Research Article

Transcriptional regulation of the protein kinase a subunits in Saccharomyces cerevisiae during fermentative growth

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Abstract

Yeast cells can adapt their growth in response to the nutritional environment. Glucose is the favourite carbon source of Saccharomyces cerevisiae, which prefers a fermentative metabolism despite the presence of oxygen. When glucose is consumed, the cell switches to the aerobic metabolism of ethanol, during the so-called diauxic shift. The difference between fermentative and aerobic growth is in part mediated by a regulatory mechanism called glucose repression. During glucose derepression a profound gene transcriptional reprogramming occurs and genes involved in the utilization of alternative carbon sources are expressed. Protein kinase A (PKA) controls different physiological responses following the increment of cAMP as a consequence of a particular stimulus. cAMP–PKA is one of the major pathways involved in the transduction of glucose signalling. In this work the regulation of the promoters of the PKA subunits during respiratory and fermentative metabolism are studied. It is demonstrated that all these promoters are upregulated in the presence of glycerol as carbon source through the Snf1/Cat8 pathway. However, in the presence of glucose as carbon source, the regulation of each PKA promoter subunits is different and only TPK1 is repressed by the complex Hxk2/Mig1 in the presence of active Snf1. Copyright © 2017 John Wiley & Sons, Ltd.

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Introduction

Saccharomyces cerevisiae can adjust its growth in response to nutritional depletion by triggering one of several alternative developmental programmes, depending on the particular nutritional conditions. S. cerevisiae cells consume glucose or any fermentable carbon source preferentially over non-fermentable carbon sources such as glycerol, ethanol or acetate, catabolized by oxidative phosphorylation. Despite the presence of oxygen, when glucose is present, fermentative metabolism is preferred and respiration is repressed. Glucose represses transcription of genes required for initial catabolism of less favourable sugars avoiding the oxidative metabolism. This phenomenon occurring during the fermentative phase of yeast growth is called glucose catabolite repression or simply glucose repression (Broach, 2012).

During the diauxic transition, a switch from fermentative to oxidative metabolism occurs and is accompanied by a major reprogramming of gene expression at both the transcriptional (DeRisi et al., 1997) and post-transcriptional levels (Munchel et al., 2011). A variety of interrelated signalling networks mediates this reprogramming.
of the metabolic and transcriptional capacity of the cell. Glucose effects on biosynthetic capacity and stress responses are mediated by several signalling pathways. One of these pathways, mediated by the protein kinase A, induces genes related with glycolytic enzymes and growth, and represses stress genes and gluconeogenic enzymes (Rolland et al., 2001, 2002). The main glucose repression pathway is mediated by Hxk2, its most important effector being the Mig1 transcription factor. This pathway represses respiratory metabolism and gluconeogenic genes. Another pathway activated by extracellular glucose sensing leads to the expression of glucose transporter genes (HXT genes) and is mediated by the inactivation of Rgt1 (Conrad et al., 2014). This transcriptional repressor, once phosphorylated by Protein kinase A (PKA), is released from its repressive upstream binding sites, leading to derepression of the HXT genes (Kim and Johnston, 2006).

The activation of genes involved in the use of alternative carbon sources is mediated predominantly by Snf1, the yeast AMP-activated kinase. At high glucose concentration Snf1 is inactive and the transcription factor Mig1 is not phosphorylated and therefore localized in the nucleus where it causes the repression of genes involved in the utilization of alternative carbon sources, together with the Ssn6/Tup1 repressor complex (Gancedo, 1998; Carlson, 1999; Hedbäcker and Carlson, 2008). When glucose concentration is limited, Snf1 is active and phosphorylates Mig1, allowing the release of glucose repression and the expression of glucose-repressed genes (Piškur and Compagno, 2014).

Mig1 also interacts with Hxk2 to suppress glucose-repressed genes and Hxk2 interacts with Snf1, resulting in a stable complex in both the presence and absence of glucose. When glucose is abundant Hxk2 interacts with Mig1 at Ser311, a site that is also targeted by Snf1 for phosphorylation (Ahuatzi et al., 2007; Peláez et al., 2010). In this way, Hxk2 avoids Mig1 phosphorylation by Snf1 and thus its removal from the nucleus. Furthermore, when Snf1 is active (glucose depleted), it phosphorylates Hxk2 at Ser14, preventing its nuclear localization and its interaction with transcription factors (Fernández-García et al., 2012). Yeast has two other zinc finger proteins that are closely related to Mig1: Mig2 and Mig3. Mig1 and Mig2 can both upregulate or downregulate several genes and can be partially or completely redundant in its repression or activation function (Westholm et al., 2008). Mig2 has a minor role in glucose repression. Some glucose-repressed genes are synergistically repressed by Mig1 and Mig2 while others are repressed only by Mig1. No genes have been shown to be repressed only by Mig2. Mig3 contributes to glucose repression only on a small number of genes (Lutfiyaa and Johnston, 1996; Lutfiyya et al., 1998; Kaniak et al., 2004). Although MIG2 gene is induced by glucose, the nuclear localization of Mig2 is not controlled by glucose, and Mig2 is not regulated by Snf1 (Kaniak et al., 2004). However Mig3 level is under glucose control. The MIG3 gene is glucose induced (Kaniak et al., 2004), and the Mig3 protein is subjected to Snf1-dependent phosphorylation and subsequent degradation in the absence of glucose (Dubacq et al., 2004).

Cat8 and Sip4 are two transcription factors positively regulated by Snf1 that induce genes containing carbon source-responsive element in their promoter sequences (Hedges et al., 1995; Lesage et al., 1996). Cat8 and Sip4 activation by Snf1 phosphorylation allows (Hedges et al., 1995; Randez-Gil et al., 1997) the upregulation of genes involved in the utilization of non-fermentable carbon sources.

The cAMP–PKA pathway in Saccharomyces cerevisiae controls a variety of essential cellular processes associated with fermentative growth, the entrance into stationary phase, stress response and developmental pathways (Conrad et al., 2014; Rødkaer and Faergeman, 2014; Gancedo, 2008; Palecek et al., 2002). PKA from S. cerevisiae is a tetrameric holoenzyme composed of two regulatory and two catalytic subunits resembling the mammalian counterparts. The regulatory subunit is encoded by only one gene, BCY1, while there are three genes encoding the catalytic subunit: TPK1, TPK2 and TPK3 (Toda et al., 1987a, b). PKA activity negatively regulates its own subunits expression, contributing to control of the specificity of the cAMP–PKA pathway. Although the expression of all the subunits is autoregulated by PKA activity, the global result is that each subunit is expressed differentially during growth on glucose and during heat shock and saline stress (Pautasso and Rossi, 2014).

In this work we study the transcriptional behaviour of PKA subunit promoters in two different
growth conditions: respiratory and fermentative metabolism. We demonstrate that the four promoters share a high transcriptional upregulation in non-fermentable conditions and the regulation through the Snf1/Cat8 pathway. However, in the presence of fermentable carbon source only the TPK1 promoter is regulated by the Hxk2/Mig1 repressor complex through Snf1. Thus, PKA subunit promoters share a mechanism of regulation in the non-fermentable metabolism although they are regulated by different mechanisms under fermentative metabolism.

**Experimental procedures**

**Strains and culture conditions.**

*S. cerevisiae* strains used in this study are summarized in Table 1. Strains were cultivated at 30°C to log phase in synthetic media (SD) containing 0.67% yeast nitrogen base without amino acids, 2% glucose or 2% glycerol plus the necessary additions to fulfill auxotrophic requirements. The cultures were grown until an OD_{600} of 1 at 30°C (~0.5 × 10^7 to 1 × 10^8 cells/mL).

**Plasmids**

The plasmids used to measure the promoter activities were derived from the YEp357 plasmid (Myers et al., 1986). The TPK1-lacZ, TPK2-lacZ, TPK3-lacZ and BCY1-lacZ fusion genes contain the 5' regulatory region and nucleotides of the coding region of each gene (positions –800 to +10 with respect to the ATG initiation codon in each case). The pOV84 plasmid contains the wild-type (WT) version of the SNF1 gene (pl Snf1), and its derivative, the pKH43 plasmid, contains the T210A mutant version of the SNF1 gene (pl Snf1 T210A) (Hedbacker et al., 2004).

**β-galactosidase assays**

Cells were grown on SD medium up to an OD_{600} of 1. Aliquots (10 mL) of each culture were collected by centrifugation and resuspended in 1 mL buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM MgSO_4). β-Galactosidase activity measured according to Miller (1972) was expressed as Miller units. The results are expressed as the means ± SD of the total replicate samples (n = 4, n = 6 or n = 8) coming from independent assays (two, three or four experiments). *tpk1w1BCY1* and *TPK1bcy1Δ* strains have a SP1 genetic background, while in the rest of the mutants used in this study the genetic background corresponds to BY4741. Promoter activities assessed in the WT strains of the different genetic backgrounds showed no differences (data not shown).

**Chromatin immunoprecipitation assays**

Chromatin immunoprecipitation (ChIP) was performed as described previously with modifications (Kuras and Struhl, 1999). Rpb1, Mig1 or Cat8-TAP tagged strains (50 mL culture) were grown in glucose- or glycerol-containing media to an

| Table 1. Yeast strains. |
|------------------------|
| **Strain**          | **Genotype**                   | **Reference or source**     |
| SP1 (WT)             | *Matα his3 leu2 ura3 trp1 ade8* | Toda et al. (1987a, b)       |
| S18-1D (tpk1w1BCY1)  | (SP1) tpk1w1 tpk2::HIS3 tpk3::TRP1 | Nikawa et al. (1987)         |
| 133 (TPK1 bcy1Δ)     | (SP1) tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 | Cameron et al. (1988)       |
| BY4741 (WT)          | *Matα his3Δ1 leu2Δ10 met15Δ0 ura3Δ0* | EUROSCARF                   |
| mig1Δ                | (BY4741) mig1::KanMX4           | EUROSCARF                   |
| mig2Δ                | (BY4741) mig2::KanMX4           | EUROSCARF                   |
| mig3Δ                | (BY4741) mig3::KanMX4           | EUROSCARF                   |
| snf1Δ                | (BY4741) snf1::KanMX4           | EUROSCARF                   |
| cat8Δ                | (BY4741) cat8::KanMX4           | EUROSCARF                   |
| sip4Δ                | (BY4741) sip4::KanMX4           | EUROSCARF                   |
| rgt1Δ                | (BY4741) rgt1::KanMX4           | EUROSCARF                   |
| Cat8-TAP             | (BY4741) Cat8-TAP::HIS3MX       | Open Biosystems             |
| Mig1-TAP             | (BY4741) Mig1-TAP::HIS3MX       | Open Biosystems             |
| Rpb1-TAP             | (BY4741) Rpb1-TAP::HIS3MX       | Open Biosystems             |

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OD$_{600}$ 1 and fixed for 20 min at room temperature with 1% formaldehyde (final concentration). Glycine was then added to give a final concentration of 125 mM and incubated for 5 min. Cells were harvested, washed with ice-cold Tris buffered saline and resuspended in 1 mL of FA lysis buffer (50 mM HEPES/KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and 2 mM phenylmethylsulphonyl fluoride). An equal volume of glass beads (0.5 mm diameter; Sigma) was added and the cells were disrupted by vortexing for 40 min at 4°C. The lysates were separated from the glass beads, and chromatin was then pelleted by centrifugation (17 000 g for 15 min) and resuspended in 1 mL of fresh FA lysis buffer. Samples were sonicated to obtain DNA fragments with an average size of 500 bp (Branson Sonifier; 3–10 s at 15% amplitude) and clarified by centrifugation at 17 000 g for 15 min. A 1 mg aliquot of protein was used for each immunoprecipitation. IgG-Sepharose 6 Fast Flow (GE Healthcare) was washed with PBS plus 5 mg/mL BSA. Samples were incubated with the resin in a rotator overnight at 4°C. Immune complexes were sequentially washed four times with FA lysis buffer, four times with FA lysis buffer containing 500 mM NaCl, four times with wash buffer (10 mM Tris–HCl pH 8, 0.25 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate) and once with Tris–EDTA buffer. Bound proteins were eluted from the resin by adding elution buffer (50 mM Tris–HCl pH 8, 10 mM EDTA, 1% SDS) and incubated for 1 h at 37°C with proteinase K (0.25 mg/mL). Cross-linking was reversed, incubating 5 h at 65°C. DNA was purified using a QIAquick PCR purification kit (Qiagen). Real-time quantitative PCR was carried out with an Opticon Monitor 3 (Bio-Rad), using primers that amplified different regions of the TPK1 promoter. DNA detection was performed with SYBR Green. A pair of primers that amplify a region located outside TPK1 promoter was used as an unbound control (POL1 region). ChIP DNA was normalized to input DNA and calculated as a signal-to-noise ratio over a non-TAP tagged strain control ChIP. The $\Delta \Delta CT$ method was used to calculate the fold change of binding to the promoter of interest (Livak and Schmittgen, 2001). Error propagation was handled using standard root mean square methods. Primers used on TPK1 promoter were: forward, 5’ AGCTGTGCTGCTATTCCGTT CT 3’; reverse, 5’ ACTTTTACGGAGATCCCCGTT TT 3’, for Mig1 and Rpb1 binding; and forward, 5’ GAGCAATGCTTTGGTAAAGGT 3’; reverse, 5’ TGGAAAGATGGCAACAAATG 3’, for Cat8 binding. Control ORF POL1 was: forward, 5’ CTGCACTGCAAAACAGAAA 3’; reverse, 5’ TCTTAAAC GACGGCAATAGA 3’.

Quantitative real-time PCR

Total RNA was prepared from different yeast strains, grown up to the same OD$_{600}$ as for $\beta$-galactosidase assays, using standard procedures. The relative levels of specific TPK1, TPK2, TPK3 and BCY1 mRNAs were measured by quantitative RT-PCR. Aliquots (~10 μg) of RNA were reverse-transcribed into single-stranded complementary cDNA using an oligo-dT primer and Superscript II reverse transcriptase (Invitrogen). The single-stranded cDNA products were amplified by PCR using gene-specific sense and antisense primers (mRNA TPK1: forward, 5’ CGAGCA GACCATGTGTC GAC 3’; reverse, 5’ GTACCACGACCTGGTGC 3’; mRNA TPK2: forward, 5’ GCTTGT GGAGCATCCGTTC 3’; reverse, 5’ CACTAAACATGGGTGAC 3’; mRNA TPK3: forward, 5’ CGTTGGAACAAG ACATTCTG 3’; reverse, 5’ GTCCGTATAC 3’; mRNA BCY1: forward, 5’ CGAACGGACAC TACCTACGAC 3’; reverse, 5’ GCCTGATTCG 3’; mRNA TUB1: forward, 5’ TUB1; reverse, 5’ CACCAAGTCTGGTCTA CCCATTC 3’; reverse, 5’ GGATAAGACTG GAGAANATGAAC 3’). The PCR products were visualized using SYBR Green. The relative mRNA levels of TPK1, TPK2, TPK3 and BCY1 were first normalized to those of TUB1 (a-tubulin gene) and then compared with each other. Quantitative data were obtained from three independent experiments and averaged.

Results

TPK1, TPK2, TPK3 and BCY1 are upregulated in the presence of glycerol as carbon source

TPK1, TPK2, TPK3 and BCY1 promoter activities were first assessed using promoter-lacZ-based reporter assay to measure the expression of lacZ
driven by each of the four non-coding upstream sequences (−800 to +10 with respect to the ATG initiation codon), in WT cells grown in 2% glycerol or glucose as carbon sources. Figure 1(a) shows the β-galactosidase activity for each promoter measured in the presence of glycerol in comparison with the same measurement in the presence of glucose. The promoters were upregulated when the cultures were grown in glycerol-containing medium. This was surprising since it is known that there is low activity of the PKA pathway when yeast cells are grown in the presence of a poor carbon source or during stationary phase (Rubio-Texeira et al., 2010). mRNA levels of each subunit analysed by quantitative real-time PCR (qRT-PCR) (Figure 1b) were in agreement with the promoter activities. The results indicate that there is a high level of transcriptional activity in a situation in which low PKA activity levels are required as in the nutritional stress of growth in glycerol.

PKA activity regulates TPKs promoter activities in the presence of glycerol as carbon source

Recently published results from our group indicate that PKA subunit promoters are downregulated by PKA activity under fermentative growth conditions (Pautasso and Rossi, 2014). In order to evaluate whether this regulation also occurs during respiratory metabolism, we assessed the promoter activity of each TPK-lacZ and BCY1-lacZ fusion gene in different TPKs yeast strains: WT, tpk1wt/BCY1 strain (carrying an attenuated form of Tpk1 and deletion of TPK2 and TPK3 genes, resulting in very low PKA activity) and TPK1bcy1Δ strain (strain with a deregulated PKA activity). TPK2 and TPK3 promoters showed induction of their activities in the tpk1wt/BCY1 strain, which was completely reverted in the TPK1bcy1Δ strain (Figure 2a). TPK1 and BCY1 promoters were not upregulated in tpk1wt/BCY1 strain but were downregulated in TPK1bcy1Δ strain. A possible explanation for this particular behaviour is that in the presence of glycerol TPK1 and BCY1 promoters are already at the maximum of their activities and insensitive to a further attenuation of PKA activity, although they are sensitive to downregulation by high PKA activity. These results support the idea that PKA activity generates the downregulation of PKA subunits expression in the presence of glycerol as occurs during fermentative metabolism. TPK1 and BCY1 mRNA levels (when present) were measured by qRT-PCR (Figure 2b) in the WT and mutant strains and the results were consistent with the promoter activities.

PKA subunit promoters are regulated by Snf1

Taking into account that glycerol is regulating the activity of PKA promoters, we decided to analyse the participation of the protein kinase Snf1 in this regulation, since this kinase is known to regulate the expression of genes involved in the use of alternative carbon sources. Snf1 works through different mediators such as Mig1, Cat8 and Sip4. As a first approach we analysed the promoter sequences in silico using the Yeastract server (Teixeira et al., 2006, 2014; Abdulrehman et al., 2011), looking...
for the presence of DNA consensus sequences for the binding of these transcription factors in the TPKs and BCY1 promoters. However no common clear picture could be derived from this analysis regarding putative binding sequences shared by these promoters, nor from published array genomic results (Westholm et al., 2008; Reimand et al., 2010; Yeastrec server, Spell from SGD http://www.yeastgenome.org; Chua et al., 2006).

We therefore decided to test the promoters in strains lacking various transcription factors involved in carbon source metabolism such as mig1Δ, mig2Δ, mig3Δ, cat8Δ, sip4Δ, rgt1Δ strains, as well as in a snf1Δ strain. These strains were transformed with the plasmids containing the different promoter-lacZ-based reporters and the promoter activity measured in cultures grown in the presence of glycerol or glucose (Figure 3). The first conclusion from Figure 3 is that the activity of the promoters of the three TPKs genes is positively regulated by the Snf1 signal transduction pathway, particularly when glycerol is used as carbon source although also during growth in glucose. The promoter of BCY1 seems to be absolutely dependent on Snf1 for its activity in both carbon sources. The results of an effect of Snf1 under glucose growth such as the one shown by BCY1 promoter, and less severely by TPK1 promoter, is particularly interesting because Snf1 is supposed to be inactive under fermentative conditions. When the carbon source is glycerol it is very evident that Cat8, a transcriptional activator of genes from respiratory metabolism regulated by Snf1, is involved as an activator of the four promoters activities. It is known that the two zinc cluster transcription factors Cat8 and Sip4 play central roles in gene expression during adaptation to alternative carbon source and that both share the recognition of a Carbon Source Responsive Element in the genes they regulate (Turcotte et al., 2009). However in this case, although the four promoters are strongly repressed in the absence of Cat8, particularly when glycerol is the carbon source, the deletion of Sip4 has no effect except for TPK2, in glucose, in which case Sip4 seems to be a repressor, since its deletion increases TPK2 promoter activity. The results indicate that Sip4 and Cat8 have non-overlapping functions on the four promoters (Figure 3g and h).

We chose the TPK1 promoter to further investigate the mechanism through which Snf1 might up-regulate its activity in glucose. Figure 3(a) shows that the deletion of the repressive transcription factor Mig1 produces an upregulation of the TPK1 promoter, an effect observed only for this promoter. This result is in accordance with the known inverse interrelationship between Snf1 and Mig1 regarding glucose repression of gene regulation. However no effect was observed when the closely related transcription factors Mig2 and Mig3 were deleted, indicating in this case that the three proteins do not have the same function in the regulation of the expression of TPK1 promoter. Since in glucose

Figure 2. TPKs and BCY1 promoters are regulated by PKA activity in glycerol-containing medium. (A) β-Galactosidase activity was determined in WT (BY4741), tpk1ΔBCY1 and TPK1bcy1Δ strains carrying TPKs-lacZ or BCY1-lacZ fusion genes. Cultures of each strain were grown in glycerol containing medium to log phase (OD600 1). For each reporter the values measured in each mutant strain were expressed relative to the value obtained in the WT strain. The results are expressed as fold induction means ± SD from replicate samples (n = 8) from independent experiments. (B) TPK1 and BCY1 endogenous mRNA levels were determined by qRT-PCR in WT (BY4741), tpk1ΔBCY1 and TPK1bcy1Δ strains cultures grown in the presence of glycerol and normalized to TUB1 mRNA.
Figure 3. Snf1 kinase regulates TPKs and BCY1 promoter activities. β-Galactosidase activity was determined in WT (BY4741), mig1Δ, mig2Δ, mig3Δ, snf1Δ, snf1Δ + pl Snf1, snf1Δ + pl Snf1 T210A, cat8Δ, sip4Δ, rgt1Δ and hxx2Δ strains carrying TPK1-lacZ (A and B), TPK2-lacZ (C and D), TPK3-lacZ (E and F) or BCY1-lacZ (G and H) fusion genes. Cultures of each strain were grown to log phase (OD_{600} 1) in either glucose (A, C, E and G) or glycerol (B, D, F and H) medium. For each condition, the values measured in each mutant strain were normalized to the value obtained for the WT strain. The results are expressed as fold induction means ± SD from replicate samples (n = 6) from independent experiments.
Mig1 acts with Hxk2, the results presented so far could indicate that Snf1 is regulating the promoter through the Mig1-Hxk2 complex (Ahuatzi et al., 2007). We analysed the participation of Snf1, Hxk2 and the direct participation of Mig1 on TPK1 promoter regulation in the presence of glucose as a carbon source. It has been reported that during growth in glucose the phosphatase Gclc7Reg1 inactivates Snf1 kinase by dephosphorylating hr210 in the catalytic loop (Momicilovic et al., 2008). However some reports indicate that Snf1 could be active in the presence of high glucose (Ciriacy, 1977; Denis, 1984; Ahuatzi, et al., 2007; Pessina et al., 2010; Busnelli et al., 2013; Nicostratos et al., 2015). Published reports also suggest a role for the non-phosphorylated form of Snf1 during glucose growth, since Hxk2 is required to inhibit Mig1 phosphorylation by Snf1 kinase in high glucose growth conditions (Ahuatzi et al., 2007). We therefore assessed the effect of the deletion of Hxk2 (Figure 3a), and observed an expected upregulation of TPK1 promoter in agreement with the upregulation in the mig1Δ strain. These results suggest that, as a consequence of Hxk2 deletion, Snf1 can phosphorylate Mig1, resulting in the liberation of repressive conditions on TPK1 promoter when cells are grown in the presence of glucose.

Since the results suggest a role for Snf1 under repressive conditions, we assessed whether the non-phosphorylated or the phosphorylated form of Snf1 was involved in this effect. The snf1Δ-p-TPK1-lacZ strain was transformed with a plasmid expressing the WT version of Snf1 kinase (snf1Δ + pl Snf1 strain) or with a plasmid expressing a mutant version of Snf1 in which the Thr210 is replaced by Ala and therefore cannot be phosphorylated (snf1Δ + pl Snf1 T210A strain; Hedbacker et al., 2004). The results in Figure 3 (a) show that only the WT version of Snf1 could revert the snf1Δ strain defect on TPK1 promoter activity to the one of the control. The strain carrying the Snf1 T210A mutant plasmid and the snf1Δ strain showed the same downregulation in comparison with WT strain. These results indicate that a phosphorylatable and active Snf1 was necessary for the upregulation of TPK1 promoter in the presence of glucose. The results therefore indicate that the Mig1–Hxk2–Snf1 complex participates in the repression of TPK1 promoter by glucose: Hxk2 avoids the inhibitory phosphorylation of Mig1, allowing this transcription factor to act as a repressor. Snf1 would be active in the complex, performing its regulation through other regulators, as Cat8 transcription factor.

Although all the promoters have in common the regulation by the Snf1/Cat8 pathway, each one has individual features. TPK3 was shown to be upregulated by Mig1 in glycerol (Figure 3f); however, in the presence of glucose, TPK3 promoter activity was not modified in any of the migΔ strains (Figure 3e). This result is in contrast with published results from microarrays pointing to the downregulation of TPK3 mRNA levels in a migΔ strain in comparison with WT strain (Westholm et al., 2008). BCY1 promoter displays a regulation quite different from the catalytic subunit promoters, since it is downregulated by Mig3 in glucose and by Rgt1 in glycerol (Figure 3g and h). Regarding Hxk2 regulation on each promoter, TPK2, TPK3 and BCY1 showed a different behaviour in the presence of glucose. BCY1 and TPK3 did not show any change in the hxx2Δ strain but TPK2 promoter was upregulated in this strain (Figure 3c).

mRNA levels of TPK1, TPK2, TPK3 and BCY1 measured in the snf1Δ, mig1Δ and cat8Δ strains by qRT-PCR were consistent with the promoter activities in the same strains (Figure 4).

Finally, the direct participation of Mig1 and Cat8 on TPK1 promoter regulation was analysed by ChIP assay (Figure 5), using yeast strains containing TAP-tagged versions of Mig1, Cat8 or Rpb1. We measured an elevated occupancy of Mig1 transcription factor on TPK1 promoter in the presence of glucose and the opposite result for Cat8, an increase in its occupancy in the presence of glycerol. The higher Rpb1 occupancy (Figure 5) is in agreement with a greater promoter activity under glycerol growth conditions. Taken together, these results indicate that during glucose repression Mig1 downregulates directly TPK1 promoter in a complex with Hxk2, while Cat8 does not. The activator effect of Cat8 on TPK1 promoter in the presence of glucose is probably the result of an indirect effect, possibly through the transcriptional regulation of other gene or group of genes.

**Discussion**

In this study we have investigated the regulation of PKA subunit promoters TPK1, TPK2, TPK3 and
Figure 4. TPKs and BCY1 mRNA levels are regulated by Snf1. TPK1 (A and B), TPK2 (C and D), TPK3 (E and F) and BCY1 (G and H) endogenous mRNA levels were determined by qRT-PCR in WT (BY4741), mig1Δ, snf1Δ and cat8Δ strains grown in the presence of glucose (A, C, E and G) or of glycerol (B, D, F and H). Values are normalized to TUB1 mRNA. For each condition, the values measured in each mutant strain were expressed relative to the value obtained for the WT strain taken as 1. The results are expressed relative to wild-type strain ± SD from replicate samples (n = 4) from independent experiments.
BCY1 in the presence of glycerol or glucose as carbon source. We demonstrate that the involvement of the Snf1/Cat8 pathway is a common feature in the regulation of the four promoters (Figure 6). A striking point in this regulation is the role of Snf1 in glucose growth (Figure 3), a condition in which Snf1 should not be active. Under low-glucose conditions, Snf1 kinase is phosphorylated and therefore is activated (Turcotte et al., 2009). Snf1 kinase forms different complexes with the γ-regulatory subunit Snf4, which protects active Snf1 (Mayer et al., 2011) and with different β-subunits Sip1, Sip2, or Gal83 which determine the localization of Snf1 to distinct subcellular locations (Vincent et al., 2001). When glucose levels increase, Snf1 is dephosphorylated in Thr210 by Gclc and the kinase becomes inactive; the presence of Hxk2p protein contributes to this situation (Treitel et al., 1998; Sanz et al., 2000; McCartney and Schmidt, 2001). The active Snf1 protein kinase phosphorylates Mig1 protein in low-glucose growth conditions (Treitel et al., 1998). Under these conditions Mig1 translocates from the nucleus to the cytoplasm. In high glucose, Mig1 is dephosphorylated and localizes in the nucleus, where it represses genes transcription.

The Hxk2 nuclear localization is also modulated by the availability of glucose, and Mig1 is required to maintain Hxk2 in the nucleus (Dolz-Edo et al., 2013) through direct interaction (DeVit et al., 1997; Ahuatzi et al., 2004). A key residue for the interaction of Mig1 with Hxk2 is Ser311, which is also the target for inhibitory phosphorylation by Snf1. It has been demonstrated that Snf1 interacts constitutively with Hxk2, forming a complex in high- and low-glucose conditions (Ahuatzi et al., 2007). It was also demonstrated that Snf1 binds to Mig1 under low-glucose conditions and this binding is largely avoided after a shift to high-glucose medium (Ahuatzi et al., 2007). Therefore, it is proposed that Hxk2 interacts both with Mig1 and Snf1 in a complex that is maintained in high glucose, inhibiting Snf1 to phosphorylate the Mig1 Ser311 residue (Ahuatzi et al., 2007).

Our results indicate that the putative Snf1-Mig1-Hxk2 complex regulates TPK1 promoter activity during high glucose (Figure 3a), as snf1Δ mutant strain showed a downregulation and hxxk2Δ and mig1Δ, an upregulation of the promoter activity. A well-known effector of Snf1, the Cat8 transcription factor, also participates in controlling TPK1 promoter activity (Figure 3a); cat8Δ and snf1Δ strains exhibited the same effect on TPK1 in glucose medium. Sip4, another transcription factor downstream of Snf1, has no effect on TPK1 promoter in the corresponding mutant deletion strain (Figure 3a). It should be noted that Cat8 and Sip4 have been proposed to bind to the same type of promoters although with different affinities, Sip4 being a weaker activator than Cat8 (Portillo et al., 2005). Our finding suggests that, at least in glucose medium, Sip4 has a separate function from that of Cat8. The same result has been also proposed for the regulation of TRK1 (potassium transporter gene) and HAL5 (activating protein kinase gene) (Portillo et al., 2005).

There is a hypothetical model postulated to explain the participation of Snf1 in the activation of genes implicated in functions like K + transport...
(Portillo et al., 2005). In this model it is considered that the Snf1 protein kinase complex has a dual role in yeast cells depending on glucose availability and, therefore, on the phosphorylation status of Thr210. In glucose-starved cells, Snf1 has the Thr210 phosphorylated, and hence the kinase complex is fully active, and is able to regulate a set of transcriptional factors which promote the utilization of alternative carbon sources. In high-glucose cells, Snf1 is non-phosphorylated, but still has some activity level. Snf1 can form a complex that could activate a different set of transcriptional factors, which in turn would activate the expression of other genes implicated in other functions that adapt the cell to the growth in fermentable sugar. This model could be applied to TPK1 promoter regulation since, although all the promoters respond to Snf1 both in glucose-starved medium and in high-glucose medium, only the TPK1 promoter seems to be regulated by the Snf1–Hxk2–Mig1 complex (Figure 3). Our results suggest that, in the presence of glucose, the phosphorylatable form of Snf1 (Figure 3a) acts through Cat8 to indirectly activate the transcription of TPK1 gene. In this growth condition, the amount of active Snf1 would not be sufficient to achieve the inactivation of Mig1. Thus, this transcription factor contributes to the downregulation of TPK1 promoter when cells are growing in the presence of glucose.

Hxk2 is not involved in the regulation of TPK3 and BCY1 promoters; however the disruption of Hxk2 has an effect on TPK2 promoter. There are other antecedents of genes which show a similar repressive regulation by Hxk2 as the TPK2 promoter, in which Mig1 is dispensable (Kartasheva et al., 1996; Hu et al., 2000).

As we have mentioned, the PKA subunit promoters are upregulated when the cultures are grown in glycerol-containing medium. However, an unregulated PKA activity has been shown to impair the establishment of stationary phase (Boy-Marcotte et al., 1987). Published results

Figure 6. Summary of the regulation of TPKs and BCY1 promoters. For more details see the text. The thick \( \rightarrow \) indicates a higher expression level than in glucose (thin arrow). (\( \rightarrow \)) indicates activation and (\( \leftarrow \)), in dicates repression.
and previous results from our group (Portela et al., 2003; Rubio-Texeira et al., 2010) have also shown that the endogenous PKA activity is lower in cells from stationary-phase or respiratory-phase compared with exponentially glucose growing cells. These results seem to be paradoxical since the PKA subunit promoters are upregulated but the PKA holoenzyme activity is low. In cells growing on glycerol it was observed a Bcy1 expression level higher than the one of the catalytic subunits, and an increase in Tpk1 and Tpk2 levels as compared with glucose, whereas Tpk3 levels always remain low, under glucose and glycerol growth conditions. It is calculated that, in cells growing in glucose and glycerol, the amount of Bcy1 is stoichiometrically equivalent to the amount of total Tpk. The compartmentalization of Tpk2 and Tpk3 in cytoplasmic granules could be an alternative to sequester the excess catalytic amount of total Tpk. The compartmentalization of Tpk2 and Tpk3 in cytoplasmic granules could be an alternative to sequester the excess catalytic subunits in glycerol growth conditions (Tudisca et al., 2012). This apparent contradiction in glycerol conditions in which PKA protein is present when the activity is low is possibly the result of a cell strategy aiming to rapidly stimulate fermentative growth. The specificity of cAMP-signal transduction is maintained by several levels of control acting all together. One of these levels is the regulation of the expression of each PKA subunit gene. Previously we have shown that the four promoters share a negative mechanism of isoform-dependent auto-regulation, which directs TPKs and BCY1 gene expression. The promoter of each TPK isoform and of BCY1 is differentially activated during the growth phase and only TPK1 promoter activity is positively regulated during heat shock and saline stress (Pautasso and Rossi, 2014). Here we demonstrate that, during the growth in a non-fermentable carbon source, the promoters share a pathway of regulation that is dependent on Snf1/Cat8. In addition, Snf1 regulates the four promoters in the presence of high glucose, indicating that the kinase would be active in this condition. Finally, only TPK1 seems to be regulated by the putative Mig1–Hxk2–Snf1 complex. These, and another individual features on TPKs and BCY1 promoter regulation contribute to the idea that each PKA subunit is regulated in a particular way, therefore allowing the maintenance of the specificity in the cAMP–PKA pathway.

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The authors declare that there is no conflict of interest.

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**Supporting information**

Additional Supporting Information may be found online in the supporting information tab for this article.

**Figure S1.** β-Galactosidase activity was determined in WT (BY4741) and in SP1 strains carrying TPK1-lacZ. Cultures of each strain were grown to log phase (OD600 1) in either glucose (white bars) or glycerol (grey bars) medium. The results are expressed as fold induction means ± SD from replicate samples (*n* = 3) from independent experiments.