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Citation for published version (APA):

Schuurmans, J. M. (2008). *The effect of altered expression of transcriptional regulators of catabolism on the transcription profile and physiology of *Saccharomyces cerevisiae**.

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Chapter 4

A transcriptome analysis of an *HAP4* overexpression mutant under different physiological conditions

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Abstract

In this chapter we analyze the effects of *HAP4* overexpression on the transcriptome of *Saccharomyces cerevisiae*. Four specific conditions, namely batch growth (μ_{\max}) nitrogen-limited growth ($\mu=0.1 \text{ h}^{-1}$) and glucose limited growth ($\mu=0.1$ and 0.32 h^{-1}) of both the wild-type and the *HAP4* ↑ strain were analyzed. Besides the changes in central carbon metabolism, several target groups of genes under control of a specific regulator were identified. As expected the Hap4p element was overrepresented in conditions where cells were growing under glucose repressed conditions. The Mig1p element and the Cat8p elements were identified under conditions of glucose de-repressed growth. Both these elements are involved in regulation of transcription under conditions of glucose exhaustion whereas the Zap1p and Rcs1p elements (involved in zinc respectively iron homeostasis) were identified under quite varying conditions. The former was primarily present in batch grown wild-type cells but largely underrepresented in the *HAP4* ↑ strain under the same conditions. The Rcs1p element was found in glucose limited *HAP4* ↑ cultures at low growth rates and in the batch grown cultures compared to the other conditions. Additionally an Msn2-4p element was found under low-growth conditions ($\mu=0.1 \text{ h}^{-1}$). Together these results show an interesting overview of the specific effects on the transcriptome of *Saccharomyces cerevisiae*, when *HAP4* is overexpressed.

Introduction

The ability of the yeast *Saccharomyces cerevisiae* to ferment sugars to ethanol and carbon dioxide even in the presence of oxygen has led to its wide use in applications, such as the brewing of beer and the raising of dough. However, this organism is also used in biotechnological processes concerned with the production of fine chemicals, specialties and others compounds, including proteins. In such processes, formation of ethanol is highly undesirable as it dramatically lowers the yield on glucose. Optimization of biomass yield has been attempted in many ways, mostly by searching for conditions or genetic modifications that lower ethanol production and increase the respiratory capacity of the organism. One way to achieve this is by altering the expression of genes encoding enzymes involved in respiration and fermentation. For instance Flikweert *et al.* (Flikweert, et al., 1999) attempted to block flux towards ethanol by deleting the functional pyruvate decarboxylase genes. These strains however needed addition of acetate or ethanol to grow, which is not desirable in a production process. With the knowledge obtained the last decades with regard to the extent to which control over a metabolic pathway is distributed over the enzymes involved, it is to be foreseen that increasing the activity of a single enzyme in the pathway will hardly ever result in a similar increase in pathway fluxes due to redistribution of control (Schaaff, et al., 1989). Indeed, studies on overexpressing glycolytic enzymes have shown this to be the case (Smits, et al., 2000).

More promising is the approach where one alters the expression of global regulators (transcription factors) that control a certain pathway. In baker's yeast, the HAP2/3/4/5 complex is such a global regulator controlling activation of the genes involved in respiration (de Winde and Grivell, 1993; Forsburg and Guarente, 1989). This occurs at very low levels of glucose or when glucose is depleted and respiration is the major catabolic mode and fermentation is virtually absent. Hap2p, Hap3p and Hap5p are members of this complex that are continuously expressed, and as such appear to be only structural components. Expression of the Hap4p activator, however, is controlled by the Mig1p and Mig2p repressors which respond negatively to the availability of glucose (Gancedo, 1998). Under high glucose conditions Hap4p is actively repressed and the protein is hardly present in the cell. When glucose availability becomes scarce, repression is relieved and Hap4p binds to the Hap2/3/5p components to form a complex, which activates genes that contain a CCAATCA like promoter element (Dang, et al., 1994). These include both genes involved in the respiratory chain (e.g. Qcr8p) and in the TCA cycle (e.g. Mdh1p). This makes Hap4p the ideal candidate for overproduction as a tool to increase respiration as a whole, bypassing the stage of needing to adapt the levels of each glycolytic enzyme to relieve their control. Studies on a strain that overexpressed Hap4p indeed showed an increase in biomass yield on glucose and an increased respiratory capacity in batch cultures (Blom, et al., 2000). Other studies showed changes in the respiratory capacity under various continuous conditions, but failed in relating this to a concomitant increase in biomass (van Maris, et al., 2001). This is most likely due to strain and medium changes. Considering the complexity of regulation of glycolytic fluxes, of the expression levels of the genes involved therein and the kinetics of the glycolytic enzymes, it is to be expected that varying levels of Hap4p will have a severe effect on the transcriptome of the cell as a whole. Here we compare transcriptional profiles and

physiological data of a Hap4p overproducing strain with the wild-type under 4 different conditions in order to make a further step in the elucidation of the regulatory network underlying glycolysis in baker's yeast.

Materials & Methods

Strains and growth conditions

Strains used were CEN.PK113-7D (MATa URA3 LEU2 HIS3 TRP1 SUC2 MAL MEL GAL) provided by Dr. P. Koetter (Frankfurt, Germany), a *HAP4* overexpression strain previously described in Van Maris *et al.* (van Maris, et al., 2001). For the data on a glucose-limited culture at a dilution rate of 0.32 h^{-1} , strains were grown in laboratory fermenters (L.H. Engineering, Maidenhead, UK) under aerobic glucose-limited conditions. A defined mineral medium described by Verduyn *et al.* (Verduyn, et al., 1992) was used. The concentration of glucose in the feed was $5 \text{ g}\cdot\text{l}^{-1}$. The working volumes used varied between 0.65 and 0.8 l. pH was kept at 5.0 ± 0.1 by automatic addition of 1 M NaOH. The growth temperature was set at 30°C . BDH Laboratory Supplies Silicone anti-foaming agent was used in a $50 \mu\text{l/l}$ concentration to prevent foam development. The cultures were sparged with air at a flow rate of approximately 1 volume of air per culture volume per minute. The cultures were stirred with double impellers (LH Engineering) at 750 rpm and were grown in continuous mode by steady addition of medium while the overflow medium was disposed as waste. After 6-7 volume refreshments, a steady-state was considered to be reached as biomass and CO_2 and O_2 did not vary more than 5% after this time. Oxygen consumption and carbon dioxide production were determined by passing the effluent gas through a paramagnetic oxygen analyzer type 1100 (Servomex, Crowborough, UK) and an IR gas analyzer type 4100 (Servomex, Crowborough, UK), respectively. For the batch cultivation of the CEN.PK113-7D and *HAP4* overexpressing strain the following setup was used. The strains were precultured overnight at 30°C in 0.67% YNB 2% glucose 0.1 M phtalic acid, pH=5.0 using KOH. Cells were inoculated to an OD_{600} of 0.2 in the same medium in batch fermentors with a 0.5 liter working volume, aerated with 1 vvm and stirred at 500 rpm. Growth was followed in time both by measuring OD_{600} and biomass. For the glucose-limited and nitrogen-limited cultures standard conditions used in the Delft laboratory were used (Boer, et al., 2003). All results are typical results representing at least two identical of experimental setups.

Sample extraction and analysis

Samples taken for metabolite analysis were immediately treated with 35% cold (4°C) perchloric acid. Samples were stored at -20°C . Upon analysis samples were neutralized with 7M KOH and filtered through a $0.45 \mu\text{m}$ filter. Dry weight was determined by putting $2 \times 10 \text{ ml}$ of culture in pre-weighed tubes, the culture was then spun down at 5000 rpm (Sorvall, SS-34 rotor) at 4°C , then washed with demineralized water, spun down again and dried overnight at 100°C . The difference in weight was used to determine the dry-weight of the culture.

Metabolites were determined by means of high performance liquid chromatography (LKB, Bromma, Sweden) with a Rezex organic acid analysis column with an 8- μ m particle size, 8% cross-linking and a hydrogen ionic form (Phenomenex, Torrance, CA, USA) at a temperature of 45°C and with 7.2 mM H₂SO₄ as eluent. Detection was done with an RI1530 refractive index detector (Jasco, Tokyo, Japan). Peak integration and data processing were done with BORWIN (Le Fontanil, France) chromatography software.

Preparation of total RNA and labeled cRNA

Samples for RNA isolation were collected each hour from a batch fermentor's culture at appropriate time points, flash-frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using the method of Llinas, by the procedure described by Zakrzewska and co-workers (Zakrzewska, et al., 2005). The concentration and quality of RNA was determined by measuring absorbance at 260, 280, and 230 nm on a Nanodrop spectrophotometer. The purity and integrity of the RNA samples were further validated with RNA LabChip on a 2100 Bioanalyzer from Agilent Technologies.

Total RNA was labeled according to the manufacturer's protocol (Affymetrix). Twenty μ g of total RNA was used for first strand cDNA synthesis. This was followed by synthesis of second strand cDNA. cDNA was purified using the GeneChip Sample CleanUp Module from Qiagen. The cDNA was used for synthesis of biotin-labeled cRNA, which was performed with the ENZO BioArray HighYield RNA Transcript Labeling Kit from Affymetrix. The synthesized cRNA was purified with the GeneChip Sample CleanUp Module from Qiagen. The concentration and quality of labeled cRNA was tested using a Nanodrop spectrophotometer. Subsequently, the cRNA fragmentation reaction was carried out according to the manufacturer's protocol. The degree of fragmentation was confirmed with RNA LabChip on a 2100 Bioanalyzer from Agilent Technologies. The samples were stored at -20°C prior to hybridization. For the continuous cultures at a dilution rate of 0.1 h⁻¹ the conditions described by Boer *et al.* 2003 were used (Boer, et al., 2003)

Hybridization and scanning of the DNA microarrays

The biotin-labeled cRNA samples were hybridized to the Affymetrix GeneChip[®] Yeast Genome S98 Array according to Affymetrix protocols (<http://www.affymetrix.com>). This chip contains 25-mer oligonucleotide probes for approximately 6400 *S. cerevisiae* ORFs. Each ORF is represented by approximately 16 probes, covering different parts of its sequence. Every probe is neighbored by a probe that is identical, except for one nucleotide in the middle of its sequence. This probe is called the 'mismatch' probe (MM), as opposed to the 'perfect match' probe (PM). The arrays were scanned with the GeneArray Scanner System on standard settings at 3 mm resolution. The data were extracted from the scanned images with MAS 5.0 (Microarray Suite 5.0). For the continuous cultures at a dilution rate of 0.1 h⁻¹ the conditions described by Boer *et al.* were used (Boer, et al., 2003)

Data processing

DChip was used to normalize the raw data. This is a software package implementing model-based expression analysis of oligonucleotide arrays and several high-level analysis procedures (Li and Wong 2001). The model-based approach allows probe-level analysis on multiple arrays. The arrays were normalized by adjusting the overall brightness of the arrays to a similar level. Background subtraction was performed prior to calculating the expression values. Expression values were calculated using the perfect match model only, as this is unaffected by adverse effects of mismatch probes. A more detailed description of the used procedure is given by Zakrzewska *et al.* (Zakrzewska, et al., 2005). For the continuous cultures at a dilution rate of 0.1 h^{-1} the conditions described by Boer *et al.* 2003 were used (Boer, et al., 2003)

T-profiler analysis of DNA microarray data

To assess the contribution of the expression of genes from specific gene classes to the total gene expression, T-profiler was used (Boorsma, et al., 2005). This algorithm uses an unpaired *t* test to classify a difference between the mean of a set of a specific class of genes and the mean of the remaining genes of the total gene expression profile. We have looked at classes of genes containing a similar promoter element. The elements used are within 600 basepairs of the start codon and are based on literature and computational analysis results. The experiments are separately analyzed and no arbitrary cutoffs were applied prior to T-profiler analysis. T-profiler and additional information can be found on <http://www.t-profiler.org>.

Results and Discussion

In this study four different cultivations were used to analyze the effects of *HAP4* overexpression on the level of the transcriptome. Both wild type and the mutant were grown in batch culture and in chemostat culture under ammonium-limited respectively carbon-limited conditions at a specific growth rate of 0.1 h^{-1} . In addition, the mutant strains were grown glucose-limited at a specific growth rate of 0.32 h^{-1} (being the critical growth rate where ethanol production begins).

Physiology

The most relevant physiological parameters of the strains grown under 4 different conditions are summarized in Table 1. The data, both physiological as transcriptional, presented for batch and glucose-limited at a dilution rate of 0.32 h^{-1} are identical to Chapter 2 and Chapter 3 respectively. When wild-type and *HAP4* ↑ are grown to steady-state in glucose-limited aerobic chemostat cultures at low dilution rate (0.1 h^{-1}), both strains show fully respiratory growth as can be deduced from the ratio of the specific CO_2 production rate and the specific O_2 consumption rate ($q_{\text{CO}_2}/q_{\text{O}_2}$, RQ) of approximately 1. This was confirmed by the absence of ethanol in the cultures. In accordance with the identical catabolic behaviour, under these conditions overproduction of hap4p has no

effect on the biomass yield on glucose (Y_{glc}). Under glucose limitation, both strains switched to respiro-fermentative catabolism at a dilution rate of 0.32 h^{-1} (μ_{crit} for the wild type is 0.31 h^{-1}) but with the wild type a significantly higher flux to ethanol, was seen. Surprisingly, this seemingly less efficient energy conservation catabolic mode was not reflected in the Y_{glc} values ($0.41 \text{ g}\cdot\text{g}^{-1}$ respectively $0.42 \text{ g}\cdot\text{g}^{-1}$). A further anomaly was observed: even though the q_{ethanol} of the wild type significantly exceeds that of the *HAP4* ↑ strain, the RQ values are quite close despite the fact that carbon recoveries were close to 100%.

In nitrogen-limited aerobic steady-state chemostat cultures, large differences are seen between the behaviour of the strains. Now, the wild-type shows respiro-fermentative catabolism, shuttling pyruvate to both the respiratory pathway and the fermentative pathway, as indicated by an RQ value of 2.9, with more emphasis on the fermentative pathway. Here no such anomalies as mentioned above were found. The effects on yield value are in accordance with what would be expected. The lower ATP/glucose stoichiometry of the wild type is reflected by an increased glucose consumption rate to meet the energy demands for growth and maintenance and therefore a lower Yield value ($0.11 \text{ g}\cdot\text{g}^{-1}$ compared to $0.21 \text{ g}\cdot\text{g}^{-1}$ in the *HAP4* ↑ strain).

Table 1: Physiology of the wild-type and the *HAP4* ↑ strain.

	$\mu \text{ (h}^{-1}\text{)}$	q_{glucose} ($\text{mmol}\cdot\text{g}^{-1}$ $\text{dw}\cdot\text{h}^{-1}$)	q_{etoh} ($\text{mmol}\cdot\text{g}^{-1}$ $\text{dw}\cdot\text{h}^{-1}$)	RQ ($q_{\text{CO}_2}/q_{\text{O}_2}$)	$Y_{\text{glc}} \text{ (g}\cdot\text{g}^{-1}\text{)}$
CEN.PK113-7D					
Climited	0.1	1.1	0	1.1	0.51
Nlimited	0.1	5.3	6.2	2.9	0.11
Climited 0.32	0.32	4.4	7.1	1.3	0.41
Batch	0.36	10.13	17	3.4	0.18
<i>HAP4</i> ↑					
Climited	0.1	1.1	0	1	0.52
Nlimited	0.1	2.6	1.1	1.2	0.21
Climited 0.32	0.32	4.3	2.7	1.4	0.42
Batch	0.35	7.41	11.3	2.9	0.24

Strains were grown in glucose-limited aerobic chemostat at two dilution rates, in nitrogen-limited aerobic chemostats and in batch. Fluxes were determined for glucose, ethanol, CO₂ and O₂. The biomass yield on glucose is also shown. q_{O_2} and q_{CO_2} are not shown, instead the respiratory quotient (RQ) is shown. The given values are the mean of two independent experiments in the cases of Batch and C-limited 0.32 and three independent experiments in the cases of Climited and Nlimited.

Grown in aerobic batch cultures, the same effect of *hap4p* overproduction was observed as outlined above for nitrogen-limited aerobic conditions, be it less outspoken. No effect of overproduction was observed with respect to the specific growth rate (μ_{max} of 0.36 h^{-1} for the wild-type and a μ_{max} of 0.35 h^{-1} for the *HAP4* ↑ strain) but again the RQ of the *HAP4* ↑ was reduced compared to the wild-type due to lower specific ethanol production rate and hence a higher yield value ($0.24 \text{ g}\cdot\text{g}^{-1}$ versus $0.18 \text{ g}\cdot\text{g}^{-1}$).

Transcriptome analysis

From all conditions tested RNA samples were taken during steady-state (in chemostat cultures) or early-mid log phase ($OD_{600} \sim 1$, in the batch culture). These RNA samples were treated according to the Affymetrix™ protocol and hybridized to Affymetrix™ YG-S98 *Saccharomyces cerevisiae* chips. Instead of using individual expression of genes, we used T-profiler to get a global overview of the events going on in these cells under the conditions applied. This analysis resulted in a few specific groups of genes that all have a common promoter element, which is associated with a transcriptional activator or repressor. Although T-profiler recognizes several promoter elements for the same transcriptional activator or repressor, which are usually complementary, only one element is shown each time. The first two elements that were checked were the Hap4p element CCAATCA and the Mig1p element TWCCCCM. These results are shown in figure 1.

A positive T-score of the Hap4p element shows that genes that contain the CCAATCA element are upregulated more or less as a whole module but it should be mentioned here that individual expression of genes belonging to the module may differ. Taking into account that stringent glucose-limited conditions result in *HAP4* expression, it is not unexpected to see that only aerobic batch cultures and nitrogen-limited chemostat cultures show a positive contribution. The other conditions do not show such changes in Hap4p mediated expression. This is consistent with the observation that these conditions resulted in significantly different physiology between the strains.

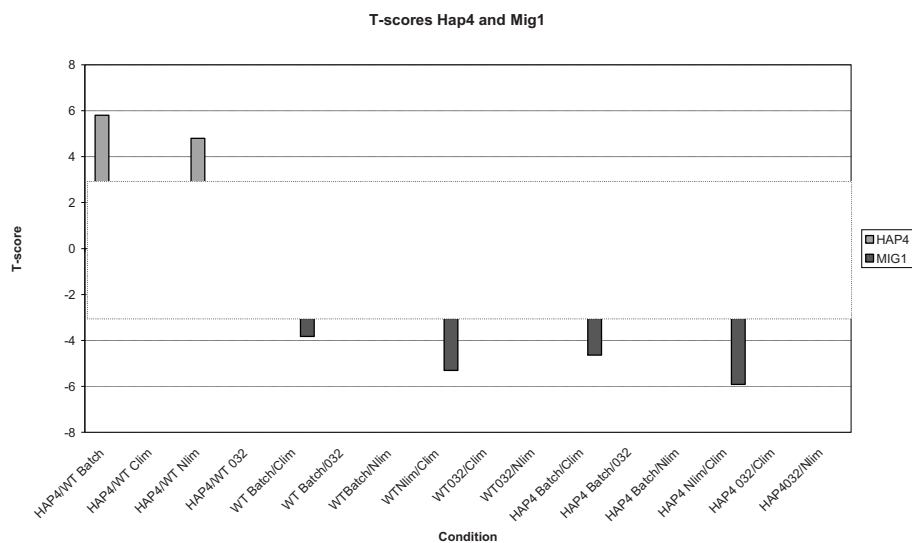


Figure 1: T-scores for the promoter elements Hap4p and Mig1p. Shown are the significant T-scores for the CCAATCA (Hap4p) binding element and the TWCCCCM (Mig1p) binding element. Only scores with a corresponding e-value of <0.05% are shown.

Other cross-comparisons can be made, now for one strain with the various conditions tested. Some conditions turn out to have no significant t-score for Hap4p, even though there is a large change in respiratory activity (*e.g.* comparing the glucose-limited aerobic culture with the nitrogen-limited aerobic culture with both wild-types or both *HAP4* ↑ strains.) and despite the fact that the elements are present in these cases. Another indicator for the respiratory activity is the transcriptional repressor Mig1p, which represses expression of respiratory genes and *HAP4* itself. And indeed in the conditions where a Hap4p element was expected a negative t-score for the Mig1p element was found, indicating repressing conditions caused by binding of Mig1p to the promoter element and reducing expression as a whole. This then might also explain why under these conditions the Hap4p element is not found significant as it is under control of Mig1p. The conditions where the Hap4p element was found are all comparisons of *HAP4* ↑ over the wild-type, and in the *HAP4* ↑ overexpression strain a second copy of *HAP4* is present which is not under control of Mig1p.

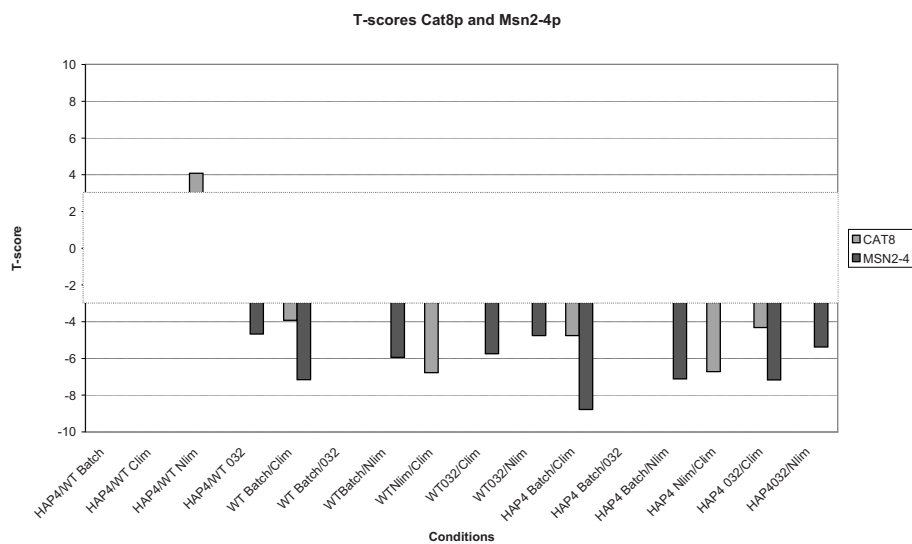


Figure 2: T-scores for the promoter elements Cat8p and Msn2-4p. Shown are the significant T-scores for the CCGNNNCCG (Cat8p) binding element and the CCCT (Msn2-4p) binding element. Only scores with a corresponding e-value of <0.05% are shown.

T-profiler also significantly scored the Cat8p promoter element CCGNNNCCG and the Msn2-4p promoter element CCCT and the results are shown in figure 2. The Cat8p transcriptional activator controls gene expression of enzymes needed for gluconeogenesis and other processes which typically occur in glucose depleted environments. Cat8p is under control of both the Mig1p repressor and the Hap2/3/4/5 activator complex. Indeed the *HAP4* ↑ strain grown under aerobic nitrogen-limited conditions scores significantly for the Cat8p relative to the wild type but does not so when grown in aerobic batch cultures. This might not be expected but it may be hypothesized that relatively high

respiratory activity per se triggers the cell to adapt to seemingly low glucose condition, despite the proper functioning of the glucose sensing machinery in this strain.

The Cat8p element shows up in the wild type strain under the conditions tested as expected. For instance, in batch grown cells Cat8p controlled expression is much lower than under glucose-limited condition. The same is found when comparing a nitrogen-limitation to a glucose limitation. Comparing the *HAP4* ↑ strains to each other under all condition revealed the same results.

The only comparison where a significant score for Cat8p lacks is between the batch cultures or nitrogen-limited cultures to the glucose-limited cultures at a dilution rate just over the μ_{\max} . Although these cultures are still glucose limited, they do ferment, implying that they are no longer fully glucose de-repressed. Of course, here the glucose limitation is less stringent, since the cells grow at a rate close to their maximal attainable growth rate. This causes the Mig1p repressor to block transcription of genes under control of Cat8p and as such the lack of significant scores is consistent.

A fair amount of significant Msn2-4p controlled elements were found. Msn2-4p is a transcriptional activator complex that is mostly associated with various kinds of stress, such as continuous heat stress (Mensonides, et al., 2002). In these series all the conditions were similar with respect to temperature and other environmental parameters (pH, stirring, aeration) (with the exception of 100 mM phthalic acid in the batch cultures). This finding rules out any responses of Msn2-4p to these parameters. Interestingly all the conditions in which Msn2-4p scores significantly are comparisons where the growth rate varies largely (e.g. batch wild-type (0.36 h^{-1}) versus glucose-limited cultures at a growth rate of 0.1 h^{-1}). It has been described that PKA activity and Msn2/4p expression antagonize one another controlling the cells between growth and non-growth under various conditions (starvation, heat stress, osmotic stress) (Smith, et al., 1998). This explanation would fit very well with the observed results of higher expression of genes under control of Msn2/4p under nutrient limiting conditions. The absence of this category in either of the comparisons between unrestricted growth or very low growth rate with the glucose-limited culture at 0.32 h^{-1} seems contradictory to this explanation. It must be remarked here that at this growth rate the organisms have switched to respiro-fermentative metabolism, which suggests positive PKA signaling and as a consequence lower expression of Msn2/4p controlled genes.

Two other categories also scored significantly in this analysis, namely genes under control of the transcriptional activator Rcs1p with a common promoter element of RVACCCTD, and genes with the common promoter element ACCNNNNNGGT, which is recognized by the transcriptional activator Zap1p (shown in figure 3). Rsc1p is a transcription factor, which is involved in iron homeostasis in yeast. As such the appearance of this category might not be surprising as increased respiration requires more iron containing haeme.

Yet, when comparing the *HAP4* ↑ strain with the wild-type under glucose-limited conditions at a low growth rate (where both strains grow fully respiratory) this category shows up. Under these conditions, the two strains respire at the same rate, but it may well be that due to the Mig1p independent expression of *HAP4* a signal is triggered which results in increased haeme synthesis, or even increased synthesis of other components of the respiratory chain. It would be interesting to investigate this hypothesis by a biochemical analysis of the haeme content of the overproducer and its respiratory

capacity. Noteworthy, the analysis showed a slight upregulation of the QCR genes in the *HAP4* ↑ strain encoding the ubiquinol cytochrome c oxidoreductase proteins under glucose-limited conditions (data not shown).

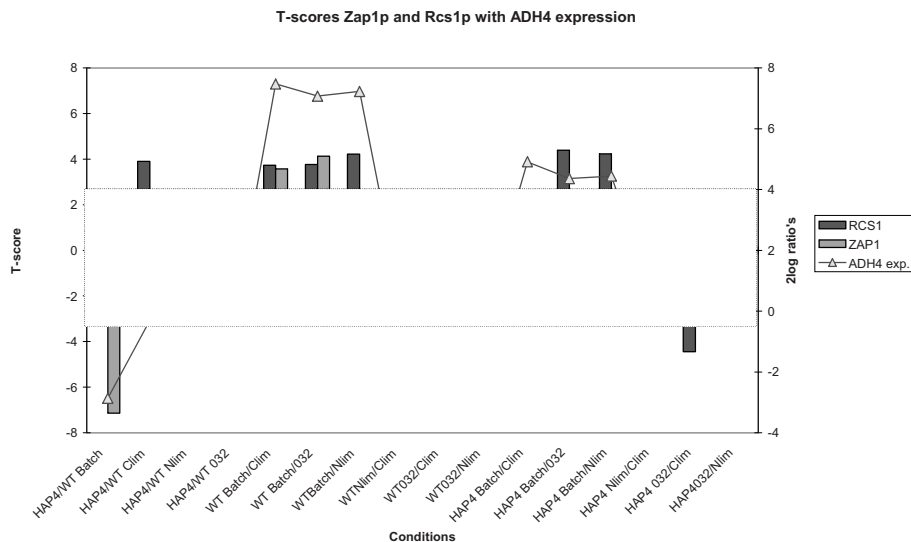


Figure 3: T-scores for the promoter elements Rcs1p and Zap1p and the expression ratio's of ADH4. Shown are the significant T-scores for the RVACCCTD (Rcs1p) binding element and the ACCNNNNNGGT (Zap1p) binding element. Only scores with a corresponding e-value of <0.05% are shown. Additionally the $2\log$ ratios of ADH4 expression are shown on the second y-axis.

Interesting results are obtained by comparing the Rcs1p element for the same strain grown under various conditions. In most analyses a *higher* expression of Rcs1p controlled genes was seen under the conditions where there is *less* respiration. Only for the *HAP4* ↑ low versus high growth rate glucose-limited analysis this was not the case. This may indicate some opposing regulatory events: the strong and overruling glucose repression network results in low respiration rates that in turn give rise to changes in some cellular state parameter (e.g. the redox or energy state). These latter changes may be signals to the cell to enhance its respiration and thus to provide sufficient iron. Indeed, this seems in line with the absence of this relationship in the hap4p overproducing strain which, due to increased respiration activity under all circumstances, lacks the signal for Rcs1p controlled genes.

Throughout all analysis, it was surprising to find a significant impact of the category under control of Zap1p, controlling zinc homeostasis in *S. cerevisiae*. The results seem consistent with the idea that increased respiration results in more Reactive Oxygen Species being formed, and therefore more superoxide dismutase is needed. In yeast these are encoded by *SOD1* and *SOD2*, of which Sod1p requires zinc to be functional. However, a further analysis showed no increased expression of both Sod proteins under enhanced respiration. An alternative explanation for the involvement of Zn-related processes may reside in the differential expression of *ADH* genes. *ADH4* is the only

alcohol dehydrogenase which requires zinc. Indeed, significant upregulation of *ADH4* and the Zap1 scores correlated in a number of analyses, but it must be noted that this correlation was not always found. This raises the question whether other Zn dependent proteins play some role in the increase in Zap1 controlled gene expression.

It has been shown previously that increased levels of Hap4p result in a more oxidative catabolism in *S. cerevisiae* (Schuurmans, et al., 2008[van Maris, 2001 #104]). Our analyses show that the major effect of the overproduction is on those genes that are involved in respiration rather than on those involved in glycolysis. Thus, the hap regulatory network seems to work on a different metabolic level, with an important difference with the physiological and genetic impact, that controlled by of *hvk2* (Schuurmans, et al., 2008). Although the absence of the latter global regulator redistributes catabolism towards oxidative pathways, it has many other effects as well, including loss of glucose repression as a whole. This leads to a loss of response to both the concentration and nature of the carbohydrates in the environment. The respiration-specific effect of *HAP4 ↑* overproduction is not absolute though, as has been shown here, and it remains to be seen whether the side effects are due to a completely other and as yet unknown role of the regulator or are somehow related to respiration and hence indirect consequences of the functioning of Hap4p.