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Schuurmans, J.M.

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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 2

On the role of *HXK2* deletion and *HAP4* overproduction on the respiro-fermentative flux distribution and gene expression in *S. cerevisiae*

**J. Merijn Schuurmans[§], André Boorsma[§], Romeo Lascaris[§], Klaas J. Hellingwerf[§]
and M. Joost Teixeira de Mattos[§]**

[§]*Department of Molecular Microbial Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands*

Keywords: *Saccharomyces cerevisiae*, Hap4p, Hxk2p, Glucose repression, Yield, fermento-respirative metabolism, T-profiler

Published in modified form in FEMS Yeast Research (2008) **8**: 26-34 as “Physiological and transcriptional characterization of *Saccharomyces cerevisiae* strains with modified expression of catabolic regulators”

Abstract

We present a comparative physiological and transcriptional study on wildtype *Saccharomyces cerevisiae* and mutants with altered levels of catabolic regulators: *hxx2Δ* lacking hexokinase2, *HAP4* ↑ overproducing Hap4p and *hxx2ΔHAP4* ↑. Relative to the wildtype, *HAP4* ↑ expressed the same growth rate with some increased yield on glucose, *hxx2Δ* grew 28% slower but with a dramatically improved yield. *Hxx2ΔHAP4* ↑ grew 14% slower but virtually fully oxidative. Higher yield correlated with increased respiration.

For both *hxx2Δ* strains glucose-repression was relieved, (upregulation of high-affinity sugartransporters, invertase and oxidative phosphorylation). T-profiler analysis showed that genes under control of the hap2/3/4/5 binding motif were significantly altered in expression in all strains. *HAP4* overexpression, directly or in *hxx2* knockouts, led to repression of the genes containing the Zap1p-motif including *ZAP1* itself, indicating altered zinc metabolism. Whereas *HAP4* overexpression results in a shift towards oxidative metabolism only, deletion of *HXX2* results in a strain that besides being oxidative, almost completely lacks the ability to sense glucose. Since the double mutant has an energetic efficiency close to the maximum even with excess glucose and is derepressed to a larger extent and over a broader range, we conclude that the functioning of the two regulators is in general synergistic.

Introduction

Saccharomyces cerevisiae is a unicellular fungus, which in its natural habitat encounters rapidly environmental changes. In its original habitat, either ripening or decomposing fruits, especially the concentration of various sugars changes dramatically. It is therefore not surprising that to effectively cope with these changes, *S. cerevisiae* has developed a very complex regulatory network to control their uptake and utilization as carbon and energy source. *S. cerevisiae* favors glucose (or fructose) for the latter purpose above other sources, and the presence of these sugars will have the effect that genes encoding enzymes needed to utilize other sugars, such as maltose, saccharose and galactose, will remain repressed. Additionally, the concentration of glucose per se is important, as at high concentrations (>30 mM), the organism also represses genes encoding enzymes needed for respiration (Gancedo, 1998). Thus at high glucose concentrations, *S. cerevisiae* partly ferments glucose to ethanol even in the presence of an excess oxygen, the so-called Crabtree effect (De Deken, 1966).

In some industrial processes fermentation is undesirable since it significantly reduces the biomass yield on glucose, and ethanol at high concentrations can become a growth inhibitor. Hence the catabolic regulatory network, also known as the glucose repression pathway, has been studied quite extensively. Many of the proteins involved in this regulation network have been identified and characterized (Gancedo, 1998). Signaling pathways can be divided into three parts, namely the G-coupled protein Gpr1p (Kraakman, et al., 1999), the Snf3p and Rgt2p glucose sensors (Ozcan, et al., 1996a) and the glucose transport systems plus hexokinase II (Teusink, et al., 1998). Gpr1p senses glucose and can activate the Ras-cAMP pathway (Rolland, et al., 2002). Since this pathway also controls many genes involved in nitrogen source utilization, Gpr1p links glucose availability to the nitrogen status of the cell. Snf3p and Rgt2p are involved in the control of expression of various hexose transporter (*HXT*) genes (Ozcan, et al., 1998), thereby directly exerting control on the glucose influx rate, as each individual hexose transporter has a different affinity for glucose transport (Diderich, et al., 1999). With respect to glucose transport itself and hexokinase II (Elbing, et al., 2004; Moreno and Herrero, 2002), the exact signal received and transmitted is not exactly known. It should be realized that the above three systems are intricately linked and interact with each other at various levels. For instance, changes in *HXT* expression mediated by Snf3p or Rgt2p may change the glucose uptake rate, which again will affect the glycolytic flux and Hxk2p activity and this subsequently may affect the expression of *HXT* genes again.

Hexokinase II is not only the primary glucose phosphorylating enzyme, it also has a regulatory function in glucose repression (Moreno and Herrero, 2002). It has been shown that in the presence of high glucose concentrations (>30 mM), hexokinase II migrates to the nucleus to exert its regulatory function, together with Mig1p (Ahuatzi, et al., 2004). It has also been shown that a strain lacking the *HXX2* gene is largely shifted towards respiratory metabolism (Diderich, et al., 2001a) and shows co-consumption of various sugars and even ethanol (Raamsdonk, et al., 2001). The genes needed for utilization of these alternative sugars are normally repressed most predominantly by Mig1p and to a lesser extent by Mig2p. These latter proteins also repress genes involved in respiration. A *mig1Amig2Δ* strain indeed shows an increase in respiration (Klein, et al., 1999), although not as profound as might have been expected due to the absence of the main repressor

genes. For a fully functional respiratory chain and TCA-cycle, genes in the corresponding genes involved in these processes also need to be properly induced and activated. The transcriptional activator Hap4p mediates this process. It has been shown indeed that overproduction of this protein results in a considerably higher respiration rate and an increased biomass yield for *S. cerevisiae* grown on glucose (Blom, et al., 2000; van Maris, et al., 2001).

Clearly, the regulatory mechanisms that result in the net glycolytic, fermentative and respiratory activity of the yeast cell are complex and the contributions of the various components of the network to achieve the final output are still largely unknown. Each of the regulators has its own characteristics, often studied in isolation (Gancedo, 1998; Santangelo, 2006). The question arises to what extent the various regulatory proteins communicate with each other within the regulatory network. It may be that each of them affects gene expression and hence the physiology of the cell for some specific adaptive response or they may act in a concerted manner to provide the cell with a means to cope with a variety of conditions. In this study we quantitatively analyze the separate contribution of two of the key regulators (Hap4p and Hxk2p) to the metabolic activity of baker's yeast as well their synergistic functioning by a comparative study of the wild type strain, and single and double mutants.

Materials & Methods

Strains and cultivation

Strains used were CEN.PK113-7D (*MAT α URA3 LEU2 HIS3 TRP1 SUC2 MAL MEL GAL*), a *HAP4* overexpression strain (van Maris, et al., 2001), a *hxk2 Δ :KanMX4* mutant (Diderich, et al., 2001a)), and a *hxk2 Δ :KanMX4 HAP4* overexpression strain (Lascaris, et al., 2004). All mutants are derived from the CEN.PK113 background

The strains were precultured overnight at 30° C in 0.67% YNB 2% glucose 0.1 M phthalic acid, pH=5.0 using KOH. Cells were inoculated to an OD₆₀₀ 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 OD₆₀₀ and biomass. All results are typical results representing a series of experiments.

Metabolite and flux analysis

Extracellular metabolites were measured every hour by HPLC. 1 ml of culture was mixed with 100 μ l 35% (v/v) cold PCA. After 10 min., the PCA was precipitated by adding 55 μ l 7 M KOH. After centrifugation, the supernatant was filtered and analyzed for glucose, ethanol, glycerol, acetate, succinate, phosphate and pyruvate by HPLC (Phenomenex type Rezex Organic Acid column; eluent, 7.2 mM H₂SO₄ at 45° C). From the data on changes in dry weight and on metabolites the specific flux of compound x during a given time span could be calculated, using the following formula:

$q_{[x]} = \Delta[x] \cdot (\Delta t)^{-1} \cdot (\text{average}) DW^{-1}$ expressed in mmol•g dry weight⁻¹•h⁻¹.

Biomass yield on glucose (Y_{glc} g dry weight.g glucose⁻¹) was calculated from

$Y = 1000 \cdot (\mu \cdot q_{glc}^{-1}) / 180$ and is presented in percentages.

The Y_{ATP} value was calculated assuming that net 1 mole ATP per mole ethanol produced was synthesized and that 2.4 mole of ATP per mole of O_2 reduced (Verduyn, et al., 1991) was synthesized. The following relations were used:

$$q_{ATP} = q_{ethanol} + 2.4 \cdot q_{O_2} \text{ (mmol} \cdot \text{g dry weight}^{-1} \cdot \text{h}^{-1}\text{)} \text{ and } Y_{ATP} = \mu \cdot q_{ATP} \text{ (g} \cdot \text{mol}^{-1}\text{)}$$

Preparation of total RNA and labeled cRNA

Samples for RNA isolation were collected from a batch fermentor's culture at early-mid log phase, flash-frozen in liquid nitrogen and stored at -80°C . Total RNA was extracted using the method of Llinas, in the same way as was done 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 μg 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.

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).

Data processing

DChip was used to normalize the raw data. dChip is a software package implementing model-based expression analysis of oligonucleotide arrays and several high-level analysis procedures (Li, 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 (Zakrzewska, et al., 2005).

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.). 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. GO: Ontology (<http://www.yeastgenome.org>) has been used to classify genes into specific categories. Further analysis was done to see whether specific GO categories are overrepresented within our arrays.

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

Physiological characterization

In order to characterize the effects of mutations in the glycolytic regulatory network on the physiological and transcriptional level, wild type and mutant cells were grown in batch fermentors and growth was followed from the early to mid-log phase. During the growth phase samples were withdrawn and quickly quenched (see material and methods) to determine metabolite concentrations. Off-gas was analyzed for CO₂ and O₂ just prior to sampling for micro-arrays. From these data the physiological parameters as given in Table 1 were calculated. At an OD₆₀₀ of approximately 1, the cells were harvested for micro-array analysis. The WT strain was found to grow on the medium used (see methods and materials) at a maximal specific growth rate (μ) of 0.36 h⁻¹, with a respiratory quotient (RQ, defined as the q_{CO2}/q_{O2} ratio and indicative for the fermentative catabolism over the oxidative catabolism ratio with a value of approx. 1 representing fully oxidative metabolism) of 3.4, showing both respiration and fermentation. A biomass yield on glucose of 18 % was calculated. The data obtained for the *HAP4*↑ strain are in accordance with a previous report (Blom, et al., 2000): it showed a similar growth rate as the wild-type, with a slight reduction in ethanol production, glucose consumption and RQ, resulting in a biomass yield value on glucose that is 30% higher than the value found for the wild type. The absence of the other major global glycolytic regulator, *HXX2*, resulted on the one hand in a growth rate that was significantly lower (0.26 h⁻¹) but on the other hand in a much higher catabolic efficiency, i.e. catabolism was shifted towards respiration as can be deduced from the lower RQ ratio. Indeed, under the conditions tested this strain showed nearly full respiration, concomitant with a doubling of the biomass yield on glucose. Virtually maximal energetic efficiency, i.e. a completely oxidative catabolism resulting in the highest energy conservation rate (Bruinenberg,

1986), with little decrease in growth rate capacity, was obtained with the *hxx2ΔHAP4* strain. This strain had a growth rate of 0.31 h^{-1} and an insignificant ethanol flux and hence an even higher biomass yield of 47%, which is comparable to values found for fully respiring wild type cultures, e.g. as found in glucose-limited chemostat cultures (Bruinenberg, 1986). For all strains, a Y_{ATP} value of $11.5 \pm 1.0 \text{ g} \cdot \text{mol}^{-1}$ was calculated assuming a P/O ratio of 1.2 (Verduyn, et al., 1991). Carbon balances were consistently found to be $110 \pm 1\%$ and therefore suggest a technical error. Even if this is due to a systematic overestimation of the gas flow analysis, none of the calculations can be affected to any significant extent.

Table 1: Physiological characteristics of the wild type and the mutants as determined in aerobic batch cultures.

Strain	$\mu \text{ (h}^{-1}\text{)}$	$q_{\text{etoh}} \text{ (mmol g dw}^{-1} \text{ h}^{-1}\text{)}$	$q_{\text{O}_2} \text{ (mmol g dw}^{-1} \text{ h}^{-1}\text{)}$	RQ	$Y_{\text{glc}} \text{ (\%)}$
Wt	0.36	17.00	7.16	3.4	18
<i>hxx2</i>	0.26	2.40	8.43	1.3	36
<i>hap4</i>	0.35	11.30	6.04	2.9	24
<i>hxx2.hap4</i>	0.31	0.84	9.95	1.1	47

The mutants and the wild-type were grown in aerobic cultures in batch-fermentors as described in Methods and Materials. Fluxes and the maximal specific growth rate were determined during early log growth. The data present typical results as observed for various time spans for one growth experiment and for separate growth experiments. No other products were detectable. The given values are the mean of two independent experiments.

Transcriptome analysis

Transcript profiles were made of samples taken from the mid-log phase at an optical density between 0.8 and 1.0. A comparative analysis of these profiles of the mutants and the wild type strain was carried out. With respect to the central carbon metabolism, (sugar transport, glycolysis, the pentose phosphate pathway, the tricarboxylic acid cycle and the glyoxylate cycle, shown in figure 1) the following interesting points can be seen. In strains lacking *HXX2*, an upregulation of expression of the high affinity glucose transporters *HXT6* and *HXT7*, and a concomitant decrease in expression of the low affinity transporter *HXT1* is seen. Expression of *HXT4*, a glucose transporter with a rather high affinity increased as well, whereas *HXT3* (medium to low affinity) mRNA levels remained unchanged. Apparently, the modification of the regulatory network results in a shift from low-affinity glucose transport to high affinity glucose transport. In addition *HXT5* is upregulated in the *hxx2* strains, whereas under these conditions *HXT5* is completely shut off in the wild-type.

Quite in contrast to the significant changes in the glucose transport machinery, it is seen that the expression of most glycolytic genes is hardly changed by the modification of the level of the general regulators. The only exception seems to be the expression levels of the sugar phosphorylating enzymes *HXX1* and (to a minor extent) *GLK1*, both being strongly upregulated when *HXX2* is absent, but not when only *HAP4* is overproduced.

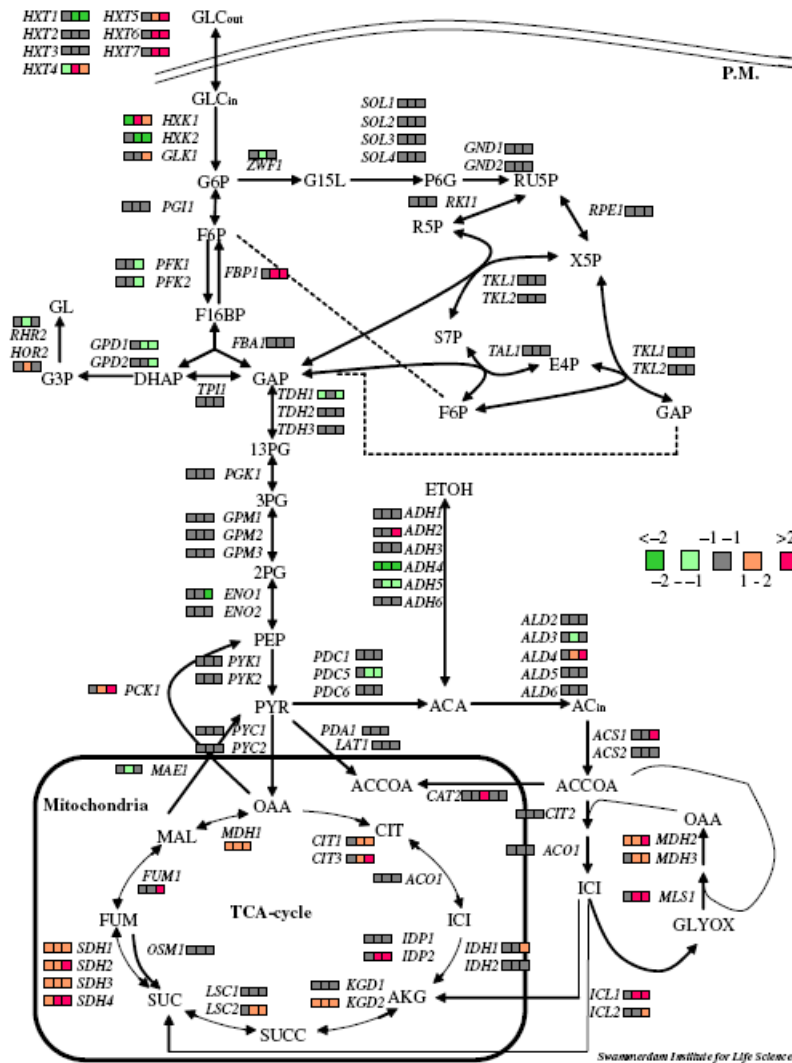


Figure 1: Expression of individual genes within glycolysis/gluconeogenesis, the PP pathway, the glyoxylate cycle and the TCA cycle. Transcriptome data from the early log phase of the mutants and the wild-type were normalized and expression ratios were calculated relative to the Wild-type. Shown here are the expression ratio's of most of genes involved in the depicted pathways. The values are expressed as log₂-ratio's and are relative to the Wild-type. The first squares show the *HAP4*↑/WT ratio, the middle squares show the *hxk2*Δ/WT ratio and the right squares show the *hxk2*Δ*HAP4*↑/WT ratio. The given values are the mean of two independent experiments.

Further noteworthy changes in the upper part of glycolysis include upregulation of *PFK1* and *PFK2* on the one hand and downregulation of *ENO1*, both in the *hvk2ΔHAP4* ↑ strain. More dramatic effects of altered levels of HAP4p and/or *hvk2p* are observed for the lower part of glycolysis; here, of all the alterations, the change in expression of *ADH2* in the *hvk2ΔHAP4* ↑ strain stands out most. It should be noted that *ADH2* is the alcohol dehydrogenase that in the wild type only is expressed after glucose depletion and is associated with gluconeogenic growth. As exemplified by *ADH2* and also *FBP1* there is an upregulation of genes associated with gluconeogenic growth in strains lacking *hvk2p* but not in the strain that only overproduces HAP4p. *PDC5*, *ADH4* and *ADH5* are also downregulated in the *hvk2Δ* strains. *ALD4* is strongly upregulated in the latter cells, presumably to facilitate strains reshufling acetate into the TCA-cycle. The other *ALD* genes remain mainly constant with the exception of a 2-fold downregulation of *ALD3* in the *hvk2Δ* strain.

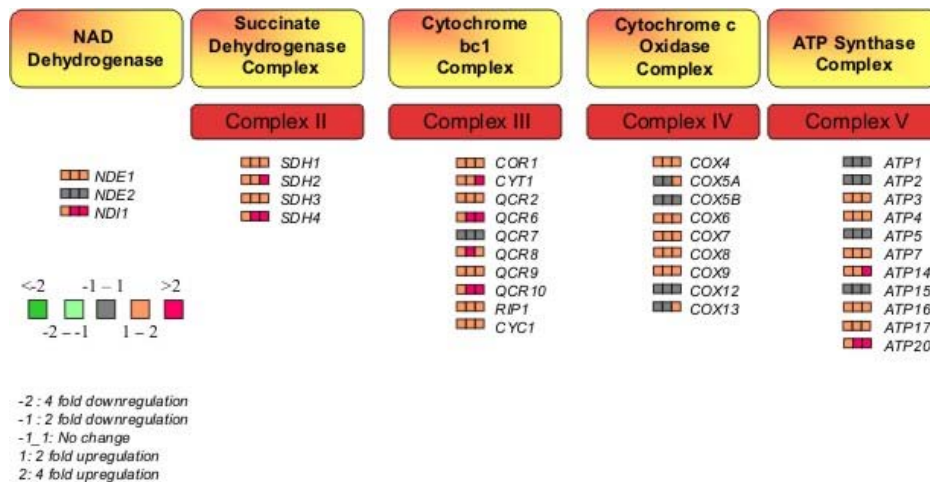


Figure 2: Expression of individual genes within the respiratory chain. Transcriptome data from the early log phase of the mutants and the wild-type were normalized and expression ratios were calculated relative to the Wild-type. Shown here are the expression ratio's of most of genes involved in the depicted pathways. The values are expressed as \log_2 -ratio's and are relative to the Wild-type. The first squares show the *HAP4*↑/WT ratio, the middle squares show the *hvk2Δ*/WT ratio and the right squares show the *hvk2ΔHAP4*↑/WT ratio. The given values are the mean of two independent experiments.

In line with the physiological data, there is a huge increase in mRNA levels of genes encoding the TCA cycle enzymes. In each mutant many of these genes are strongly upregulated. Also in most cases it is a gene encoding a single iso-enzyme, which is strongly increased, whereas the other remains unchanged, due to different repressors and/or inducers interacting with a specific iso-enzyme only. In addition, a strong upregulation of almost all those genes that are involved in oxidative phosphorylation and expressed from the nucleus is observed. As shown in figure 2, although some specific genes remain expressed at the same level, the total expression levels of genes related to each individual component of the respiratory chain (NADH dehydrogenase, complex II, III and IV) and the ATP synthetase is increased. This correlates well with the increased

respiration and to the lowering of ethanol production. Also genes in the glyoxylate cycle were upregulated primarily in the *hvk2Δ* strains, most likely enabling use of fermentation products to re-enter the respiratory chain.

Regulatory Motifs (T-profiler)

A T-profiler analysis was carried out to score the activity of gene groups based on shared regulatory motifs. Essentially, this analysis quantifies the correlation between changes in expression of genes sharing common DNA motifs in their promoter region. Statistical analysis yields so-called *t*-scores to be assigned to such a group of genes with a positive value indicating that these genes are upregulated on average. A *p*-value is assigned by comparing the amount of genes in a group against the total amount of genes. A *p*-value below 0.05 is considered to be significant. A detailed description of the analysis can be found in Boorsma *et al.* 2005.

Table 2: Regulatory motifs identified using T-profiler.

Consensus motif	TF	<i>t</i>	ORFs
<i>hvk2ΔHAP4</i> ↑			
ccgnnnnnccg	CAT8	+5.8	139
ccccrnwwwww	MIG1	+5.05	157
cggggta	MIG1	+4.83	26
ccaatca	HAP4	+4.15	126
cggnnnnnnnnnnnnnnnnccg	ORE	+4.13	124
ctawwwwtg	RLM1	-4.19	97
tcgttta	UPC2	-4.56	245
rvaccctd	RCS1	-4.61	453
accnnnnnggt	ZAP1	-5.38	195
<i>hvk2Δ</i>			
cggggta	MIG1	+5.36	26
ccaatca	HAP4	+4.47	126
cgatgag	PAC	+4.35	245
ccccrnwwwww	MIG1	+4.23	157
accnnnnnggt	ZAP1	-4.04	195
tcgttta	UPC2	-4.08	245
<i>HAP4</i> ↑			
ccaatca	HAP4	+5.41	126
cgatgag	PAC	+5.3	245
accnnnnnggt	ZAP1	-7.48	195

Transcriptome data from the early log phase of the mutants and the wild-type were normalized and expression ratios were calculated relative to the Wild-type. These data were used in T-profiler to identify common motifs in promoter regions that appear to contribute more to expression than the mean. All the motifs that scored at least a *t*-score of -4 or 4, in at least one of the mutants tested are shown

Using T-profiler, it was found that the *hvk2Δ* and *HAP4* ↑ mutant strains had increased levels of transcription of genes containing the general transcriptional activator motifs, known to be bound by PAC. The largest contribution to this increase was found to be due to genes needed for mitochondrial biogenesis and function. As expected we found the HAP4 (CCAATCA) motif in all the mutants and the MIG1 (CGGGGTA) motif was found in all strains, except in the *HAP4* ↑ strain (see table 2). Further, we found strong contributions of the CAT8 and ORE (oleate responsive element) motifs in the *hvk2ΔHAP4* ↑ strain, but surprisingly these motifs are not significantly present in either the *hvk2Δ* or *HAP4* ↑ strains. These two motifs are associated with the derepressed status as found during growth on poor carbon sources or after the diauxic shift. Most surprising, the transcriptomes of the tested mutants showed a very strong negative contribution of the transcription factor of ZAP1, a transcription factor controlling zinc transport and homeostasis.

Analysis of non-redundant functional categories

A further analysis was carried out by using the GO Ontology categories in T-profiler. This methodology analyzes the extent to which genes within assigned functional categories are coregulated. Assignments may be based on the GO, MIPS or KEGG databases. A T-profiler analysis is presented based on the GO Ontology database in table 3.

A strong positive upregulation for both the mitochondrial category and oxidative phosphorylation for all the strains was observed, again showing the direct involvement of *HAP4* in this process. Similarly a strong upregulation of the hexose transporter category in all the strains (except the *HAP4* ↑ strain) was seen, mainly due to the induction of high-affinity hexose transporters (*HXT6-7*). Further, glucose catabolism is significantly downregulated as a whole in the *hvk2Δ* strains, but not in the other strains. This suggests that *HAP4* ↑ does not interfere with glucose catabolism as a functional group and that it is dependent on other regulators under the control of Hxk2p. Finally, it should be noted that the finding of downregulation of genes under control of Zap1p and Rcs1p, is supported by a significant downregulation of the categories involved in transition metal ion transport and siderochrome transport, respectively in the *hvk2Δ* and in the *hvk2ΔHAP4* ↑ mutants.

Table 3: Functional categories according to GO Ontology contributing to the changes in expression identified using T-profiler.

GO Ontology category	<i>t</i>	ORFs
<i>HAP4</i> ↑		
Mitochondrion	+29.88	624
Organellar ribosome	+15.8	73
Nucleolus	+10.32	189
Oxidative phosphorylation	+10.18	27
Ribonucleoprotein complex	+4.85	367
Mitochondrial electron transport chain	+4.68	21
Cellular component unknown	- 6.21	743
<i>hvk2Δ</i>		
Mitochondrion	+17.36	624
Organellar ribosome	+9.92	73
Nucleolus	+9.79	189
Hexose transport	+8.11	16
Oxidative phosphorylation	+6.99	27
Main pathways of carbohydrate metabolism	+4.86	65
Energy pathways	+4.83	180
Hydrolase activity, hydrolyzing O-glycosyl compounds	+4.48	31
Glucose catabolism	- 4.15	23
Amino acid and derivative metabolism	- 6.42	186
Siderochrome transport	- 6.89	9
<i>hvk2ΔHAP4</i> ↑		
Mitochondrion	+21.37	624
Organellar ribosome	+8.2	73
Peroxisomal matrix	+6.98	14
Hexose transport	+5.59	16
Cytoplasm organization and biogenesis	+5.09	670
Electron transport	+5.06	18
Carbohydrate metabolism	+4.62	182
Glucose catabolism	- 4.32	23
Transition metal ion transport	- 4.96	36
Siderochrome transport	- 7.34	9

Transcriptome data from the early log phase of the mutants and the wild-type were normalized and expression ratios were calculated relative to the Wild-type. These data were used in Quontology to identify functional categories that appear to contribute more to expression than the mean. Shown are all the functional categories according to GO Ontology, which scored at least a *t*-score of -4 or 4, in at least one of the mutants tested. *t*-scores of 4 or more and -4 or less are considered significant.

Discussion

The rather subtle regulation of glucose catabolism in *Saccharomyces cerevisiae* is brought about by a complex regulatory network that still is a challenge to understand, despite the fact that it has been a subject of many studies. Here we have quantified the effects of two important catabolic regulators, Hap4p and Hxk2p on the physiology of *S. cerevisiae*. Their overproduction, respectively absence, results in a major redistribution of the carbon flux over fermentative and respiratory pathways. Moreover, their effects are to some extent additive: both in the *hxk2Δ* and the *HAP4* ↑ strain a shift to respiratory catabolism is seen under excess glucose conditions and this is the case even more in the *hxk2ΔHAP* ↑ mutant. Accordingly, the increased energetic efficiency due to respiration results in considerable gain in the biomass yield on glucose (up to 3-fold). Increased yield values on the energy source may either indicate a more efficient mode of energy conservation or a lowered energetic demand for biosynthesis and maintenance. As there is no reason to assume the latter (cell composition presumably does not change and growth conditions being identical), the same Y_{ATP} value for the various strains should be obtained. Assuming a P/O ratio of 1.2 (Verduyn, et al., 1991), indeed, for all strains a value of $11.5 \pm 1 \text{ g} \cdot \text{mol}^{-1}$ was calculated.

Although under the conditions tested here, the growth rate in the *hxk2Δ* strain was significantly affected, it has also been shown that under other conditions (glucose-limited chemostat cultures with simple salt medium) this mutant can grow at a similar rate as the wild type. Apparently the mechanisms to maintain a high μ_{max} are less robust in this strain and it has been shown indeed that the μ_{max} of *hxk2Δ* strain depends on the external glucose concentration (Diderich, et al., 2002). In addition, *HAP4* overexpression appears to restore the growth rate to almost that of the wild-type. The *HAP4* ↑ strain expresses an increased amount of respiratory genes, though this increase is not as massive under these conditions as in the *hxk2Δ* strain. The additive effect of overexpression of *HAP4* in the *hxk2Δ* background suggests that in the latter background the genes needed for mitochondrial biogenesis and function are not induced to their fullest extent, and thus can be increased even more by addition of more active *HAP4*. This increased respiratory capacity may cause the μ_{max} to increase.

Many of the physiological effects seen in the various strains are in good agreement with the analysis of the transcriptome profiles. Thus, the upregulation of gene expression related to the TCA cycle and oxidative phosphorylation corresponds with the increased respiration. More complex relations are seen with respect to the large changes in the glucose transport step, with a clear shift from low affinity transport to high affinity transport. The fact that this shift is absent in the *HAP4* ↑ strain could be explained by induction of the high affinity transport system(s) being brought about by relieving the repression due to increased phosphorylation levels of Mig1p and Mig2p (indirectly caused by the absence of Hxk2p) (Treitel, et al., 1998). Downregulation of low-affinity transport could be interpreted as a response to the increased expression of high-affinity transporters. It can not be excluded though that the control of the glucose sensors Rgt2p/Snf3p may be altered or absent due to this mutation, resulting in a low glucose signal regardless of the extracellular glucose concentration.

It should be emphasized that observed changes in expression levels are not necessarily due to a direct (absence of) interaction of the regulator *per se*: if a regulator acts upstream

in the catabolic pathways, the resulting changes in metabolite concentrations may in turn affect expression of catabolic genes encoding enzymes anywhere in the pathway. For example, increased respiration may cause glycolysis to be somewhat downregulated in all mutants by the accompanying changes in the redox state (or the NADH pool) and the energy state (or the ATP and ADP pool).

Interestingly, both the absence of *hvk2p* and the overexpression of *hap4p* results in a very significant increase in genes associated with growth on non-fermentable carbon sources, e.g. *ADH2* and *FBP1*. This suggests that in the *hvk2* mutants glycolysis and gluconeogenic enzymes, the glyoxylate cycle and the TCA cycle are all present and may be active given proper intermediate levels. In this connection it should be mentioned that co-consumption of ethanol and glucose in the *hvk2* strain has been reported. (Raamsdonk, et al., 2001). The occurrence of simultaneous expression of genes involved in processes with opposing functions but considered to be in the same categories illustrates the need for proper defining functional categories (Koerkamp, et al., 2002).

With respect to the PP Pathway, only minor changes in expression were observed.

Mass flux analyses showed that the flux through the PP pathway is stoichiometrically related to the growth rates due to the need for NADPH as a biosynthetic redox carrier (Maaheimo, et al., 2001) As there are minor changes in growth rate for the various strains tested, PP-fluxes should have changed according to this relationship, however gene expression of the PP Pathway has not significantly changed, suggesting that the necessary changes are either too small to be noted experimentally or they are not regulated at the transcriptional level. More drastic changes were seen for the glyoxylate cycle. Whereas in the wild-type this cycle seems almost non-active during growth on glucose, the *hvk2Δ* strains show an increased expression level of most of the genes involved. Moreover only in the *hvk2ΔHAP4* ↑ strain there's a strong induction of *ACSI* which is needed for formation of Acetyl-CoA to be used either in the TCA-cycle or in the glyoxylate cycle, which also suggests this cycle is active in both strains and is actually both producing and consuming ethanol simultaneously, as has been reported earlier (Raamsdonk *et al.* 2001) On a more global scale we found many DNA-motifs contained in promoter regions of genes changed in expression according to expectation. For instance genes with the CCAATCA (Hap2/3/4/5p) motif were mostly upregulated in all the mutants. On the other hand, genes containing the Mig1 specific CGGGGTA motif were mostly upregulated in all the strains, except the *HAP4* ↑ strain. These findings are well in agreement with the proposed regulatory network involving Mig1p and Hap4p.

A surprisingly strong negative contribution was seen for the motif known to be bound by the Zap1p regulator, which controls zinc transport and zinc homeostasis. This suggests that there is a much lower demand for Zn in strains that overexpress *HAP4*. In accordance, it is observed that the zinc-dependent alcohol dehydrogenase *ADH4* is downregulated in this strain. In addition, the manganese dependent superoxide dismutase *SOD2* was upregulated, thereby diminishing the need for the zinc-dependent *SOD1*, although it should be mentioned that the latter was not found to be downregulated.

In general, the results point towards an additive effect of the two regulators studied.

Again specifically for the *hvk2Δ* and *hvk2ΔHAP4* ↑ strains we found categories associated with growth on alternative carbon sources, such as galactose and also maltose. All the above suggests that glucose repression, and preference for glucose as the carbon and energy source are completely abolished in strains lacking Hvk2p. In other words, the

HXK2 gene is crucial in maintaining glucose repression and its activity forms the basis for the Crabtree-positive phenotype. The absence of repression, respiratory activity and co-consumption of other carbon sources than glucose, combined with the maintenance of high affinity glucose transport characteristics under excess glucose conditions justifies the conclusion that a lack of Hxk2p renders *S. cerevisiae* rather inapt to respond appropriately to extracellular glucose and turns *S. cerevisiae* in a non-adaptive yeast. Our data indicate that the mechanism of control by Hap4p is quite different. Changed levels of this regulator do decrease the Crabtree-positive behavior but do not affect glucose sensitivity. The effect of increased energetic efficiency seems to be solely due to increased expression levels, and hence capacity, of oxidative catabolism.