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RESEARCH ARTICLE

Identification of novel transcriptional regulators of PKA subunits in *Saccharomyces cerevisiae* by quantitative promoter–reporter screening

Constanza Pautasso[1], Sol Reca[1], Kate Chatfield-Reed[2], Gordon Chua[2], Fiorella Galello[1], Paula Portela[1], Vanina Zaremberg[2] and Silvia Rossi[1,*]

[1]Departamento de Química Biológica, IQUIBICEN- CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires C1428EGA, Argentina and [2]Department of Biological Sciences, University of Calgary, Calgary, AB T2N 1N4, Canada

*Corresponding author: Departamento de Química Biológica, IQUIBICEN- CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria Pabellon II, 1428 Buenos Aires, Argentina. Tel: 541145763342; E-mail: srossi@qb.fcen.uba.ar
One sentence summary: Novel modulators were identified by quantitative promoter-reporter screen: transcription factors, lipid and phosphate metabolism regulates PKA subunits transcription.
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ABSTRACT

The cAMP-dependent protein kinase (PKA) signaling is a broad pathway that plays important roles in the transduction of environmental signals triggering precise physiological responses. However, how PKA achieves the cAMP-signal transduction specificity is still in study. The regulation of expression of subunits of PKA should contribute to the signal specificity. *Saccharomyces cerevisiae* PKA holoenzyme contains two catalytic subunits encoded by *TPK1*, *TPK2* and *TPK3* genes, and two regulatory subunits encoded by BCY1 gene. We studied the activity of these gene promoters using a fluorescent reporter synthetic genetic array screen, with the goal of systematically identifying novel regulators of expression of PKA subunits. Gene ontology analysis of the identified modulators showed enrichment not only in the category of transcriptional regulators, but also in less expected categories such as lipid and phosphate metabolism. Inositol, choline and phosphate were identified as novel upstream signals that regulate transcription of PKA subunit genes. The results support the role of transcription regulation of PKA subunits in cAMP specificity signaling. Interestingly, known targets of PKA phosphorylation are associated with the identified pathways opening the possibility of a reciprocal regulation. PKA would be coordinating different metabolic pathways and these processes would in turn regulate expression of the kinase subunits.

Keywords: PKA; transcription regulation; Tpks; Bcy1; *Saccharomyces cerevisiae*

INTRODUCTION

The great variety of cellular processes regulated by the cAMP-protein kinase (PKA) pathway must be strictly controlled to maintain specificity in the response. Different regulatory mechanisms must exist to ensure the phosphorylation of the correct substrate in response to the proper stimulus. Several combined factors determine the differential effects of the signaling cascade initiated by cAMP. In mammals, different biochemical properties and substrate specificity displayed by PKA isoenzymes and their localization through the association with anchoring proteins (AKAPs) have been shown to contribute to specificity in the cAMP pathway (Skålhegg and Taskén 1997).

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Transcriptional regulation of the PKA subunits and their level of expression should also have a critical impact on signal specificity, but little is known about how these processes are regulated.

The regulatory (R) and catalytic (C) subunits of mammalian PKAs are transcriptionally regulated by hormones and mitogens acting through different receptors like G-protein coupled receptors (Jahnsensg *et al*. 1985; Pariset *et al*. 1989; Landmark *et al*. 1993) or tyrosine kinases-associated receptors (Skålhegg and Taskén 1997). PKA subunits have shown differential expression patterns at different developmental and differentiation stages as well as in different tissues (Cadd and Mcknight 1989; Beebe *et al*. 1990; Hougel 1992; Landmark *et al*. 1993; Reinton *et al*. 1998; Cumming, Fidler and Vaux 2007). cAMP positively modulates transcription of PKA subunits involving stabilization of their mRNAs as well as on both R and C protein stability after dissociation of the holoenzyme (Houge, Vintermyr and Døskeland 1990; Knutsen *et al*. 1991; Taskén *et al*. 1991; Hougel 1992).

In the model organism *Saccharomyces cerevisiae*, PKA controls a variety of essential cellular processes associated with fermentative growth, entrance into stationary phase, stress responses and development (Thevelein and Winde 1999; Thevelein *et al*. 2008; Smets *et al*. 2010). This pleiotropic role of PKA also needs a tight regulation. The structure of the PKA holoenzyme is conserved from mammals to yeast, and consists of a heterotetramer composed of a regulatory subunit homodimer and two associated catalytic subunits. The yeast catalytic subunits are encoded by three genes, *TPK1*, *TPK2* and *TPK3*, and the regulatory subunit, by the *BCY1* gene. Among the factors contributing to the cAMP-PKA pathway specificity in yeast are the synthesis, breakdown and spatial localization of cAMP, Tpk isoform-dependent phosphorylation of substrates and subcellular localization of the holoenzyme (Griffioen and Thevelein 2002; Vandamme, Castermans and Thevelein 2012; Engelberg, Perlman and Levitzki 2014).

We are interested in understanding how transcriptional regulation of the PKA subunits contributes to the specificity of the cAMP-PKA signaling by modification of molecular balance between the catalytic isoforms and regulatory subunits. We have previously conducted investigations aimed at characterizing the promoter activity of the *BCY1* and *TPK* genes and have demonstrated that the promoter of each isoform of *TPK* and of *BCY1* is differentially regulated during the growth phase and stress conditions (Pautasso and Rossi 2014). *TPK1* promoter activity is positively regulated during heat shock and saline stress but *TPK2*, *TPK3* and *BCY1* promoters, unlike *TPK1*, are not activated under these stress conditions. Therefore, the expression of each PKA subunit involves different mechanisms in response to heat shock or saline stress. However, the four promoters of PKA subunits share an inhibitory autoregulatory mechanism since all of them are downregulated by PKA activity (Pautasso and Rossi 2014). Taking into account these antecedents, our aim in this study was to identify novel transcriptional activators and inhibitors of PKA subunits. Taking advantage of the unique tractability of yeast, we used an unbiased high-throughput approach to uncover regulators of the promoters of the *BCY1* and *TPK* genes. We performed a reporter-synthetic genetic array (R-SGA) screen to assess the effect of viable deletion mutants on the activity of *TPK1*, *TPK2*, *TPK3* and *BCY1* promoters. The R-SGA screen makes use of a two-color promoter–reporter system that is delivered to the array of viable haploid deletion mutants using high-throughput genetics (Kainth *et al*. 2009). This reporter-based screen has shown to be a powerful strategy for identification of regulatory proteins and upstream signals involved in promoter regulation (Kainth and Andrews 2010). Using this approach, we were able to identify unique pathways that differentially regulate the activity of the *BCY1*, *TPK1*, *TPK2* and *TPK3* promoters. Clustering analysis of the genes identified revealed enrichment in genes with roles in several GO biological processes. Lipid and phosphate metabolism, and regulation of transcription categories were further characterized and validated using β-galactosidase reporter assay and qRT-PCR. The results of our genetic screen pointed to inositol, phosphate and inositol-polyphosphates as novel upstream signals that modulate transcription of PKA subunit genes.

RESULTS AND DISCUSSION

Validation of the R-SGA genetic screen

We used the dual-reporter functional genomic screen, R-SGA approach (Kainth *et al*. 2009) to survey the viable deletion collection of mutants that affect the transcription of promoters of the each PKA subunits, (*prTPK1*-GFP, *prTPK2*-GFP, *prTPK3*-GFP and *prBCY1*-GFP). In this system, these GFP-fusion constructs each carried on plasmids are separately introduced into the yeast deletion collection together with an integrated fusion control promoter fused to the dsRed variant tdTomato (referred to as RFP) (Kainth *et al*. 2009). Differential GFP expression was assessed by scanning fluorescence intensities (GFP and control RFP) directly from colonies arrayed on agar plates, obtaining in this way a genome-wide result of the effect of viable deletion mutants on the activity of the promoters of PKA subunits. The R-SGA assay was performed from colonies arrayed in defined media grown for 4 days with glucose as carbon source. The GFP:RFP ratios were normalized and the log2 values calculated as described (Fig. S1, Supporting Information). Decreased GFP:RFP ratios correspond to deletion of putative activators, while increased ratios reveal deletion of putative repressors (Kainth *et al*. 2009). Taking into account values of log2 (GFP:RFP) $\pm$ 1.5 standard deviation from the main of all values for each PKA pr-GFP, a list of deletion strains was obtained (Table S1, Supporting Information) allowing the identification of putative repressors and activators.

Clustering analysis of the genes identified in each case was done using the online software FunSpec, with a $P < 0.05$. We considered the possibility that the genome-wide screen gave false positives using a $P < 0.05$, which could be adjusted using a more strict $P < 0.01$. Although not much is known about transcriptional regulation of these genes, we have previously demonstrated that *TPK*s promoters are inhibited by PKA activity, and that Tpk2 had a stronger inhibitory effect on *TPK1* and *TPK3* promoters when cells were grown in liquid cultures (Pautasso and Rossi 2014). Thus, we expected the deletion of *TPK2* to result in increased *prTPK1*-GFP and *prTPK3*-GFP expression compared to its effect on the control RPL39pr-RFP gene. We found that *TPK2* deletion did in fact cause a change in *TPK1* transcription (Table S1, Supporting Information). The MIPS and GO categories of cAMP signaling were enriched for *TPK2* and *TPK1* respectively and appeared using a $0.01 < P < 0.05$ value. (Table S1, Supporting Information). Reducing to $P < 0.01$, Tpk2 was no longer detected; thus, even though the false positives could be reduced using a lower cut-off, this lower p-value could discard true regulators. The putative transcriptional regulators identified with a $P < 0.05$ are summarized in Table S1 (Supporting Information). Altogether, the results of our screen were consistent with previous findings, successfully identifying some of the expected modulators, therefore validating the use of this approach for the unbiased identification of novel regulators of the gene expression of PKA subunits.

Novel transcriptional regulators of PKA subunits

We focused our analysis on the results obtained from R-SGA screens performed from colonies arrayed on defined medium with glucose as carbon source grown 4 days at 30°C. The log2 ratios were transformed to Z scores and p-values assigned on the basis of a normal distribution (see Materials and Methods). This analysis identified 469 putative transcriptional modulators for *TPK1* promoter, 437 for *TPK2* promoter, 307 for *TPK3* promoter and 299 for *BCY1* promoter (Table S1, Supporting Information).

Clustering analysis of the identified genes revealed a discrete number of GO categories significantly enriched in each screen. The categories that were found to be enriched for *TPK1, TPK2*, *TPK3* and *BCY1* promoters with a cut-off of a $P < 0.05$ are listed in Fig. 1 and Table S1 (Supporting Information). Several GO categories differentially affected the expression of *TPKs* and *BCY1*, while many others were common to all (Table S1, Supporting Information). A total of 151 modulators of PKA subunits gene transcription were associated to the categories of transcriptional factors from which 34 were regulators of *TPK1* promoter, 22 of *TPK2* promoter, 49 of *TPK3* promoter and 46 of *BCY1* promoter. A total of seven candidates were shared by all four PKA subunits promoters reflecting the overlapping regulation of *TPKs* and *BCY1*. However, several transcriptional regulators were specific to one of the three isoforms of *TPKs* or *BCY1*, suggesting their potential specialized functions (Fig. 2).

Positive modulators Swi4 for *TPK2* and *TPK3*, Cbf1 for *TPK1* and *BCY1* and Rtg2 for *TPK1*, *TPK2* and *TPK3*, and negative modulators Mot3 for *TPK2*, *TPK3* and *BCY1* and Flo8 for *TPK3* and *BCY1* were further evaluated by qRT-PCR (Fig. 3). The results showed in Fig. 3 suggest that the impact that the lack of these transcription factors had on promoter activities did not necessarily correlate with mRNA levels. This is not surprising as growing evidence indicates that mRNA life is tightly regulated by proteins that control its storage and degradation, including transcription factors (Bregman *et al*. 2011; Haimovich *et al*. 2013; Medina *et al*. 2014).

The case of Flo8 transcription factor is noteworthy since it was identified as regulator of *TPK3* and *BCY1* transcription in our R-SGA screens but the absence of this transcription factor affected the levels of all four mRNAs studied (Fig. 3). It is well known that yeast strains derived from S288c, like BY4741 used in our screen, are incompetent for filamentous growth due to a mutation in the *FLO8* gene (*flo8-1*) that produces a truncated transcription factor altering expression of *FLO11* (Liu, Styles and Fink 1996). The initial combined results from our screens suggested a role for this truncated protein and were further confirmed by measuring the levels of mRNA in the *flo8*Δ strain (Fig. 3). This deletion mutant displayed robust changes in all *TPK* and *BCY1* mRNAs, with the greatest effects on *TPK1, TPK3* and *BCY1*. Our findings are in agreement with previous study reporting mRNA changes in the *flo8*Δ mutant for all *TPKs* and *BCY1*, analyzed using microarray technology (Hu, Killion and Iyer 2007). Interestingly, it has been recently reported that the C-terminal region of Flo8 has a novel transcriptional activation domain with a crucial role in activating transcription (Kim *et al*. 2014).

Effect of inositol and choline

Lipid metabolism in *S. cerevisiae* is altered by growth phase and by supplementation with inositol or choline. This regulation is controlled by genetic and biochemical mechanisms (Carman and Henry 2007). The regulation of genes related to phospholipid biosynthesis in *S. cerevisiae* through cis-acting upstream activating sequence by inositol supplementation (UAS_{INO}) is thoroughly known (Rupwate, Rupwate and Rajasekharan 2012). In addition, inositol-polyphosphates, product of the reaction catalyzed by the phosphatidylinositol-specific phospholipase C (Plc1), have also been proposed to regulate gene transcription (Alcázar-Román and Wente 2008). Interestingly, phosphate and polyphosphate metabolism was a category enriched for all *TPK* and *BCY1* promoters ($P < 0.05$).

Among the genes identified in the GO category of lipid metabolism (summarized in Table S1, Supporting Information), there were many known to be regulated by inositol and choline, such as those involved in glycerolipid synthesis and regulation or transcription factors that respond to UAS_{INO} elements, as well as genes involved in inositol polyphosphate metabolism. In addition, genes involved in sphingolipid metabolism were identified.

All these results pointed to a possible novel role of inositol as an upstream regulator of PKA subunits expression. To test this, the *TPK1*, *TPK2* and *TPK3* promoter activities were assessed using promoter-lacZ-based reporter assays (Fig. 4A, upper panel), and their endogenous mRNA levels were also measured (Fig. 4A, lower panel) in wild-type (WT) cells (strain BY4741) grown to late log phase in minimal medium containing glucose but lacking or supplemented with 75 μM inositol and/or 1 mM choline. In fact, the activity of all three promoters was sensitive to the presence of inositol. Interestingly, addition of choline to inositol depleted medium also activated these promoters, and no major differences were found when inositol and choline were added together. This is somehow surprising as the effect of exogenous choline is usually much less dramatic than the effects of exogenous inositol in UAS_{INO} containing genes (Carman and Henry 2007). In addition, no classic UAS_{INO} sequences can be detected in any of the promoters analyzed. Altogether this may suggest that the effect of inositol and choline on the *TPKs* promoter activities is indirect by modulating the levels of signaling lipids and signaling soluble products of phospholipid turnover like inositol polyphosphates (Lee *et al*. 2008).

PKA subunit promoters were activated by inositol in contrast with promoters of many genes involved in yeast phospholipid metabolism where inositol usually causes repression. Yet, in several promoters of genes coding for enzymes of lipid metabolism and signaling, inositol had the opposite effect. For example, *INM1* (encoding the inositol 3-phosphate phosphatase), *DPP1* and *PAH1* (encoding lipid phosphate phosphatases) and *PIS1* (encoding the phosphatidyl-inositol synthase) are derepressed in the presence of inositol and in stationary phase (Murray and Greenberg 1997; Oshiro *et al*. 2000; Jani and Lopes 2008). In addition to these antecedents, microarray analysis has also revealed many additional genes, which are regulated in response to the presence or absence of inositol and choline which are not involved in lipid metabolism (Santiago and Mamoun 2003; Jesch *et al*. 2005). In order to understand the mechanism involved in the regulation of PKA subunits expression, we further tested the effect that deletion of various genes forming the inositol/phosphatidic acid (PA) sensing core had on the promoter activities of the PKA subunits. The localization of the transcriptional repressor Opi1 depends on the levels of inositol and PA (Alvarez-Vasquez *et al*. 2005). Opi1 is localized to the endoplasmic reticulum when inositol is low by the binding to the membrane-associated protein, Scs2 (Loewen, Roy and Levine 2003) and PA (Loewen *et al*. 2004). In the presence of high levels of inositol, PA is consumed and Opi1 rapidly translocates to the nucleus, where it represses the expression of genes required for phospholipid biosynthesis (Loewen *et al*. 2004; Jesch *et al*. 2005). The expression of these genes is dependent on both Ino2 and Ino4, which bind as a heterodimer to the UAS_{INO} element

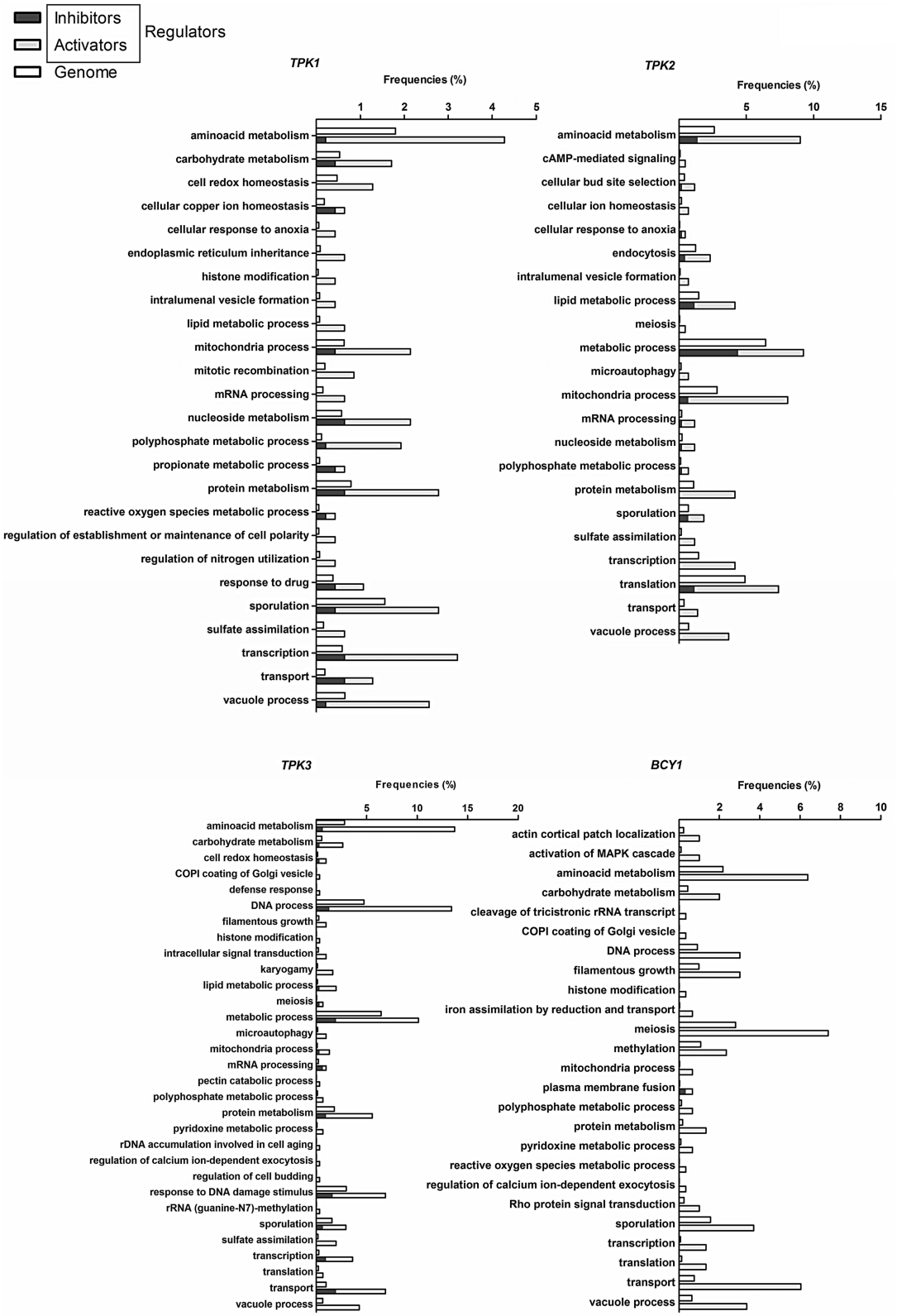

Figure 1. Functional categorization of genes regulators identified in the screen for *TPK1*, *TPK2*, *TPK3* and *BCY1* promoters. Classifications were performed based on functional category according to the gene annotation database of FunSpec (http://funspec.med.utoronto.ca/), using a $P < 0.05$. Black and gray bars represent inhibitor and activator regulator fractions identified, respectively, and white bars represent the genome gene fraction in these categories.

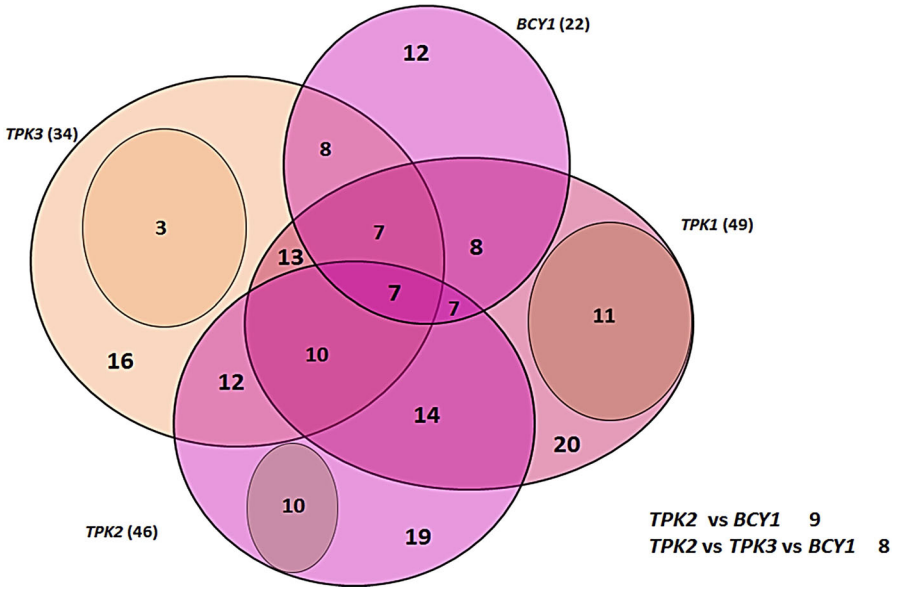

Figure 2. Venn diagrams of PKA subunits transcriptional regulators. The number of total transcriptional factors, activators and inhibitors of each subunit is indicated between brackets. The number of transcriptional inhibitors (which are not shared in any case) is indicated in a circle into the diagram corresponding to each subunit. The caption indicates the number of total regulators shared between *TPK2* vs *BCY1* and *TPK2* vs *TPK3* vs *BCY1* promoters.

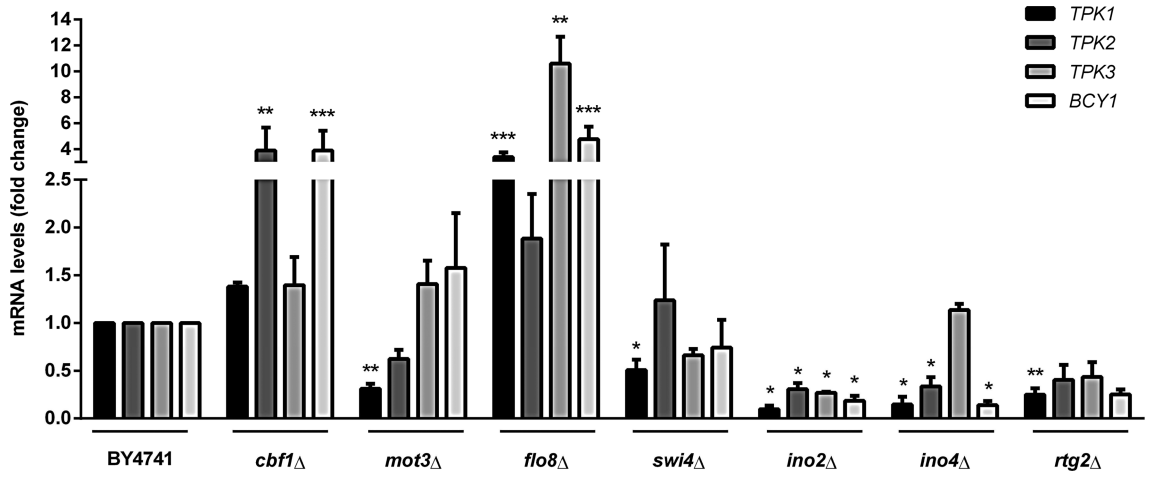

Figure 3. PKA subunits transcription regulators. *TPKs* and *BCY1* endogenous mRNA levels were determined in WT (BY4741), *cbf1*Δ, *mot3*Δ, *flo8*Δ, *swi4*Δ, *ino2*Δ, *ino4*Δ and *rtg2*Δ strains by qRT-PCR and normalized to *TUB1* mRNA. The mRNA level for each subunit in the WT strain was defined as 1. Results are expressed as the mean ± SD $n = 3$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

present in the promoters of Opi1 target genes (Ambroziak and Henry 1994). Opi1 mediates the repression of UAS_{INO}-containing genes through a direct interaction with Ino2 (Gardenour, Levy and Lopes 2004; Heyken *et al.* 2005).

The endogenous mRNA levels of each *TPK* gene were measured by RT-qPCR in mutant strains lacking Ino2, Ino4, Opi1, Scs2 and the DAG kinase, *DGK1*, which produces PA (Fig. 5). The results showed that the mRNA levels of *TPK1* and *TPK2* were downregulated in the strains lacking the Ino2 and Ino4 transcription factors, while they were upregulated when the Opi1 repressor was deleted. Interestingly, the mRNA levels of *TPK3* were only affected by the deletion of Ino2, but did not change due to lack of Ino4 or Opi1. Furthermore, mRNA levels of *TPK2* were slightly altered when Dgk1 was deleted. Altogether these results indicate that although inositol affects promoter activity and mRNA levels of all three *TPK* genes, alterations in the signaling circuit that senses inositol mostly impact *TPK1* and *TPK2* at the mRNA level. The fact that inositol is a positive regulator of *TPK* transcription while deletion of Ino2 and Ino4 induces downregulation of *TPK* mRNAs indicates that the regulatory mechanism is not by repression by inositol and choline. It has been proposed that aside from their role in regulating directly the transcription of genes that are repressed in the presence of inositol, Ino2 and Ino4 may also indirectly affect the transcription of genes that are activated in the presence of inositol by functioning through a transcriptional regulatory network (Jesch *et al.* 2005). *PIS1* is an example of a gene upregulated by inositol and that its expression is dependent on Ino4 (Jani and Lopes 2008).

Effect of phosphate

Another GO category which showed significant gene enrichment was phosphate metabolism. The complex formed by the regulatory proteins Pho80, Pho81 and the cyclin kinase Pho85 is known to regulate the response to phosphate limitation (Huang *et al.*

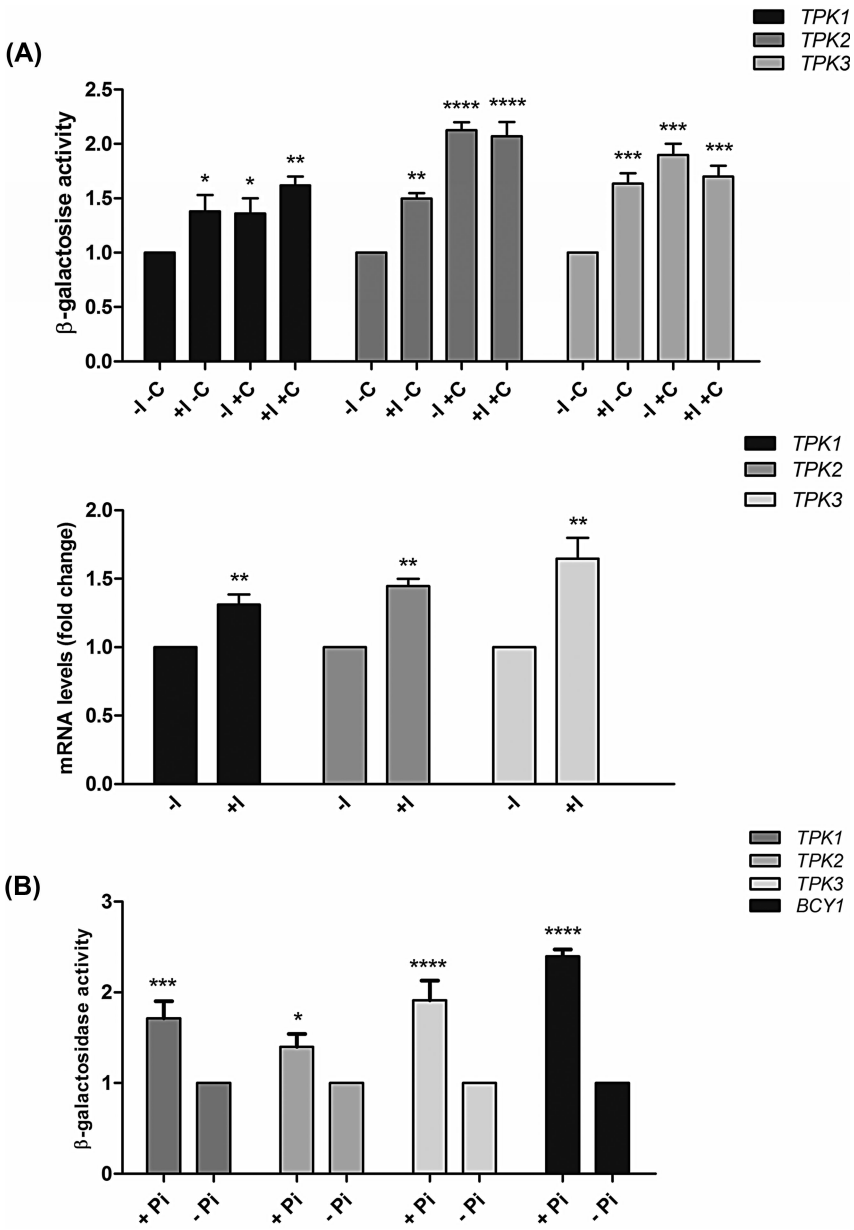

Figure 4. PKA subunits transcription modulated by inositol, choline and phosphate. **(A)** Upper panel, β-galactosidase activity was determined in WT cells (BY4741 strain) carrying the *TPKs-lacZ* fusion gene. Cells were grown in complete synthetic medium with glucose lacking or supplemented with inositol (I) and choline (C). β-Galactosidase activity is expressed in Miller units. Results are expressed as the mean ± SD $n = 3$ within a representative assay and normalized to the values without I and C for each *TPK*. Lower panel, *TPKs* endogenous mRNA levels were determined in WT strain (BY4741) grown in the same conditions described above. Results are expressed as the mean ± SD from triplicates within a representative assay. The values were normalized to *TUB1* mRNA. The ratio *TPK1*/*TUB1* for each subunit in the WT strain was defined as 1, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. **(B)** β-Galactosidase activity was measured as in A but using complete synthetic with high phosphate (+Pi) or low phosphate (–Pi). Results are expressed as the mean ± SD $n = 3$ within a representative assay, * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

2007). Similar to inositol and choline, the presence of high phosphate (7.35 mM KH_2PO_4) increased the activity of all promoters compared to low phosphate (0.15 mM KH_2PO_4) (Fig. 4B). Therefore, promoter activity was lower in conditions of phosphate limitation. The effect of phosphate may be linked to its regulatory role on inositol metabolism and the generation of inositol polyphosphates, which are known regulators of gene expression in the phosphate-sensing pathway (Lee *et al.* 2008). In addition, it was reported that phospholipid biosynthesis is coordinated with phosphate utilization via the bHLH proteins, Ino2/4 and Pho2/4 (He, Swaminathan and Lopes 2012). We have observed that the PKA subunit promoters responded to the presence of inositol, choline and also Pi, suggesting a possible cross-regulation between these pathways.

Yeast Pi sensing, acquisition and storage are mediated mainly by the phosphate-responsive signaling pathway, referred as the PHO pathway (Mouillon and Persson 2006). The four Pi transporters are the low-affinity Pi transporters, Pho87 and Pho90, and the two high-affinity Pi transporters, Pho84 and Pho89. Under limiting Pi conditions, the activation of the PHO pathway is mediated by the transcription factors Pho4 and Pho2 (Magbanua *et al.* 1997; Shao, Creasy and Bergman 1998). The

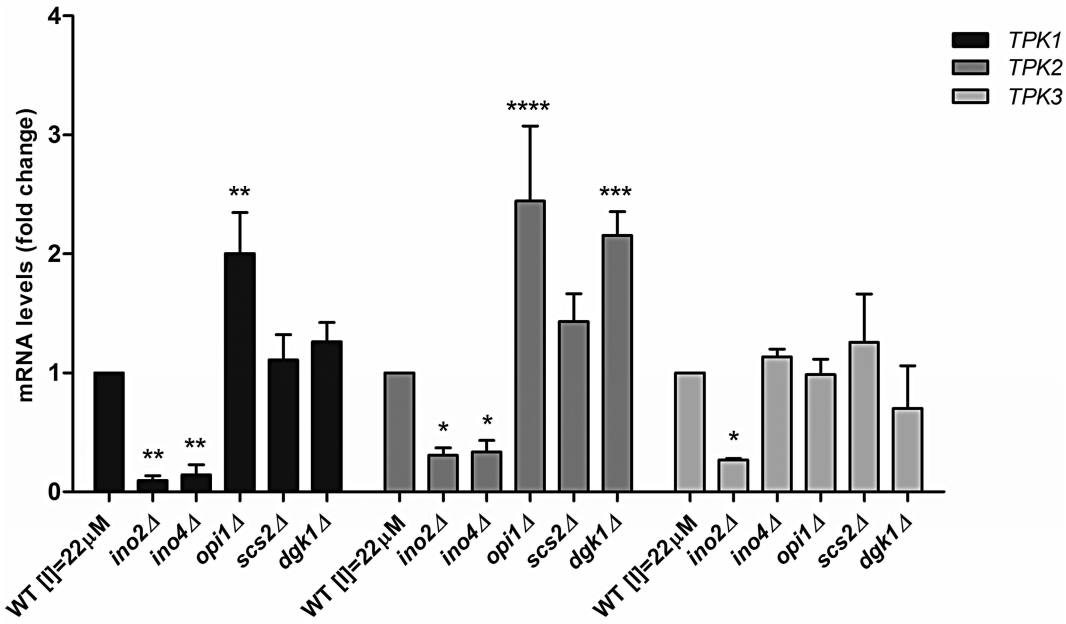

Figure 5. PKA catalytic subunits transcription and lipid metabolism. *TPKs* endogenous mRNA levels were determined in WT (BY4741), *opi1*Δ, *ino2*Δ, *ino4*Δ, *scs2*Δ or *dgk1*Δ strains by qRT-PCR and normalized to *TUB1* mRNA. Cells were grown in complete synthetic medium with glucose. Results are expressed as the mean ± SD $n = 3$. The ratio of mRNA levels for each subunit in the WT strain was defined as 1, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

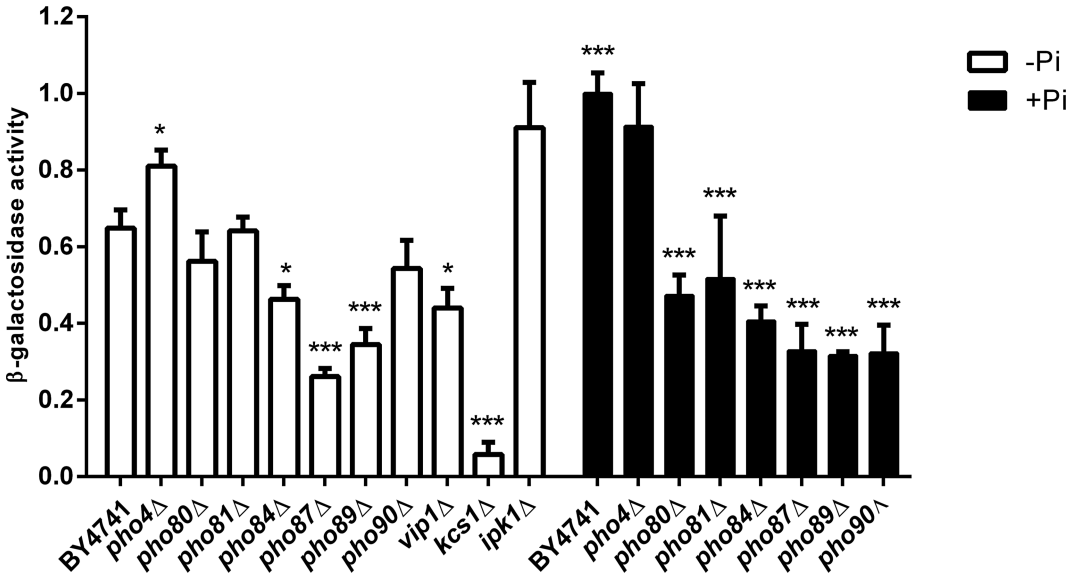

Figure 6. *TPK1* transcription and phosphate metabolism. (**A**) β-Galactosidase activity was determined in WT (BY4741), *pho4*Δ, *pho80*Δ, *pho81*Δ, *pho84*Δ, *pho87*Δ, *pho89*Δ, *pho90*Δ, *vip1*Δ, *kcsip1*Δ and *ipk1*Δ strains carrying the *TPK1-lacZ* fusion gene. Cells were grown as indicated in Fig. 4B. β-Galactosidase activity is expressed in Miller units. Results are expressed as the mean ± SD $n = 3$ within a representative assay and normalized to the WT strain grown in high phosphate (+Pi) values. (B) *TPK1* endogenous mRNA levels were determined by qRT-PCR in the strains grown in the same conditions described above. The values were normalized to *TUB1* mRNA. The ratio *TPK1*/*TUB1* in the WT strain grown in +Pi was defined as 1, * $P < 0.05$, *** $P < 0.001$.

activity of Pho4 has been shown to be negatively regulated through phosphorylation by the Pho80–Pho85 cyclin-dependent kinase (CDK) complex (Komeili and O'Shea 1999) in the presence of Pi. To corroborate the involvement of the Pi signaling in *TPKs* and *BCY1* promoters regulation, *TPK1* subunit was chosen as an example, and β-galactosidase reporter assays were measured in mutant yeast strains lacking different PHO genes from the pathway (*pho4*Δ, *pho80*Δ, *pho81*Δ, *pho84*Δ, *pho87*Δ, *pho89*Δ *and pho90*Δ) in the presence and absence of added Pi (Fig. 6). The results showed that WT cells increased *TPK1* promoter activity upon Pi addition. The fact that the *TPK1* promoter activity was upregulated under limiting Pi conditions in cells lacking the transcription factor Pho4 suggests that the effect is indirect. It is also observed a downregulation upon Pi addition in all the other mutants indicating that the alteration in any component of Pi pathway affects *TPK1* promoter activity.

Inositol and phosphate: effect of polyphosphates

Inositol, once in the cytoplasm, is available for incorporation into lipids and subject to multiple rounds of phosphorylation

and subsequent dephosphorylation. The pool of inositol-polyphosphates has important roles in the control of gene expression (Rupwate, Rupwate and Rajasekharan 2012; Banfic *et al.* 2013; Galdieri *et al.* 2013). We have found that several genes included in the pathway of biosynthesis of inositol-polyphosphates were functionally enriched, indicating the possible importance of these metabolites in the regulation of the activity of PKA subunit promoters. The hydrolysis of $PI(4,5)P_2$ by Plc1 yields two prominent eukaryotic second messengers: 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (Divecha and Irvine 1995; Carman and Han 2011). Plc1 and four inositol polyphosphate kinases (Ipk2/Arg82, Ipk1, Kcs1 and Vip1) constitute a nuclear signaling pathway that affects transcriptional control (Odom *et al.* 2000), export of mRNA from the nucleus (Monserrate and York 2010), homologous DNA recombination, cell death and telomere length (Banfic *et al.* 2013). All of these genes have been identified in our screen as regulators of PKA subunit promoters. Interestingly, it has been demonstrated that upon Pi starvation, the levels of inositol heptakisphosphate (IP7) increases and that IP7 inhibits the activity of Pho81–Pho85–Pho80 complex (Lee *et al.* 2008). We observed that impairing IP7 synthesis, as in *ipk1*Δ and *vip1*Δ or positively affecting IP7 synthesis as in *kcs1*Δ, resulted in the upregulation or downregulation of the *TPK1* promoter activity, respectively (Fig. 6). These results support the involvement of inositol polyphosphates in the transcriptional regulation of PKA subunits. Dissecting the molecular mechanisms operating in this circuit will be the focus of future investigations.

Regulatory loops

In a recent study aimed at identifying positive and negative upstream regulators of PKA activity focused only on Tpk1-Bcy1 and Tpk2-Bcy1 holoenzyme isoforms (Filteau *et al.* 2015), the authors identified 494 high-confidence candidate regulators, with 64 of these (13%) proposed to have physical associations with PKA isoforms similar to mammalian AKAPs. Modulators from a wide range of cellular processes were identified including amino acid biosynthesis, protein acetylation, DNA repair, export from the nucleus, chromatid cohesion, autophagy and nucleobase biosynthesis. Interestingly, only two categories involved in the direct regulation of PKA activity overlap with those identified in our screen: sulfate assimilation and amino acid biosynthesis including methionine and serine metabolism. Therefore, except for genes in these metabolic pathways, the striking lack of overlap between direct regulators of PKA activity and the list of genes identified in our study indicates that our screen was enriched in transcriptional modulators of PKA subunits independent of an effect they may have on PKA activity. From our results, changes in transcriptional levels of the subunits could have potentially affected the formation of PKA complexes identified by Filteau *et al.* (2015). Additional conclusions regarding an extensive level of feedback mechanisms operating on PKA complex formation can be drawn from our study on PKA subunits transcriptional regulation. Many regulatory loops can be envisioned based on the categories enriched in our study, similar to the autoregulatory loop by which PKA activity regulates the promoter activities of its subunits.

For example, a regulatory loop in which the expression of PKA subunits is controlled by lipid metabolic pathways and PKA activity regulates in turn enzymes involved in phospholipid synthesis by phosphorylation could be proposed. Indeed, many enzymes involved in phospholipid synthesis are known substrates of PKA including PS synthase, CTP synthetase, choline kinase and PA phosphatase (Carman and Kersting 2004). In addition, the transcriptional repressor Opi1 is also regulated by PKA phosphorylation (Sreenivas and Carman 2003).

Regulatory loops can also be proposed to categories related to mitochondria, vacuole, transport and sporulation known to be regulated by PKA. It is well documented that the cAMP pathway regulates mitochondria function, as unregulated PKA activity can lead to the production of mitochondria that are prone to the production of ROS, and to an apoptotic form of cell death (Feliciello, Gottesman and Avvedimento 2005; Aun, Tamm and Sedman 2013; Galello, Moreno and Rossi 2014). Progression of sporulation is also known to be regulated from an interconnected signaling network that includes the Ras/cAMP pathway (Honigberg and Purnapatre 2003). Although it is clear how PKA activity regulates sporulation and mitochondria functioning, it is more difficult to envision how their dysfunction would deregulate the expression of PKA subunits. We observed an upregulation of the promoters of PKA subunits during growth in the presence of glycerol as carbon source (results to be published elsewhere). Thus, during a respiratory metabolism, when proper mitochondrial functioning is necessary, activation of the PKA subunit promoters is observed. Here, our SGA results are suggesting that defective mitochondria deregulate PKA subunits promoter activities. Therefore, a reciprocal regulation could be proposed by which PKA regulation plays a physiological role in coordinating respiratory function with nutritional status in budding yeast and the respiratory metabolism would upregulate PKA subunit promoter activities.

Another interesting example is the regulation of vacuolar function and proton acidification. Mutants lacking several subunits of the vacuolar-ATPase (*vma2, vma4, vma5, vma7, vma13, vma21* and *vma22*) have been identified in our screen. The V-ATPase is a proton pump that regulates cytosolic pH by pumping protons into the vacuole. There are at least two possible explanations of the participation of the proton pump in the PKA subunits transcription regulation. One is based in recent studies that suggest that the V-ATPase is required for the glucose-mediated stimulation of PKA and that cytosolic pH serves as a second messenger in this regulatory pathway (Dechant *et al.* 2010). Activation of V-ATPase is required for full activation of PKA upon glucose stimulation, thereby transducing, at least in part, the pH signal to PKA. As PKA activity autoregulates the activity of its own subunit promoters, malfunction of V-ATPase could indirectly impact the activity of the promoters by affecting the expression level of PKA subunit and therefore the kinase activity. The other is based on research that indicates that intracellular acidification stimulates cAMP synthesis *in vivo* (Thevelein 1991; Colombo *et al.* 1998). Mutants for any vacuolar-ATPase subunit should have altered proton pumping to the vacuole and will therefore display low intracellular pH. In this situation, the cAMP synthesis is stimulated through activation of the Ras proteins. High cAMP would activate PKA activity and therefore would also affect the expression of *TPK* genes.

CONCLUSIONS

The results of our screen were successful in identifying some of the expected transcription factors as modulators, while pointing to inositol and inositol-polyphosphates, choline and phosphate as novel upstream signals that regulate transcription of PKA subunit genes. The results also suggest the existence of regulatory loops where PKA controls by phosphorylation enzymes involved in various metabolic pathways, which in turn control somehow the level of expression of PKA subunits by a

Table 1. Yeast strains.

Strain	Genotype	Reference or source
BY4256	*MATα can1Δ::STE2pr-his5 lyp1Δ HO::RPL39prtdTomato::hphMX*	Kainth *et al.* (2009)
BY4741 (WT)	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
ino2Δ	(BY4741) *ino2::KanMX4*	EUROSCARF
ino4Δ	(BY4741) *ino4::KanMX4*	EUROSCARF
dgk1Δ	(BY4741) *dgk1::KanMX4*	EUROSCARF
opi1Δ	(BY4741) *opi1::KanMX4*	EUROSCARF
rtg2Δ	(BY4741) *rtg2::KanMX4*	EUROSCARF
flo8Δ	(BY4741) *flo8::KanMX4*	EUROSCARF
swi4Δ	(BY4741) *swi4::KanMX4*	EUROSCARF
snf2Δ	(BY4741) *snf2::KanMX4*	EUROSCARF
snf5Δ	(BY4741) *snf5::KanMX4*	EUROSCARF
mot3Δ	(BY4741) *mot3::KanMX4*	EUROSCARF
cbf1Δ	(BY4741) *cbf1::KanMX4*	EUROSCARF

mechanism which deserves further intensive investigations. Taking as a whole, these results strengthen our hypothesis about the role of transcription regulation to collaborate with cAMP-PKA specificity signaling since each subunit has common and specific regulators.

EXPERIMENTAL PROCEDURES

Strains, plasmids and culture conditions

Table 1 lists the genotype of the strains used in this study. For the β-galactosidase reporter assays and RNA purification, the strains were cultivated at 30°C to late log phase (OD_{600} = 3.5, 0,5 × 10^7–1 × 10^8 cell ml^{-1}) in synthetic media containing 0.67% yeast nitrogen base without amino acids, 2% glucose plus the necessary additions to fulfill auxotrophic requirements. In the assays to measure inositol and choline effects, yeast cultures were grown at 30°C in a complete synthetic medium lacking inositol, choline (Klig and Henry 1984) and uracil (in the case of reporter plasmids). Where indicated, 75 μM inositol (I) and/or 1 mM choline (C) was added. The assays to analyze the phosphate effect were performed in low-Pi medium containing 0.15 mM KH_2PO_4, and high-Pi medium containing 7.35 mM KH_2PO_4. High-throughput functional assays were performed with plasmids containing the 5′ regulatory region and nucleotides of the coding region of *TPK1*, *TPK2*, *TPK3* and *BCY1* genes (positions –800 to +10 with respect to the ATG initiation codon in each case), cloned into pBA1926 controlling GFP expression. The plasmids used in β-galactosidase reporter assays 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 same 810 bp regulatory fragments included in the pBA1926 constructs.

High-throughput functional assay

The *S. cerevisiae* array of 4500 viable strains, each missing a non-essential gene (BY4741 background, Euroscarf) was used in the R-SGA screen (Kainth *et al.* 2009) to detect genes that affect the expression of a GFP reporter system under the control of *TPK1*, *TPK2*, *TPK3* and *BCY1* promoters. The–800 to +10 sequences from each promoter were cloned upstream the GFP reporter gene. The constructs were used to transform the BY4256 strain carrying the RPL39pr–tdTomato reporter (RFP). After mating, the collection strain with the BY4256 MATα strain carrying each of the *pr*GFP constructs, diploids were selected and sporulated followed by the selection of MATa xxxΔ haploids containing the *pr*GFP plasmid. Robotic manipulation of the collection was performed using a Biomatrix robot and plate imaging system (S&P Robotics Inc.). Colony size for each arrayed mutant was analyzed, and positions on the array with no or slow colony growth were eliminated from further analysis. Colony fluorescence was assayed following 3 days of incubation on minimal glucose medium using PharosFX Molecular Imager (Bio-Rad). The data were analyzed with Array Gauge V1.2 software. The log2 GFP/RFP ratio from each colony on the array was calculated as described (Kainth *et al.* 2009). A large number of deletion mutants were represented twice in the array, and each screen was performed in duplicate, resulting in quadruplicate measurements for some deletion mutants. In order to testing for normality of the distribution of the log2(GFP:RFP) data, samples were standardized and compared with a standard normal distribution using the Shapiro–Wilkins test and the Kolmorogov indicating the normality of the distribution. These log2 ratios were transformed to robust Z scores using median and median absolute deviation, and p-values were assigned to these Z scores based on a normal distribution. A p-value cut-off of 0.01 and 0.05 was set. A list of 260 deletion mutants that usually appear as hits in several screens (Henny Goettert and Brenda Andrews, personal communication) was removed from further analysis of specific *TPK*s and *BCY1* promoter regulators.

GO analysis

The distribution of regulators into functional groups was assessed by measuring the enrichment for genes in the same functional category according to the gene annotation database of FunSpec (http://funspec.med.utoronto.ca/). The enrichment of the genes belonging to the GO biological process, GO molecular function and MIPS functional classification categories was calculated with respect to the total gene number and the total number of genes belonging to the GO category, with a p-value of 0.05 or 0.01. The hypergeometric distribution was used to calculate a p-value for this fraction, and took $P < 0.05$ or $P < 0.01$ to be significant. Only the results for stationary growth phase are shown.

β-Galactosidase assays

Cells were grown on SD medium at 30°C up to an OD_{600} of 3.5. 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 was measured and expressed as Miller units (Miller 1972). Results shown correspond to a representative experiment which was repeated at least three times, each one performed in duplicates or triplicates.

qRT-PCR

Total RNA was prepared from different yeast strains, grown at 30°C to the same OD_{600} as for β-galactosidase assays, using standard procedures. To determine the relative levels of specific *TPK1*, *TPK2*, *TPK3* and *BCY1* mRNAs, a qRT-PCR experiment was carried out. Aliquots (~10 μg) of RNA were reverse-transcribed into single-stranded complementary cDNA using an oligo-dT primer and Superscript II reverse transcriptase (Life Technologies). The single-stranded cDNA products were amplified by real-time PCR using gene-specific sense and antisense primers (mRNA *TPK1*: Fw: 5′ CCGAAGCAGCCACATGTCAC 3′, Rv: 5′ GTACTAACGACCTCGGGTGC 3′; mRNA *TPK2*: Fw: 5′ GCTTGTGGAGCATCCGTTTC 3′, Rv: 5′ CACTAAACCATGGG TGAGC 3′; mRNA *TPK3*: Fw: 5′ CGTTGGACAAGACATTCCTG 3′, Rv: 5′ GTCGGT TATCTTGATATGGCC 3′; mRNA *BCY1*: Fw: 5′ CGAACAGGACACTCACCAGC 3′, Rv: 5′ GGTATCCAGTGCATCGGCAAG 3′; mRNA *TUB1* (α-Tubulin gene): Fw: 5′ CAAGGGTTCTTGT TTACCCATTC 3′, Rv: 5′ GGATAAGACTGGAGAATATGAAAC 3′). The PCR products were visualized using SYBR® Green (Life Technologies). The relative mRNA levels of *TPK1*, *TPK2*, *TPK3* and *BCY1* were first normalized to those of *TUB1* and then relativized as indicated in each figure. Quantitative data were obtained from three independent experiments and averaged.

Statistics analysis

All the experiments were replotted several times (indicated in each figure) with independent cultures. Figure 4 upper panel, Fig. 3 and Fig. 5 were analyzed using the two- way ANOVA-Dunnett´s multiple comparisons test (α 0.05). Figure 4 lower panel and Fig. 4B were analyzed using one-way ANOVA-Sidak´s multiple comparisons test.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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