

# Hap4 Is Not Essential for Activation of Respiration at Low Specific Growth Rates in *Saccharomyces cerevisiae*\*<sup>§</sup>

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In *Saccharomyces cerevisiae*, the heme-activated protein complex Hap2/3/4/5 plays a major role in the transcription of genes involved in respiration. Thus, overexpression of *HAP4* has been shown to result in a 10% increase in the respiratory capacity. Here the physiology of a *HAP4*-deleted *S. cerevisiae* strain was investigated, and we found that the *hap4Δ S. cerevisiae* exhibited poor growth on ethanol, although the growth rate on glucose was indifferent from the wild type in aerobic as well as anaerobic cultures. Moreover, it exhibited a large (75%) reduction in the critical glucose uptake rate at which fermentative metabolism is onset, indicating a substantial reduction in respiratory capacity. We also performed whole genome transcription analysis for the *hap4Δ* and the wild type, grown in carbon-limited chemostat cultures operated at a dilution rate of 0.05 h<sup>-1</sup>. Although both strains exhibited respiratory metabolism, there was significant change in expression of many genes in the *hap4Δ* strain. These genes are involved in several different parts of the metabolism, including oxidative stress response, peroxisomal functions, and energy generation. This study strongly indicates that Hap4 activation only occurs at intermediate specific growth rates, below which the transcription of genes responsible for respiration is dependent on the Hap2/3/5 complex and above which the Hap4 protein augments the transcription. Furthermore, statistical analysis of the transcription data and integration of the data with a genome scale metabolic network provided new insight and evidence for the role of Hap4 in transcriptional regulation of mitochondrial respiration.

Respiration plays a central role in providing Gibbs free energy required for growth and overall cellular function. The respiratory chain is closely coupled with the operation of the tricarboxylic acid cycle as it ensures oxidation of NADH generated in this cycle. Proper functioning of respiration is therefore essential not only for providing Gibbs free energy but also for operation of the tricarboxylic acid cycle. Consequently, many metabolic diseases result either directly or indirectly from altered functioning of respiration. Moreover, respiration and energy metabolism are of particular interest in metabolic engineering, where the objective may be to design new cell factories for production of chemicals that demand energy for their synthesis. The yeast *Saccharomyces cerevisiae* is not only an important cell factory for production of fuels, chemicals, and pharmaceuticals but also serves as an important

eukaryotic model organism for studying human diseases, thus making it an attractive model for studying respiration.

In *S. cerevisiae*, the heme-activated protein complex Hap2/3/4/5 plays a major role in orchestrating the transcription of genes involved in the tricarboxylic acid cycle, the electron transport chain, ATP generation, and mitochondrial biogenesis by binding to the CCAAT box at the upstream activation sequence of genes encoding enzymes of these pathways (1). Binding is believed to take place at the consensus sequence TNRTTGGT, and it is primarily found to occur at relatively low glucose uptake rates, *i.e.* when there is solely respiratory metabolism on glucose, and during growth on carbon sources that require respiratory metabolism, *e.g.* ethanol, acetate, and glycerol (2). The DNA binding capability of the Hap complex is conferred by the Hap2/3/5 proteins, and Hap4 enhances their activity via its activation domain. Hap4 has also been found to possess at least two distinct trans-activation domains, each of which has different levels of dependence on co-activation proteins (3). Furthermore, *in vitro* experiments have shown that the mechanism involved in translation of *HAP4* mRNA shifts from the cap-dependent to completely IRES<sup>2</sup> (4)-dependent translation at derepressed conditions. *In vivo* experiments have confirmed that the IRES-dependent translational efficiency was at a low level at the beginning of the stationary growth phase and was enhanced during the glucose-exhausted phase, indicating that there is an increase in the level of the Hap4 protein under glucose-derepressing conditions due to a shift toward IRES-dependent translation (5).

Although the other members of the complex are constitutively expressed, the expression of *HAP4* is regulated by the carbon source and is up-regulated manifold upon glucose exhaustion (6). Glucose represses the expression of *HAP4* via the Mig1 pathway and thereby activation of respiration is prevented at high glucose concentrations. However, loss of function of Mig1 alone does not result in constitutive activation of respiration and the tricarboxylic acid cycle<sup>3</sup> (7). Under fermentative conditions, yeasts have compromised mitochondrial function, and the control of the tricarboxylic acid cycle genes might take place by a synergistic action of the retrograde genes (retrograde signaling becomes operative when cells sense dysfunctional mitochondrion) and the Hap complex. The transcription factor Rtg2, for example, is known to up-regulate three genes of the tricarboxylic acid cycle, *viz.* *CIT1*, *ACO1*, and *IDH1*, and the anapleurotic reaction toward oxaloacetate encoded by *PYC2*, to match the increased requirement for precursor metabolites for amino acid biosynthesis (8–10).

Even though the name of the gene suggests that Hap4 is activated by heme, neither the heme nor the oxygen regulation of the Hap complex is clearly understood. Tai *et al.* (11) report that *HAP4* mRNA is present in carbon-limited cultivations even under anaerobic conditions where

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<sup>2</sup> The abbreviations used are: IRES, internal ribosome entry site; WT, wild type.

<sup>3</sup> S. L. Westergaard, A. P. Oliveira, C. Bro, L. Olsson, and J. Nielsen, submitted for publication.

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<http://www.jbc.org/cgi/content/full/M512972200/DC1>

Hap4 has no obvious role. The promoter of *HAP4* has three carbon source responsive elements (12), which require a functional Cat8 for the activation (a transcription factor that activates gluconeogenic genes); however, deletion of *CAT8* had no effect on the steady state level of *HAP4* expression (13). It has not been proven *in vivo* that Mig1 binds to the *HAP4* promoter even though it is generally accepted that *HAP4* expression is under glucose repression via Mig1. Moreover, deletion of *MIG1* together with *HAP4* overexpression was found to result in further derepression of the *SUC2* gene compared with a *mig1Δ* control (14), indicating a possible role of Hap4 in Mig1-mediated glucose repression.

Overexpression of *HAP4* has been shown to result in a 10% increase in the respiratory capacity (seen as an increase in the dilution rate in a chemostat culture at which there is onset of fermentation), implying that Hap4 activity is limiting the respiratory metabolism (15). The increased level of respiration because of overexpression of *HAP4* has been shown further to result in an extended life span of yeast (16). Homologues of *HAP4* have also been found in many related and distant yeasts. In *Kluyveromyces lactis*, expression of *HAP4* is constitutive, and deletion resulted in no distinct phenotype on respiratory substrates (17), whereas in *S. cerevisiae* deletion of *HAP4* almost abrogates growth on glycerol or ethanol. By genome-wide transcription analysis of deletion mutants of *HAP2* and *HAP4*, it has been found that a number of genes related to mitochondrial biogenesis and translation had changed expression (17). The study was based on shake flask cultivations in YPGalactose medium, to prevent repression of the respiratory genes by the use of glucose. However, there have not been any studies on the effect of deleting *HAP4* during growth on glucose. We therefore conducted a characterization and transcription analysis of a *HAP4* deletion mutant grown on a minimal medium at defined physiological states (chemostat cultures). The microarray data were also analyzed by using an integrative algorithm (18) for identification of reporter metabolites (metabolites around which the most significant expression changes occur, see "Materials and Methods" for detailed description) and metabolic subnetworks (connected metabolic subnetworks with significant collective transcriptional response, see "Materials and Methods" for detailed description).

## MATERIALS AND METHODS

**Strains**—*S. cerevisiae* strains employed in the study were CEN.PK 113-7D (wild type (WT); *MATa MAL2-8c SUC2*) and CEN.PK 517-1A (*MATa MAL2-8c SUC2 HAP4::loxP-Kan-loxP*). The strains were stored at  $-80^{\circ}\text{C}$  suspended in yeast extract, peptone, and dextrose medium containing 20% glycerol. Before each experiment, cells from the stock were plated out to YPD medium and incubated at  $30^{\circ}\text{C}$  for 24 h before use.

**Batch Fermentations**—The shake flask and the fermentation medium employed were the same and were prepared according to Ref. 19. A single colony was inoculated from a fresh YPD plate to the shake flask and kept in an orbital shaker set at 150 rpm and at a temperature of  $30^{\circ}\text{C}$  for 24 h. The medium used in the fermentor was exactly the same as the inoculation medium except that the batch cultivations had 20 g/liter glucose and the anaerobic medium was supplemented with ergosterol and Tween 80 (20). Batch experiments were carried out in a Braun Biostat B reactor with a working volume of 2 liters. The temperature was controlled at  $30^{\circ}\text{C}$ . The aeration rate employed for the batch cultivation was 0.5 volume of air/volume of liquid volume/min, whereas the anaerobic culture was sparged with  $\text{N}_2$ , both of which were filtered through a  $0.22\text{-}\mu\text{m}$  sterile membrane filter. The pH was controlled at 5 by the automatic addition of 2 M KOH. The agitation was maintained at 800 rpm. The culture vessel was inoculated with an initial absorbance ( $\lambda =$

600 nm) of 0.02 from an exponentially growing shake flask culture. The exhaust gas was passed through a condenser, maintained at a temperature of  $6^{\circ}\text{C}$  by circulating cooled water, before entering the gas analyzer. 15-ml samples were taken for biomass, optical density, and metabolite analysis. The concentration of carbon dioxide and oxygen in the exhaust gas was determined by use of an acoustic gas analyzer (Brüel & Kjær, Denmark).

**Chemostat Cultivations**—The chemostat experiments were also carried out in Braun Biostat B (with all the process variables maintained same as mentioned in the aerobic batch) but with a working volume of 1 liter, initially as a batch mode for 24 h until glucose depletion and then switched to a chemostat mode, at a dilution rate of  $0.05\text{ h}^{-1}$  by continuous addition of medium (feed concentration of glucose, 10 g/liter). 15-ml samples were taken for biomass, optical density, and metabolite analysis every 20 h (time taken for 1 volume change). Steady state was assumed to be reached after at least 5 volume changes had passed since the last change in growth conditions, and when the  $\text{CO}_2$  evolution and the biomass concentration had remained constant during at least two volume changes ( $\pm 3\%$ ).

**Productostat**—A productostat was used to obtain a steady state dilution rate that corresponds with a given ethanol concentration. Ethanol was measured online by a sensor placed in the outlet gas stream. The sensor used (measuring the content of reduced gases) was the Figaro TGS 822 (Hammer Electronic, Elsinore, Denmark). The set point (1 V) for the voltage of the sensor was found to correlate with an ethanol concentration in the cultivation broth of 30–60 mg/liter. The dilution rate controller was a proportional-integral controller with a nonlinear error term to account for the nonlinear behavior of the ethanol concentration around the critical dilution rate (21). The bioreactor was operated as batch cultivation for  $\sim 24$  h until glucose depletion, and then it was shifted to chemostat operation. When there was no more ethanol in the fermentation medium, operation of the bioreactor shifted to productostat mode. In this mode the controller increased the dilution rate until there was a stable low ethanol measurement, which could be maintained for several residence times with a very low standard deviation.

**Biomass Determination**—A 5-ml sample in duplicate was filtered using a pre-dried, pre-weighed  $0.45\text{-}\mu\text{m}$  filter and washed with distilled water. The filter with wet biomass was dried in a microwave oven at 150 watts for 15 min. The biomass concentration was calculated from the difference of the weights.

**Extracellular Metabolite Determination**—The glucose concentration during the course of the fermentations was analyzed using a 1-ml sample from the reactor, which was filtered immediately through a  $0.45\text{-}\mu\text{m}$  filter (Frisenette ApS, Denmark). Glucose, glycerol, succinate, and ethanol were detected by measurement of the refractive index, whereas acetate and pyruvate were determined by a UV detector, in a Waters high pressure liquid chromatography fitted with a Bio-Rad HPX-87H column and maintained at a temperature of  $60^{\circ}\text{C}$ , using an autosampler. The eluent used was 5 mM  $\text{H}_2\text{SO}_4$ , at a flow rate of 0.6 ml/min.

**Transcription Analysis**—Metabolic oscillations were persistent in the *HAP4*-deleted strain, and we were unable to avoid the oscillations. All samples were therefore taken when the  $\text{CO}_2$  partial pressure in the exhaust gas exhibited a maximum. To evaluate the possible effect of the oscillations, a single sample was also taken when the  $\text{CO}_2$  partial pressure in the exhaust gas exhibited a minimum. Three 20-ml samples were taken from each steady state (and as mentioned one additional sample for the oscillating culture) using a syringe, injected directly into a Falcon tube (precooled in liquid  $\text{N}_2$ ), and spun for 5 min at 4000 rpm. The supernatant was discarded, and the pellet was stored at  $-80^{\circ}\text{C}$  until further use. Total RNA extraction was performed with FastRNA<sup>®</sup> Pro

TABLE 1

Physiological parameters obtained from duplicate batch cultivations with the *hap4Δ* mutant

In the exponential growth phase, the yield coefficients for biomass, ethanol, pyruvate, glycerol, and acetate were determined from linear regressions of their concentration as a function of the residual glucose concentration. ND indicates not detected.  $\mu_{\max}$  indicates the maximum specific growth rate at  $\text{h}^{-1}$ .  $Y_{\text{sx}}$  indicates grams of biomass per g of glucose consumed.  $Y_{\text{sac}}$  indicates grams of acetate per g of glucose consumed.  $Y_{\text{se}}$  indicates grams of ethanol per g of glucose consumed.  $Y_{\text{sppyr}}$  indicates grams of pyruvate per g of glucose consumed.  $Y_{\text{sg}}$  indicates grams of glycerol per g of glucose consumed.

Strain	Condition	$\mu_{\max}$	$Y_{\text{sx}}$	$Y_{\text{sac}}$	$Y_{\text{se}}$	$Y_{\text{sppyr}}$	$Y_{\text{sg}}$	Ref.
<i>hap4Δ</i>	Aerobic	0.27	0.11	0.0022	0.38	0.0049	0.081	
<i>hap4Δ</i>	Anaerobic	0.3	0.11	ND	0.41	0.0027	0.1	
WT	Aerobic	0.31	0.10	0.0042	0.34	ND	0.073	37
WT	Anaerobic	0.33	0.10	0.0058	0.36	0.0029	0.097	38

Red kit (Qiagen) following the manufacturer's instructions with minor modifications. cRNA was synthesized as described in the Affymetrix GeneChip® Expression Analysis Manual, after which 15  $\mu\text{g}$  were hybridized to Yeast Genome S98 oligonucleotide arrays (Affymetrix). Microarrays were scanned in an Agilent gene array scanner (Affymetrix), followed by raw data processing, which was performed with the Microarray Suite software, version 5.0, with a global scaling factor for target intensity equal to 500. Average difference values, representing the absolute hybridization intensities, were then calculated for each probe set. The microarray data were first normalized using dChip software suite (dChip, version 1.3; Wong Laboratory, Harvard School of Public Health and Dana-Farber Cancer Institute, Boston). Expression levels of all 9335 probe sets were calculated with the Perfect Match model using dChip version 1.3. From the 9335 probe sets in the array, the expression level of 6079 annotated unique open reading frames from the *Saccharomyces* Genome Data base were extracted. Using the absent/present call as calculated by GeneChip® Operation software, transcripts found to be absent in all arrays were excluded. Hence, the forthcoming analyses were performed on the remaining 5814 transcripts. Significance of expression change was calculated in terms of  $p$  values for all the genes using Student's  $t$  test.

**Reporter Metabolite and Subnetwork Analysis**—Gene expression changes in response to *HAP4* deletion were mapped on the genome scale metabolic model of *S. cerevisiae* (22) in order to identify metabolic hot spots with respect to transcriptional regulation. The analysis involved identification of so-called reporter metabolites, defined as metabolites around which significant transcriptional changes were observed, and enzyme subnetworks with maximum transcriptional response. All enzymes/reactions in the genome scale metabolic model were scored based on the significance value of change in the expression of the corresponding gene by using the  $t$  test. Metabolites were then scored based on the average score of its  $k$  neighboring enzymes, and corrected for the number of neighbors and background distribution by subtracting the mean and dividing by the standard deviation of average scores of 10,000 randomly selected enzyme groups of size  $k$ . The corrected score was then converted to the  $p$  value by using normal cumulative distribution. Top scoring metabolites were selected for further analysis. To uncover the global coordinated response of the genes across different pathways, the genome scale model was first converted to an enzyme interaction graph where enzymes were represented as nodes and metabolites as interaction. Enzyme subnetwork(s) (any connected set of enzymes) with maximum average score (corrected for the background distribution and size) were then identified by using a simulated annealing algorithm.

## RESULTS

**HAP4 Deletion Mutant Is Phenotypically Indifferent from WT in Aerobic and Anaerobic Batch Cultivation for Growth on Glucose**—To evaluate whether Hap4 influences unrestricted growth on glucose, duplicate batch fermentations with the *hap4Δ* strain under aerobic as well as anaerobic conditions were carried out with 20 g/liter glucose in a min-

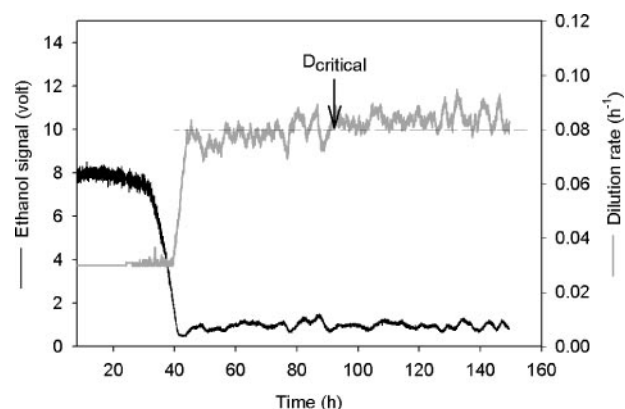


FIGURE 1. Dilution rate and ethanol sensor signal as a function of time in a productostat experiment. The signal from off-gas ethanol sensor was used to control the operation of a chemostat at the critical dilution rate. Dilution rate was slowly increased starting from a low value ( $0.03 \text{ h}^{-1}$ ) and then maintained at the value where ethanol was barely detected in the off-gas. This dilution rate corresponds to  $D_{\text{crit}}$ .

imal medium (see "Materials and Methods"). From these experiments the maximum specific growth rate and the overall yield coefficients, *i.e.* biomass formation and ethanol production per unit of glucose consumed, were determined. The results (Table 1) show the characteristic growth behavior of *S. cerevisiae* at high glucose uptake rates, *i.e.* there is fermentative metabolism with most of the carbon being directed toward ethanol with concomitant secretion of pyruvate, acetate, glycerol, and succinate. The yield of biomass was about 0.1 g per g of glucose consumed, both at aerobic and anaerobic growth conditions. The results show that deletion of *HAP4* had little or no effect on the specific growth rate, compared with the wild type both aerobically as well as anaerobically. Similarly, as the yield coefficients are almost the same for the two strains, there is no major shift in overall flux distribution upon deletion of *HAP4*.

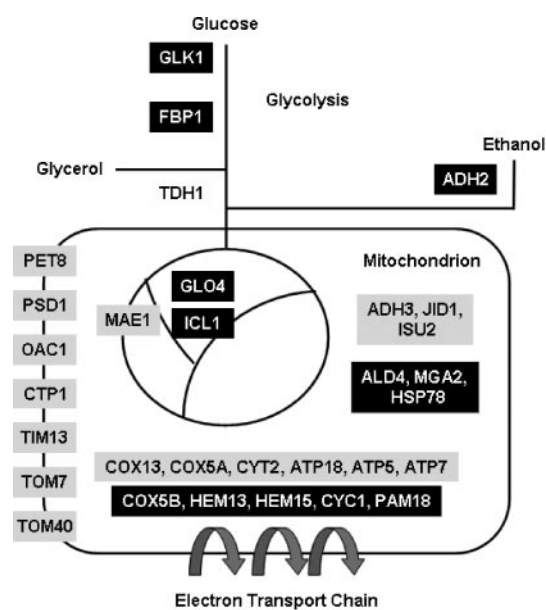
**HAP4 Deletion Reduces the Critical Growth (Dilution) Rate by Almost 75%**—Productostat experiments revealed that the *hap4Δ* strain has a critical dilution rate of  $0.083 \text{ h}^{-1}$  (Fig. 1) compared with  $0.3 \text{ h}^{-1}$  for the WT (15). However, as the *hap4Δ* strain could show completely respiratory metabolism at very low glucose uptake rates, it can be concluded that Hap4 is not solely responsible for activation of respiration.

***hap4Δ* Cells Exhibit Respiratory Growth at a Dilution Rate of  $0.05 \text{ h}^{-1}$** —To understand better the respiratory behavior of the mutant at dilution rates below the critical dilution rate, we performed whole genome transcription analysis in glucose-limited chemostat cultures operated at a dilution rate of  $0.05 \text{ h}^{-1}$ . The respiratory growth at this dilution rate is characterized by a low glucose uptake rate, production of biomass and  $\text{CO}_2$ , and not measurable amounts of ethanol and other metabolites (Table 2). In the chemostat cultures with the *hap4Δ* mutant there was sustained oscillations (observed from the  $\text{CO}_2$  measurements in the off gas) within a period of about 5.5 h (oscillations were not observed at dilution rates above the critical dilution rate). Oscillations

**TABLE 2****Physiological parameters observed during the chemostat cultivations**

Values represent the mean  $\pm$  S.D. of data from three independent chemostat cultivations for *hap4* $\Delta$  and from two independent chemostat cultivations for wild type.  $Y_{sx}$  indicates grams of biomass per g of glucose consumed.  $q_s$  indicates millimoles of glucose consumed per g of biomass per h.  $q_{CO_2}$  indicates millimoles of carbon dioxide formed per g of biomass per h.  $q_{O_2}$  indicates millimoles of oxygen consumed per g of biomass per h.  $D$  indicates dilution rate per h.  $RQ$  indicates respiratory quotient,  $q_{CO_2}/q_{O_2}$ .

	$Y_{sx}$	$q_s$	$q_{CO_2}$	$q_{O_2}$	$D$	$RQ$
WT	0.48 $\pm$ 0.00	0.51 $\pm$ 0.00	1.2 $\pm$ 0.00	1.2 $\pm$ 0.04	0.045 $\pm$ 0.00	0.98 $\pm$ 0.02
<i>hap4</i> $\Delta$	0.48 $\pm$ 0.00	0.57 $\pm$ 0.00	1.46 $\pm$ 0.00	1.69 $\pm$ 0.12	0.049 $\pm$ 0.00	0.87 $\pm$ 0.05



(A)

Process / Function / Component	Genes
Amino acid metabolism	AAT2, GCV3, BTS1, HIP1, FSH1, SER1, GDH1, MET12
Peroxisome	EHD3, FAA1, IDP3, POX1, YPC1
	BTS1, CRD1, EHT1, ELO1, GPI16, LCB1, PSD1, STT4, SUR2, SUT2, TUB1, AR07
Cytoskeleton	ARC15, FUS2, FUS3, MDM30, MSB1, BIG1, AXL2, PAN1
Regulators	SHP1, MIG2, NRG2, HAP4
	GAL80, CBS2, CBP1, PET111
Genome maintenance	HML1, UTH1, RAD59, POL32, EXO1, REX2
Response to Stress	ALD3, DAK2, GAD1, GRE1, HSP26, HSP78, MGA2, MSH2, POL4, SOD2, TDP1, CUP1-1, GTT1
	ORM1, UBC4, ADH4, SMF2

(B)

**FIGURE 2. Schematic representation of selected genes that were up- or down-regulated in different parts of the metabolism as a consequence of HAP4 deletion in *S. cerevisiae*.** A, genes belonging to the central carbon metabolism are placed in a schematic sketch of the glycolysis, the tricarboxylic acid cycle, and the glyoxylate cycle (which is located in the cytosol). B, other genes are placed in the table with indication of their corresponding cellular function, location, or process. Up-regulated genes are placed inside gray boxes, and down-regulated genes are placed in black boxes.

started after the washout of ethanol that was produced during the initial batch phase. Normally oscillations occur at low dilution rates in a carbon-limited chemostat, even in WT, and are prevented by briefly imposing oxygen limitation. With the *hap4* $\Delta$  mutant it was not possible, however, to avoid the sustained oscillations by introducing small perturbations to the system.

**Transcriptional Profiling during Respiring Conditions**—The use of chemostat cultures enabled analysis of the role of Hap4 in respiratory metabolism at low glucose uptake rates. For a  $p$  value cut-off of 0.01 and 0.05, there were 90 and 390 genes significantly changed, respectively. Of the 390 genes, 122 had no known function (supplemental Table S1). The significantly changed genes with known functions are depicted in Fig. 2. Changes in mRNA expression were found to span several different parts of the metabolic network, including carbohydrate metabolism, energy generation, mitochondrial dynamics, amino acid metabolism, stress response and detoxification, and DNA repair.

**Reporter Metabolites and Subnetwork Analysis Reveal Changes Associated with Redox, Tricarboxylic Acid Intermediates, and Ethanol**—To further analyze the transcription data in the context of the whole metabolism, we used an algorithm reported by Patil and Nielsen (18) to identify small but biologically significant and coordinated changes following deletion of HAP4. Reporter metabolites were calculated as the metabolites around which the most significant and coordinated expression changes occurred in the HAP4-deleted mutant as compared with the WT (Table 3). This list highlights metabolites involved in the tricarboxylic acid cycle (e.g.  $\alpha$ -keto-

glutarate and isocitrate) and metabolites playing an important role in redox balancing and fermentative pathway in yeast (e.g. NADH, acetaldehyde, and ethanol). Interestingly, the top scoring metabolite found is  $CO_2$ , indicating major transcriptional changes in the genes involved in the oxidation/respiration. Certain metabolites related to amino acid and nucleotide metabolism also appear on the list, showing the multitude of effects HAP4 deletion caused in reprogramming the mRNA expression of the metabolic network.

Consistent with these findings, the significantly co-regulated metabolic subnetwork (Table 4) also consisted of many genes related to mitochondrial respiration and redox metabolism. This subnetwork spans many branches of the metabolism indicating the tight connection of regulatory mechanisms across them. This is expected, as changes in redox genes will demand readjustment of many cellular pathways to adjust metabolite levels to the new steady state or to maintain homeostasis. Notably, certain transport-related genes (*YOR071C*, *HIP1*, and *HXT16*) are also part of this subnetwork supporting this hypothesis.

**Possible Effect of Oscillations on the Transcription Levels**—Although we analyzed only one sample from the bottom (groove) of the oscillation period, the high correlation of the transcription levels in this sample with the transcription levels in the other replicates (supplemental Fig. S4) enabled us to compare, at least qualitatively, the changes in the expression levels during oscillations. Among the metabolic genes, only about 30% of the genes showed more than 2-fold change in the expression when expression at the peak and the groove are compared (supplemental Fig. S2). Of these only 14% of the genes (thus, around 4% of all

**TABLE 3**

Reporter metabolites with significant normalized transcriptional response of the neighboring genes (see "Materials and Methods" for detailed description) while comparing expression in wild type and *hap4Δ* strain

Only the top scoring 20 metabolites in the decreasing order of their scores are shown.

Metabolite	No. of neighbors
CO <sub>2</sub>	37
( <i>RS</i> )-Lactoylglutathione	1
Glutathione	1
5'-Phosphoribosyl- <i>N</i> -formylglycinamide	2
Acetaldehyde	12
NADH	41
Ethanol	5
5'-Phosphoribosylglycinamide	2
Acetaldehyde	3
Coproporphyrinogen	2
Cytosine	4
Citrate	2
Isocitrate	5
2-Oxoglutarate	21
Methylglyoxal	1
Guanine	6
D-Glucosamine 1-phosphate	1
Heme	1
Malate	5
Palmitoyl-CoA	2

metabolic genes) have *p* values less than 0.05 in comparison between wild type and peak. Moreover, about 60% of those genes also showed more than 2-fold expression change in comparison between wild type and groove. We therefore conclude that there is no (or only little) possible oscillation-dependent bias reflected in the results.

**DISCUSSION**

From the physiological data it was found that the *hap4Δ* mutant exhibited only a subtle difference in the maximum specific growth rate under both aerobic as well as anaerobic conditions. During the batch cultivations the external glucose was sufficiently high to cause repression of the respiratory genes, and glucose was therefore primarily metabolized by the fermentative pathway producing ethanol and other byproducts. When glucose is exhausted, the metabolism is remodeled (known as diauxic shift) so that the ethanol present in the medium can be utilized as carbon and energy source. Hap4 is required for activation of genes required for ethanol catabolism. However, as the mutant lacks Hap4, it did not consume ethanol even 10 h after the shift (data not shown), confirming the role of Hap4 in activating catabolism of respiratory substrates like ethanol.

To evaluate the effect of *HAP4* deletion on the respiratory capacity, we determined the critical dilution rate of the *hap4Δ* mutant. It was found that the respiratory capacity was substantially reduced, resulting in a critical dilution rate of about 0.08 h<sup>-1</sup>, compared with 0.3 h<sup>-1</sup> for the WT (15). Thus, in the mutant overflow metabolism toward ethanol occurs at much lower glycolytic fluxes compared with the WT. The onset of overflow metabolism at lower glycolytic fluxes might be triggered by the inability of the cells to oxidize cytosolically derived NADH due to lack of Hap4-induced respiration. However, as the cells are able to respire even without Hap4 at dilution rates below 0.08 h<sup>-1</sup>, it is compelling to infer the following: 1) Hap4 might play a role in activating respiration only for specific growth rates between 0.08 and 0.3 h<sup>-1</sup>; 2) Hap4 is not essential for complete on/off expression of tricarboxylic acid cycle genes, as deletion of *HAP4* does not result in complete shutting down the expression of tricarboxylic acid cycle and respiratory genes. However as the growth on ethanol for the *HAP4* mutant is abrogated, it is evident that Hap4 plays a role in the catabolism of respiratory substrates.

The *hap4Δ* mutant had sustained oscillations in glucose-limited chemo-

**TABLE 4**

Genes identified as part of the metabolic subnetwork with maximum normalized transcriptional response (see "Materials and Methods" for detailed description) while comparing expression in wild type and *hap4Δ* strain

Gene name/open reading frame	Enzymatic function	<i>p</i> value
<i>AAT2</i>	Aspartate aminotransferase	0.0052
<i>ADE5,7</i>	Phosphoribosylamine-glycine ligase	0.1504
<i>ADE8</i>	Phosphoribosylglycinamide formyltransferase	0.0034
<i>ADH2</i>	Alcohol dehydrogenase II	0.0023
<i>ADH4</i>	Alcohol dehydrogenase IV	0.0278
<i>ALD4</i>	Mitochondrial aldehyde dehydrogenase	0.0005
<i>CAR2</i>	Ornithine aminotransferase	0.0027
<i>CPA2</i>	Carbamyl-phosphate synthetase	0.0320
<i>CTP1</i>	Mitochondrial inner membrane citrate transport protein	0.0240
<i>DAK2</i>	Dihydroxyacetone kinase	0.0004
<i>FAA1</i>	Long chain fatty acid CoA ligase	0.0198
<i>FCY1</i>	Cytosine deaminase	0.0180
<i>FDH1</i>	Formate dehydrogenase	0.0010
<i>GLK1</i>	Glucokinase	0.0361
<i>HEM13</i>	Coproporphyrinogen III oxidase	0.0201
<i>HIP1</i>	Histidine permease	0.0136
<i>HXT16</i>	Hexose transporter	0.0264
<i>IDP3</i>	NADP-dependent isocitrate dehydrogenase	0.0099
<i>LCB1</i>	Serine C-palmitoyltransferase	0.0007
<i>LYS1</i>	Saccharopine dehydrogenase	0.0099
<i>MAE1</i>	Mitochondrial malic enzyme	0.0112
<i>MET12</i>	Methylenetetrahydrofolate reductase	0.0302
<i>MET2</i>	Homoserine O-acetyltransferase	0.0262
<i>OAC1</i>	Mitochondrial oxaloacetate transporter	0.0067
<i>PMP1</i>	Plasma membrane proteolipid (ATPase)	0.0259
<i>PNP1</i>	Purine nucleoside phosphorylase	0.0206
<i>PSD1</i>	Phosphatidylserine decarboxylase	0.0174
<i>SAH1</i>	Adenosylhomocysteinase	0.0226
<i>SER1</i>	3-Phosphoserine transaminase	0.0198
<i>SUR2</i>	Hydroxylase involved in sphingolipid metabolism	0.0253
<i>TDH1</i>	Triose-phosphate dehydrogenase	0.0086
<i>TRR1</i>	Thioredoxin reductase	0.0118
<i>YOR071C</i>	Thiamine transporter	0.0102
<i>YPL276W</i>	Formate dehydrogenase	0.0163

stat cultivations, and we were unable to prevent these. Liu *et al.* (23) showed that the level of the glycolytic enzyme glyceraldehyde dehydrogenase fluctuated in concert with metabolic oscillation, and deletion of the gene *TDH1* encoding this enzyme resulted in the disappearance of the oscillation. In this study we found that *TDH1* is up-regulated in the mutant compared with the WT, and deletion of *HAP4* may therefore destabilize the energy metabolism resulting in a stress response under respiratory conditions. This may result in the appearance of sustained oscillations.

Transcriptional analysis of the *hap4Δ* strain also provided information on the role of Hap4 in heme-dependent transcriptional regulation in *S. cerevisiae*. Heme is a critical cofactor for all living cells as it is involved in many biological processes, including oxidative metabolism, xenobiotic detoxification, gene regulation at the level of transcription, protein translation and targeting, and maintaining protein stability (24). Most living organisms synthesize heme in response to oxygen, although there are exceptions like the pathogenic prokaryote *Staphylococcus aureus* and the eukaryotic helminth *Caenorhabditis elegans*, which extract heme from their host (25, 26). *S. cerevisiae* makes heme in response to oxygen, and it is proposed that heme then activates the Hap complex which in turn reduces the expression of heme biosynthetic genes to dampen the expression of

HEM genes (24). This is supported by our study where several HEM genes were up-regulated upon disruption of HAP4.

Among the other genes up-regulated in the mutant are *OAC1*, *MAE1*, and *AAT2*. These three genes code for an inner membrane oxaloacetate transporter, mitochondrial malic enzyme, and cytosolic aspartate aminotransferase that uses  $\alpha$ -ketoglutarate and aspartate as substrates to yield glutamate and oxaloacetate, respectively. The combined action of these three enzymes may serve as a redox system for formation of NADPH in the mitochondria. Thus, cytosolic oxaloacetate formed by the action of Aat2 can be transported into the mitochondria with the help of Oac1, where the mitochondrial malic enzyme could convert malate (formed from oxaloacetate) to pyruvate with co-current formation of NADPH.

Several genes directly involved in respiration were also found to be up-regulated. Thus, the hypoxic genes, *COX5B* and *COX13*, and two genes in the heme biosynthetic pathway, viz. *HEM13* and *HEM15*, were found to have increased expression in the mutant. Another gene in this group is *CYC1*, which is a known target of both HapI and the HapII/3/4/5 complex. *CYT2* (cytochrome  $c_1$  heme lyase) expression was, however, decreased in the HAP4-deleted cells, but this gene is also known to be regulated by the carbon source. However, in contradiction to our findings, Zollner *et al.* (27) reported that neither the Hap complex nor Mig1 play a role in its regulation, but this may be due to the fact that our study was performed at derepressed conditions. Hypoxic genes are known to be repressed by Rox1 whose expression is positively regulated by HapI, when cells have abundant oxygen supply. The fact that the hypoxic isoforms of respiration had increased expression when HAP4 was deleted shows that the cells respond as they experience oxygen limitation, even though the cells are actively respiring and there is excess oxygen available. It implies that there is some type of cross-talk between HapI and Hap4, *i.e.* the transcription of the hypoxic isoforms of their aerobic counterparts helps to increase the turnover number of enzymes participating in the electron transport chain (28).

A defect in respiration generally causes down-regulation of most of genes involved in the defense mechanisms counteracting free radicals resulting from respiration and consequent cellular damage. Some of these regulatory genes also had altered expression levels in the HAP4 deletion mutant. *SOD2*, which encodes a superoxide dismutase, was down-regulated in the mutant. *SOD2* is a known target of HapI and HapII/3/4/5 and is also known to be regulated in a heme-dependent manner (29). Down-regulation of Thi4, which has a role in thiamine biosynthesis as well as in mitochondrial DNA maintenance (30), might indicate that a defect in respiration could cause mitochondrial genome instability. Four DNA repair genes were also found to be significantly up-regulated (supplemental Table S1) thus supporting this hypothesis.

Certain key transcriptional regulators were also found to be responding to the deletion of HAP4, including the transcriptional repressors Mig2 and Nrg2 that are known to be active only under glucose-repressing conditions (31, 32). Even though both the WT and the mutant were grown in glucose-derepressing conditions and expression of Mig2 and Nrg2 was at a low level, both genes were significantly down-regulated in the mutant compared with the WT. Furthermore, some of the targets of Adr1 and Cat8 were down-regulated significantly. This could indicate that Hap4 has an indirect role in the regulation of Adr1- and Cat8-regulated genes. It has been shown that the carbon source responsive elements of HAP4 require a functional Cat8, but the deletion of *CAT8* had no effect on the transcription of HAP4 *per se* (13). Our study on the other hand shows that Hap4 plays a role in the controlling the expression of genes regulated by Adr1 and Cat8.

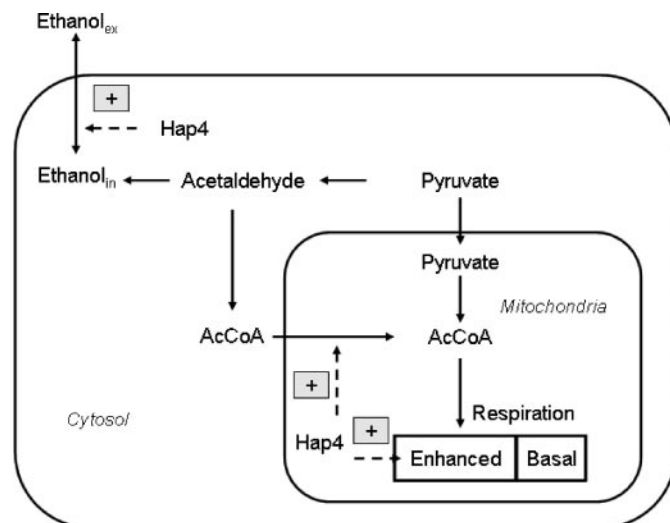


FIGURE 3. Proposed model for the transcriptional regulation of respiration in *S. cerevisiae*. Until a certain glucose flux, Hap4-independent respiration (denoted as basal) occurs in yeast, after which the Hap4-dependent respiration augments the respiratory capacity (denoted as enhanced). Hap4 might positively regulate catabolism of ethanol as well as the entry of acetyl-CoA (AcCoA) into the mitochondrion. The dashed arrow represents the proposed regulation based on indirect evidence.

In line with what is found from the analysis of a few selected genes, identification of reporter metabolites and metabolic subnetworks collectively point toward a key role of Hap4 in the transcriptional reprogramming of metabolic processes involved in respiration and redox balancing. The emergence of  $\text{CO}_2$  and NADH as reporter metabolites is consistent with the notion that Hap4 is involved in the regulation of tricarboxylic acid cycle-related genes. The ethanol node, which is directly related to controlling the redox balance in the cell, also has significantly changed expression. Although both the list of reporter metabolites and the identified subnetworks strongly indicate that Hap4 might play a key role in regulating the respiration, the changes in other parts of metabolism might be a consequence of the altered respiratory capacity of the cell, *i.e.* a secondary effect of deletion of HAP4. This hypothesis is also consistent with the observation of persistent oscillations in the chemostat culture of the HAP4-deleted strain and the observation that the mutant was unable to grow on ethanol as a carbon source. The change in the redox balance is also strongly correlated to the respiratory processes in the yeast and may have resulted in changes in the  $\text{NAD}^+/\text{NADH}$  ratio. Remarkably, certain genes in the subnetwork are directly involved in transporting metabolites across the mitochondrial membrane, indicating that there may have been changes in metabolite levels across the mitochondria membrane in the *hap4* $\Delta$  strain, which strongly supports the hypothesis of control of mitochondrial respiratory capacity and hence redox balance by Hap4.

Based on these findings and data analysis using statistical and integrative computational tools, we hereby propose a model (Fig. 3) where the oxidation of acetyl-CoA occurs by Hap4-dependent and Hap4-independent means. For low glucose uptake rate, only a part of the pyruvate originating from glycolysis is shunted toward the tricarboxylic acid cycle and respiration. There is a basal level of respiration that is independent of Hap4. The remaining pyruvate is shunted via the bypass. Acetyl-CoA originating from the shunt is transported back into the mitochondrion for oxidation, and Hap4 might positively regulate this step. When the glucose flux exceeds a threshold at which the Hap4-independent respiration can no longer take care of oxidation of pyruvate, the Hap4-dependent activation is required, and this provides an excess respiratory capacity allowing for respiratory metabolism at

higher glucose fluxes. It may well be such that Hap4 plays a role in the catabolism of ethanol produced in the fermentative pathway at higher glucose fluxes. Various lines of evidence from other experiments point toward the validity of our proposed model. From genome-wide transcription analysis at different dilution rates,<sup>4</sup> it has been found that expression of the ethanol consumption genes (*ADH2*, *ICL1*, *ALD4*, and *FBP1*) is high for dilution rates below the critical dilution rate, whereas the genes are down-regulated at higher dilution rates where there is onset of fermentative metabolism. The *hap4Δ* mutant has significant down-regulation of genes involved in C2 metabolism, and this may partly explain the onset of fermentative metabolism at low glycolytic fluxes in the mutant. According to the Kappeli model (33), yeast uses respiratory metabolism for increasing glycolytic fluxes, but because of the presence of a maximum respiratory capacity, ethanol is formed as an overflow metabolite at high glycolytic fluxes. Deletion of *HAP4* deprives the cells of its excess respiratory capacity, and hence there is ethanol formation at lower glycolytic fluxes. In agreement with our hypothesis, deletion of both *NDE1* and *NDE2* (external mitochondrial NADH dehydrogenases encoded by *NDE1* and *NDE2*, which oxidize cytosolic NADH and couple it to the respiratory chain) reduced the  $D_{crit}$  only by 23% (34, 35), which is in sharp contrast to 75% reduction for the *hap4Δ* mutant observed here. dos Santos *et al.* (36) reported that overexpression of malic enzyme leads to a decrease in the critical dilution rate by as much as 54% of the WT. Malic enzyme produces pyruvate from malate in mitochondria, and the fact that *MAE1* is up-regulated in the mutant might also partly explain the early onset of overflow metabolism in the mutant.

Although there are still many open questions regarding *HAP4*-dependent regulation, *e.g.* how oxygen and heme control expression of *HAP4*, our work provides evidence that cells do not require Hap4 activity for respiration until a certain specific growth rate, and it further provides evidence that Hap4 plays a key role in controlling the expression of genes involved in mitochondrial respiration and reductive pathways. Moreover, the physiology and gene expression results together with findings of Tai *et al.* (11) show that the transcriptional regulatory network controlling the mitochondrial respiration in yeast has clearly a distinct hierarchical organization with respect to growth regimes.

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