Gis1 is required for transcriptional reprogramming of carbon metabolism and the stress response during transition into stationary phase in yeast

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Transition from growth to the stationary phase in yeast is still poorly understood. Previously, we identified a group of yeast genes that are universally upregulated upon starvation for different macronutrients. Here, we demonstrate that the Gis1 transcription factor and the Rim15 kinase are responsible for the upregulation of many of these genes. In chemostat cultures, *gis1* or *rim15* mutant cells are outcompeted by their wild-type parents under conditions resembling the later stages of diauxie (glucose-limiting) and post-diauxie (ethanol as a carbon source). Whilst Gis1p and Rim15p have distinct functions in gene repression, the growth defects of *gis1* or *rim15* deletants can be accounted for by the overlapping functions of their protein products in promoting the expression of genes involved in glutamate biosynthesis, the glyoxylate cycle, the pentose phosphate pathway and the stress response. Further, we show that the sets of *GIS1*- and *RIM15*-dependent genes and the degree of their regulation change in response to the identity of the carbon source, suggesting the likely dynamics of gene regulation exerted by Rim15p and Gis1p during different phases of the transition into stationary phase. In particular, Rim15p is required for the expression of genes involved in gluconeogenesis/glycolysis and glycerol biosynthesis only when ethanol is used as the carbon source. In agreement with this, Rim15p is shown to act in parallel with Hog1p to defend cells against osmotic stress.

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INTRODUCTION

Cell growth is modulated in response to the availability of nutrients and growth factors. When grown in a glucose-based batch culture, yeast cells consume glucose and proliferate rapidly by fermentation, producing ethanol. As glucose becomes limiting, cells enter the diauxic shift and reprogramme their metabolism from fermentation to respiration. During the post-diauxic phase, the fermentation product, ethanol, is used as the carbon source. Eventually, all of the carbon sources are exhausted and yeast cells cease mitotic division and arrest at the G0 phase of the cell cycle. The key feature of entering the diauxic shift is that glucose repression is released and cells express a large set of genes involved in the utilization of alternative carbon sources, gluconeogenesis, the tricarboxylic acid (TCA) cycle, respiration and peroxisomal functions (Johnston & Carlson, 1992). In order to survive an extended period of starvation, during the transition phase,

cells also acquire a set of characteristics typical of the stationary phase, including accumulation of storage carbohydrates (glycogen and trehalose), increased resistance to a variety of environmental stresses, and thickening of the cell wall (Herman, 2002).

Transcriptional reprogramming during the diauxic and post-diauxic phases involves at least one-quarter of the yeast genome (DeRisi *et al.*, 1997) and is controlled by the Snf1 kinase pathway and a myriad of transcription factors, including the general glucose repressor Mig1p, the Hap2/3/4/5 complex, the Rtg3–Rtg1 complex, Adr1p, Oaf1p/Pip2p and Cat8p/Sip4p (Schuller, 2003). Two other conserved signalling pathways, Ras/cAMP and TOR, have also been implicated in the regulation of growth and entry into stationary phase in response to nutrient deprivation (Inoki *et al.*, 2005; Thevelein & de Winder, 1999; Wilson & Roach, 2002; Wullschleger *et al.*, 2006). Both PKA and TOR have been shown to regulate entry into the G0 phase of the cell cycle, converging on common targets, the Rim15 kinase and its downstream effectors, Msn2/4 and Gis1 (Swinnen *et al.*, 2006). As a member of the PAS family, Rim15p integrates signals from TOR through the Sch9 kinase (Pedruzzi *et al.*, 2003; Urban *et al.*, 2007; Wanke *et al.*,

Abbreviations: GO, gene ontology; PDS, post-diauxic shift; UES, universally expressed at starvation.

The ArrayExpress accession number for the the microarray data generated in this study is E-MAXP-1139.

2008), PKA (Pedruzzi *et al.*, 2000) and the Pho80p–Pho85p (Wanke *et al.*, 2005) signalling pathways. It functions as a key controller of many aspects of the stationary-phase programme – including cell-cycle arrest, storage-carbohydrate accumulation and activation of Gis1p- and Msn2/4p-dependent transcription (Swinnen *et al.*, 2006).

The *GIS1* gene was originally isolated as a multicopy suppressor of the Gal[−] phenotype of an *snf1/mig1/srb8* triple mutant (Balciunas & Ronne, 1999). It has since been shown to function as both a transcription repressor (Jang *et al.*, 1999; Oshiro *et al.*, 2003) and a transcription activator (Pedruzzi *et al.*, 2000). Both transcriptional repression and activation by Gis1p seem to be mediated largely via the PDS (post-diauxic shift) element present in the promoters of its target genes. By comparing the transcriptome of exponentially growing cells with that from cells exhausted for glucose in batch cultures, Cameroni *et al.* (2004) showed that the entire set of genes that require Rim15p for induction during the diauxic shift is included within the combined set of Gis1p- and Msn2/4p-dependent genes, suggesting that the functions of Gis1p and Msn2/4p are controlled by the Rim15 kinase. However, it remains to be found what set of genes is regulated by Gis1p and/or Rim15p in the later stages of the transition, and how they change from the diauxic (growth on limited glucose) to the post-diauxic (growth on ethanol) phase. It is also interesting to determine whether the repression functions of Gis1p are also controlled by Rim15p.

In a previous study, using chemostat cultures limited for different macronutrients, we identified 17 yeast genes (*SSA3*, *AUT7*, *HXT5*, *SML1*, *TBF1*, *MOH1*, *UGX2*, *YDL218w*, *YGR201c*, *SPG1*, *YHR097c*, *YIL055c*, *YIL057c*, *YJL144w*, *SPG4*, *UIP4* and *GRE1*) whose transcription is activated significantly (>5-fold) by starvation for any of the macronutrients (Wu *et al.*, 2004). We named them UES genes, for universally expressed at starvation. Here, we demonstrate that the Gis1 transcription factor and the Rim15 kinase are responsible for activation of many of the UES genes from nutrient limitation to nutrient starvation. Gis1p, as well as Rim15p, is also required for optimal cell growth in glucose- or ethanol-limited chemostat cultures, which resemble the later stages of diauxie and post-diauxie, respectively. These phenotypes can be accounted for by the overlapping functions of Gis1p and Rim15p in regulating expression of genes involved in glutamate biosynthesis, the glyoxylate cycle, the pentose phosphate pathway and the stress response.

The groups of *GIS1*- or *RIM15*-dependent genes under the two carbon-limited conditions, although largely overlapping, change in accordance with the identity of the carbon source. In particular, we demonstrate that Rim15p regulates the expression of genes involved in glycolysis/gluconeogenesis and glycerol biosynthesis upon increased respiratory demand (ethanol as a carbon source) and that Rim15p acts in parallel with Hog1p in defending cells against hyperosmolarity. These data indicate the likely dynamics of the gene regulation exerted by Gis1p and

Rim15p during the transition into the stationary phase. Furthermore, a set of stress-response genes that are upregulated in *gis1* deletants are either downregulated or unchanged in *rim15* deletion mutants, suggesting that the repression function of Gis1p is independent of the control exerted by Rim15p.

METHODS

Strains. Yeast deletion strains were based on BY4743 (*MATα/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0*) and generated by the *Saccharomyces* Genome Deletion Project (Winzler *et al.*, 1999). The *hog1Δrim15Δ* double mutant was generated by crossing single deletion mutants of the opposite mating types (BY4741 and BY4742) and sporulation of the resulting diploid. The genotype of the desired spore carrying both deletions is the same as that of BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). YPD, YP, SD and SMM (supplemented minimal medium) media (Amberg *et al.*, 2005) were used to grow yeast cells at 30 or 37 °C, as indicated. A stock solution (1 mg ml^{−1}) of rapamycin (Sigma) was made up in 90 % (v/v) ethanol and 10 % (v/v) Tween 20, and used at a final concentration of 200 ng ml^{−1}.

Chemostat cultures and competition experiment. Chemostat cultures were established at a dilution rate (*D*) of 0.1 h^{−1} in 2 l fermenters (Applikon Biotechnology) with both temperature (30 °C) and pH (4.5) controlled. Dissolved oxygen was maintained at the maximal concentration. Glucose-, ammonium- and ethanol-limiting media were formulated, and competition experiments were performed, as reported previously (Baganz *et al.*, 1998). Competition between the wild-type and mutant cells was carried out in chemostat cultures. Briefly, each strain was grown at steady state (*D*=0.1 h^{−1}) in separate fermenters under carbon (glucose or ethanol) limitation. Cultures of wild-type and mutant cells were then mixed in equal volumes and growth was allowed to continue at the same dilution rate. The ratio of mutant:wild-type cells was determined from the percentage of cells displaying geneticin resistance due to their possession of the *kanMX4* replacement marker.

Microarray and specific transcript analyses. Total RNA was isolated from cultures as described previously (Wu *et al.*, 2004). Individual transcripts were assayed by Northern analysis according to Brown *et al.* (2001) or RT-PCR (using a kit from ABgene Inc.). Microarray experiments were carried out by using yeast genome S98 oligonucleotide arrays (Affymetrix, Inc.) according to the manufacturer's instructions. Normalization and statistical analyses were carried out by using Partek Genomics software (<http://www.partek.com>). Microarray data were generated by using two independent samples from cells grown at steady state at *D*=0.1 h^{−1} under either glucose- or ethanol-limiting conditions. The correlation coefficients between the duplicated microarray experiments were between 0.973 and 0.996. In compliance with MIAME guidelines, the data from this study have been deposited at the ArrayExpress repository (<http://www.ebi.ac.uk/arrayexpress/>) at the European Bioinformatics Institute under accession no. E-MAXP-1139.

RESULTS

Gis1p is required for induction of UES genes in response to starvation for different nutrients

In an attempt to find possible upstream activating sequences that might help to identify the transcription

factors involved in regulating the 17 UES genes, we used regulatory sequence analysis (RSA) tools (<http://rsat.ulb.ac.be/rsat/>) to retrieve over-represented oligonucleotide and dyad sequences present in their promoters. Whilst there were no significant dyad sequences found, a few hexanucleotides with high statistical significance were found. The most significant hexanucleotide, AAGGGA, is present in the promoter regions of 14 UES genes. This hexamer resembles the previously identified PDS element, which mediates transcriptional activation in response to nutritional limitation (Boorstein & Craig, 1990). PDS element-driven expression has been shown to be strongly dependent on the Rim15 kinase and almost entirely dependent on the transcriptional activator Gis1p (Pedruzzi *et al.*, 2000).

To confirm the dependence of the expression of these UES genes on Gis1p and Rim15p, wild-type, *gis1Δ* and *rim15Δ* cells were grown in a glucose-limited chemostat to steady state. The residual glucose concentration in the glucose-limited chemostat culture is about 30 mg l⁻¹. The medium feed was then switched off and the cells were allowed to enter a glucose-starved stationary phase. As shown in Fig. 1(a), all of the transcripts analysed were strongly dependent on functional *GIS1* and *RIM15* genes, except for *SSA3*, which showed a weaker and delayed transcriptional response in both *gis1Δ* and *rim15Δ* mutants. Similar results were seen when exponentially growing cells in an N-limited chemostat were starved of ammonium (Fig. 1b). Under these conditions, abundant glucose was present for the first 12 h after switching off the medium feed (data not shown). The expression of these genes was also shown to be dependent on Gis1p or Rim15p in batch cultures treated with rapamycin (Fig. 1c). Induction of a similar set of genes upon rapamycin treatment has been shown previously (Pedruzzi *et al.*, 2003). Swinnen *et al.* (2005) confirmed that expression of these genes is also activated when phosphate is starved, and that their activation is independent of Pho4p, but relies on the Pho81 and Rim15 kinases. Thus, Rim15p and Gis1p both play a central role in regulating the transcriptional response to different nutrient-starvation signals.

***GIS1* and *RIM15* have overlapping functions in gene activation**

Epistasis tests have put the Rim15 kinase upstream of Gis1p in the pathway that activates the nutrient-starvation response (Pedruzzi *et al.*, 2000). To find out whether the Rim15 kinase controls all of the functions of the Gis1 transcription factor, we first determined whether all of the phenotypes revealed by a *gis1Δ* strain are shared by *rim15Δ* cells. The *gis1* mutant cells are temperature-sensitive when grown on rich medium (YP) with ethanol as a carbon source (Balciunas & Ronne, 1999). Grown on supplemented minimal medium, *rim15* deletants display a more severe temperature-sensitive phenotype on ethanol than do *gis1* cells (Fig. 2a). On glycerol, both mutants show similar temperature-sensitive defects, compared with the wild-

Fig. 1. Activation of UES genes is dependent on *GIS1* and *RIM15*. (a) Glucose starvation (Northern analysis). Cells were grown to steady state in glucose-limited chemostat culture. Samples were taken 0, 4, 10, 24 and 48 h after the medium feed was switched off to effect glucose starvation. (b) Ammonium starvation (RT-PCR). Cells were grown to steady state in ammonium-limited chemostat culture. Samples were taken 0, 6, 12, 18, 24 and 48 h after the medium feed was switched off to effect nitrogen starvation. (c) Rapamycin treatment (Northern analysis). Cells were grown in SD medium until mid-exponential phase (OD₆₀₀ of approx. 0.4). Samples were taken 0, 1, 2 and 4 h after adding rapamycin.

type. When grown at 30 °C, only the *rim15* mutant cells have a moderate growth defect on ethanol (Fig. 2b). No growth defects were observed for these mutants when they were grown on glucose (2.0 or 0.2%) at different temperatures (30 or 37 °C).

To delineate further the phenotypic differences shown on non-fermentable carbon sources, we conducted competition experiments at a normal growth temperature (30 °C) under glucose- or ethanol-limiting conditions (see Methods). Both *gis1Δ* and *rim15Δ* cells were outcompeted by the wild-type under either ethanol-limited (Fig. 2c) or glucose-limited (Fig. 2d) conditions, indicating that they grew more slowly than their isogenic parents. This stringent phenotypic analysis suggests that Gis1p, as well as Rim15p, is functionally required for cells to adapt to both the diauxic (limited glucose as the carbon source) and the post-diauxic (ethanol as the carbon source) phases involved in the transition to stationary phase.

To find out why the *gis1Δ* and *rim15Δ* mutants were less fit than the wild-type when grown in glucose- or ethanol-

Fig. 2. Phenotypic analysis of *gis1Δ* and *rim15Δ* mutants. (a, b) Temperature-sensitivity test. Cells were spotted, in a 7-fold dilution series, onto SMM agar plates containing different carbon sources. Plates were incubated at either 37 °C (a) or 30 °C (b) for 3–6 days. Glu, Glucose; Et, ethanol; Gly, glycerol. (c, d) Competition between the wild-type and mutant cells grown in either glucose-limited (c) or ethanol-limited (d) chemostat cultures. Samples were taken when the two strains were first mixed and at the time points indicated. Diluted cell suspensions were plated onto YPD agar and then replicated onto YPD plates containing 200 μg geneticin ml^{-1} , in order to determine the relative proportions of mutant and wild-type cells in the populations. Mean $\pm$ SD was calculated from triplicate samples. $\blacktriangle$, *gis1/gis1Δ*; $\bullet$, *rim15/rim15Δ*.

limited chemostat cultures, we went on to determine whether *GIS1* and *RIM15* regulate the same set of genes under these conditions. Hybridization-array analyses of their transcriptomes (see Methods) demonstrated that the transcript levels of 202 ORFs were significantly different ($P < 0.01$) in the *gis1Δ* and/or *rim15Δ* mutants compared with the wild-type grown under the same conditions. Of these transcripts, 123 changed their level by more than 1.5-fold (up- or downregulated). These fell into two main functional categories: ‘carbohydrate metabolism’ (22 genes, $P = 2.15 \times 10^{-11}$) and ‘response to stimulus’ (24 genes, $P = 3.64 \times 10^{-5}$).

There is a significant overlap between the sets of genes whose transcription is downregulated in the two mutants compared with the wild-type, under either glucose- or ethanol-limited conditions (Fig. 3a). For example, 23 genes are downregulated more than 1.5-fold by the *gis1Δ* deletion when cells are growing under glucose-limited conditions. The majority of them are regulated similarly in *rim15Δ* cells (Table 1). These include genes involved in the stress response (*HSP26*, *SIP18* and *GRE1*), the pentose phosphate pathway (*TKL2*, *GND2*), glutamate synthesis (*IDP2*, *GDH3*) and the glyoxylate cycle (*MLS1*, *ICL1*). Expression of the gluconeogenic genes (*FBP1*, *PCK1*) is downregulated significantly only in *gis1Δ* cells grown under

glucose-limited conditions. These pathways are essential for cells to grow by respiration, on limited glucose or on non-fermentable carbon sources such as ethanol (Schuller, 2003). This may also explain the compromised fitness of either *gis1Δ* or *rim15Δ* cells in glucose- or ethanol-limited chemostat cultures (Fig. 2c, d). Of the 23 genes downregulated in the *gis1Δ* deletant, 13 have one or more near-consensus PDS elements (WAGGGA) in their upstream regions and the pentamer ‘AGGGA’ is the most over-represented motif in their promoters (Table 1). These results indicate that the Gis1 transcription factor has important roles in the activation of gene expression under conditions where there is no carbon-catabolite repression.

Among the above set of genes, expression of the majority of them (19 out of 23) is also induced when exponentially growing cells are treated with rapamycin (Table 1). The transcript level of *GDH3*, encoding the minor form of the NADP^+ -dependent glutamate dehydrogenase, was also determined in *gis1Δ* and *rim15Δ* cells treated with rapamycin. As seen in Fig. 3(b), the induction of *GDH3* was severely reduced in the *gis1Δ* deletant and completely abolished in the *rim15Δ* mutant. *GDH3* has been shown to be repressed in cells growing by fermentation, but induced in those using respiration for growth (Avendano *et al.*, 2005). Furthermore, a few genes sensitive to nitrogen-

Fig. 3. Microarray analysis of *gis1Δ* and *rim15Δ* mutants grown in chemostat cultures. (a) Venn diagram showing the overlap between the genes downregulated in *gis1Δ* and *rim15Δ* cells growing in glucose (Glu)- or ethanol (Et)-limited chemostats. (b) Northern analysis of the *GDH3* transcript in wild-type (BY4743), *gis1Δ* and *rim15Δ* cells treated with rapamycin for 0, 1, 2 and 4 h. (c) Venn diagram showing the overlap between the genes upregulated in *gis1Δ* and *rim15Δ* cells growing in glucose (Glu)- or ethanol (Et)-limited chemostats. (d) Venn diagram showing the overlap of the downregulated genes in *gis1Δ* (left) and *rim15Δ* (right) cells.

Table 1. Genes downregulated in *gis1* deletion cells grown in glucose-limited chemostat culture

Abbreviations: BY, wild-type; *gis1*, *gis1Δ/gis1Δ*; *rim15*, *rim15Δ/rim15Δ*; G, glucose-limited; E, ethanol-limited.

ORF	Description	<i>gis1</i> / BY(G)	<i>rim15</i> / BY(G)	<i>gis1</i> / BY(E)	<i>rim15</i> / BY(E)	rap +/rap- (2 h)*	PDS/ STRE†
YAL062W	<i>GDH3</i> , NADP ⁺ -dependent glutamate dehydrogenase	-1.72	-0.92	-1.35	-0.15	3.94	2/0
YBR072W	<i>HSP26</i> , small heat-shock protein with chaperone activity	-0.61	-3.03	-0.79	-5.25	2.47	0/4
YBR117C	<i>TKL2</i> , transketolase	-1.16	-2.25	-1.64	-2.68	1.05	2/2
YDL204W	<i>RTN2</i> , protein of unknown function	-0.61	-0.55	-0.53	-0.66	0.58	1/1
YDL223C	<i>HBT1</i> , substrate of the Hub1p ubiquitin-like protein	-0.66	-0.79	-1.71	-1.89	1.08	0/2
YDR070C	<i>FMP16</i> , localized to mitochondria	-1.42	-1.80	-2.03	-2.61	1.71	1/1
YER015W	<i>FAA2</i> , long-chain fatty acyl-CoA synthetase	-0.61	0.28	0.09	0.53	0.63	0/0
YER065C	<i>ICL1</i> , isocitrate lyase	-1.20	-0.71	-0.08	-0.02	0.79	2/0
YFL030W	<i>AGX1</i> , alanine: glyoxylate aminotransferase	-0.92	-0.34	-0.32	0.06	0.82	0/1
YGL121C	<i>GPG1</i> , proposed γ subunit of the heterotrimeric G protein	-1.97	-0.83	-1.81	-1.36	2.05	2/0
YGR256W	<i>GND2</i> , 6-phosphogluconate dehydrogenase	-2.08	-1.81	-0.39	-1.01	1.99	2/2
YIL117C	<i>PRM5</i> , pheromone-regulated protein	-0.59	-0.23	0.01	0.32	0.79	0/0
YIR032C	<i>DAL3</i> , ureidoglycolate hydrolase	-0.76	-0.86	-0.60	0.37	0.63	0/1
YJR152W	<i>DAL5</i> , allantoin permease	-0.67	-0.48	-1.71	1.61	3.92	0/0
YKL163W	<i>PIR3</i> , O-glycosylated covalently bound cell-wall protein	-0.71	-0.78	0.93	-1.28	1.89	2/1
YKR097W	<i>PCK1</i> , phosphoenolpyruvate carboxykinase	-1.53	-0.43	-0.42	-0.27	-0.10	1/0
YLL057C	<i>JLP1</i> , Fe(II)-dependent sulfonate/ α -ketoglutarate dioxygenase	-0.80	0.76	-0.50	0.83	0.35	0/1
YLR174W	<i>IDP2</i> , cytosolic NADP-specific isocitrate dehydrogenase	-1.61	-0.68	-0.16	0.26	0.83	1/0
YLR377C	<i>FBP1</i> , fructose-1,6-bisphosphatase	-1.26	-0.26	-0.09	0.53	-0.08	0/2
YML131W	Hypothetical protein	-0.61	0.36	-0.47	0.33	0.09	0/1
YMR175W	<i>SIP18</i> , induced by osmotic stress	-1.77	-2.71	-4.25	-5.17	2.35	3/3
YNL117W	<i>MLS1</i> , malate synthase	-1.49	-0.86	-0.06	0.05	3.02	1/1
YPL223C	<i>GRE1</i> , stress-induced	-0.66	-1.07	-2.69	-3.67	2.22	6/0

*Comparison of transcript levels in cells treated with (+) and without (-) rapamycin (rap) for 2 h. The whole dataset was published by Castrillo *et al.* (2007).

†No. of PDS elements/no. of STRE elements.

catabolite repression, such as *DAL3* and *DAL5*, were downregulated in the *gis1* and *rim15* deletants. Put together, these data suggest that *RIM15* and *GIS1* are important regulators of the carbon metabolism pertaining to nitrogen assimilation in the diauxic and post-diauxic phases.

***GIS1* and *RIM15* have distinct functions in gene repression**

Gis1p was shown previously to act as a transcriptional repressor of *DPP1* (encoding diacylglycerol pyrophosphate phosphatase) in exponentially growing cells, and the PDS elements in the *DPP1* promoter are required for this repression (Oshiro *et al.*, 2003). As seen in Fig. 3(c), there is very little overlap between the sets of genes upregulated in the two mutants under the same carbon-limited conditions. Specifically, 28 genes are upregulated >1.5-fold in the *gis1* deletants compared with the wild-type when grown under glucose-limiting conditions. The transcription of only one of these genes is also upregulated in *rim15Δ* cells. In fact, the expression of the majority of them remains downregulated or unchanged in *rim15Δ* cells. A similar pattern for the same group of genes is also

observed when gene expression in the two mutants is compared under ethanol limitation (Table 2). These results indicate that the function of Gis1p as a transcriptional repressor is independent of that of the Rim15 kinase.

Gene ontology (GO) analysis did not reveal any significant GO term among the 28 genes upregulated in *gis1Δ* cells. Most of them encode stress-response proteins (*YCR061W*, *GPD1*, *HOR2*, *CTT1* and *DDR48*) or proteins of unknown functions (*BOP3*, *PHM8*, *PNS1*, *YCR061W*, *YGR066C*, *YHL044W*, *YHR140W*, *YJL163C*, *YMR173W-A* and *YMR181C*). Interestingly, seven genes (*PHM8*, *YGR066C*, *YGR248W*, *TPK1*, *MSN4*, *GSP2* and *FRE3*) are toxic to cell growth when overexpressed (Sopko *et al.*, 2006), suggesting that one of the functions of Gis1 as a transcription repressor is to regulate properly the expression of genes important for growth. Promoter analysis was carried out to determine whether Gis1p represses their expression directly. STRE elements (AG₄, targeted by Msn2/Msn4 transcription factors), rather than the PDS elements, are the most over-represented motifs in the promoters of these genes ($P=3.8 \times 10^{-9}$). The transcription of *MSN4* itself is also activated by the deletion of *GIS1* and there are two near-consensus PDS elements, AAGGGA (approx. -700 to -695 and -276 to -271), in the promoter of *MSN4*. This

Table 2. List of genes upregulated in *gis1* deletion cells grown in glucose- or ethanol-limited chemostats

Abbreviations: BY, wild-type; *gis1*, *gis1Δ/gis1Δ*; *rim15*, *rim15Δ/rim15Δ*; G, glucose-limited; E, ethanol-limited.

ORF	Description	<i>gis1</i> / BY(G)	<i>rim15</i> / BY(G)	<i>gis1</i> / BY(E)	<i>rim15</i> / BY(E)	STRE/ PDS*
YCR061W	Protein that may be involved in response to heat and high salinity	0.67	-0.37	0.88	-1.31	1/0
YDL022W	<i>GPD1</i> , NAD-dependent glycerol-3-phosphate dehydrogenase	0.74	-0.45	1.09	-0.85	4/4
YDL214C	<i>PRR2</i> , protein kinase potentially involved in pheromone response	1.62	-0.25	0.22	-0.16	2/2
YER037W	<i>PHM8</i> , strong similarity to hypothetical protein YGL224c	1.22	-0.97	0.84	-1.09	4/0
YER044C	<i>ERG28</i> , hypothetical protein; endoplasmic reticulum membrane protein	0.60	0.32	0.31	-0.52	0/0
YER062C	<i>HOR2</i> , DL-glycerol-3-phosphatase	0.66	-0.28	1.09	-1.03	1/0
YGR066C	Similarity to hypothetical protein YBR105c	0.92	-0.17	0.99	-0.39	1/1
YGR088W	<i>CTT1</i> , cytoplasmic catalase T	1.24	-0.50	0.45	-2.24	4/0
YGR248W	Possible 6-phosphogluconolactonase	0.60	-0.48	0.37	-1.01	1/2
YHL044W	Member of the duplication (DUP240) family	0.64	-0.12	1.05	-0.12	0/0
YHR016C	<i>YSC84</i> , protein involved in the organization of the actin cytoskeleton	0.64	0.19	0.75	-0.18	1/1
YHR140W	Protein that contains six predicted transmembrane segments	0.63	-0.08	0.19	-0.26	1/1
YIL099W	<i>SGA1</i> , intracellular glucoamylase	0.65	-0.38	1.07	-0.44	5/0
YJL163C	Protein that contains ten potential transmembrane segments	0.90	-0.26	0.59	-0.59	0/1
YJL164C	<i>TPK1</i> , subunit of cytoplasmic cAMP-dependent protein kinase	1.17	0.33	1.09	-0.32	3/1
YKL062W	<i>MSN4</i> , transcriptional activator related to Msn2p	1.02	-0.22	1.03	-0.27	0/2
YMR105C	<i>PGM2</i> , phosphoglucomutase	0.76	-0.11	0.26	-1.61	5/0
YMR173W	<i>DDR48</i> , induced by heat shock, DNA damage or osmotic stress	0.81	-0.75	0.57	-1.08	2/1
YMR173W-A	Protein of unknown function	0.73	-0.57	0.29	-1.03	2/0
YMR181C	Protein of unknown function	0.62	0.02	0.81	0.28	2/0
YMR319C	<i>FET4</i> , low-affinity Fe(II)-transport protein	1.50	0.07	0.34	-0.05	2/2
YNL042W	<i>BOP3</i> , bypass of PAM1; protein of unknown function	0.73	-0.17	0.59	-0.13	2/0
YOR161C	<i>PNS1</i> , mitochondrial protein of unknown function	1.75	-0.19	1.74	-0.50	2/1
YOR185C	<i>GSP2</i> , Ran, a GTP-binding protein member of the ras superfamily	0.75	-0.04	1.08	-0.37	2/1
YOR381W	<i>FRE3</i> , similar to FRE2; ferric reductase	0.93	0.15	0.03	-0.36	0/2

*No. of STRE elements/no. of PDS elements.

suggests that, at least in actively growing cells, Gis1p may target either the PDS elements directly, or the STRE elements indirectly via Msn4p, to repress the expression of a set of stress-response genes.

Gene regulation exerted by Gis1p and Rim15p changes in response to the identity of the carbon source

Deletion of either *GIS1* or *RIM15* leads to growth-fitness defects in both the glucose- and ethanol-limited media (Fig. 2c, d), suggesting that *GIS1* and *RIM15* control different stages of the transition to stationary phase. Correspondingly, >50 % of the genes downregulated in the glucose-limited mutant cells are regulated similarly when the deletants are grown in ethanol-limited chemostats (Fig. 3d). However, the degree of regulation for most of these genes changes in response to the identity of the carbon source. For example, the effect of *gis1* or *rim15* deletions on the expression of three stress-response genes (*HSP26*, *SIP18* and *GRE1*) is more pronounced under ethanol-limiting conditions (Table 1). Moreover, some of the *GIS1*- or *RIM15*-dependent genes are regulated specifically under either glucose- or ethanol-limiting conditions. Thus, the expression of *PIS1* (phosphatidylinositol synthase), *CDA1* (chitin deacetylase) and a few others is not affected significantly by the deletion of *GIS1* in glucose-limited cells, but loss of *GIS1* leads to down-regulation under the ethanol-limited condition. Similarly, the expression of 28 genes was downregulated in the *rim15Δ* strain only when cells were grown in ethanol-limited chemostat cultures. This set of 28 includes genes involved in defence against oxidative damage (*CTT1* and *AHP1*), glycolysis/gluconeogenesis (*CDC19*, *ENO1*, *TDH3*, *GPM1* and *PGM2*) and glycerol biosynthesis (*HOR2*, *RHR2* and *GPD1*). This indicates that Rim15p controls the expression of both the damage-defence genes and glycerol-biosynthetic genes in response to increased oxidative stress when cells are grown on ethanol as a carbon source. These data also suggest that Rim15 and Gis1 regulate the expression of different sets of genes at different stages of the transition to stationary phase.

Production of glycerol is required to protect yeast cells from oxidative stress (Pahlman *et al.*, 2001), which is generally triggered by reactive oxygen species produced as a result of an increase in mitochondrial respiratory activity (Costa & Moradas-Ferreira, 2001). Glycerol production in

yeast is also known to have fundamental physiological roles in adaptive responses to osmoregulation and anaerobic redox control (Maeda *et al.*, 1994). The involvement of Rim15p in glycerol biosynthesis suggests that Rim15p may be required for cells to defend themselves against osmotic stress. As shown in Fig. 4, whilst the *hog1* mutants were sensitive to osmotic stress at all concentrations of sorbitol tested, the *rim15* deletants displayed a growth defect only at the high concentration of sorbitol (2.8 M). The double mutant displayed a more severe growth defect at the low concentration of sorbitol (0.7 M) than either single mutant when incubation was extended to 5 days. These data suggest that Rim15p may be required to coordinate central carbon metabolism and glycerol production in response to different stresses and that Rim15 and Hog1 act in parallel to defend cells against osmotic shock.

Previously, Cameroni *et al.* (2004) identified a number of genes that are regulated by *RIM15*, *GIS1* and *MSN2/4* during the diauxic shift by comparing the transcriptome from cells in the exponential phase of batch culture with those from cells that have just exhausted the glucose in the medium. Interestingly, there is very little overlap between the gene sets identified in their experiments and those that we have uncovered. For example, among the 57 genes shown to be downregulated in *rim15* mutant cells by Cameroni *et al.* (2004), only four of them are regulated similarly in the same cells grown in glucose- or ethanol-limited chemostats. In fact, the majority are not regulated significantly by *RIM15* under either glucose- or ethanol-limiting conditions (data not shown). Similarly, no significant correlation is observed between the set of *GIS1*-dependent genes revealed by Cameroni *et al.* (2004) and those shown in our study (data not shown). Whilst Cameroni *et al.* (2004) studied the effects of deletions of *RIM15*, *GIS1* and *MSN2/4* on gene expression during early diauxie, the physiological conditions of the glucose- and ethanol-limited chemostat cultures used in this study are more similar to the later stages of diauxie and post-diauxie, respectively. These data may further support the notion that Rim15p and its downstream effectors regulate the expression of changing sets of genes during different phases of the transition into stationary phase.

DISCUSSION

We have demonstrated, by phenotypic and transcriptomic analyses, that both Gis1p and Rim15p are required for cells

Fig. 4. Phenotypic analysis of *rim15*-deletion cells exposed to hyperosmolarity. Cells were spotted, in a 7-fold dilution series, onto YPD plates and incubated at 30 °C. Sorbitol concentration and incubation time are indicated.

to grow on limited glucose or ethanol as a carbon source. Such conditions resemble the later stages of diauxie and post-diauxie, respectively. Both *GIS1* and *RIM15* are also required for activation of a number of UES genes when cells are starved of different macronutrients. Deletion of either of them has a deleterious effect on cell survival in the stationary phase (Pedruzzi *et al.*, 2000). Overall, the data demonstrate that Rim15p and Gis1p both participate in an important pathway that regulates yeast gene expression during the transitions that precede entry into the stationary phase.

Compared with the transcriptome analyses conducted during the diauxic shift of yeast in batch culture (Cameroni *et al.*, 2004), our chemostat studies have shown very little correlation between the two sets of genes regulated by either *RIM15* or *GIS1* (data not shown). The sets of *RIM15*- or *GIS1*-dependent genes and the degree of their regulation change according to the identity of the limiting carbon source (Fig. 3d). These data suggest that there are likely dynamic changes of gene regulation exerted by Gis1p and Rim15p during different phases of the shift into the stationary phase. Whilst Gis1p and Rim15p have overlapping functions in regulating the expression of genes involved in central carbon metabolism and the stress response, we have revealed that a set of genes repressed by Gis1p was either downregulated or unchanged in *rim15Δ* cells, suggesting that the action of Gis1p as a transcription repressor is independent of the control of the Rim15 kinase. This repression activity of Gis1p may be mediated directly, by its binding to PDS elements, or indirectly via Msn4p – given that STRE elements are the most-represented motifs in the promoters of these genes and the expression of *MSN4* is, itself, regulated by *GIS1* (Table 2).

However, it cannot be ruled out that *GIS1* may mediate gene activation or repression by binding to STRE elements directly. Firstly, among the downregulated genes in the *gis1* mutants, not all contain PDS elements and the majority also have STRE elements in their promoters (Table 1). Secondly, 95 % of Gis1p-dependent genes are included within the larger set of Msn2/4p-dependent genes (Cameroni *et al.*, 2004). Furthermore, Gis1p and Msn2/4p were demonstrated to have partially redundant functions in regulating the expression of genes containing only PDS or STRE elements in their promoters (Roosen *et al.*, 2005). It will be interesting to find out how *GIS1* and *MSN2/4* cooperate to both activate and repress gene transcription under the same physiological conditions.

Rim15p was shown to control the expression of several genes that are classically involved in defence against oxidative stress (Cameroni *et al.*, 2004). In this study, we have extended the understanding of the role of Rim15p in the oxidative-stress response by demonstrating that it regulates the expression of glycerol-biosynthetic genes under conditions of increased mitochondrial respiratory activity (Fig. 3d). These data suggest that Rim15p coordinates carbon metabolism and the oxidative-stress

response during the transition from nutrient limitation to stationary phase. A number of studies have demonstrated that the Rim15 kinase receives nutritional signals from both the PKA (Pedruzzi *et al.*, 2000) and TOR (Pedruzzi *et al.*, 2003; Urban *et al.*, 2007; Wanke *et al.*, 2008) signalling pathways. The PAS domain at the N-terminal region of Rim15p was suggested to sense the oxidative-stress signal (Cameroni *et al.*, 2004). However, how Rim15p integrates both the nutrient- and oxidative-stress signals and how the activity of its downstream effectors is regulated to coordinate metabolism and stress responses remain to be determined. Recently, the general stress-response transcription factor Msn2/4p was shown to receive signals from both Hog1 and other pathways to activate the expression of a large number of genes upon osmotic shock (Capaldi *et al.*, 2008). Given that deletion of both *RIM15* and *HOG1* renders cells more sensitive to hyperosmotic conditions than either of the single mutants (Fig. 4) and that the expression of Msn2/4p-dependent genes is also controlled by Rim15p under a number of physiological conditions (Cameroni *et al.*, 2004; Roosen *et al.*, 2005; Swinnen *et al.*, 2006), it is tempting to suggest that the Hog1 and Rim15 kinases function cooperatively to activate the osmotic-stress response programme.

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