Hap4 Is Not Essential for Activation of Respiration at Low Specific Growth Rates in Saccharomyces cerevisiae

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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 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⁻¹. 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.

In vitro experiments have shown that the mechanism involved in translation of HAP4 mRNA shifts from the cap-dependent to completely IRES² (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 cycle³ (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 mitochondrial) 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 anaplerotic 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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2 The abbreviations used are: IRES, internal ribosome entry site; WT, wild type.

3 S. L. Westergaard, A. P. Oliveira, C. Bro, L. Olsson, and J. Nielsen, submitted for publication.
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 SLIC2 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 abolishes 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 SLIC2 and CEN.PK 517-1A (MATA MAL2-8c SLIC2 HAP4loxP-Kan-loxP). The strains were stored at −80 °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 °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 °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 °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 N2, both of which were filtered through a 0.22-μm 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 (A = 0.02) from an exponentially growing shake flask culture. The exhaust gas was passed through a condenser, maintained at a temperature of 6 °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 (Bruel & 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 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 CO2 evolution and the biomass concentration had remained constant during at least two volume changes (±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 fermentation 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 ~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-μm 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-μm 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 °C, using an autosampler. The eluent used was 5 mM H2SO4, 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 CO2 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 CO2 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 N2), and spun for 5 min at 4000 rpm. The supernatant was discarded, and the pellet was stored at −80 °C until further use. Total RNA extraction was performed with FastRNA® Pro
Red kit (Qbiogene) following the manufacturer’s instructions with minor modifications. cRNA was synthesized as described in the Affymetrix GeneChip® Expression Analysis Manual, after which 15 μg 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 Database 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.

**Table 1**

| Strain | Condition | Y_{sx} | Y_{soc} | Y_{seq} | Y_{spr} | Y_{so} | Ref. |
|--------|-----------|--------|---------|---------|---------|-------|-----|
| hap4Δ  | Aerobic   | 0.27   | 0.11    | 0.0022  | 0.38    | 0.0049| 0.81 |
| hap4Δ  | Anaerobic | 0.3    | 0.11    | ND      | 0.41    | 0.0027| 0.1  |
| WT     | Aerobic   | 0.31   | 0.1     | 0.0042  | 0.34    | ND    | 0.073| 37   |
| WT     | Anaerobic | 0.33   | 0.1     | 0.0058  | 0.36    | 0.0029| 0.097| 38   |

**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 minimal 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 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 h⁻¹ (Fig. 1) compared with 0.3 h⁻¹ for the WT (15). However, as the hap4Δ strain could show complete 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 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 h⁻¹. The respiratory growth at this dilution rate is characterized by a low glucose uptake rate, production of biomass and CO₂, 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 CO₂ 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

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**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 h⁻¹) and then maintained at the value where ethanol was barely detected in the off-gas. This dilution rate corresponds to D_{crit}.
TABLE 2
Physiological parameters observed during the chemostat cultivations

Values represent the mean ± S.D. of data from three independent chemostat cultivations for hap4Δ and from two independent chemostat cultivations for wild type. Yxx indicates grams of biomass per g of glucose consumed. q indicates millimoles of glucose consumed per g of biomass per h. qCO2 indicates millimoles of carbon dioxide formed per g of biomass per h. qO2 indicates millimoles of oxygen consumed per g of biomass per h. D indicates dilution rate per h. RQ indicates respiratory quotient. qCO2/qO2.

|          | Yxx   | q    | qCO2  | qO2   | D    | RQ    |
|----------|-------|------|-------|-------|------|-------|
| WT       | 0.48 ± 0.00 | 0.51 ± 0.00 | 1.2 ± 0.00 | 1.2 ± 0.04 | 0.045 ± 0.00 | 0.98 ± 0.02 |
| hap4Δ    | 0.48 ± 0.00 | 0.57 ± 0.00 | 1.46 ± 0.00 | 1.69 ± 0.12 | 0.049 ± 0.00 | 0.87 ± 0.05 |

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. 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). 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 hap4Δ 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. α-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 CO2, 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
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### TABLE 3

| Metabolite                                 | No. of neighbors |
|--------------------------------------------|------------------|
| CO₂                                        | 37               |
| (R)-Lactoylglutathione                     | 1                |
| Glutathione                                | 1                |
| 5’-Phosphoribosyl-N-formylglycinamide       | 2                |
| Acetaldehyde                               | 12               |
| NADH                                       | 41               |
| Ethanol                                    | 5                |
| 5’-Phosphoribosylglycinamide               | 2                |
| Acetaldehyde                               | 3                |
| Coprotoporphinogen                         | 2                |
| Cytosine                                   | 4                |
| Citrate                                    | 2                |
| Isocitrate                                  | 5                |
| 2-Oxoglutarate                             | 21               |
| Methylglyoxal                              | 1                |
| Guanine                                    | 6                |
| N-Glucosamine-1-phosphate                  | 1                |
| Heme                                       | 1                |
| Malate                                     | 5                |
| Palmitoyl-CoA                               | 2                |

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

### 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 remodelled (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⁻¹, compared with 0.3 h⁻¹ for the WT (15). Thus, in the mutant the oxygen 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⁻¹, 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⁻¹; 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 chemostat 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 stabilize the energy metabolism resulting in a stress response under respiratory conditions. This may result in the appearance of sustained oscillations.

### TABLE 4

| Gene name/open reading frame | Enzymatic function | p value |
|-----------------------------|-------------------|---------|
| AAT2                        | Aspartate aminotransferase | 0.0052 |
| ADES,7                      | Phosphoarginine-glycine ligase | 0.1504 |
| ADE8                        | Phosphoarginine-glycine formyltransferase | 0.0034 |
| ADH2                        | Alcohol dehydrogenase II | 0.0023 |
| ADH4                        | Alcohol dehydrogenase IV | 0.0278 |
| ALD4                        | Mitochondrial aldehyde dehydrogenase | 0.0005 |
| CAR2                        | Ornithine aminotransferase | 0.0027 |
| CPA2                        | Carbamyl-phosphate synthetase | 0.0320 |
| CTP1                        | Mitochondrial inner membrane citrate transport protein | 0.0240 |
| DAK2                        | Dihydroxyacetone kinase | 0.0004 |
| FAA1                        | Long chain fatty acid CoA ligase | 0.0198 |
| FCY1                        | Cytosine deaminase | 0.0180 |
| FDH1                        | Formate dehydrogenase | 0.0010 |
| GKL1                        | Glucokinase | 0.0361 |
| HEM13                       | Coproporphyrinogen III oxidase | 0.0201 |
| HIP1                        | Histidine permease | 0.0136 |
| HXT16                       | Hexose transporter | 0.0264 |
| IDP3                        | NADP-dependent isocitrate dehydrogenase | 0.0099 |
| LCB1                        | Serine O-palmitoyltransferase | 0.0007 |
| LYS1                        | Saccharopine dehydrogenase | 0.0099 |
| MAE1                        | Mitochondrial malic enzyme | 0.0112 |
| MET12                       | Methyleneetrahydrofuran reductase | 0.0302 |
| MET2                        | Homoserine O-acetyltransferase | 0.0262 |
| OAC1                        | Mitochondrial oxaloacetate transporter | 0.0067 |
| PMP1                        | Plasma membrane proteolipid (ATPase) | 0.0259 |
| PNP1                        | Purine nucleoside phosphorylase | 0.0206 |
| PSD1                        | Phosphatidylserine decarboxylase | 0.0174 |
| SAH1                        | Adenosylhomocysteinease | 0.0226 |
| SER1                        | 3-Phosphoserine transaminase | 0.0198 |
| SUR2                        | Hydroxynitrile involved in spinagolipid metabolism | 0.0253 |
| TDH1                        | Triose-phosphate dehydrogenase | 0.0086 |
| TRR1                        | Thioredoxin reductase | 0.0118 |
| YOR071C                     | Formate dehydrogenase | 0.0163 |

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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 α-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 Hap1 and the Hap1I/II/2/3/4/5 complex. CYT2 (cytochrome c1, 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, Zoller 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 Hap1, 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 Hap1 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 Hap1 and Hap1I/II/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-repressing 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.

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 CO2 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 NAD+/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 hap4Δ 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

**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.
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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, 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 provide evidence that Hap4 plays a key role in controlling the expression of genes involved in mitochondrial respiration and reductive path-ways. 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 a distinct hierarchical organization with respect to growth regimes.

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