Deficiency of Pkc1 activity affects glycerol metabolism in *Saccharomyces cerevisiae.*

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Abstract

Protein kinase C is apparently involved in the control of many cellular systems: the cell wall integrity pathway, the synthesis of ribosomes, the appropriated reallocation of transcription factors under specific stress conditions and also the regulation of N-glycosylation activity. All these observations suggest the existence of additional targets not yet identified. In the context of the control of carbon metabolism, previous data demonstrated that Pkc1 p might play a central role in the control of cellular growth and metabolism in yeast. In particular, it has been suggested that it might be involved in the derepression of genes under glucose-repression by driving an appropriated subcellular localization of transcriptional factors, such as Mig1 p. In this work, we show that *pkc1Δ* mutant is unable to grow on glycerol because it cannot perform the derepression of *GUT1* gene that encodes for glycerol kinase. Additionally, active transport is also partially affected. Using this phenotype, we were able to isolate a new *pkc1Δ* revertant. We also isolated two transformants identified as the nuclear exportin Msn5 and the histone deacetylase Hos2 extragenic suppressors of this mutation. Based on these results, we postulate that Pkc1 p may be involved in the control of the cellular localization and/or regulation of the activity of nuclear proteins implicated in gene expression.

1. Introduction

Glycerol, besides being the compatible solute that *Saccharomyces cerevisiae* accumulates under osmotic/salt stress, is also a key metabolite which pathway plays a central role in regulation processes such as redox balance, Pi availability and lipid synthesis. Glycerol can also be used as carbon source and is transported simultaneously by the constitutively expressed Fps1p channel [3, 32, 54] and by a proton symporter repressed by glucose and inducible by growth on non fermentable carbon sources [24, 28]. Furthermore, glycerol is catabolized in *S. cerevisiae* through two subsequent enzymatic steps. The first leads to the production of glycerol 3-P, an important lipid synthesis intermediate, by glycerol kinase encoded by *GUT1* [40, 51]. The second metabolic step connects glycerol consumption to glycolysis through the production of dihydroxyacetyone-P by a FAD-dependent glycerol 3-P dehydrogenase encoded by *GUT2* [42]. This enzyme is localized in the outer membrane of mitochondria and plays, together with the cytosolic NADH-dependent glycerol 3P dehydrogenase (Gpd1p, Gpd2p), a determinant role on the redox shuttle between mitochondria and cytoplasm [30].

Consistent with these multiple roles a major signalling pathway has been named upon glycerol synthesis modulation through the transcriptional control of the *GPD* genes*, i.e.,* the High Osmolarity Glycerol (HOG) pathway. This plays an important role in vital responses to osmotic, salt or temperature stress [57]. Furthermore it interplays with other signalling pathways involved in cell shaping, polarization, mating and integrity, from which we stress the PKC MAP Kinase pathway [6, 23].

Both *GUT1* and *GUT2* have long been known to be under glucose repression [17, 42]. *GUT1* repression is independent of Mig1p and occurs through Opi1p, a repressor involved in inositol metabolism. The Adr1p and Ino2/Ino4p are responsible for more than 90% of *GUT1* expression during derepression conditions [17].

Recently, it was demonstrated that chromatin binding of Adr1p is controlled by Snf1 protein kinase [58], whereas Opi1p activity is controlled by phosphorylation in a PKC dependent mechanism [52]. These data are in accordance with the existence of a complex net acting in the control of glycerol metabolism in yeast, which is consistent with the multiple roles mentioned above.

The role of Pkc1p in the control of carbon metabolism seems to be rather broad, since *pkc1Δ* mutant displays defects on fermentation initiation and the derepression of different enzymes upon glucose starvation [43]. In addition, *pkc1Δ* has low respiratory capacity. This mutant is also unable to use glycerol as sole carbon source. In this context, *pkc1Δ* inability to grow on glycerol may be due to a respiratory defect or to a link between protein kinase C and glycerol metabolism regulation. Moreover, *pkc1Δ*, when compared to the wild type strain, shows poor or slow growth in media containing galactose or raffinose, as well as a defect in derepression of invertase activity, *i.e., SUC2* expression upon transfer of cells from glucose to raffinose [5, 43]. All these results suggest that Pkc1p activity could be connected to the glucose repression mechanism in *S. cerevisiae*. It has been suggested that Pkc1p may control the cellular localization of Mig1p transcriptional factor [43] independently of Snf1p activation [19, 25, 35].

Besides the role in the control of carbon metabolism, Pkc1p pathway is essential for the maintenance of cellular integrity by controlling the expression of genes encoding enzymes involved in cell wall construction [21]. It seems to be connected to other signal transduction pathways and/or cellular processes such as the mating MAP kinase pathway [6], cell fusion [41], polarized growth [2], regulation of actin cytoskeleton polarization [12; 33], control of morphogenesis checkpoint during cell cycle [20] and several other events that affect cell membrane [46]. Accordingly, it has been suggested the involvement of PKC pathway, in connection with calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase, in the

101 temperature-induced expression of *FKS2,* a gene encoding the catalytic subunit of
102 the 1,3-β-glucan synthase [59]. This enzyme is a dimer with Rhop1p as its
103 regulatory subunit, acting upstream Pkc1p in the correspondent-signalling pathway
104 [48].

105 Moreover, the PKC MAP Kinase pathway cascade regulates the expression of
106 yet another subset of yeast genes targeting the formation of one of the two complex
107 forms of RNA polymerase II [9].

108 Concerning carbon metabolism, in a previous work [43] it had been shown that
109 the *pkc1Δ* mutant could not grow on glycerol. This could be due to the genes
110 involved in this substrate consumption being under PKC pathway control. In order to
111 evaluate this possibility, the two first steps needed for glycerol catabolism, transport
112 and phosphorylation by glycerol kinase were studied. In this work, we demonstrate
113 that Pkc1p controls glycerol consumption, affecting the glycerol kinase activity by
114 regulating the *GUT1* gene expression via a MAP Kinase–independent pathway. This
115 reinforces the idea that there is a bifurcation eventually at the level of Pkc1p with
116 consequent alternative targets. We also demonstrate that the growth phenotype is
117 not due to a defect on glycerol symporter activity, which is affected but not abolished
118 in *pkc1Δ* strain.

119 Furthermore, a new mutant from the *pkc1Δ* strain presenting a constitutive
120 derepression phenotype has been found, as well as two extragenic suppressors of
121 this mutation that were identified as the nuclear exportin Msn5p and the histone
122 deacetylase Hos2p. Considering previous data, the results presented here support
123 the idea that Pkc1p could be involved in the control of the cellular localization and/or
124 regulation of the activity of nuclear proteins implicated in gene expression.

2. Materials and methods

2.1 Strains and growth conditions

The *Saccharomyces cerevisiae* strains used in this study were: W303-1A (*Mat a ade2-1 his3*-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 GAL mal SUC2), YSH813 (W303-1A + *bck1 ⊿::LEU2*), YSH850 (W303-1A + *pkc1⊿::HIS3*) and CLY3 (isogenic to W303 but *gut1* [24]). It was also used the TOP10F *E. coli* strain (*F', mcr A, ⊿ (mrr-hadRMS - mcrBC) Ø 80⊿ lacZ ⊿M15, ⊿lacx74, deoR, rec ⊿1, araD139, ⊿ (ara, leu), 7697, gal IU, galK, λ⁻ rs2p, end ⊿1, nupG).* Yeast cells were grown at 30ºC in YP medium (2% (w/v) peptone and 1% (w/v) yeast extract) supplemented with variable concentrations of the carbon sources glycerol, glucose, galactose, raffinose or fructose and 1M sorbitol as indicated. Growth was monitored by measuring OD at 600 nm or by drop test on solid media *E. coli* cells were grown at 37ºC in LB medium (1% (w/v) triptone; 0.5% (w/v) yeast extract; 0.5% NaCl (w/v); 1.5% agar (w/v); pH).

2.2 Determination of invertase and glycerol kinase activities

Invertase activity was measured as described before [16] with the modification introduced by Celenza and Carlson [8] except that the assay was carried out at pH 5.1 and 37ºC. Glycerol kinase activity was determined in cell free extracts obtained with 50 mM imidazol buffer without sorbitol. Measurement of specific activity of glycerol kinase was performed according to Castro and Loureiro-Dias [7] by following ADP formation and using 1 unit. ml^{-1} pyruvate kinase and lactate dehydrogenase in the coupled reaction. The reaction was started by the addition of 0.1 mM of glycerol. An extinction coefficient of 6.22 (l. $mmol^{-1}$. cm^{-1}) for NADH was used for calculations. Enzyme specific activities were expressed in relative units of cell free extracts total protein. Protein was estimated by the method of Lowry [31] using bovine serum albumin as standard.

2.3 Glycerol transport studies

Initial rates of glycerol uptake as well as in/out accumulation ratios, were determined as previously described [28]. The intracellular volume value used to determine intracellular glycerol molarity has been determined by Lages and co-workers [29].

2.4 RNA isolation and Northern-blot analysis

For a shift from growth on glucose to growth on glycerol, yeast cells were grown in 20 ml YP with glucose (4% w/v) up to OD ± 0.8-1.2. The sample was split in two and washed quickly by centrifugation with 1M sorbitol. One of the cellular pellets was used for RNA extraction (repressive state). The other was resuspended in YP glycerol (3%, w/v) and glucose (0.05%) plus 1M sorbitol, rapidly mixed and incubated as before. After two hours, the sample was washed in the same way and the pellet used for RNA extraction (derepressed state). Total yeast RNA was isolated by the hot acid phenol method [44]. 15 µg of total RNA was separated on 1% (w/v) agarose in 50 mM boric acid, 1mM sodium citrate, 5 mM NaOH, pH 7.5; containing 1% (w/v) formaldehyde. Subsequently RNA was blotted onto Hybond-N membranes in 10X SSC (1.5M NaCl, 0.15 M sodium citrate, pH 7.0) and hybridized with gene-specific probes. These were obtained by PCR with the following primers: f5'-AATAGTTATATGTTTCCC-3' and r5'-GCTATTTATGTTGTTATTGG-3' for *GUT1* and f5'-GCTGCTTTGGTTATTGATAAC-3' and r5'-GATAGTGGACCACTTTCGTCG-3' for *ACT1* (constitutive endogenous control). Probes were radioactively labelled using the Rediprime™ II labelling kit (Pharmacia). The RNA levels were visualized by exposing the membrane to CL-X Posure™ Film from Pierce.

176 *2.5 Mutagenesis of yeast cells using UV radiation*

177 Yeast cells were grown overnight (OD_{600nm} ± 1), collected by centrifugation,
178 washed and resuspended in 4 ml 1M sorbitol. Appropriated dilutions of this
179 suspension (1/10; 1/100 and 1/1000) were plated and exposed to a different UV dose
180 (0, 2, 10 and 15 mJ). Immediately after, the cells were wrapped in aluminum foil to
181 avoid photo reactivation and incubated at 30ºC 3 to 5 days. The mutants obtained in
182 this process were selected on YPglycerol plates plus 1M sorbitol. They were
183 subsequently transformed with a multi-copy yeast genomic DNA library (YEP13
184 multi-copy vector, ATCC 37329), using the lithium acetate procedure [26]. The
185 transformants were selected in SD-glucose + 1M sorbitol [40] according to the
186 auxotrophic marks. In a second selection round, the colonies were plated in medium
187 YPglycerol + 1M sorbitol. Revertants of the *pkc1* Δ mutant phenotype on glycerol
188 were selected and tested for invertase activity after growth (OD ± 1.5) on 4% glucose
189 and 2% raffinose.

190

191 *2.6 DNA manipulation*

192 Plasmid DNAs were rescued from yeast transformants [11] and amplified in
193 *Escherichia coli* TOP10F'. Suitable restriction fragments of the selected clone were
194 subcloned and sequenced [45]. All reactions were carried out using the ABI Prism
195 BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kits and the ABI^{R} 310 DNA
196 Analyser. After editing, sequences were compared in the *Saccharomyces* Genome
197 Database to identify the over expressed sequences.

198 To test mutations introduced through mutagenesis in the genes *MSN5* and
199 *HOS2,* approximately 0.3 µg of the genomic DNA extract from strain 335 were used
200 as template in standard PCR reactions using specific primers. In each amplification,
201 the products were analyzed by agarose gel electrophoresis. The amplicons were
202 sequenced and compared with the published *MSN5* and *HOS2* sequences. Three

different amplicons for each gene were sequenced to ensure that mutations were not introduced into DNA fragments by the PCR.

2.7 Subcellular localization of Mig1p by analysis of GFP fluorescence

To study the subcellular localization of Mig1p, we used yeast cells (wild type and mutant strains *pkc1* Δ and 335) transformed with a plasmid containing the transport domain of *Mig1* fused to *GFP* gene [11]. Cells were grown on YP medium supplemented with 4% (w/v) glucose until mid-exponential phase. Samples (1 ml) were harvested and washed by centrifugation (13000 rpm in a microcentrifuge) with a cold solution of sorbitol (1M) plus glucose (4%, w/v). The remaining cell suspension was transferred to YP-raffinose (2%, w/v) medium for 1 hour. The cells were harvested and washed by centrifugation with a cold solution containing sorbitol (1M), plus glucose (2%, w/v) (repressed cells) or glycerol (3%, w/v) (derepressed cells). Finally 10-15 µl of these cell suspensions were mixed with 10-15 µl of agarose (1%, w/v), at 37ºC for microscopy observations. Images were registered by using an Olympus BX51 TRF microscope with accessory apparatus for fluorescence. Nuclei were visualized using a DNA dye solution containing 2% Hoechst 33342 (Molecular Probes).

221 **3. Results**

222

223 The strain deleted in *PKC1* gene is unable to grow on non-fermentable
224 carbon sources [43]. This growth defect is not present in the *bck1Δ* (the first protein
225 kinase of the MAP Kinase pathway) and wild type (W303-1A) strains, indicating that it
226 is not dependent on the downstream components of the MAP kinase pathway [6]. It
227 is well known that glycerol kinase (encoded by *GUT1*) is subjected to glucose
228 repression and required for glycerol metabolism [24]. Therefore, we investigated
229 whether glycerol kinase activity could be constitutively repressed in the *pkc1Δ*
230 mutant. In Fig. 1A, we show that, contrarily to the W303-1A (wild type) and *bck1Δ*
231 strains, the *pkc1Δ* mutant presented a very low glycerol kinase activity after cells
232 have been shifted from glucose to glycerol. Accordingly, Northern blot analysis (Fig.
233 1B) evidenced that the *GUT1* gene was less efficiently derepressed in the *pkc1Δ*
234 mutant than in wild type and *bck1Δ* strains.

235 Considering that growth defect on glycerol might be caused not only by a
236 defect on *GUT1* expression but also by a defect on active transport activity [24], we
237 measured glycerol uptake in the *pkc1Δ* mutant. This strain presented a radiolabelled
238 glycerol accumulation capacity approximately half of the wild type strain assayed in
239 derepression conditions identical to the ones above mentioned for Gut1p activity
240 assays, *i.e.,* grown in YPD with 1M sorbitol and subsequently incubated in YPG in
241 the presence of 1M sorbitol (Fig. 2). Consistently, uptake V_{max} in the *pkc1 Δ* mutant
242 was 288±37 μmoles h^{-1} g^{-1} (n=3), while the estimated V_{max} in wild type was 392±25
243 μmoles h^{-1} g^{-1} (n=3), a value within the range of the ones determined previously in
244 the same strain [24, 36]. The affinity of the carrier (K_m) in either strain remained
245 within the predicted interval according to previous statistical validation [36]: 3.4±0.6
246 mM (n=4) and 3.5±1.3 mM (n=6) in wild type and *pkc1Δ,* respectively [24, 29].
247 Additionally, *bck1Δ* strain was also assayed, presenting, consistently with the results

above, transport-unchanged ability (V_{max} 329±31 µmoles h^{-1} g^{-1} (n=3); K_m 2.1±0.5 mM (n=3)) (Fig. 2).

Additionally, glycerol uptake V_{max} was determined in the *gut1Δ* strain grown and subsequently incubated in identical conditions as mentioned above. This was performed as a control for *pkc1Δ* mutant, taking into consideration previous data that showed glycerol kinase to have an effect on the initial rates of glycerol uptake used for transport kinetic constants determination [24, 36]. The value obtained for V_{max} was 350±41 µmoles h^{-1} g^{-1} (n=3), once more, in the range of the wild type V_{max} . This indicates that the reduced transport velocity determined in the *pkc1Δ* mutant is not an indirect consequence of the inability to express the Gut1p.

In order to get more information on the mechanism by which Pkc1p controls glycerol metabolism, a strategy involving the generation of new mutants was developed. The *pkc1Δ* mutant was exposed to UV irradiation originating eleven new mutants that recovered the capacity to grow on glycerol (not shown). However, they still did not grow in the absence of sorbitol (not shown), suggesting that the new mutation probably did not occur in the downstream components of the PKC MAP Kinase pathway. In this way, a new strategy was applied to obtain mutants able to revert *pkc1Δ* defect of growth on non-fermentable carbon sources. One of mutants was chosen, strain 335, for showing reversion of *pkc1Δ* phenotype on raffinose (slow growth) and/or glycerol (no growth) (Fig. 3).

In Fig. 1A, we show that strain 335 presented a higher glycerol kinase activity after cells have been shifted from glucose to glycerol. In the same way, Northern blot analysis (Fig. 1B) revealed that the *GUT1* gene was constitutively derepressed in this strain when compared to the wild type and *bck1Δ* strains. Furthermore, mutant strain 335 presented uptake with a V_{max} of 324 (n=2), in the range of the values found for wild type strain. Accordingly, accumulation ability was also not significantly different from the one measured in wild type (Fig. 2).

On the other hand, in Fig. 3, it is shown that this strain grows on glycerol and therefore seems to have recovered the respiratory growth ability of the wild type. Consistently, in Fig. 4 we show that, when grown in liquid medium having glucose as carbon source, strain 335 presents diauxic cellular growth, which second phase is inhibited by antimycin, known to block the respiratory chain.

Considering that invertase is one of enzymes well known to be down regulated by *pkc1* deletion, the invertase activity of this mutant was measured in cells grown on glucose and raffinose (Fig. 5) and compared with wild type and *pkc1Δ* strains. As expected, in the wild type strain the classical pattern was observed: low level of activity in glucose (or fructose-grown cells) and a high level in raffinose-grown cells, while in the *pkc1*Δ mutant, a low level of invertase activity was observed in both media. The mutant strain 335 presented a very interesting phenotype, being apparently constitutively derepressed, since the invertase activity was high in both carbon sources (Fig. 5).

Mig1p was previously shown to remain in the nucleus in the *pkc1Δ* mutant, even under derepression [43]. For this reason, we decided to investigate the cellular localization of Mig1p to see if the constitutive derepressive phenotype observed in the 335 mutant was due to the fact that Mig1p would remain outside the nucleus. The results presented in Fig. 6 show Mig1p to be localized inside the nucleus in both strain 335 and *pkc1Δ* either after repressive or derepressive growth conditions. This suggests that strain 335 derepressed phenotype, inferred from the results above, cannot be explained by the control of the cellular localization of Mig1p.

Taking into account all these results, and considering the possibility that 335 mutant could present a mutation in a gene encoding for a protein originally under the control of Pkc1p, we complemented the strain 335 with two different yeast genomic libraries. Our selection strategy was based on the recovery of the inability to grow on glycerol. We were able to recover five transformants using the multi-copy library

YEP13, all of which did not grow in the absence of 1M sorbitol (not shown). We chose two of these strains, 342 and 345, for showing partial reversion phenotypes: these transformants cannot grow on glycerol, such as *pkc1Δ* (Fig. 3); nevertheless they can still grow on raffinose, although with certain difficulty. Moreover, when grown in liquid medium having glucose as carbon source, they did not present growth diauxic phase (data not shown). When the invertase activity was measured, these strains presented an intermediary activity (Fig. 5) compared to the ones observed in the *pkc1 Δ* mutant and strain 335.

The insert isolated from strain 342 presented around 4600 base pairs in length, containing an open reading frame of 3675 bp in length corresponding to *MSN5* gene sequence, which encodes for a nuclear exportin. On the other hand, the DNA fragment extracted from strain 345 presented 2300 bp, and contained *HOS2* gene complete sequence, encoding for a Class I histone deacetylase. These two genes were isolated by PCR from strain 335 and further sequenced, showing no mutation. We can therefore consider that they apparently encode putative extragenic suppressors of the derepression phenotypes from strain 335.

4. Discussion

The Pkc1p seems to be involved in many important cellular functions beyond controlling cell wall synthesis through the PKC MAP Kinase pathway. There are evidences indicating the participation of Pkc1p in the control of ribosomal synthesis, the appropriated reallocation of transcriptional factors under specific stress conditions [34] and also the regulation of N-glycosylation activity [39]. Pkc1p seems to be essential to the glucose-induced activation of the plasma membrane ATPase [50] and also for the derepression mechanism of the glucose-repressible genes [5; 43]. Together, all these data reinforce the idea that Pkc1p is involved in many important functions in yeast cells. The present work elucidates deeper into the mechanisms underlying the involvement of Pkc1p in the control of different glucose mediated effects.

S. cerevisiae can utilize glycerol as sole carbon and energy source whereas *pkc1Δ* mutant is unable to grow on this substrate [5; 43]. Glycerol uptake can be performed through Fps1p channel [37, 53] or by a glycerol/proton symport [24; 28]. Once inside the cell, glycerol is first converted into glycerol-3-phosphate by the enzyme glycerol kinase (Gut1p) and this is oxidized to dihydroxyacetone phosphate by the mitochondrial enzyme glycerol-3-phosphate dehydrogenase (Gut2p). Cells presenting deletions in both *GUT1* and *GUT2* genes are unable to grow in media containing glycerol as carbon source suggesting that this is the unique pathway operating for the glycerol catabolism in *S. cerevisiae* [51].

In this work, we demonstrated that a *pkc1Δ* mutant could not grow on glycerol because the transcription of *GUT1* gene and consequently glycerol kinase activity are abolished. Furthermore, we showed that glycerol active transport in this strain is affected, since its accumulation capacity is considerably reduced in relation to wild type, concomitantly with a reduction of approximately 30% in the corresponding V_{max}.

This effect appears to be independent of the downstream components of the PKC MAP kinase pathway since in the *bck1Δ* mutant, both glycerol kinase activity as well as *GUT1* expression and transport activity present the same pattern observed in the wild type strain. These results seem to confirm the existence of bifurcation at the level of Pkc1p in this pathway. Moreover, the *pkc1Δ* mutant shows low invertase activity, regardless of the carbon source used for cultivation. On the other hand, when glucose is used as carbon source, this mutant did not present the respiratory growth phase observed in wild type.

For this reason, we decided to try the identification of new putative components of a pathway under direct control of Pkc1 p and involved in the derepression of glucose-repressed genes. The isolated mutant (strain 335) reverted the *pkc1Δ* phenotypes: it recovered the ability to grow on glycerol and presented a constitutive derepression of glycerol kinase as well as invertase activity. Concomitantly, mutant 335 was still able to grow following glucose exhaustion, supposedly consuming respirable substrates like ethanol produced during glucose consumption.

Pkc1p seems to be responsible for the phosphorylation of Opi1p that is a negative regulator of *GUT1* expression [17, 52]. It is still not clear how the phosphorylation of Opi1p affects its function. However, Pkc1p is involved in the reallocation of transcription factors [34, 43], therefore, it seems reasonable to imagine that Opi1p phosphorylation might be important in the control of its subcellular localization. Indeed, we have previously demonstrated that Pkc1p is important for an appropriate reallocation of Mig1 transcription factor [43] which action is essential for the glucose repression of several genes [38]. According to this, the constitutive repression exhibited by *pkc1Δ* mutant could be at least partially explained by the permanent nuclear localization of Mig1p regardless the carbon source used. This finding agrees with the previous suggestion of Pkc1p being essential to the control of

372 subcellular localization of transcription factors [34]. In this context, Mig1p localization
373 could be predicted as essentially extranuclear in the strain 335. However, our results
374 demonstrate that Mig1p remains in the nucleus in the mutant 335 in either repressive
375 or derepressive conditions indicating that Mig1p is not the agent responsible for the
376 derepression observed in this mutant. These results add a higher level of complexity
377 to a hypothetical pathway where Pkc1p appears to have a pivotal role in the
378 derepression of different genes.

379 We were able to isolate two extragenic suppressors of the 335 strain
380 phenotype: the ß exportin Msn5, involved in the traffic of transcriptional factors such
381 as Mig1p from the nucleus to the cytoplasm [13, 47], and the deacetylase Hos2 that
382 acts on chromatin structures enabling the induction of some genes [55]. It has been
383 suggested that Pkc1p may control the cellular localization of Mig1p transcriptional
384 factor [43] independently of Snf1p activation [19, 25, 35]. Apparently, this
385 transcriptional repressor contains a nuclear export signal that is phosphorylated by
386 Snf1 provoking its recognition by Msn5p [13]. Msn5 p was originally identified as a
387 high-copy suppressor of the *snf1* mutation [14; 15]. It seems to be a nuclear protein
388 involved in different cellular processes such as carbon source utilization, calcium
389 tolerance, mating, and cyclin specific functions [1]. Moreover, class I histone
390 deacetylases are involved in the transcription regulation of many genes by affecting
391 the chromatin organization [27]. Particularly, the deacetylation of lysine residues in
392 the H3 and H4 histone tails is required for interaction with Tup1p/Ssn6p, the co-
393 repressor complex involved in the regulation of many glucose-repressed genes [4,
394 10, 49, 56]. However, it has been demonstrated that Hos2p and Rpd3p, both class I
395 histone deacetylases, seem to have antagonic actions. Deacetylation triggered by
396 Hos2p seems to be necessary for transcription induction of genes such as *GAL* and
397 *INO1* genes, while the Rpd3p activity is required for repression. Apparently,
398 differences between Hos2p and Rpd3p in the dynamic chromatin binding, as well as

399 the additional deacetylation of H2A and H2B histones by Rpd3, would be responsible
400 for the different roles in the gene regulation [55].

401 Although these proteins are direct or indirectly involved in mechanisms
402 controlling gene expression, the results obtained in this work do not allow us to
403 establish a conclusive relationship between Pkc1p activity and Msn5p or Hos2p.
404 However, they still allow us to propose a model mechanism in which Pkc1p would
405 regulate a protein necessary for the activation and/or translocation of nuclear
406 proteins essential for the derepression process. Thus, the mutations in the strain 335,
407 would have affected this protein turning the derepression control independent of
408 Pkc1p. Following this model, the overexpression of *MSN5* gene (strain 342) would
409 promote unspecific translocation of nuclear factors involved in derepression. On the
410 other hand, the partial reversion of the original phenotype observed in strain 335 by
411 the overexpression of *HOS2* gene (strain 345) is most probably a very different issue
412 relating to the putative unspecific action of this histone deacetylase on target sites of
413 other similar enzymes like for example Rpd3, which deacetylation is clearly involved
414 in glucose repression [10].

415 Since the proteins Mig1p and Hxk2p are directly involved in the glucose
416 repression of genes like *SUC2* [22], it is not possible to discard the hypothesis that
417 the mutation in the strain 335 occurred in the genes *MIG1* and *HXK2*. However, it
418 does not seem probable because there are no evidences indicating their participation
419 in either glycerol metabolism regulation [18] or in the transcriptional regulation of
420 genes encoding for components of the respiratory apparatus [47]. Still, other
421 transcription factors like Sko1 have been suggested to act either as positive or
422 negative regulator of transcription according to promoter specificity [23].

423 In conclusion, our findings do not allow us to propose a precise me chanism
424 by which Pkc1p participates in the derepression process in yeast cells. However, we
425 can postulate that Pkc1p appears to be involved in the control of the cellular
426 localization and/or regulation of the activity of proteins implicated in glycerol

metabolism, in particular the glycerol symporter as well as the glycerol kinase. Still further investigation will be needed to better understand the molecular basis for these assumptions.

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Legends

Figure 1 – Glycerol kinase activity (Panel A) and *GUT1* expression levels (Panel B) on *S. cerevisiae* wild type and mutant strains. Cells were grown in YPD supplemented with 1M sorbitol, repression conditions (R), and subsequently incubated in YPG with the same amount of sorbitol for derepression (D), for 6 hours (Panel A) and 2 hours (Panel B). The expression was determined by Northern blot analysis using a probe for the constitutive actin gene *ACT1* as control.

Figure 2 – Radiolabelled glycerol accumulation ratios on *S. cerevisiae* cells cultivated on YPD supplemented with 1M sorbitol and subsequently incubated for 6 hours in YPG with the same amount of sorbitol for derepression: (□, wild-type W303-1A; ■, *bck1Δ*; O, *pkc1Δ*; ●, strain 335 (new mutant isolated from UV radiation on *pkc1 Δ*). Results are medium values of at least three independent experiments.

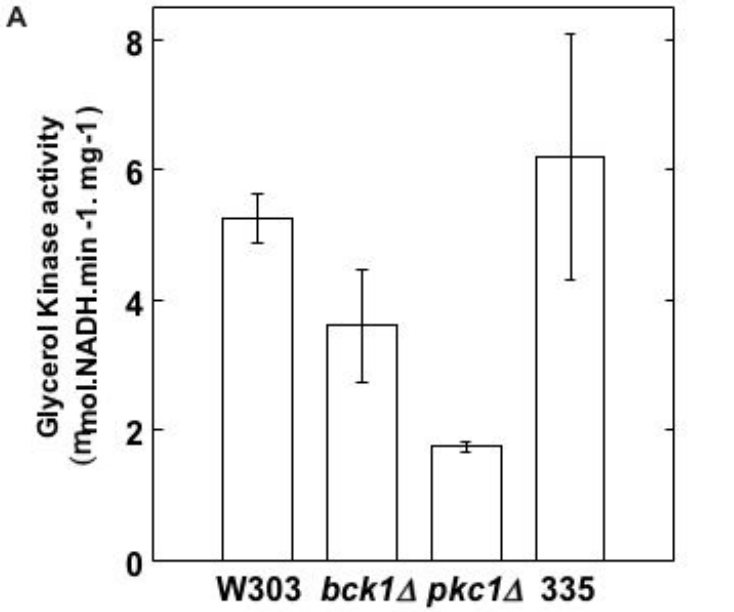
Figure 3 – Growth of S. *cerevisiae* strains on YP media containing different carbon sources: A – YPD; B – YPD + 1M sorbitol; C – YPGlycerol + 1M sorbitol; D - YPRaffinose + 1M sorbitol. Strain 335 is a mutant isolated from UV radiation on *pkc1 Δ. Strains* 342 and 345 are transformants from strain 335.

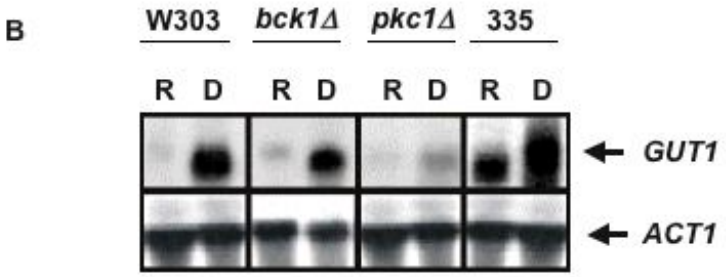
Figure 4 – Growth of *S. cerevisiae* strains in YPD medium supplemented with 1 M sorbitol in the absence (open symbols) and in the presence of 2 µg. ml^{-1} antimycin (closed symbols): (A) wild type, (B) *pkc1 Δ* and (C) strain 335.

Figure 5 – Invertase activity in *S. cerevisiae* strains: wild type W303-1A; *pkc1 Δ*, 335 mutant, 342 and 345 transformants. Cells were grown in (A) YPD or (B) YPraffinose with 1M sorbitol.

644 **Figure 6** - Subcellular localization of Mig1p in *S. cerevisiae* wild type, *pkc1* Δ and 335
645 mutant strains. Cells were transformed with a plasmid containing the *MIG1* transport
646 domain fused with *GFP* gene. After growth on YPD (with sorbitol), cells were
647 transferred to YP-raffinose for 1 h. Images were taken through an Olympus BX51
648 TRF microscope with accessory apparatus for fluorescence detection. Nuclei were
649 visualized using a DNA dye solution containing 2% Hoechst 33342 (Molecular
650 Probes). The white arrowheads indicate the position of the nuclear region observed
651 in the yeast cells.

Figure 1


A
Glycerol Kinase activity
(mmol.NADH.min -1. mg-1)
8
6
4
2
0
W303
bck1Δ
pkc1Δ
335


B
W303
bck1Δ
pkc1Δ
335
R D R D R D R D
GUT1
ACT1

652

653

Figure 2

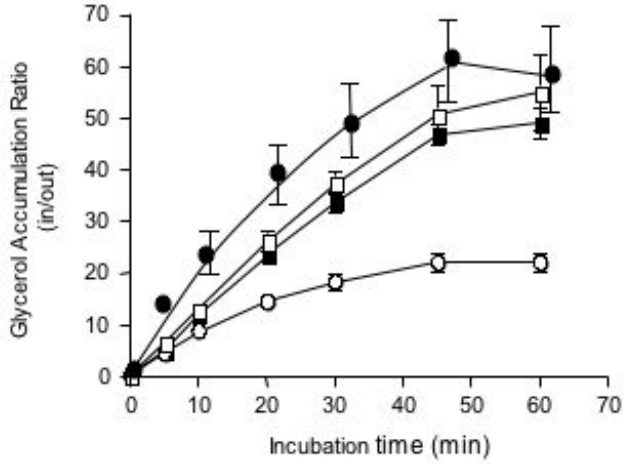


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Figure 3

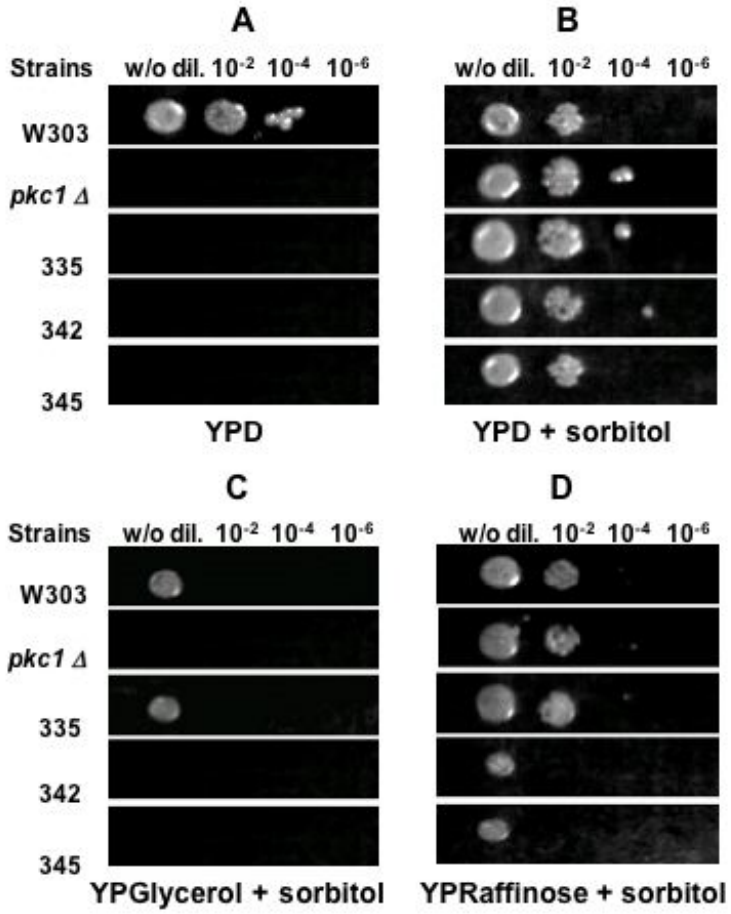

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Figure 4

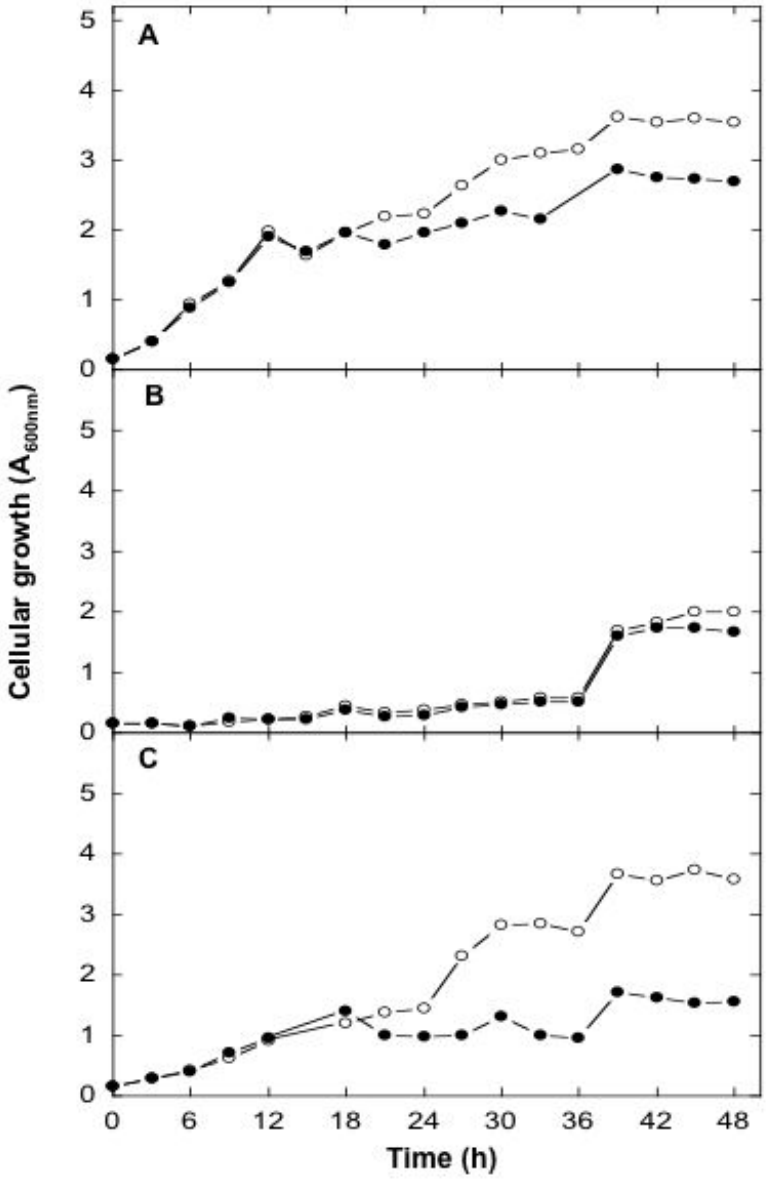

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Figure 5

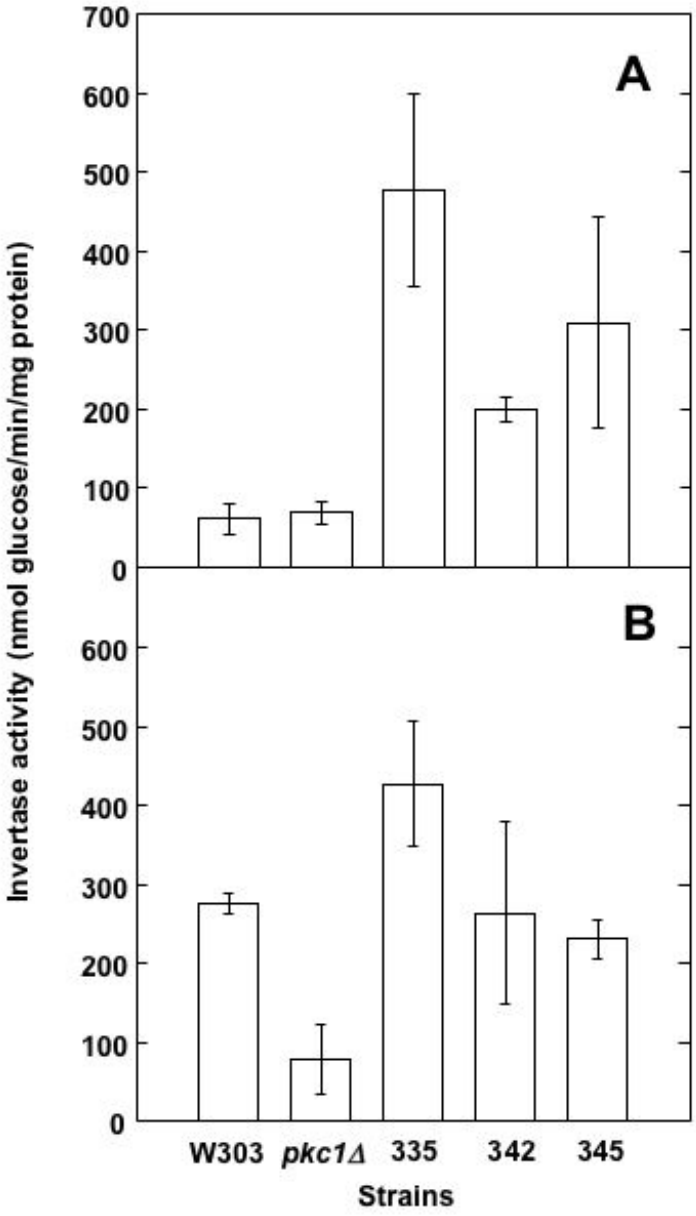

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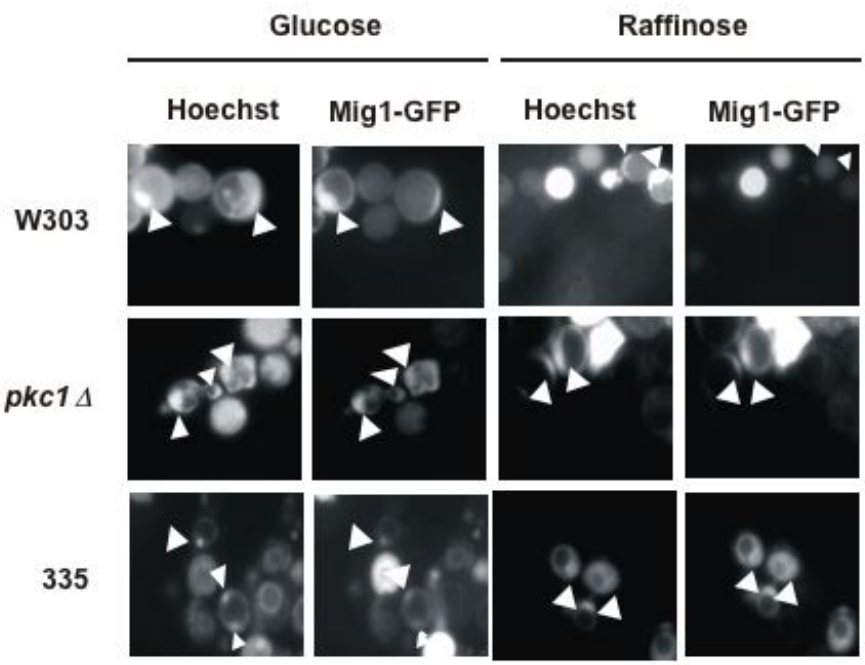
Figure 6



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