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 favorite carbon source of *Saccharomyces cerevisiae* that 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 signaling. 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.

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INTRODUCTION

Saccharomyces cerevisiae can adjust its growth in response to nutritional depletion by triggering one of several alternative developmental programs, depending on the particular nutritional condition. *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 favorable 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 signaling 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 signaling 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; Rolland *et al.*, 2002). The main glucose repression pathway is mediated by Hxk2 being its most important effector 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 PKA, is

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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 (AMPK). At high glucose concentration Snf1 is inactive, the transcription factor Mig1 is not phosphorylated and therefore localized in the nucleus where it exerts repression of genes involved in the utilization of alternative carbon sources, together with the Ssn6/Tup1 repressor complex (Gancedo, 1998; Carlson, 1999; Hedbacker 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 both in the presence or absence of glucose. When glucose is abundant Hxk2 interacts with Mig1 at Ser311, a site that is also targeted by Snf1 for phosphorylation (Ahuatzi *el 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 (Lutfiyya 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

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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 (CSRE) 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.

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). 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*, 1987). PKA activity negatively regulates its own subunits expression, contributing to control 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 behavior 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

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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. *S*trains 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 (≈$0.5x10^7$ – $1x10^8$ cells/ml).

2.2. 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 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).

2.3. 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

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Miller (Miller J H, 1972) was expressed as Miller Units. The results are expressed as the mean ± SD of the total replicate samples (n=4, n=6 or n=8) coming from independent assays (2, 3 or 4 experiments). *tpk1^{w1}BCY1* and *TPK1bcy1*Δ 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).

2.4. Chromatin immunoprecipitation (ChIP) 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 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 (TBS) 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 phenylmethylsulfonyl 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 x *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 sec at 15% amplitude) and clarified by centrifugation at 17,000 x *g* for 15 min. 1 mg 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,

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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 one time with Tris-EDTA (TE) 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 1h 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 (qPCR) was carried out with an Opticon Monitor 3 (Bio-Rad), using primers that amplified different regions of *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 $\Delta\Delta C_T$ 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: Fw: 5' AGCTGTGCTGCTATTCGTTCT 3'; Rv: 5' ACTTTTACCAGATCCCGCCTT 3', for Mig1 and Rpb1 binding, and Fw: 5' GAGCAATGGCTTTGGTAAGGT 3'; Rv: 5' TAGGAAGAATGTCAACAAGATG 3', for Cat8 binding. Control ORF *POL1*: Fw: 5' CTGCACTGGCAAAACAGAAA 3', Rv: 5' TCTTAAAC GACGGCCAATAGA 3'.

2.5. qRT-PCR.

Total RNA was prepared from different yeast strains, grown up to the same OD_{600} as for β-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

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TPK1: Fw: 5' CCGAAGCAGCCACATG TCAC 3', Rv: 5' GTACTAACGACCTCGGGTGC 3'; mRNA *TPK2*: Fw: 5' GCTTGT GGAGCATCCGTTTC 3', Rv: 5' CACTAAACCATGGGTGAGC 3'; mRNA *TPK3*: Fw: 5' CGTTGGACAAGACATTCCTG 3', Rv: 5' GTCGGTTATCTTGATATGGCC 3'; mRNA *BCY1*: Fw: 5' CGAACAGGACACTCACCAGC 3', Rv: 5' GGTATCCAG TGCATCGGCAAG 3'; mRNA *TUB1*: Fw: 5' CAAGGGTTCTTGTTTACCCATTC 3', Rv: 5' GGATAAGACTGGAGAATATGAAAC 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* (α-Tubulin gene) and then compared to 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. Fig. 1A 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 analyzed by quantitative real time PCR (qRT-PCR) (Fig. 1B) were in agreement with the promoter activities. The results indicate that there is a high level of transcriptional activity in

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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 (wild type), $tpk1^{w1}$*BCY1* (strain carrying an attenuated form of Tpk1 and deletion of *TPK2* and *TPK3* genes, resulting in very low PKA activity), and *TPK1bcy1*Δ strain (strain with a deregulated PKA activity). *TPK2* and *TPK3* promoters showed induction of their activities in the $tpk1^{w1}$*BCY1* strain, completely reverted in the *TPK1bcy1*Δ strain (Fig. 2A). *TPK1* and *BCY1* promoters were not upregulated in $tpk1^{w1}$*BCY1* strain but were downregulated in *TPK1bcy1*Δ strain. A possible explanation for this particular behavior 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 (Fig. 2B) in the WT and mutant strains and the results were consistent with the promoter activities.

PKA subunit promoters are regulated by Snf1

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Taking into account that glycerol is regulating the activity of PKA promoters, we decided to analyze 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 as Mig1, Cat8 and Sip4. As a first approach we analyzed the promoter sequences *in silico* using the Yeastract server (Teixeira *et al.*, 2006; Abdulrehman *et al.*, 2011; Teixeira *et al.*, 2014), looking for the presence of DNA consensus sequences for the binding of these transcription factors in the *TPK*s 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, Yeastract server, Spell from SGD http://www.yeastgenome.org, Chua *et al.*, 2006) .

We therefore decided to test the promoters in strains lacking different transcription factors involved in carbon source metabolism such as *mig1*Δ, *mig2*Δ, *mig3*Δ, *cat8*Δ, *sip4*Δ, *rgt1*Δ strains, as well as in a *snf1*Δ 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 (Fig. 3). The first conclusion from Fig. 3 is that the activity of the promoters of the three *TPK*s genes is positively regulated by Snf1 signal transduction pathway, particularly when glycerol was 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

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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 (CSRE) in the genes they regulate (Turcotte *et al.*, 2010). 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 *TPK*2, in glucose, in which case Sip4 seems to be a repressor, since its deletion increases *TPK*2 promoter activity. The results indicate that Sip4 and Cat8 have non overlapping function on the four promoters (Fig. 3G and H). .

We chose the *TPK1* promoter to further investigate the mechanism through which Snf1 might upregulate its activity in glucose. Fig. 3A 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 *TPK*1 promoter. Since in glucose 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 analyzed 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 described that during growth in glucose the phosphatase Glc-7Reg1 inactivates Snf1 kinase by dephosphorylating hr210 in the catalytic loop (Momcilovic *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; Nicastro *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

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therefore assessed the effect of the deletion of Hxk2 (Fig. 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 can not be phosphorylated (*snf1Δ*+pl Snf1 T210A strain) (Hedbacker *et al.*, 2004). The results in Fig. 3A 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* showed to be upregulated by Mig1 in glycerol (Fig. 3F); however, in the presence of glucose, *TPK3* promoter activity was not modified in any of the *mig*Δ strains (Fig. 3E). This result is in contrast with published results from microarrays pointing to the downregulation of *TPK3* mRNA levels in a *mig2*Δ strain in comparison to WT

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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 (Fig. 3G and H). Regarding Hxk2 regulation on each promoter, *TPK2, TPK3* and *BCY1* showed a different behavior in the presence of glucose. *BCY1* and *TPK3* did not show any change in the *hxk2*Δ strain but *TPK2* promoter was upregulated in this strain (Fig. 3C).

mRNA levels of *TPK1*, *TPK2*, *TPK3* and *BCY1* measured in the *snf1*Δ, *mig1*Δ and *cat8*Δ strains by qRT-PCR were consistent with the promoters activities in the same strains (Fig. 4).

Finally, the direct participation ofMig1 and Cat8 on *TPK1* promoter regulation was analyzed by ChIP assay (Fig. 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 (Fig. 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 *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. A striking point in this regulation is the role of Snf1 in glucose growth (Fig. 3), a condition in which Snf1 should not be active. Under low glucose

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conditions, Snf1 kinase is phosphorylated and therefore is activated (Turcotte *et al.*, 2010). Snf1 kinase forms different complexes with the γ-regulatory subunit Snf4, that protects active Snf1 (Mayer *et al.*, 2011) and with different β-subunits Sip1, Sip2, or Gal83 that determine the localization of Snf1 to distinct subcellular locations (Vincent *et al.*, 2001). When glucose levels increase, Snf1 is dephosphorylated in Thr210 by Glc7 and the kinase becomes inactive; the presence of Hxk2 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 (De Vit *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 at 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 (Fig. 3A), as *snf1*Δ mutant strain showed a downregulation and *hxk2*Δ and *mig1*Δ, an upregulation of the promoter activity. A well-known effector of Snf1, the Cat8 transcription factor, also participates in controlling *TPK1* promoter activity (Fig. 3A); *cat8*Δ and *snf1*Δ strains exhibited the same effect on *TPK1* in

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glucose medium. Sip4, another transcription factor downstream of Snf1, has no effect on *TPK1* promoter in the corresponding mutant deletion strain (Fig. 3A). It should be noted that Cat8 and Sip4 have been proposed to bind to the same type of promoters although with different affinities, being Sip4 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 (Fig. 3). Our results suggest that in the presence of glucose, the phosphorylatable form of Snf1 (Fig. 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.

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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 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 to 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 to 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 of 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, possibly the result of a cell strategy aimed to rapidly stimulate fermentation and regulate properties linked to rapid 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 autoregulation, which directs *TPKs* and *BCY1* genes expression. The

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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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TABLE 1

YEAST STRAINS

Strain	Genotype	Reference or source
SP1 (WT)	*Matα his3 leu2 ura3 trp1 ade8*	(Toda *et al.,* 1987)
S18-1D (*tpk1w1BCY1*)	(SP1) *tpk1w1 tpk2::HIS3 tpk3::TRP1*	(Nikawa *et al.,* 1987)
133 (*TPK1bcy1Δ*)	(SP1) *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*	(Cameron *et al.,* 1988)
BY4741 (WT)	*Mata his3Δ1 leu2Δ0 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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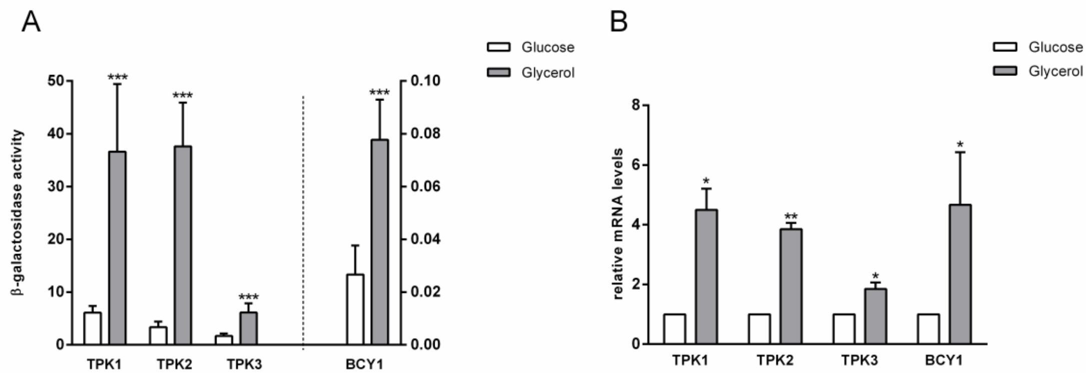

Figure 1. Yeast PKA subunits are upregulated in glycerol containing medium. (A) β-Galactosidase activity was determined in WT (BY4741) strains carrying *TPK1*-lacZ, *TPK2*-lacZ, *TPK3*-lacZ or *BCY1*-lacZ fusion genes. Cultures of each strain were grown in glucose or glycerol containing medium to log phase (OD_{600} 1). The results, shown in Miller Units, are expressed as the mean ± SD from replicate samples (n=8) from independent experiments. (B) *TPKs* and *BCY1* endogenous mRNA levels were determined by qRT-PCR in WT (BY4741) strain cultures grown in glucose or glycerol containing medium and normalized to *TUB1* mRNA. The mRNA levels from each subunit in the glucose cultures were defined as 1.

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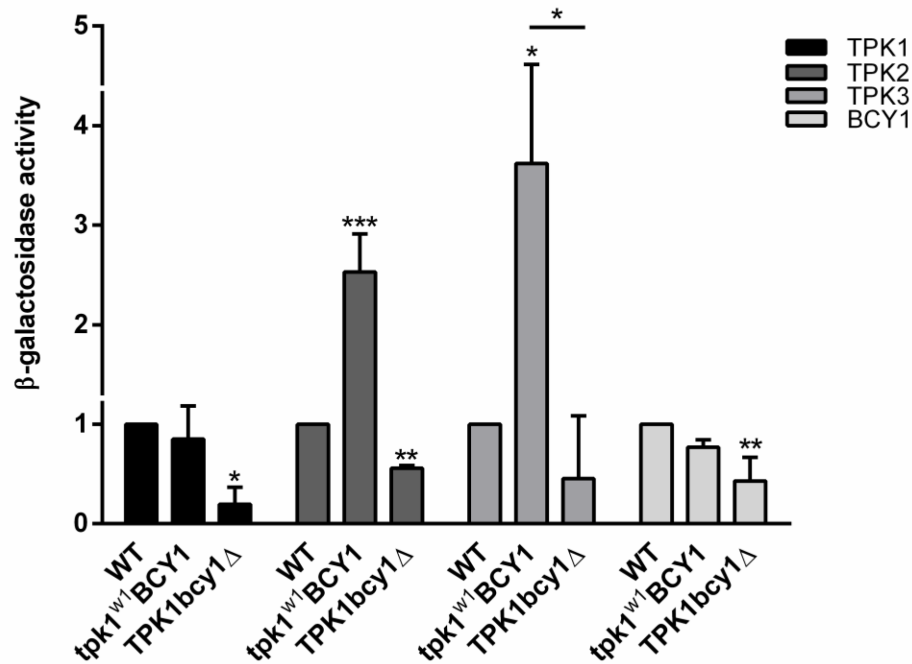
Figure 2



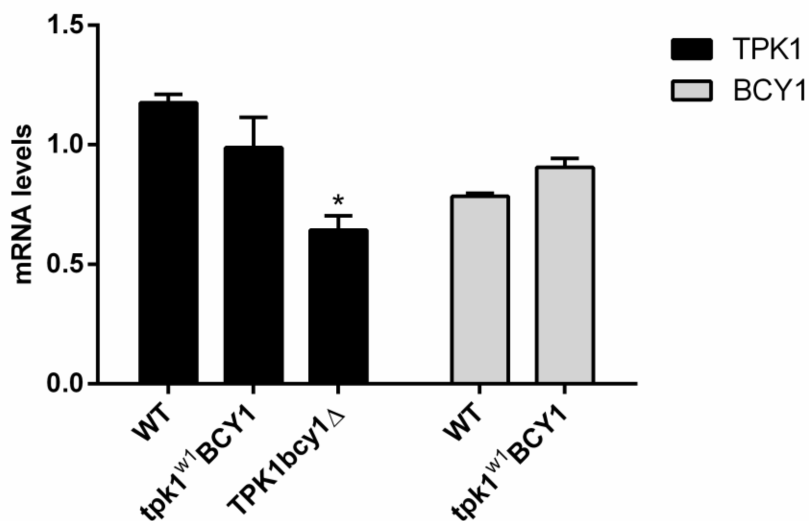


Figure 2. *TPKs* and *BCY1* promoters are regulated by PKA activity in glycerol containing medium. (A) β-Galactosidase activity was determined in WT (BY4741), *tpk1*^{w}*BCY1* and *TPK1bcy1*Δ strains carrying *TPKs*-lacZ or *BCY1*-lacZ fusion genes. Cultures of each strain

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were grown in glycerol containing medium to log phase (OD_{600} 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), *tpk1*^{w}*BCY1* and *TPK1bcy1*Δ strains cultures grown in the presence of glycerol and normalized to *TUB1* mRNA.

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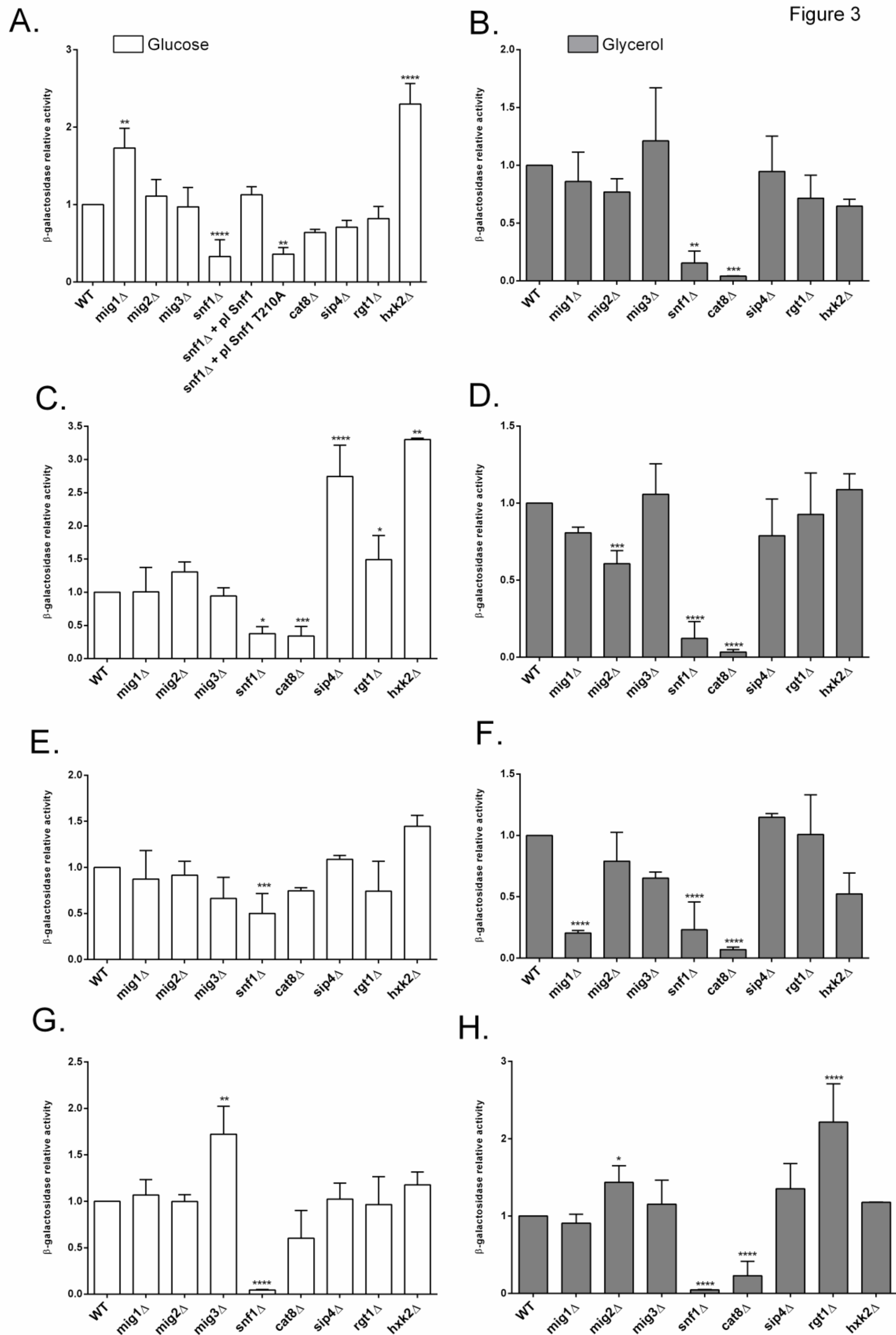

Figure 3. Snf1 kinase regulates *TPKs* and *BCY1* promoter activities. β-Galactosidase activity was determined in WT (BY4741), *mig1*Δ, *mig2*Δ, *mig3*Δ, *snf1*Δ*, snf1*Δ *+ pl Snf1, snf1*Δ *+ pl*

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Snf1 T210A , *cat8*Δ, *sip4*Δ, *rgt1*Δ and *hxk2*Δ 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.

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Figure 4

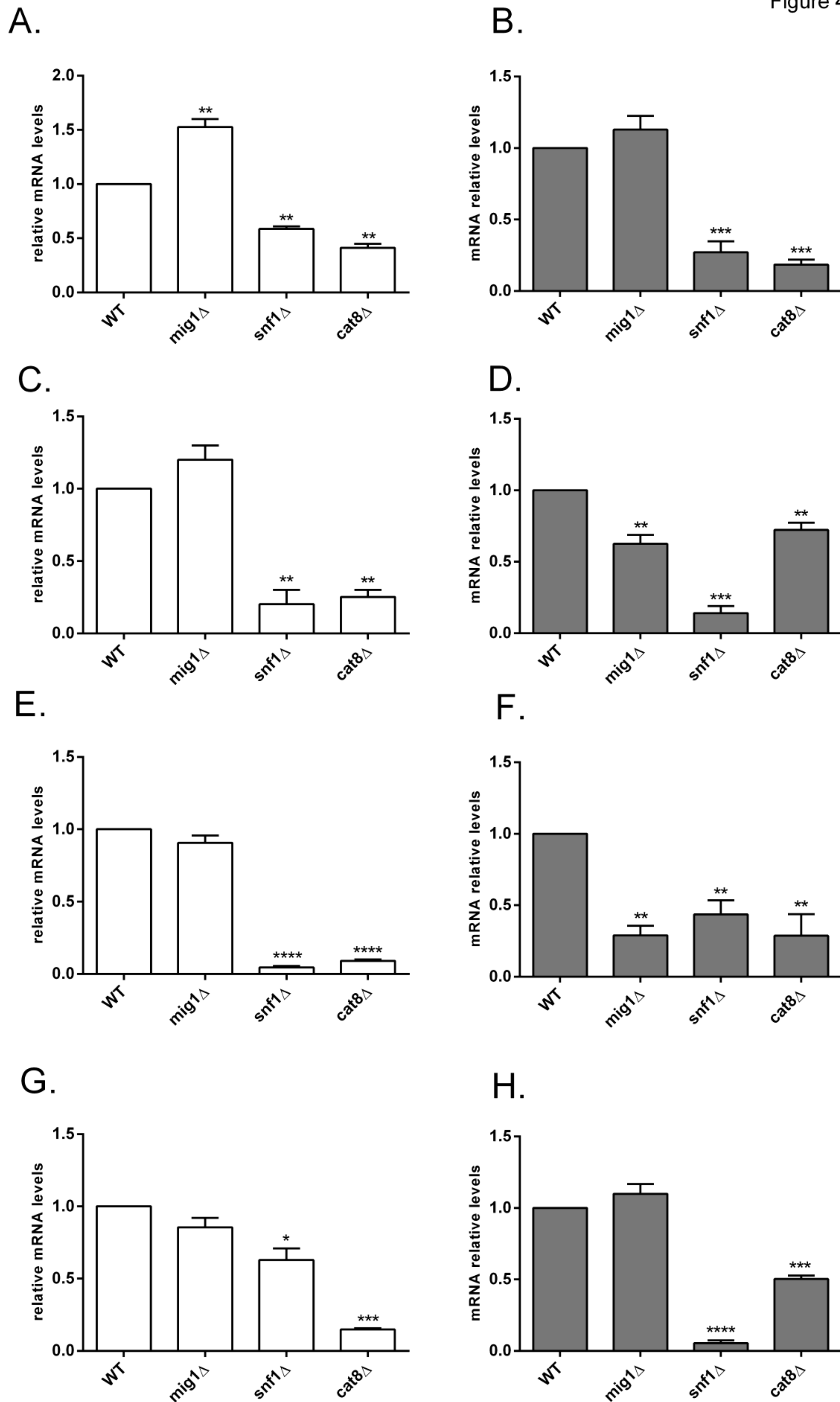

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Figure 4. *TPKs* and *BCY1* mRNA levels are regulated by Snf1. *TPK1* (A and B), *TPK2* (C and D), *TPK3* (E and F) and *BCY1* (G and H) endogenous mRNA levels were determined by qRT-PCR in WT (BY4741), *mig1*Δ, *snf1*Δ and *cat8*Δ strains grown in 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.

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Figure 5

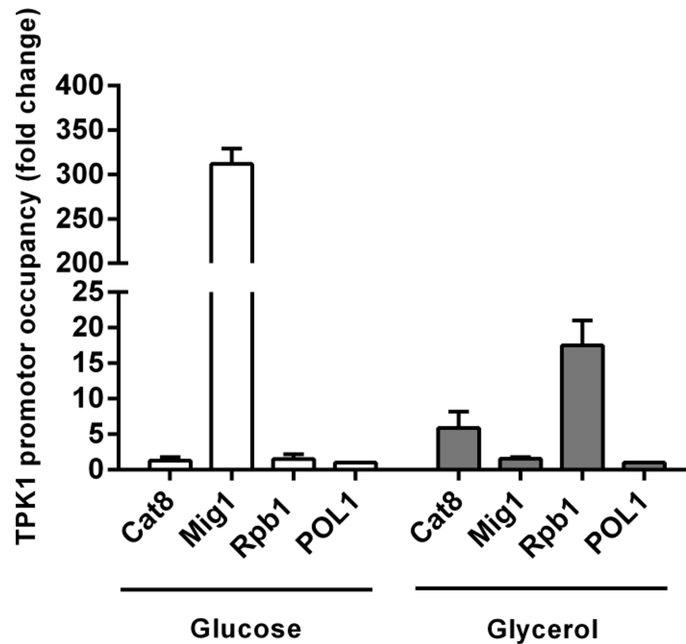

Figure 5. Mig1 and Cat8 bind *TPK1* promoter in a carbon source-dependent manner. WT (BY4741) cells expressing Mig1-TAP, Cat8-TAP and Rpb1-TAP fusion proteins were grown in glucose or glycerol containing medium to log phase (OD_{600} 1). ChIP assays were carried out using IgG-Sepharose. qPCR was performed with specific primers that cover the whole *TPK1* promoter, with an overlap of 150 bp. Only the results for the primer pairs positive for ChIP is represented: Cat8, -696 a -545; Mig1 and Rpb1, -330 a -109. ChIP DNA was normalized to input DNA and calculated as a signal-to-noise ratio to the background values of the non-tagged strain. Results are expressed as *TPK1* promoter occupancy means ± SD (n=3) relative to unspecific *POL1* region.

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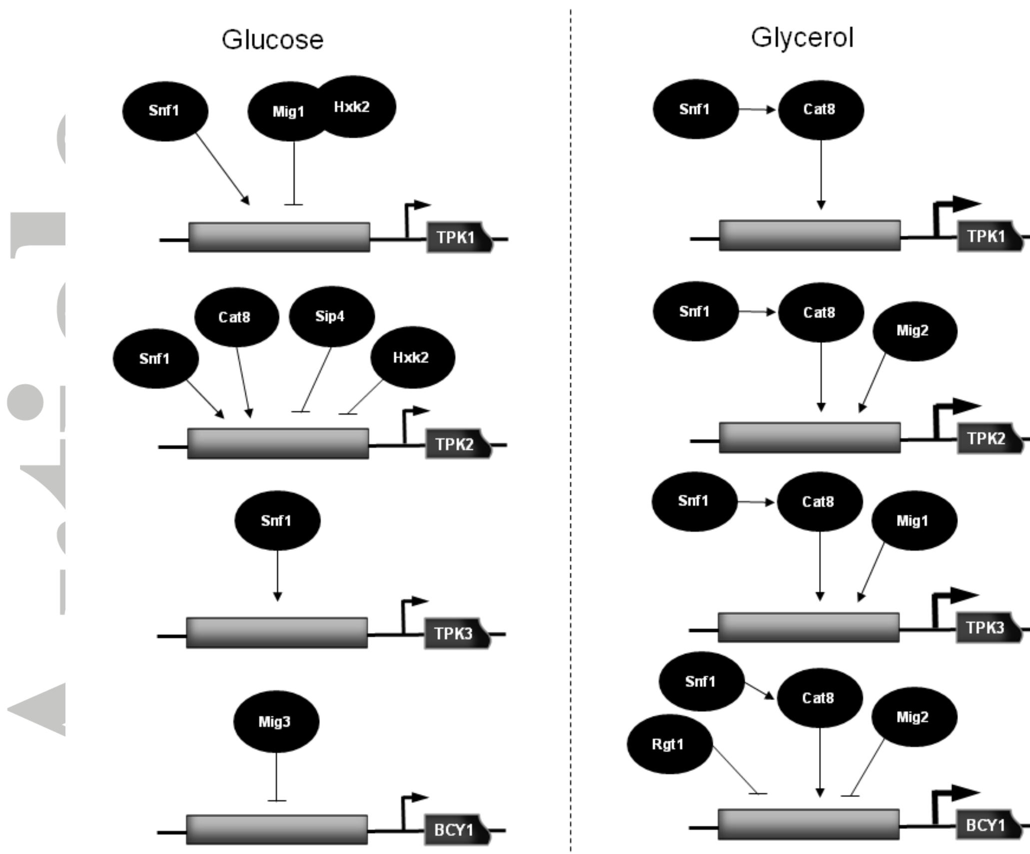

Figure 6. Summary of the regulation of *TPKs* and *BCY1* promoters. For more details see the text. The thick arrow on the promoters in glycerol indicates a higher expression level than in glucose (thin arrow). (→) indicates activation and (⊣), indicates repression.

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