclins (Clb1, Clb2, Clb3, Clb4 and Clb5) are ubiquitinated by the anaphase promoting complex (APC).
B) Sic1 inhibits the activity of Clbs-Cdc28 complexes. Cln–Cdc28 complexes phosphorylate Sic1, promoting SCF^{Cdc4}-mediated ubiquitination and subsequent degradation of Sic1, thus allowing for Clb-Cdc28 activation and S-phase entry. Clbs-Cdc28 complexes also phosphorylate Sic1 to induce its proteolysis.
(From Bloom & Cross, 2007).

Clb5 and Clb6

The transcription of the *CLB5* and *CLB6* genes (encoding for two 50% identical cyclins) peaks in late G1, earlier than the other B-type cyclins, and is as part of the G1/S transcriptional program, (Wittenberg & Reed) from a certain point of view, Clb5 and Clb6 might be considered as G1 cyclins (Mendenhall & Hodges, 1998): consistent with such classification, their overexpression (Epstein & Cross, 1992, Schwob & Nasmyth, 1993, Basco et al., 1995) restores viability in a *cln1 cln2 cln3* triple mutant, whereas the remaining B-type cyclins do not posses this ability (Epstein & Cross, 1992, Lew et al., 1991). Furthermore, *cln1 cln2 clb5 clb6* cells are inviable (Schwob & Nasmyth, 1993).
CLB5 and *CLB6* are expressed at the same time as *CLN1* and *CLN2*; initially, the newly formed Clb5,6/Cdc28 complexes are maintained in an inactive state by their interaction with Sic1, a potent stochiometric inhibitor (Schwob et al. 1994; Knapp et al. 1996). Recent evidences also suggest that Sic1 facilitates nuclear accumulation of Clb5, 6 (Rossi et al., 2005). Cln1,2-Cdk1 complexes initiate the phosphorylation of Sic1, promoting the proteolysis of the inhibitor via an ubiquitin dependent process (Verma et al., 1997; Nash et al., 2001). Upon Clb5,6-Cdc28 activation, Sic1 phosphorylation can be carried out directly by the Clbs-CDK complexes ("positive feedback loop").
Clb5 and Clb6 have prominent roles in activating DNA synthesis (Schwob & Nasmyth, 1993; Schwob et al., 2004; Toone et al., 1997) and in preventing reinitiation on replication origins that have already "fired" (Dahmann et al., 1995; Mendenhall & Hodges, 1998). The length of the S phase is extended in cells lacking

Clb5 (Epstein & Cross, 1992, 310, Schwob & Nasmyth, 1993). The inactivation of *CLB6* reduces the length of G1 phase and the average cell size, likely as a consequence of a premature Start transition, whereas overexpression of *CLB6* represses the transcription of *CLN2* and *CLB5* (Basco et al., 1995). Conversely, *CLB5* overespression promotes the transcription of several *START* specific genes (Oehlen et al., 1998). In a *clb5 clb6* double mutant the S-phase initiation (but not bud emergency) is strongly delayed, but once initiated the duration of the process is apparently normal (Kuhne & Linder, 1993; Schwob & Nasmyth, 1993). Since this mutant can progress into S phase other B-type cyclins (Clb1-4) expressed later in the cell cycle must posses a latent ability to promote DNA synthesis (Bloom & Cross, 2007). Strains lacking all six B-type cyclins are unable to enter S phase, but can perform other Start-dependent functions such as budding (Schwob et al., 1994).
Apparently, Clb5 and Clb6 have also a role in downregulating the activity of Clns/Cdc28 complexes (Basco et al., 1995): both Clb5 and Clb6 negatively influence the formation of Cln2-Cdc28 complexes (Basco et al., 1995). In addition, Clb6/Cdc28 phosphorylates Swi6 to promote its nuclear export (Bloom & Cross, 2007).
Analyses of multiple *CLBs* and *CLNs* deletion mutants revealed that both Clb5 and Clb6 may play a role in spindle formation (Schwob & Nasmyth, 1993), although the two cyclins are not sufficient to form the bipolar spindles required for mitosis (Amon et al., 1993; Fitch et al., 1992; Richardson et al., 1992).
Clb6 is the only B-type cyclin degraded in an SCF-dependent manner: its proteolysis occurs earlier than Clb5 and is mediated by an SCF complex containing the F-box protein Cdc4 (SCF_{Cdc4}) (Fig. 35; Jackson et al., 2006; Bloom & Cross, 2007). Clb5, like the other B-type cyclins, is degraded after ubiquitination promoted by the Anaphase Promoting complex (APC: Peters, 2006; Bloom & Cross, 2007): during metaphase, APC is bound to Cdc20 and targets Clb5 and the mitotic B-type cyclins for degradation (Shyrayama et al., 1999; Wasch et al., 2002); later in mitosis, APC binds to the adaptor protein Cdh1 and completes the degradation of mitotic B-type cyclins, including the main mitotic cyclin, Clb2 (Bloom & Cross, 2007). The contrasting modes of Clb5 and Clb6 proteolysis might explain the observation that despite substantial overlap of Clb5 and Clb6 function in DNA replication, cells lacking *CLB5* activate predominantly early origins of replication (Donalson et al., 1998): Clb6 might be already depleted by the time later origins are normally activated (Bloom & Cross, 2007).

Sic1

Sic1 is a potent stoichiometric inhibitor of Cdc28-Clbs complexes that prevents premature DNA replication and ensures correct timing for the G1/ S transition, thus maintaining genome integrity (Schwob et al., 2004). *SIC1* transcription is dependent on the Swi5 transcription factor and peaks at the G1/M-phase border (Toyn et al., 1997).
Sic1 expression is confined from late mitosis to the G1/S phase transition (Donovan et al., 1994; Verna et al., 1997). During G1 phase Sic1 protein is stable and inhibits the newly formed Clb5,6-Cdc28 complexes, preventing a premature entry into S phase. Sic1 binding motif to Clbs/CDK complexes has been mapped to the C-terminal half of the inhibitor (Verma et al., 1994b). At *START*, Cln1,2/Cdc28 complexes phosphorylate Sic1 on multiple sites, allowing the recognition of the inhibitor by the SCF_{Cdc4} ubiquitin ligase and its subsequent proteolytic degradation

(Fig. 35; Feldman et al., 1997; Verma et al., 1997). Sic1 proteolysis requires phosphorylation of at least six of the nine Cln1,2/ Cdc28 consensus sites (Nash et al., 2001b).
The removal of Sic1 relieves the inhibition from Clb5,6–Cdc28 complexes allowing the entry into S phase and the onset of DNA replication (Schwob et al., 2004). Apparently, Sic1 degradation is the only essential function of the G1 cyclins*:* consistently*,* a *cln1 cln2 cln3 sic1* multiple null mutant is viable (Epstein & Cross, 1994; Schneider et al., 1996, Tyers, 1996).
SIC1 is a nonessential gene (Donovan et al., 1994, Nugrobo et al., 1994), although *sic1* null cells often undergo a permanent G2 arrest (Nugrobo et al., 1994). Loss of *SIC1* causes premature DNA replication from fewer origins, extension of the S phase length and inefficient separation of sister chromatids during anaphase (Lengronne & Schwob, 2002); conversely, in strains expressing Sic1 variants resistant to proteolysis DNA synthesis is delayed, indicating that the timing of Sic1 degradation is crucial for the onset of DNA replication (Schwob et al., 2004).
An additional major function proposed for Sic1 is the downregulation of Clb-Cdc28 activity at the exit from mitosis in late anaphase to telophase (Lengronne & Schwob, 2002; Calzada et al., 2001; Donovan et al., 1994).
The amount of Sic1 is modulated by nutrient availability, in particular by the carbon source: Sic1 content is higher in cells cultivated in ethanol than in glucose grown cells (Rossi et al., 2005). Furthermore, rapamycin treatment induces a G1 arrest by a dual mechanism consisting of downregulation of the G1-cyclins Cln1-3 and upregulation of Sic1 (Zinzalla et al., 2007).
Subcellular localization of Sic1 is also carbon source modulated: in glucose grown cells, Sic1 is mainly nuclear, whereas a sizeable amount of the inhibitor is detected also in the cytoplasm during growth on ethanol (Rossi et al., 2005). Nuclear import of Sic1 is dependent upon a bipartite localization sequence and is essential for correct cell cycle progression in a carbon-source dependent manner (Rossi et al., 2005).
Similarly to Cip/Kip proteins (Sic1 mammalian counterparts (Barberis et al., 2005)) Sic1 facilitates nuclear accumulation of its cognate cyclin Clb5 (and possibly Clb6), thus playing also a positive role in promoting the G1/ S transition (Rossi et al., 2005). Consistent with this notion, phenotypes of cells expressing a nuclear exporting signal (NES).fused to Sic1fusion are more severe than those observed in *sic1* null mutants, indicating that when excluded from the nucleus Sic1 can act as a cytoplasmic retention device for Clb5-Cdc28 complexes and thus reduce the Clb5 nuclear pool. The aberrant morphologies observed in cells expressing the NES-Sic1 fusion are similar to those observed in strains expressing stabilized versions of Sic1 (Schwob et al., 2004; Petrosky & Deshaies, 2003) or in *cdc34ts* strains at the restrictive temperature, in which Sic1 cannot be degraded due to the inactivation of the catalytic subunit of the SCF complex (Schwob et al., 2004).
In addition to Cln- and Clb/Cc28 complexes, Sic1 can be phosphorylated by other kinases, such as the Pcl1/Pho85 complex (Nishizawa et al., 1998) or CK2 (Coccetti et al., 2004; Coccetti et al., 2006; Tripodi et al., 2007).
Loss of the CK2 phosphorylation site (Ser^{201}) alters the coordination between growth and cell cycle progression by increasing the critical size at the onset of DNA replication (Coccetti et al., 2004).
Phosphorylation of Sic1 by the Hog1 kinase has been shown to be essential for arrest of cell-cycle progression in response to osmotic stress (Escoté et al., 2004).

Cell size control and the modular nature of G1

Coherency of the G1 regulon: the linear model vs. the positive feedback module

START is a fundamental checkpoint where several physiological inputs (such as

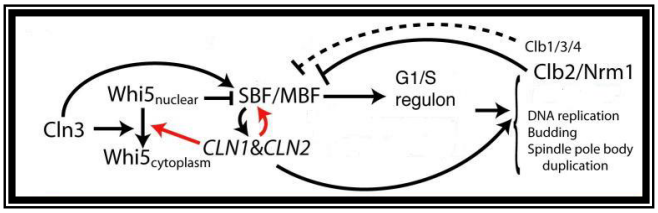


Figure 36. The Cln1,2 feedback loop ensures coherent expression of the G1/S regulon.
(From Skotheim et al., 2008)

nutrients, mating factor, cell size) are integrated to produce an all-or-none decision to enter a new round of mitotic division. In order to ensure a proper entry into the cell cycle, the events at start must be kept temporally coherent.

According to the prevailing model, the precise timing of the G1/S transition relies mainly on Cln3, which acts as the sole trigger in a linear, feedback-free cascade that culminates in the transcription of the G1/S regulon (see Fig. 36, omitting red arrows; Dirick & Nasmith, 1995; Stuart et al., 1995).

The activation of the G1/S regulon does not occur in a triple mutant *cln1 cln2 cln3*, whereas the presence of any one of the *CLNs* cyclin is sufficient to restore the transcriptional program (Tyers et al., 1993). This has led to the proposal that Cln1 and Cln2 could form a positive feedback loop to enhance their own expression, as well as that of all SBF/MBF-regulated genes, thus accelerating the progress into S phase. According to this "feedback-model", Cln3, which is already present in early G1, would act as initial trigger for the Cln1/Cln2 auto-activation loop when cells reach a critical size (Tyers et al., 1993), while the timing of *START* execution would be primarily dictated by Cln1 and Cln2. The weak kinase activity associated with Cln3 is also consistent with the idea that this cyclin does not participate directly in driving cell cycle progression (*"Cln3 is genetically strong, biochemically weak."*. Tyers et al., 1993)

This attractive hypothesis was initially found to be incorrect: experiments with cell populations showed that *cln1 cln2* double mutants activated the transcription of the G1/S regulon with nearly identical kinetic and at the same size as wild type strains (Stuart & Wittenberg, 1995; Dirick & Nasmyth, 1995); despite this, the other Start-related events (such as budding, DNA replication, acquisition of pheromone resistance) were all delayed in cells lacking functional *CLN1* and *CLN2*, thus explaining their increased size (Dirick & Nasmyth., 1995). In contrast, *cln3* deficient cells delayed the transcription of SBF/MBF target genes until they reached a very large size (Stuart & Wittenberg, 1995; Dirick et al., 1995). This led to the conclusion that the putative Cln1/Cln2 feedback loop had only negligible importance in wild type cells: instead, under physiologic conditions, Cln3 acted as a size sensor and was the sole activator of the G1/S transcriptional program, while Cln1 and Cln2 were essential to regulate the other *START* events (Dirick et al., 1995; Stuart & Wittenberg, 1995).

However, a recent study conducted on single-cells using a real-time approach has completely turned the tide, demonstrating that the Cln1 Cln2 loop is central in the

coordination of *START* (Skotheim et al., 2008; Santos & Ferrel, 2008) Apparently, in *cln1 cln2* deficient cells the expression of the entire G1/S regulon is not only delayed, but also desynchronized and incoherent: while all the genes of the G1 cluster are transcribed synchronously in the wild type cells, the interval elapsing between the induction of two selected marker genes is highly variable in a *cln1 cln2* double mutant. Furthermore, the most "strongly incoherent" *cln1 cln2* cells fail to bud and arrest in G1 phase, likely as a consequence of insufficient expression of the G1/S regulon before the inactivation of the transcriptional program by the Clbs cyclins (Skotheim et al., 2008).
The Cln1/Cln2 feedback loop seems to operate by promoting the rapid nuclear exclusion of Whi5. In fact, the exit of Whi5 from the nucleus in wild type cells is quite abrupt and tightly correlated with induction of *CLN2*; in contrast, the redistribution of Whi5 to the cytoplasm occurs more slowly in a *cln1 cln2* mutant and, accordingly, the activation of the *CLN2* promoter is delayed. Furthermore, a version of Whi5 ($Whi5^{6A}$) lacking several of the Cdk phosphorylation sites reduces the coherence of the regulon transcription both in wild type and in *cln1 cln2* cells: this result is likely a consequence of the impaired nuclear export of $Whi5^{6A}$ which interferes with the feedback loop mechanism (Skotheim et al., 2008).
Taken together, these results suggest that the positive feedback loop involving Cln1 and Cln2 makes the nuclear exclusion of Whi5 rapid, thus allowing the synchronous expression of the G1/S regulon. This mechanism contributes decisively to the irreversible activation of the *START* switch ("sharpness of the *START* switch") and to the proper entry into cell cycle. The role of Cln3 would be limited to trigger the activation of the feedback circuit: once feedback is initiated, the rapid accumulation Cln1 and Cln2, whose associated kinase activity is much stronger, would render Cln3, the rate-limiting upstream activator, unimportant (Skotheim et al., 2008; Santos & Ferrel, 2008).
These new evidences can be reconciled with previous reports arguing against the importance of positive feedback in wild type strains because measurements averaged over a population of cells necessarily entail loss of information (Skotheim et al., 2008).

The modular architecture of the G1 phase: the "timing" and "sizing" modules

A recent analysis has showed that the structure of the G1 phase is modular and consists of a "sizing module" and a "timing module", each controlled by a different G1 cyclin (Fig. 37; Di Talia et al., 2007).

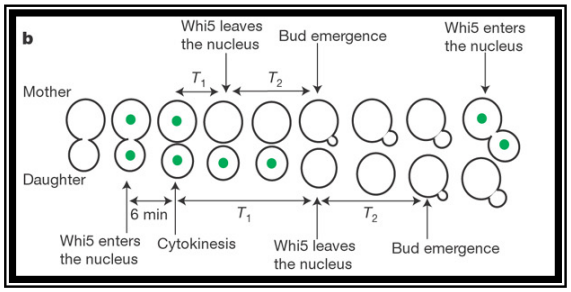


Figure 37. The modular architecture of the G1 phase in yeast
(From Di Talia et al., 2007)

The nuclear exit of the transcriptional repressor Whi5, which coincides with the activation of the Cln1/Cln2 feedback loop (Skotheim et al., 2008), demarks the boundary between these two independent steps, which are temporally uncorrelated and functionally distinct (Di Talia et al., 2007).
The upstream sizing module, which covers the period between citokinesis and nuclear exit of Whi5 (T1), is responsible for cell size control in small daughter cells, but is of modest duration in mother cells. An increased *CLN3* gene dosage as well as the inactivation of *WHI5* shorten T1 length and essentially eliminate size control in daughter cell, whereas the impact on mother cell is negligible; in contrast, loss of *CLN3* significantly increases T1 length in both mother and daughter cells (Di Talia et al., 2007).
The downstream timing module, which encompasses the interval from the nuclear exit of Whi5 to budding (T2), is unaffected by cells size and *CLN3* gene dosage and its duration is similar in mother and daughter cells. Increasing *CLN2* copy number modestly decreases T2 length in both mother and daughter cells (Di Talia et al., 2007).
The G1 size control is restricted to T1, the period of Whi5 nuclear residence: accordingly, this interval is very short in mother cells, which have already reached the critical size (Di Talia et al., 2007).
The overall length of the G1 phase in mother cells is dominated by the "timing module" (T2), which is independent on size and whose duration is nearly identical in mother and daughter cells. (Di Talia et al., 2007): thus, mother-daughter asymmetry in G1 overall length is almost entirely confined to the cell cycle interval (T1) during which Whi5 is nuclear (Di Talia et al., 2007; Bean et al., 2006).
The existence of a "sizer plus timer" mechanism also explains the fact that parent cells do not but immediately after cytokinesis (although they have already achieved in the previous round of mitotic division the critical size required for cell cycle entry) and thus increase their size with genealogical age (Johnston et al., 1979; Barberis et al., 2007; Alberghina et al., 2009
In addition, the timing of G1 in budding yeast shows substantial variability that is independent of the critical cell size control: the leading source of cell cycle variability is "molecular noise" in gene expression (Di Talia et al., 2007; Samailov et al., 2006; Bean et al., 2006).
G1 variability is reduced in both mothers and daughters by a factor of $\sqrt{2}$ for each ploidy doubling (Di Talia et al., 2007). Temporal variability of the "size module" is due to the natural variability in cell size at birth coupled with size control, as well as "molecular noise" (possibly due to variability in *CLN3* expression). The variability of the "timing module" is affected by the expression of *CLN2*, but not by cell size or *CLN3*.
Changing the gene dosage of either *CLN3* or *CLN2* has different effects on G1 variability in mothers and daughters cells: increasing *CLN3* copy number reduces G1 variability in daughter, but has little effect in mother cells where T1 is brief. In contrast, G1 variability in parent cells is quite affected by *CLN2* dosage, which regulates the T2 interval. Consistent with the independence of the sizing and timing modules, the concomitant increase in copy number of both *CLN3* and *CLN2* lead to very low G1 variability in both mother and daughter cells (Di Talia et al., 2007).

How yeast cells measure their size

Yeast cells maintain size homeostasis over multiple generations by enforcing their growth to a critical size threshold before committing themselves to a new round of cell division. This coordination between growth and cell cycle progression takes place at *START*, the narrow interval in G1 phase that regulates the G1/S transition.

Besides the requirement of a critical size, progression through *START* depends on the nutritional supply and on a minimal translation rate (Jorgensen et al., 2004; Moore, 1988; Popolo et al., 1982).

Critical size increases with ploidy and is modulated by nutrients, so that cells growing in rich media are larger than cells growing on poor media.

Although several compelling experimental evidences support the validity of this "critical size model" (Schneider et al, 2004; Jorgensen et al., 2004; Alberghina et al., 2004), many questions remains unsolved.

It is still uncertain how yeast cell actually gauges its size, whether by measuring measure its volume, its mass (protein and /or RNA content), or its biosynthetic capacity (rate of protein synthesis) (Jorgensen & Tyers, 2004).

Above all, the molecular connections between cell size and the execution of the *START* program remain enigmatic: how does cell sense it have reached the appropriate size to enter a new cycle of division? And, as a subsidiary issue, how does it set the threshold size limit according to the nutritional status?

The very concept of "critical size thresholds" postulates the existence of a "cell sizer mechanism", a molecule (or a set of molecules) whose activity (abundance?) is correlated with cellular size and is influenced by diverse elements such as nutrient availability and ploidy (Rupes, 2002).

Despite these uncertainties, several convincing (although not conclusive) evidences support the idea that budding yeast may assess its size by measuring the rate of protein synthesis: bigger cells possess more ribosomes, thus their biosynthetic capacity is larger. Such a model unifies the volume, nutrient and translation requirements for *START* execution (Jorgensen & Tyers, 2004).

In fact, a good correlation exists between the cell size and the overall translation rate in active proliferating cells (Elliot & McLaughlin, 1978; Jorgensen & Tyers, 2004).

Even cells larger than the critical size threshold do not pass START upon nutrient starvation since a critical translation rate cannot be attained (Unger & Hartwell, 1976; Popolo et al., 1982; Moore, 1988; Polymenis & Schmidt, 1997). Sublethal doses of cycloheximide (an inhibitor of protein synthesis) reduce translation rate and delay *START* execution, thus extending the G1 length and increasing cell size (Jorgensen et al., 2004; Popolo et al., 1982; Moore, 1988). Nutrients influence the rate at which ribosomes function (translation rate per ribosome) by determining the amount of material and energy available for translation; furthermore, recent evidences have documented that nutrients can also regulate ribosome biogenesis and thus determine the biosynthetic capacity of the cell (i.e. the number of ribosomes per cell) (see below; Jorgensen et al, 2004; Warner, 1999; Kief & Warner, 1981; Barbet et al., 1996; Cherkasova & Hinnesbusch, 2003).

It has been proposed that the overall rate of protein synthesis reports cell size (communicates information about cell size) to the cell division machinery through unstable "translational sizers", key regulators of the cell cycle whose abundance is especially sensitive to changes in the translational capacity and thus might serve as reliable "surrogates" of cell size: the G1 cyclins (and Cln3 in particular) have been

suggested as attractive candidates for such a role (Polymenis & Schmidt, 1997; Rupes, 2002).

The karyoplasmic ratio and Cln3 nuclear accumulation: a possible model for the coordination between cell size and growth

The ultimate mechanistic basis for size control is still unknown, but several theories propose that yeast cell somehow measures the relative volumes of a growing cytoplasm versus a constant nucleus: assuming that the nuclear volume is proportional to the DNA content (and thus it does not change appreciably during G1 phase) cell division would be triggered when the cytoplasm has grown sufficiently large to alter the "karyoplasmic" ratio (ratio of nuclear volume to cell volume) (Futcher, 1996; Csikasz-Nagy et al., 2006).

A recent study has unequivocally demonstrated the requirement for a threshold level of G1 cyclin synthesis to promote cell cycle entry (Schneider et al., 2004). Cln3 functions upstream of all the other cyclins/Cdk-regulated cell cycle events by serving as the activator of the G1/S transcriptional program (Tyers et al., 1993; Dirick & Nasmyth, 1995; Stuart & Wittenberg, 1995; Skotheim et al., 2008). Passage through *START* is highly sensitive to *CLN3* gene dosage: overproduction of the cyclin shortens the G1 phase and reduces cell size, whereas inactivation of *CLN3* brings about the opposite effect. Furthermore, Cln3 is among the most unstable proteins and thus its overall abundance is primarily determined by its rate of synthesis (Schneieder at al., 2004). Consistently, Cln3 protein levels are extremely sensitive to decreases in protein synthesis rate (Hall et al., 1998; Polymenis & Schmidt, 1997; Gallego et al., 1997): *CLN3* mRNA contains in the long 5'-untranslated region an upstream micro-ORF which makes *CLN3* translation highly inefficient when few ribosomes are available (Polymenis & Schmidt, 1997). Since the number of ribosomes correlates with growth rate, this mechanism may provide a link between growth rate and the rate of Cln3 synthesis (Polymenis & Schmidt, 1997). Therefore, Cln3 is particularly well suited for playing a critical role in coupling cell growth with cell division ("translational sizer"): the synthesis of an adequate level of Cln3 protein may be the rate-limiting step for *START* execution.

The amount of Cln3 during G1 phase is proportional to cell mass: it is generally assumed that the rate of Cln3 synthesis steadily increases as cells grow larger and acquire more ribosome during the G1 phase (Polymenis & Schmidt, 1997; Alberghina et al., 2009). Cln3 accumulates in the nucleus (Edgington & Futcher, 2001) and it is highly unstable, therefore, it was proposed that the effective nuclear concentration of Cln3 during G1 rises with increasing cell size (Futcher, 96; Rupes, 2002; Schneider et al., 2004). Since the nuclear volume would be the metric against which the protein synthetic rate is measured, a crucial assumption of this model originally proposed by Futcher is that the nuclear volume remains constant throughout the G1 phase: in this way, the progressive accumulation of Cln3 in a constant volume nucleus would eventually reach the threshold value needed to trigger *START* (Futcher, 1996). However, recent observations have seriously undermined the validity of this simple model coupling cell size with the rate of Cln3 synthesis: in fact, both nuclear and cytoplasmic volume increases during the G1 phase, so that the "karyoplasmic ratio" remains relatively unaltered (Jorgensen et al., 2007).

Furthermore, (as already discussed) Cln3 nuclear translocation is regulated by a surprisingly sophisticated mechanism (Fig. 34). In fact, during early G1 the newly

synthesized Cln3 is retained on the cytoplasmic face of the endoplasmic reticulum, where it forms a tight complex with Cdc28 and the Ssa1/2 chaperones: this ER-retention mechanism likely prevents premature activation of the *START* program by blocking unscheduled nuclear transport of Cln3. In late G1, once cells has grown to the critical size, the Ydj1 chaperone unlocks the complex, thus releasing Cln3 from the ER and allowing its nuclear accumulation to trigger *START* (Verges et al., 2007; Aldea et al., 2007). According to this model, a threshold level (and thus a critical translation rate) of Ydj1 would be the limiting factor for the release of Cln3 and the timely entry into cell cycle: Ydj1 relative level would increase linearly during G1 as cell grows and expands its biosynthetic capacity, until, in late G1, a surplus of folding activity would be sufficient to release Cln3 from the ER and promote *START* (Verges et al., 2007; Aldea et al., 2007).
It is noteworthy that, despite the well deserved attention it has received, *CLN3* is not essential for *START* execution and cells lacking the cyclin can still modulate their size in response to nutrient availability (Jorgensen et al., 2004; Alberghina et al., 2004; see below): thus, although appealing, simple models connecting the rate of CLN3 translation to cell size homeostasis need to be reconsidered. Nonetheless, all available genetic evidences point to synthesis of adequate G1cyclins as the rate-limiting step in completing *START* (Zaman et al., 2008).

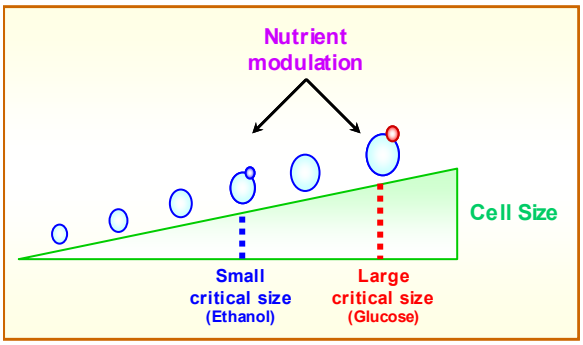


Fig. 38. Nutrient modulation of cell size in yeast.
In yeast the tight coordination between growth and cell division takes place at *START* and is achieved by enforcing growth to a critical size threshold before cell can commit to a new round of mitotic division. Nutrient status (in particular the quality and quantity of carbon source carbon source) modulate the critical size required for budding and DNA replication, so that cells growing in poor media initiate a new round of mitotic division at smaller sizes than cells growing in rich media. (Adapted from Jorgensen & Tyers, 2004).

Nutrient Modulation of Critical Cell Size: the cell sizer
The size at which yeast cells initiate a new round of mitotic division is a function of the nutrient status (Fig. 38): in rich media, cells are larger than cells grown on poor nutrients (Johnston et al., 1979; Tyson et al., 1979; Johnston et al., 1977; Lorincz & Carter, 1979). Upon nutrient shift, the critical size threshold is promptly reset (Lorincz & McCarter, 1979; Johnston et al., 1979): for example, glycerol-growing cells respond to glucose addition by immediately delaying the execution of *START* (as evidenced by the transient decrease in the budding index) and by adjusting their size according to the newly available carbon source before entering into S phase (Lorincz & Carter, 1979).

Cells rapidly growing in presence of abundant nutrients have higher ribosome content than cells cultivated on poor media and thus higher biosynthetic capacity (Kief et al., 1981). Since the overall translation rate is the (putative) parameter that cells uses to communicate its size to the cell cycle machine, cells grown in rich media might be expected to achieve the critical rate of protein synthesis required to pass *START* at lower size than cells growing in poor media: actually, the opposite situation is true, since rich and abundant nutrients appear to negatively regulate *START* by increasing the critical size required for the G1/S transition (Jorgensen et al., 2004; Jorgensen & Tyers, 2004).

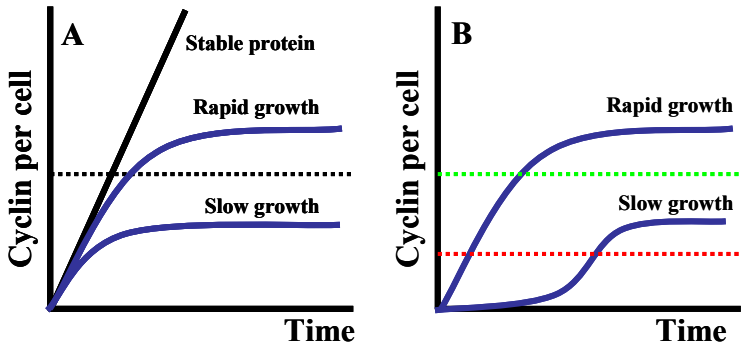


Figure 39. The slow-growth problem.
A) Unstable proteins like cyclins achieve equilibrium levels proportional to their rate of synthesis. Therefore, slowly growing cells can never accumulate the same amount of cyclins as rapidly growing cells. The abundance of a stable protein is compared with the abundance of an unstable protein at two different protein synthesis rates, rapid and slow. The dotted line represents a hypothetical critical threshold amount.
B) The critical threshold of Cln required for START execution varies with the growth rate.
(Adapted from Schneider et al., 2004)

Furthermore, the level of G1 cyclins is strongly affected by growth rate: cells slowly growing on poor media have dramatically lower amount of G1 cyclins than do rapidly growing cells (Fig. 39; Schneider et al., 2004). This enigma is resolved by the fact that the threshold level of G1 cyclins required to execute *START* is significantly lower in slow growing cells than in rapidly growing cells (Schneider et al., 2004).

Several mechanisms have been proposed to explain how nutrients modulate the critical cell size required for the G1/S transition, most of which invoke changes in the abundance of G1 cyclins (Tokiwa et al., 1994; Flick et al., 1994; Polymenis & Schmidt, 1997; Hall et al., 1998).

According to one model, carbon source modulation of the critical size at *START* is achieved, at least in part, via transcriptional repression of *CLN1*: in fact, transcription of this gene is someway delayed and reduced in media containing glucose as carbon source, thus resulting in increased size at *START* (Tokiwa et al., 1994; Flick et al., 1998); accordingly, cells lacking *CLN1* fail to appropriately adjust their size in response to glucose addition (Flick et al., 1998). The glucose-dependent transcriptional repression of *CLN1* seems to be mediated by the cAMP/PKA pathway (Tokiwa et al., 1994; Flick et al., 1998). Although exogenous cAMP

strongly represses transcription of both *CLN1* and *CLN2* (Baroni et al., 1994), the role of Cln2 in the glucose regulation of cell size is apparently negligible (Flick et al., 1998). In addition, Grr1, a F-box protein component of the SCF ubiquitin-ligase complex involved both in the degradation of Cln1 and Cln2 and in glucose signaling (Johnston & Kim, 2005; see elsewhere), is also activated in response of glucose (Barral et al., 1995; Li et al., 1997) and this could potentially reduce further the level of the G1 cyclins (Rupes, 2002). However, it is noteworthy that a more recent study has shown that *CLN1* and *CLN2* mRNA are present at similar levels in cells grown on various carbon sources, whereas the protein levels of the two cyclins dramatically differ among the diverse growth conditions: in particular, the Cln1 and Cln2 amounts are much higher in cells grown on glucose (Schneider et al., 2004). It has been suggested that Cln1 (and Cln2) abundance might regulate the length of the START interval rather than the timing of START activation (Jorgensen et al., 2004; Di Talia et al., 2008).

Many studies assign to Cln3 the prominent role in setting cell size threshold in response to nutrient. Both *CLN3* mRNA and protein levels respond quickly to changes in the nutrients availability and quality. Furthermore, multiple signal transduction pathways that control cell cycle progression converge to regulate Cln3 transcription, translation, stability, and activity.
The level of Cln3 varies dramatically with the available carbon (Hall et al., 1998; Newcomb et al., 2003; Parviz et al., 1998a; Schneider et al., 2004; Hubler et al., 1993) or nitrogen sources (Gallego et al., 1997; Schneider et al., 2004). *CLN3* transcription is repressed under starvation conditions (Gallego et al., 1997).
The *CLN3* promoter contains binding sites for Azt1, a transcription factor that stimulates *CLN3* transcription in the presence of glucose (Newcomb et al., 2002; Parviz et al., 1998b). *CLN3* translation is extremely sensitive to changes in the overall protein synthesis rate, due to the presence of a micro-ORF in the 5'untranslated region of *CLN3* mRNA that strongly reduces translation efficiency at low growth rates, when the cellular ribosome content is low (Polymenis & Schmidt, 1997). The translation efficiency of *CLN3* is further regulated by the activity of the TOR (Barbet et al., 1996) and the cAMP-PKA pathways (Hall et al., 1998), two of the most important circuits involved in nutrient signaling and affecting many metabolic and growth-related functions in yeast.

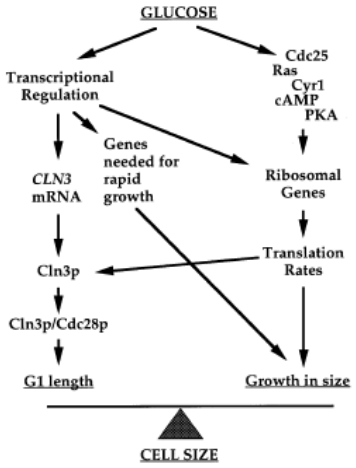


Fig. 40. The Cln3 abundance model
Mechanisms that favour rapid growth in response to glucose are also coupled via *CLN3* to rapid cell cycle progression.
(From Hall et al., 1999).

Therefore, it has been proposed that Cln3 couples the critical cell size required for *START* to nutrient availability by virtue of its translational (and transcriptional) control (Polymenis and Schmidt 1997; Hall et al. 1998). According to this "Cln3 abundance model", rapidly growing cells express high levels of Cln3 in order to accelerate (Fig. 40) cell cycle progression, whereas slowly growing cells spend more time in G1 phase by downregulating Cln3 levels (Hall et al., 1998). Conditions that favor rapid growth (i.e. presence of abundant and good quality nutrient supply) would promote Cln3 translation by increasing the cellular ribosome content: conversely, nutrient shortage or adverse conditions would slow down protein synthesis rate and thus decrease Cln3 amount in the cell. The translational control of Cln3 might also serve to halt the cell division cycle under conditions of low protein synthesis (i.e. nutrient starvation): Cln3p amount sufficient to pass Start would not accumulate when growth conditions are not optimal (Hall et al., 1998; Polymenis & Schmidt, 1997). Furthermore, ectopic expression of *CLN3* can bypass the G1 cell cycle arrest imposed by nutrient limitation (Hadwiger et al., 1989) or by inactivation of the cAMP/PKA pathway: in this latter case, cells proceed through the cell cycle, although the decrease in protein synthesis rate slows down their growth (Hall et al., 1998). The Cln3 abundance model also offer an explanation for the lengthening of G1 phase in daughter cells: as the rate of protein synthesis increases when cells becomes larger, Cln3 levels may be higher in mother cells (Laabs et al., 2003), allowing them to traverse *START* more rapidly than smaller daughter cells (Hall et al., 1998).

Although this model can successfully explains the effects of nutrients on the length of G1 phase, it does not explain why slow growing cells pass *START* at smaller size than do rapidly growing cells (Jorgensen et al., 2004). Furthermore, cells growing in poor nutrients execute *START* not only with reduced mass and translational capacity, but also with extremely low level of Cln3 (Schneider et al., 2004; Hall et al., 1998). Nutrient upshifts delay *START* (Johnston et al. 1977; Lorincz & Carter 1979) despite increases in Cln3 abundance (Hall et al., 1998; Newcomb et al., 2003; Alberghina et al., 2004). In addition, extending G1 phase does not necessarily lead to *START* entry at a smaller cell size: in fact, sublethal doses of cycloheximide increase the critical cell size threshold by reducing translation rate and delaying the G1/S transition (Hartwell & Unger, 1977; Popolo et al., 1982; Jorgensen et al., 2004). The hypothesis asserting that the cumulative effect of Cln3 may be integrated over extended G1 phase, eventually reaching some minimum sufficient to induce *START*, has been proven incorrect (Schneider et al., 2004).

Finally, both *cln3* deficient and *CLN3* overespressing cells can still modulate their threshold critical size in response to nutrients (Jorgensen et al., 2004; deBruin et al., 2004; Costanzo et al., 2004): indeed, nutrient effect on cell size are manifested even in a *cln3 bck2 whi5* triple null strain, which lacks all the upstream regulator of *START* (Jorgensen et al., 2004).

Taken together, these evidences foreshadow the existence of an uncharacterized "cell sizer" mechanism through which nutrients modulate cells size threshold; surprisingly, this mechanism may not depend (or at least, not entirely) on the known regulators of the G1/S transition (Jorgensen et al., 2004; Jorgensen & Tyers, 2004; Cook & Tyers, 2007).

The critical cell size as an emergent property of the G1 to S network

Recently, Barberis and colleagues have developed a new mathematical model which describes the molecular events taking place at the G1/S transition (Fig. 41; Barberis et al., 2007).

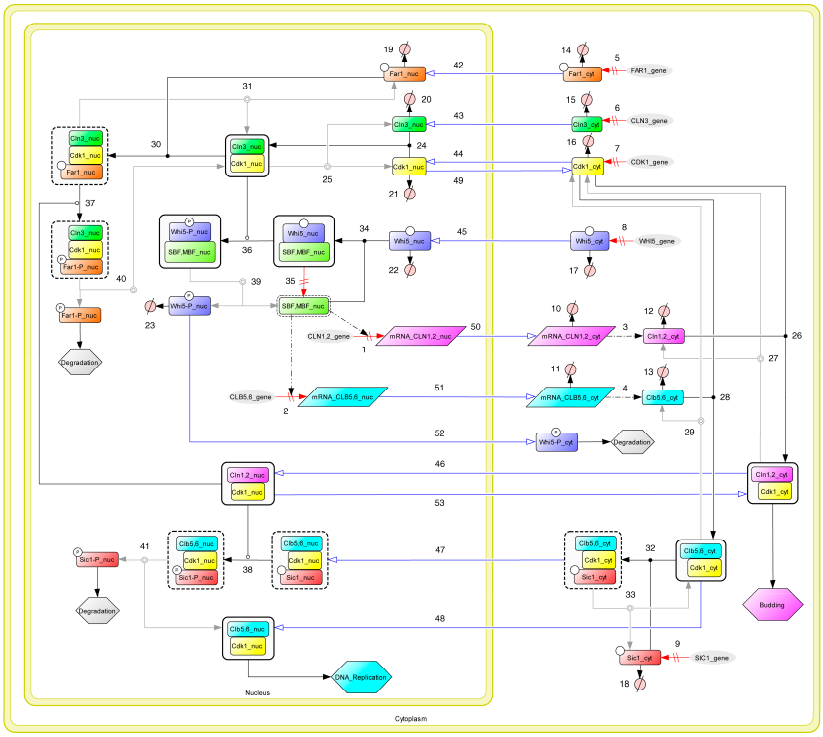


Figure 41. Processes Regulating the G1/S Transition in Yeast Cell Cycle
Model for the G1/S transition according to Barberis et al., 2007. See text for details.

Many diverse experimental evidences have been integrated into a concise mathematical model through a set of ordinary differential equations (Barberis et al., 2007). These equations describe the temporal change of the concentrations of proteins and complexes involved in the G1/S network. The model also considers the localization of these components in different cellular compartments (cytoplasm or nucleus) and cell growth during the G1 phase Barberis et al., 2007).

As a distinguishing feature, the model proposes that two sequential, nutrient modulated thresholds control the entry into S phase (Alberghina et al., 2004; Barberis et al., 2007; Vanoni et al., 2005).

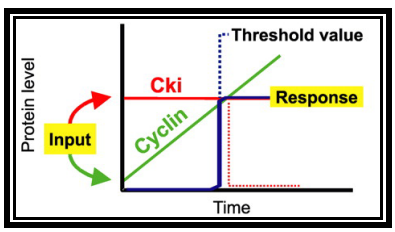


Fig. 42. A molecular threshold.
The number of molecules of the activator increases with growth. The threshold is overcome when the activator exceeds the inhibitor.
(From Alberghina et al., 2004).

Basically, a molecular "threshold" is given by the interplay between an "activator" and an inhibitor blocking its activity: when the number of molecules of the activator exceeds that of the inhibitor, the threshold is overcome (Fig. 42).
The first threshold regulating the G1/S transition involves the G1 cyclin Cln3, the Cdk inhibitor (Cki) Far1 and the cyclin-dependent kinase Cdc28, whereas the second one comprises the S phase cyclin Clb5 (and Clb6), the Cki Sic1 and Cdc28.
Far1 is a well established inhibitor of the Cln_s/Cdc28 complexes activity during the pheromone response (Tyers & Futcher, 1993; Peter et al., 1994; Chang et al., 1993); however, several convincing evidences have suggested that it also may play a relevant role in the mitotic cycle (Alberghina et al., 2004; Fu et al., 2003): in fact, ectopic expression of *FAR1* increases cell size (a phenotype more pronounced in ethanol medium), whereas bud emergences and DNA replication are partially uncoupled and occur at smaller cell size in a *far1* mutant than in the isogenic wild type strain. These observations have led to the proposal that Far1 may cooperate with Cln3 in a nutritionally modulated threshold that controls the cell cycle dynamics of the G1/S transition (Alberghina et al., 2004; Vanoni et al., 2005; Barberis et al., 2007; Alberghina et al., 2009).
According to the model, the Cln3/Far1 threshold is set by the amount of Far1, which is mostly inherited by newborn cells at the end of the previous mitotic cycle and remains roughly constant during the G1 phase. It is assumed that Far1 binds to and inhibits the nuclear Cdk1-Cln3 complexes in early G1 phase, when it is present in substantial excess relative to Cln3 (Alberghina et al., 2004; Vanoni et al., 2005; Barberis et al., 2007). As cells grows during G1 phase, Cln3 accumulates proportionally to cell mass until it exceeds Far1 levels, thus allowing to overcome the first threshold regulating the G1/S transition (Alberghina et al., 2004; Vanoni et al., 2005; Barberis et al., 2007). The overcoming of this threshold is made irreversible by Cln3–Cdc28-primed Far1 degradation (Henchoz et al., 1997; Alberghina et al., 2004). Once free from Far1 inhibition, Cln3/Cdc28 complexes trigger the SBF/MBF transcriptional program by inactivating the Whi5 inhibitor, which abandons the nucleus (Costanzo et al., 2004; deBruin et al., 2004). SBF and MBF drive the expression of ~200 genes, including those encoding the other G1 cyclins, Cln1 and Cln2, and the S-phase cyclins Clb5 and Clb6 (Wittenberg & Reed, 2005). In the cytoplasm, Cln1 and Cln2 bind to Cdc28 and promote the biochemical steps that result in bud emergence (Dirick et al., 1995; Stuart & Wittenberg, 1995; Skotheim et al., 2008). The newly formed Cdc28/Clb5,6 complexes are maintained inactive by their interaction with the Sic1 inhibitor, whose levels set the second threshold (Schwob et al., 1994; Alberghina et al., 2004; Rossi et al., 2005). Apparently, Sic1 also facilitates the nuclear accumulation of its cognate Cdc28/Clb5,6 complexes (Rossi et al., 2005). The irreversible overcoming of the second threshold requires the elimination of Sic1: the growing pool of Cdc28/Cln1,2 phosphorylates the inhibitor on multiple site, promoting its degradation via an ubiquitin mediated mechanism, (Nash et al., 2001).The now active Cdc28/Clb5,6 complexes drive the entry into S phase by promoting the onset of DNA replication (Verma et al., 1997; Deshalis & Ferrel, 2001; Toone et al., 1997).
In the model (Barberis et al., 2007), nutrient-dependent control of the G1 to S transition is distributed over the two described sequential thresholds, each one able to integrate cell signaling information coming from external and internal conditions (Alberghina et al., 2004; Rossi et al., 2005; Vanoni et al., 2005). When one or both components of each threshold are inactivated, cells largely retain their ability to

modulate their size according to carbon source availability. In contrast, concurrent loss of either *CLN3* or *FAR1* (first threshold) and *SIC1* (second threshold), abolishes glucose modulation of cell size (Alberghina et al., 2004). Consistently, nutrient status (and in particular the quantity and quality of available carbon source) influences the components of the two thresholds at the level protein abundance and sub-cellular localization. For instance, Cln3 and Far1 levels are higher in cells growing on glucose than in cell cultivated on ethanol (Hall et al., 1998; Alberghina et al., 2004). On the contrary, Sic1 content is higher in ethanol growing cells; the sub-cellular localization of Sic1 is also carbon source-modulated: the inhibitor is mostly nuclear in cells grown in glucose- media, whereas a sizeable amount of Sic1 is detected also in the cytoplasm during growth on ethanol (Rossi et al., 2005).
A simulation analysis performed with the model described above has provided a novel, intriguing conclusion: the critical cell size required for entry into S phase (as defined by the parameter Ps, the protein content at the onset of DNA replication) is an emergent property of the G1/S network and is strongly influenced by growth rate (Barberis et al. 2007; Alberghina et al., 2009). In other words, Ps is a property that individual components of the G1/S network do not possess but that emerges from their interaction.
The setting of the critical cells size (Ps) is carried out by a mechanism consisting of a "sizer "plus a "timer" (Fig. 43).

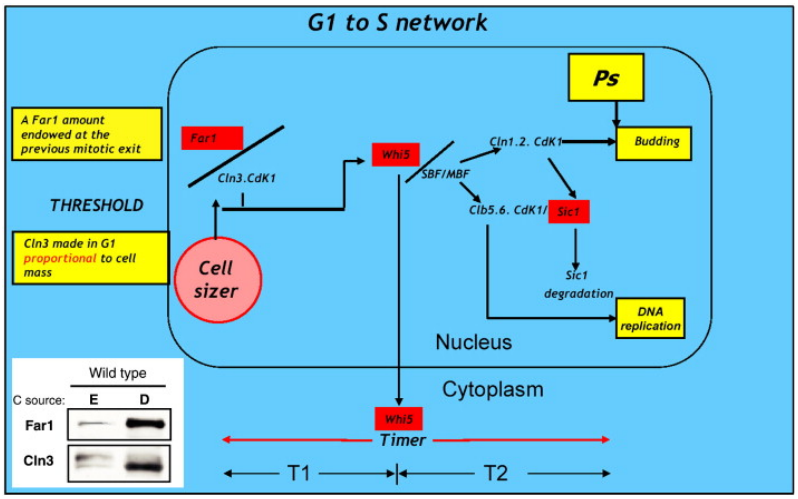


Figure 43. The "sizer + timer" mechanism underlying the G1/ S transition in S. cerevisiae
The sizer + timer mechanism is given by three couples of interactors, each comprising an inhibitor (Far1, Whi5 and Sic1, shown in red boxes). The sizer is given by the overcoming of the inhibitory effect of Far1 by the growing pool of Cln3, synthesized during G1 proportionally to cell mass. The duration of the timer (divided in two intervals, T1 and T2, according to Whi5 localization) is given by the time required to accomplish all the biochemical events from the overcoming of the sizer (first threshold) to the onset of DNA replication and budding (second threshold). The parameter Ps defines the "critical size" that the cell has achieved at the entry into S phase. In the inset: the relative amounts of Far1 and Cln3 in cells growing on ethanol (E) and glucose (D).
(From: Alberghina et al., 2009).

The Far1/Cln3 threshold acts essentially as a growth-sensitive sizer, which is activated at similar cell size both in cells growing in rich or poor media: in fact, the Cln3/Far1 ratio remains almost equimolecular in the various growth conditions, since both Cln3 and Far1 levels increase or decrease accordingly to the growth rate

(Hall et al., 1998; Alberghina et al., 2004; Alberghina et al., 2009; Barberis et al., 2007). The first Cln3/Far1 threshold and the second one involving Clb5,6 and Sic1 are temporally spaced ("timer") (Barberis et al., 2007): therefore, the actual value of Ps depends not only on the Cln3/Far1"sizer", but also on the length of the "timer", which is the period elapsing between the passage through the first threshold and the overcoming of the second one (Barberis et al., 2007).
The growth rate (which depends on nutrient availability and quality) is a major factor in determining the critical size required for budding and DNA replication (Ps): in fact, since it is the overcoming of the second threshold that actually sets Ps, its value will be much higher in fast growing cells (Fig. 44).

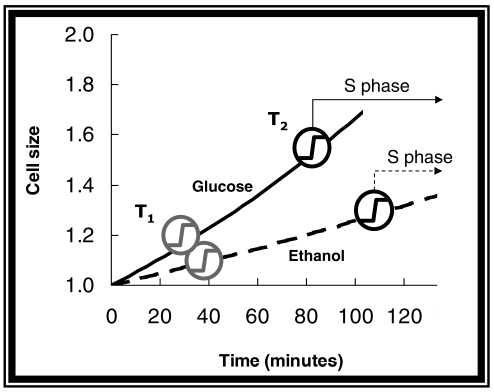


Figure 44. The G1/S transition and the setting of critical size (Ps) in yeast.
Simulation of the G1/S transition in elutriated cells according to the model by Barberis et al., 2007. Cells grow with different kinetics in glucose (solid line) and ethanol (dotted line) media. Overcoming of the first (Cln3/Far1) and second (Clb5,6/Sic1) threshold is shown by circled symbols (gray, first threshold; black, second threshold). T1 and T2 represent the threshold-overcome times. Cells overcome the first Cln3/Far1 threshold ("sizer") at similar size in ethanol and glucose media. However, since the two thresholds are temporally spaced and it is the overcoming of the second threshold that actually sets the Ps ("timer"), glucose growing cells will be larger at the onset of budding and DNA replication as a consequence of their faster growth rate.
(From: Barberis et al., 2007)

In other models of the yeast cell cycle previously proposed, the G1/S transition is controlled by a single event: a cell sizer is operative only at low-growth rates, whereas an oscillator mechanism is active at fast-growth rates (Chen et al., 2004; Csikasz-Nagy et al., 2006). In the model presented by Barberis and colleagues, a sizer mechanism is operative at all growth rates, and the presence of two distinct, temporally spaced, thresholds cooperating to set the critical size (Ps) introduces a delay that is sensitive to the growth rate (Barberis et al., 2007).
The existence of a "sizer plus timer mechanism" regulating the G1/S transition has been confirmed in a work by Di Talia and colleagues (Di Talia et al., 2007), as discussed in previous sections. In this study, several of the predictions offered by the mathematical model have been experimentally verified, thus further supporting the soundness of the model by Barberis and colleagues (Alberghina et al., 2009)

The "sizer plus timer mechanism" also offers a convincing explanation of the fact that parent cells increase their size with their genealogical age (Fig. 45) (Johnston et al., 1979; Porro et al., 2009; Alberghina et al., 2009): again, the prevision offered by the model fit with the experimental evidences (Alberghina et al., 2009).

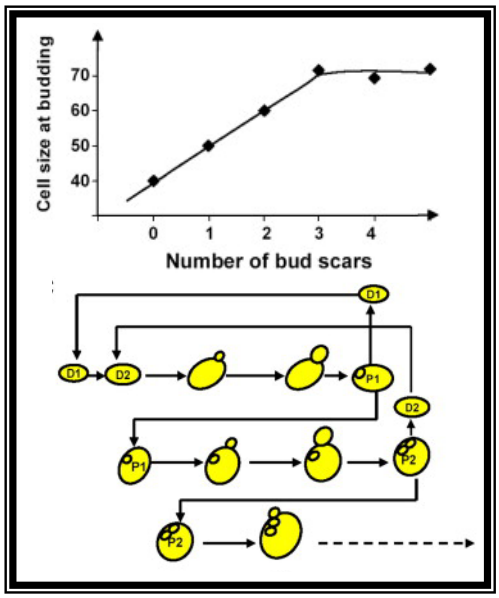


Figure 45. Increase of size in parent and daughter cells at the increase of genealogical age.
(From Alberghina et al., 2009)

In conclusion, the model of the G1/S network here discussed demonstrates the validity of a systems biology approach to understand complex biological processes (Alberghina et al., 2009; Barberis et al., 2007).

Ribosome biogenesis, growth and cell size threshold

Cell growth requires the synthesis of proteins, the synthesis of proteins requires ribosomes. Thus, the control of growth potential must somehow involve the control of ribosome synthesis (Rudra & Warner, 2004).

The budding yeast ribosome consists of 79 ribosomal proteins, encoded by 138 genes (RP regulon), and four rRNAs (5S, 5.8S, 18S, and 25S) encoded by 150 rDNA repeats existing as a tandem array in the genome. The vast majority of genes in the *RP* regulon have promoter-binding sites for Rap1, whereas a few have sites for Abf1 (Zaman et al., 2008; Warner et al., 2001).

Another 236 genes (Ribi regulon) encodes proteins involved in ribosome assembly (which takes place in the nucleolus) and activity (RNA polymerases I and III, tRNA synthetases, rRNA processing and modifying enzymes, translation factors). The promoters of these genes contain two motifs, termed RRPE and PAC. (Zaman et al., 2008)

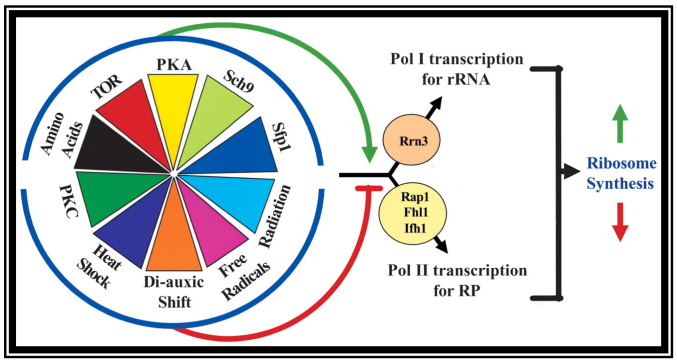


Figure 46. Regulation of ribosome biogenesis in yeast.
Ribosome biosynthesis requires the cooperation of several elements: active TOR and PKA pathways, Sch9, sufficient nutrients (carbon and nitrogen source, aminoacids), the factors Rap1, Flh1, Ifh1, Sfp1 and Rrn3 (for RNA). Repression of ribosome biogenesis can occur in response to inactivation of the TOR pathway, reduction of PKA activity, signalling through PKC, nutrient exhaustion and numerous stress conditions (heat-shock, free radicals, radiation).
(From Rudra & Warner, 2004)

To maintain robust growth in response to favourable conditions, yeast cells synthesize about 2000 ribosomes per minute (Warner, 2001). Ribosome biogenesis is the predominant biosynthetic activity in yeast cells and is extremely expensive in energetic terms; it is estimated that during exponential growth synthesis of the translation machinery accounts for 50% of total transcription and utilizes about 90% of the cellular energetic resources (Warner et al., 2001). Therefore, it is not surprisingly that yeast cells carefully adjust their ribosome biogenesis rate in response to changes in nutrient availability (Zaman et al., 2008). Two key nutrient-sensing circuits, the cAMP/PKA and TOR signaling pathways, regulate the transcription of rRNA, *RP*, and *Ribi* genes (Fig. 46-47; Jorgensen & Tyers, 2004; Wullschleger et al., 2006; Klein & Struhl, 1994; Neuman-Silberberg et al., 1995; Cardenas et al., 1999; Hardwick et al., 1999; Powers & Walter 1999; Zaman et al., 2009; Wang et al., 2004; Zaman et al., 2008).

The control of rRNA synthesis depends heavily on phosphorylation of the critical initiation factor TIF-1A/Rrn3 (Grummt, 2003). Both Rap1-binding sites (RP

regulon) and RRPE elements (Ribi regulon) render gene transcription sensitive to Ras/PKA signaling (Klein and Struhl 1994; Neuman-Silberberg et al. 1995; Wang et al. 2004). The effects of the TOR pathway on regulation of ribosome biogenesis are (at least partially) mediated by the Sch9 kinase (Jorgensen et al., 2004; Urban et al., 2007). Sch9 is specifically required for maximal expression of the RP regulon and is regulated in a nutrient-sensitive fashion by both phosphorylation and localization to the vacuolar membrane (Jorgensen et al. 2004). The abundance of Sch9 is also regulated by TOR activity (Jorgensen et al., 2004): under steady-state proliferation on different carbon sources, Sch9 levels correlate with growth rate, RP/Ribi transcription, and cell size (Jorgensen et al., 2004).

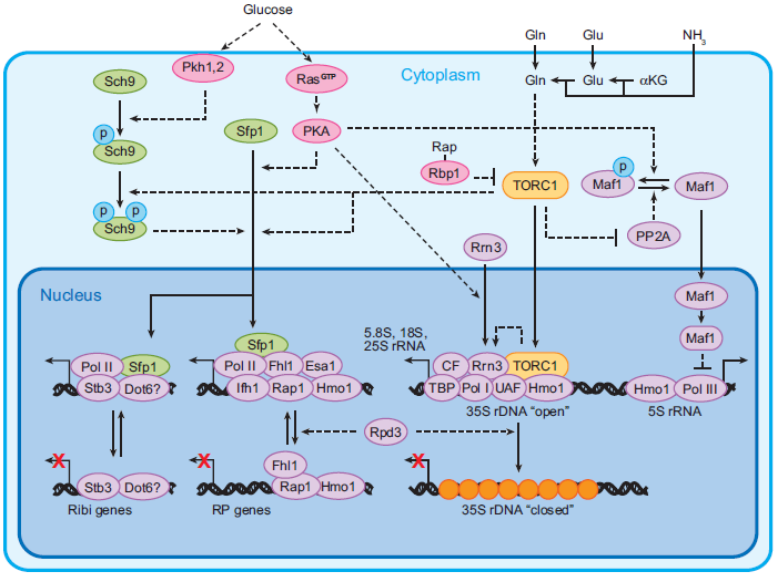


Figure 47. Nutritional control of ribosome biogenesis.
Nutrient availability regulates expression of all components of the ribosome, including the RNA polymerase II-dependent ribosomal protein (RP) and ribosomal biogenesis (Ribi) genes, the RNA polymerase I-dependent ribosomal RNA genes and the RNA polymerase III-dependent 5S RNA genes. The major participants in this regulation and their likely interactions are indicated. (From Zaman et al., 2008)

A key role for the transcription factor Sfp1 has also been documented (Jorgensen et al., 2004; Jorgensen et al., 2002). Sfp1 act as a "master regulator" controlling a large cohort of >200 genes of the Ribi regulon and (directly or indirectly) also activates the RP regulon transcription (Jorgensen et al. 2002; Fingerman et al., 2003; Jorgensen et al., 2004). The subcellular localization of Sfp1 is highly responsive to nutrient conditions: in glucose medium, Sfp1 resides in the nucleus, but upon nutrient starvation or exposure to stress, Sfp1 rapidly relocalizes to the cytoplasm (Jorgensen et al. 2004; Marion et al. 2004). Nutrient-responsive localization of Sfp1 is modulated by both TOR and cAMP/PKA pathway, although the molecular details of this regulation is not entirely known. Recent findings have shown that Mrs6, an essential Rab escort protein, regulates both Sfp1 nuclear localization and Tor activity: these results foreshadow an intriguing interplay among intracellular vesicular trafficking, Tor signaling and ribosome biogenesis in the control of cell growth (Singh & Tyers, 2009; Lempiainen et al., 2009). In addition, Sfp1 dictates

the nuclear localization of Fhl1 and Ifh1, two transcription factors implicated in RP gene expression (see also TOR section; Martin et al. 2004; Schawalder et al. 2004; Wade et al. 2004; Rudra et al. 2005; Jorgensen et al., 2004): nutrient starvation or loss of *SFP1* forces Fhl1 and Ifh1 to localize to nucleolus, concomitant with reduced RP gene transcription (Jorgensen et al., 2004). Recent evidences have demonstrated that Sfp1 is a direct substrate of the TORC1 complex, which regulates Sfp1 function via phosphorylation at multiple residues (Lempiainen et al., 2009). Sfp1, in turn, negatively regulates TORC1 phosphorylation of Sch9, the other key target of Tor in ribosome biogenesis, revealing a feedback mechanism that regulates RP and Ribi gene transcription (Lempiainen et al., 2009).

A genome-wide analysis of size control in yeast has revealed surprising connections between ribosome biogenesis and cell size (Jorgensen et al., 2004; Jorgensen et al., 2002). Mutations that accelerate cell division relative to cell growth result in a small cell size, referred to as *whi* phenotype. The systematic screens has shown that most of the mutations reducing cell size affect genes involved in respiration or ribosome biosynthesis. Notably, two of the smallest strains identified lack either the Sch9 kinase or the transcription factor Sfp1 (Jorgensen et al., 2002; Jorgensen et al., 2004); the inactivation of these genes also results in reduced expression of RP and Ribi genes and in a slow-growth phenotype, but the effects on size (~40% of wild-type strain volume) are disproportionate relative to the changes in doubling time, indicating that growth and cell division are partially uncoupled in these strains (Fig. 48: Jorgensen et al., 2002). In contrast, ectopic expression of either *SFP1* or *SCH9* leads to large cells (Jorgensen et al., 2004).

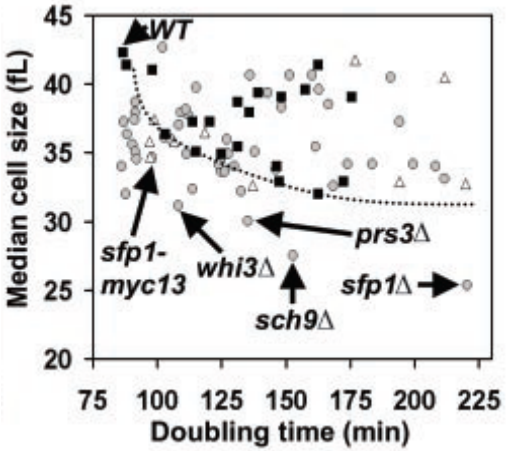


Fig. 48. Uncoupling of growth and cell division in sch9 and sfp1 strains
A plot of doubling time versus cell size for two wild type strains and several ribosomal gene deletion strains (solid squares) was used to establish a baseline correlation between doubling time and small cell size (dotted line). Another 61 *whi* strains (gray circles) are plotted, including 10 *whi* strains with deletions in genes whose products form part of a nucleolar network (open triangles). Strains that partially uncouple growth from division (i.e. *sch9* and *sfp1*) fall on or below the baseline fitted to the smallest ribosomal gene deletion strains. (From Jorgensen et al., 2002)

Consistent with a possible role of Sch9 and Sfp1 as negative regulators of *START*, the activation of the G1/S transcriptional program and the progress into S phase are accelerated in both *sfp1* and *sch9* null strains: this result suggest that Sch9 and Sfp1

may act upstream of the G1/S transcriptional network (Rudra & Warner, 2004; Jorgensen et al., 2004).
Even more interesting, both *sfp1* and *sch9* null strains are largely defective in carbon source modulation of cell size threshold, in contrast to a *cln3 bck2 whi5* mutant, which is fully responsive to carbon source despite the loss of all the known regulator of START (Jorgensen et al., 2004). Introduction of the *sfp1* mutation into a *cln3 bck2 whi5* background reduces cells size, but not as much as the single sfp1 mutant: since by criterion of cell size *sfp1* is not fully epistatic neither to the triple *cln3 bck2 whi5* null strain nor to any of the single mutant, Whi5, Cln3, and Bck2 still play a role in sfp1 null cells. Apparently, nutrients operate through Sfp1 and Sch9 to match the critical size threshold required for *START* to the rate of ribosome biogenesis: surprisingly, this mechanism seems to be largely independent of known upstream regulators of the G1/S transition (Jorgensen et al., 2004).
Tyers and colleagues (Jorgensen et al., 2004; Jorgensen & Tyers, 2004; Cook & Tyers, 2004) have proposed that the cAMP/PKA pathway, the TOR pathway, Sch9 and Sfp1 function in a nonlinear network that dictates both the critical cell size threshold and expression of the Ribi and RP regulons according to nutrients availability and stresses (Fig 49; Jorgensen et al., 2004; Jorgensen & Tyers, 2004; Cook & Tyers, 2007). Alterations in any component of this quartet has a profound impact on cell size: reduced activity of the cAMP/PKA pathway or loss of Sfp1 and Sch9 renders the cells small and impervious to carbon source regulation of their size (Baroni et al., 1989; Jorgensen et al., 2004; Tokiwa et al., 1994; Belotti et al., 2006). On the other hand, overproduction of Sfp1 and Sch9 or constitutive activation of the cAMP circuit results in large cells (Jorgensen et al., 2004; Baroni et al., 1989; Baroni et al., 1992). Like cAMP/PKA and TOR networks, Sfp1 and Sch9 are sensitive to nutrient status and to stresses (at the level of localization and abundance, respectively (Jorgensen et al., 2004; Marion et al., 2004)). Interestingly, as noted above, cAMP/PKA, Sfp1, and Sch9 all converge on ribosome biogenesis by regulating the transcription of the RP and Ribi regulons in response to nutrient (and stress) signals (Jorgensen et al., 2002; Jorgensen et al., 2004; Neuman-Silberberg et al., 1995; Wang et al., 2004; Fingerman et al., 2003; Marion et al., 2004). In addition, strains deleted for other genes implicated in ribosome synthesis are similarly (although less dramatically) uncoupled for growth and division (Jorgensen et al. 2002).
All these evidences have been unified in a model where the rate of ribosome biogenesis, which is proportional to nutrient quality and abundance, negatively regulates *START* execution, thereby linking nutrient status to the setting of the critical cell size: the current rate of ribosome biogenesis would modulate the critical cell size according to nutrients availability, whereas the overall translation rate (which depends on the current cellular ribosome content and nutrient status) would steadily report cell size to the cell division machinery (Fig. 49; Jorgensen et al., 2004; Jorgensen & Tyers, 2004; Cook & Tyers, 2007).
Ribosome biogenesis is optimally placed in the cellular network to integrate both upstream nutrient (stress) signaling pathways and feedback signals from downstream events (Cook & Tyers, 2007).
Accordingly, the rate of ribosome biogenesis parallels nutrient effects: under nutrient shortage, ribosome biogenesis rate is low and cells are small, whereas in presence of abundant and good quality nutrient supply ribosome biogenesis rate is high and cells are large (Jorgensen et al., 2004; Cook & Tyers, 2007). Moreover,

just like the critical size itself (Johnston et al., 1977), the rate of ribosome biogenesis rapidly and dynamically adapts to changes in nutrient status (Kief & Warner, 1981). By coupling the size threshold directly to ribosome biogenesis the yeast cell may anticipate future changes in its protein synthesis rate (triggered by fluctuations in nutrients availability or stresses) and thus promptly adjust its size long before these changes actually occur (Jorgensen et al., 2002; Tyers et al., 2007; Jorgensen et al., 2004; Jorgensen & Tyers, 2004).

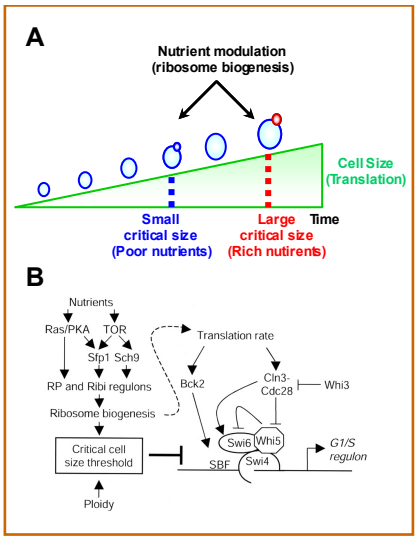


Figure 49. Oversimplified representation of the major pathways regulating growth and division at the G1/S phase transition in yeast.
A) The mechanism that sets the critical cell size required for entry into S phase in response to nutrient status (possibly ribosome biogenesis rate) appears to be distinct from the mechanism that actually determines cell size (possibly the translation of an unstable sizer). Size increases over time as cells accumulate biosynthetic capacity, whereas the critical size is determined by the current nutrient status and ploidy.
B) Cell cycle commitment (*START*) occurs in late G1 phase and is dependent on achieving a critical cell size. This size threshold correlates closely with the rate of ribosome biogenesis and is higher in rich nutrients. Nutrient signals are relayed through the TOR, PKA and Sch9 kinases, which show complex inter-dependencies. The rate of ribosome biogenesis is dictated by various mechanisms, including control of nuclear localization of Sfp1, a master regulator of ribosome biogenesis (Ribi) factor and ribosomal protein (RP) genes. Once critical cell size is achieved, Cln3-Cdc28 complexes phosphorylate and inactivate the transcriptional repressor Whi5, thereby enabling G1/S transcription mediated by SBF, expression of the G1 cyclins Cln1 and Cln2, degradation of the B-type cyclin inhibitor Sic1 and onset of DNA replication. Inhibition of translation induces transcription of the Ribi regulon and delays START execution, suggesting the existence of a sensor that detects appropriate translational rate under given nutrient status. (Adapted from Jorgensen & Tyers, 2004).

Under favorable growth conditions, the cell needs vigorous ribosome biosynthesis to enable rapid growth and at the same time is interested in delaying cell cycle entry in order to grow to an optimal size: according to the proposed model (Fig. 49), the PKA and the TOR pathway would relay nutrient (and/or stress) signals to Sfp1 and Sch9, thus promoting the transcription of RP and Ribi regulons and a resulting delay in Start execution through an unknown mechanism. Then, when environmental conditions deteriorate, as a consequence of stress or nutrient shortage, cells needs more resources to respond to the hostile situation: under these circumstances, Sfp1 rapidly abandons the nucleus and Sch9 abundance/localization are altered, ribosome synthesis slow down and the cell size threshold can be consequently reset to a lower value (accordingly) (Rudra & Warner, 2004; Cook & Tyers, 2007).
The mechanisms connecting ribosome biogenesis to *START* execution via Sfp1 and Sch9 are still unknown. As discussed above, it has been hypothesized that these effects may at least partially independent of Cln3 and Whi5, since the critical size threshold can be reset also in strains lacking these upstream regulators of the G1/S transition (Jorgensen et al., 2004). However, the issue is extremely complex and interpretation of experimental evidence is often far from straightforward.
An interesting hypothesis is that the cell cycle machinery and the ribosome biosynthetic apparatus might have something in common to compete for. Aldea and colleagues have proposed that chaperones availability might be the "missing link" between ribosome biogenesis and regulation of the critical size threshold required for cell cycle entry (Aldea et al., 2007; Verges et al., 2007). As already discussed,

their study demonstrated that the accumulation of a critical amount of the Ydj1 chaperon is crucial for the release of Cln3 from the ER and cell cycle entry (Verges et al., 2007). Ydj1 has an essential role in regulating the functions of Hsp70 chaperones and in protein translocation to the ER lumen (Caplan et al., 1992); however, an integrative analysis of high-throughput data has also predicted Ydj1 as being involved in translation and ribosome biogenesis (Kemmeren et al., 2005).
According to the proposed model, in rapidly growing cells high ribosome and protein synthesis rates might reduce the availability of Ydj1 for the ER-release of Cln3, as an inevitable consequence, lead to a delay in cell cycle entry. A simple corollary derived from this hypothesis is that the critical threshold size at Start would be proportional to the growth rate: the faster the cell grows, the larger it must grow to accumulate enough chaperone amounts to overcome constant growth demands and release Cln3 from the ER to trigger *START* (Aldea et al., 2007; Verges et al., 2007).
Although interesting, this model fails to explain how a strain lacking *CLN3* can still adjust its size in response to nutrient availability (Jorgensen et al., 2004; deBruin et al., 2004; Costanzo et al., 2004).
Consistent with the model described by Tyers and colleagues, recent studies evaluating the effect of deficiencies in ribosome biosynthesis on cell cycle progression have confirmed that changes in the rate of ribosome biogenesis can affect execution of Start long before any alterations in overall protein synthesis rates occur (Bernstein et. al., 2007). However, the effects on size observed in this case are opposite from those predicted by the model: in fact, upon depletion of an essential ribosomal protein, biogenesis rate slow down, the passage through *START* is inhibited through a Whi5 dependent mechanism and cell size increase instead of decreasing (Bernstein et al., 2007).
Thus, ribosome biogenesis seems to have multiple effects on cell cycle progression: it may delay Start by increasing the cell size threshold under favorable growth condition and independently promote START by inactivating Whi5 (Bernstein et al., 2007).

The Yeast Metabolic Cycle (YMC) and the control of cell division cycle: the "compartment hypothesis" vs. the "finishing kick to START"

Several evidences suggest a further layer of regulation of *START* as a function of carbon source availability (Futcher, 2006). During growth in glucose-rich medium, *S. cerevisiae* cells preferentially ferment glucose to support rapid growth; in contrast, when grown in continuous cultures under nutrient-limited conditions, yeast cells exhibit a robust, highly periodic metabolic cycle (YMC, yeast metabolic cycle) for energy generation by rhythmically alternating a reductive phase (glycolysis) and an oxidative phase (respiration) (Fig. 50; Tu & Mcknight, 2007; Klevecz et al., 2004; Tu et al., 2005; Chen et al., 2007; Muller et al., 2003). These metabolic oscillations (which are primarily characterized by oscillations in the redox potential of the cell) are associated with periodic changes in cellular the content of storage carbohydrates such as trehalose and glycogen (Futcher, 2006). As already discussed, yeast cells growing oxidatively on low concentrations of glucose or on non-fermentable carbon sources accumulate large internal stores of glucose as glycogen and trehalose during the long G1 phase: about 15% of the dry weight of a respiring cell is given by storage carbohydrates, whereas a cell growing via fermentation on abundant glucose contains virtually no glycogen or trehalose (Francois & Parrou,

2001; Guillou et al., 2004; Futcher, 2006). However, in late G1 phase carbohydrates storage ceases (possibly in response to a spike in the cAMP level (Muller et al., 2003)) and the reserve stores of glycogen and trehalose are rapidly metabolized (at least partially) via glycolysis (Futcher, 2006; Muller et al., 2003): shortly afterwards, cells commit to a new round of mitotic division, execute the *START* transcriptional program and progress into S phase; later on, the stores of carbohydrates become exhausted, the burst of fermentation ceases and cells reprise storing glycogen and trehalose for the next round of cell division (Futcher, 2006; Fig. 50).

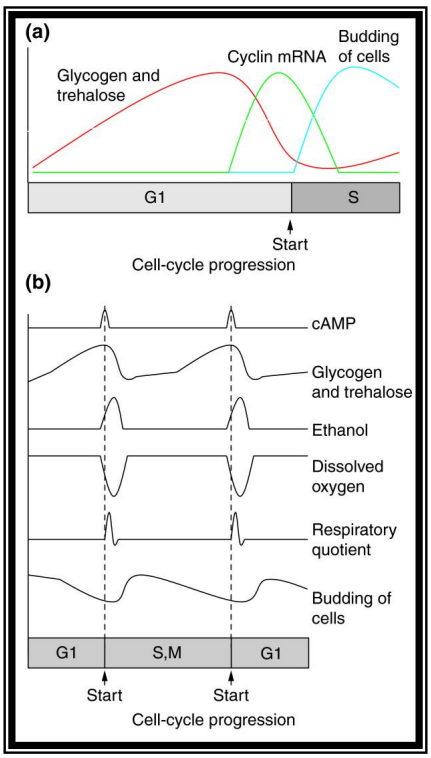


Figure 50.
The metabolic cycle in slowly growing yeast cells.
(a) The cycle of storage carbohydrates.
In slowly growing cells, glycogen and trehalose stores build up during G1, then are suddenly metabolized in late G1. Shortly after, the mRNA levels of the G1 cyclins Cln1 and Cln2 reach a peak, START is executed and budding and DNA synthesis occur as cell progress into S phase.
(b) The metabolic cycle of spontaneously synchronized cells growing in limiting glucose.
The cyclic oscillations in the levels of various indicators of metabolism are shown. A small increase of cAMP is observed in late G1 phase. Immediately afterwards, glycogen and trehalose are metabolized and ethanol appears in the medium, presumably produced by fermentation of the freed glucose. The level of oxygen dissolved in the medium drops at the same time that stored carbohydrate is being consumed, suggesting that glucose from stored carbohydrates is metabolized by respiration as well as by fermentation. The respiratory quotient rises from below 1 to about 1.2, indicating a shift from pure respiration (which would give a respiratory quotient of 1.0) to a partially fermentative metabolism. Budding occurs shortly afterwards. (From Futcher, 2006).

According to the "compartment hypothesis", the yeast metabolic cycle may serve to temporally separate biochemically incompatible processes (Tu et al, 2005 Klevecz et al., 2004; Chen et al., 2007; Tu & Mcknight, 2007;). Consistently, the YMC is accompanied by a highly organized transcriptional cycle: in fact, recent microarray studies have revealed that over half of the entire yeast genome is expressed periodically as a function of these metabolic oscillations, thereby defining an extensively orchestrated program responsible for regulating numerous cellular processes in a manner reminiscent of the circadian rhythms (Klevecz et al., 2004; Tu et al., 2005; Tu & Mcknight, 2007; Reinke & Gattfield, 2006). Not surprisingly, genes encoding proteins associated with energy production, metabolism, and protein synthesis tend to be expressed with exceptionally robust periodicity (Klevecz et al., 2004; Tu et al., 2005;). Essential cellular and metabolic events occur in synchrony with the metabolic cycle, indicating that in a simple eukaryotic microorganism like *S. cerevisiae* (potentially incompatible) key processes are compartmentalized in time (Tu et al., 2005; Klevecz et al., 2004; Chen et al., 2007). In particular, the metabolic

oscillations are apparently superimposed on the cell division cycle, which is tightly constrained to the reductive phase of the yeast metabolic cycle, when oxygen consumption is minimal (Tu et al., 2005; Klevecz et al., 2004; Chen et al., 2007): restriction of DNA replication to the glycolytic phase of the metabolic cycle may be a mechanism to protect genome integrity from damage by the reactive oxygen species (ROS) produced during periods of intense respiration (Tu et al., 2005; Klevecz et al., 2004; Chen et al., 2007). Therefore, according to this "compartment hypothesis", the burst of fermentation during late G1 phase in otherwise respiring cells may serve to temporally segregate DNA synthesis from respiration, in order to minimize the risks of oxidative damage (Tu et al., 2005; Klevecz et al., 2004; Chen et al., 2007). Consistent with this hypothesis, cell cycle mutants in which DNA replication is no more restricted to the reductive phase of the YMC exhibit substantial increases in spontaneous mutation rate (Chen et al., 2007).

An alternative (but not necessarily exclusive) view is the 'finishing kick' hypothesis, which focuses on the requirement of a minimal rate of protein synthesis for START execution and proposes that the YMC may be important to promote the G1/S transition under unfavourable growth conditions (Futcher, 2006). As already discussed, progress through START depends on the three G1 cyclins, Cln1, Cln2 and Cln3, which bind to the cyclin-dependent kinase Cdc28 and activate the G1/S transcriptional program that drives the entry into S phase (Wittenberg & Reed, 2005). The G1 cyclins are extremely unstable proteins: at low protein synthesis rate, cells do not pass through START, since the G1 cyclins cannot accumulate to the critical level required for cell cycle entry as a consequence of their rapid turn over (Schneider et al., 2004). The "finishing-kick hypothesis" proposes that the quick turnover of the G1 cyclins can only be overcome by increasing the rate of protein synthesis: therefore, slowly growing cells would steadily accumulate storage carbohydrates, until in late G1 sufficient reserves would be available to allow a short burst of fermentation, which would increase the translational capacity (as further confirmed by late-G1 peak in expression of ribosome and protein synthesis genes (Tu et al., 2005; Klevecz et al., 2004)) and promote START execution by providing enough G1 cyclins and other raw materials required for the following S phase (such as glucans for the wall of the emerging bud and deoxynucleotides for DNA synthesis). According to Futcher, this would be the "finishing kick to START and, exactly like the finishing kick of an Olympic 10,000-meter runner, it involves a lot of glycolysis".

Although the instability of G1 cyclins provides the mechanism by which the entry into S phase is delayed, the accumulation of enough cyclins is not the whole point of the metabolic burst: rather, the unstable G1 cyclins would act as a "gating device" that would limit *START* to times when storage carbohydrates, raw materials and protein synthesis rates are sufficiently high to complete of a new round of mitotic division (Futcher, 2006).

This model may explain why mutants with a hyperactive cAMP/PKA pathway, which do not accumulate storage carbohydrates, cannot grow on nonfermentable carbon sources (Thevelein & deWinde, 1999).

Furthermore, the finishing kick hypothesis also proposes a molecular basis for the critical size required for the entry into S phase (Futcher, 2006). According to the model proposed by Futcher, in slowly growing yeast cells the size-related parameter being measured by the cell would be the amount of glycogen and trehalose. Therefore, the critical size would be equivalent to the level of stored carbohydrates:

when (and only when) enough reserve of glycogen and trehalose are available for successful progress into the energy- and material-consuming S phase of the cell cycle, a signal would be generated (possibly via some glucose related metabolite like glucose 6-phosphate and the cAMP/PKA pathway), the carbohydrates would be metabolized via glycolysis, and a rapid burst of metabolism, energy, nucleotide and protein synthesis would ensue, thus allowing the G1/S transition (Futcher, 2006). The late-G1 wave of expression of genes involved in ribosome biogenesis and protein synthesis would reflect the need for an increase in translational capacity, whereas the oscillations in other metabolites and gene clusters would be explained by downstream effects of the metabolic burst and of the oscillation in stored carbohydrates levels (Futcher, 2006).
The finishing-kick hypothesis offers an explanation for the critical size required for *START* in slowly growing cells, but apparently the model is not applicable to cells rapidly growing on abundant glucose (Futcher, 2006): in fact, these cells do not accumulate glycogen or trehalose and do not need a metabolic burst for the passage through START. However, several evidences suggest that yeast cells may employ multiple mechanisms for controlling the timing of START execution (Schneider et al., 2004) and the mechanisms used by fast-growing cells may be rather different from those of slow-growing cells (Laabs et al., 2003).

In sum, nutrient-limited yeast cells exhibit a metabolic oscillation superimposed on their cell-cycle oscillation. Presently, it is still unclear whether this metabolic cycle is primarily important for temporal compartmentalization of incompatible cellular processes (Tu et al., 2005; Klevecz et al., 2004), or for execution of *START* under difficult circumstances (Futcher, 2006), or whether both possibilities are true. Future studies of mutants that do not accumulate storage carbohydrates (Guillou et al., 2004; Sillje et al., 1999), which are viable, but with aberrant cell cycles, may be useful for distinguishing between these two hypotheses

Nutrient sensing and control of cell cycle progression: the role of PKA
Yeast cells grow and proliferate rapidly when nutrients are abundant and arrest cell cycle progression under starvation conditions. The nutritional status regulates cell cycle progression at START through the signaling pathways described in the previous sections, in particular the cAMP/PKA cascade and the TOR network; however, the critical relationships between the nutrients sensing pathways and the complex machinery governing cell growth and proliferation have often resisted elucidation.
Inactivation of the cAMP/PKA pathway causes a first cycle arrest in G1 phase even in the presence of abundant nutrients (Thevelein & deWinde, 1999; Thevelein, 1994), thus suggesting that signaling through the PKA circuit is an essential requisite for START execution (Zaman et al., 1998). The terminal phenotype of cells arrested as a consequence of loss of PKA activity closely resembles that of nutrient starved, stationary phase cells (Thevelein & deWinde, 1999; Thevelein, 1994).
Upon nutrients exhaustion yeast cells enter into a non-proliferating, quiescent state, characterized by strongly diminished transcriptional and protein synthesis rate, severely reduced expression of genes encoding ribosomal proteins and induced transcription of stress responsive genes, accumulation of storage carbohydrates, thickened cell wall, enhanced stress resistance, chromosomes condensation and

autophagy (the process of engulfment of the cytoplasm into lipid vesicles which are delivered to the vacuole for degradation) (Gray et al., 2004).
Many of these phenotypes are regulated by the redundant stress response factors Msn2 and Msn4, which are activated by decreased PKA or TOR signaling and promote transcription of genes containing STRE elements (stress response element) in their promoters (Martinez-Pastor et al., 1996; Gorner et al., 1998; Gorner et al., 2002; Smith et al., 1998; Santhanam et al., 2004). In addition, many diverse environmental stresses (including heat shock, osmotic stress, oxidative stress and DNA damage) also activate Msn2/4 through different signal transduction pathways and induce at least a transient arrest of cell cycle progression: Xbp1, a transcriptional repressor with homology to Swi4 and Mbp1, is induced by stress and glucose starvation and may contribute to repress the transcription of the G1 cyclins-encoding genes, thus causing a transient cell cycle delay under stress conditions (Mai & Breeden., 1997; Mai & Breeden, 2006; Ubersax et al., 2003).
Although the cAMP/PKA circuit affects ribosome biogenesis (Jorgensen et al., 2004; see previous sections), the impact of PKA inactivation on cell cycle progression is too rapid to be the simple result of diminished cellular biosynthetic capacity (Zaman et al., 2008; Jorgensen & Tyers, 2004).
Removal of Msn2/4 or loss of the Yak1 kinase (which acts as growth antagonist) suppresses the growth defects associated with inactivation of PKA pathway (Smith et al., 1998; Garret et al., 1991; Garret et al., 1989); therefore, a simple model to conciliate all these observations proposes that the *START* transcriptional program might be under indirect control of Msn2/4, which in turn are sensitive to PKA activity (Zaman et al., 2008). However, the process is likely much more complex, and possibly involves other undefined effects of PKA activity on translation initiation, which would affect the expression of the G1 cyclins (Zaman et al., 2008).
Mutants with a hyperactive cAMP/PKA pathway exhibit a remarkable sensitivity to numerous stress conditions and fail to arrest cell cycle progression in G1 phase upon nutrient depletion, rapidly losing viability (Thevelein, 1994; Thevelein & deWinde, 1999). A common interpretation for these phenotypes is that hyperactivity of the cAMP/PKA circuit hinders mounting of an adequate cellular stress response and prevents a proper G1 arrest by forcing the constitutive execution of START, regardless of nutrient availability (Thevelein, 1994; Thevelein & deWinde, 1999; Zaman et al., 2008). However, a constitutive cAMP signalling also prevents accumulation of storage carbohydrates (such as glycogen and trehalose) and other nutrients (Thevelein, 1994; Thevelein & deWinde, 1999; Markwardt et al., 1995); therefore, as an alternative view, it has been suggested that upon starvation mutants carrying an hyperactive PKA are unable to complete the ongoing round of mitotic division due to the lack of stored nutrients and thus they simply arrest in the phase of the cell cycle where they are at the moment of nutrient exhaustion, without accumulating as G1 unbudded cells (Markwardt et al., 1995; Zaman et al., 2008). The sensitivity to nutrient starvation exhibited by these mutants might be explained in terms of metabolic effects rather than by a defective arrest of cell cycle progression: the constitutive activity of PKA would stimulate cells to use all of their resources for metabolic growth and the lack of nutritional reserves would make them vulnerable to sudden stressful conditions (Markwardt et al., 1995).

cAMP/PKA pathway and regulation of cell size

Many evidences support a prominent role for the cAMP/PKA pathway in regulation of the cell size, particularly in response to the available carbon sources (Tamaki, 2007).

Mutants cells with reduced cAMP signaling generally exhibit a consistent decrease in cell size.

A *cdc25* temperature-sensitive mutant shows a smaller volume than its isogenic wild type strain (Baroni et al., 1989). The carbon-source-dependent modulation of cell size is also lost in a strain expressing a truncated version of *CDC25* lacking the amino-terminal region or heterologous GEFs: these mutant exhibits nearly identical reduced size both in glucose and ethanol (Belotti et al., 2006).

A *tpk1*^{w} *tpk2 tpk3 bcy1* quadruple-null mutant, which possesses a weak constitutive PKA activity, also exhibits reduced cell volume (Cameron et al., 1988; Tokiwa et al., 1994).

In the presence of glucose, *gpr1* and *gpa2* single and double mutants strains display small size (Alberghina et al., 2004; Tamaki et al., 2005) and reduced protein synthesis rate (Tamaki et al., 2005), whereas no alteration is apparent during growth on ethanol (Alberghina et al., 2004; Tamaki et al., 2005). The doubling time and the length of the budded phase in glucose are unaffected by inactivation of the GPCR system, consistent with the notion that signaling through this circuit specifically modulates the critical size required for budding and DNA replication (Alberghina et al., 2004; see following sections). A slight decrease in Cln3 level was also observed during growth on glucose in both the *gpr1* and *gpa2* null strains (Alberghina et al., 2005), but another study reported the opposite result (Tamaki et al., 2005). Apparently, the mRNA levels for G1 cyclins (*CLN1*, *CLN2* and *CLN3*) are not affected by inactivation of the GPCR system (Tamaki et al., 2005) during balanced exponential growth. Gpr1 and Gpa2 are also required for the rapid adjustment of cell size in response to glucose: consistently, inactivation of the GPCR module largely eliminates the glucose-mediated increase of cell volume during a nutritional ethanol to glucose shift-up (Tamaki et al., 2005). It has been proposed that the transient delay in cell cycle progression (as evidenced by a decrease of budding index) necessary to reset cell size in response to glucose is due to repression of *CLN1* (Flick et al., 1998): consistently, the glucose induced transcriptional downregulation of *CLN1* is completely abolished when the GPCR system is inactivated and no drop in budding index is evident (Tamaki et al., 2005).

In contrast, the hyper-activation of the cAMP pathway results in dramatically large cells. *RAS2*^{V19}, a constitutively activated allele of *RAS2*, increases cellular mass (Baroni et al., 1994). The deletion of both *PDE1* and *PDE2*, which encode 3′-5′-cyclic nucleotide phosphodiesterases increase the cellular cAMP content and cell volume (Mitsuzawa et al., 1994). Furthermore, these strains respond to exogenous cAMP by increasing their size in a dose-dependent fashion (Tokiwa et al., 1994; Baroni et al., 1992). Inactivation of *BCY1* and *IRA2* also leads to increased cell size (Mitsuzawa, 1994; Jorgensen et al., 2002).

Unanswered questions

Despite recent outstanding progresses in our knowledge of nutrient sensing in yeast, many questions still remain unanswered.

Numerous missing links exist even in the best studied signal transduction pathways: for example, it is still unclear how the TOR network responds to nutrients availability, or how glucose activates the Ras branch of the cAMP/PKA pathway. In most cases, the precise interconnections among TOR, PKA and the other nutrient sensing pathways are just beginning to emerge.

Furthermore, the exact mechanisms by which nutrients signaling circuits regulate the cellular responses often remain elusive: for instance, the molecular basis of nutrient control of cell size and the connection between size and *START* execution are still unclear; it remains largely unknown how cells integrate inputs from multiple nutrient pathways to make developmental decisions; the precise connections between stress response and control of cell cycle progression are not completely understood; the complex relationship between the nutritional status and aging await further elucidations.

Abstract

Glucose and regulation of cell cycle in *S. cerevisiae*: analysis of mutants impaired in sugar uptake mechanisms

Requisito fondamentale per la sopravvivenza di microrganismi a vita libera come il lievito *S. cerevisiae* è la capacità di regolare il proprio metabolismo e la progressione del ciclo cellulare in modo tale che la crescita sia rapida in presenza di abbondanti nutrienti e si arresti all'esaurirsi degli stessi. Perché questo sia possibile, nutrienti come il glucosio devono generare segnali che vengano recepiti ed elaborati dal complesso macchinario che governa il ciclo cellulare.

S. cerevisiae possiede almeno tre meccanismi per rilevare variazioni dei livelli di glucosio nel mezzo di coltura:

- il *pathway* di Rgt2/Snf3, che controlla l'espressione dei trasportatori degli zuccheri esosi;
- il *pathway cAMP/PKA*, che regolando l'attività della protein-kinasi A promuove l'espressione di geni coinvolti nel metabolismo fermentativo e nella crescita cellulare e inibisce la trascrizione di geni coinvolti nella risposta agli stress.
- il *glucose main repression pathway*, che reprime l'espressione di geni coinvolti nella respirazione cellulare, nella gluconeogenesi e nell'utilizzo di fonti di carbonio alternative al glucosio;

L'assunzione di glucosio nel citoplasma dall'ambiente esterno avviene attraverso i trasportatori codificati dalla famiglia di geni *HXT* (*HeXose Transporter*), che comprende almeno 20 membri: *HXT1-17*, *RGT2*, *SNF3* e *GAL2*. Snf3 e Rgt2 sono incapaci di trasportare lo zucchero, ma agiscono piuttosto da sensori del livello di glucosio extracellulare: in particolare, Snf3 rileva basse concentrazioni dello zucchero inducendo l'espressione dei trasportatori ad alta affinità (codificati dai geni *HXT2-HXT4*), mentre Rgt2 rivela alte concentrazioni di glucosio promuovendo l'espressione dei trasportatori a bassa affinità (*HXT1*). Nessuno dei trasportatori è essenziale e solo la delezione di tutti i geni *HXT* (o almeno di quelli compresi tra *1-7* in alcuni background) rende la cellula di lievito incapace di crescere in presenza di glucosio come unica fonte di carbonio. L'espressione dei vari trasportatori è regolata a livello trascrizionale attraverso un complesso network che coinvolge tutti e tre pathway deputati al sensing del glucosio: come risultato, S. Cerevisiae è in grado di mantenere sempre un alto flusso glicolitico esprimendo il set di trasportatori più adatto alla quantità di glucosio disponibile.

Le connessioni tra i *pathway* deputati al *sensing* del glucosio e gli elementi di regolazione del ciclo cellulare non sono completamente definite, anche perché risulta spesso difficile scindere il duplice ruolo dello zucchero come nutriente e come molecola segnale.

Obbiettivo del presente progetto di ricerca è chiarire gli effetti di alterazioni nei meccanismi di *sensing* e (in modo particolare) di trasporto del glucosio sulla coordinazione tra crescita e divisione cellulare.

In una prima fase dello studio sono stati presi in esame alcuni mutanti con delezioni nei geni *HXT1-7*, codificanti per i principali trasportatori degli zuccheri esosi: i dati presenti in letteratura certificano infatti come queste mutazioni siano sufficienti ad abolire sostanzialmente l'assunzione (*uptake*) cellulare del glucosio impedendo la crescita dei mutanti su tale fonte di carbonio. I parametri di crescita (tempo di duplicazione, indice di gemmazione, contenuto proteico e di DNA) di ciascuno dei ceppi sono stati determinati in due condizioni sperimentali:

i) crescita esponenziale bilanciata in terreno csm/YNB addizionato di 2% etanolo o di una miscela 2% Etanolo + 2% glucosio, da cui è emerso come il glucosio possa

esercitare un effetto sulle dimensioni celulari anche nei mutanti *hxt* (indipendente quindi dal suo ruolo come nutriente). Infatti, analogamente alle cellule wild-type, anche i ceppi con i trasportatori deleti mostrano dimensioni cellulari e contenuto proteico maggiore quando fatti crescere in glucosio+Etanolo, sebbene (diversamente dal ceppo *wild type*) la loro velocità di crescita sia simile a quella registrata in terreno con solo etanolo;
ii) crescita durante shift-up nutrizionale etanolo => glucosio. All'aggiunta dello zucchero le cellule *wild-type* vanno incontro ad fase iniziale di adattamento (evidenziato dalla diminuzione transiente (10-15%) dell'indice di gemmazione), necessaria per reimpostare profilo trascrizionale, velocità di crescita e dimensioni cellulari e per il successivo passaggio ad un metabolismo energetico di tipo fermentativo. A differenza del ceppo *wild type*, dopo l'aggiunta di glucosio le cellule *hxt(1-7)* manifestano una drammatica e prolungata riduzione nell'indice di gemmazione e un forte rallentamento (arresto) nella progressione del ciclo cellulare. In seguito le cellule riprendono a dividersi con una velocità sostanzialmente identica a quella precedente lo shift, mentre volume cellulare e contenuto proteico medio aumentano sensibilmente: l'effetto del glucosio sulle dimensioni cellulari dei mutanti *hxt(1-7)* è tuttavia transiente e si esaurisce nell'arco di due/tre round di divisioni, quando le cellule tornano ad assumere le dimensioni tipiche della crescita su etanolo.
I dati finora riportati sembrano quindi suggerire che, almeno inizialmente, gli effetti del glucosio sulle dimensioni cellulari dipendano dal sensing dello zucchero e non dal suo metabolismo. Tuttavia, sebbene il ceppo *hxt(1-7)* non sia in grado di crescere su glucosio come unica fonte, rimane comunque dotato di una capacità residua di trasporto dello zucchero, che sebbene insufficiente a sostenere il metabolismo glicolitico, potrebbe comunque assumere un'importanza decisiva per la regolazione delle dimensioni cellulari.
Nel tentativo di scindere ancora più nettamente il duplice ruolo del glucosio come nutriente e come molecola segnale, in una successiva fase di studi sono stati utilizzati mutanti con delezioni in tutti i geni per i trasportatori del glucosio (*hxt(1-17)*), in cui ogni residuo trasporto dello zucchero risulta abolito. In aggiunta, si sono presi in esame una serie di mutanti con una capacità di *uptake* del glucosio progressivamente ridotta: nel dettaglio, la lista comprende (oltre ovviamente al ceppo wild type di riferimento):
i) *hxt(1-17)gal2*, in cui il trasporto del glucosio è completamente abolito;
ii) il ceppo *hxt(1-17)*) *snf3*, in cui l'inattivazione del sensore *SNF3* rispristina una trascurabile capacità di trasporto del glucosio, insufficiente comunque a garantire la crescita in terreno liquido contenente glucosio come unica fonte: l'assunzione dello zucchero in questo caso sembrerebbe avvenire attraverso un trasportatore non ancora caratterizzato, la cui trascrizione risulta derepressa in assenza di *SNF3*;
iii) il ceppo (*hxt(1-17) gal2 HXT1*, che esprime in modo costitutivo come unico trasportatore *HXT1*, un *carrier* a bassa affinità;
iv)) il ceppo (*hxt(1-17) gal2 HXT7*, che esprime in modo costitutivo come unico trasportatore *HXT7*, un *carrier* ad alta affinità;
v) il ceppo *snf3 rgt2*, in cui l'uptake del glucosio è ridotto a causa dell'inattivazione del principale pathway che regola l'espressione dei maggiori trasportatori;
vi) il triplo deleto *hxk2 hxk1 glk1*, che è in grado di trasportare glucosio nel citoplasma ma non è in grado di metabolizzarlo a causa dell'assenza di tutte e tre le chinasi che catalizzano il primo passaggio della glicolisi.

I ceppi sopra elencati sono stati sottoposti alle analisi descritte in precedenza.
Nel caso dei ceppi capaci di metabolizzare il glucosio, il tasso di crescita e le dimensioni cellulari su tale fonte sono generalmente correlate all'efficienza del sistema di trasporto dello zucchero nei vari mutanti: sembra esistere una relazione sostanzialmente lineare tra velocià di consumo del glucosio/velocità di crescita/dimensioni cellulari. Unica eccezione pare essere il ceppo *snf3 rgt2*, che manifesta dimensioni cellulari notevolmente più ridotte rispetto a quanto atteso sulla base del suo tasso di crescita: un risultato che sembrerebbe suggerire un ruolo diretto del pathway Snf3/Rgt3 nei meccanismi che regolano le dimensioni cellulari in risposta ai nutrienti.
Diversamente da quanto emerso in precedenza, la crescita dei mutanti *hxt(1-17)* risulta fortemente inibita in terreni contenenti miscele di etanolo (o altra fonte non fermentabile) e glucosio, anche quando la concentrazione dello zucchero è a livelli sub-ottimali (0.05% anziché 2%). L'aggiunta di glucosio a cellule *hxt(1-17)* in crescita su etanolo (shift-up nutrizionale) determina l'arresto permanente del ciclo cellulare in G1 (cellule vitali, non gemmate con contenuto di DNA presintetico). Sembra quindi che la semplice presenza di glucosio nell'ambiente extracellulare - ma non il trasporto dello zucchero nel citoplasma - sia sufficiente ad impedire l'utilizzo di fonti di carbonio alternative presenti nel medium: ciò spiegherebbe la mancata crescita in terreni misti glucosio+etanolo da parte di cellule *hxt(1-17)*, incapaci di effettuare l'*uptake* dello zucchero. Se tale ipotesi fosse corretta, inattivando contemporaneamente tutti i *pathway* deputati al *sensing* del glucosio dovrebbe essere possibile ripristinare la crescita di cellule *hxt(1-17)* in terreni contenenti miscele di glucosio ed etanolo.
Al momento, si è appurato che l'inattivazione del ramo del cAMP/PKA pathway passante attraverso Gpr1/Gpa2 non è sufficiente a correggere il difetto di crescita del ceppo *hxt(1-17)gal2* in fonte mista glucosio/etanolo. Al contrario, la semplice inattivazione di *SNF3* (ma non di *RGT2*)sembra sostanzialmente azzerare l'effetto citostatico del glucosio sulla crescita del ceppo *hxt(1-17) gal2 snf3*. L'interpretazione di tale risultato è ovviamente complicata dal fatto che la delezione di *SNF3* ripristina parzialmente il trasporto del gluccosio in un ceppo privo di tutti i trasportatori, sebbene, vale la pena ricordare, su scala estremamente ridotta e comunque insufficiente a sostenere la crescita, come confermato attraverso misurazioni dirette della velocità di consumo delo zucchero nel ceppo *hxt(1-17)*) *snf3*. Tuttavia, diversi dati in letteratura suggeriscono come il pathway Snf3/Rgt2 partecipi in qualche misura ai meccanismi della *glucose repression*, in particolare attrverso Mig2, un repressore trascrizionale che in presenza di glucosio collabora con Mig1 nel reprimere la trascrizione di geni richiesti per l'utilizo di fonti di carbonio alternative. La delezione di *MIG2* non è tuttavia sufficiente a ripristinare la crescita su etanolo/glucosio del ceppo *hxt(1-17)gal2*: ulteriori indagini sono dunque necessarie per chiarire quale sia il ruolo giocato da *SNF3* nell'intero processo.
In aggiunta, il comportamento manifestato dal ceppo *hxk2 hxk1 glk1* durante *shift-up* nutrizionale da etanolo a glucosio sembra ulteriormente confermare come in lievito lo zucchero sia in grado di regolare le dimensioni cellulari indipendentemente dal proprio metabolismo, almeno in una fase iniziale: le cellule *hxk2 hxk1 glk1* in crescita su etanolo rispondono all'aggiunta di glucosio aumentando considerevolmente il proprio volume, in misura paragonabile a quanto si registra nel ceppo *wild* type; tuttavia, contrariamente al ceppo wild type, nel mutante *hxk2 hxk1 glk1* l'aumento delle dimensioni celluari si accompagna ad un progressivo

rallentamento della velocità di crescita, fino ad un totale arresto del ciclo di divisione cellulare che sopraggiunge a circa 12 ore dallo shift. Dopo una fase di *lag* piuttosto prolungata ed estremamente variabile, in cui le cellule, pur non dividendosi, si mantengono gemmate, si assiste alla ripresa del ciclo di divisione cellulare: le cellule tornano a dividersi lentamente utilizzando l'etanolo residuo nel terreno e nell'arco di due/tre generazioni assumono nuovamente le tipiche dimensioni ridotte associate alla crescita su fonte di carbonio non fermentabile. Ad ulteriore conferma di come l'effetto del glucosio sia solo temporaneo, dimensioni e contenuto proteico di cellule *hxk2 hxk1 glk1* in crescita bilanciata su etanolo o su fonte mista etanolo/glucosio sono sostanzialmente identiche.
Nonostante il sorprendente effetto citostativo dello zucchero, l'aumento delle dimensioni cellulari in risposta all'aggiunta di glucosio si manifesta anche nel ceppo privo di tutti i trasportatori (*hxt(1-17) gal2*)., sebbene in misura meno eclatante rispetto al triplo mutante *hxk2 hxk1 glk1*.
Nell'insieme, tali risultati sembrano confermare come il glucosio sia in grado di modulare le dimensioni della cellula di lievito in maniera (almeno in parte) indipendente dal proprio ruolo come nutriente, funzionando in buona sostanza come un "ormone".
Per chiarire le basi molecolari di tale fenomeno è necessario chiarire le connessioni tra i pathway deputati al sensing del glucosio e gli elementi di regolazione del ciclo di divisione cellulare in *S. cerevisiae*. A tal fine, si è ultimata la costruzione di una serie di mutanti esprimenti versioni "taggate" (*HA-tag*) di alcuni dei principali regolatori coinvolti nella transizione G1/S (nello specifico Cln3, Cln2, Far1, Sic1 e Clb5), così da facilitare l'analisi dei loro livelli di espressione e di localizzazione subcellulare. Gli studi in questo senso sono tuttavia in una fase ancora troppo preliminare per poter trarre conclusioni definitive.
Da utlimo, si è cercato di valutare il contributo relativo di sensing, trasporto e metabolismo del glucosio alla regolazione trascrizionale del gene *SUC2*, uno dei marcatori più comunemente utilizzati per valutare il fenomeno della *glucose repression* in *S. cerevisiae*. *SUC2* codifica per l'invertasi, un enzima chiave per l'utilizzo del disaccaride saccarosio e la sua espressione risulta completamente bloccata in presenza di alti livelli di glucosio mentre viene indotta da raffinosio o da bassi livelli di glucosio. I risultati ottenuti con i vari mutanti hanno evidenziato come in presenza di abbondante glucosio il livello basale di attività invertasica sia generalmente proporzionale alla velocità del flusso glicolitico, che dipende in larga misura dalla capacità di trasporto dello zucchero: nei ceppi aventi un sistema di *uptake* per il glucosio ad efficienza ridotta l'invertasi risulta parzialmente o addirittura competamente derepressa, come nel caso del mutante privo di tutti i trasportatori. Il ceppo *snf3 rgt2* sfugge invece a questa regola, in quanto l'attività invertasica risulta sì parzialmente derepressa in presenza di alte concentrazioni di glucosio, ma non più inducibile da bassi livelli di glucosio o raffinosio. In aggiunta, l'inattivazione di *SNF3* e di *RGT2* abolisce completamente l'induzione dell'attività invertasica nel ceppo *hxt(1-17)gal2* in presenza di glucosio. Nell'insieme, i dati appena descritti sembrano suggerire per il pathway Snf3/Rgt2 un ruolo decisamente più rilevante nella regolazione di *SUC2* rispetto a quanto gli viene comunemente attribuito. Esperimenti futuri permetteranno di chiarire meglio la questione.

Experimental procedures

Construction of yeast strains

DNA manipulations were performed according to standard techniques. Strains used in this study are listed in Table I.

The *hxt(1-7)gal2* strain (MC996A genetic background) and the *hxt(1-17)gal2* strain (CEN.PK genetic background) were a kind gift from prof. Eckard Boles.

Auxotrophic strains were made prototrophic by integration of the appropriated *URA3*, *LEU2*, *HIS3* and *TRP1* cassettes, obtained by digestion with *BamHI* of the YDp plasmids (Berben et al., 1991).

Deletion mutants were generated by the PCR-mediated gene disruption method (short flanking homology *loxP::marker::loxP/Cre* recombinase system: Guldener et al., 1996; Guldener et al., 2002). Cells were transformed by the classic lithium acetate procedure (Schiestl & Gietz 1989). After growth of transformants on selective media, deletion of the targeted gene sequences was routinely confirmed by colony PCR using the primer sets listed in Table II. When necessary, markers used for disruption were removed by inducing the recombination of the flanking *loxP* sequences through expression of the Cre recombinase (Guldener et al., 1996; Guldener et al., 2002). In Table II primers for generations of multiple disruption cassettes containing different heterologous selectable markers using the pUGXX series of plasmids (Euroscarf) as template are shown. Primers were designed using the PerlPrimer software and purchased from Primm.

gpa2 null strains were constructed by one-step gene replacement using a *gpa2::LEU2* disruption cassette obtained by digestion with *PstI* of the pUC19-*gpa2::LEU2* plasmid (Colombo et al., 1998).

For inactivation of *HXK1*, an *hxk1::HIS3* disruption cassette was amplified by PCR using genomic DNA from a *hxk1*-null strain as template (de Winde et al., 1996).

GLK1 was disrupted using a *glk1::LEU2* cassette obtained by digesting the PWA40 plasmid with *NcoI/SacI* (de Winde et al., 1996).

Plasmids PYX022-*HXT1* and PYX022-*HXT7* were a kind gift from Dr.ssa Paola Barduardi's Lab. *HXT1* and *HXT7* coding sequences were amplified by PCR using genomic DNA as template and cloned into the PYX022 integrative plasmid (R&D systems) under the control of the strong constitutive *TPI* promoter. The constructs were digested with *PstI* and integrated at the *HIS3* locus in the *hxt(1-17)gal2* strain, yielding the *HXT1*- and *HXT7*-only strains.

Plasmid pTet-*CLN3-3HA* was used to obtain *CLN3* overexpression (Alberghina et al., 1994).

A complete set of isogenic strains expressing C-terminal HA-epitope tagged versions of key cell cycle regulators (Cln3, Cln2, Clb5, Sic1, Far1) were constructed by in-locus 3'in-frame insertion, according to the procedure described by Longtine et al., 1998). The *4HA-KANMX* fragments used for the tag of the various proteins were generated by PCR using the pDHA plasmid (Tripodi et al., 2007). Successful genomic-tag was verified in yeast transformants by PCR colony and subsequent western blot analysis.

To obtain double-tagged strains, the pDHA-hph plasmids was constructed by replacing the .*KANMX* marker of pDHA (*BglII-PmeI* fragment) with a *BamHI-EcoRV* segment from pAG26 (Goldstein et al., 1999), containing the *hph* gene (*Klebsiella pneumoniae)* that confers resistance to the antibiotic hygromycin B.

Tagged strains were phenotypically indistinguishable from their parent strain in all the tested growth conditions.

Growth conditions

All strains cells were grown in shake flask at 30°C on a rotary shaker (160rpm) in synthetic medium containing 0.67 g/L YNB (Formedium), supplemented with appropriate quantities of "drop-out" mixture (CSM, Formedium). Carbon source were either 2% glucose (w/v), 2% ethanol (v/v), or 2% Ethanol (v/v) + 2% glucose (w/v) mixtures. Growth media also contained 0.2% (v/v) glycerol to improve growth in presence of ethanol. Solid media contained 2% (w/v) agar. Growth of liquid cultures was monitored as increase in cell number using a Coulter Counter model Z2 (Coulter Electronics, Inc.).

The fraction of budded cells was scored by direct microscopic observation on at least 300 cells, fixed in 3.6% formalin and mildly sonicated. For growth plate assay, serially diluted cellular suspensions were spotted on plates and incubated at 30°C. The length of the budded phase was calculated according to the formula

$$T\mathrm{B} = \log_2(1 + F\mathrm{B})T$$

where FB is the percentage of the budded cells and T is the population doubling time ($T = \ln 2/\alpha$, where α is the experimentally determined growth rate).

Analysis of the cell size distribution was performed on asynchronous cultures during log-phase growth using a Coulter Z2 Particle Cell Analyzer (Beckman-Coulter). Cell size distribution was analyzed with the Z2 AccuComp software (Beckman-Coulter).

For shift-up experiments, glucose was added to ethanol growing cells at a final concentration of 2% (w/v). Samples were taken at regular intervals to check cell number, budding index, mean cellular size and for cytofluorimetric analysis.

Flow cytometric analysis

Samples of growing cultures (about 2×107 cells) were collected and fixed in 70% ethanol before analysis. (Coccetti et al., 2004). Cells were washed twice times with PBS (3.3 mM NaH_2PO_4, 6.7 mM Na_2HPO_4, 127 mM NaCl, 0.2 mM EDTA, pH 7.2), resuspended in RNAse solution (1 mg/ml RNAse (Roche) in PBS) and incubated over night at 30°C.

To obtain protein distribution, cells were washed once with PBS, resuspended in 1mL of freshly prepared FITC staining solution (50 µg/mL fluorescein isothiocianate (Sigma-Aldrich) in 0.5 M $NaHCO_3$) and incubated at 4°C in the dark for 1h. Cells were then washed three times with PBS and resuspended in 1 ml PBS before the analysis.

For DNA staining, cells treated with RNAse were washed once with PBS, resuspended in 1 ml of DNA staining solution (1 M Sytox Green (Molecular Probes) in 50 mM Tris pH 7.5) and incubated in ice and dark for at least 30 min.

All samples were mildly sonicated for 20s before FACS analysis, which was performed on at least 2×10^5 cells with a BD FACStarPlus fluorescence-activated cell sorter equipped with a Coherent Innova 70 Ion-Argon laser with a 488-nm laser emission. Plot generation and data analysis were performed using the WinMDI 2.9 software.

Heat-shock resistance

For determination of heat-shock resistance, samples were taken from exponentially growing phase cultures. Cells were collected by centrifugation, resuspended in sterile water ($2*10^8$ cells/mL) and heated at 51°C for 10 minutes. After cooling,

cells were serially diluted and spread on nutrient plates. Resulting colonies were counted after 3 days at 30°C.

Protein extraction and western blot analysis

For preparation of crude extracts, exponentially growing cells (about $4*10^8$) were collected and lysated in cold 20% TCA buffer with glass beads, according to the procedure described in Reid & Schatz, 1982, with minor modifications. Protein concentration was determined by UV dosage at 280nm. Typically 50 µg of total extract (150-200 µg for Cln3-4HA) were used for western blot analysis. As a loading control, blotted membranes were stained with Ponceau Red (Sigma) before immunodecoration. Anti-HA monoclonal antibodies (12CA5) were purchased from Roche. Enhanced chemioluminescence system (ECL, Amersham Biosciences) was used for immunoblot detection according to the manufacturer's instructions. Protein levels were quantified by densitometry analysis of raw scanned images using the Scion Image software (Scion Corporation). Images were resized and eventually adjusted for brightness/contrast for figures preparation.

Glucose consumption and ethanol production

Unless otherwise specified, cells were grown overnight to exponential phase in synthetic medium supplemented with 1%glucose/1%ethanol mixture. Cells were collected by filtration , washed twice with 2 volumes of medium without carbon source and resuspended in fresh medium containing 50mM glucose at a final cellular density of $4*10^6$ cells/mL. Alternatively, to measure the residual glucose consumption in strains with extremely reduced sugar uptake capacity, cells were resuspended at extremely high density ($4*10^8$ cells/mL). Cultures were incubated at 30°C on a rotatory shaker and samples were taken at regular intervals to determine. Glucose consumption and ethanol production were determined by standard enzymatic assays (Sigma-Aldrich; Megazyme).

Invertase assay

Cells growing in ethanol medium (unless otherwise stated) were harvested by filtration, washed once and resuspended in 2%ethanol medium (basal condition), 2% glucose plus 2% ethanol medium (repressing condition) and 0.1% glucose + 2% ethanol (inducing conditions) at a final cellular density of $3\text{-}4*10^6$ cells/mL. After 5 hours at 30°C $2*10^8$ cells were harvested by filtration, washed once with ice cold 10mM sodium azide and resuspended in 1mL sodium azide/acetate buffer (10mM NaN_3, 50mM Na-acetate). 1 mL of 200mM saccharose was added and cellular suspensions were incubated at 37°C for 30 min. Reaction was stopped by addition of 250 µl Tris 1M pH 8.8 and by heating at 90°C for 3 minutes. Samples were centrifugated and the glucose concentration in the surnatant was measured by the glucose oxidase-peroxidase method (Sigma-Aldrich).

Invertase activity was calculated as

$(\mu mol\ min^{-1})/10^8$cells (micromoles of glucose released from saccharose per minute by 10^8 cells).

Table I. List of strains

Strains	***Relevant genotype***	***Reference***
MC996A background		
MC996A	*MATa ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2 GAL MEL*	Reifenberger et al., 1995
hxt(1-7)gal2	*hxt2::HIS3 hxt5::LEU2 hxt1:::HIS3::hxt4 hxt3::LEU2::hxt6::hxt7 gal2::URA3*	Reifenberger et al., 1997
hxt(1-7)gal2 gpa2	*hxt(1-7)gal2 gpa2::URA3*	Rolland et al., 2000
gpa2	MC996A *gpa2::LEU2*	This study
CEN.PK background		
CEN.PK2-1C	*MATa leu2-3,112 ura3-52 trp1-289 his3-1 MAL2-8^{c} SUC2 hxt17*	Entian & Koetter, 2007
snf3	*snf3::his5^{Sp}*	This study
rgt2	*rgt2::KANMX*	This study
snf3 rgt2	*snf3::his5^{Sp} rgt2::KANMX*	This study
gpa2	*gpa2::LEU2*	This study
gpa2 gpr1	*gpa2::LEU2 gpr1::his5^{Sp}*	This study
snf3 rgt2 gpa2 gpr1	*snf3::loxP rgt2::KANMX gpa2::LEU2 gpr1::his5^{Sp}*	This study
hxt (1-17) gal2	*hxt13 ::loxP hxt15::loxP hxt16::loxP hxt14 ::loxP hxt12::loxP hxt9::loxP hxt11::loxP hxt10::loxP hxt8::loxP hxt514::loxP hxt2::loxP hxt367 ::loxP gal2 stl1::loxP agt1::loxP ydl247w::loxP yjr160c::loxP*	Wieczorke et al., 1999
hxt (1-17) gal2 snf3	*hxt (1-17) gal2 snf3::LEU2^{KI}*	This study
hxt (1-17) gal2 rgt2	*hxt (1-17) gal2 rgt2::KANMX*	This study
hxt (1-17) gal2 snf3 rgt2	*hxt (1-17) gal2 snf3::loxP rgt2::loxP*	Wieczorke et al., 1999
hxt gpa2 gpr1	*hxt (1-17) gal2 gpa2::LEU2 gpr1::his5^{Sp}*	This study
hxt gpa2 gpr1 snf3 rgt2	*hxt (1-17) gal2 snf3::loxP rgt2::loxP gpa2::LEU2 gpr1::his5^{Sp}*	This study
HXT1	*hxt (1-17) gal2 TPI$_{pr}$-HXT1::HIS3*	This study
HXT7	*hxt (1-17) gal2 TPI$_{pr}$-HXT7::HIS3*	This study
hxk2	*hxt (1-17) gal2 hxk2::LEU2^{KI}*	This study
hxk2 hxk1	*hxt (1-17) gal2 hxk2::LEU2^{KI} hxk1::HIS3*	This study
hxk2 hxk1 glk1	*hxt (1-17) gal2 hxk2::loxP hxk1::HIS3*	This study
hxt (1-17) gal2 mig2	*hxt (1-17) gal2 mig2:: LEU2^{KI}*	This study
Tagged-strains		
CLN3-4HA	CEN.PK2-1C *CLN3-4HA::KANMX*	This study
snf3 rgt2 CLN3-4HA	*snf3::loxP rgt2::loxP CLN3-4HA::KANMX*	This study
hxt (1-17) gal2 CLN3-4HA	*hxt (1-17) gal2 CLN3-4HA::KANMX*	This study
hxt (1-17) gal2 snf3 CLN3-4HA	*hxt (1-17) gal2 snf3::LEU2^{KI} CLN3-4HA::KANMX*	This study
HXT1CLN3-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT1::HIS3 CLN3-4HA::KANMX*	This study
HXT7 CLN3-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT7::HIS3 CLN3-4HA::KANMX*	This study
hxk2 hxk1 glk1 CLN3-4HA	*hxt (1-17) gal2 hxk2::loxP hxk1::HIS3 CLN3-4HA::KANMX*	This study
CLN3-4HA^{OE}	CEN.PK2-1C [pTET-*CLN3-4HA*]	This study
hxt (1-17) gal2 CLN3-4HA^{OE}	*hxt (1-17) gal2* [pTET-*CLN3-4HA*]	This study
FAR1-4HA	CEN.PK2-1C *FAR1-4HA::HPH*	This study
snf3 rgt2 FAR1-4HA	*snf3::loxP rgt2::loxP FAR1-4HA::HPH*	This study
hxt (1-17) gal2 FAR1-4HA	*hxt (1-17) gal2 FAR1-4HA::HPH*	This study
hxt (1-17) gal2 snf3 FAR1-4HA	*hxt (1-17) gal2 snf3::LEU2^{KI} FAR1-4HA::HPH*	This study
HXT1 FAR1-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT1::HIS3 FAR1-4HA::HPH*	This study
HXT7 FAR1-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT7::HIS3 FAR1-4HA::HPH*	This study
hxk2 hxk1 glk1 FAR1-4HA	*hxt (1-17) gal2 hxk2::loxP hxk1::HIS3 FAR1-4HA::HPH*	This study

(to be continued)

Strains	***Relevant genotype***	***Reference***
CLN3-4HA FAR1-4HA	CEN.PK2-1C *CLN3-4HA::KANMX FAR1-4HA::HPH*	This study
snf3 rgt2 CLN3-4HA FAR1-4HA	*snf3::loxP rgt2::loxP CLN3-4HA::KANMX FAR1-4HA::HPH*	This study
hxt (1-17) gal2 CLN3-4HA FAR1-4HA	*hxt (1-17) gal2 CLN3-4HA::KANMX FAR1-4HA::HPH*	This study
hxt (1-17) gal2 snf3 CLN3-4HA FAR1-4HA	*hxt (1-17) gal2 snf3::LEU2^{KI} CLN3-4HA::KANMX FAR1-4HA::HPH*	This study
HXT1 CLN3-4HA FAR1-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT1::HIS3 CLN3-4HA::KANMX FAR1-4HA::HPH*	This study
HXT7 CLN3-4HA FAR1-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT7::HIS3 CLN3-4HA::KANMX FAR1-4HA::HPH*	This study
hxk2 hxk1 glk1 CLN3-4HA FAR1-4HA	*hxt (1-17) gal2 hxk2::loxP hxk1::HIS3 CLN3-4HA::KANMX FAR1-4HA::HPH*	This study
CLN2-4HA	CEN.PK2-1C *CLN2-4HA::KANMX*	This study
snf3 rgt2 CLN2-4HA	*snf3::loxP rgt2::loxP CLN2-4HA::KANMX*	This study
hxt (1-17) gal2 CLN2-4HA	*hxt (1-17) gal2 CLN2-4HA::KANMX*	This study
hxt (1-17) gal2 snf3 CLN2-4HA	*hxt (1-17) gal2 snf3::LEU2^{KI} CLN2-4HA::KANMX*	This study
HXT1 CLN2-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT1::HIS3 CLN2-4HA::KANMX*	This study
HXT7 CLN2-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT7::HIS3 CLN2-4HA::KANMX*	This study
hxk2 hxk1 glk1 CLN2-4HA	*hxt (1-17) gal2 hxk2::loxP hxk1::HIS3 CLN2-4HA::KANMX*	This study
CLB5-4HA	CEN.PK2-1C *CLB5-4HA::KANMX*	This study
snf3 rgt2 CLB5-4HA	*snf3::loxP rgt2::loxP CLB5-4HA::KANMX*	This study
hxt (1-17) gal2 CLB5-4HA	*hxt (1-17) gal2 CLB5-4HA::KANMX*	This study
hxt (1-17) gal2 snf3 CLB5-4HA	*hxt (1-17) gal2 snf3::LEU2^{KI} CLB5-4HA::KANMX*	This study
HXT1 CLB5-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT1::HIS3 CLB5-4HA::KANMX*	This study
HXT7 CLB5-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT7::HIS3 CLB5-4HA::KANMX*	This study
hxk2 hxk1 glk1 CLB5-4HA	*hxt (1-17) gal2 hxk2::loxP hxk1::HIS3 CLB5-4HA::KANMX*	This study
SIC1-4HA	CEN.PK2-1C *SIC1-4HA::KANMX*	This study
snf3 rgt2 SIC1-4HA	*snf3::loxP rgt2::loxP SIC1-4HA::KANMX*	This study
hxt (1-17) gal2 SIC1-4HA	*hxt (1-17) gal2 SIC1-4HA::KANMX*	This study
hxt (1-17) gal2 snf3 SIC1-4HA	*hxt (1-17) gal2 snf3::LEU2^{KI} SIC1-4HA::KANMX*	This study
HXT1 SIC1-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT1::HIS3 SIC1-4HA::KANMX*	This study
HXT7 SIC1-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT7::HIS3 SIC1-4HA::KANMX*	This study
hxk2 hxk1 glk1 SIC1-4HA	*hxt (1-17) gal2 hxk2::loxP hxk1::HIS3 SIC1-4HA::KANMX*	This study
CLB5-4HA SIC1-4HA	CEN.PK2-1C *CLB5-4HA::KANMX SIC1-4HA::HPH*	This study
snf3 rgt2 CLB5-4HA SIC1-4HA	*snf3::loxP rgt2::loxP CLB5-4HA::KANMX SIC1-4HA::HPH*	This study
hxt (1-17) gal2 CLB5-4HA SIC1-4HA	*hxt (1-17) gal2 CLB5-4HA::KANMX SIC1-4HA::HPH*	This study
hxt (1-17) gal2 snf3 CLB5-4HA SIC1-4HA	*hxt (1-17) gal2 snf3::LEU2^{KI} CLB5-4HA::KANMX SIC1-4HA::HPH*	This study
HXT1 CLB5-4HA SIC1-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT1::HIS3 CLB5-4HA::KANMX SIC1-4HA::HPH*	This study
HXT7 CLB5-4HA SIC1-4HA	*hxt (1-17) gal2 TPI$_{pr}$-HXT7::HIS3 CLB5-4HA::KANMX SIC1-4HA::HPH*	This study
hxk2 hxk1 glk1 CLB5-4HA SIC1-4HA	*hxt (1-17) gal2 hxk2::loxP hxk1::HIS3 CLB5-4HA::KANMX SIC1-4HA::HPH*	This study

Table II. List of primers

Name	*Sequence (5'→3')*	*Comments*
5'-CLN3-HA-K	ACTGAAAAAGAGATCAACTTCCTCTGTGGATTGTGATTTTAATGA TAGTAGCAACCTCAAGAAAACTCGCcggccgcatggatcctatcc	*CLN3-HA::KANMX*
3'-CLN3-HA-K	ATGTATGTTAACGTATTTGCTTTGCAAATTTTAATTTATTTGTTG TTAAATGCATTTTTTTTTTTGTCGTTggatggcggcgttagtatcg	*CLN3-HA::KANMX*
5'-SIC1-HA-K	AAGGTTAACGGATGAAGAAAAGAGAAGATTCAAGCCAAAGGCATT GTTTCAATCTAGGGATCAAGAGCATcggccgcatggatcctatcc	*SIC1-HA::KANMX* *SIC1-HA::hph*
3'-SIC1-HA-K	TTGCAAATAAATGTAGAATAAGTAAGTAAATAAAATATAATCGTT CCAGAAACTTTTTTTTTTCATTTCTggatggcggcgttagtatcg	*SIC1-HA::KANMX (*
3'-SIC1-HA-HPH	TTGCAAATAAATGTAGAATAAGTAAGTAAATAAAATATAATCGTT CCAGAAACTTTTTTTTTTCATTTCTATCGATGAATTCGAGCTC	*SIC1-HA::hph*
5'-FAR1-HA-K	GATAGAAATAGAATATTTTGACCTGGTAAAGCAGCAAAGAATTCA TCAGACCCTGGAAGTTCCCAACCTCcggccgcatggatcctatcc	*FAR1-HA::KANMX* *FAR1-HA::hph*
3'-FAR1-HA-K	ATAGACGTGGAGAAACGAAAAAAAAAAAAAGGAAAAGCAAAAGCCT CGAAATACGGGCCTCGATTCCCGAAggatggcggcgttagtatcg	*FAR1-HA::KANMX*
3'-FAR1-HA-HPH	ATAGACGTGGAGAAACGAAAAAAAAAAAAAGGAAAAGCAAAAGCCT CGAAATACGGGCCTCGATTCCCGAAATCGATGAATTCGAGCTC	*FAR1-HA::hph*
CLB5-HAFor	TTATTTCCAAACTTTCAAGTGGTGTACATCCGAAATGCATAGCAA CTTTCAAAATCTATTTAATCTTAAGcggccgcatggatcctatcc	*CLB5-HA::KANMX*
CLB5-HARev	CCTTTTAGTTCAGCAAAAAGAAAAGAAAATGTAAAGAGTATGCGA ATTCATGAGCATTACTAGTACTAATggatggcggcgttagtatcg	*CLB5-HA::KANMX*
5'-CLN2-HA-K	ATAAATAGCGGTAAATCTAGCAGTGCCTCATCTTTAATTTCTTTT GGTATGGGCAATACCCAAGTAATAcggccgcatggatcctatcc	*CLN2-HA::KANMX*
5'-CLN2-HA-K	CTCTCTTTTCCCGCAGAATATGAAAGCTTTTCTTTTATAAATCTT ATAATATTGGTCTCTTTTTGGTACggatggcggcgttagtatcg	*CLN2-HA::KANMX*
gpr1::loxP-FW	CGACAAACAAGTGATCCGAAGTGTGACGAATAAAGCAAACTCTCC AACTCcagctgaagcttcgtacgc	*gpr1* deletion
gpr1::loxP-RE	GTCAATTTGTATTACGTTCCTTACTTTCCATTTTCAAACATCGCG ATACgcataggccactagtggatctg	*gpr1* deletion
snf3::HPH-FW	CAGAAGGATATGCCTTTGTTGGCATAGAAAGAAGAATTTATAAca gctgaagcttcgtacgc	*snf3* deletion
snf3::HPH-RE	GCACGTCCGCTTAATTAATACATCGAATAACATTAAATTAAgcat aggccactagtggatctg	*snf3* deletion
rgt2::KAN-FW	CAGAAACCACTATATATATATGGAAATATCTCGAATATTGCTTGT cagctgaagcttcgtacgc	*rgt2* deletion
rrgt2::KAN-RE	CGGTTTATAAGACCTCGAACGATCGTAAGATGCTATTGGTTTgca taggccactagtggatctg	*rgt2* deletion
FW-hxk2::loxP	GTAGGAATATAATTCTCCACACATAATAAGTACGCTAATTAAATA AAcagctgaagcttcgtacgc	*hxk2* deletion
RE-hxk2::loxP	AAGGGCACCTTCTTGTTGTTCAAACTTAATTTACAAATTAAGTTT Agcataggccactagtggatctg	*hxk2* deletion
FW-mig2::loxP	CTTTTTTCAACTTTTATTGCTCATAGAAGAACTAGATCTAAAcag ctgaagcttcgtacgc	*mig2* deletion
RE-mig2::loxP	CTTATGAAGAAAGATCTATCGAATTAAAAAAATGGGGTCTAgcat aggccactagtggatctg	*mig2* deletion
gpa2α	GCGCATCTTCAGAAAAGAACG	Control *gpa2* deletion
gpa2β	TGATGGCGGCAAATACTAATC	Control *gpa2* deletion
FW-gpr1(-152ATG)	TTGTCTACATCCCTTTCTCTACG	Control *gpr1* deletion
RE-gpr1(+155STOP)	ACTTATCGAGGAATCACATTGC	Control *gpr1* deletion
FW-SNF3(-210ATG)	CTAGACAATAGTCCTATCCTCGGCA	Control *snf3* deletion
RE-SNF3(+245STOP)	TAATGACTTCCGACGTTGACCG	Control *snf3* deletion
RGT2-CNT::KAN-FW	GAGCAGATCAGGAATAGTATC	Control *rgt2* deletion
FW-HXK2(-218ATG)	CAAATATCGTGTCCAATTCCGTG	Control *hxk2* deletion
RE HXK2(+152STOP)	TATCGTCACGAATAAATCCCGTG	Control *hxk2* deletion
FW-HXK1(-276ATG)	GAGAGGAATAGTAACAAGTGAACG	Control *hxk1* deletion
RE-HXK1(+204STOP)	CGGAGAACAAAGTAAGTGGA	Control *hxk1* deletion
FW-GLK1(-825ATG)	TCAGGAGCCATGTTCTTACAG	Control *glk1* deletion
FW-GLK1(-196ATG)	CATTATAAGTGGTGTGCCGA	Control *glk1* deletion

(to be continued)

Name	***Sequence (5'→3')***	***Comments***
RE-GLK1(+245STOP)	TGATAAAGGAAGACCTAGCA	Control *glk1* deletion
FW-MIG2-CNT	ACCTTGGAGATAACAGAAACTAG	Control *mig2* deletion
3'-CNT-HA-KAN	ATTCTGGGCCTCCATGTCG	Control *KANMX* deletion Control *HA::KANMX* TAG
KAN-CNT-RE	CCTGGAATGCTGTTTTGCCG	Control *KANMX* deletion Control *HA::KANMX* TAG
HPH-CNT-RE	CACTATCGGCGAGTACTTC	Control *hph* deletion Control *HA::hph* TAG
KAN/HIS5Sp-FW	CCTCGACATCATCTGCCC	Control *KANMX* deletion Control *his5*^{Sp} deletion
KAN/HIS5Sp-RE	GGATGTATGGGCTAAATG	Control *KANMX* deletion Control *his5*^{Sp} deletion
HIS5-RE(1126bp)	TTACAACACTCCCTTCGTGC	Control *his5*^{Sp} deletion
LEU2Kl-FW	ATCTCATGGATGATATCC	Control *LEU2*^{Kl} deletion
LEU2Kl-RE	AGTTATCCTTGGATTTGG	Control *LEU2*^{Kl} deletion
URA3Kl-FW	CAGACCGATCTTCTACCC	Control *URA3*^{Kl} deletion
URA3Kl-RE	TTGGCTAATCATGACCCC	Control *URA3*^{Kl} deletion
*FW-SIC1(-187STOP)	TGAACTGGTCACTCAGGAAATTAG	Control *SIC1-HA*
RE-SIC1(+231STOP)	CTCGCTTTGACGAAATACTACAATG	Control *SIC1-HA*
*FW-FAR1(-183STOP)	GATGTAACTCTTCGTCTACCAC	Control *FAR1-HA*
RE-FAR1(+174STOP)	CCAATAGGTTCTTTCTTAGGCA	Control *FAR1-HA*
*FW-CLB5(-306STOP)	CATTTACCTCCATCTACCGT	Control *CLB5-HA*
RE-CLB5(+536STOP)	TTCTCACTAATAACACCACACC	Control *CLB5-HA*
*FW-cln3(-280STOP)	TTCAGGTTCGTTCTCTTCTACC	Control *CLN3-HA*
CNT-cln3-RE	TAATGTGACTAGAGGAAGTAAGGAG	Control *CLN3-HA*
*FW-CLN2(-363STOP)	CAATCATCACCAATCACTCCA	Control *CLN2-HA*
RE-CLN2(+362STOP)	ATATGTCGTCGCTTCTTATCC	Control *CLN2-HA*

Aim of the study

Cell proliferation requires the tight coordination of different processes, such as mass accumulation, DNA replication and cell division. This coordination, which heavily relies on the ability of cell to integrate environmental and metabolic signals with the activity of key regulators of the cell cycle progression, ensures the maintenance of cell size homeostasis over multiple generations and the faithful partitioning of the genetic material.

An essential requisite for the survival of free living microorganism like the budding yeast *Saccharomyces cerevisiae* is the capacity to regulate growth and cell cycle progression according to the frequent changes in the nutrient status, so that proliferation is rapid when large supplies of nutrients are available and ceases when these becomes exhausted. Nutrients like glucose must therefore generate signals that are somehow received and elaborated by the complex machinery governing growth and cell cycle progression. Besides being the favorite carbon and energy source for *S. cerevisia*e, glucose can act as a signaling molecule ("hormone") to regulate multiple aspects of yeast physiology: addition of glucose to quiescent or ethanol growing cells triggers a fast and massive reconfiguration of the transcriptional program, which enables the switch to fermentative metabolism and promotes an outstanding increase of the cell biosynthetic capacity.

S. cerevisiae possesses at least three mechanisms to monitor changes of glucose level in the growth medium:

- the cAMP/PKA pathways (with its two branches comprising Ras and the Gpr1/Gpa2 module), which plays the major role in the cell response to glucose by inducing the transcription of genes required for fermentative metabolism and ribosome biogenesis and by repressing genes involved in the stress response;
- the Rgt2/Snf3-Rgt1 pathway, which regulates the expression of the transporters for hexose sugars encoded by the *HXT* genes;
- the main repression pathway involving the kinase Snf1, which represses the expression of genes involved in respiratory metabolism and in the utilization of alternatives carbon sources when glucose is available.

Glucose import into the cytoplasm occurs through a series of transporters encoded by the *HXT* genes. Yeast possesses at least 18 glucose carriers (*HXT1-17* plus *GAL2*), each with a different transport capacity and affinity for the sugar. The expression of the diverse transporters is tightly regulated at the transcriptional level: in this way, *S cerevisiae* can utilize glucose efficiently over a broad range of concentrations (from a few micromolar to a few molar) and maintains a high glycolytic flux by expressing the set of transporters most suitable to the available amount of glucose.

Glucose signaling in *Saccharomyces cerevisiae* requires in most cases at least partial metabolism of the sugar (Gancedo, 2008; Zaman et al., 2008; Santangelo, 2006): as a result, the roles of glucose as nutrient and signaling molecule are closely intertwined and it is difficult to separate the two functions.

Therefore, a central issue in this study was to determine whether (and possibly, to which extent) the regulatory function of glucose can be separated from its nutrient function. To this aim, we characterized yeast strains in which glucose metabolism is strongly reduced or even prevented due to the absence of a functional transport system (*hxt*-null strain) or to the loss of the three kinases catalyzing the first step in glycolysis (*hxk2 hxk1 glk1* strain).

In addition, since the precise connections between the glucose sensing pathways and the elements involved in the regulation of the cell cycle progression and size homeostasis remain largely obscure, we checked for possible effects of alterations in glucose sensing and uptake mechanisms on the coordination between growth and cell division.

Results

Results

Glucose dependent modulation of cell size in strains impaired for sugar uptake

The first obligatory step in the utilization of glucose is its transport across the plasma membrane. Glucose uptake in *S. cerevisiae* occurs via facilitated diffusion and is catalyzed by a series of glucose carriers encoded by the *HXT* genes. Although more than 20 *HXT* genes have been identified (Boles & Hollenberg, 1997; Ozcan & Johnston, 1999), previous studies have shown that loss of the major hexose transporter (encoded by the *HXT1-HXT7* genes) in the wild type MC996A strain substantially abolishes the sugar import and thus prevents growth on this carbon source (Reifenberger et al., 1997).

Table III. Growth parameters of strains impaired in glucose uptake (MC996A background)

2%Ethanol						
Strains	**T** (min)	**FB** (%)	**TB** (min)	**FΣ** (%)	**V** (fL)	**P** (Ch.#)
***wild type* (MC996A)**	457 ± 21	34.4 ± 1.1	195	31.5 ± 3.1	32.5 ± 0.3	305 ± 15
hxt(1-7)gal2	443 ± 17	32.6 ± 1.1	180	30.0 ± 4.4	31.3 ± 0.6	324 ± 18
gpa2	434 ± 27	34.2 ± 0.7	184	32.2 ± 5.3	32.8 ± 0.7	298 ± 13
hxt(1-7) gal2 gpa2	450 ± 15	31.3 ± 0.7	177	32.7 ± 4.6	31.1 ± 0.4	308 ± 7
2%Ethanol + 2%Glucose						
Strains	**T** (min)	**FB** (%)	**TB** (min)	**FΣ** (%)	**V** (fL)	**P** (Ch.#)
***wild type* (MC996A)**	118 ± 3	62.6 ± 3.1	83	65.2 ± 4.2	49.0 ± 0.4	557 ± 22
hxt(1-7)gal2	499 ± 36	35.7 ± 2.5	220	36.7 ± 2.5	35.6 ± 1.1	348± 12
gpa2	116 ± 4	61.7 ± 1.4	80	66.3 ± 3.7	46.5 ± 0.3	433 ± 28
hxt(1-7) gal2 gpa2	502 ± 72	35.8 ± 1.8	222	37.1 ± 5.0	34.5 ± 0.9	325 ± 16

T = doubling time (min); **FB** = budding index (%); **TB** = length of the budded phase (min); **FΣ** = fraction of cells with DNA content >1c (%); **V** = mean cell volume (fL); **P** = mean protein content (arbitrary fluorescence units).

Growth parameters were monitored during growth at 30°C in SC/YNB medium supplemented with 2% ethanol or 2% glucose/ethanol mixtures.

Cell volumes were determined with a Coulter particle analyzer, whereas DNA and protein content were quantified by cytofluorimetric analysis.

Values reported are mean ± standard deviation (SD) of at least 5 independent experiments.

Therefore, the *hxt(1-7)gal2* null mutant, together with its isogenic wild type reference MC996A strain, was initially employed in our study (Table I).

In a first set of experiments, several common growth parameters (including doubling time, budding index, length of the budded phase, mean cell volume, protein content, sensitivity to heat stress) were evaluated during balanced growth in synthetic complete medium supplemented with either ethanol or an ethanol/glucose mixture (Table III; Fig. 1) as carbon sources.

As expected, no significant differences between the two strains were detected during respiratory growth on ethanol: under this condition, both the wild type and the *hxt(1-7)*-null cells were small and grew slowly showing a considerable resistance to heat stress.

In the presence of glucose, the wild type strain exhibited the typical distinctive features (traits) associated with a fermentative metabolism: the growth rate and the budding index were remarkably high, cells were large and stress sensitive (Table II, Fig. 1).

In contrast, most of the growth parameters of the *hxt(1-7)gal2* strain were quite similar in the two growth conditions (Table III, Fig. 1), although the lag phase was somewhat longer when cells where cultivated in presence of glucose. Interestingly, a minor but consistent increase in cellular volume was detected during growth in ethanol/glucose medium: significantly, the larger size of *hxt(1-7)gal2* cells was accompanied by a parallel increase in the average protein content (Fig. 1; Table III).

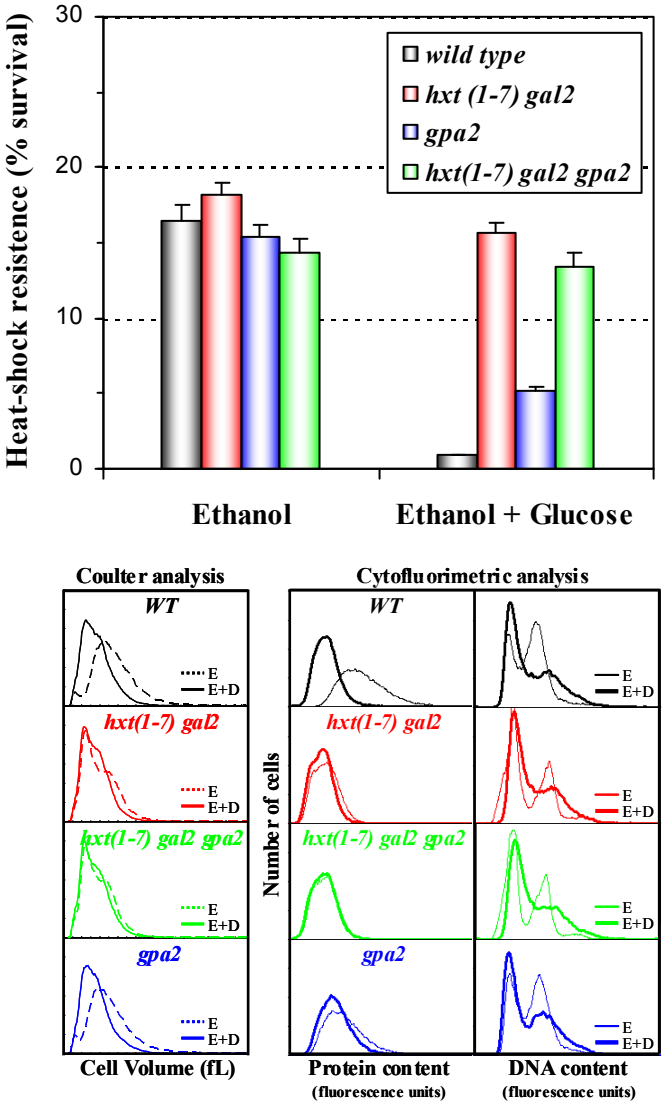


Figure 1.
Heat shock resistance and size alterations in mutants impaired for glucose sensing and uptake.
Cells were grown in media containing ethanol or glucose/ethanol mixtures at 30°C.
Heat-shock resistance (upper panel) was determined by heating aliquots of cultures at 51°C for 10 minutes. After cooling, treated and untreated cells were serially diluted and spread on plates. Resulting colonies were counted after 3 days at 30°C.Values reported are means ± SD of at least three independent experiments.
Cell volumes (lower panels) were determined by a Coulter particle analyzer, whereas DNA and protein content were quantified by cytofluorimetric analysis.

These observations apparently suggest that glucose can partially affect cell size even in a strain where sugar metabolism is absent as a consequence of the loss of the major HXT_s carriers. In other words, glucose might modulate cell size by acting as a signaling molecule, in a way (at least partially) independent from its role as nutrient. Consistent with this notion, inactivation of the Gpr1/Gpa2 branch of the cAMP/PKA glucose sensing system in the wild type strain reduces the average cell size without affecting the cell cycle parameters (doubling time, length of the budded phase: Table III; Fig. 1; (Alberghina et al., 2004; Tamaki et al., 2005)); in contrast, deletion of *GPA2* in the *hxt*-null mutant essentially abolishes the small effect of glucose on cell size (Table II; Fig. 1). Such a result was rather surprising, since previous studies had demonstrated that sugar uptake and phosphorylation are an essential prerequisite for cAMP signaling through the Gpr1/Gpa2 module (Rolland et al., 2000).

In order to take a deeper look into this issue, growth parameters were analyzed during an ethanol to glucose nutritional shift-up, which consists in the addition of glucose to ethanol growing cells. Several informative parameters (cell number, budding index, cell volumes and average protein content) were monitored at regular intervals after the glucose pulse (Fig. 2A-B).

Soon after glucose addition, the wild type strain underwent a transient delay in cell cycle progression (as highlighted by the transitory decrease (-15%) of the budding index (Fig. 2A-B): as widely described in the available literature, this delay is necessary to reconfigure the transcriptional profile and the biosynthetic machinery for the switch from a respiratory to fermentative metabolism (Johnston et al., 1979; Kief & Warner, 1981; Alberghina et al., 1998). During this transitory phase, cells rapidly increased their growth rate, their volume and the average protein content, reaching the higher values characteristic of the novel steady state condition. Loss of *GPA2* rendered the cells partially defective in adjusting their size during an ethanol-glucose nutritional shift-up: no drop in budding index was observed after the sugar addition and cells substantially failed to rapidly increase their volume and proteins content (Fig. 2A-B; Tamaki et al., 2005; Alberghina et al., 2004).

In contrast to wild type cells, the strain lacking the major hexose transporters exhibited a dramatic decrease of the budding index and a prolonged G1 arrest of the cell cycle progression (about 5 hours) upon glucose addition (Fig. 2A-B). At the cell cycle reprise, cell resumed dividing at the same rate as before the arrest, whereas both their volume and the average protein content increased appreciably. However, in the *hxt(1-7)gal2* mutant the effect of glucose on modulation of size was only transient, as it run out within 2-3 generations, when the cells again adopted the small volume and the low protein content typical of the growth on ethanol medium (Fig. 2A-B).

Taken together, the results reported so far might indicate that the initial effects of glucose on cell size rely on sugar sensing and are (at least partially) independent of sugar metabolism. Consistent with this hypothesis, inactivation of *GPA2* in the *hxt(1-7)gal2* strain strongly diminished the increase of cell size following the addition of glucose; on the other hand, the cell cycle arrest after the nutritional shift still occurred in the *hxt(1-7)gal2 gpa2* strain , although the drop of the budding was apparently less marked (Fig. 2A-B).

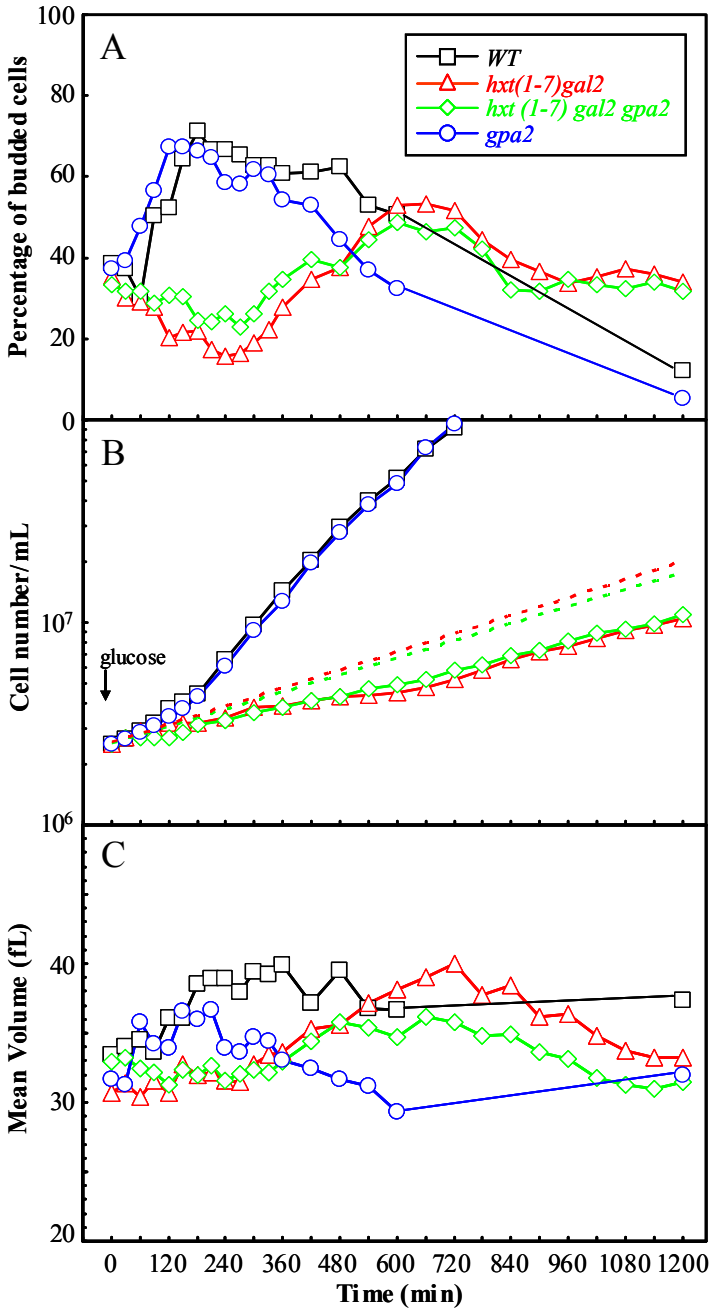


Figure 2.
Nutritional ethanol-glucose shift-up with strains deleted in the major glucose transporters (HXT1-7)
Glucose was added at a final concentration of 2% to ethanol growing cells. Samples were taken at the indicated intervals in order to evaluate budding index (Panel A), cell density (Panel B), cellular mean volumes (determined by a Coulter particle analyzer: Panel C) and for cytofluorimetric analysis (Panel D; see following page).

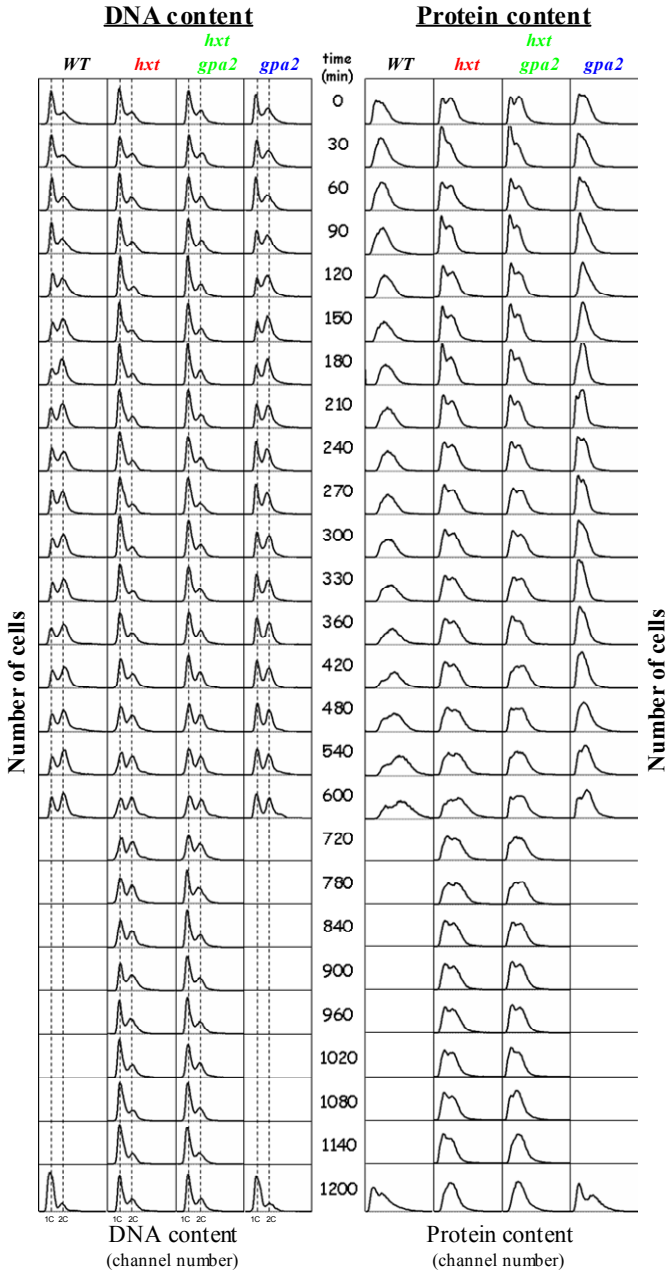

DNA content
Protein content
WT
hxt
gpa2
hxt gpa2
time (min)
0
30
60
90
120
150
180
210
240
270
300
330
360
420
480
540
600
720
780
840
900
960
1020
1080
1140
1200
Number of cells
Number of cells
1C 2C
DNA content
(channel number)
Protein content
(channel number)

Figure 2D

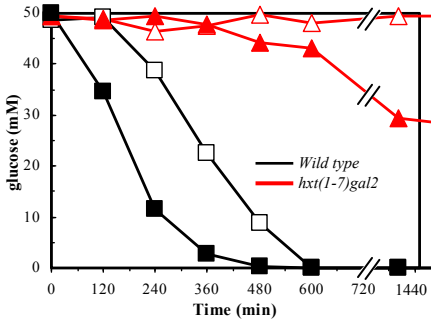


Figure 3.
Residual glucose consumption of the hxt(1-7)gal2 strain.
Wild type (black squares) or *hxt(1-7)gal2* (red triangles) cells were grown overnight in glucose/ethanol mixtures and resuspended in fresh medium containing 50mM glucose at low ($4*10^6$cells/mL, open symbols) or high ($4*10^8$cells/mL, closed symbols) cellular densities. At the indicated time points samples were taken to measure the glucose consumption.

Nevertheless, a clear and unambiguous interpretation of these data is hindered by the residual glucose uptake capacity exhibited by the *hxt(1-7)gal2* strain (Fig. 3, closed red triangles; Reifenberger et al., 1997; Ozcan, 2002): although we confirmed that this negligible sugar transport activity is absolutely insufficient to sustain an adequate glycolytic flux (and thus growth on medium containing glucose as sole carbon source was prevented), still it may play a decisive role in the glucose signaling events that regulate cell size.

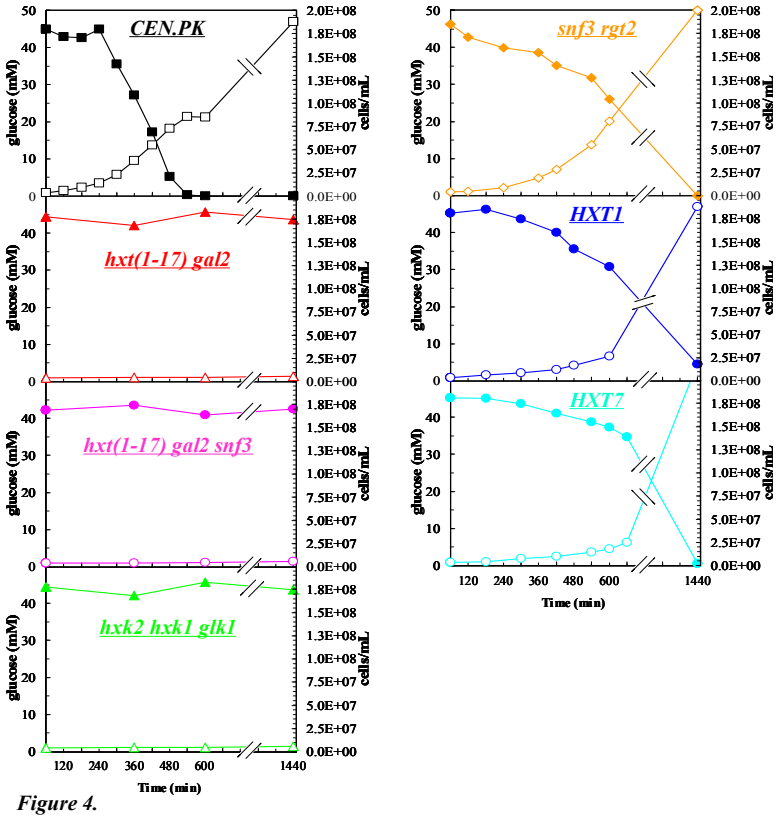


Figure 4.
Glucose consumption in strains impaired for sugar metabolism.
Cells were grown overnight in glucose/ethanol mixtures and resuspended in fresh medium containing 50mM glucose at a final cellular density of about $4*10^6$cells/mL. At the indicated time points samples were taken to measure the glucose consumption and the cellular densities of the cultures.

Effects of glucose on physiology of strains with null or reduced sugar uptake activity

In an attempt to fully separate the dual function of glucose as nutrient and signaling molecule we subsequently employed mutants carrying deletions in all the known glucose transporter encoding genes ((*hxt1-17)gal2*)), where any residual sugar uptake activity was completely abolished.

In addition, we included in our analysis a set of mutants exhibiting a progressively reduced glucose uptake capacity: in detail, besides the CEN.PK wild type reference strain, the new strains list (see Table I) included the following mutants:

i) the *hxt(1-17)gal2* strain, where glucose uptake activity is completely abolished (Wieczorke et al., 1999);

ii) the *hxt(1-17) gal2 snf3* strain, where the inactivation of the sensor encoded by *SNF3* restores a negligible sugar transport activity, which is still insufficient to sustain growth in liquid medium supplemented with glucose as sole carbon source: in this strain, sugar transport likely occurs via a still uncharacterized carrier, whose expression is derepressed in absence of *SNF3* (Wieczorke et al., 1999).

iii) the *hxt(1-17) gal2 HXT1* strain, which constitutively expresses *HXT1*, a low affinity carrier, as its only glucose transporter (Reifenberger et al., 1997);

iv) the *hxt(1-17) gal2 HXT7* strain, which constitutively expresses *HXT7*, a high affinity carrier, as its only glucose transporter (Reifenberger et al., 1997);

v) the *snf3 rgt2* strain, which exhibits a moderately reduced glucose uptake capacity as a consequence of the inactivation of the Rgt2/Snf3 pathway required to induce the expression of the *HXT* transporters in the presence of the sugar (Ozcan et al., 1998);

vi) the triple null mutant *hxk2 hxk1 glk1*, which imports glucose in the cytoplasm but still is not able to metabolize it due to the loss of all the three kinases catalyzing the fist step in glycolysis.

Table IV. Relative glucose consumption and ethanol production in mutants in glucose uptake/metabolism.

Strains	Glucose consumption[a]	Ethanol production[a]
wild type (CEN.PK2-1C)	1.000 ± 0.050	1.000 ± 0.080
snf3 rgt2	0.291 ± 0.063	0.25 ± 0.071
hxt(1-17)gal2	ND[b,d]	ND[b,d]
hxt (1-17)gal2 snf3	0.002 ± 0.001[c]	ND[c,d]
HXT1	0.270 ± 0.073	0.224 ± 0.056
HXT7	0.144 ± 0.040	0.152 ± 0.041
hxk2 hxk1 glk1	ND[d]	ND[d]

[a] The glucose consumption and ethanol production rates were determined in cells grown overnight in ethanol/glucose medium and resuspended in medium containing 50mM glucose at a final density of about $4*10^6$cells/mL.

[b] The *hxt(1-17)gal2* strain was grown in ethanol due to the cytostatic effect of glucose in this mutant. Cells were harvested and resuspended in ethanol medium supplemented with 50mM glucose at a final density of $4*10^7$cells/mL.

[c] to measure the extremely low glucose consumption rate of the *hxt(1-17)gal2 snf3* was resuspended in 50mM glucose medium at a cellular density of $4*10^7$cells/mL.

The values of glucose consumption and ethanol production here reported are relative to the value measured in the wild type strain. Mean values plus standard deviations of at least two independent experiments are given.

[d] ND = not detectable

The glucose consumption and the ethanol production rate of the diverse mutants are shown in Fig. 4 and Table IV. Interestingly, decreases in the glucose consumption rate were accompanied by proportional decreases in the ethanol yield (Table IV). These findings are compatible with previous reports (Elbing et al., 2004a,b; Otterstedt et al., 2004; Henricsson et al., 2005), which demonstrated that, at high external glucose concentration, sugar uptake can play a dominant role in controlling the glycolytic flux only in strains where the overall transport capacity is diminished: in these studies, reduced glucose uptake limited fermentative growth, thereby leading to a partial respiratory utilization of sugars and thus to a decrease in ethanol yield. However, although it would be interesting to know if our strains exhibiting diminished glucose consumption can actually adopt a partial respiratory metabolism at high sugar concentrations, this issue was not further investigated.

In a preliminary analysis, the strains listed above were tested for growth on solid media containing different carbon sources.
All the strains grew similarly on ethanol and maltose (a disaccharide consisting in two glucose molecules which is taken up by a separate transport system and converted to glucose by cytoplasmic maltase); the only exception was the triple *hxk2 hxk1 glk1* mutant, which could not grow on maltose due to the lack of the kinases required to phosphorylate the glucose generated by maltase.
Neither the *hxt(1-17) gal2* nor the *hxk2 hxk1 glk1* grew on glucose, saccharose (a fructose-glucose disaccharide) and raffinose (a trisaccharyde galactose-glucose-fructose), whereas loss of *SNF3* partially restored growth of the *hxt*-null strain growth of the cells on these carbon sources. The growth of strain with reduced sugar uptake capacity on glucose was slightly diminished, although the phenotype was not pronounced. In contrast with other genetic backgrounds, where the inactivation of the Snf3/Rgt2 pathway produces a dramatic growth defect on glucose (Ozcan et al., 1998), the loss of the two sensors encoded by *SNF3* and *RGT2* in the CEN.PK strain had only marginal effects on growth on glucose solid media, as already described (see Ramakrishnan et al., 2003).
As expected from previous studies (Reifenberger et al., 1995), the *HXT1*-only strain, which expresses a low-affinity carrier, failed to grow on raffinose and sucrose, which are hydrolyzed outside the cell, providing very low extracellular amounts of glucose and/or fructose. The wild type, the *snf3 rgt2* and the triple null-kinase strains grew on galactose, which is phosphorylated by galactokinase (encoded by *GAL1*), whereas the *hxt*-null, the *HXT1* and *HXT7* strains did not as a consequence of the inactivation of *GAL2*.

Subsequently, the strains were characterized during growth in liquid medium essentially as previously described (Table V).
Again, no significant differences in the considered growth parameters were detected when the various strains were cultivated in ethanol (or maltose) medium (Table IV, Fig. 5-6). With regard to strains able to metabolize glucose (wild type strain, *HXT1*- and *HXT7*-only strains, *hxt(1-17) gal2 snf3* strain and *snf3 rgt2* double null mutant), both the growth rate and the average cell size (determined by using a Coulter particle analyzer or by cytofluorimetric quantification of the protein content) in glucose media were generally correlated with the overall efficiency of the sugar uptake system (Table IV, Fig. 5-6): in fact, we discovered that a rough linear relation apparently exists between the doubling time (which in turn is proportional to the glucose consumption rate) and the cell size in the diverse mutants (Fig. 7).

One exception to this rule was the *snf3 rgt2* mutant, whose size in glucose media was reduced slightly more than expected on the basis of the growth rate exhibited by this mutant (Fig. 7: yellow circle and squares): this result might indicate that the Snf3/Rgt2 pathway plays a direct, active role in the mechanisms regulating the cell size in response to glucose. Another exception was the *HXT7*-only strain, which exhibited high protein content despite growing quite slowly in medium containing glucose as sole carbon source (Fig. 7, turquoise circle).

Table V. Growth parameters of strains impaired in glucose metabolism (sugar uptake or phosphorylation).

2%Ethanol						
Strains	**T (min)**	**FB (%)**	**TB (min)**	**FΣ (%)**	**V (fL)**	**P (Ch.#)**
***wild type* (CEN.PK)**	210 ± 10	58.4 ± 2.9	139	61.6 ± 2.5	36.0 ± 0.8	256 ± 13
snf3 rgt2	215 ± 13	59.3 ± 2.4	144	62.2 ± 3.1	34.3 ± 0.6	249 ± 15
hxt(1-17) gal2	208 ± 12	57.6 ± 2.9	137	61.9 ± 4.3	34.8 ± 0.7	257 ± 15
hxt(1-17)gal2 snf3	212 ± 15	58.0 ± 3.5	140	62.7 ± 3.1	35.3 ± 0.9	259 ± 10
hxt(1-17)gal2 HXT1	218 ± 15	59.8 ± 2.4	147	60.2 ± 4.8	35.5 ± 0.7	246 ± 12
hxt(1-17)gal2 HXT7	216 ± 11	60.2 ± 1.8	147	63.5 ± 3.2	34.7 ± 1.0	260 ± 8
hxk2 hxk1 glk1	207 ± 14	60.5 ± 3.0	141	59.3 ± 3.6	33.8 ± 1.6	252 ± 10
2%Ethanol + 2%Glucose						
Strains	**T (min)**	**FB (%)**	**TB (min)**	**FΣ (%)**	**V (fL)**	**P (Ch.#)**
***wild type* (CEN.PK)**	113 ± 3	75.1 ± 2.3	91	72.1 ± 2.9	49.1 ± 1.7	366 ± 18
snf3 rgt2	128 ± 10	63.5 ± 1.3	91	64.4 ± 3.0	42.2 ± 2.7	306 ± 15
hxt(1-17) gal2	NG	NG	NG	NG	NG	NG
hxt(1-17)gal2 snf3	222 ± 19	57.5 ± 2.3	145	55.8 ± 1.7	37.6 ± 1.8	273 ± 16
hxt(1-17)gal2 HXT1	131 ± 13	67.5 ± 2.6	97	65.3 ± 3.8	46.2 ± 2.7	345 ± 14
hxt(1-17)gal2 HXT7	154 ± 11	65.0 ± 3.4	111	61.8 ± 3.1	44.0 ± 3.1	332 ± 13
hxk2 hxk1 glk1	215 ± 13	61.7 ± 3.1	149	58.0 ± 3.5	36.1 ± 1.6	260 ± 8
2%Glucose						
Strains	**T (min)**	**FB (%)**	**TB (min)**	**FΣ (%)**	**V (fL)**	**P (Ch.#)**
***wild type* (CEN.PK)**	92 ± 3	79.6 ± 3.2	79	75.8 ± 2.2	55.2 ± 1.3	404 ± 12
snf3 rgt2	118 ± 5	67.8 ± 3.4	88	65.1 ± 2.6	46.1 ± 2.4	338 ± 15
hxt(1-17) gal2	NG	NG	NG	NG	NG	NG
hxt(1-17)gal2 snf3	NG	NG	NG	NG	NG	NG
hxt(1-17)gal2 HXT1	122 ± 10	70.4 ± 2.1	94	67.8 ± 2.0	50.4 ± 2.9	361 ± 18
hxt(1-17)gal2 HXT7	187 ± 21	56.4 ± 4.1	121	54.9 ± 3.3	52.4 ± 1.5	363 ± 23
hxk2 hxk1 glk1	NG	NG	NG	NG	NG	NG

T = doubling time (min); **FB** = budding index (%); **TB** = length of the budded phase (min); **FΣ** = fraction of cells with DNA content >1c (%); **V** = mean cell volume (fL); **P** = mean protein content (arbitrary fluorescence units).

NG = no growth

Growth parameters were monitored during growth at 30°C in SC/YNB medium supplemented with the indicated carbon sources. Cell volumes were determined with a Coulter particle analyzer, whereas DNA and protein content were quantified by cytofluorimetric analysis.

Values reported are mean ± standard deviation of at least 3 independent experiments.

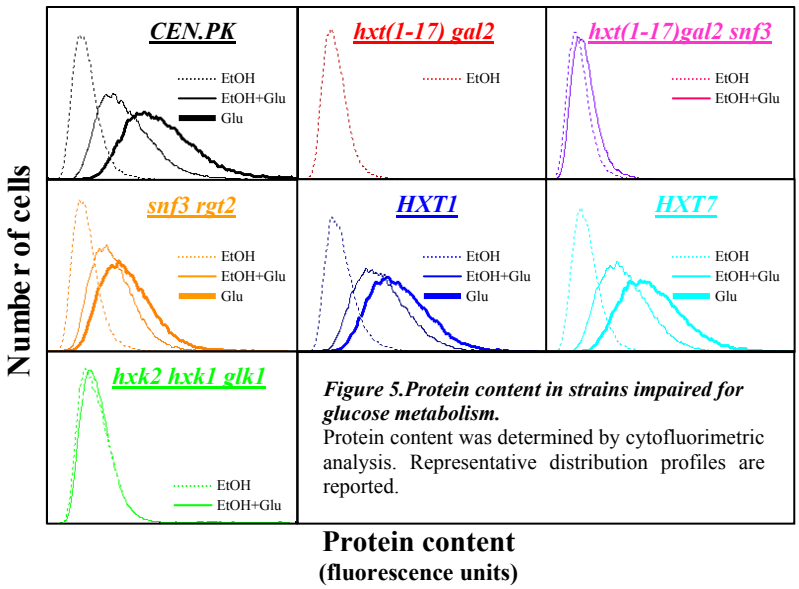


Figure 5.Protein content in strains impaired for glucose metabolism.
Protein content was determined by cytofluorimetric analysis. Representative distribution profiles are reported.

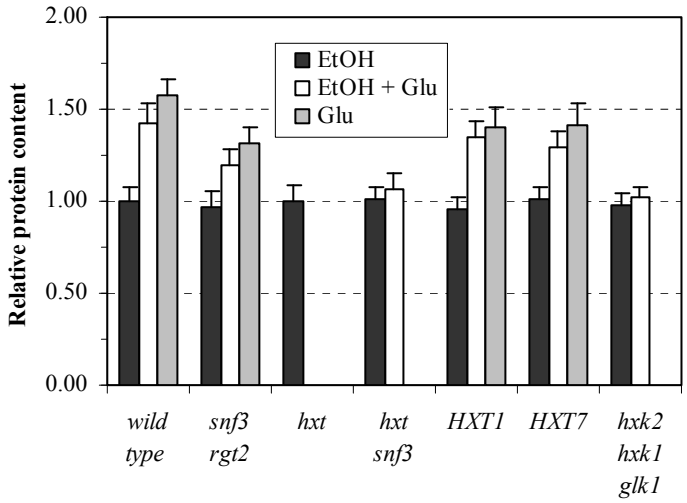


Figure 6.
Relative protein content in strains impaired for glucose metabolism under various growth conditions.
Protein contents were normalized by dividing for the value measured in wild-type cells grown in ethanol medium. Values reported are mean ± standard deviation of at least 3 independent experiments.

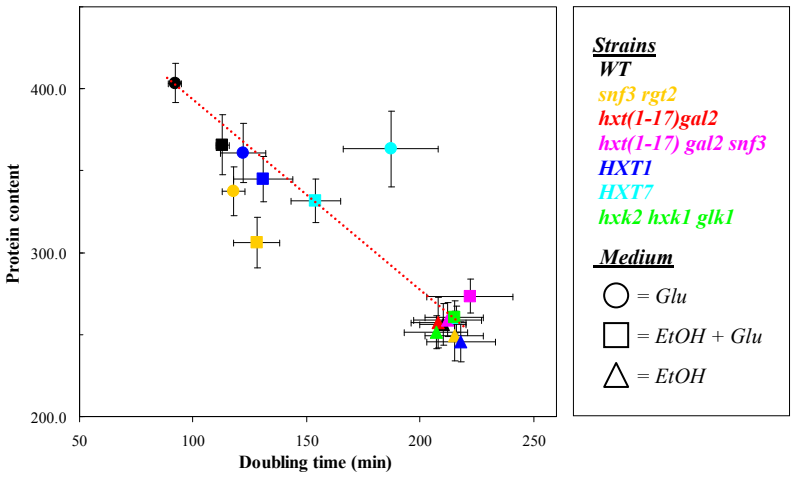


Figure 7.
Linear relation between protein content and growth rate in strains with reduced glucose consumption rates.
Doubling times were plotted versus the average protein contents measured in three diverse growth conditions (glucose, ethanol + glucose, ethanol). Dotted line represents the baseline correlation.
Values reported are mean ± standard deviation of at least 3 independent experiments. A similar result was obtained by plotting doubling time versus mean cellular volumes.

Interestingly, both the wild type and the *snf3 rgt2* strains exhibited a longer lag phase (data not shown), a slightly reduced growth rate and significantly smaller size when cultivated in medium containing glucose/ethanol mixture instead of glucose alone (Table IV, Fig. 6-7): this behavior is likely the consequence of some toxic effects of ethanol. In contrast, the *HXT7*-only strain grew surprisingly faster on ethanol /glucose medium than on medium containing glucose as sole carbon source (Table V): this finding might indicate that due to its reduced glucose uptake capacity the *HXT7* mutant adopts a respiratory/fermentative metabolism even in presence of high concentrations of sugar and thus can co-consume glucose and ethanol when both the carbon sources are available. However, this issue was not further investigated.

For the *hxt(1-17) gal2 snf3* strain, which exhibited an extremely low glucose consumption rate (Fig. 4; Table IV), the growth parameters in ethanol and ethanol/glucose media were quite similar (Table V, Fig. 5-6): although small increases in cell volume and average protein content were sometimes observed when cells were cultivated in the presence of glucose, statistical analysis revealed that such differences were not significant. No appreciable growth was detectable for this mutant in liquid medium containing glucose as sole carbon source.

In contrast to the results described in the previous section, which were obtained with the strain lacking only the *HXT1* to *HXT7* hexose transporters (MC996 background)), the new analysis showed that the growth of the *hxt(1-17)gal2* mutant was substantially abolished in media containing a mixture of ethanol (or equivalent non-fermentable carbon source) and glucose (Table V): this surprising phenotype was observed even in the presence of sub-optimal concentrations of glucose (0.1% instead of 2%), both on plates and in liquid medium.

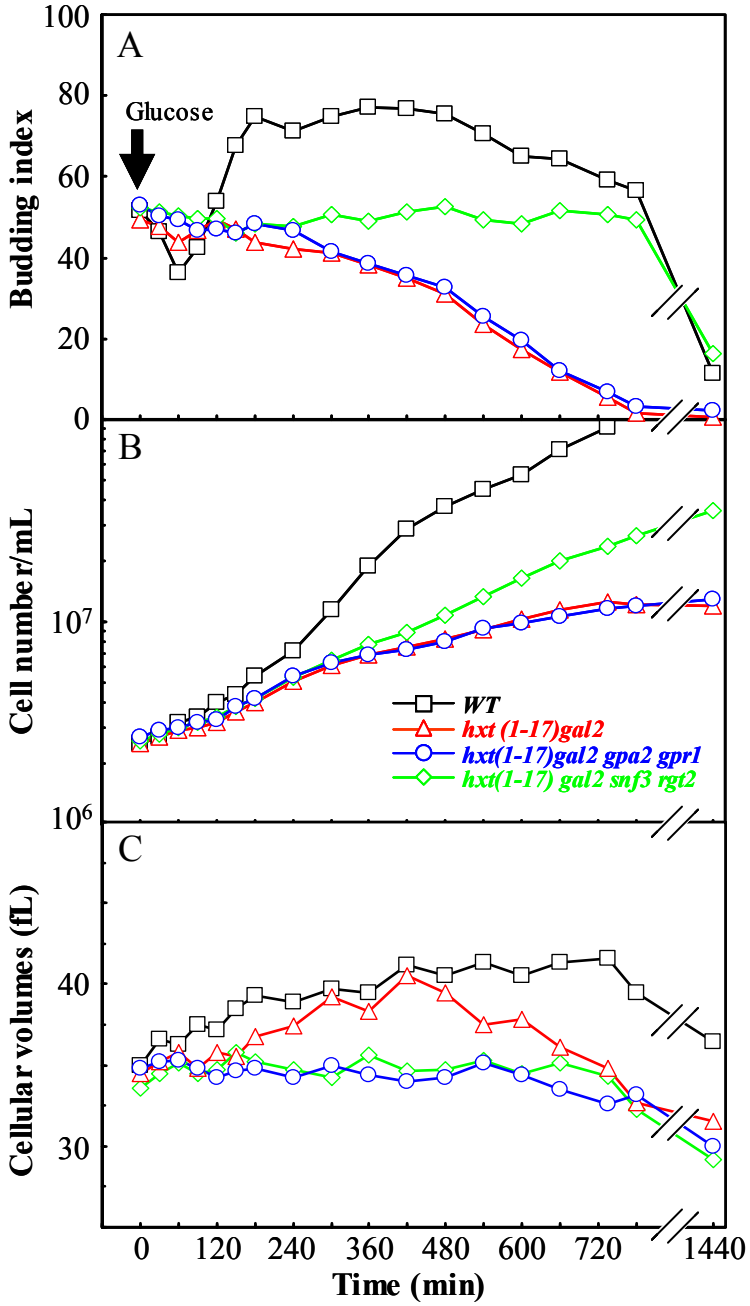


Figure 8.
Nutritional ethanol-glucose shift-up with strains impaired for glucose transport.
Glucose was added to ethanol growing cells. Samples were taken at the indicated intervals in order to evaluate budding index (Panel A), cell density (Panel B) and cellular mean volumes (determined by a Coulter particle analyzer: Panel C). Representative size distributions (acquired with a Coulter particle analyzer) are shown in Panel D (see following page).

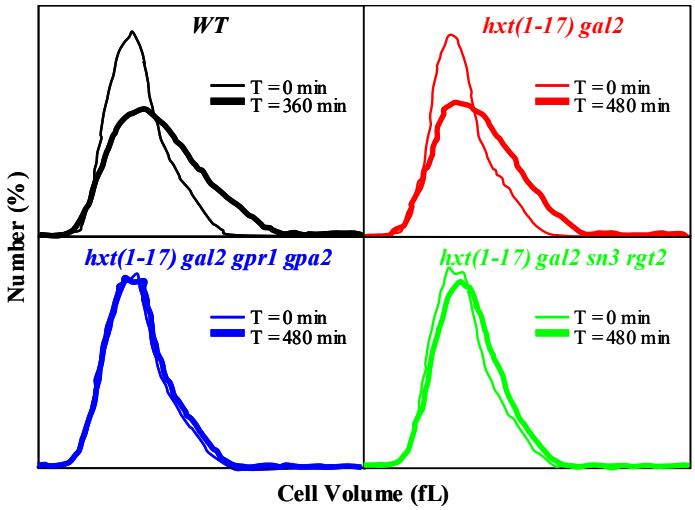


Figure 8D

Despite its cytostatic effect, glucose transiently modulates cell size even in a strain where sugar transport is completely abolished

Addition of glucose to the *hxt(1-17)gal2* strain growing on ethanol (nutritional shift-up) caused a permanent arrest as round, unbudded cells with pre-synthetic DNA content (G1 arrest) within 3 rounds of cell division (Fig. 8A-B, red triangles); in minimal medium, the cessation of cellular division was even more rapid. The G1-arrested cells maintained viability, as judged by viable count with methylene blue, and promptly resumed growth once inoculated in fresh medium containing ethanol as sole carbon source, but not an ethanol /glucose mixture. An inhibitory effect of glucose on growth of the *hxt*-null strain was also observed when cell were cultivated on maltose media, although in this case the phenotype was far less evident, likely as a consequence of the constitutive expression of *MAL* genes in the CEN:PK background (.

The apparent inconsistency between these observations and the results described in previous sections may be attributed to the different genetic backgrounds of the strains employed in the two sets of experiments: for instance, previous studies showed that a *hxt(1-7) gal2* mutant constructed in the CEN.PK background was still able to grow on glucose (although slowly), possibly as a consequence of the higher respiratory capacity which enables CEN.PK cells to metabolize glucose even at extremely low uptake rates (Wieczorke et al., 1999).

Nevertheless, during an ethanol/glucose shift-up, just before the definitive arrest of cell cycle progression in G1 phase, a slight but significant increase of cell size was observed also in the *hxt(1-17)gal2* mutant (CEN:PK background) lacking all the known hexose carriers (Fig. 8C-D, red triangles and thick line). Although in this case the effect of glucose on size was partially masked by the growth inhibitory effect of the sugar, such a result perfectly reconciles with the behavior exhibited under the same experimental conditions by the *hxt(1-7)gal2* (MC996 background) strain, which is devoid of only the major glucose transporters. Furthermore, the transient modulation of cells size was completely abolished when the Gpr1/Gpa2

pathway was inactivated in the hxt-null strain (Fig. 8C-D, blue circles and thick line).
Therefore, despite the surprising cytostatic effect that glucose seems to exert on the *hxt(1-17)gal2* strain, these findings substantially reinforce the hypothesis that glucose can modulate yeast cell size even in strains where sugar transport activity (and thus sugar metabolism) is dramatically reduced or completely absent.

Inactivation of MIG2 does not suppress the cytostatic effect of glucose in the hxt-null strain

The data reported in the previous sections suggest that the simple presence of glucose in the extracellular environment is apparently sufficient to prevent a strain devoid of sugar transport activity from utilizing other available carbon sources, such as ethanol. Thus, in ostensible contrast with the current view (Gancedo, 1998; Zaman et al., 2008; Santangelo, 2006), the glucose mediated repression of genes involved in ethanol metabolism seems to require only sugar sensing, but not sugar uptake, at least in our *hxt*-null strain. If this hypothesis is correct, by concurrently inactivating all the signaling pathways involved in glucose sensing it should be possible to restore growth of *hxt(1-17)gal2* cells in medium containing glucose/ethanol mixtures.
We thus decided to construct a set of isogenic mutants in a *hxt(1-17) gal2* background, where two of the major glucose sensing pathway (the Gpr1/Gpa2 branch of the cAMP/PKA cascade and the Rgt2/Snf3 circuit) were inactivated in various combinations (Table I). Despite abolishing the transient effect of glucose on size (Fig. 8C-D, blue circles), loss of the Gpr1/Gpa2 branch of the cAMP/PKA pathway did not suppress the growth defect of *hxt(1-17) gal2* cells in presence of glucose (Fig. 8A-B, blue circles). In contrast, inactivation of the glucose sensor encoded by *SNF3* (but not *RGT3* deletion) substantially eliminated the cytostatic effect of glucose on growth of the *hxt*-null mutant (Fig. 8A-B, green diamonds). Obviously, an unambiguous interpretation of this result is complicated by the fact that loss of *SNF3* function also restores a partial glucose transport activity in our *hxt*-null strain, as already discussed (Table IV; Wieczorke et al., 1999).
Nevertheless, recent studies have discovered the existence of numerous crosstalks among the diverse glucose sensing systems (Kaniak et al., 2004; Kim et al., 2006; Pasula et al., 2007; Gadura et al., 2006; Palomino et al., 2006; Zaman et al., 2009; Zaman et al., 2008; Gancedo et al., 2008). In particular, it has been demonstrated that the Snf3/Rgt2 pathway can contribute to the glucose repression mechanisms by inducing the expression of *MIG2*, encoding a transcriptional repressor which collaborates with Mig1 in the glucose-mediated repression of several genes required for the metabolism of alternative carbon sources (Kaniak et al., 2004; Luftiyya et al., 1998; Zaman et al., 2009). However, inactivation of *MIG2* was insufficient to suppress the growth defect of the *hxt*-null strain in presence of ethanol/glucose mixture (Fig.9, purple circles). Therefore, further investigations will be required to better clarify the role of Snf3 in determining the cytostatic effect of glucose in *hxt(1-17) gal2* cells.

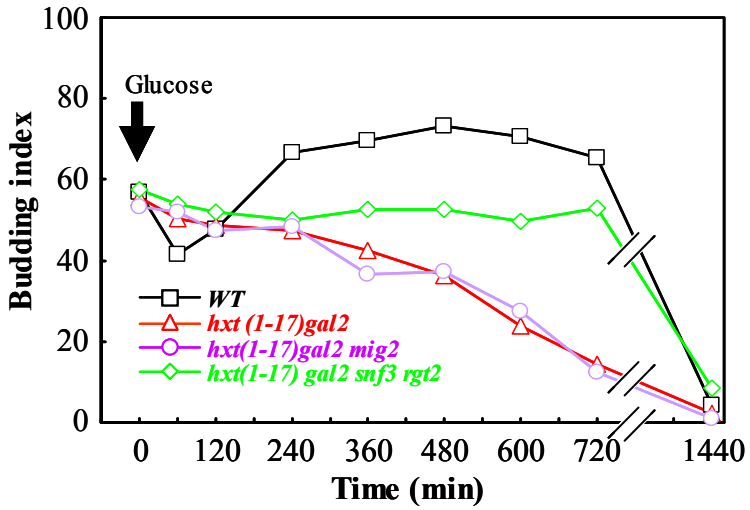


Figure 9.
Inactivation of MIG2 does not suppress the cytostatic effect of glucose in the hxt-null strain.
Potential effects of *mig2* deletion were checked during a nutritional ethanol-glucose shift-up. Growth conditions were as previously described.

Glucose partially modulates cell size in a hxk2 hxk1 glk1 triple null mutant strain
Apparently, the behavior exhibited by the *hxk2 hxk1 glk1* strain during a nutritional shift-up further confirmed that in yeast glucose can modulate cell size even in absence of sugar metabolism, at least in an initial stage: in fact, similarly to their isogenic wild type counterparts, the *hxk2 hxk1 glk1* cells initially responded to glucose addition by considerably increasing both their mean volume and their average protein content (Fig. 10C-D, green triangles and thick lines). However, in contrast to wild type strain, the glucose-induced cell size increase of the triple-null kinase mutant was accompanied by a progressive slow-down of the cell cycle progression, which culminated in a complete arrest of cellular division about 10 hours after the sugar addition (Fig. 10A-B, green triangles). After this prolonged and quite variable "adaptation phase", during which cells did not divide despite remaining budded, cell cycle progression started again: *hxk2 hxk1 glk1* cells slowly resumed growth by consuming the residual ethanol in the medium and within three generations again adopted the typical small size associated with growth on non-fermentable carbon source (Fig. 10C-D, green triangles and dotted line). As a further confirmation that the effect of glucose in the *hxk2 hxk1 glk1* triple-null mutant was only transient, we found that the values of mean cell volumes and average protein contents for this strain were nearly identical during balanced growth on either ethanol or glucose/ethanol mixture (Table V, Fig. 5-6).

In sum, our result seem to indicate that glucose can induce an transient increase of cell size even in strains where sugar metabolism is completely abolished, due to the absence of a functional transport system (*hxt*-null strain) or to the loss of the three kinases catalyzing the first step in glycolysis (*hxk2 hxk1 glk1* strain).
Therefore, glucose can apparently modulate yeast cell size by acting as signaling molecule ("hormone"), in a way at least partially independent from its role as nutrient.

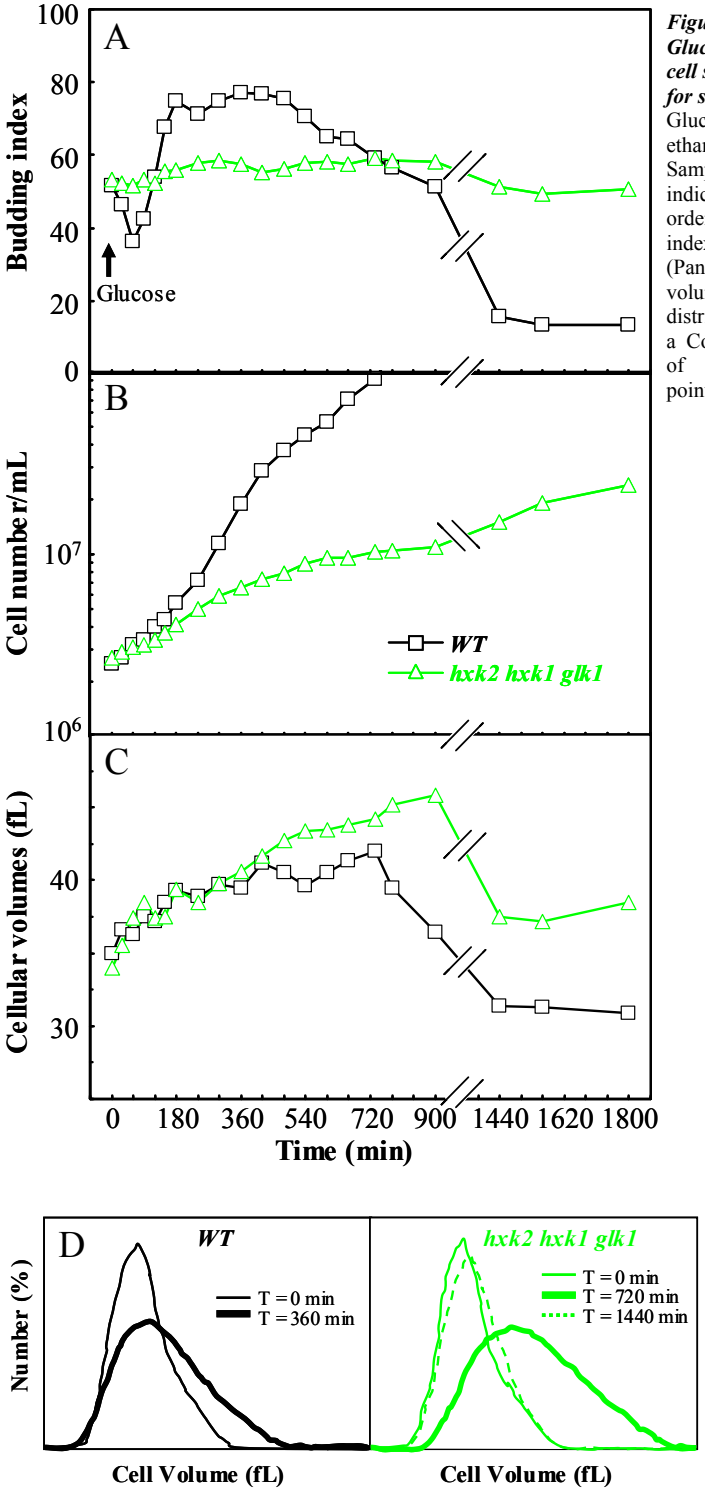


Figure 10.
Glucose transiently increase cell size in a strain impaired for sugar phosphorylation.
Glucose was added to ethanol growing cells. Samples were taken at the indicated time points in order to evaluate budding index (Panel A), cell density (Panel B) and cellular mean volumes (Panel C). Size distributions (acquired with a Coulter particle analyzer) of several relevant time points are shown in Panel D.

Expression levels of cell cycle regulators in strains impaired for glucose metabolism: a preliminary analysis

In *Saccharomyces cerevisiae,* coordination between growth and cell division takes place at Start, a short interval in late G1 phase and requires that yeast cell reaches a critical size before entering into S phase. The critical size at which cell initiates a new round of mitotic division is regulated by the nutrient status, in particular by the available carbon source: in fact, cells growing on glucose are larger than cells growing on ethanol (Johnston et al., 1979; Tyson et al., 1979; Johnston et al., 1977; Lorincz & Carter, 1979).

According to the model recently proposed by Barberis and colleagues (Barberis et al., 2007), carbon source modulation of the critical cell size is distributed over two sequential "thresholds" that control the G1/S transition. Each threshold consists of an activator and an associated inhibitor blocking its function: the first one involves the G1 cyclin Cln3, the Cdk inhibitor (Cki) Far1 and the cyclin-dependent kinase Cdc28, whereas the second one comprises the S phase cyclin Clb5 (and Clb6), the Cki Sic1 and Cdc28 (Barberis et al., 2007; Alberghina et al., 2009; Alberghina et al., 2004). Carbon source affects the expression level of the components of both thresholds: for instance, Cln3 and Far1 levels are higher in cells growing on glucose than on ethanol (Hall et al., 1998; Alberghina et al., 2004), whereas Sic1 content is increased in non-fermentable carbon sources (Rossi et al., 2005). The two thresholds cooperate to adjust the critical cell size according to the available carbon source: consistent with this notion, when both the thresholds are inactivated yeast cells lose the capacity to increase their size in presence of glucose (Alberghina et al., 2004).

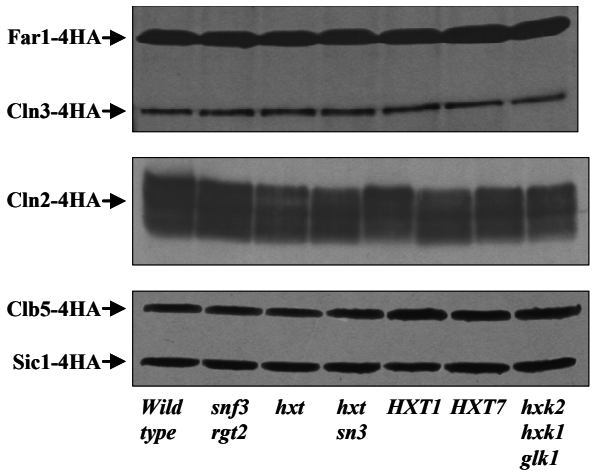
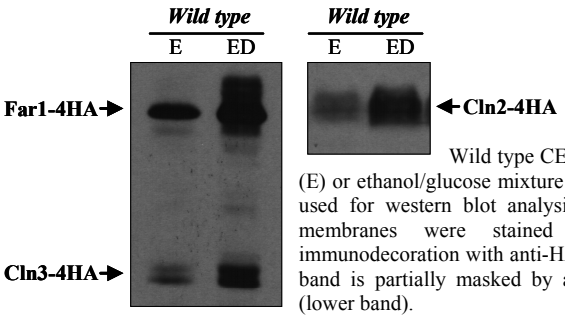


Figure 11
Expression levels of cell cycle regulators in strains impaired for glucose metabolism.
Cells were grown in maltose medium (with the exception of the *hxk2 hxk1 glk1* triple null strains, which were grown or galactose).
50 µg (150 µg for *CLN3HA-FAR1HA* cells) of crude extract were loaded on each lane for western blot analysis.

In order to evaluate if alterations in glucose uptake mechanisms could affect the expression levels of the major cell cycle regulators involved in the G1/S transition, we constructed a complete set of strains (CEN.PK genetic background) expressing HA-C-terminal tagged versions of Cln3, Far1, Sic1, Clb5, Cln2 (Fig. 11; Table I). Attempts to express a functional Whi5-HA protein were unsuccessful (data not shown). The amounts of the various cell cycle regulators (quantified by western blot analysis) were monitored during balanced growth in synthetic complete media supplemented with 2% maltose, 2% ethanol or a 2% ethanol /glucose mixture.

The levels of the various proteins were substantially identical in all the tested strains during growth on media supplemented with ethanol or maltose (Fig. 11).
As expected from previous studies, the expression levels of the Cln3 and Cln2 cyclins in the wild type strain were significantly higher when cells were grown in presence of glucose than in medium containing ethanol as sole carbon source (Fig. 12; Hall et al., 1998; Schneider et al., 2004; Alberghina et al., 2004). Analogously, Far1 content was increased during growth on glucose (Fig. 12; Alberghina et al., 2004). Interestingly, a preliminary analysis suggested that in mutants with reduced glucose uptake capacity the amounts of the Cln3 and Cln2 cyclins might be similarly low both during growth in absence or presence of glucose. However, the experimental data collected so far are unfortunately too unreliable to draw any definitive conclusion: therefore these data were not included in this context and will not be discussed in detail.

Figure 12
Expression levels of CLn3, Far1 and Cln2 in the wild type strain during growth in ethanol or ethanol/glucose mixtures.



Wild type CEN.PK cells were grown in ethanol (E) or ethanol/glucose mixture (ED). 150µg of total extract were used for western blot analysis. As a loading control, blotted membranes were stained with Red Ponceau before immunodecoration with anti-HA antibodies. Note: the Cln3-4HA band is partially masked by a degradation form of Far1-4HA (lower band).

When cells were cultivated on ethanol, no significant differences in the Sic1and Clb5 levels were detected among the various strains (Fig. 13). Consistent with previous findings, the Sic1 content in wild type cells cultivated in glucose containing medium was about 1.5/2-fold lower than the one of ethanol growing cells (Rossi et al., 2005); a similar result was obtained with the *snf3 rgt2* mutant (Fig. 13). Conversely, in strains where glucose metabolism (and thus growth rate on glucose medium) was strongly reduced or even absent (i.e. *HXT7* only strains*, hxt(1-17) gal2 snf3* strain and triple *hxk2 hxk1 glk1* null mutant), the Sic1 levels from cells cultivated either on ethanol or glucose/ethanol mixtures were nearly indistinguishable (Fig. 11): this result is quite interesting, since it might indicate a potential link between glucose metabolism and the coordination of growth and cell division.
Interestingly, neither Clb5 nor (surprisingly) Sic1 specific bands could be detected in extracts from *hxt*-null cells (*hxt(1-17) gal2*) arrested in G1 phase after an ethanol/glucose shift-up (data not shown); furthermore, Cln3 and Cln2 were apparently no longer expressed in the same mutant following glucose exposure (Fig. 14). We therefore hypothesized that the glucose-induced G1 arrest of cell cycle progression in the *hxt*-null strain could arise from its failure to express the Cln3 cyclin, the most upstream activator of *START*; however, the sustained expression of *CLN3* under the control of a strong constitutive promoter did not prevent the arrest of the cell cycle after glucose addition, although in this case *hxt*-null cells did not

accumulate in G1 phase (Fig 15). Therefore, other mechanisms must be responsible for the cytostatic effect of glucose on growth of the *hxt*-null mutant.

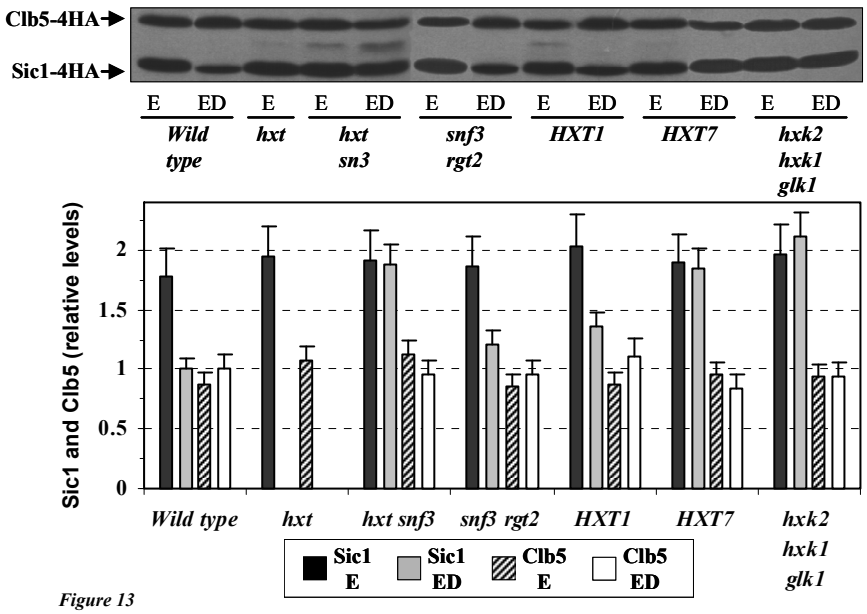


Figure 13
Expression levels of Sic1 and Clb5 in mutants for glucose metabolism during growth in ethanol or ethanol/glucose mixtures
Wild type CEN.PK cells were grown in ethanol (E) or ethanol/glucose mixture (ED). 50µg of total extract were used for western blot analysis. As a loading control, blotted membranes were stained with Red Ponceau before immunodecoration and only equally stained membranes were further processed for densitometric analysis. Blot shown (upper panel) is representative of experiments repeated twice with similar results. Expression levels reported (lower panel) are relative to the Clb5 or Sic1 content quantified by densitometric analysis in wild type cells during growth on ethanol/glucose medium.
Data reported are means ± standard deviation of three independent experiments.

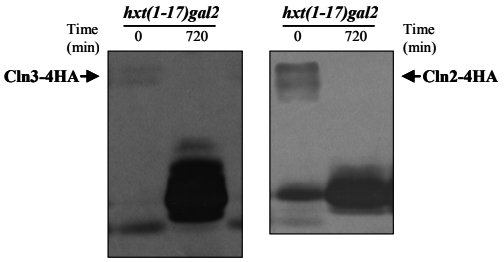


Figure 14
Cln3 and Cln2 are not expressed in the hxt-null strain after the glucose induced cell cycle arrest.
Glucose was added to *hxt(1-7)gal2* cells growing in ethanol and samples were collected at time point 0min (actively dividing cells) and 720min after sugar addition (G1 arrested cells).
150µg of total extract were used for western blot analysis. The experiment was repeated twice with identical results.

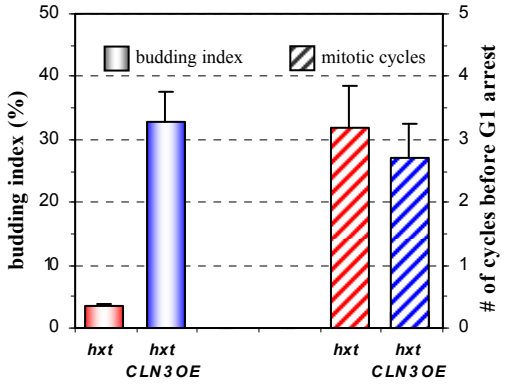


Figure 15
CLN3 overespression does not prevent cell cycle arrest of hxt-null cells in glucose media
Glucose was added to *hxt(1-7)gal2* cells (*hxt*, red bars) and *hxt(1-7)gal2*[pTET-CLN3HA] cells (*hxt CLN3OE*, blue bars) growing in ethanol.
The final budding indexes (left bars) and the rounds of mitotic division completed before the G1 arrest of the cell cycle progression (right bars) are shown.
The number of cell division cycles completed from glucose addition to the definitive arrest of the mitotic cycle was evaluated according to the formula

$$n = \log_2 (N_F/N_0),$$

with N_0 = initial cell density at the moment of glucose addition
and
N_F = final cell density of the culture.
Data reported are means ± SD of two independent experiments.

Glucose mediated regulation of invertase activity is partially defective in strains impaired for sugar metabolism

Previous studies have demonstrated that the glucose-dependent repression of many different genes is correlated with the capacity of the yeast cells to import glucose, but not with the presence of any specific glucose transporter (Reifenberger et al., 1997). Furthermore, a good correlation between the glycolytic flux rate and the degree of glucose repression has also been reported (Reifenberger et al., 1997; Otterstedt et al., 2004; Elbing et al., 2004).
The *SUC2* gene encodes secreted invertase and is paradigmatic in the study of glucose repression. Low levels of glucose (or raffinose) induce *SUC2* expression (Ozcan et al, 1997), whereas in presence of high glucose concentrations *SUC2* transcription is inhibited primarily by the Snf1–Mig1 pathway (Gancedo,, 2008; Luftiya & Johnston, 1996).
Since our strains exhibited different degrees of sugar consumption rate (Fig. 4), we evaluated the state of glucose repression by assaying the invertase activity under different growth conditions.
During balanced growth on 2% ethanol a low basal level of invertase activity was measured in all the mutant strains (Fig 16A, black bars). Similarly, .no significant differences were observed in cells exponentially growing on maltose or galactose media (Fig. 16D, Fig. 16E, black bars). However, the situation changed dramatically when cells were shifted to high glucose (5%: repressing conditions) or low (0.1% glucose: derepressing conditions) media.

The wild type strain behaved as expected from existing literature: upon transfer to media containing a high concentration of glucose, invertase activity became completely repressed within 6 hours (Fig 16A, white bar; Table VI); in contrast, the presence of a low amount of glucose in growth medium strongly induced invertase expression (Fig. 16A, grey bars; Table VI).

Table VI.
Invertase activity relative to the basal value measured in ethanol medium
The values reported are the ratio between the invertase activity in 2% or 0.1% glucose and ethanol. Standard deviation were calculated according to formula

$$\sigma(x/y) = \{\sqrt{[(\mu_y^2 * \sigma_x^2) + (\mu_x^2 * \sigma_y^2)]}/\} \mu_x^2$$

with μ= mean value and σ= standard deviation

Strains	2%glucose	0.1%glucose
wild type (CEN.PK2-1C)	0.08 ± 0.01	5.15 ± 0.40
snf3 rgt2	1.39 ± 0.13	1.11 ± 0.09
hxt-null	5.93 ± 0.55	6.24 ± 0.49
hxt snf3 rgt2	1.32 ± 0.12	0.89 ± 0.07
HXT1	0.33 ± 0.03	11.0 ± 0.86
HXT7	0.46 ± 0.04	7.16 ± 0.56
hxk2 hxk1 glk1	3.36 ± 0.31	3.33 ± 0.26
snf3	0.26 ± 0.02	3.84 ± 0.30
rgt2	0.26 ± 0.04	4.71 ± 0.37
gpa2 gpr1	0.10 ± 0.01	3.19 ± 0.25
snf3 rgt2 gpa2 gpr1	1.68 ± 0.16	1.35 ± 0.11
hxt snf3	7.10 ± 0.65	7.73 ± 0.60
hxt rgt2	6.22 ± 0.57	6.07 ± 0.47
hxt gpa2 gpr1	4.57 ± 0.42	5.68 ± 0.44
hxt gpa2 gpr1 snf3 rgt2	1.15 ± 0.11	1.01 ± 0.08

A similar behavior was exhibited by the strains expressing *HXT1* or *HXT7* as their sole glucose carriers. Nevertheless, the glucose-mediated repression of invertase activity was slightly but significantly defective in these mutants (Fig 16A, black bars; Table VI), which under derepressing conditions also displayed higher invertase expression relative to their isogenic wild type strains (Fig. 16A, grey bars; Table VI). In particular, the strong induction of invertase activity observed in the *HXT1* strain upon shift to low glucose medium (two fold higher than the corresponding value of the wild type strain under the same growth conditions; see Table VI) may be due to the fact that *HXT1* encodes a low affinity hexose carrier which cannot sustain an adequate glycolytic flux when glucose levels are low: consistent with this notion, the *HXT1* strain grows poorly under these conditions. Taken together, these results further support the notion that the extent of glucose repression correlates with the glucose consumption rate (Reifenberger et al., 1997; Otterstedt et al., 2004; Elbing et al., 2004).
Consistent with the hypothesis that induction of *SUC2* expression can take place even in absence of glucose import, a strong increase of invertase activity was detected when the strain lacking all the known hexose transporters (*hxt(1-17)gal2*) was shifted to both high and low glucose media (Fig. 16B, Fig. 16E, white and grey bars; Table VI). An analogous result was reported in early works by Reifenberger and colleagues (Reifenberger et al., 1997), but not in a more recent study, although

in this latter case the experimental set-up was quite different from ours (Elbing et al., 2004a). Surprisingly, the degree of invertase induction in the *hxt*-null strain did not correlate with the glucose concentration in the growth medium, but required the presence of the Rgt2 and Snf3 sensors: in fact, concomitant inactivation of *SNF3* and *RGT2* in a *hxt*-null strain completely abolished the effect (Fig. 16B, Fig.16E, white and grey bars; Table VI). These observations indicate that the role of the Rgt2/Snf2 system in the glucose-dependent derepression mechanisms may be even more relevant than previously suspected (Belinchon et al., 2007; Belinchon et al., 2006; Ozcan, 2002; Gancedo, 2008; Schmidt et al., 1998; Ozcan et al., 1997).
Therefore, in order to deepen our knowledge about the regulation of *SUC2* expression, we extended our analysis to a set of congenic mutants in glucose sensing, transport and phosphorylation mechanisms.
Inactivation of the *GPR1*/*GPA2* branch of the cAMP/PKA pathway (GPCR module) in a wild type background had a modest impact on induction of invertase under derepressing conditions, whereas repression of *SUC2* in high glucose was relatively normal (*gpa2 gpr1* strain: Fig. 16B, white and grey bars; Table VI); no effects on invertase expression were detected upon inactivation of the GPCR system in the *hxt*-null mutant (Fig. 16C; Table VI).
Similarly, the glucose-dependent control of invertase activity was substantially unaffected by single deletions of the glucose sensors encoded by *SNF3* and *RGT2*, aside from a minor defect in invertase derepression exhibited by the *snf3* mutant (Fig. 16B, 16C; Table VI ;Liang & Gaber, 1996; Ozcan et al., 1997; Smith et al., 1998).
In contrast, loss of both the Snf3 and Rgt2 glucose sensors resulted in a severe defect in invertase regulation in both the wild type and (as discussed above) the *hxt*-null background. In fact, invertase activity was not repressed upon shift to 5% glucose in the *snf3 rgt2* strain, whereas the induction of invertase at low glucose levels was essentially abolished in this mutant (Fig. 16B, 16C; Table VI). No additive effect was detected in the quadruple null mutant *snf3 rgt2 gpr1 gpa2* (Fig. 16B, 16C; Table VI).
Apparently, the derepression of *SUC2* in low glucose media does not even require sugar metabolism, since it can occur in a *hxk2 hxk1 glk1* triple mutant strains, which lacks all the three glucose phosporylating enzymes (Fig. 16E). A similar result was previously described by Belinchon and coworkers (Belinchon et al., 2006). In addition, the *hxk2 hxk1 glk1*, as well as all the strains lacking a functional Hxk2 enzyme, exhibited a constitutive high invertase activity both in high and low glucose media, consistent with the widely documented role of Hxk2 in the establishment of the long-term glucose repression (Fig.16D) (de Winde et al., 1996).

In sum, our findings confirm the importance of glucose metabolism in the establishment of the catabolyte repression; however, the contribute of the glucose sensing system should not be underestimated.

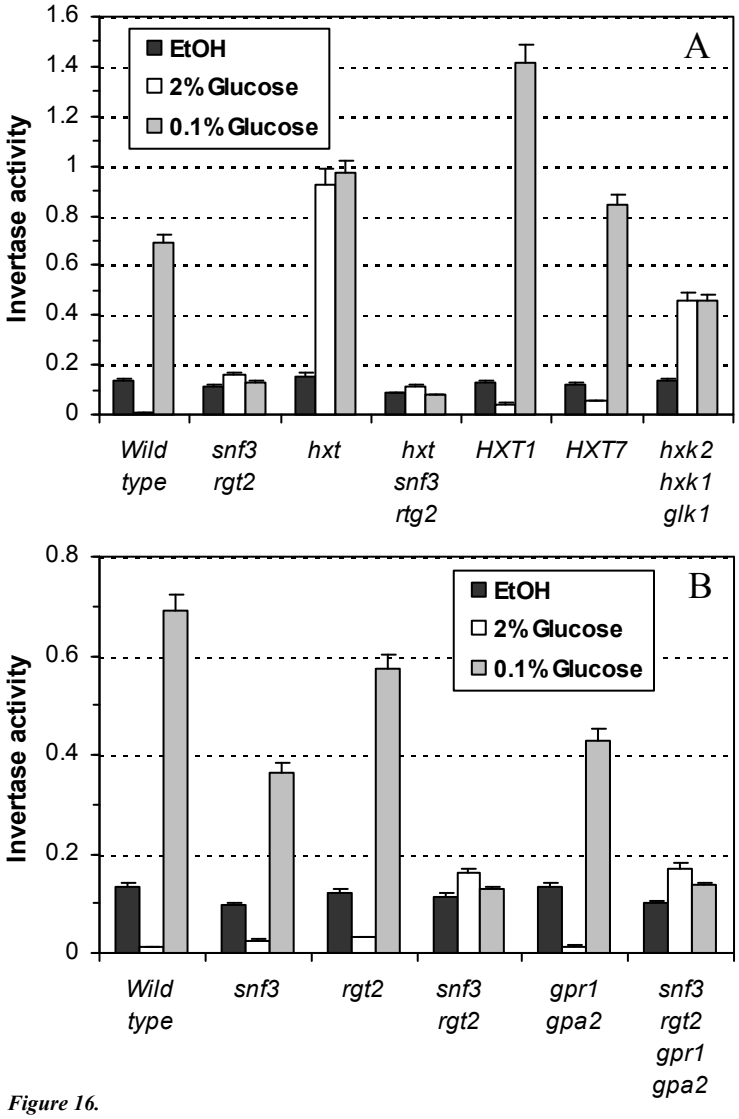


Figure 16.
Invertase activity in strains impaired in glucose metabolism
Invertase activity is expressed as µmol of glucose released from saccharose per minute per 10^8cells ((µmol glucose liberated *min^{-1})/10^8cells).
Mean values ± standard deviations of at least three independent experiments are reported.
Cells were grown in YNB/csm medium supplemented with ethanol as carbon source (panel A-D, except in panel E) and harvested by filtration at cell density <1*10^7cells/mL. Cells were transferred to medium containing 2% ethanol (basal condition, black bars), 2% ethanol plus 2% glucose (repressing condition, white bars) or 2% ethanol +0.1% glucose (derepressing condition, grey bars). After 5h at 30°C, 2*10^8cells were harvested by filtration for the invertase assay.

Figure 16A-B

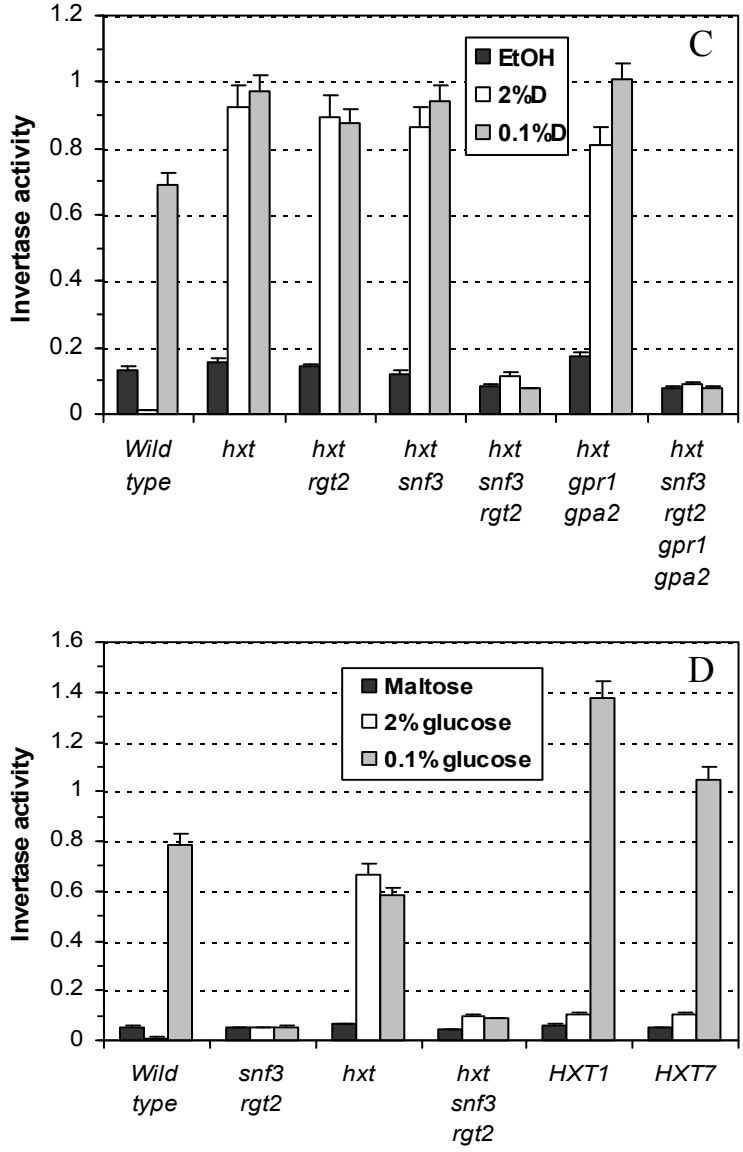

C
EtOH
2%D
0.1%D
Invertase activity
1.2
1
0.8
0.6
0.4
0.2
0
Wild type
hxt
hxt rgt2
hxt snf3
hxt snf3 rgt2
hxt gpr1 gpa2
hxt snf3 rgt2 gpr1 gpa2
D
Maltose
2% glucose
0.1% glucose
Invertase activity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Wild type
snf3 rgt2
hxt
hxt snf3 rgt2
HXT1
HXT7

Figure 16C-D

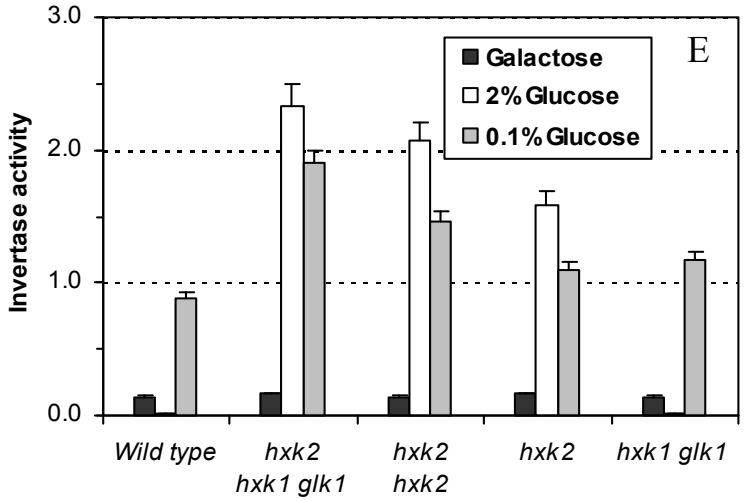

3.0
2.0
1.0
0.0
Invertase activity
Galactose
2% Glucose
0.1% Glucose
E
Wild type
hxk2 hxk1 glk1
hxk2 hxk2
hxk2
hxk1 glk1

Figure 16E

Discussion

Discussion

An essential requisite for the survival of free-living microorganisms like *S. cerevisiae* is the capacity to rapidly adapt to continuous changes in nutrient availability. Not surprisingly, budding yeast has developed sophisticated mechanisms to constantly monitor the nutrients status in its habitat and to respond appropriately by adjusting its transcriptional profile and its metabolic machinery.

Yeast cells set their generation time and the rate of mass accumulation according to nutrient availability: in this way, both growth and proliferation are rapid when large supplies of nutrients are available, whereas they slow down or completely cease when nutrients become limiting or exhausted (Zaman et al., 2008). A closely related issue is the maintenance of cell size homeostasis, which requires a tight and precise coordination between growth (continuous accumulation of cell mass) and cell division (discontinuous events governing duplication and segregation of the genetic material). Yeast cells maintain a constant size over multiple generations by rendering the transition from G1 to S phase of the cell division cycle ("*START*") dependent on the achievement of a "critical size" (Hartwell \et al., 1974; Jorgensen & Tyers, 2004; Cook & Tyers, 2007).

Besides regulating the passage through *START*, nutrient availability influences the size at which cells initiate a new round of mitotic division by modulating the critical size required for cell cycle entry: in fact, yeast cells growing in presence of abundant and good quality nutrients are larger than cells growing under nutrient shortage (Jorgensen & Tyers, 2004).

An extensively characterized example of how yeast cells adapt to changes in nutrient availability is the response to glucose (Santangelo, 2006; Gancedo, 2008; Zaman et al., 2008). Besides being the favorite carbon and energy source for *S. cerevisiae*, glucose can act as a signaling molecule to regulate multiple aspects of yeast physiology. Addition of glucose to yeast cells slowly growing on a non-fermentable carbon source triggers a rapid and massive restructuring of the transcriptional profile, which enables the switch from respiratory to fermentative metabolism and promotes a dramatic increase of the cellular biosynthetic capacity (Zaman et al., 2008). Within minutes from sugar addition, the induction of genes required for sugar uptake, glycolysis and mass accumulation (ribosome biogenesis) occurs, accompanied by the repression of genes involved in respiration, gluconeogenesis, utilization of alternative carbon sources and stress resistance (Zaman et al., 2009; Wang et al., 2004; Slattery et al., 2004). As a result of the glucose-induced reconfiguration of the transcriptional and metabolic profiles, sugar uptake and the glycolytic flux are strongly stimulated, whereas gluconeogenesis and respiration are inhibited. Furthermore, transition to fermentative growth on glucose also coincides with a drastic increase of ribosome production, which enables faster mass accumulation, increase in growth rate and cell size, loss of stress resistance and mobilization of storage carbohydrates (Gancedo, 2008; Santangelo, 2006, Rolland, Thevelein & deWinde, 1999).

The response of yeast cell to glucose is mediated by several sugar sensing systems which organize interconnected and overlapping processes: the cAMP/PKA pathways (with its two branches comprising Ras and the Gpr1/Gpa2 module), the Rgt2/Snf3-Rgt1 pathway and the main repression pathway involving the kinase Snf1 (Zaman et al., 2008; Santangelo, 2006). The cAMP/PKA pathway plays the prominent role in responding to changes in glucose availability and initiating the signaling processes that promote cellular growth and division; the Snf1 and the Rgt2/Snf3-Rgt1

contribute to the cellular response to glucose by regulating the expression of two groups of genes specialized in respiratory/alternative carbon sources metabolism and glucose uptake, respectively (Zaman et al., 2009; Wang et al., 2004; Slattery et al., 2008).
Many interconnections exist between the diverse glucose sensing systems (Gancedo, 2008; Zaman et al., 2008; Santangelo, 2006). Furthermore, glucose signaling requires in most cases at least partial metabolism of the sugar (the only apparent exception being the Snf3/Rgt2 pathway) (Gancedo, 2008; Zaman et al., 2008; Santangelo, 2006): as a result, the roles of glucose as nutrient and signaling molecule are closely intertwined and it is often difficult to separate the two functions.
Therefore, a central issue in our study was to determine whether (and possibly, to which extent) the regulatory function of glucose can be separated from its nutrient function.
In addition, since the precise connections between the glucose sensing pathways and the elements involved in the regulation of the cell cycle progression and size homeostasis are not completely defined, we tried to evaluate possible effects of alterations in glucose sensing and uptake mechanisms on the coordination between growth and cell division.

Glucose transiently modulates size even is strains defective in sugar uptake and phosphorylation

Our findings indicate that glucose might modulate yeast cell size by acting as a signaling molecule ("hormone"), in a way partially independent from its role as nutrient. Consistent with this notion, glucose induces a transient increase of cell size even in strains where sugar metabolism is dramatically reduced or completely abolished, due to the absence of a functional uptake system (*hxt*-null strains) or to the loss of the three kinases catalyzing the first step in glycolysis (*hxk2 hxk1 glk1* strain): in fact, during an ethanol/glucose nutritional shift-up, both the *hxt*-null mutant and the triple *hxk2 hxk1 glk1* null mutant initially respond to glucose addition by increasing their cellular volumes and their average protein content (Fig. 2; Fig. 8; Fig. 10), analogously to wild-type cells. However, the time frame and the kinetic of the "adaptation phase" to glucose in the two mutants are quite different form those of the wild-type strain (Fig. 2; Fig. 8; Fig. 10). Furthermore, in absence of sugar metabolism, the glucose-dependent modulation of cell size is only transient: the effect of sugar addition on both cell volumes and protein content runs out within few generations, when the triple *hxk2 hxk1 glk1*mutant strain again adopt the usual small size typical of the growth on ethanol medium, whereas the *hxt*-null strain undergoes a permanent G1-arrest of the cell cycle progression (Fig. 2; Fig. 8; Fig. 10; see below).
A reasonable explanation for our results may be that the initial effect of glucose on cell size mainly relies on sugar sensing and is partially independent of sugar metabolism; in contrast, long term maintenance of "large size phenotype" requires glucose uptake and metabolism. Consistent with this hypothesis, inactivation of the Gpr1/Gpa2 branch of the cAMP/PKA glucose sensing pathway in the wild type strain reduces the average cell size during balanced growth on glucose media without affecting the other growth parameters (Fig. 1; Table III; Fig. 5-6; Table V; Alberghina et al., 2004; Tamaki et al., 2005)) and strongly delays the reset of cell size during an ethanol/glucose nutritional shift-up (Fig. 2; Fig. 8; Tamaki et al.,

2005; Alberghina et al., 2004). Inactivation of the Snf3/Rtg2 circuit in a wild type background also reduces the average size of glucose growing cells (Fig. 5-6; Table V), although in this case part of the effect is likely due to the decrease in sugar metabolism, as a consequence of the impaired glucose uptake capacity in the *snf3 rgt2* mutant (Fig. IV, Table IV). Even more interestingly, loss of either the Gpr1/Gpa2 or the Snf3/Rgt2 pathways activities in the *hxt*-null mutant substantially abolishes the transient increase of cell size in response to glucose addition (Fig. 8). In order to complete the picture, we are presently trying to evaluate the impact of mutations in the various glucose sensing pathway in the triple *hxk2 hxk1 glk1 null* mutant strain.

Taken together, the available evidences strongly support our proposal that the regulatory function of glucose can be (at least partially) separated from its role as nutrient, since the sugar can modulate cell size even in yeast strains where sugar metabolism is dramatically reduced or completely absent. This conclusion holds true despite the surprising inhibitory effect of glucose on growth of the *hxt*-null mutant (Fig. 8; see below).

Several recent microarray analyses have evaluated the relative contributions of the known glucose sensing pathway to the global transcriptional response triggered by glucose (Zaman et al., 2008; Slattery et al., 2004; Wang et al., 2004): in particular, these studies have demonstrated that it is possible to mimic most of the glucose-induced transcriptional changes even in the complete absence of the sugar by artificially activating the cAMP/PKA signaling circuit (Zaman et al., 2008; Slattery et al., 2004; Wang et al., 2004). Consistent with our proposal that glucose modulation of cell size is partially independent of sugar metabolism, induction of many genes involved in mass accumulation (ribosome biogenesis, protein synthesis) can occur even in a *hxt*-null strain (Slattery et al., 2008) as long as the cAMP/PKA pathway remains functional (Slattery et al., 2008; Zaman et al., 2008; Wang et al., 2004). Such observations are rather surprising, since previous studies demonstrated that glucose signaling requires in most cases sugar uptake and phosphorylation (Gancedo, 2008; Santangelo, 2006): for instance, no cAMP increase was detected in a *hxt*-null mutant or in a triple *hxk2 hxk1 glk1* null strain upon glucose addition (Rolland et al., 2000). Despite these inconsistencies, the results from transcriptomic studies, which point out the key role of the cAMP/PKA circuit in the cellular response to glucose, help us to explain why the inactivation of the Gpr1/Gpa2 pathway eliminates the partial effect of glucose on size of our mutant strains where sugar metabolism is prevented. Furthermore, these recent transcriptomic analyses might offer a plausible interpretation of the cytostatic effect of glucose on growth of the *hxt*-null strain.

Cytostatic effect of glucose in strains impaired for sugar uptake

Apparently, our data indicate that the simple presence of the sugar in the growth medium is sufficient to prevent a strain devoid of sugar transport activity from utilizing other available carbon sources, such as ethanol (Fig. 8): therefore, the glucose dependent repression of genes involved in ethanol metabolism seems to require only sugar sensing, but not sugar uptake and metabolism, at least in our *hxt*-null strain. According to Zaman and colleagues, in *S. cerevisiae* the transcriptional profile is mostly dictated by glucose sensing and not by glucose metabolism: a yeast cell adjust its transcriptional program, its metabolic machinery and its growth rate solely on the basis of its perception of the nutrient status, not on the basis of

metabolites actually produced from the available nutrients (Zaman et al., 2009; Slattery et al., 2008). Under most conditions, this kind of regulation is quite efficient, since the nutrients which the cell recognizes as being present in its living environment are actually available. However, a mismatch between what cell perceives and the real nutrient status (as a result of drug treatment or genetic manipulation) can have dramatic consequences: for instance, activation of the cAMP/PKA pathway in absence of glucose in ethanol-growing cells leads to a rapid decrease in growth rate followed by complete arrest of cell cycle progression (Zaman et al., 2009). Similarly, addition of glucose to our *hxt*-null cells during growth on ethanol might repress transcription of genes required for respiration, thus preventing utilization of ethanol and leading to a permanent G1 arrest (Fig. 8). Nevertheless, the issue is likely more complex: in fact, in contrast to the *hxt*-null strain, the arrest of cell cycle progression in the triple *hxk2 hxk1 glk1* null strain during an ethanol/glucose shift-up is only transient: somehow, this latter mutant can bypass the inhibitory effect imposed by glucose and resume growth by metabolizing ethanol even when glucose is present in the growth media. Therefore, further experiments will be needed to clarify the different behaviors of the two mutants in response to glucose addition.

Surprisingly, inactivation of the *SNF3* sensor is sufficient to suppress the cytostatic effect of glucose on growth of the *hxt*-null mutant (Fig. 8). Unfortunately, an unambiguous interpretation of such result is rather difficult, since loss of *SNF3* function also restores a partial glucose transport activity in the *hxt*-null strain, although not sufficient to sustain growth in liquid medium containing glucose as sole carbon source (Fig. 4; Table IV; Wieczorke et al., 1999). As aforementioned, recent studies have highlighted the existence of numerous crosstalks among the various glucose sensing pathways (Zaman et al., 2009; Zaman et al., 2008; Gancedo et al., 2008; Kaniak et al., 2004; Kim et al., 2006). In particular, it has been shown that the Snf3/Rgt2 pathway contributes to the glucose repression mechanisms by regulating the expression of the Mig2 transcriptional repressor, which participates together with Mig1 to the repression of genes required for the metabolism of alternative carbon sources (Kaniak et al., 2004; Luftiyya et al., 1998; Zaman et al., 2009). However, our data demonstrate that inactivation of *MIG2* does not restore growth of the *hxt*-null strain in medium containing glucose/ethanol mixtures. Therefore, further investigations will be required to clarify if Snf3 actually plays a role in the cytostatic effect of glucose on *hxt*-null cells.

Coordination of growth and cell division in mutants for glucose metabolism: a preliminary analysis

In yeast, the coordination between cell growth and cell division is established by imposing the requirement of a critical size for the passage through *START*, the narrow interval in late G1 phase that regulates the G1/S cell cycle transition. The critical size required for entry into S phase increases with ploidy and responds dynamically to nutrient status, in particular to carbon sources; so that cells grown on ethanol medium are smaller than those grown on glucose; shifting cells between different nutrient conditions leads to a rapid reset of the critical size (Jorgensen and Tyers, 2004; Alberghina et al., 1998; Johnston et al., 1979).

Recently, a new mathematical model which describes the molecular events occurring at the G1/S transition has been developed (Barberis et al., 2007). As a distinguishing feature, the model proposes that two sequential, nutrient modulated

thresholds control the entry into S phase (Alberghina et al., 2004; Barberis et al., 2007). Basically, a molecular "threshold" is given by the interplay between an "activator" and an inhibitor blocking its activity: when the number of molecules of the activator exceeds that of the inhibitor, the threshold is overcome.
The first threshold regulating the G1/S transition comprises the G1 cyclin Cln3 and the Cdk inhibitor (Cki) Far1, whereas the second one involves the S phase cyclin Clb5 (and Clb6) and its associated inhibitor Sic1 (Barberis et al., 2007; Alberghina et al., 2004).
The critical cell size required for budding and DNA replication (as defined by the parameter Ps, the protein content at *START*) is an emergent property of the G1/S network and is strongly influenced by growth rate: in other words, Ps is a property that individual components of the G1/S network do not possess but that emerges from their interaction (Barberis et al. 2007; Alberghina et al., 2009).
According to the model proposed by Barberis and colleagues, the setting of the critical cells size is carried out by a mechanism consisting of a "sizer "plus a "timer" (Barberis et al., 2007; Alberghina et al., 2009). The Far1/Cln3 threshold acts essentially as a growth-sensitive sizer, which is activated at similar cell size during growth in rich (i.e. glucose) or poor (i.e. ethanol) media, since the Cln3/Far1 ratio remains almost equimolecular in the various growth conditions (Alberghina et al., 2004; Hall et al., 1998; Barberis et al., 2007). The first Cln3/Far1 threshold and the second one involving Clb5,6 and Sic1 are temporally spaced (Barberis et al., 2007): therefore, the actual critical size depends not only on the Cln3/Far1"sizer", but also on the length of the "timer", defined as the period elapsing between the crossing of the first threshold and the overcoming of the second one (Barberis et al., 2007). The growth rate (which depends on nutrient availability and quality) is a major factor in determining the critical size required for the G1/S transition: in fact, since it is the passage through the second threshold that actually sets the critical size, fast growing cells will enter into S phase at larger size than slow growing cell (Barberis et al., 2007). Concurrent inactivation of both the thresholds substantially abolishes the carbon source modulation of cell size, indicating that glucose dependent setting of the critical cell size largely (if not entirely) relies on these two cyclin/Cki thresholds (Alberghina et al., 2004).
Recent experimental evidences have confirmed the existence of a "sizing" and a "timing" modules regulating the G1/S transition (Di Talia et al., 2007; Skotheim et al., 2008), thus supporting the validity of the mathematical model by Barberis and colleagues (Barberis et al., 2007).
The growth rate strictly depends on nutrient availability and quality. Furthermore, the nutrient status (and in particular the quantity and quality of carbon source) influences the components of the two thresholds at the level protein abundance and sub-cellular localization (Alberghina et al., 2004; Rossi et al., 2005; Vanoni et al., 2005). For instance, both Cln3 and Far1 levels are higher in cells growing on glucose than in cell cultivated on ethanol (Hall et al., 1998; Alberghina et al., 2004). Conversely, Sic1 content is higher in ethanol growing cells. The sub-cellular localization of Sic1 is also carbon source-modulated: the inhibitor is mostly nuclear in glucose grown cells, whereas partly relocalizes into the cytoplasm during growth on ethanol (Rossi et al., 2005).
We thus decided to investigate if alterations in glucose uptake mechanisms could affect the expression levels of the major cell cycle regulators involved in the G1/S transition. Cln3, Far1, Clb5, Sic1 and Cln2 levels were evaluated in our mutants

during growth in ethanol or ethanol/glucose mixture. In the wild type strain, the pattern of expression for the various regulators in the two growth conditions faithfully reflects previous results: Cln3, Cln2 and Far1 amounts are significantly higher when cells are grown in presence of glucose (Fig. 12; Hall et al., 1998; Schneider et al., 2004; Alberghina et al., 2004). In contrast, our preliminary analysis indicates that in mutants with reduced glucose metabolism the expression levels of the Cln3 and Cln2 cyclins might be extremely low even during growth in glucose containing media; although interesting, the reliability of this data is presently too scarce to draw any definitive conclusion: further analyses to verify these preliminary observations are currently underway.
Interestingly, Cln3, Cln2, Clb5 and (surprisingly) Sic1 specific bands become undetectable in extracts from *hxt*-null cells (*hxt(1-17) gal2*) arrested in G1 phase after an ethanol/glucose shift-up (Fig. 14): this finding might indicate that the glucose-induced G1 arrest of the *hxt*-null strain partly arises from the failure to express Cln3, the most upstream activator of the G1/S transition; however, ectopic expression of *CLN3* in the *hxt*-null mutant does not prevent the arrest of the cell cycle after glucose exposure, suggesting that other mechanisms may be responsible for the cytostatic effect of glucose in this strain.
Again consistent with previous studies, the Sic1 content in wild type cells growing in ethanol medium are about two-fold higher than in glucose grown cells (Fig. 13; Rossi et al., 2005). In contrast, no clear differences between the Sic1 levels in the two growth conditions are detectable in most strains where glucose consumption is reduced/absent (with the exceptions of the *snf3 rgt2* and *HXT1* mutants; Fig. 13). Although promising, these results await further confirmations.

Glucose sensing/metabolism, ribosome biogenesis and coordination of growth and cell division: a future perspective

Analyses to validate these and other observations are currently underway. Furthermore, since the effects of glucose size modulation in our mutants appear to be largely transient, we are presently evaluating the dynamic of the expression levels for the various cell cycle regulators during an ethanol/glucose nutritional shift up.
In addition, several recent studies have revealed surprising connections between nutrient sensing, ribosome biogenesis and cell size (Jorgensen et al., 2004; Jorgensen et al., 2002; Bernstein et al., 2008; Jorgensen & Tyers, 2004; Cook & Tyers, 2007; Zurita-Martinez & Cardenas, 2005). Two of the major nutrient sensing systems, namely the cAMP/PKA pathway (which responds to carbon source) and the TORC1 network (responsive to nitrogen source) regulate the subcellular localization of Sfp1, a "master regulator" controlling a large cohort of genes involved in ribosome biogenesis. The localization of Sfp1 is highly responsive to nutrient conditions: in glucose medium, Sfp1 resides in the nucleus, but it rapidly relocalizes to the cytoplasm upon nutrient starvation or exposure to stress, (Jorgensen et al. 2004; Marion et al. 2004).
According to the model proposed by Tyers and colleagues, the rate of ribosome biogenesis, which is proportional to nutrient quality and abundance, negatively regulates *START* execution, thereby linking the nutrient status to the setting of the critical cell size required for entry into S phase (Jorgensen et al., 2004; Jorgensen & Tyers, 2004; Cook & Tyers, 2007). The rate of ribosome biogenesis parallels nutrients effects: under nutrient shortage, ribosome biogenesis rate is low and cells are small, whereas in presence of abundant and good quality nutrient supply

ribosome biogenesis rate is high and cells are large (Jorgensen et al., 2004; Jorgensen & Tyers, 2004; Cook & Tyers, 2007). Moreover, just like the critical size itself the rate of ribosome biogenesis dynamically and rapidly adapts to changes in nutrient status (Cook & Tyers, 2007; Jorgensen & Tyers, 2004). By coupling the critical size directly to ribosome biogenesis yeast cells can anticipate future changes in their protein synthesis rate (triggered by fluctuations in nutrient availability or stresses) and thus promptly adjust their size long before these changes actually occur (Jorgensen et al., 2002; Tyers et al., 2007; Jorgensen et al., 2004; Jorgensen & Tyers, 2004). Under favorable growth conditions, cells need vigorous ribosome biosynthesis to enable rapid growth and at the same time are interested in delaying cell cycle entry in order to grow to an optimal size: according to the model proposed by Tyers and coworkers, the PKA and the TOR pathway would relay nutrient (and/or stress) signals to Sfp1, thus promoting the transcription of genes involved in ribosome biogenesis and delaying Start execution through an unknown mechanism. Then, when environmental conditions deteriorate, as a consequence of stress or nutrient shortage, cells needs more resources to respond to the hostile situation: under these circumstances, Sfp1 rapidly exits the nucleus, ribosome synthesis slow down and the critical cell size can be consequently reset to a lower value (Jorgensen & Tyers, 2004; Jorgensen et al., 2004; Cook & Tyers, 2007 Rudra et al., 2004;)..
The molecular mechanisms connecting ribosome biogenesis to *START* execution via Sfp1 are largely unknown. It has been hypothesized that these effects are at least partially independent of Cln3 and Whi5, since the critical size can be reset also in strains lacking these upstream regulators of the G1/S transition (Jorgensen et al., 2004; Jorgensen & Tyers, 2004; de Bruin et al., 2004; Costanzo et al., 2004).
The analysis of the subcellular localization of Sfp1 in strains were glucose metabolism is reduced or absent and the main glucose sensing system are inactivated may be helpful to better define this issue.

Regulation of invertase activity in strains with reduced glucose uptake capacity: the critical role of the Snf3/Rgt2 signalling pathway

Numerous studies have documented the existence of a good correlation between the glucose transport capacity and the degree of glucose-mediated repression of various yeast genes, including those involved in respiration, gluconeogenesis and utilization of alternative carbon sources (Gamo et al., 1994; Ozcan et al., 1997; Ozcan et al., 1998; Schmidt et al., 1999; Reifenberger et al., 1997; Ye et al., 1999; Schulte et al., 2000; Ozcan et al., 2002; Elbing et al., 2004a,b; Otterstedt et al., 2004).
For instance, by employing yeast mutants expressing single hexose transporters, it was early demonstrated that the strength of the glucose repression signal on *SUC2* (encoding invertase) *GAL1* (galactokinase) and the *MAL* genes (required for maltose metabolism) did not depend on the presence of a specific carrier, but instead correlated with the different glucose consumption rates exhibited by the various strains (Reifenberger et al., 1997).
A similar result was obtained by Ye and colleagues: by modulating the expression levels of *HXT7* in a strain with no other hexose carriers, their study showed that both the invertase activity and the rate of oxidative metabolism increased proportionally with the reduction of glucose transport capacity (Ye et al., 1999).
A *snf3 rgt2* double mutant strain, which lacks the glucose sensors required to induce the hexose transporters encoding genes (*HXTs*) and thus grows poorly in glucose media, also exhibits a substantial defect in glucose repression that is likely due to

impaired sugar uptake and metabolism (Ozcan et al., 1998; Schmidt et al., 1999; Ozcan et al., 2002): in fact, ectopic expression of the *HXT1* transporter in the *snf3 rgt2* strain is sufficient to restore both growth and glucose repression to wild type level (Ozcan, 2002).

More recent studies have characterized a *S. cerevisiae* strain expressing a chimera between Hxt1 and Hxt7 as its sole hexose carrier: as a consequence of its extremely low levels of sugar import, this strain maintains a full respiratory metabolism even in presence of high glucose concentrations and switches to fermentation only when oxygen is removed, in contrast to wild-type strain which mainly ferments glucose to ethanol and carbon dioxide under the same conditions. (Otterstedt et al., 2004; Elbing et al., 2004a,b; Henricsson et al., 2005; Bosch et al., 2008; Bonander et al., 2008).

Taken together, these evidences strongly support the notion that the signal for glucose repression requires glucose uptake and metabolism (Ozcan, 2002; Belinchon et al., 2007; Gancedo, 2008; Elbing et al., 2004a, Lafuente et al., 2000; Ye et al., 1999). Furthermore, glucose transport seems to play a decisive role in determining the relative activities of the fermentative and respiratory pathways for glucose metabolism, both by dictating the glycolytic flux rate and by influencing the glucose repression status of various metabolic activities (Ye et al., 1999; Otterstedt et al., 2004; Elbing et al., 2004a,b).

Nevertheless, as already discussed elsewhere, several recent studies have shown that activation of the cAMP/PKA signaling circuit in ethanol growing cells is sufficient to trigger at least 90% of the glucose dependent transcriptional changes (both induction and repression), even in complete absence of the sugar (Zaman et al., 2009; Wang et al., 2004; Slattery et al., 2008). Furthermore, a large fraction of the cellular response to glucose can occur even in absence of glucose uptake (Slattery et al., 2008). Therefore, these results apparently downsize the absolute importance of sugar metabolism in the cellular response to glucose.

The status of long-term glucose repression in our strains was determined by measuring their invertase activity after a nutritional shift-up from ethanol to glucose medium (Fig. 16A-E). Consistent with the existing literature, our results confirm that the glucose uptake capacity can significantly affect the extent of glucose repression: in fact, under repressing conditions (i.e. growth in high glucose media) all the strains with reduced glucose consumption rate exhibited a slight but reproducible defect in the repression of invertase activity compared to the wild type reference strain (Table VI).

Furthermore, the glucose dependent repression of invertase activity was completely abolished in strains where glucose metabolism is absent, such as the *hxt(1-17) gal2* mutant strain, which lacks a functional sugar transport system, or the *hxk2 hxk1 glk1* triple null mutant, which is devoid of all the glucose phosphorylating enzyme (Table VI; Fig. 16A-C). Taken together, these observations further underscore the crucial importance of glucose metabolism in the establishment of the catabolite repression: apparently, the presence of high concentrations of glucose in the growth medium is not sufficient to block transcription of glucose-repressed genes; instead, the sugar must be imported in the cytoplasm and phosphorylated in order to trigger the repression mechanisms (Gancedo, 2008; Belinchon & Gancedo, 2007a,b; Gamo et al., 1994; Ye et al., 1999; Otterstedt et al., 2004; Elbing et al., 2004a).

Inactivation of the glucose sensing system comprising Gpa2 and its cognate receptor Gpr1 had no measurable effect on the repression of invertase in presence of high

glucose levels (Table VI; Fig. 16B-C). Furthermore, as discussed above, the defective repression exhibited by the *snf3 rgt2* (Table VI; Fig. 16A-C) strain may be interpreted as a consequence of the diminished glucose uptake in this mutant rather than an evidence of a direct involvement of the Snf3/Rgt2 sensors in the repression mechanisms (Table IV).

Therefore, our findings may indicate that the signal for glucose repression of invertase is generated mainly inside the cell by the glucose metabolism rather than by plasma membrane-localized receptors.

A recent study has also drawn a similar conclusion by demonstrating that most of the glucose effects require sugar uptake and phosphorylation but are largely unaffected by the loss of the glucose sensors Snf3/Rgt2 and the G-protein coupled receptor Gpr1 (Belinchon & Gancedo., 2007b).

However, the issue is likely more complex and several evidences suggest that the mechanism for glucose repression may not be universal, since exceptions are common. For instance, previous studies have demonstrated that glucose repression of several stress responsive genes (*SSA3*, *HSP12*) is delayed in a *gpr1* mutant (Kraakman et al., 1999). Inactivation of *GPR1* also reduces the degradation of fructose 1,6-bisphosphatase in the presence of glucose (Belinchon & Gancedo, 2007b). Furthermore, as discussed above, recent findings have demonstrated that the Snf3/Rgt2 pathway directly contributes to glucose repression by inducing the *MIG2* transcriptional repressor, which collaborates with *MIG1* in the repression of many genes, including *SUC2* (Kaniak et al., 2004; Lutfiyya & Johnston, 1996; Lutfiyya et al., 1998). Therefore, the role of the glucose membrane receptors in the catabolyte repression should not be underestimated.

On the other hand, the regulation of *SUC2* transcription is particularly complex: unlike the *GAL* genes, whose repression in presence of glucose involves Mig1 as the primary (and possibly sole) transcriptional repressor, *SUC2* is regulated by a variety of proteins, including Mig1, Mig2, Ngr1/2, Med8, Gcr1, Sfl1, Sko1 and possibly Rgt1 (Gancedo, 2008; Zhou and Winston, 2001; Herrero et al., 1998; Turkel et al., 2003; Hazburn & Fields, 2002; Kaniak et al., 2004; Luftiyya et al., 1998; Lutfiyya & Johnston, 1996; Chaves et al., 1999; Bu & Schmidt, 1998).

In addition, the picture is further complicated by the fact that the expression of *SUC2* is not only repressed by high glucose levels, but also induced at low glucose levels (Ozcan et al., 1997). As demonstrated by our study, glucose induction of invertase activity can take place even in absence of glucose metabolism and strongly relies on glucose sensing pathways (Table VI; Figure 15A-C): in fact, inactivation of the Gpr1/Gpa2 module reduces the stimulation of *SUC2* expression in the wild type strain under derepressing conditions, whereas loss of the Snf3/Rgt2 sensors substantially eliminates the effect.

The finding that induction of invertase occurs in a strain devoid of any glucose transport activity is quite intriguing, although not completely unexpected. Previous works by Reifenberger and colleagues reported similar results; surprisingly, their study also showed that during growth on ethanol the invertase activity of an *hxt*-null mutant was significantly increased compared with wild type strain, indicating that glucose induction might operate even in absence of external glucose. As an explanation for this result, the authors suggested that under these growth conditions internal glucose might arise by dephosphorylation of glucose 6-phosphate derived from gluconeogenesis: since mutants devoid of sugar transport activity cannot export sugar excess, glucose would accumulate in the cytoplasm in sufficient

amount to trigger induction of invertase (Reifenberger et al, 1997). Although interesting, this model cannot explain why loss of the Snf3 and Rgt2 sensors in our *hxt*-null strain completely abolishes the effect of glucose addiction on invertase activity (Fig 15C; Table VI). Instead, our findings may indicate that in absence of glucose metabolism, the glucose signal for invertase induction is entirely generated by the sugar sensing systems relying on membrane receptors, in particular by the Snf3/Rgt2 pathway. Interestingly, although in most cases glucose signaling is (at least partially) dependent on sugar metabolism (Santangelo, 2006; Gancedo, 2008; Rolland et al., 2002), the activity of the Snf3 and Rgt2 sensors does not require the transport and metabolism of glucose (Ozcan et al., 1996a; Ozcan et al., 1998; Ozcan & Johnston, 1999; Johnston & Kim, 2005; Ozcan, 2002).

The pattern of *SUC2* expression closely resembles the regulation of the *HXT2* and *HXT4* genes (Ozcan & Johnston, 1999): in fact, the expression of all three genes is repressed at high glucose concentrations and induced by low levels of glucose. Since glucose induction of *HXT2* and *HXT4* is mediated by the Snf3/Rgt2 sensors through the Rgt1 repressor, a simple model to explain the effect of the Rgt2/Sfn3 signaling on invertase activity might be that the Rgt1 repressor directly regulates *SUC2* expression. However, invertase activity is not significantly affected by the *RGT1* gene deletion either under repressing or inducing conditions (Palomino et al., 2005; Ozcan et al., 1997).

Recently, it has been proposed that the protein Gis4 may be specifically required for the derepression of *SUC2* in presence of low glucose (La Rue et al., 2005). According to the proposed model, Gis4 is ubiquitinated in a Grr1 dependent fashion, possibly in response to a glucose signal generated by the Snf3/Rgt2 sensors. The ubiquitinated form of Gis4 can interact with the Snf1 kinase, a key player in the glucose repression mechanisms: at low glucose levels, the active Snf1 phosphorylates the transcriptional repressor Mig1 and, together with Gis4, inhibits other negative regulators, allowing the expression of glucose-repressed genes such as *SUC2* (La Rue et al., 2005). Therefore, Gis4 is apparently part of the cross-talk linking the Snf3/Rgt2 pathway to the Snf1 repression pathway.

In a recent study, Belinchon & Gancedo presented convincing evidence that the glucose induction of *SUC2* expression in the *hxk2 hxk1 glk1* triple null mutant is entirely dependent on the presence of a functional Gpr1 receptor (Belinchon & Gancedo, 2007a). Thus, it would be interesting to verify if inactivation of the *SNF3*/*RGT2* sensors may also affect the glucose dependent induction of invertase in the *hxk2 hxk1 glk1* background. Further study to better elucidate this issue are currently underway.

In sum, although our findings confirm that glucose uptake and metabolism play the prominent roles in the regulation of *SUC2* expression, the overall contribute of the sugar sensing systems based on membrane receptor, such as the Snf3/Rgt2 pathway, should not be underestimated.

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"..non c'è più niente da bruciare, solo le bianche ceneri.."