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

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27 **Abstract**

28

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

46 **1. Introduction**

47

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

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

70 Both *GUT1* and *GUT2* have long been known to be under glucose repression
71 [17, 42]. *GUT1* repression is independent of Mig1p and occurs through Opi1p, a
72 repressor involved in inositol metabolism. The Adr1p and Ino2/Ino4p are responsible
73 for more than 90% of *GUT1* expression during derepression conditions [17].

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

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

92 Besides the role in the control of carbon metabolism, Pkc1p pathway is
93 essential for the maintenance of cellular integrity by controlling the expression of
94 genes encoding enzymes involved in cell wall construction [21]. It seems to be
95 connected to other signal transduction pathways and/or cellular processes such as
96 the mating MAP kinase pathway [6], cell fusion [41], polarized growth [2], regulation
97 of actin cytoskeleton polarization [12; 33], control of morphogenesis checkpoint
98 during cell cycle [20] and several other events that affect cell membrane [46].
99 Accordingly, it has been suggested the involvement of PKC pathway, in connection
100 with calcineurin, a Ca²⁺/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 *pkc1 Δ* 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 *pkc1 Δ* strain.

119 Furthermore, a new mutant from the *pkc1 Δ* 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.

125 **2. Materials and methods**

126

127 *2.1 Strains and growth conditions*

128 The *Saccharomyces cerevisiae* strains used in this study were: W303-1A
129 (*Mat a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 GAL mal SUC2*),
130 YSH813 (W303-1A + *bck1 Δ::LEU2*), YSH850 (W303-1A + *pkc1Δ::HIS3*) and CLY3
131 (isogenic to W303 but *gut1* [24]). It was also used the TOP10F *E. coli* strain (*F'*, *mcr*
132 *A*, Δ (*mrr-hadRMS - mcrBC*) \emptyset 80 Δ *lacZ ΔM15*, Δ *lacx74*, *deoR*, *rec Δ1*, *araD139*, Δ
133 (*ara*, *leu*), 7697, *gal IU*, *galK*, λ : *rs2p*, *end Δ1*, *nupG*). Yeast cells were grown at 30°C
134 in YP medium (2% (w/v) peptone and 1% (w/v) yeast extract) supplemented with
135 variable concentrations of the carbon sources glycerol, glucose, galactose, raffinose
136 or fructose and 1M sorbitol as indicated. Growth was monitored by measuring OD at
137 600 nm or by drop test on solid media *E. coli* cells were grown at 37°C in LB medium
138 (1% (w/v) triptone; 0.5% (w/v) yeast extract; 0.5% NaCl (w/v); 1.5% agar (w/v); pH).
139

140 *2.2 Determination of invertase and glycerol kinase activities*

141 Invertase activity was measured as described before [16] with the
142 modification introduced by Celenza and Carlson [8] except that the assay was carried
143 out at pH 5.1 and 37°C. Glycerol kinase activity was determined in cell free extracts
144 obtained with 50 mM imidazol buffer without sorbitol. Measurement of specific activity
145 of glycerol kinase was performed according to Castro and Loureiro-Dias [7] by
146 following ADP formation and using 1 unit. ml⁻¹ pyruvate kinase and lactate
147 dehydrogenase in the coupled reaction. The reaction was started by the addition of
148 0.1 mM of glycerol. An extinction coefficient of 6.22 (l. mmol⁻¹. cm⁻¹) for NADH was
149 used for calculations. Enzyme specific activities were expressed in relative units of
150 cell free extracts total protein. Protein was estimated by the method of Lowry [31]
151 using bovine serum albumin as standard.

152 *2.3 Glycerol transport studies*

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

157

158 *2.4 RNA isolation and Northern-blot analysis*

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

176 *2.5 Mutagenesis of yeast cells using UV radiation*

177 Yeast cells were grown overnight ($OD_{600nm} \pm 1$), 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 30°C 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 ($OD \pm 1.5$) 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™ 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 μ g 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

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

205

206 *2.7 Subcellular localization of Mig1p by analysis of GFP fluorescence*

207 To study the subcellular localization of Mig1p, we used yeast cells (wild type
208 and mutant strains *pkc1* Δ and 335) transformed with a plasmid containing the
209 transport domain of *Mig1* fused to *GFP* gene [11]. Cells were grown on YP medium
210 supplemented with 4% (w/v) glucose until mid-exponential phase. Samples (1 ml)
211 were harvested and washed by centrifugation (13000 rpm in a microcentrifuge) with a
212 cold solution of sorbitol (1M) plus glucose (4%, w/v). The remaining cell suspension
213 was transferred to YP-raffinose (2%, w/v) medium for 1 hour. The cells were
214 harvested and washed by centrifugation with a cold solution containing sorbitol (1M),
215 plus glucose (2%, w/v) (repressed cells) or glycerol (3%, w/v) (derepressed cells).
216 Finally 10-15 μ l of these cell suspensions were mixed with 10-15 μ l of agarose (1%,
217 w/v), at 37°C for microscopy observations. Images were registered by using an
218 Olympus BX51 TRF microscope with accessory apparatus for fluorescence. Nuclei
219 were visualized using a DNA dye solution containing 2% Hoechst 33342 (Molecular
220 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 $288 \pm 37 \mu\text{moles h}^{-1} \text{g}^{-1}$ (n=3), while the estimated V_{max} in wild type was 392 ± 25
243 $\mu\text{moles h}^{-1} \text{g}^{-1}$ (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

248 above, transport-unchanged ability (V_{\max} $329 \pm 31 \mu\text{moles h}^{-1} \text{g}^{-1}$ ($n=3$); K_m $2.1 \pm 0.5 \text{ mM}$
249 ($n=3$)) (Fig. 2).

250 Additionally, glycerol uptake V_{\max} was determined in the *gut1Δ* strain grown
251 and subsequently incubated in identical conditions as mentioned above. This was
252 performed as a control for *pkc1Δ* mutant, taking into consideration previous data that
253 showed glycerol kinase to have an effect on the initial rates of glycerol uptake used
254 for transport kinetic constants determination [24, 36]. The value obtained for V_{\max} was
255 $350 \pm 41 \mu\text{moles h}^{-1} \text{g}^{-1}$ ($n=3$), once more, in the range of the wild type V_{\max} . This
256 indicates that the reduced transport velocity determined in the *pkc1Δ* mutant is not an
257 indirect consequence of the inability to express the Gut1p.

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

268 In Fig. 1A, we show that strain 335 presented a higher glycerol kinase activity
269 after cells have been shifted from glucose to glycerol. In the same way, Northern blot
270 analysis (Fig. 1B) revealed that the *GUT1* gene was constitutively derepressed in this
271 strain when compared to the wild type and *bck1Δ* strains. Furthermore, mutant strain
272 335 presented uptake with a V_{\max} of 324 ($n=2$), in the range of the values found for
273 wild type strain. Accordingly, accumulation ability was also not significantly different
274 from the one measured in wild type (Fig. 2).

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

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

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

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

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

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

318 4. Discussion

319

320 The Pkc1p seems to be involved in many important cellular functions beyond
321 controlling cell wall synthesis through the PKC MAP Kinase pathway. There are
322 evidences indicating the participation of Pkc1p in the control of ribosomal synthesis,
323 the appropriated reallocation of transcriptional factors under specific stress conditions
324 [34] and also the regulation of N-glycosylation activity [39]. Pkc1p seems to be
325 essential to the glucose-induced activation of the plasma membrane ATPase [50]
326 and also for the derepression mechanism of the glucose-repressible genes [5; 43].
327 Together, all these data reinforce the idea that Pkc1p is involved in many important
328 functions in yeast cells. The present work elucidates deeper into the mechanisms
329 underlying the involvement of Pkc1p in the control of different glucose mediated
330 effects.

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

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

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

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

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

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

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431

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439

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617 **Legends**

618

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

625

626 **Figure 2** – Radiolabelled glycerol accumulation ratios on *S. cerevisiae* cells
627 cultivated on YPD supplemented with 1M sorbitol and subsequently incubated for 6
628 hours in YPG with the same amount of sorbitol for derepression: (□, wild-type W303-
629 1A; ■, *bck1*Δ; ○, *pkc1*Δ; ●, strain 335 (new mutant isolated from UV radiation on
630 *pkc1* Δ). Results are medium values of at least three independent experiments.

631

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

636

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

640

641 **Figure 5** – Invertase activity in *S. cerevisiae* strains: wild type W303-1A; *pkc1* Δ, 335
642 mutant, 342 and 345 transformants. Cells were grown in (A) YPD or (B) YPrffinose
643 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

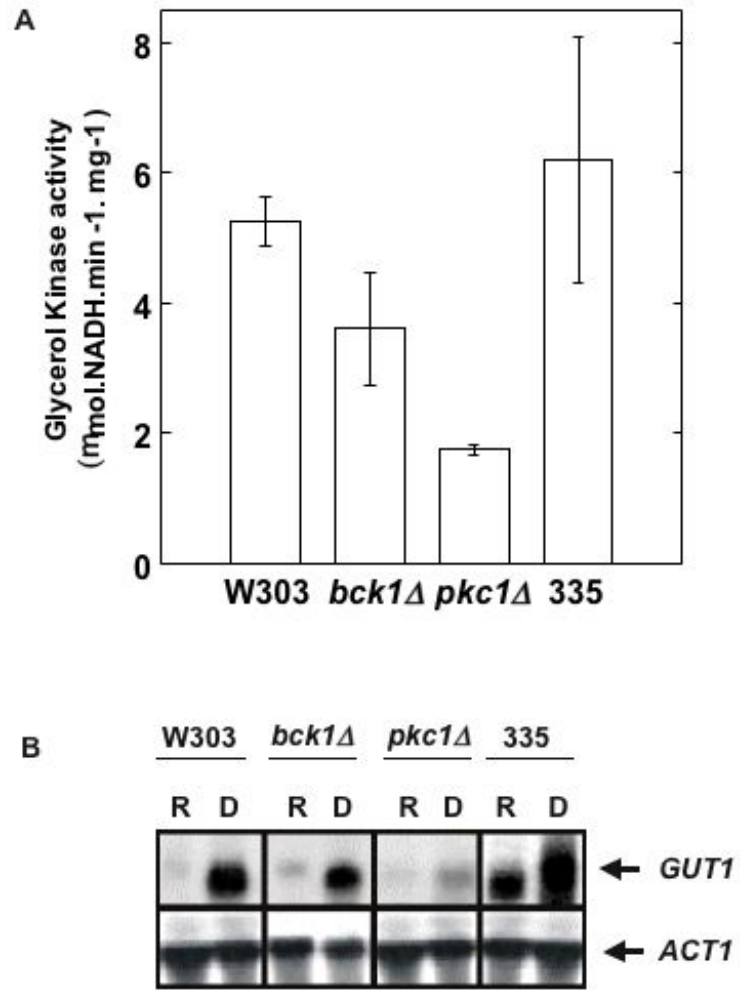


Figure 2

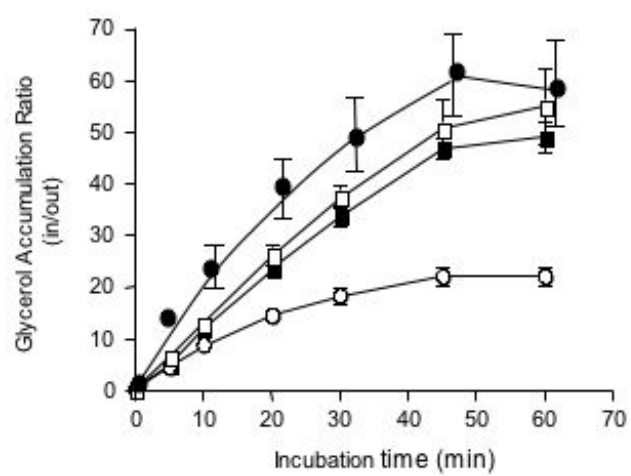


Figure 3

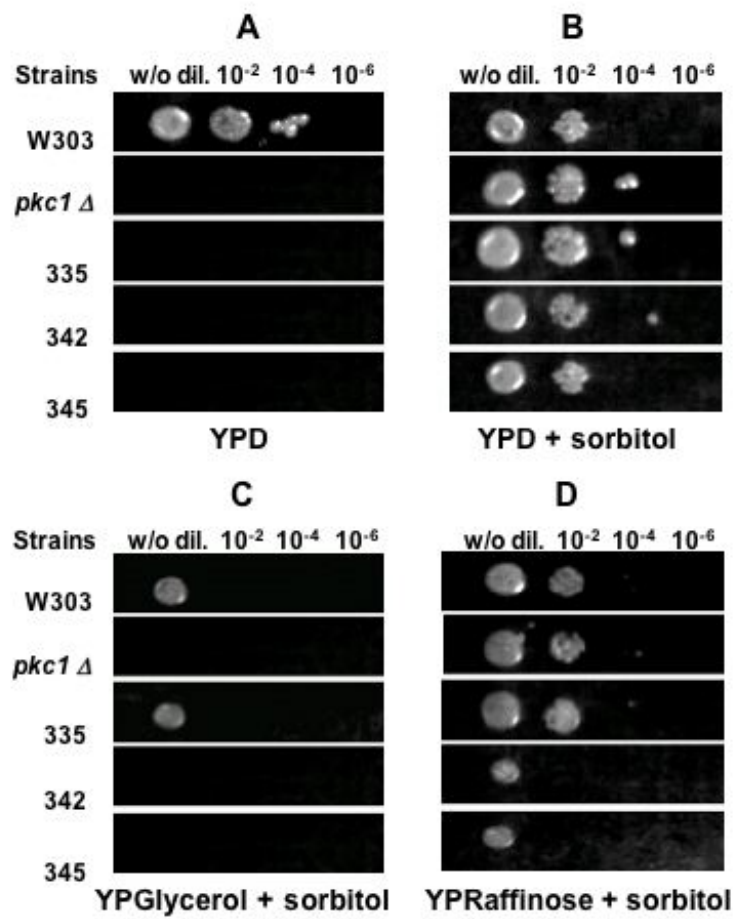


Figure 4

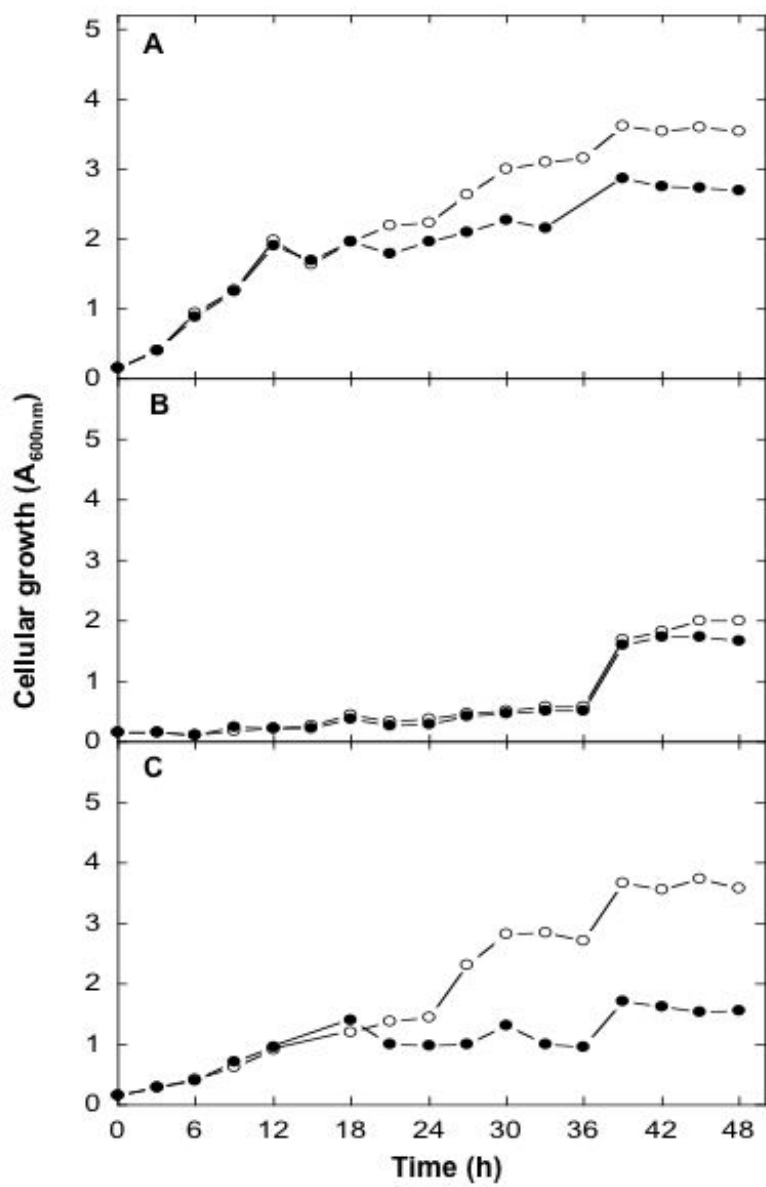


Figure 5

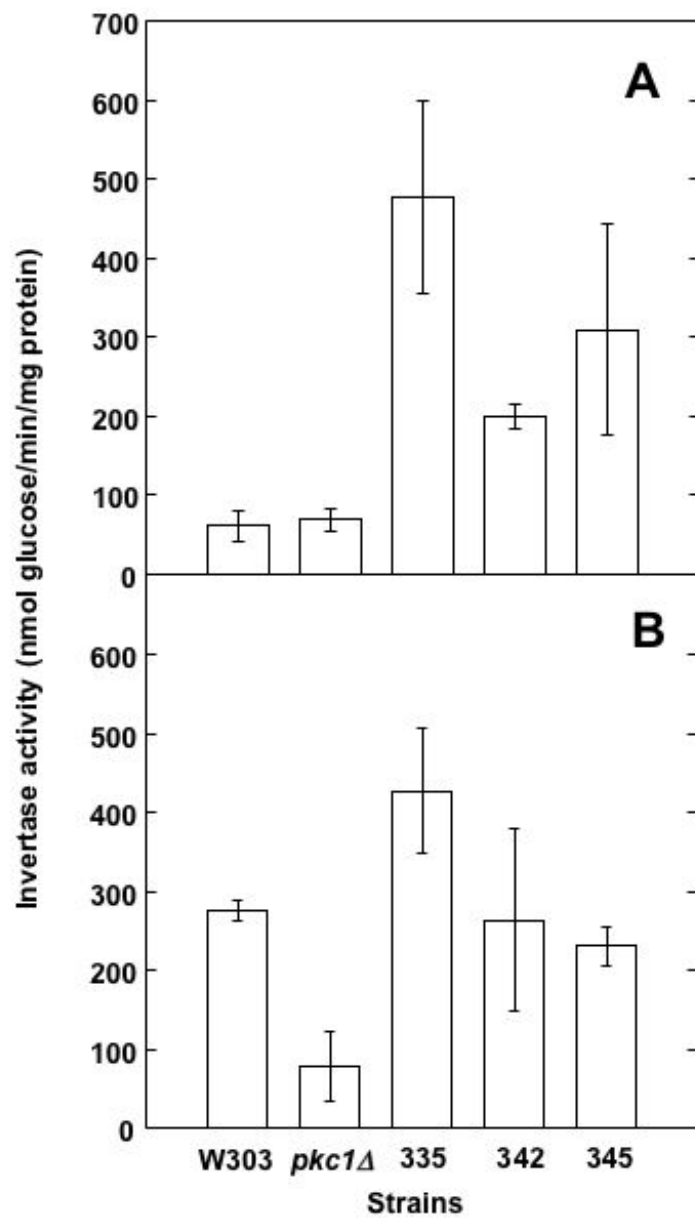


Figure 6

