Hap4p overexpression in glucose-grown Saccharomyces cerevisiae induces cells to enter a novel metabolic state

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Abstract

Background: Metabolic and regulatory gene networks generally tend to be stable. However, we have recently shown that overexpression of the transcriptional activator Hap4p in yeast causes cells to move to a state characterized by increased respiratory activity. To understand why overexpression of \( HAP4 \) is able to override the signals that normally result in glucose repression of mitochondrial function, we analyzed in detail the changes that occur in these cells.

Results: Whole-genome expression profiling and fingerprinting of the regulatory activity network show that \( HAP4 \) overexpression provokes changes that also occur during the diauxic shift. Overexpression of \( HAP4 \), however, primarily acts on mitochondrial function and biogenesis. In fact, a number of nuclear genes encoding mitochondrial proteins are induced to a greater extent than in cells that have passed through a normal diauxic shift: in addition to genes required for mitochondrial energy conservation they include genes encoding mitochondrial ribosomal proteins.

Conclusions: We show that overproduction of a single nuclear transcription factor enables cells to move to a novel state that displays features typical of, but clearly not identical to, other derepressed states.
HAP4 overexpression system on the other hand is relatively simple: one factor that is known to be required for induction of respiratory activity can indeed induce a physiological change that resembles that of the diauxic shift [2].

Metabolic and regulatory gene networks have generally evolved to allow physiological and developmental processes to compensate for the effects of potentially deleterious mutations, termed robustness [3]. Important questions are how equilibrium states are maintained and how the transition between states is implemented by the cell. Depending on where a particular regulatory protein is located in the hierarchical global regulatory network of the cell, the response to a change in its activity may be either localized or pleiotropic. To identify the various components of the pleiotropic response to HAP4 overexpression, that is, to ‘fingerprint’ the changes in the regulatory network, we use two different methods. First, we use the algorithm REDUCE [4] to infer the regulatory activity of transcription factors from the mRNA expression of their target genes. Second, we use a new and related algorithm, named Quontology, to identify classes of genes with similar function that are significant induced or repressed. Both methods have the property that they can detect small-amplitude but coordinated changes in the average expression level of a set of genes, even if the expression of individual genes is not changing significantly.

In this paper we report detailed analysis of the changes that occur as a result of overexpression of HAP4. Using a number of techniques it could be shown that overexpression of HAP4 enhances transcription of a large set of mitochondrial protein genes, leading to increased mitochondrial biogenesis and enabling cells to move to a distinct and novel state.

**Results**

**Cells overexpressing HAP4 upregulate mitochondrial biogenesis and activity even in the presence of glucose**

To determine the effect of overexpression of HAP4 on mitochondria, several different experimental approaches were used to quantify mitochondrial components and mitochondrial structures in the cell.

First, the quantity and quality of the respiratory-chain complexes were analyzed by subjecting mitochondrial extracts to two-dimensional gel electrophoresis. As shown in Figure 1, in the glucose-grown wild-type strain (WT YPD in Figure 1) most of the various respiratory chain or OXPHOS (oxidative phosphorylation) complexes are undetectable or barely detected as a result of the glucose-mediated repression of respiratory function. Only complex V (the ATP synthase) can be clearly distinguished, in particular an intense spot that consists of the α- and β-subunits of the F1 subcomplex. Glucose-grown cells that overexpress HAP4, on the other hand, show very distinct OXPHOS complexes (see HAP4 YPD in Figure 1). The pattern of spots of the glucose-grown HAP4 overproducer is similar to wild-type cells that grow in fully respiratory mode on a non-fermentable carbon source (compare HAP4 YPD and WT YPEG in Figure 1). The increased abundance of OXPHOS complexes in the glucose-grown HAP4 overproducer is further shown by the fact that mitochondrial suspensions from the HAP4 overexpression strain contain approximately twofold more protein per gram wet weight compared to its wild-type counterpart (Figure 2a).
The cellular content of cytochromes $c + c_1$ and $b$ is also markedly increased, 50% and 65% respectively, when the $HAP4$ gene is overexpressed in glucose-grown cells (Figure 2b). The cytochrome $aa_3$ content is also increased in the $HAP4$ strain, although the corresponding part of the difference spectra does not allow proper quantification.

The mitochondrial content of cells was further analyzed by fluorescence microscopy using the fluorescent dye DASPMI, which specifically stains mitochondria [5]. To compare mitochondrial DASPMI fluorescence between strains directly, glucose-grown wild-type and $HAP4$-overexpressing cells were mixed. Wild-type and $HAP4$ cells were identified in situ by also staining one of the strains with the cell-wall dye CalcoFluorWhite (CFW [6]) before mixing the cells. As shown in Figure 3, $HAP4$-overexpressing cells contain more mitochondrial structures per cell ($HAP4$ cells are identified by CFW fluorescence in the left panels and by the absence of CFW fluorescence in the right panels). This is consistent with the fact that more membranous vesicles are present in mitochondrial suspensions extracted from $HAP4$-overexpressing cells (data not shown). Interestingly, $HAP4$-overexpressing cells also exhibit increased DASPMI fluorescence. Although this may suggest an increased membrane potential over the mitochondrial inner membrane [7], we favor the idea that the inner membrane has a more compact structure in these mitochondria [5].

Nuclear DNA encodes the majority of mitochondrial proteins. Several mitochondrial components, such as a number of subunits of enzymes of the respiratory chain, are encoded by the mitochondrial genome. Figure 4 shows that overexpression of $HAP4$ in glucose-growth conditions not only results in increased transcription of nuclear-encoded mitochondrial protein genes ([2], and see below) but also leads to enhanced transcription of all mitochondrial genes tested. In particular, COX2 mRNA was induced to high levels. It should be noted that the increase in transcription in the mitochondrion is not accompanied by strong alterations in the copy number of mitochondrial DNA (mtDNA) (data not shown), indicating that the increase in mitochondrial transcription is mainly due to increased activity of the mitochondrial transcription machinery.

The strong increase in OXPHOS complex abundance and in mitochondrial protein and cytochrome content per cell, together with the evidence that shows that $HAP4$ cells contain more mitochondrial structures per cell, conclusively shows that overexpression of $HAP4$ in glucose-growth conditions results in increased mitochondrial biogenesis.

**Fingerprinting the regulatory network of $HAP4$ overexpressing cells**

Genome-wide expression was monitored with Affymetrix GeneChips using RNA from wild-type and $HAP4$ cells grown aerobically on minimal glucose medium (YNB) in a batch

![Figure 2](http://genomebiology.com/2002/4/1/R3)
Figure 3
HAP4-overexpressing cells contain more mitochondrial structures with greater DASPMI fluorescence than wild-type cells. DASPMI fluorescence of glucose-grown wild-type and HAP4-overproducing cells was compared in microscopic preparations of mixed cells. To identify HAP4-overexpressing cells or wild-type cells in situ, microscopic preparations were analyzed in which only one of the strains was additionally stained with CalcoFlourWhite (CFW), a dye that stains the cell wall, in particular the bud-neck and bud scars [6]. (a) Top panels show a mixture of HAP4-expressing cells stained with CFW + DASPMI and wild-type cells stained with DASPMI only. Left, CFW fluorescence; right, phase contrast. (b) Top panels show a mixture of wild-type cells stained with CFW + DASPMI and HAP4-overexpressing cells stained with DASPMI only. Left, CFW fluorescence; right, phase contrast. In both (a) and (b) the large bottom panels show a mixture of wild-type and HAP4-overexpressing cells stained with DASPMI only.

Because the HAP4-overexpression system can induce a physiological change that resembles that of the diauxic shift [2], we first analyzed whether the state of the regulatory activity network in the HAP4 overpressor is similar to one or more of the transient states of the diauxic shift [1]. For this, the regulatory activity network was fingerprinted using the Quontology algorithm (see Materials and methods). This algorithm enables the identification of functional groups of genes that have changed in expression as a result of increased expression of HAP4. A Z-score is calculated for the functional categories from the MIPS database [8], which is a measure of the changes that occur in the regulatory activity network. Categories with a Z-score larger than 4 can be considered to be induced or repressed significantly. Figure 5 shows all functional categories that were induced or repressed significantly in the HAP4-overexpressing strain or in one of the expression profiles from the diauxic shift.

From Figure 5 it is evident that overexpression of HAP4 results in alterations in the regulatory network that resembles the time points of the diauxic shift with respect to several important functional categories (see categories marked with a filled circle).

The alterations in the regulatory activity network of the diauxic shift mainly occur in three phases, as indicated in Figure 5: high glucose concentrations; strongly decreasing glucose concentration; and glucose depletion where cells grow in respiratory mode on the remaining ethanol. The HAP4 overexpression profile is similar in a number of respects to the profiles of cells growing in decreasing glucose concentrations (13.5 and 15.5 hours; see Figure 5). However, in this case also, the changes that occur in the diauxic shift are much more extensive, in particular with respect to the categories ‘metabolism of energy reserves’ and ‘rRNA transcription’. Hence, the regulatory activity network of the HAP4-overexpressing strain has moved to a state that resembles one of the transition states of the diauxic shift, but at the same time differs in a number of important aspects.

Hap4p is a global regulator that coordinately and specifically upregulates mitochondrial function and mitochondrial biogenesis

All functional categories that are significantly increased (Z-score > 4) in the HAP4-overexpressing strain are related to mitochondrial function (see Figure 5), including the main category ‘protein synthesis’ which consists largely of mitochondrial protein genes. Consistently, the vast majority of the 139 nuclear genes that are induced more than twofold in the HAP4-overexpressing strain (see genes listed in Figure 6 and Materials and methods) encode proteins that localize to mitochondria. It is striking that if the expression of these genes is compared to the expression in respiratory-state cells (columns 2-8 in Figure 6) or in glucose-repressed cells (column 9), it can be observed in the right part of Figure 6...
that there are a large number of genes that are strongly upregulated in the HAP4 overproducer but not upregulated in respiring cells. As these genes also encode mitochondrial proteins, this suggests that overexpression of HAP4 leads to a novel state in which the expression of mitochondrial proteins goes beyond levels that are required in normal physiological conditions.

The two clusters of genes in Figure 6 fall into different functional categories. The group of genes that is responsive to changes in the respiro-fermentative growth mode consists mainly of genes encoding proteins that are directly involved in respiration (see genes listed in the left part of Figure 6). The second, larger and non-responsive group mainly consists of genes that encode proteins involved in mitochondrial protein synthesis (see genes listed in the right part of Figure 6). Apparently, overexpression of HAP4 not only results in increased expression of genes encoding proteins required for mitochondrial energy conservation (or generation) but also of genes that are required for the synthesis of functional mitochondria.

The upstream activation sequences (UAS) of the genes of the non-responsive group - but not those of the responsive group - generally all lack the typical Hap2/3/4/5p-binding element CCAATCA. This is interesting, as this particular element strongly correlates with the changes in expression that occur as a result of overexpression of HAP4 ([4], REDUCE: p < 10^-6). Furthermore, with a more in-depth computational search for potential Hap2/3/4/5p sites using a nucleotide-distribution matrix, no difference could be observed between promoters of the non-responsive group and random-sequence DNA (Figure 7a). In contrast, in the gene promoters of the responsive gene cluster a large number of potential Hap2/3/4/5p sites can be detected that are distributed over the promoter according to a typical yeast UAS (100-400 bp upstream of the translational start codon ATG). These data strongly suggest that the Hap2/3/4/5p complex does not directly regulate the vast majority of genes of the non-responsive gene cluster, namely genes important for mitochondrial proliferation.

Overexpression of HAP4 negatively affects transcription of a small set of genes involved in zinc metabolism

In addition to the above-mentioned genes, which are upregulated and lead to increased mitochondrial biogenesis, it should be noted that the expression of 23 genes is reduced by overexpression of HAP4. These genes are RPR1 (-2.3), EUG1 (-2.3), ZRT1 (-17.0), ADHA4 (-7.9), YGL258W (-3.9), YHR048W (-2.5), HXT4 (-2.7), TDH1 (-3.0), ZAP1 (-6.8), CWP1 (-3.0), ZRT3 (-3.4), ZRT2 (-2.9), TFS1 (-2.4), THI7 (-2.9), CAR2 (-2.7), SNO1 (-3.8), SNZ1 (-2.9), FET4 (-2.7), YNL254C (-5.3), FRE4 (-3.0), COS10 (-2.4), YOL154W (-27.3), YOR387C (-18.4). Interestingly, a large number of these genes are involved in zinc metabolism. Genes indicated
in bold are most strongly affected by overexpression of HAP4 (-3) and have been proposed to be targeted by zinc-specific transcription factor Zap1p [9]. In fact, the expression of ZAP1 itself is strongly repressed by overexpression of HAP4 and, furthermore, the repressive motif ACC-5-GGT, the DNA-binding site of Zap1p [10], was identified by the REDUCE algorithm with high significance, \( p < 10^{-12} \). Hence, we conclude that the main repressive effect of overexpression of HAP4 is mediated through decreased expression of the Zap1p transcription factor. Genes that are typically not altered in expression on zinc depletion or zap1-deletion [9] are given in non-italic type above, and are suggested to be repressed through a different mechanism, possibly through the same mechanism that initially represses the ZAP1 gene.

### Discussion

In this report we show that overexpression of the gene encoding the activation moiety of the Hap2/3/4/5p transcription complex causes glucose-grown cells to move to a novel state that is characterized by a shift to higher respiratory activity. HAP4-overexpressing cells contain more mitochondrial structures, increased amounts of mitochondrial proteins/cytochromes and enhanced transcription of the mitochondrial energy metabolism genes.

#### Figure 6

Genes that show more than twofold induction in the HAP4 overexpression dataset can be divided in two distinct groups using k-means clustering analysis (Cluster software [22]). The gene-expression profile of HAP4-overexpressing cells was compared to datasets derived from cells that have a particular and/or shifted balance between respiration and fermentation. These were: the 18.5 h and 20.5 h time points of the diauxic shift at which glucose has been exhausted [1]; the ‘adaptive evolution’ datasets, which are derived from strains selected for growth in aerobic, glucose-limited chemostats for more than 250 generations (denoted as pare1, pare2 and pare3 [28]); stationary phase cells (2-day stationary phase); and expression profiles from cells grown either on ethanol or glucose [11]. It should be emphasized that mitochondrial transcripts are not represented in this analysis as these transcripts do not contain a 3' poly(A) tail and hence are not labeled properly.
Figure 7
Non-responsive gene promoters rarely contain Hap2/3/4/5p regulatory elements and respond to signals that depend on growth rate. (a) Frequency distribution of potential Hap2/3/4/5p sites in the promoters of genes from the responsive gene cluster (red) and the non-responsive gene cluster (blue) from Figure 6. The frequency of occurrence of potential Hap2/3/4/5p sites as detected by a Hap2/3/4/5p nucleotide-distribution matrix was calculated in 100 bp intervals (threshold = 0.85 [24]). The distribution of Hap2/3/4/5p sites in the promoters of genes from the responsive gene cluster localize between -400 and -100 bp from the ATG, a common distance for yeast UAS. Putative Hap2/3/4/5p sites extracted from the non-responsive gene cluster show a random distribution similar to that found for random-sequence DNA (A/T 31%, C/G 19%), and are therefore likely to be non-functional matches to the matrix. (b) The correlation between Z-scores and growth rate of the constitutive (left) and regulated (right) clusters calculated with the Quontology algorithm (see also Figure 5) using experimental data from a large number of deletion mutants [15]. Z-scores larger than 4 or smaller than -4 have significantly altered expression (p < 0.1). The running mean of the absolute Z-scores was calculated to determine to what extent regulation is correlated to the growth rate.
mitochondrial genome. This suggests that overexpression of HAP4 results in a general increase in mitochondrial biogenesis. Consistently, whole-genome expression profiling and subsequent computational analyses do indeed show that overexpression of HAP4 specifically alters transcription of a very large set of mitochondrial protein genes. HAP4-overexpressing cells resemble cells that undergo a physiological diauxic shift, but at the same time differ from these cells in a number of important respects. First, in the HAP4 overexpressor no increase occurs in the functional category ‘metabolism of energy reserves’ and in functional categories that are involved in transcription, mainly ‘rRNA transcription’. Second, genes involved in zinc metabolism are downregulated, and third, genes involved in protein synthesis in mitochondria are strongly upregulated.

With respect to the latter it should be noted that the effects we show in Figure 6 might to some extent be affected by differences in the set-up of the experiments, that is, differences in the genetic background of the strains and/or of the array platforms used. However, other previously published expression profiles show induction of this non-responsive group of genes, which contains mainly mitochondrial ribosomal protein genes (MRP genes). These studies looked at the effects of oxidative stress [11], addition of rapamycin [12], mitochondrial dysfunction [13,14], a ‘pulse’-like induction at the diauxic shift (see lanes 2 in Figure 6), and at the diauxic shift that precedes the stationary phase [11]. Furthermore, if the Z-scores are calculated for the genes of the non-responsive and the responsive group using the expression profiles of 300 null mutants from Hughes et al. [15], we find that the expression of the non-responsive group alters more strongly in mutants that have a decreased growth rate (Figure 7b). As the growth rate of the HAP4 overproducer is not affected, we suggest that the transcriptional response of MRP genes is not only dependent on the growth rate itself, but also on whether cells respire more and/or ferment less.

The fact that genes involved in zinc metabolism are primarily downregulated by overexpression of HAP4 is surprising. Complex (auto)regulation of the zinc regulator Zap1p and the central role of mitochondria in metal metabolism are likely to be important in this downregulation. However, as zinc is an essential component of more than 300 enzymes - including, paradoxically, cytochrome oxidase - particular zinc-dependent factors may be involved. Candidate factors that require zinc are: the mitochondrial metalloproteases Afg3p, Rca1p and Yem1p; the transcription factors Cat8, Mig1p, Mig2p, Mal63 and Hap1p; and alcohol dehydrogenases.

We have shown here that increased expression of a single transcription factor enables the alteration of a particular branch of the yeast regulatory activity network, leading to remarkably specific alterations in expression of specific functional sets of genes and revealing only minor cross-talk with other parts of the regulatory activity network. Hence, the regulatory activity network that determines mitochondrial function appears to be highly modular. This is an important notion, as the regulatory activity network may consist of similar more-or-less isolated modules, which may enable the construction of a simple but realistic computational model of the in silico cell.

**Materials and methods**

**Strains and growth conditions**

The wild-type strain CEN.PK113-7D and its HAP4-overproducing counterpart 436 GH [16], in which the HAP4 gene is overexpressed more than fivefold in glucose-growth conditions, were used. The latter contains a genomically integrated HAP4-overproduction cassette in which the TDH3 promoter regulates the HAP4 gene. Cells were grown aerobically in YPD (1% yeast extract, 1% Bacto-peptone and 2 or 3% D-glucose) or YPEG (YP containing 2% ethanol and 2% glycerol). In all experiments cells were harvested at early logarithmic phase.

**Isolation of mitochondria and blue native PAGE**

Yeast cells were grown in 100 ml YPD or YPEG medium until OD_{600} of 0.7-1.0 and mitochondria were isolated according to [17]. Protein concentration was determined using Bradford. Two-dimensional gel electrophoresis was carried out according to [18]; the first dimension consisted of blue native, 5-13% gradient polyacrylamide gel electrophoresis (PAGE), and the second of denaturing PAGE on gels containing 10% SDS. Protein was visualized by Coomassie staining of the gels. The identity of a constitutive spot at the upper-left part of the gels was determined using mass spectrometry [17].

**Spectral analysis and fluorescence microscopy**

Cells were grown to OD_{600} = 1.0-1.2, frozen in liquid nitrogen and stored in -20°C. The cell pellet was resuspended in a final volume of 2 ml using a buffer containing 100 mM KPO_{4}, pH 7.3, 250 mM sucrose and 0.5% Na-cholate. Difference spectra were derived from dithionite-reduced cells minus ferricyanide-oxidized cells. Spectral measurements were carried out at room temperature in a DW2000 dual-beam spectrophotometer. Concentrations of cytochromes were determined essentially as in [19].

Mitochondrial biogenesis in living cells was analyzed by DASPMI staining of cells grown logarithmically in liquid medium (YPD). YPD culture samples of 5 ml were incubated at 28°C in the presence of 10 μl of 1 mg/ml CalcoFluorWhite [6], centrifuged, resuspended in 20 μl YPD. CFW stained cells (2 μl) and 2 μl unstained cells were mixed, 1 μl of 1 mg/ml DASPMI and 15 μl 0.1 M Tris-Cl pH 8.0 were added. After 5 min, cells were washed in 0.1 M Tris-Cl pH 8.0, centrifuged and resuspended in 0.1 M Tris-Cl pH 8.0. After addition of 1 volume YPD, cells were aerated by pipetting and analyzed microscopically.
Northern analysis and microarray experiments

RNA isolation, northern blotting and hybridizations were carried out essentially as in [20]. Probes for mitochondrial transcripts COX1, COX2 and SCD1 and the loading control PDA1 were obtained by [32P]ATP labeling of PCR-generated DNA fragments. For microarray analyses, a pre-culture was grown overnight in YNB medium (0.67% yeast nitrogen base) containing 2% raffinose. The main culture was inoculated at OD600 = 0.1 in a batch fermenter containing 500 ml YNB medium buffered at pH 5.0 with 100 mM sodium phthalate and supplemented with 2% glucose, stirred at 500 rpm and aerated at 1vvm (vessel volume per minute). Both strains grew equally well, having growth rates of μ = 0.36/h and 0.34/h for wild-type and HAP4, respectively. The respiratory quotient (RQ), decreased from RQ = 3.5 in wild-type cells to 2.6 in the HAP4-overexpressing strain. This shows that the latter strain grows in a more respiratory mode, similar to [2], which is accompanied by a decrease in the glucose consumption and ethanol production rates: From qglucose = 15.6 mmol/g/h and qethanol = 19.8 mmol/g/h for the wild-type strain, to qglucose = 11.1 mmol/g/h and qethanol = 15.6 mmol/g/h for the HAP4-overexpressing strain. The cultures were grown until OD600 = 1.0 (approximately 8 h), cells were chilled rapidly by addition of ice, centrifuged and stored at -70°C. RNA was extracted using hot phenol according to [21] and poly(A)+ mRNA was isolated using Oligotex (Qiagen). Labeling and hybridization of GeneChip Yeast Genome S98 Arrays were carried out according to Affymetrix.

Computational analysis

The average ratio was calculated from log2 expression ratios that derive from two independent experiments of the HAP4 overexpressor relative to two independent wild-type experiments. ORFs for which the expression ratios diverged twofold in duplicate experiments were excluded from the analysis. Using interpolated variance analysis, 110 genes were calculated to be regulated significantly (p < 0.01; data not shown). In this analysis genes are selected that show a twofold change. k-means clustering was carried out using Cluster [22], corrected for a minor bug that inappropriately displays the gene of the second cluster as the final gene of the first cluster. Matrix searches were carried out using the Hap2/3/4/5/p nucleotide-distribution matrix from the TransFac database [23] and the MatInspector program [24]. Matches to the Hap2/3/4/5/p matrix were analyzed in random-sequence DNA (A/T 31%, C/G 19% [25]) that contained the same number of promoters of the non-responsive gene cluster.

Quontology algorithm

Several publications have used genome-wide expression patterns to score functional gene categories, by considering the overlap between each category and the set of genes induced or repressed above a certain threshold. We take a very different approach that is essentially identical to the REDUCE algorithm for scoring promoter elements [4] but with manually defined gene categories from an ontology replacing the set of genes whose promoter contains a particular DNA motif. Again, a Z-score can be calculated for each category that measures the deviation of the average log-ratio for genes in the category from the genome-wide average, in units of the standard deviation. A similar method has recently been discussed by Pavlidis et al. [26].

Additional data files

The original data used to carry out this analysis is available as an Excel file with the online version of this article. This contains the following columns: Affymetrix identifier, ORF names, four columns containing the Affymetrix cross-experiment comparison (log2) of HAP4 versus wild type, and one column containing the average log ratio of four data points (log2).

Acknowledgements

We thank Leo Nijtmans, Marta Artal, Monique van Galen, Jan Berden, Conrad Woldringh, Matthew Piper, Jack Pronk, Betsie Voetdijk, Elzo de Wit and Joost Texeira de Mattos for help, advice and technical support. This work was financially supported by the Dutch Ministry of Economic Affairs (EET program EETK-99020).

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