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PKA and Sch9 control a molecular switch important for the proper adaptation to nutrient availability

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Summary

In the yeast *Saccharomyces cerevisiae*, PKA and Sch9 exert similar physiological roles in response to nutrient availability. However, their functional redundancy complicates to distinguish properly the target genes for both kinases. In this article, we analysed different phenotypic read-outs. The data unequivocally showed that both kinases act through separate signalling cascades. In addition, genome-wide expression analysis under conditions and with strains in which either PKA and/or Sch9 signalling was specifically affected, demonstrated that both kinases synergistically or oppositely regulate given gene targets. Unlike PKA, which negatively regulates stress-responsive element (STRE)- and post-diauxic shift (PDS)-driven gene expression, Sch9 appears to exert additional positive control on the Rim15-effector Gis1 to regulate PDS-driven gene expression. The data presented are consistent with a cyclic AMP (cAMP)-gating phenomenon recognized in higher eukaryotes consisting of a main gatekeeper, the protein kinase PKA, switching on or off the activities and signals transmitted through primary pathways such as, in case of yeast, the Sch9-controlled signalling route. This mechanism allows fine-tuning various nutritional responses in yeast cells, allowing them to adapt metabolism and growth appropriately.

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Introduction

For the yeast *Saccharomyces cerevisiae* nutrients are the prime environmental factors controlling growth, proliferation and metabolism. For instance, the addition of glucose to respiring cells triggers the necessary adaptations that reset metabolism to fermentation (Jiang *et al*., 1998). One of the key signalling cascades involved in this process is the Ras/cyclic AMP (cAMP) pathway. Addition of fermentable sugars triggers activation of adenylate cyclase and the production of a pronounced cAMP spike (Mbonyi *et al*., 1988). Cyclic AMP in turn activates PKA by binding to the two *BCY1*-encoded regulatory subunits allowing their dissociation from the two catalytic subunits, which are encoded by one of three partially redundant *TPK* genes (i.e. *TPK1*, *TPK2* and *TPK3*) (Toda *et al*., 1987a,b; Doskeland *et al*., 1993). Cyclic AMP is quickly degraded by the low- and high-affinity phosphodiesterases Pde1 and Pde2 (Nikawa *et al*., 1987a; Wilson and Tatchell, 1988). Furthermore, glucose-induced activation of the Ras/cAMP pathway is strictly controlled through negative feedback mechanisms (Nikawa *et al*., 1987b; Ma *et al*., 1999). Consequently, activation of cAMP synthesis beyond the basal level is a transient effect, limited to the short period of the onset of fermentation. Glucose-induced activation of PKA has been reported to trigger additional adaptations associated with optimal growth conditions, like the mobilization of reserve carbohydrates and the reduction of the overall stress resistance (Thevelein *et al*., 2000). Some of these physiological effects appear by changes in enzyme activities. Fairly well-established examples are phosphofructokinase 2, the low-affinity phosphodiesterase and the trehalose-degrading enzyme trehalase (Ortiz *et al*., 1983; Uno *et al*., 1983; Francois *et al*., 1984; Thevelein and Beullens, 1985; Zahringer *et al*., 1998). Other PKA-mediated effects can be accounted for by changes in transcription. For instance, PKA has been shown to modulate the activity of the multifunctional transcription factor Rap1 for the induction of ribosomal protein (rp) genes (Klein and Struhl, 1994). In addition, PKA counteracts the general stress response by compromising nuclear translocation of the partially redundant zinc-finger transcription factors, Msn2 and Msn4 (Gorner *et al*., 1998; Garreau *et al*., 2000). These factors bind to stress-responsive elements (STRE) in the promoter of many genes such as *HSP12*, *CTT1* and genes

encoding different subunits of the trehalose synthase complex (Marchler *et al*., 1993; Ruis and Schuller, 1995; Winderickx *et al*., 1996; Boy-Marcotte *et al*., 1998; Moskvina *et al*., 1998). Expression of stress-responsive genes also largely depends on Rim15, a protein kinase immediately downstream of and negatively regulated by PKA (Reinders *et al*., 1998; Pedruzzi *et al*., 2000). Consistently, deletion of *MSN2* and *MSN4* or *RIM15* can suppress the lethality of mutants with compromised PKA activity (Reinders *et al*., 1998; Smith *et al*., 1998). Rim15 appears to be specifically required for proper entry into stationary phase by inducing typical G0 traits such as accumulation of glycogen, trehalose and Gis1-dependent post-diauxic shift (PDS) element-driven gene expression (Reinders *et al*., 1998; Pedruzzi *et al*., 2000). Interestingly, Msn2/4 and Gis1 cooperatively mediate almost the entire Rim15-dependent transcriptional response at the diauxic shift (Cameroni *et al*., 2004).

The protein kinase Sch9 is structurally related to the catalytic subunits of PKA. It was initially isolated as a high-copy suppressor of the growth defect resulting from disruption of PKA signalling (e.g. following loss of Ras or PKA activity) (Toda *et al*., 1988). Conversely, the slow growth phenotype associated with loss of Sch9 can be suppressed by enhanced PKA activity, suggesting that both kinases may act, at least in part, redundantly or that overexpression of one kinase compensates for the loss of function of the other via promiscuous phosphorylation (Toda *et al*., 1988). More recently, Sch9 has been implemented in the fermentable growth medium-induced (FGM) pathway as a glucose- and nitrogen-responsive regulator that acts independently of cAMP to control phenotypic characteristics known to be affected by PKA such as stress resistance (Crauwels *et al*., 1997; Thevelein and de Winde, 1999 and references therein). However, the precise relationship between PKA and Sch9 has not been established yet as it was not unambiguously demonstrated whether Sch9 functions in parallel to the Ras/cAMP pathway. Furthermore, Sch9 is also involved in the regulation of cell size (Jorgensen *et al*., 2002) and longevity (Fabrizio *et al*., 2001; 2003). Although several putative substrates have been identified for PKA, none have been described for Sch9. Some candidate substrates were identified for both kinases with overlapping substrate specificities (Zhu *et al*., 2000). Furthermore, given their functional redundancy, it is difficult to differentiate properly between specific target genes.

This study further addresses the relationship between PKA and Sch9 at the transcriptional level. Results are presented demonstrating that PKA and Sch9 are key elements of separate signalling cascades. Our data point to a Sch9- and Rim15-dependent molecular switch involving Gis1 and Msn2/4 to control STRE- and PDS-driven gene expression. As Rim15 is negatively regulated by PKA on glucose medium, its role is to reset the molecular switch as a function of PKA activity and the available carbon source. This mechanism closely resembles a phenomenon known as cAMP-gating in higher eukaryotes in which PKA modulates the signal flow through primary pathways (Iyengar, 1996; Jordan and Iyengar, 1998).

Results

Sch9 contributes nutritional information independently of PKA

In order to elucidate in more detail the functional relationship between PKA and Sch9, we initially introduced the deletion of *SCH9* in the *tpk1Δ tpk2Δ tpk3Δ msn2Δ msn4Δ* strain that lacks PKA activity but is able to grow resulting from the suppressive effect obtained by deletion of the two STRE-binding transcription factors Msn2 and Msn4 (Smith *et al*., 1998). However, the deletion of *SCH9* in this background caused growth failure (data not shown), which is consistent with the previous observations that Sch9 is essential in strains with reduced activity of the Ras/cAMP pathway (Lorenz *et al*., 2000). Therefore, we shifted strategy and constructed a strain where the activity of PKA is specifically dependent on the addition of extracellular cAMP. To this end, the genes *PDE2* and *CYR1*, encoding, respectively, the high-affinity cAMP phosphodiesterase and adenylate cyclase, were deleted in a W303-1A wild type. As reported, the lack of Pde2 makes the cells responsive to extracellular cAMP (Mitsuzawa, 1993; Wilson *et al*., 1993) and permits to bypass the lethality caused by loss of Cyr1 (Fig. 1A). Hence, such a strain grows solely on cAMP-containing medium. Similar to the alleviation of the growth defect of the PKA-deficient mutant described above, the loss of the highly redundant transcription factors Msn2 and Msn4 or the overexpression of Sch9 allows the *pde2Δ cyr1Δ* mutant to grow in the absence of cAMP, although to different extents. Note that addition of cAMP or the additional deletion of *MSN2* and *MSN4* in the *pde2Δ cyr1Δ* mutant supported solely fermentative growth whereas enhanced Sch9 activity supported respiratory growth as well.

Next, we introduced the deletion of *SCH9* in the quadruple *pde2Δ cyr1Δ msn2Δ msn4Δ* mutant and found that loss of Sch9 abolished the ability of the corresponding mutant to grow fermentatively on glucose in the absence of cAMP (Fig. 1A). This is again consistent with the observations that the deletion of *SCH9* is essential in strains with reduced activity of PKA or the Ras/cAMP pathway (Kraakman *et al*., 1999; Lorenz *et al*., 2000). However, although the quintuple *pde2Δ cyr1Δ msn2Δ msn4Δ sch9Δ* mutant did not grow in the absence of cAMP, the strain retained its viability after cAMP starvation for up to 24 h and it responded to the re-addition of cAMP by resumption

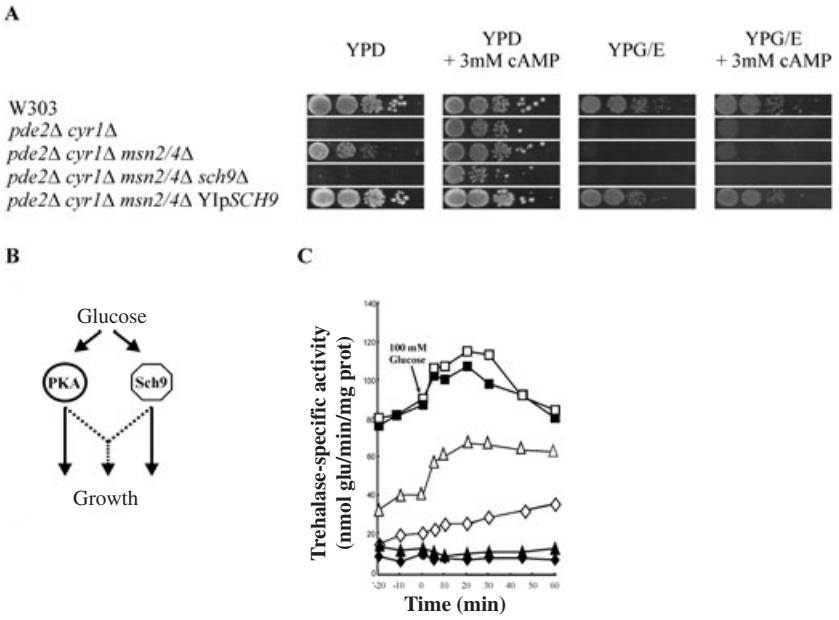

Fig. 1. PKA and Sch9 define separate signalling cascades.
A. Growth of mutant strains in the absence and presence of 3 mM cAMP on rich glucose or glycerol/ethanol-containing medium.
B. Summary figure of the parallel and converging effector branches of the PKA and Sch9 signalling pathways.
C. PKA and Sch9 requirement for proper glucose-induced trehalase activation. Strains *pde2Δ cyr1Δ msn2Δ msn4Δ* (△, ▲), *pde2Δ cyr1Δ msn2Δ msn4Δ sch9Δ* (◇, ◆) and *pde2Δ cyr1Δ msn2Δ msn4Δ* YIp*SCH9* (□, ■) with (open symbols) or without (closed symbols) pretreatment of 0.5 mM cAMP were supplemented with 100 mM glucose at time 0 to induce activation of trehalase. Average values over three independent measurements are shown with an average standard deviation of 2.19.

of growth (data not shown). Interestingly, we also noticed that even in the presence of cAMP, the *pde2Δ cyr1Δ msn2Δ msn4Δ sch9Δ* mutant still displays a severe growth defect on minimal glucose-containing medium (Fig. S1A in *Supplementary material*) and this phenotype could only be restored by reintroduction of *SCH9* and not by overexpression of *TPK1* or *BCY1* (Fig. S1B in *Supplementary material*). These data are consistent with Sch9 being important for transmitting additional nutritional signals besides those generated by the presence of a fermentable carbon source (Thevelein and de Winde, 1999). Also the overexpression of *SCH9* in the quadruple *pde2Δ cyr1Δ msn2Δ msn4Δ* mutant indicated that cAMP-activated PKA cannot compensate for all functions of Sch9 because enhanced Sch9 activity improved growth on glycerol-containing media independently of cAMP. In summary, our data indicate that Sch9 controls more growth regulatory functions than those regulated by cAMP-activated PKA or Msn2/4 and hence, they favour a model in which Sch9 and PKA control partially overlapping signalling networks. Consequently, at least one of the Sch9-effector branches should converge on a component downstream of PKA while others function in parallel as illustrated in Fig. 1B.

PKA is required but not sufficient for proper glucose-induced trehalase activation

The glucose-induced activation of the trehalose degrading enzyme trehalase is one of the best-established examples in which yeast PKA has an important regulatory function (Uno *et al.*, 1983; Zahringer *et al.*, 1998). We monitored glucose-induced trehalase activation in the different strains constructed above under conditions with or without pretreatment with 0.5 mM cAMP. As shown in Fig. 1C, addition of 100 mM glucose did not induce trehalase activation in the *pde2Δ cyr1Δ msn2Δ msn4Δ* or *pde2Δ cyr1Δ msn2Δ msn4Δ sch9Δ* strains in the absence of cAMP. Also the supplementation of 0.5 mM cAMP (data points −20, −10, 0 in Fig. 1C) failed to trigger activation of trehalase although it elevated the basal activity. Only the combined treatment, i.e. addition of glucose to cAMP-treated cells, triggered a fast increase in trehalase activity but solely in the quadruple *pde2Δ cyr1Δ msn2Δ msn4Δ* mutant and not in the quintuple *pde2Δ cyr1Δ msn2Δ msn4Δ sch9Δ* mutant. The very modest increase in trehalase activity that was still present in the quintuple mutant resulted entirely from the cAMP treatment as it was also observed when only cAMP was given without a subsequent addition of glucose (results not shown). Thus, it can be concluded that cAMP-induced activation of PKA is required but not sufficient to mediate the glucose-dependent change in trehalase activity and that an additional requirement depends on Sch9. The overexpression of *SCH9* in the *pde2Δ cyr1Δ msn2Δ msn4Δ* strain, on the other hand, not only increased dramatically the basal trehalase activity but also rendered the glucose-induced trehalase activation independent of cAMP. This confirms that overactivation of Sch9 indeed compensates for the loss of PKA activity. These data are consistent with the results obtained for growth as they show different but converging functions of Sch9 and PKA.

Identification of target genes for cAMP-activated PKA and Sch9 using genome-wide expression analysis

In order to identify target genes controlled by cAMP-activated PKA and Sch9, we performed a genome-wide

expression analysis with the strains *pde2Δ cyr1Δ msn2Δ msn4Δ*, *pde2Δ cyr1Δ msn2Δ msn4Δ sch9Δ* and *pde2Δ cyr1Δ msn2Δ msn4Δ* YIp*SCH9* that did or did not receive 3 mM cAMP after a 6 h cAMP starvation period on galactose-containing medium. As mentioned, all strains remained viable upon cAMP starvation and responded to the re-addition of cAMP by resumption of growth (data not shown). Using these conditions, we retrieved a limited number of genes that are predominantly regulated by either PKA or Sch9 and a larger number of genes that are controlled by both kinases, as described in more detail below. The validity of the genome-wide expression data was confirmed by conventional Northern blot analysis for several randomly selected genes starting from RNA samples prepared independently of those used for the genome-wide expression analysis (see Fig. S2 in *Supplementary material*). In addition, many genes known to be affected by PKA, such as rp genes and genes involved in reserve carbohydrate metabolism and the stress response were consistently retrieved in our analysis (Marchler *et al*., 1993; Griffioen *et al*., 1994; Klein and Struhl, 1994; Winderickx *et al*., 1996; Boy-Marcotte *et al*., 1998) (and Table S1 in *Supplementary material*). Furthermore, there is a significant overlap in the genes retrieved by our microarray analysis and those identified by genome-wide expression analyses of glucose-induced effects in *tpk*-attenuated strains and strains with overexpression of *RAS2* and *GPA2* (Wang *et al*., 2004).

It should be noted that the results obtained for the expression of genes in the strain with overexpression of *SCH9* are difficult to interpret. Similarly to the activation of trehalase, the increase in Sch9 activity rendered many of the genes unresponsive to cAMP. Although consistent with the observations that overexpression of Sch9 compensates for the loss of PKA, one cannot rule out the possibility that this effect simply results from aberrant phosphorylation of proteins at epitopes that in wild-type cells are normally not a substrate of the Sch9 kinase. Therefore, this strain was not taken into account for the interpretation of PKA- and Sch9-mediated transcriptional effects.

Specific target genes of cAMP-activated PKA and Sch9

In order to retrieve those genes that can be considered as specific targets for either cAMP-activated PKA or Sch9, i.e. genes of which transcription predominantly depends on only one of the kinases, we applied the following selection procedure. Genes were deemed PKA specific when their fold change difference in expression exceeded 2.0 resulting from addition of cAMP to the strains *pde2Δ cyr1Δ msn2Δ msn4Δ* and *pde2Δ cyr1Δ msn2Δ msn4Δ sch9Δ* and of which the fold change difference resulting from deletion of *SCH9* was lower than 1.5, i.e. the comparisons '*pde2Δ cyr1Δ msn2Δ msn4Δ* without cAMP' versus '*pde2Δ cyr1Δ msn2Δ msn4Δ sch9Δ* without cAMP' and '*pde2Δ cyr1Δ msn2Δ msn4Δ* with cAMP' versus '*pde2Δ cyr1Δ msn2Δ msn4Δ sch9Δ* with cAMP'. This selection procedure thus retrieves those genes of which the expression is changed significantly resulting from the addition of cAMP irrespective of the presence of Sch9 but of which the expression does not change significantly by deletion of *SCH9* irrespective of the absence or presence of cAMP. Conversely, genes were deemed Sch9 specific when their fold change difference in expression exceeded 2.0 in the comparisons '*pde2Δ cyr1Δ msn2Δ msn4Δ* without cAMP' versus '*pde2Δ cyr1Δ msn2Δ msn4Δ sch9Δ* without cAMP' and '*pde2Δ cyr1Δ msn2Δ msn4Δ* with cAMP' versus '*pde2Δ cyr1Δ msn2Δ msn4Δ sch9Δ* with cAMP' and of which the fold change difference resulting from addition of cAMP in both strains was lower than 1.5. This selection procedure thus retrieves those genes of which the expression is changed significantly resulting from the deletion of *SCH9* irrespective of the absence or presence of cAMP but of which the expression does not change significantly by addition of cAMP (Table 1). Note that the fold change cut-off values were statistically determined and that they correspond to the values that allow for selection of differentially expressed genes with a confidence level of 99% or for genes of which the expression was largely unchanged with a confidence level of 90%. In this way, we identified 11 genes for which the transcription was predominantly regulated by cAMP-activated PKA and largely independent of Sch9 with functions in cell cycle and DNA processing (*NOP7*, *HCA4* and *HAS1*) and stress response (*HSP26* and *THI4*) (Table 1). Twenty-four genes were retrieved of which transcription was predominantly controlled by Sch9 and largely independent of cAMP-activated PKA (Table 1). These genes mostly play central roles in amino acid biosynthesis such as *ARO4*, *ASN2*, *BAP3*, *GLY1*, *ILV2*, *MET3*, *MET6*, *MET10* and *MET17* and other metabolic pathways such as glycolysis and gluconeogenesis (*PYC2*, *PFK1* and *SDH1*)

Expression profiling reveals synergistic and opposing effects of PKA and Sch9 on transcription of common target genes

We applied the Adaptive Quality Based Clustering (AQBC) algorithm (De Smet *et al*., 2002) to compare and cluster the expression profiles of all the genes that responded significantly to changes in the activity of both PKA and Sch9. This allowed sorting of 918 genes into 14 different clusters (Fig. 2A). These clusters could be further classified into five major classes when only the first four conditions were taken into consideration, i.e. expression in the *pde2Δ cyr1Δ msn2Δ msn4Δ* (1) and the *pde2Δ cyr1Δ msn2Δ msn4Δ sch9Δ* (2) mutants in the absence

Table 1. PKA- and Sch9-specific target genes.

		Effect of cAMP[a]		Effect of SCH9[a]		
ORF	Gene	*SCH9*[b]	*sch9Δ*[b]	–cAMP[b]	+cAMP[b]	Biological function
PKA specific (11 genes)						
Induced						
YGR103W	*NOP7*	**2.86**	**2.15**	1.01	1.34	Nucleolar protein
YGR160W	*FYV13*	**2.52**	**3.00**	1.39	1.16	Hypothetical protein
YJL033W	*HCA4*	**2.88**	**2.18**	1.17	1.54	RNA helicase CA4
YKL044W		**2.15**	**3.01**	1.02	–1.37	Hypothetical protein
YLR089C		**2.41**	**2.51**	1.31	1.26	Putative alanine aminotransferase
YMR290C	*HAS1*	**3.41**	**2.17**	–1.20	1.31	RNA helicase
Repressed						
YBR072W	*HSP26*	**–6.13**	**–4.07**	1.49	–1.01	Heat shock protein
YDR155C	*CPH1*	**–3.69**	**–2.86**	1.05	–1.23	Cyclophilin (peptidylprolyl isomerase)
YGR144W	*THI4*	**–2.82**	**–2.42**	–1.17	–1.37	Thiamine-repressed protein
YHL035C		**–2.18**	**–2.41**	1.10	1.21	Transporter activity
YIR036C		**–2.32**	**–2.58**	1.22	1.36	Reductase
Sch9 specific (24 genes)						
Induced						
YBR218C	*PYC2*	1.15	1.25	**2.48**	**2.27**	Pyruvate carboxylase 2
YBR249C	*ARO4*	1.23	1.39	**3.07**	**2.72**	2-Dehydro-3-deoxyphosphoheptonate aldolase
YDL081C	*RPP1A*	1.31	1.38	**2.66**	**2.52**	Acidic ribosomal protein P1A
YDR046C	*BAP3*	1.26	1.21	**2.34**	**2.45**	Branched chain amino acid permease
YDR399W	*HPT1*	1.30	1.03	**2.19**	**2.76**	Hypoxanthine phosphoribosyl transferase
YEL046C	*GLY1*	1.39	1.11	**2.76**	**3.45**	L-threonine aldolase, low specific
YER091C	*MET6*	–1.26	–1.23	**2.46**	**2.39**	Methionine synthase
YFR030W	*MET10*	–1.36	–1.06	**3.77**	**2.93**	Sulphite reductase flavin-binding subunit
YGR124W	*ASN2*	1.13	1.43	**2.99**	**2.36**	Asparagine synthetase
YGR240C	*PFK1*	1.08	1.40	**3.26**	**2.51**	6-Phosphofructokinase, α-subunit
YGR271W	*SLH1*	1.42	1.25	**2.43**	**2.77**	'SKI2-like helicase'
YJL008C	*CCT8*	–1.20	1.46	**4.00**	**2.28**	Component of TCP ring complex
YJL101C	*GSH1*	–1.46	–1.42	**3.04**	**2.94**	γ-Aglutamylcysteine synthetase
YJR010W	*MET3*	1.19	–1.24	**2.95**	**4.37**	Sulphate adenylyltransferase
YKL054C	*DEF1*	1.35	1.29	**2.49**	**2.60**	RNA PolII degradation factor 1
YKL148C	*SDH1*	–1.46	–1.43	**3.10**	**3.03**	Succinate dehydrogenase flavoprotein subunit
YLR130C	*ZRT2*	–1.02	–1.18	**2.49**	**2.87**	Low-affinity zinc transporter
YLR302C		1.22	1.06	**6.76**	**7.77**	Protein of unknown function
YLR303W	*MET17*	1.04	1.32	**6.01**	**4.73**	O-acetylhomoserine sulphydrylase
YML002W		–1.34	–1.32	**2.67**	**2.62**	Hypothetical protein
YML123C	*PHO84*	1.10	1.02	**4.27**	**4.59**	High-affinity inorganic phosphate symporter
YMR108W	*ILV2*	–1.20	1.38	**4.26**	**2.57**	Acetolacetate synthase
Repressed						
YFR026C		1.30	1.29	**–2.11**	**–2.09**	Protein of unknown function
YIL058W		1.16	–1.27	**–3.32**	**–2.25**	Protein of unknown function

a. Variable parameter: effect of cAMP or presence of *SCH9* in the strain *pde2Δ cyr1Δ msn2/4Δ*. Values represent fold change difference in expression. See text and *Experimental procedures* for selection procedures.
b. Constant parameter.
Genes were deemed PKA specific when their fold change difference in expression exceeded 2 resulting from addition of cAMP in both strains and when the fold change difference in expression did not exceed 1.5 resulting from the presence of *SCH9*. Genes were deemed Sch9 specific when their fold change difference in expression exceeded 2 resulting from the presence of *SCH9* and when the fold change difference in expression did not exceed 1.5 resulting from addition of cAMP.

(a) or presence (b) of cAMP. The different classes were determined based on the effect triggered by the addition of cAMP (condition 1a versus 1b and condition 2a versus 2b in Fig. 2A) combined with the effect obtained for the deletion of *SCH9* (condition 1a versus 2a and condition 1b versus 2b in Fig. 2A). Thus, the subclusters in each class differed mainly in the expression profiles obtained with overexpression of *SCH9* (conditions 3a and 3b in Fig. 2A), which, as mentioned, were difficult to interpret and not used in further descriptions. As deduced from the MIPS (Munich Information Centre for Protein Sequences) functional categories, many of the genes within each cluster encoded proteins that are involved in the same physiological or metabolic process (Fig. 2B).

To get more insight in the transcriptional mechanisms governed by PKA and Sch9, we then searched for the presence of over-represented motifs using the genome-wide screening programs REDUCE (Bussemaker *et al*., 2001) and MOTIF FINDER (Thijs *et al*., 2002). In this way, we identified 14 putative *cis*-acting DNA elements that may contribute to PKA- and/or Sch9-dependent regulation of expression (Table 2). Subsequently, we screened each element against the Transfac database (Heinemeyer *et al*., 1998) to identify corresponding DNA-binding

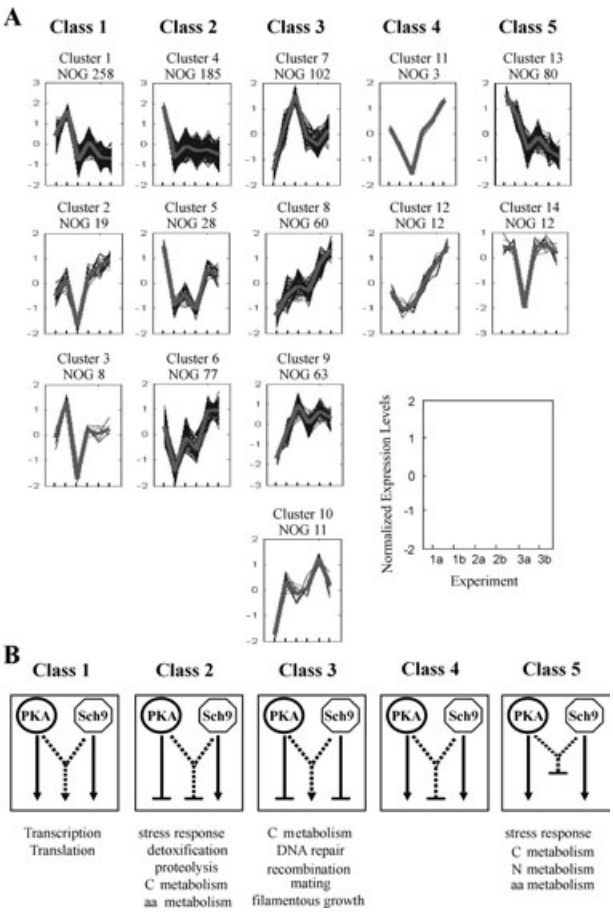

Fig. 2. Genome-wide expression profiling of normalized intensities using the AQBC clustering algorithm.
A. Genes were clustered based on their normalized intensities in the strains (1) *pde2Δ cyr1Δ msn2Δ msn4Δ* (2) *pde2Δ cyr1Δ msn2Δ msn4Δ sch9Δ* and (3) *pde2Δ cyr1Δ msn2Δ msn4Δ* YIp*SCH9* grown in the absence (a) or in the presence (b) of 3 mM cAMP. Output of AQBC clustering – normalized intensity profiles of the genes – is shown (black lines). For each cluster, the mean expression profile is also shown (bold grey line). NOG stands for number of genes assigned to each cluster. Chart axes are shown in the empty chart on the lower right side.
B. Schematic representation for each class depicting the effect on expression in the different strains under conditions with and without 3 mM cAMP. Filled lines represent the effects of PKA and Sch9 when considered separately (2a versus 2b and 1a versus 2a respectively) while dashed lines represent the combined effects of both kinases (1a versus 1b and 1b versus 2b). The most highly enriched MIPS functional categories for each class are shown.

proteins. The different classes are described in detail below.

Class 1 contains three clusters which are enriched for genes that encode proteins with a function in translation and protein synthesis. These include rp genes (*RPL* and *RPS* genes), translation initiation and elongation factors (e.g. *HYP2*, *GCD11*, *EFB1*, *EFT2*), tRNA synthetases (e.g. *ILS1*, *GRS1*), proteins involved in rRNA transcription (e.g. *NHP2*, *DBP3*), nucleotide metabolism (e.g. *IMD2-4*, *URA7*, *URA5*, *PRS1*, *PRS3*) and amino acid metabolism (e.g. *AGP1*, *BAP3*). These genes are not only induced by addition of cAMP-activated PKA but they are also positively regulated by the Sch9 protein kinase, independently of cAMP because deletion of *SCH9* dramatically reduces their expression. In agreement with previously reported data (Griffioen *et al*., 1996; Crauwels *et al*., 1997), the effects triggered by PKA and Sch9 appeared to be largely independent of each other (compare condition 1a versus 2a and 1b versus 2b in Fig. 2A). REDUCE and MOTIF FINDER identified three known DNA elements, i.e. RPG, RRPE and PAC, and three unknown DNA elements, designated U1-U3, in genes of class 1 and more in particular those of cluster 1 (Table 2 and Table S2 in *Supplementary material*) (Tavazoie *et al*., 1999). REDUCE also assigned positive *F*-scores for both PKA and Sch9 indicating a positive correlation between transcriptional regulation through these elements and enhanced PKA and Sch9 activity (Table 2). For the known elements, the RPG box was reported to be bound by Rap1, a PKA-dependent multifunctional transcription factor required for expression regulation of rp genes (Griffioen *et al*., 1994; Klein and Struhl, 1994). The RPPE (rRNA processing element) (Tavazoie *et al*., 1999; Hughes *et al*., 2000) and the PAC (P and C box) elements (Dequard-Chablat *et al*., 1991) are putative recruitment sites for the histone deacetylase Rpd3, which reverses histone H4 acetylation in response to nutrient limitation thereby causing repression of rp genes and genes encoding proteins involved in rRNA and tRNA synthesis (Kurdistani *et al*., 2002; Rohde and Cardenas, 2003). Very recently, PKA was described to negatively influence the histone deacetylase HDAC8, the mammalian homologue of yeast Rpd3, through phosphorylation (Lee *et al*., 2004). As so far, however, no data are available for the involvement of PKA or Sch9 in histone acetylation and deacetylation in yeast, we confirmed the mathematical calculations of REDUCE by *in vivo* β-galactosidase activity measurements using a minimal promoter construct containing one single RRPE element. As shown, both PKA and Sch9 were indeed required to obtain maximal induction of the β-galactosidase reporter (Fig. 3A). REDUCE also identified a fourth unknown DNA element, designated U4, which based on the calculations should have no correlation with PKA but a positive correlation with Sch9 (Table 2). Also this could be confirmed *in vivo* using a U4-driven β-galactosidase reporter construct (Fig. 3B). Thus, although the physiological relevance of the U4 motif remains unclear, this DNA element might be considered a prime candidate for Sch9-specific signalling in the control of cluster 1 genes. Taken together, both PKA and Sch9 positively and synergistically control transcription of class 1 genes presumably through separate yet unidentified mechanisms (Fig. 2B). Our data suggest that this may involve modulation of Rap1 activity or histone deacetylase activity.

Table 2. *Cis*-acting promoter elements identified by REDUCE (R) and/or MOTIF FINDER (M).

Method	Element	Name	Consensus sequence	Transcription factor	Cluster[a]	PKA correlation[b]	Sch9 correlation[b]
R/M	AGGGG	STRE	AG4	Msn2/4	4,5,6,9,14	–	0
R	GATGAG	PAC	AKCTCATCKC	Unknown	1,7	+	0
R	(C)(C)(T/A)AAGG(G)	PDS	TWAG3AT	Gis1	4,5,6	–	+
R/M	(C)ACCCAT(A)	RPG	RMACCANNCAYY	Rap1	1,10,13	+	+
R	TGAAAAA	RRPE	WGAAAAAWWTT	Unknown	1	+	+
R	AAAATTT(T)	RRPE	AAAATTT	Unknown	1	+	+
M	TGAAAC	PRE	A/TGAACA	Ste12	7,8,9	ND	ND
M	CATTCT	FRE	CATTCC/T	Tec1	7,8,9	ND	ND
R	CCG repeat	URS1	TAGCCGCCGA	Ime1/Ume6	1,13	0	+
R	(T)CCGTAC(A)	U1	Unknown	Unknown	1,14	+	+
R	(G)(V)CTGG(A)(C)	U2	Unknown	Unknown	1,4,14	–	+
R	CAGGCCG	U3	Unknown	Unknown	1,7,11,13	+	+
R	AAACG	U4	Unknown	Unknown	1	0	+
R	GGATTCC	U5	Unknown	Unknown	7,12	+	–

a. The distribution for each element in each cluster relative to its genome-wide distribution was calculated according to Tavazoie *et al.* (1999). Elements were deemed significantly enriched when the minus log2(*P*-values) exceeded 1.
b. +, positive correlation; –, negative correlation; 0, no correlation. The PKA and/or Sch9 correlation was determined based on the *F*(Multi)-scores of the tested DNA motif in a given comparison as calculated by REDUCE (Bussemaker *et al.*, 2001). *F*(Multi)-score: the contribution of the identified DNA motif to the log2ratio of gene Ag relative to the baseline level C according the formula $Ag = C + \Sigma(F \times N)$. Negative values correspond to a drop in expression whereas positive values correspond to higher expression as function of a particular DNA motif.
ND, not detected by REDUCE.

Class 2 contains three clusters that are enriched for genes encoding proteins required for the general stress response (e.g. *SSA3*, *HSP12*, *GRE1*, *CTT1*), cell growth (e.g. *TPD3*, *ABP1*, *ARP2*), detoxification (e.g. *SOD2*, *CCP1*, *GPX1*, *GPX2*), proteolysis (e.g. *RPN5*, *RPN8*, *RPN12*, *RPT2*, *RPT4*), reserve carbohydrate metabolism (e.g. *TPS1*, *TSL1*, *PPG1*, *GLC3*), the tricarboxylic acid (TCA) cycle (e.g. *SDH2*, *ACO1*, *KGD2*), amino acid metabolism (e.g. *HIS5*, *SER1*, *GDH3*) and carbohydrate metabolism (e.g. *GLK1*, *HXT5*, ml*S1*, *PDA1*, *PDC6*). These genes are all repressed by cAMP-activated PKA (compare condition 1a versus 1b and 2a versus 2b in Fig. 2A), which is consistent with previously published data (Belazzi *et al.*, 1991; Werner-Washburne *et al.*, 1993; Mager and De Kruijff, 1995; Winderickx *et al.*, 1996; Boy-Marcotte *et al.*, 1998). Sch9, on the other hand, exerts a positive effect on these genes in the absence of cAMP because its deletion reduces their expression although not to the same extent as cAMP-activated PKA does (compare condition 1a versus 2a in

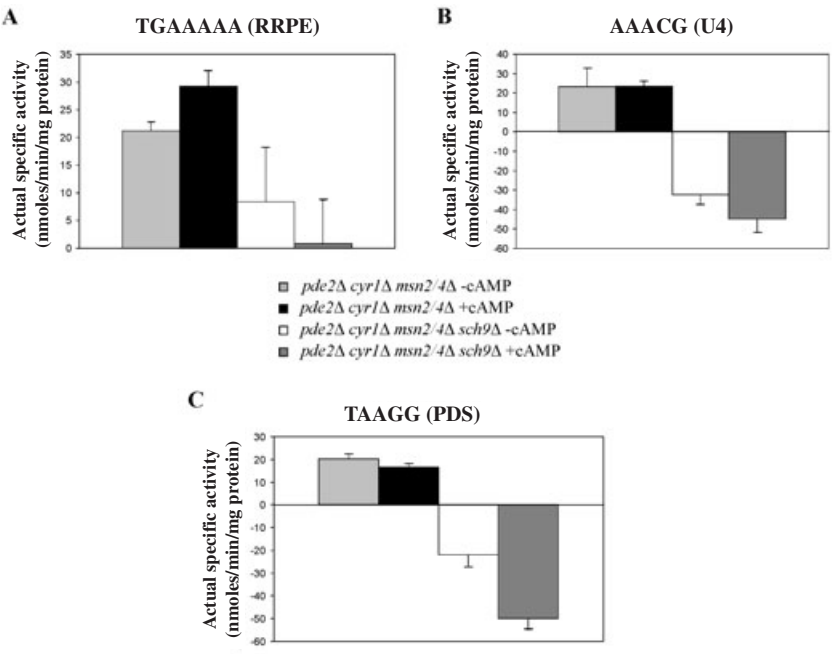

Fig. 3. *In vivo* confirmation of the mathematical predictions by REDUCE. β-Galactosidase assays in the strains indicated using promoter reporter constructs pJS205XN1B, pJS205XN4B, pJS205XN3B containing one single element of the DNA elements (A) TGAAAAA (RRPE), (B) AAACG (U4) or (C) TAAGG (PDS) respectively. Values are represented as actual specific activity (nmoles per minute per mg protein) with baseline zero representing the background activity of the empty vector. Averages of three independent measurements are shown.

Fig. 2A). Interestingly, however, the lack of Sch9 prevented to some extent the repression by cAMP-activated PKA for most genes, i.e. clusters 4 and 6 (compare condition 2a versus 2b in Fig. 2A). This apparent dual function of Sch9 has been noticed before, when deletion of the kinase was described to induce a high-PKA phenotype in derepressed cells while compromising maintenance of high-PKA phenotypes in repressed cells (Crauwels *et al*., 1997). REDUCE identified the STRE and PDS elements which are significantly enriched in class 2 genes (Table 2). Consistent with the literature (Marchler *et al*., 1993; Pedruzzi *et al*., 2000), both elements scored negative *F*-values and hence negatively correlated with cAMP-activated PKA, indicating that PKA mediated repression through these elements. For Sch9, there was no strict correlation with the STRE element while a positively correlation was found with the PDS element. The latter was not only consistent with the marked drop in expression of the PDS-controlled genes upon activation of PKA or deletion of *SCH9* (Fig. 2 and Table 3), but it was also in line with the β-galactosidase studies using a PDS reporter construct (Fig. 3C). Thus, class 2 genes are negatively regulated by cAMP-activated PKA and positively controlled by Sch9. How both kinases indepen-

Table 3. PKA and Sch9 oppositely regulate STRE/PDS-driven gene expression.

ORF	Gene	cAMP[a]	*SCH9*[b]	Biological function	STRE	PDS
Stress response						
YDR353W	*TRR1*	–13.71	4.82	Thioredoxin reductase	0	1
YDR513W	*TTR1*	–4.69	3.73	Glutaredoxin	2	1
YER103W	*SSA4*	–4.33	5.11	Heat shock protein	3	3
YFL014W	*HSP12*	–7.74	6.92	Heat shock protein	7	3
YGR088W	*CTT1*	–9.11	5.05	Cytosolic catalase T	4	4
YHR008C	*SOD2*	–6.52	5.63	Manganese superoxide dismutase	1	1
YJR104C	*SOD1*	–4.22	4.88	Copper-zinc superoxide dismutase	1	2
YLL026W	*HSP104*	–10.18	4.53	Heat shock protein	3	2
YLL060C	*GTT2*	–5.32	4.30	Glutathione transferase	0	1
YML028W	*TSA1*	–4.59	3.33	Thioredoxin peroxidase	1	1
YML070W	*DAK1*	–8.49	6.68	Dihydroxyacetone kinase	0	3
YMR096W	*SNZ1*	–3.38	3.80	Putative pyridoxin	0	1
YMR175W	*SIP18*	–5.96	3.82	'Salt-induced protein'	3	1
YNL160W	*YGP1*	–6.56	3.87	'Yeast Glycoprotein'	2	0
YOL053C-A	*DDR2*	–9.89	3.96	'DNA-damage responsive'	4	3
YOR027W	*STI1*	–4.53	3.34	'Stress Inducible'	0	2
YPL223C	*GRE1*	–7.82	8.66	Stress-responsive gene	0	4
YPL240C	*HSP82*	–5.37	3.03	Heat shock protein	2	0
Energy						
YCL040W	*GLK1*	–3.25	3.98	Glucokinase	3	0
YDR074W	*TPS2*	–3.18	3.35	Trehalose 6-phosphate phosphatase	5	2
YER178W	*PDA1*	–4.01	3.04	Pyruvate dehydrogenase, α-subunit	2	1
YFL056C	*AAD6*	–6.05	6.56	Aryl alcohol dehydrogenase	0	0
YGR087C	*PDC6*	–6.96	6.77	Pyruvate decarboxylase isoenzyme 3	0	2
YGR256W	*GND2*	–9.17	3.60	6-Phosphogluconate dehydrogenase	2	1
YML100W	*TSL1*	–3.64	3.33	Trehalose 6-phosphate synthase complex, regulatory subunit	3	1
YNL241C	*ZWF1*	–3.65	5.29	Glucose 6-phosphate dehydrogenase	5	3
Metabolism						
YAL062W	*GDH3*	–6.64	3.46	Glutamate dehydrogenase	0	0
YBR026C	*ETR1*	–7.29	4.40	2-Enoyl thioester reductase	1	2
YDR368W	*YPR1*	–6.22	3.25	2-Methylbutyraldehyde reductase	0	0
YHR037W	*PUT2*	–3.34	3.35	P5C dehydrogenase	0	1
YKL157W	*APE2*	–6.90	6.06	Aminopeptidase 2	0	1
YOL058W	*ARG1*	–3.30	3.31	Argininosuccinate synthetase	2	2
YOL151W	*GRE2*	–4.93	5.53	α-Acetoxy ketone reductase	0	4
YOR374W	*ALD4*	–5.37	3.81	Mitochondrial aldehyde dehydrogenase	2	0
Other						
YBR008C	*FLR1*	–8.40	6.96	'Fluconazole resistance protein'	1	2
YDL126C	*CDC48*	–4.75	4.67	Component of ERAD system	1	1
YHR139C	*SPS100*	–7.43	4.63	Sporulation-specific protein	3	8
YJL056C	*ZAP1*	–3.70	3.30	Zinc-responsive transcriptional activator	0	1
YKR076W	*ECM4*	–12.28	7.16	'Extracellular Mutant'	2	1
YPL036W	*PMA2*	–9.45	6.98	P-type ATPase	4	3

a. Genes repressed by addition of cAMP in the strain pde2Δ cyr1Δ msn2/4Δ.
b. Genes induced by *SCH9* in the strain *pde2Δ cyr1Δ msn2/4Δ* in the absence of cAMP.
Only genes with a known function and of which the fold change difference in expression exceeded 3 across both comparisons ([a] and [b]) were retained (see *Experimental procedures* for details on data normalization and analysis). The number of STRE (AG4) and PDS (TAAGG) elements within 1000 base pairs (bp) upstream of the transcription start sites (non-coding sequences only) of the corresponding ORFs is indicated (overlapping matches were allowed).

dently and oppositely influence expression of the genes in class 2 is described in more detail in the section below.

Class 3 contains four clusters with genes that encode proteins with a function in glycolysis and gluconeogenesis (e.g. *ENO2*, *TDH2*, *FBA1*), transcription regulation (*GCN4*, *ADA2*), utilization of alternative carbon sources (e.g. *GAL1*, *GAL7*, *GAL10*), respiration, mitochondrial biogenesis and mitochondrial transport (e.g. *QCR9*, *MRS5*, *IMG2*, *TOM37*, *MFT1*), recombination and DNA repair (e.g. *EXO1*, *PES4*, *REV3*), protein modification (e.g. *STE14*, *APC11*, *HAT1*), mating type determination, pheromone response and filamentous and invasive growth (e.g. *BAR1*, *OPY2*, *GPA1*, *STE2*, *STE5*, *STE12*, *STE18*, *MFA1*, *MFA2*, *BMH2*). These genes are induced by cAMP-activated PKA in the presence of Sch9 (compare condition 1a versus 1b in Fig. 2A) but repressed by cAMP-activated PKA in the absence of Sch9 (compare condition 2a versus 2b in Fig. 2A). Conversely, Sch9 exerts negative regulation on the expression of these genes in the absence of cAMP (compare condition 1a versus 2a in Fig. 2A), but this Sch9-mediated effect is largely over-ruled by the presence of cAMP-activated PKA (compare condition 1b versus 2b in Fig. 2A) resulting in the overall induction of the genes (Fig. 2B). As deduced by MOTIF FINDER, the genes of class 3 were enriched for the Ste12 binding sites known as the sterile response element (SRE) and pheromone response element (PRE). Often these sites are found in close proximity to a presumed Tec1 consensus binding site to constitute the so-called filamentous response element (FRE) (reviewed in Gancedo, 2001). Ste12 has been shown to enhance transcription of genes encoding proteins required for pseudohyphal and invasive growth (Gancedo, 2001) and of genes that are in close proximity to Ty transposable elements (Ciriacy *et al*., 1991; Bilanchone *et al*., 1993; Laloux *et al*., 1994). Not surprisingly, the MIPS database annotated many of the genes in class 3 as so-called Ty-ORFs (open reading frames) or described them to encode proteins involved in the pheromone response, mating and the pseudohyphal and invasive growth pathway. Consistent with our data and more in particular the finding that Sch9 exerts a repressive effect on *STE12*, cells deficient for Sch9 were described to display hyperactivation of the pheromone mitogen-activated protein kinase (MAPK) pathway and up to five-fold higher transcription from a PRE-driven reporter construct even in the absence of pheromone (Morano and Thiele, 1999). In addition, the deletion of *SCH9* was also reported to induce hyperinvasive growth in strains of the Σ1278 background (Lorenz *et al*., 2000) and it should be noted that this phenotype may result from the repressive effect triggered by Sch9 on different components, including not only the transcription factor Ste12 but Gcn4 as well (Braus *et al*., 2003). Also consistent with our data are the observations that PKA plays an essential compensatory role with respect to the MAPK pathway for the induction of filamentous and haploid invasive growth (Gancedo, 2001 and references therein). Although this compensatory role depends on different transcription factors, our data point to a more direct connection between PKA and Ste12- and/or Gcn4-dependent transcription as suggested previously (Mösch *et al*., 1999; Braus *et al*., 2003).

Class 4 contains two small clusters of which only a few genes have a known function. Cyclic AMP-activated PKA represses these genes provided the presence of Sch9 (compare condition 1a versus 1b in Fig. 2A) but it induces them when Sch9 is absent (compare condition 2a versus 2b in Fig. 2A). The latter can be regarded as a compensation for the loss of Sch9 as Sch9 appears indeed to be required to maintain basal expression (Fig. 2B).

Finally, class 5 consists of two clusters with genes encoding proteins involved in amino acid metabolism (e.g. *LYS21*, *TRP1*, *MET10*, *LEU4*), glycolysis, gluconeogenesis and the citric acid cycle (e.g. *TPI1*, *ICL1*, *PCK1*, *PDC5*, *HAP4*, *IDP2*), nitrogen metabolism (e.g. *GAT1*, *ISU2*) and the stress response (e.g. *SSA2*, *SSA4*, *ZDS1*, *RSP5*). These genes display only a minor reduction in expression when cAMP is added to the *pde2Δ cyr1Δ msn2Δ msn4Δ* strain and hence they do not respond significantly to cAMP if Sch9 is present (compare condition 1a versus 1b in Fig. 2A). In contrast, these genes show a marked drop in expression when *SCH9* is deleted, indicating that they are primarily regulated by the Sch9 kinase (compare condition 1a versus 2a in Fig. 2A). Interestingly, however, cAMP-activated PKA could restore expression to some extent in the absence of Sch9 (compare condition 2a versus 2b in Fig. 2A), which is indicative for the compensation for the loss of Sch9 function (Fig. 2B).

Taken together, based on the expression profiles (Fig. 2A) it appears that Sch9 is required to maintain basal expression levels for most genes in the absence of cAMP-activated PKA. Indeed, upon deletion of *SCH9*, the expression dropped to a minimum level for the 392 genes found in classes 1, 4 and 5 while it increased to maximal levels for the 236 genes of class 3. For most of these genes, expression is restored, at least in part, when cAMP is supplemented. Also for the 290 genes of class 2, expression decreased in the *sch9*Δ mutant but in many of these cases, the lack of Sch9 compromised further repression by cAMP-activated PKA. Most interestingly, the effects exerted on expression by the combined action of both Sch9 and cAMP-activated PKA were reversed for one (i.e. Sch9 in class 2) or both kinases (classes 3–5) if they were acting alone. These opposed effects are summarized in Fig. 2B and they led us to conclude that PKA and Sch9 most probably control a molecular switch that triggers opposed transcriptional effects of the genes in each of these classes.

Sch9 positively controls PDS-driven gene expression through Gis1, independently of Rim15

As mentioned above, we found the so-called STRE element to be enriched in class 2 genes. This STRE element is known to be bound by the PKA-controlled Msn2 and Msn4 transcription factors in response to a variety of stresses (Martinez-Pastor *et al*., 1996). However, it was rather unexpected that so many of the STRE-controlled genes were still found in our genome-wide expression analysis as the strains used in this study carried deletions for both transcription factors. This strongly indicated that other factors contribute to the regulation of the STRE-controlled genes. For some of these genes, additional regulation may involve the transcriptional activator Gis1 as its corresponding DNA binding element, known as post-diauxic shift (PDS) (Boorstein and Craig, 1990; Pedruzzi *et al*., 2000), was also over-represented in the same class (Table 2 and Table S2 in *Supplementary material*). The PDS element confers derepression during the diauxic shift when glucose becomes limiting and thus provides expression regulation similar to that conducted by the STRE element (Pedruzzi *et al*., 2000). Its core closely resembles the STRE-consensus sequence and therefore it is plausible that Gis1 and Msn2/4 may have partially overlapping functions dependent on the promoter context and thus that Gis1 may account for the regulation on the STRE sites in the absence of Msn2/4. Such a functional redundancy is further supported by the observation that both Gis1 and Msn2/4 are not only positively controlled (Pedruzzi *et al*., 2000) but also mediate almost the entire transcriptional response regulated by the protein kinase Rim15, with a significant overlap between the targets affected by each of the transcription factors (Cameroni *et al*., 2004). Rim15 is immediately downstream and negatively controlled by PKA (Reinders *et al*., 1998) and it defines the convergence of PKA, Sch9 and TOR signalling (Pedruzzi *et al*., 2003). Interestingly, the latter study reported that although being a negative regulator of Rim15 nuclear accumulation, Sch9 also acts independently of Rim15 as an activator of the Rim15-controlled transcriptional responses.

Based on the genome-wide expression data and as mentioned above, REDUCE calculated that Sch9 does not correlate with the STRE motif while it shows a positive correlation with the PDS motif as opposed to the negative correlations calculated for PKA (Tables 2 and 3). Therefore, it would be more than likely that the Rim15-independent role of Sch9 for transcription activation would be mediated through Gis1. To test this possibility, we monitored expression of prototype Gis1-dependent PDS-driven genes (*GRE1*, *SSA3*) and Msn2/4-dependent STRE-driven genes (*DDR2*, *HSP12*). These genes are all repressed in wild-type cells exponentially growing on glucose-containing medium and become induced or derepressed when the cells are shifted to glycerol-containing medium, a condition that mimics the diauxic shift (Fig. 4A). In agreement with the data previously reported, derepression of the PDS-controlled *SSA3* gene was comparable to wild-type cells in the *msn2Δ msn4Δ* strain (Martinez-Pastor *et al*., 1996), significantly reduced in the single *rim15Δ* strain and completely absent in strains bearing a deletion of *GIS1* (Pedruzzi *et al*., 2000). Similar effects were observed for the PDS-controlled *GRE1* gene (Garay-Arroyo and Covarrubias, 1999) although expression levels were lower in the *msn2Δ msn4Δ* strain than in the wild-type strain, indicating that Msn2/4 might also

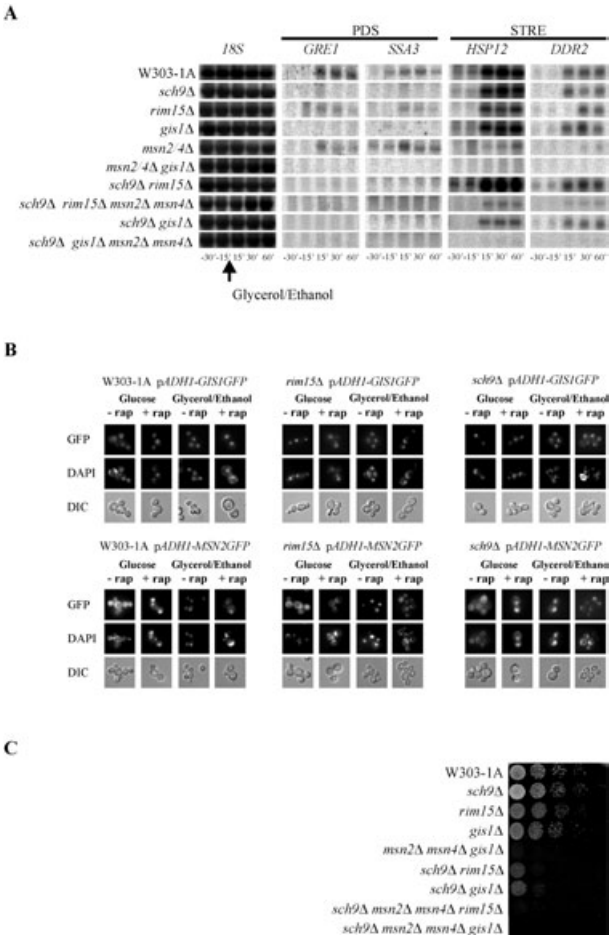

Fig. 4. Sch9 positively regulates PDS-driven gene expression.
A. Northern blot analysis of typical PDS (*GRE1*, *SSA3*) and STRE (*HSP12*, *DDR2*) genes upon shift from glucose- to glycerol-containing rich medium in various mutants. *18S* is used as a reference. Samples were taken at the indicated time points.
B. Nucleocytoplasmic localization of Gis1–GFP and Msn2–GFP is not affected by Rim15 or Sch9. Localization was visualized either with or without addition of 1 μg ml^{-1} DAPI in the absence or presence of 100 nM rapamycin. Images represent at least three independent observations.
C. Growth on glycerol-containing medium of mutants affected in the activities of Sch9, Rim15, Msn2/4 and/or Gis1.

regulate PDS-driven transcription. As predicted based on the REDUCE calculations, the strains with a deletion of *SCH9* completely failed to derepress *SSA3* and *GRE1* after the carbon source shift, indicating that Sch9 is essential to maintain Gis1-mediated transcription of the PDS-driven genes. Noteworthy, this effect was stronger than that triggered by the lack of Rim15 which confirms that the requirement of Sch9 for derepression of the PDS-driven genes cannot solely be explained based on its reported control of the nucleocytoplasmic distribution of Rim15 but that additional Rim15-independent mechanisms have to be involved. Also in line with the REDUCE calculations is that neither Gis1 nor Sch9 appeared to be essential for the derepression of the STRE-driven genes *HSP12* and *DDR2* as there was no significant effect in the single *gis1Δ* or *sch9Δ* mutants as compared with the wild-type strain. Nonetheless, the residual expression of *HSP12* and *DDR2* observed in the *msn2Δ msn4Δ* strain was completely absent in the *msn2Δ msn4Δ gis1Δ* strain suggesting that Gis1, Msn2 and Msn4 cooperatively regulate STRE/PDS-driven gene expression. This cooperative effect of Gis1 was further confirmed by comparison of the expression levels of *HSP12* and *DDR2* between the *sch9Δ rim15Δ msn2/4Δ* and the *sch9Δ gis1Δ msn2/4Δ* quadruple mutants where the expression was again completely annihilated in the latter strain (Fig. 4A). Also for Sch9, its role in the expression regulation of the STRE-driven genes becomes more apparent when multiple deletion strains are taken into account but, consistent with the data obtained from the genome-wide expression analysis, this role seems to be dual. Indeed, the expression levels of *HSP12* and *DDR2* were significantly lower in the *sch9Δ gis1Δ* mutant when compared with the single *gis1Δ* mutant, indicating that Sch9 may exert a positive function, whereas these expression levels were dramatically induced in the *sch9Δ rim15Δ* mutant when compared with the single *rim15Δ* mutant, which would be consistent with Sch9 acting as negative regulator. Note that the strongly increased expression of *HSP12* and *DDR2* in the *sch9Δ rim15Δ* strain is predominantly accounted for by Msn2 and Msn4 as only a low expression was observed in the *sch9Δ rim15Δ msn2/4Δ* strain (Fig. 4A). Thus, while our data indicate that Sch9 controls the expression of the PDS-driven genes independently of Rim15, they suggest that Sch9 may function as a positive or negative regulator for the expression of STRE-driven genes dependent on the presence or absence of Rim15.

To elaborate on the roles of Rim15 and Sch9 on STRE/PDS-driven expression, we examined whether these kinase would influence the cellular distribution of Gis1 or Msn2/4. Therefore, strains were transformed with green fluorescence protein (GFP)-tagged versions of these transcription factors and the transformants were tested under conditions known to influence nuclear accumulation of Msn2/4, i.e. glucose exhaustion and rapamycin addition to glucose-grown cells (Fig. 4B). The data showed that neither the deletion of *SCH9* nor that of *RIM15* has any effect on the nucleocytoplasmic translocalization of Msn2/4. For Gis1, our results showed that this transcription factor is always nuclear localized independent of whether or not Sch9 or Rim15 is present (Fig. 4B). Therefore, the requirements of Sch9 or Rim15 to regulate Gis1- and Msn2/4-mediated transcription should either be direct through changes in phosphorylation of these factors or indirect via alterations in global transcription complexes. Concerning the latter, our genome-wide expression analysis identified *CAF4*, *NOT5*, *ADA2*, *SPT7* and *TRA1* as targets of Sch9 and cAMP-activated PKA.

Taken together, our Northern blot analyses provide an explanation for our initial observation that PKA and Sch9 oppositely control PDS-driven gene expression (class 2 in Fig. 2A; Table 3) as they point to an independent positive regulatory effect of Sch9 and Rim15 to sustain proper Gis1 activity. They also corroborate the apparent PKA-dependent conversion of the function of Sch9 from a positive to a negative regulator as concluded from the genome-wide expression analysis (class 2 in Fig. 2A) and demonstrate the importance of Rim15 for this phenomenon. Consequently, this conversion may explain the lack of a strict correlation between Sch9 and the control of STRE-driven genes as calculated by REDUCE (Table 2). Finally, the Northern blot analyses substantiated the redundancy between Gis1 and Msn2/4, which is further supported by the fact that the triple *msn2Δ msn4Δ gis1Δ* mutant as well as the quadruple mutants *sch9Δ msn2Δ msn4Δ rim15Δ* and *sch9Δ msn2Δ msn4Δ gis1Δ* displayed a pronounced synthetic growth defect on rich glycerol-containing medium while the double mutants *msn2Δ msn4Δ*, *sch9Δ rim15Δ* and *sch9Δ gis1Δ* displayed no or only a partial growth phenotype (Fig. 4C).

Discussion

In this study, the relationship between the AGC protein kinases PKA and Sch9 in *S. cerevisiae* was investigated. Although it was previously suggested that Sch9 might act as an upstream cAMP-independent nutritional regulator of PKA (Crauwels *et al.*, 1997), our data indicate that both kinases control separate but partially redundant signal transduction pathways: (i) Sch9 controls growth regulatory functions independently of PKA and its downstream effectors Msn2/4, (ii) cAMP-activated PKA and Sch9 are separate and distinguishable requirements for glucose-induced trehalase activation and (iii) PKA and Sch9 appear to have a limited number of specific targets genes and both kinases trigger synergistic as well as opposed effects on the expression of a larger group of common target genes.

Sch9 and Rim15 control a molecular switch

When we first described the involvement of Sch9 in nutrient signalling, we demonstrated that cells lacking Sch9 showed phenotypic characteristics during growth on a non-fermentable carbon source that are usually associated with higher PKA activity while they could not maintain these high-PKA phenotypes during fermentative growth (Crauwels *et al*., 1997). Given that growth of wild-type cells on a non-fermentable carbon source is usually described as to correspond with low PKA activity and fermentative growth of wild-type cells with high PKA activity, one may conclude that deletion of *SCH9* triggers a switch that seems to reverse the correlation between PKA and the available carbon source. This switch is consistently reflected in our microarray data where indeed the combined action of both Sch9 and PKA (comparison 1a versus 1b in Fig. 2A) often triggers opposite transcriptional responses when compared with the responses triggered by either PKA (comparison 2a versus 2b in Fig. 2A) or Sch9 (comparison 1a versus 2a in Fig. 2A). More recently, we showed that PKA- and Sch9-dependent signalling converges on the protein kinase Rim15 and we demonstrated that the role of Sch9 was to prevent nuclear accumulation of Rim15 as to keep the kinase in the cytoplasm where it can be inactivated by PKA phosphorylation. We then also noticed that besides its role as a negative regulator of Rim15 nuclear import, Sch9 exerted additional control on Rim15 responses independently of Rim15 (Pedruzzi *et al*., 2003). The data presented in this article confirm this and point to the involvement of the Rim15-effector Gis1 as we found Sch9 to be absolutely required for Gis1-dependent transcription of PDS-driven genes. On the other hand, Sch9 appeared not to be essential for derepression of STRE-driven genes, which is largely accounted for by Msn2/4. Nonetheless, as mentioned above, comparison of the single *gis1Δ* deletion strain with the *sch9Δ gis1Δ* indicated a positive control of Sch9 on STRE transcription while the comparison of the single *rim15Δ* with the *sch9Δ rim15Δ* strain suggested a negative control of Sch9 on the same genes. Thus, dependent on the presence or absence of Rim15, Sch9 appears to switch from a positive to a negative regulator of STRE-driven gene expression. This altered effect of Sch9 on STRE-driven expression, however, may involve both Msn2/4 and Gis1 and it can be direct or indirect but so far we can only guess about the underlying mechanisms. One explanation would be that Sch9 and/or Rim15 regulate the interaction between Msn2/4 or Gis1 with global transcription complexes as to sustain proper STRE- and PDS-driven transcription. This may, indeed, require not only changes of Msn2/4 or Gis1 but additionally or alternatively also adaptations in the global transcription complexes as to make them compatible for interaction with Msn2/4 or Gis1. Although we did not study the mode-of-action of Sch9 and Rim15 in detail, several observations support the involvement of the latter mechanism. First, Sch9 and Rim15 do not alter the subcellular localization of Msn2 or Gis1. Second, we identified several genes encoding subunits of diverse global transcription complexes to be targets of Sch9 and cAMP-activated PKA. Third, a mode-of-action on general transcription complexes would be in line with previously reported data showing that a subunit of Ccr4-Not, i.e. Not5, a subunit shared between TFIID and SAGA, i.e. Taf25, as well as components of the histone deacetylase (HDAC) complex, i.e. Sin3 and Rpd3, have all been described as possible effectors of Rim15 (Kirchner *et al*., 2001; Lenssen *et al*., 2002; Pnueli *et al*., 2004). Thus, the combined action of Rim15 and Sch9 on the regulation of PDS/STRE-driven gene expression might be far more complicated than the simple activation or inactivation of Msn2/4 and Gis1.

Gis1 and Msn2/4 have partially redundant functions

Compelling evidence suggests that the Gis1 and Msn2/4 transcriptional activators cooperatively regulate STRE/PDS-driven gene expression. We noticed before that Msn2/4 and Gis1 largely control the Rim15 regulon upon nutrient limitation (Cameroni *et al*., 2004). In this study we observed a significant cAMP-mediated decrease of STRE-driven genes in strains devoid of *MSN2/4* (class 2 genes). Most of these genes harboured additional or partially overlapping PDS elements in their promoter and for two of those, i.e. *HSP12* and *DDR2*, we could demonstrate that Gis1 indeed mediated their transcription (Tables 2 and 3). Given the similarities between the STRE and PDS consensus sites, one may consider to extend the transcriptional regulation by Gis1 to genes containing only perfect STRE elements. This would not be without precedent as Gis1 has been reported to modulate the expression of *PHR1* gene and a derived reporter construct containing the STRE-consensus sequence (Jang *et al*., 1999). Conversely, Msn2/4 may as well be able to regulate PDS-driven gene expression as we observed for the *GRE1* gene, a gene that contains only PDS-consensus sequences in its promoter. However, it cannot be excluded that in both cases of cross-regulation, i.e. STRE-driven genes by Gis1 and PDS-driven genes by Msn2/4, other transcription factors and yet unidentified DNA elements may be involved. Nevertheless, the redundant function of Msn2/4 and Gis1 is further exemplified by the observation that the *msn2/4Δ gis1Δ* strain displays a synthetic lethal phenotype on glycerol-containing medium while the *msn2/4Δ* and *gis1Δ* mutants do not show an apparent growth defect under these conditions.

PKA, Sch9 and the Tor kinases as constituents of a nutritional integrator mechanism

We previously reported that rapamycin-induced inhibition of the Tor kinases and deletion of *SCH9* both triggered nuclear accumulation of Rim15 (Pedruzzi *et al*., 2003). In this article, we show that the deletion of *SCH9* or *RIM15* does not affect the nucleocytoplasmic distribution of Gis1 or the nuclear translocation of Msn2 upon glucose starvation or rapamycin supplementation. Especially the latter is interesting because it further discriminates between the mode-of-action of the Sch9 and TOR kinases, which have both been implicated in sensing of glucose and other nutrients, especially the availability of nitrogen. Indeed, in contrast to Sch9, the Tor kinases are known to promote nuclear export of Msn2 (Beck and Hall, 1999; Mayordomo *et al*., 2002). Hence, this observation not only confirms that Sch9 is operating in a parallel pathway with respect to the Tor kinases, but it further supports the idea that Sch9 controls changes in transcription of the stress-responsive genes mainly via Gis1 and Rim15 while TOR may control the expression of stress-responsive genes mainly via Msn2/4 and Rim15. This would further be consistent with data obtained on longevity showing that the increased life span of an *sch9Δ* strain can be partially suppressed by the additional deletion of *RIM15* but not by the additional deletion of *MSN2* and *MSN4* whereas the increased life span of a *cyr1Δ* mutant can be suppressed by both deletion of *RIM15* or *MSN2/4* (Fabrizio *et al*., 2001). It is also in agreement with our initial observation that the combined deletion of *MSN2* and *MSN4* suppresses only the requirement of PKA, but not the requirement of Sch9 for growth (Fig. 1A). Here it should also be noted that the additional deletion of *MSN2* and *MSN4* and activation of PKA by supplementation of extracellular cAMP do only support fermentative growth of the *pde2Δ cyr1Δ* mutant while it requires overexpression of *SCH9* to obtain growth under both fermentative and non-fermentative conditions. This Sch9-dependent phenotype appears to be independent of the activity of PKA or the presence of Msn2/4. As in the absence of Msn2/4, Gis1 becomes essential for growth on non-fermentable carbon sources, perhaps it requires overexpression of Sch9 to obtain enough Gis1 to force transcription of otherwise Msn2/4-controlled genes. Expression profiles from our microarray analysis that would fit best this description are those found in class 2, clusters 5 and 6. These clusters contain indeed STRE-driven genes that show dramatically reduced expression in the *pde2Δ cyr1Δ msn2Δ msn4Δ* mutant under conditions of cAMP-activated PKA as well as deletion of *SCH9* but highly enhanced expression upon overexpression of Sch9. Based on their functional classification, these genes encode proteins with roles in a variety of processes or with yet unknown functions (see http://www.kuleuven.ac.be/bio/mcb/allratio). Of particular interest are *UBC1*, *MSW1* and *SDS22* of which the null mutants display pronounced growth defects.

Given the partial redundancy between Gis1 and Msn2/4 (this study and Cameroni *et al*., 2004), and their differential regulation by the Sch9 pathway and the TOR pathway, one may look at the Sch9 pathway and the TOR pathway as counterbalancing systems that are part of a nutritional integrator allowing to fine-tune transcription of stress-responsive genes. This led us to the model presented in Fig. 5. In this model, a central position is hold by PKA, which inactivates the cytoplasmic localized Rim15, i.e. the common target of Sch9 and TOR signalling (Pedruzzi *et al*., 2000; 2003). As the activity of PKA is dramatically increased at the beginning of fermentation via a glucose-induced boost of cAMP (Thevelein *et al*., 2000) and decreased at the end of fermentation resulting from enhanced expression of its regulatory subunit, Bcy1 (Werner-Washburne *et al*., 1993), it is feasible to assume that the PKA-Rim15 module is providing contextual information regarding the presence of a fermentable carbon source. As such, this system resembles a phenomenon recognized in higher eukaryotes and described as cAMP-gating (Iyengar, 1996; Jordan and Iyengar, 1998) where a main gatekeeper, the protein kinase PKA, enhances, blocks or redirects signal flow through primary signal transduction cascades. In yeast, the phenomenon of

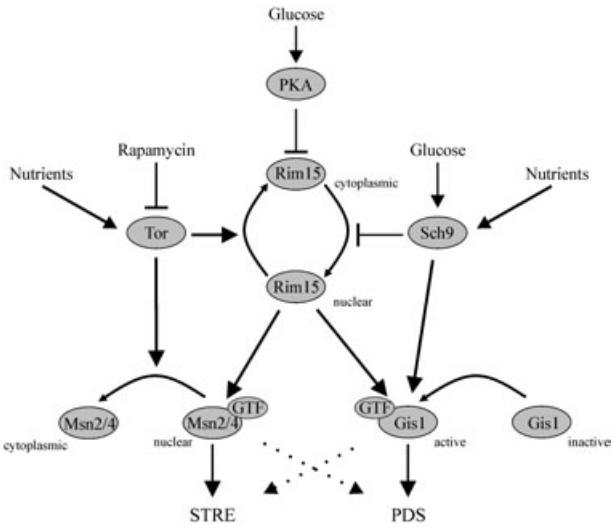

Fig. 5. Sch9 and TOR signalling are subject to cAMP-gating in yeast. Model for cAMP-gating effect in yeast consisting of a main gatekeeper, the protein kinase PKA, switching on or off the activities and signals transmitted through primary pathways such as Sch9 and TOR. Sch9 positively controls PDS-driven gene expression mainly via Gis1 and Rim15. TOR and PKA control STRE-driven gene expression mainly via Msn2/4 and Rim15. GTF stands for general transcription complex. Note that the effect of PKA on Msn2/4 localization is not depicted here. Arrows and bars refer to positive and negative interactions. Dashed lines refer to potential cross-regulation. See text for details.

cAMP-gating is supported by the data described in this article showing that transcriptional responses mediated by Sch9 are in many cases counteracted or reversed when PKA became activated by cAMP. Also for TOR there is evidence for cAMP-gating as constitutive activation of the RAS-cAMP signalling pathway confers resistance to rapamycin and prevents, similar to deletion of *RIM15*, several rapamycin-induced responses (Pedruzzi *et al*., 2003; Schmelzle *et al*., 2004).

Similar principles of nutritional integration may as well apply to the formation of pseudohyphae and the invasive growth in reaction to nutrient limitation, most notably nitrogen limitation. Pseudohyphal and invasive growth is believed to facilitate foraging for nutrients under adverse conditions. The control of this transition involves several signal transduction pathways, including the Ras/cAMP pathway and a MAPK pathway that shares components of the pheromone pathway. Consistent with their reported roles in nitrogen sensing, also the TOR and Sch9 pathway appear to be involved in the control of this morphological switch (Lorenz *et al*., 2000; Cutler *et al*., 2001). TOR plays a positive role as its inhibition after rapamycin treatment was found to prevent pseudohyphal and invasive growth of diploid cells. The role of Sch9, on the other hand, is more ambiguous as it was reported only to be a minor player in the transition of diploids while it acts as an inhibitor of invasive growth of haploid cells. The latter is consistently reflected in our genome-wide expression analysis where Sch9 was found to repress the expression of several key components required to make the transition to invasive growth (see genes of class 3). Although the function of the Sch9 kinase in haploids appears thus to be the opposite of that of the Tor kinases in diploids, in both cases the changes were associated with the inhibition or deletion of these kinases corrected by increased PKA activity (this study; Cutler *et al*., 2001; Gancedo, 2001). It should be mentioned that our microarray results clearly demonstrated that deletion of *SCH9* results in a dramatic increase of several *STE* genes under conditions of low PKA activity, a phenomenon that is compensated by cAMP activation of PKA. For *STE12*, for instance, it has been shown that cells cannot tolerate high levels of expression (Dolan and Fields, 1990). Therefore, the observed dramatic enhanced transcription and deregulation of other pheromone pathway components upon deletion of *SCH9* in the *pde2Δ cyr1Δ msn2Δ msn4Δ* mutant may provide another explanation why the lack of Sch9 in combination with low PKA may cause growth arrest.

Conclusion

To summarize, this article provides further evidence that PKA and Sch9 function in parallel signalling pathways that converge on the protein kinase Rim15 and its downstream effectors Msn2/4 and Gis1. In addition to the previously reported role of Sch9 as negative regulator of nuclear import of Rim15, our data demonstrate that Sch9 positively regulates Gis1-dependent PDS-driven gene expression independently of Rim15. Furthermore, the data presented confirm the notion that Msn2/4 and Gis1 cooperatively regulate STRE/PDS-driven gene expression. The knowledge that TOR-signalling controls nuclear export of Rim15 as well as of Msn2/4 led us to postulate the existence of a nutritional integrator system to fine-tune the expression of stress-responsive genes. This system is controlled by the two counterbalancing pathways, the Sch9 pathway on one hand, and the TOR pathway on the other, and with PKA and Rim15 as central core. Also other phenotypic read-outs appear to be regulated by the concerted action of Sch9, PKA and the Tor kinases and thus they may be regulated by a similar principle of nutritional integration.

Experimental procedures

Strains, plasmids and growth media

Yeast strains are listed in Table 4 (Thomas and Rothstein, 1989; Martinez-Pastor *et al*., 1996; Smith *et al*., 1998). Deletions were made using either plasmid-derived or polymerase chain reaction-derived disruption cassettes as described previously (Brachmann *et al*., 1998). The plasmid YIp*SCH9* expresses *SCH9* under control of the strong *TPI* promoter while YCplac111/*SCH9* expresses *SCH9* from its own promoter. Plasmids YCp*ADH1-GIS1* (Pedruzzi *et al*., 2000), YCpADH1-*RIM15* (Reinders *et al*., 1998) and pADH1-*MSN2GFP* (Gorner *et al*., 1998) were previously described. Plasmids expressing *BCY1* (181pBGHB) and *TPK1* (33pAGT) have been described (Griffioen *et al*., 2000). GFP-tagged version of Gis1 was expressed under the control of the *ADH1* promoter in a low-copy-number plasmid pNP305. For β-galactosidase assays, plasmid pJS205XXB (kindly provided by J. Schüller) (Myers *et al*., 1986) containing a truncated minimal *CYC1* promoter in front of LacZ was used. Primers N1a (TCGAGGCTAGCTGAAAAAA), N1b(GATCT TTTTTCAGCTAGCC), N3a (TCGAGGCTAGCTAAGGA), N3b (GATCTCCTTAGCTAGCC), N4a (TCGAGGCTAGC AAACGA) and N4b (GATCTCGTTTGCTAGCC) were heated and annealed by slow cooling to generate the double-stranded oligos N1, N3 and N4 that contain the DNA motifs corresponding to RRPE, U4 and PDS (underlined), respectively, flanked by *Xho*I and *Bam*HI overhangs and a unique *Nhe*I restriction site. After 5′-phosphorylation, these double-stranded oligos (T4 polynucleotide kinase) were ligated (T4 DNA ligase) into the *Xho*I/*Bam*HI-cut vector pJS205XXB generating plasmids pJS205XN1B, pJS205XN3B and pJS205XN4B respectively. Constructs were checked by *Nhe*I restriction and DNA sequencing for proper oligo-incorporation. Yeast cells were grown at 30°C in rich medium YP (yeast extract-peptone) supplemented with either 2% galactose (YPGal), 2% glycerol/2% ethanol (YPGE) or 2% glucose (YPD, ScD) as described (Sherman *et al*., 1986). Cyclic AMP was added in excess at a concentration of 3 mM

Table 4. Strains used in this study.

Strain	Genotype	Reference
W303-1A	*MATa ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1*	Thomas and Rothstein (1989)
Wmsn2msn4	*MATa* W303 with *msn2-3::HIS3 msn4-1::TRP1*	Martinez-Pastor *et al.* (1996)
CDV115	*MATa* W303 with *rim15::KanMX2*	Pedruzzi *et al.* (2003)
CDV122	*MATa* W303 with *gis1::KanMX2*	Pedruzzi *et al.* (2003)
CDV124	*MATa* Wmsn2msn4 with *gis1::KanMX2*	Pedruzzi *et al.* (2003)
TVH301	*MATa* W303 with *sch9::TRP1*	This study
BD7	*MATa* W303 with *sch9::TRP1*	This study
BD2	*MATa* Wmsn2msn4 with *rim15::KanMX2*	This study
BD3	*MATa* seggregant of Wmsn2msn4 and BD7	This study
BD9	*MATa* seggregant of BD2 and BD3	This study
BD13	*MATa* CDV124 with *sch9::LEU2*	This study
GG103	*MATa* W303 with *pde2::TRP1 cdc35::KanMX2*	This study
GG104	*MATa* GG103 with *msn2::HIS3 msn4::TRP1*	This study
ASY62	*MATa tpk1::ADE8 tpk2::HIS3 tpk3::TRP1 msn2::HIS3 msn4::LEU2*	Smith *et al.* (1998)
RJ_100A	*MATa* GG104 with *sch9::URA3*	This study
RJ_100B	*MATa* GG104 with *sch9::LEU2*	This study
RJ_101	*MATa* GG103 with YIp*SCH9*	This study
RJ_102	*MATa* GG104 with YIp*SCH9*	This study
RJ_201	*MATa* CDV115 with *sch9::LEU2*	Pedruzzi *et al.* (2003)
RJ_202	*MATa* CDV122 with *sch9::LEU2*	This study

on agar plates or at a concentration of 0.5 mM in liquid medium. Transformation of strains was performed as described previously (Gietz *et al.*, 1995).

Viability tests, growth assay

To test the viability after cAMP starvation, cells of the strains *pde2Δ cyr1Δ msn2/4Δ* (GG104) and *pde2Δ cyr1Δ msn2/4Δ sch9Δ* (RJ_100 A) were starved for cAMP for 0, 6 and 24 h. Dilution series (starting OD_{600} at 0.2) were spotted on YPD plates with or without cAMP and incubated at 30°C for 2 days and the numbers of colonies were compared. For the other growth assays, strains were grown overnight in 5 ml of preculture YPD, OD_{600} was measured and 10 μl of a 10-fold 1/10 000 dilution series with starting OD_{600} of 0.2 was plated out and incubated for 2 days at 30°C.

Genome-wide gene expression analysis

Experiment and sample taking were performed twice for two independent colonies. Strains were grown to mid-exponential phase (OD 0.5) in 100 ml of YPGal with 0.5 mM cAMP. Subsequently, the cells were washed twice with YPGal, resuspended and allowed to grow further in 100 ml of YPGal. After 6 h, the culture was divided into two equal fractions (2×50 ml) and 3 mM cAMP was added to one fraction. Both fractions were then grown for another hour. RNA was extracted using RNApure™ (GeneHunter® Corporation, Nashville, USA, Cat No. P501) according the protocol supplied by the company and diluted to a final concentration of 1 μg μl^{-1} for cDNA preparation and labelling (1 μg of RNA; [α-^{33}P]-dCTP) using Superscript II (Invitrogen, Cat No. 18064-014). Serial hybridization on Yeast Index GeneFilters (Research Genetics/Invitrogen, Huntsville, GF100) was performed according delivered protocol (Research Genetics/Invitrogen). Images were scanned (FUJIX Bas1000) and analysed using the program PATHWAYS 3.0 (Research Genetics). Duplicate experiments were performed on new filter sets. Selected genes were manually flagged for hybridization artefacts. Gene annotations were derived from the MIPS functional categories. Analysis of the control spots, present on the Yeast Gene Filters® Microarrays, showed a pronounced multiplicative error in the data (i.e. the absolute error on measurements increases with the measured intensity). Therefore, raw intensity measurements were log-transformed. To allow for across-filter comparisons of expression values, these log-transformed measurements were mean-centred per filter. Statistical analysis of the resulting data for the control spots led us to assume the error on the log-transformed data was normally distributed with variance 0.10006 and mean 0. The fold changes of 1.5, 2 and 3, as used throughout the article for selecting genes with differential expression between two conditions, thus correspond to confidence levels of 0.90, 0.99 and 0.9995 respectively. Data are freely available at http://www.kuleuven.ac.be/bio/mcb/allratio following the MIAME recommendation. Enrichment factors for each functional category were calculated as described previously (Tavazoie *et al.*, 1999). Genes with the highest variance across their expression profile (approximately one-third of all genes) were clustered using the AQBC clustering algorithm (significance parameter was set to 0.80) as described by De Smet *et al.* (2002). This resulted in 918 genes being assigned to 14 different clusters.

The program REDUCE (Bussemaker *et al.*, 2001) was used to discover oligonucleotide motifs whose occurrence in the promoter region of a gene correlates with changes in its expression level. All oligonucleotides up to length 7 were tested and matches were counted in a window of 600 nt upstream of each ORF (a truncated window was used whenever necessary to avoid overlap with upstream ORFs on either strand). Putative motifs were screened against the Transfac database (Heinemeyer *et al.*, 1998). Using a *P*-value cut-off of 0.01, their PKA and/or Sch9 dependency was evaluated for each of the pair-wise comparisons based on the *F*-scores for each element. The most significant motifs (Table 2) were tested for their abundance in each gene cluster (Fig. 3A) using regulatory sequence analysis (RSA) tools

(van Helden *et al*., 2000). Distribution of these elements for each class relative to their genome-wide distribution was calculated according the algorithm described previously (Tavazoie *et al*., 1999). Motifs were deemed to be statistically over-represented when the minus log2(*P*-value) exceeded 1.

To detect statistically over-represented motifs in the sets of coexpressed genes (clusters), we used Motif Sampler, a motif detection algorithm based on Gibbs sampling (Thijs *et al*., 2002). Yeast intergenic sequences were retrieved from RSA tools (van Helden *et al*., 2000). By using multiple runs of the algorithm and testing different parameter settings, motifs with a high consensus score, a high number of occurrences in the set of coexpressed genes and those retrieved consistently, were retained. These putative motifs were screened against the Transfac database (Heinemeyer *et al*., 1998) and only motifs for which a description was available in Transfac were further investigated.

Northern blot analysis

For microarray confirmation, strains were grown in the same way. Hybridization was performed using cDNA probes of randomly chosen genes of different clusters. For diauxic shift experiment, strains were grown on YPD medium. At mid-exponential phase ($OD_{600} = 0.5$), samples were taken at 30 min and 15 min before the cells were collected and washed in YPGE medium and resuspended in YPGE. Then, samples were taken after 15 min, 30 min and 60 min incubation in YPGE medium. RNA extraction and hybridization was performed using the protocol described previously (Crauwels *et al*., 1997). Hybridized filters were exposed to phosphorimager screens and images were scanned with FUJIX Bas1000. Quantification of Northern blot images was performed with TINA 2.0.

Trehalase enzyme assays

Strains GG104, RJ_100 A and RJ_102 were grown to exponential phase on YPGal in the presence of 0.5 mM cAMP. Cells were collected by centrifugation, washed twice with YPGal and resuspended in fresh YPGal supplemented with or without 0.5 mM cAMP. Other strains were grown on YPGal. Glucose (100 mM) was added after a 20 min pre-incubation period at 30°C. Trehalase activity (nmol glucose per minute per mg protein) in crude cell extracts was determined as described previously (Mitsuzawa, 1993). Average values over at least three independent measurements are shown with an average standard deviation of 2.19.

Galactosidase assays

Exponential growing cells were collected by centrifugation and resuspended in ice-cold breaking buffer containing 1/10 solution of dissolved proteinase inhibitor cocktail tablets (Complete Mini, EDTA free, Roche Diagnostics). Cells were broken using glass beads. Clarified protein extract (10 μl) was diluted to a total volume of 0.5 ml of breaking buffer and incubated at 30°C for 5 min and the reaction was initiated by adding 0.1 ml of 4 mg ml^{-1} ONPG (o-Nitrophenylgalactoside). At precise time the reaction was terminated by addition of 0.25 ml of 1 M Na_2CO_3. OD_{420} was measured (BioPhotometer, Eppendorf) and protein concentration was determined according the Bradford method. Specific activity was calculated by the formula $(OD_{420} \times 1.7)/(0.0045 \times \text{protein conc.} \times \text{extract volume} \times \text{time})$. The specific background activity (empty vector) was subtracted from the specific activity of the sample (reporter vector) giving the samples actual specific activity (nmoles per minute per mg protein). Experiments were performed in triplicate for independent colonies. Error estimation was calculated as the square root of [(sum of squares of standard deviations)/number of measurements].

Green fluorescence protein fluorescence microscopy

Cells expressing GFP-tagged Msn2 or Gis1 were used directly without fixation. Nuclei were stained by addition of 1 μg of DAPI (4′,6′-diamidino-2-phenylindole) per ml cell culture. Cells were viewed using a Zeiss Axioplan 2 fluorescence microscope. Images were taken with an AxioCam HRm charge-coupled device camera using AxionVision 3.0 software.

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4429/mmi4429sm.htm

Fig. S1. Cyclic AMP dependency for growth.
Fig. S2. Northern blot analysis of randomly selected genes confirmed the genome-wide expression profiles.
Table S1. The strain *pde2Δ cyr1Δ msn2/4Δ* exhibits a normal transcriptional cAMP response.
Table S2. Expected frequency of occurrence.

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