Ras-pathway has a dual role in yeast galactose metabolism

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Abstract In the yeast *Saccharomyces cerevisiae* the genes involved in galactose metabolism (GAL1,7,10) are transcriptionally activated more than a 1000-fold in the presence of galactose as the sole carbon source in the culture media. In the present work, we monitored the activity of the GAL10 gene promoter in different Ras-cAMP genetic backgrounds. We demonstrate that overexpression of C-terminus of the nucleotide exchange factor Cdc25p stimulates GAL10 transcription in yeast strains carrying the contemporary deletion of both RAS genes. Moreover, the deletion of the chromosomal CDC25 gene provokes impaired growth on galactose based media in yeast strain lacking both RAS genes and adenylate cyclase (whose viability is assured by the presence of the Bcy1-11 allele). Surprisingly, reconstitution of the Ras-pathway inhibits GAL10-promoter activation. Activation of GAL10 gene promoter is indeed possible in the presence of Ras protein but only in strains with chromosomal deletion of adenylate cyclase. These results indicate a dual role of Ras-pathway on galactose metabolism and suggest that Cdc25p has a Ras-independent role in cellular metabolism.
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1. Introduction

The Leloir pathway is the main enzymatic pathway for the conversion of galactose to glucose in organisms philogenetically distant as *Escherichia coli* and humans [1]. This conversion is mainly realized by three enzymes codified by genes collectively referred to as *GAL* genes in yeast: galactokinase (EC 2.7.1.6, GALK) corresponding to *GAL*1 in yeast, phosphorylates intracellular galactose to galactose-1-phosphate; galactose-1-phosphate uridyltransferase (EC 2.7.7.12 GALT), corresponding to *GAL*7, transfers UMP from UDP-glucose to gal-1P forming UDP-gal, and, finally, UDP galactose 4′-epimerase (EC 5.1.3.2, GALE), whose yeast counterpart is *GAL*10, converts UDP-gal to UDP-glc, via a NAD^+ dependent reaction [2].

Yeast cells respond to the presence of galactose as the sole carbon source with a several 1000-fold increase of the *GAL* genes expression [1–3]. These genes undergo a dual transcriptional regulation. They are repressed in the presence of glucose and activated in response to galactose [4]. Several experiments have demonstrated that activation and repression of the *GAL* genes act via independent pathways [5–7]. The major transcriptional activator of the *GAL* genes is the transcription factor Gal4p [8]. Gal4p is in turn subjected to two levels of regulation. It responds to the presence of intracellular galactose by 4–5-fold increase of its transcriptional level [6,8,9], and by post-translational modification of its interaction with the repressor Gal80p [10].

Many studies, in yeasts, have related the c-AMP-PKA pathway to diauxic shift [11–14]. Genetic manipulations of the Ras-cAMP pathway often result in galactose deficient phenotypes. Yeast strains expressing an hyperactivated *RAS*2 allele (*RAS*2G19V) show growth impairment on galactose-based media [15]. Furthermore, in strains with an activated allele of the adenylate cyclase (CYR1T1651I), contemporary deletions of both *RAS* genes or deletion of *RAS*1 combined with the presence of an attenuated *RAS*2 allele result in temperature-sensitive phenotype in galactose [16]. The Ras pathway has been demonstrated to be positively affected by two different nucleotide exchange factors: the essential Cdc25p and the non essential Sdc25p [17,18]. The overexpression of the former provokes growth inhibition in galactose [19] and enhanced transcription of the *GAL*4 gene [20]. Cdc25p has also been demonstrated to be essential for growth in galactose-based media [21]. We investigated the role of *CDC*25 and *RAS*2 gene products in galactose metabolism, monitoring the activity of the *GAL*10 gene promoter fused to human phenylalanine hydroxylase cDNA used as a reporter gene. This reporter was expressed in various yeast strains differing for the Cdc25p-Ras-cAMP pathway and the resulting activity of the *GAL*10 promoter measured.

2. Materials and methods

2.1. Media and yeast manipulation
YPD was used as rich medium (2% bacto peptone, 1% yeast extract and 2% glucose). Selective synthetic media were prepared with yeast nitrogen base without aminoacid at 0.67% final concentration, with the appropriate mixture of aminoacids and nucleic acid bases at the concentration indicated by Sherman [22], with glucose at 2% final concentration. Standard media containing carbon source different from glucose were obtained with 2% galactose in place of glucose. Genetic manipulation of yeasts were as described by Mortimer and Schild [23]. Yeasts transformations were carried out as described by Ito [24]. All components of media were from DIFCO (Becton Dickinson, CA, USA).

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Standard molecular biology methods have been performed as indicated by Sambrook et al. [25].

List of strains		
AAT3B	a ade2 can1-100 CRI4 his3 leu2-3,112 lys1-1 ras1Δ ras2::URA3 ura3-52	Mirisola et al. [16]
AAT3BΔ1	a ade2 can1-100 cdc25::LEU2 CRI4 his3 leu2-3,112 lys1-1 ras1Δ ras2::URA3 ura3-52	Mirisola et al. [16]
ABH7C	α ade2 bcy1-11 can1-100 cyr1::HIS3-Δ1 his3 leu2-3, 112 Lys1-1 ras1Δ ras2::URA3 ura3-52	Mirisola et al. [16]
ABH7CΔ1	α ade2 bcy1-11 can1-100 cdc25::LEU2 cyr1::HIS3-Δ1 his3 leu2-3, 112 Lys1-1 ras1Δ ras2::URA3 ura3-52	This work
	ras1Δ allele with ras1 deletion without any marker was obtained after gene conversion of the Ras1::URA3 allele in the presence of the plasmid Yep13Hras1Δ and selection with 5-FOA as described by Mirisola et al. [16]	

WOF6.1 (ras1Δras2Δcyr1::URA3 bcy1-11) strain was obtained transforming the ras1Δras2Δcyr1::HIS3 bcy1-11 (ABE2A, Verrotti et al., 1992) strain with the EcoRI/BamHI fragment of the pWOF6 vector (Fasano O. unpublished).

2.2. PAH reporter construction

The 1.8 kb SmaI/HpaI fragment of the American type culture collection (ATCC) clone ATCC 61604, containing the entire coding region of the human phenylalanine hydroxylase (PAH) has been cloned into the unique SalI restriction site of the YEp51 yeast shuttle vector [26], after Klenow treatment to fill it in. The proper orientation of the insert has been verified by restriction analysis and joint ends have been verified by sequencing.

2.3. RNA preparation

The desired yeast strain was grown at 30 °C, on the appropriate selective glucose-based medium, to $OD_{560\,nm} = 0.8/1$. Cells were collected by centrifugation and resuspended in the same medium, except for induced cultures, where galactose replaced glucose at the same concentration, and cultured at 30 °C for additional 24 h. After centrifugation, the pellet, resuspended in acetate buffer (50 mM sodium acetate, 10 mM EDTA, pH 5), was vortexed for 5′ at 65 °C in the presence of one volume of acid-washed glass beads (SIGMA G-1277, Milano, Italy) and one volume of phenol pH 5. The aqueous phase was extracted twice with chloroform and RNA was finally precipitated with 2.5 V of absolute ethanol, 0.3 M sodium acetate, pH 5.5. After centri-

fugation, the pellet was resuspended in TE 10:1. The RNA quality was verified evaluating the ribosomal RNA appearance after gel electrophoresis in denaturing conditions. Poly (A)+ RNA was obtained using a commercially available kit (QuickPrep™ Micro mRNA Purification Kit, Amersham, Milano, Italy), according to the manufacturer's instruction.

2.4. RT-PCR

Poly (A)+ (1 μg) or total RNA, obtained as described above from the indicated yeast strain, was subjected to retro transcription in the presence of the RT primer indicated below in the presence of 1 μl of the Improm (Promega, Madison, WI, USA) reverse transcriptase at 42 °C for 1 h, according to the manufacturer's instructions. Different amounts of each cDNA were used for the subsequent PCR. The primers for the each PCR are indicated as For and Rev primers. PCR conditions for hPAH; GAL4; GAL80 were as follow: 94 °C 30″; 51 °C 30″, 72 °C 45″ for two cycles and 94 °C 30″ 60 °C 30″ 72 °C 45″ for additional 28 cycles. PCR conditions for CAP were 94 °C 30″ 51 °C 30″ 72 °C 1′15″ for 30 cycles. PCR conditions for CDC25 were 94 °C 30″ 58 °C 30″ 72 °C 1′ for 30 cycles. PCR conditions for TYR1 were 94 °C 30″ 54 °C 30″ 72 °C 1′30″ for 30 cycles.

2.5. Northern blot hybridisation

Total RNA (10 μg) were fractionated in a 1.0% agarose–formaldehyde gel and blotted onto nitrocellulose membrane (Schleicher & Schuell, PROTRAN BA 83, Dassel, Germany), via capillary action as described by Sambrook [25]. Filters were baked at 80 °C for 2 h under vacuum. RNA blots were prehybridized with prehybridization buffer

Primer name:	DNA sequence 5′ → 3′
hPAH-RT	gagggcactgcaaaggattcc
hPAH probe For	aaacctgacccacattgaatctagacc
hPAH probe Rev	tgccccatgttttcttttcttcctc
GAL4-RT	ccctgtagtgattccaaacg
GAL4-For	gcggggttttttcagtatctacga
GAL4-Rev	gssgcaagcctgaaagatga
GAL80-For	tccttgccgaccagcgtatac
GAL80-Rev	gcatgcacgaaaaagggaaat
GAL80-RT	gcatgcacgaaaaagggaaat
CAP-For	cgcggtcgacatatgcctgactctaagtacaca
CAP-Rev	cgcggatccaagcttacacatgaaaaaaaccttt
CAP-RT	cgcggatccaagcttacacatgaaaaaaaccttt
Cdc25-RT	ttgcgatttttagtcctcttgctg
Cdc25-For	tggcgggacaaaagaggcactgat
Cdc25-Rev	cgtcacggatcgcaacagctctcta
TYR1-RT	gcctaatattataggaaatcagcatt
TYR1-For	gaaggaaagaggacagcatatcca
TYR1-Rev	gcctaatattataggaaatcagcatt

(0.5 M Na_2HPO_4, pH 7.2, 7% SDS, 1 mM EDTA) at 65 °C for 2 h. The filters were then hybridized consecutively with random primer-labelled human PAH cDNA probes (Readyprime Kit, Amersham). After hybridisation, RNA filters were washed for 30 min with 50 ml of 40 mM Na_2HPO_4 pH 7.2, 1% SDS at 65 °C. The filters were blotted dry and exposed to X-ray film (X-OMAT, Eastman Kodak, Rochester, NY, USA) for 24–48 h at −80 °C.

2.6. Western blotting and immunodetection

Western blot of the human PAH protein has been performed as described by Mirisola et al. [27].

Western blot of Cdc25p has been performed using a 1:200 dilution of a commercially available anti Cdc25p antibody (Cdc25 (yC-19): sc-6761, Santa Cruz Biotechnology, CA, USA).

Western blot of Ras2p has been done using a polyclonal antibody obtained as described by De Vendittis et al. [45] following the procedure previously described.

3. Results and discussion

3.1. Constructing the reporter plasmid

The coding sequence of the human phenylalanine hydroxylase gene (hPAH) has been cloned under the control of the GAL10 gene promoter of the shuttle vector YEp51 [26] to generate the reporter plasmid YEp51-PAH (for details, see Section 2).

The use of human PAH gene as a reporter has various advantages. PAH protein, normally expressed in mammalian liver cells, is a key enzyme in the production of tyrosine by means of phenylalanine hydroxylation [28]. Yeast cells produce tyrosine throughout a pathway that does not involve any hydroxylating reaction [29]. In addition, BLAST analysis, even querying with the otherwise conserved PAH catalytic domain alone, revealed no significant homology between PAH sequence and the whole yeast genome. These two features guarantees minimal interference of reporter expression on phenotype analysis and virtually no cross-hybridisation effect in Northern or reverse transcriptase-polymerase chain reaction (RT-PCR) experiments.

Western blot using a commercial monoclonal antibody raised against PAH (PH8) of crude protein extracts confirmed the ability of the reporter to drive the expression of human PAH (Fig. 1). The in vivo neutrality of the PAH gene expression was confirmed in various genetic backgrounds (results not shown) and in different growth conditions.

3.2. Effect of the CDC25 gene overexpression on GAL10 promoter activity

A positive relationship between CDC25 gene overexpression and GAL genes transcriptional derepression has been previously reported [20,21]. We tested if Cdc25p acts on galactose metabolism via its well established role as nucleotide exchange factor (GEF) of the Ras proteins [30,17] or by an unknown pathway. Full length CDC25 gene (pIND25-2H) or a truncated, but still in vivo active, C-terminus form (AA. 907–1589, pIND25-1H, [19]) were overexpressed in a recipient yeast strain lacking both RAS genes (AAT3B; for the relevant genotype, see Section 2). It is known that deletion of both RAS genes is lethal in yeast [31]. We overcame this problem using a mutated allele of the adenylate cyclase gene (CYR1T1651I), which is able to produce a basal level of cAMP even in the absence of any Ras protein [32,16] but it is still sensitive to Ras function [16]. Competent cells of this recipient yeast strain,
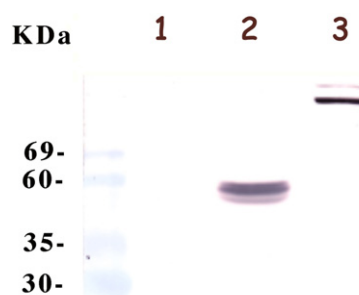

Fig. 1. Expression of the reporter construct YEp51-PAH. Western blot of whole protein extracts from the AAT3B strain (see the list of strains for relevant genotype), transformed with the reporter construct YEp51-PAH (lane 2) expressing the human PAH under the control of the GAL10 gene promoter, or with the empty vector (lane 1). Blots were immunostained with anti-PAH antibodies. Both strains were galactose-induced as described in Section 2. Purified maltose binding protein-PAH fusion protein has been used as positive control (lane 3). Protein content of each extract was determined by Bradford assay.

expressing episomal reporter PAH gene under the control of the yeast GAL10 gene promoter (YEp51-PAH) were transformed with CDC25 expression plasmids, either full length or C-terminus (pIND25-2H and pIND25-1H, respectively) or with the empty vector. Total RNA extracted from selected transformants grown on galactose, as described in Section 2, were analysed by RT-PCR, using PAH-specific oligonucleotides. We demonstrate that in the absence of both RAS genes, overexpression of the truncated form of Cdc25p is capable to activate the GAL10-dependent transcription of the PAH reporter gene (Fig. 2a) while full length Cdc25p has only a marginal effect. The different activity between full length and truncated version of Cdc25p can be explained by the different amount of the two proteins. Only the truncated form of Cdc25p can be efficiently overexpressed [20]. It has been demonstrated that full length Cdc25p, compared to its truncated version, has a reduced stability [34] probably caused by the presence of a cyclin disruption box in its N-terminus moiety [44].

To further confirm that C-terminus of Cdc25p is capable to activate GAL10-driven transcription we performed Northern blot analysis of poly (A)$^+$ RNA extracted from the same yeast strains with or without the overexpression of the truncated Cdc25p. Radioactively labelled PAH coding sequence has been used as a probe in stringent hybridisation conditions (for details, see Section 2). A strong enhancement of the PAH-specific hybridisation signal resulted in the presence of Cdc25 overexpression (Fig. 2b). In addition, whole protein extracts (for details, see Section 2) from the same yeast strains grown in the same conditions were subjected, after SDS–PAGE fractionation, to immunoblot with a commercial anti-PAH antibody (PH8). We confirmed (Fig. 2c) the ability of the C-terminus of Cdc25p nucleotide exchange factor to stimulate GAL10-dependent transcriptional activity. This result clearly indicates, for the first time, that Cdc25p has a Ras-independent role on galactose metabolism. Since GAL genes are normally activated by a 4–5-fold transcriptional increasing of GAL4 mRNA [6,8,9] we measured the mRNA level of GAL4 and GAL80 in the presence and in the absence of C-terminus Cdc25p. Total RNA from strains lacking both RAS genes and overexpressing
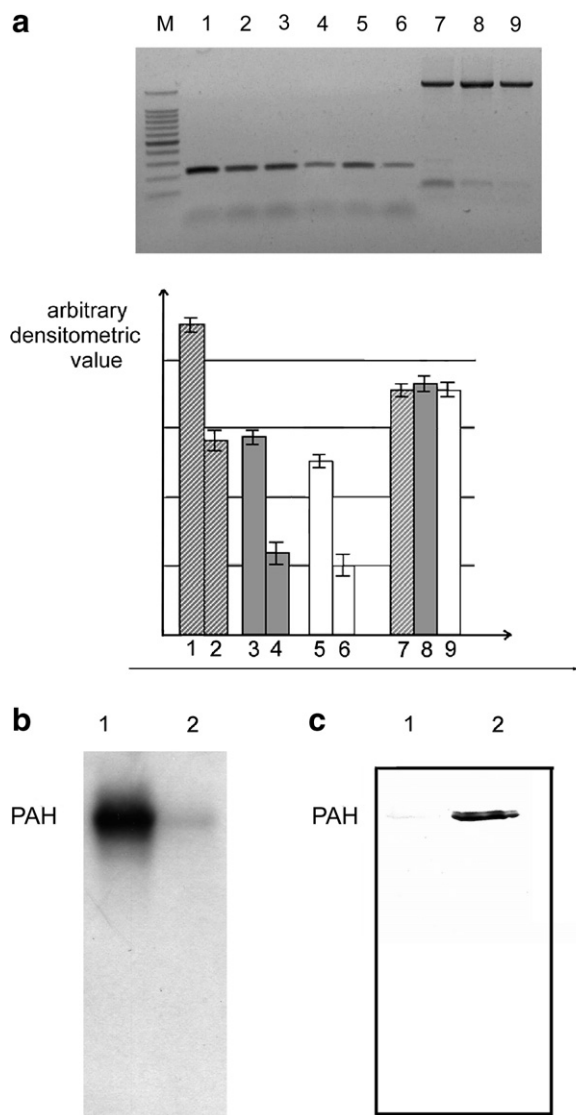
Fig. 2. Effect of full length or C-terminus Cdc25 overexpression in a strain lacking both *RAS* genes. (a) Reporter RT-PCR Upper panel: RT-PCR of PAH mRNA in the presence of C-terminus (AA. 907–1589, lanes: 1 and 2); full length (lanes 3 and 4) or without episomal *CDC25* (lanes 5 and 6). Different dilution of PAH cDNA were used for subsequent PCR: 1 μl (lanes: 1, 3, and 5); 0.1 μl (lanes: 2, 4, and 6). RT-PCR of CAP mRNA in the presence of C-terminus (lane 7); full length (lane 8) or absence (lane 9) of Cdc25p overexpression was used as a control. Amount and quality of RNA used were determined by electrophoresis of ribosomal RNA (not shown). Lower panel: densitometric analysis of upper panel. The results are the average of three independent experiments. (b) Northern blot. Autoradiography of 1 μg poly (A)[+] RNA extracted from AAT3B (see list of strains for relevant genotype) yeast strain cotrasformed with YEp51-PAH and C-terminus Cdc25 (lane 1), with YEp51-PAH and YEp13H (lane 2) were fractionated in denaturing conditions, blotted and hybridised in stringent conditions with a radioactively labelled PAH specific probe (for details see Section 2). (c) Western blot Immunostaining of PAH protein on Western blot obtained after SDS–PAGE fractionation of whole protein extract from the AAT3B strain overexpressing (lane 2), or not expressing (lane 1) the carboxy-terminus (AA. 907–1589) of the Cdc25 protein. Protein content of each extract was normalized by Bradford assay.

the truncated form of Cdc25p or the corresponding empty vector have been subjected to RT-PCR using oligonucleotides

specific for GAL4 and GAL80 RNAs. From this experiment GAL4 transcript level results almost doubled in the presence of Cdc25p while GAL80-specific RNA appears unaffected (Fig. 3). This suggests that the Cdc25-dependent activation mechanism of *GAL* genes acts, directly or indirectly, enhancing the transcription and/or mRNA stability of the *GAL4* gene. Van Aelst and coworkers have previously demonstrated that Cdc25p overexpression abolishes glucose repression of invertase activity in yeast cells [47]. Unfortunately, the plasmids we have used to overexpress Cdc25p are under the control of the GAL10 gene promoter limiting the possibility to observe the role of Cdc25p on glucose-based media. It would be interesting to overexpress Cdc25p under the control of a different gene promoter to see if Cdc25p is capable to relief glucose repression of GAL genes expression both with and without the presence of *RAS* function.

The possibility of physical interaction of Cdc25p with proteins other than Ras has been suggested several times. Frascotti and colleagues [19] suggested a Cdc25-dependent stimulation of a GTPase, other than Ras proteins, with a role in galactose metabolism. Physical interaction and modulation between Cdc25p and chaperones has been detected [33] and a role of Cdc25p on stress response in a cAMP-independent pathway has been postulated [34]. In addition several CDC25-interactors have been identified by two hybrid screens. Further studies will be necessary to identify which protein of the galactose metabolism Cdc25p interacts to.

It must be noted that the truncated version of Cdc25p used in this study contains a domain (AA. 907–1589) of the Cdc25p, sufficient to enhance dissociation of the nucleotide bound to Ras protein [35]. For this reason, it is likely that this Ras-independent role of Cdc25p in galactose metabolism is due to its ability to enhance the nucleotide exchange of a different GTP binding protein rather than to an undiscovered biochemical function of the Cdc25p.

3.3. Phenotype of CDC25 gene deletion in strains lacking adenylate ciclase and/or both RAS genes

The chromosomal *CDC25* gene disruption is normally lethal [30,36]. Herein, we evaluated the effect of the same gene deletion in strains lacking both *RAS* genes (AAT3B) or lacking both *RAS* genes and adenylate cyclase (ABH7C). The suppression of the lethality by an allele of the adenylate cyclase in the strain AAT3B is discussed in the previous paragraph. The lethality of the contemporary deletion of both *RAS* genes and/or adenylate cyclase (ABH7C) can be rescued by the presence of the bcy1-11 allele which allows sufficient kinase A activity, even in the absence of adenylate cyclase [37]. The AAT3B and ABH7C strain (for the relevant genotype, see Section 2) were engineered by replacing the *CDC25* gene with a disrupted cdc25::LEU2 allele, using the disruption plasmid and procedure described by Baroni et al. [38], thus generating the AAT3BΔ1 and ABH7CΔ1 strains, respectively. Selected transformants were individually analysed by replica plating on different carbon sources at different temperatures of incubation. The results shown in Table 1 indicate that deletion of *CDC25* in strain lacking both *RAS* genes (AAT3B) displays no phenotype while the same gene disruption in a strain (ABH7C) lacking both *RAS* genes and adenylate cyclase is always associated with an impaired growth on glycerol based media at 30 and 37 °C and on galactose based media at 37 °C for all colonies tested (Table 1 and Fig. 4). More exper-
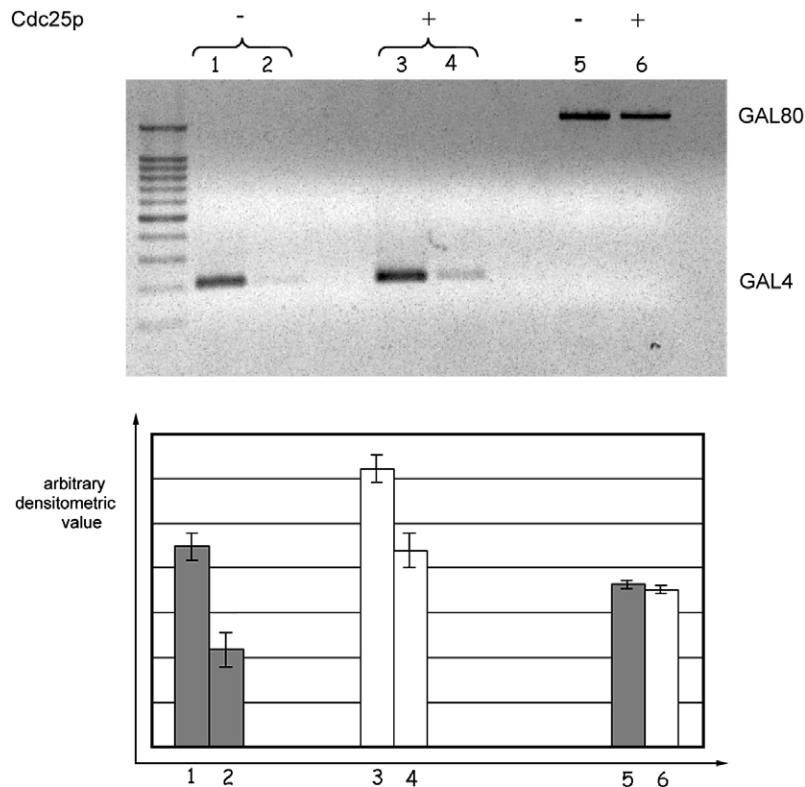
Fig. 3. Effect of Cdc25 overexpression on GAL4 and GAL80 mRNA level. Upper panel: RT-PCR of GAL4 mRNA (lanes 1–4) or GAL80 mRNA (lanes 5 and 6) were performed in the absence (lanes: 1, 2, and 5) or presence (lanes: 3,4, and 6) of Cdc25p carboxy-terminus (AA. 907–1589) overexpression. Different dilution of cDNA were used for subsequent PCR: 1 µl (lanes: 1, 3, 5, and 6); 0.1 µl (lanes: 2 and 4). Lower panel: densitometric analysis of upper panel. The results are the average of three independent experiments.

Table 1
Phenotypes have been judged after three days of growth

	YPD		YPGly		YPGal	
	30 °C	37 °C	30 °C	37 °C	30 °C	37 °C
AAT3B	+	+	+	−	+	+/−
AAT3BΔ1	+	+	+	−	+	+/−
ABH7C	+	+	+	−	+	+/−
ABH7CΔ1	+	+	−	−	+	−

iments would be necessary to discriminate if the different sensitivity of the two yeast strains to the *CDC*25 gene deletion is due to the presence/absence of the adenylate cyclase or to unidentified differences between the two yeast strains. However, the response to nutrients of yeast cells has been demonstrated to be modulated by Ras proteins and more recently by the Gα Gpa2 [39,40]. This latter protein has also been demonstrated to act in a Ras independent pathway [41] responding to extracellular nutrients [40]. Furthermore, in the fission yeast a direct interaction of Gpa2 with adenylate cyclase has been assessed [42,43]. It is therefore possible that the deletion of adenylate cyclase in the ABH7C strain abolishes both Ras dependent and Gpa2p dependent activation of adenylate cyclase resulting in the observed enhanced sensitivity to *CDC*25 gene deletion. It is interesting to note that while the phenotype resulting from the episomal expression of C-terminus Cdc25p may be due to non physiological interaction between the overexpressed Cdc25p and an unknown protein, the phenotype resulting from the chromosomal deletion of
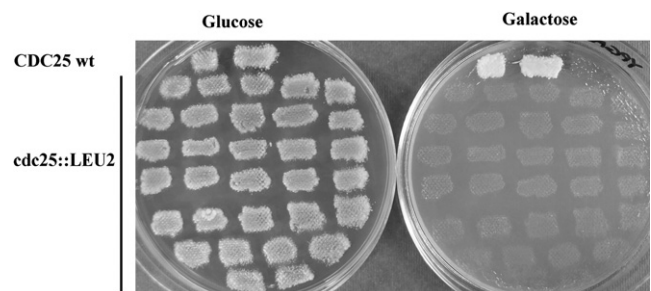

Fig. 4. Phenotypic effect of *CDC*25 deletion in a strain lacking both *RAS* genes. Replica plating of patches deriving from individual colonies of the ABH7C yeast strain carrying a wild type (first two patches) or a disrupted (the remaining 31 patches) allele of CDC25 (cdc25::LEU2, ABH7CΔ1) in glucose and galactose based media. Plates were incubated at 37 °C and analysed after 72 h.

*CDC*25 in a strain lacking both *RAS* genes and adenylate cyclase implies a physiological role of this exchange factor in galactose metabolism via a Ras-cAMP-independent pathway.

3.4. GAL10 promoter activity and RAS2 function

To further characterize the role of Ras pathway in galactose metabolism, wild type *RAS2* or attenuated *RAS2R80DN81D* alleles were re-introduced by homologous recombination [16] in a strain containing the PAH reporter plasmid and overexpressing the C-terminus of the Cdc25 protein. RT-PCR were performed using PAH-specific oligonucleotides in total RNA fractions extracted from glucose or galactose grown cells.

Surprisingly the *RAS2* re-introduction completely abolishes the Cdc25-dependent activation of GAL10 promoter (result not shown). To shed a light on this Ras-dependent effect we measured the amount of exchange factor protein Cdc25p by immunoblot in different yeast strains. Whole protein extracts from strains expressing *RAS2* wild type, *RAS2R80DN81D* or the null allele in combination with C-terminus Cdc25p were subjected to SDS–PAGE and immunostained with a commercial anti-Cdc25p antibody (see Section 2 for details). The result shown in Fig. 5 indicates that the amount of Cdc25p strongly decreases in the presence of a wild type *RAS2* allele compared to strains expressing a *ras2* null allele or a *ras2*-attenuated allele (*RAS2R80DN81D*). To discriminate if Ras2p exerts its action on the expression of the truncated form of Cdc25 at transcriptional or posttranscriptional level we performed RT-PCR using Cdc25-specific oligonucleotides on total RNA extracts from strains containing wild type, attenuated or null *RAS2* alleles. *CDC25* mRNA level, as judged by RT-PCR (Fig. 6), strongly decreases in the presence of active Ras2p while it is unaffected by the presence of attenuated Ras2p (Ras2R80DN81D). Since the episomal expression of both PAH and *CDC25* (pIND25-1H) is under the control of the GAL10 gene promoter these results indicate that reintroduction of wild type *RAS2* inhibits the transcription of *GAL* genes while reintroduction of the attenuated allele of *RAS2* (*RAS2R80DN81D*) has no effect.

We further studied if the inhibitory role of Ras2p reintroduction on GAL10 promoter activity was due to the presence of Ras2p itself or to the activation of the adenylate cyclase. Yeast strain lacking both adenylate cyclase and *RAS* genes (WOF6.1) was used as a recipient strain to express wild type and activated (G19V) Ras2 proteins under the control of the GAL10 gene promoter of the YEp51 high copy number shuttle

Fig. 6. RT-PCR analysis of *CDC25* mRNA in the presence of different *RAS2* alleles. Upper panel: RT-PCR of *CDC25* in a strain lacking both *RAS2* and C-terminus *CDC25* (AAT3B YEp13H, lane 1), lacking *RAS2* but in the presence of C-terminus *CDC25* (AAT3B pIND25-1H, lane 2), in the presence of wild type *RAS2* and C-terminus *CDC25* (AAT3BR2 pIND25-1H, lane 3), in the presence of an mutated *RAS2* allele and C-terminus *CDC25* (AAT3B R2 D80D81 pIND25-1H, lane 4) and in the absence of *RAS2* in the presence of C-terminus CDC25 but in the absence of chromosomal *CDC25* (AAT3B Δcdc25 PIND25-1H, lane 5). Lower panel: RT-PCR of total RNA extracted from the same yeast strains of the upper panel using oligonucleotides and reaction conditions specific for *TYR1* gene.

vector (see Section 2 for details). Recombinant clones were transformed with an expression vector of the adenylate cyclase (YACW, [46]) or with the corresponding empty vector. Total protein extracts from individual clones, after SDS–PAGE, were immunoblotted with a polyclonal anti-Ras antibody (see Section 2) confirming (Fig. 7) the possibility to overexpress both wild type and mutated forms of Ras2 protein. Adenylate cyclase reintroduction strongly diminished the amount of both wild type and activated Ras2 protein as judged
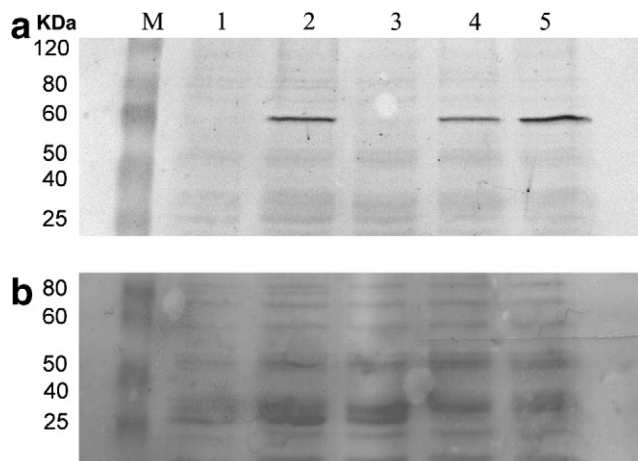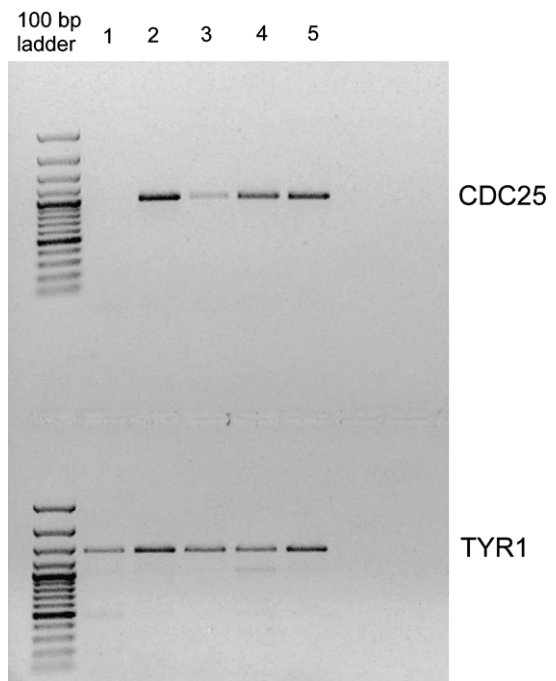

Fig. 5. Quantification of Cdc25p in the presence of different *RAS2* alleles. (a) Immunostaining of Cdc25 protein on Western blot obtained after SDS–PAGE of whole protein extract from a strain lacking both RAS2 and C-terminus *CDC25* (AAT3B YEp13H, lane 1), lacking *RAS2* but in the presence of C-terminus *CDC25* (AAT3B pIND25-1H, lane2), in the presence of wild type *RAS2* and C-terminus *CDC25* (AAT3BR2 pIND25-1H, lane 3), in the presence of an mutated *RAS2* allele and C-terminus *CDC25* (AAT3B R2 D80D81 pIND25-1H, lane 4) and in the absence of RAS2 in the presence of C-terminus CDC25 but in the absence of chromosomal CDC25 (AAT3B Δcdc25 PIND25-1H, lane 5). (b) Red Poinceau staining of the same filter before immunostaining.
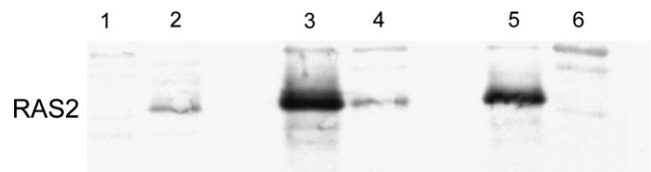

Fig. 7. Quantification of Ras2 protein in the presence/absence of the adenylate cyclase. Immunostaining of Ras2 protein on Western blot obtained after SDS–PAGE of whole protein extract of yeast strains grown on galactose and episomally expressing under the control of GAL10 promoter Ras2 wild type (WOF6.1-Yep51-R2, lanes 3 and 4) or Ras2G19V (WOF6.1-Yep51-R2V19 lanes 5 and 6) in the absence (Yep13H, lanes 3 and 5) or in the presence (YACW lanes 4 and 6) of episomally encoded adenylate cyclase. An yeast strain lacking both RAS genes (ABH7C, lane 1) and an yeast strain (19D, lane 2) with a wild type chromosomal copy of *RAS2* were used as a control.

by immunoblot. These results indicate that reconstitution of the Ras pathway inhibits GAL10 driven transcription and that this inhibition is dependent upon activation of the Ras-cAMP-PKA pathway and not by the presence of Ras2 protein itself.

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