Research

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

Romeo Lascaris*[†], Harmen J Bussemaker*[‡], André Boorsma*, Matt Piper[§], Hans van der Spek*, Les Grivell*[¶] and Jolanda Blom*

Addresses: *Swammerdam Institute for Life Sciences, University of Amsterdam, 1098 SM Amsterdam, The Netherlands. [§]Kluyver Laboratory of Biotechnology, TU Delft, Julianalaan 67, 2628 BC Delft, The Netherlands. Current addresses: [†]Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. [‡]Department of Biological Sciences and Columbia Genome Center, Columbia University, 1212 Amsterdam Avenue MC 2441, New York, NY 10027, USA. [¶]EMBO, Meyerhofstrasse 1, D-69117 Heidelberg, Germany.

Correspondence: Romeo Lascaris. E-mail: r.lascaris@nki.nl

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.

Background

Unraveling and understanding the complex regulatory networks in a cell is an important step in genomic research, since it should enable us to understand how the cell modifies its behavior in response to both internal and external signals.

A diauxic shift occurs when yeast cells have consumed all glucose fermentatively and convert their metabolism to oxidative catabolism of the ethanol remaining in the medium. This shift has been extensively studied [1] and is a good example of how cells move from one 'equilibrium' state, for example, fermentative physiology, to another 'equilibrium' state, for example, respiratory physiology. The transition from fermentative physiology to respiratory physiology, in the wild or in the laboratory, involves many changes in gene expression that contribute to the final phenotype. The

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
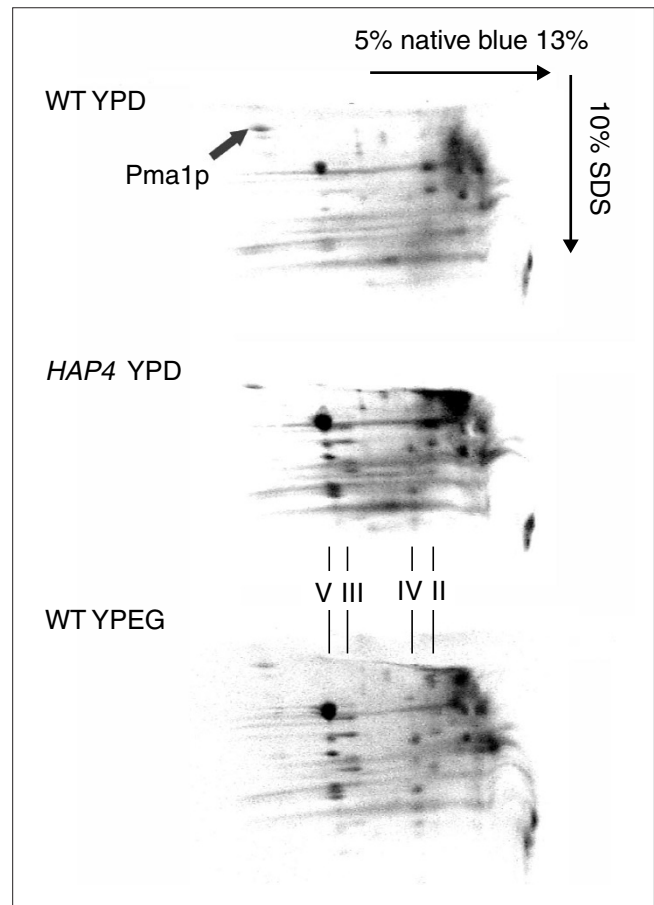

Figure 1
Cells that overexpress *HAP4* contain more respiratory chain complexes than wild-type cells. Two-dimensional blue native gel electrophoresis of mitochondrial proteins isolated from glucose-grown wild-type (WT YPD, rich glucose medium) or *HAP4*-overexpressing cells (*HAP4* YPD). Mitochondrial proteins from ethanol-grown, respiring wild-type cells are shown as control (WT YPEG, rich medium containing non-fermentable carbon sources: 2% ethanol and 2% glycerol). Respiratory-chain complexes V, III, IV and II from mitochondria isolated from 40 mg (wet weight) wild-type or *HAP4* cells were separated in the first dimension (from left to right) by blue native, 5-13% gradient gel electrophoresis [18]. These complexes were subsequently fractionated in the second dimension (from top to bottom) by denaturing gel electrophoresis (10% SDS) and proteins in the gel were visualized by Coomassie staining. The arrow indicates a spot that was determined to be Pma1p, a non-mitochondrial, H$^+$-transporting ATPase which is localized in the plasma membrane [27], and which was used as control for the amount of cells fractionated.

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
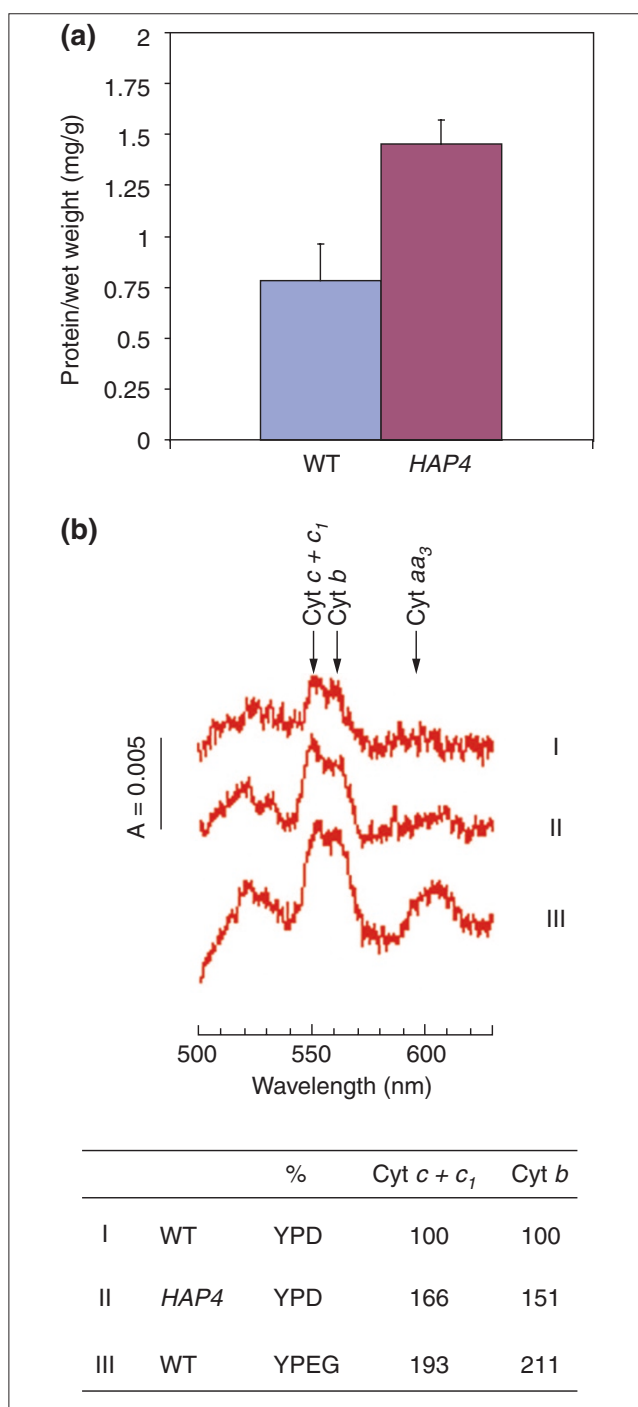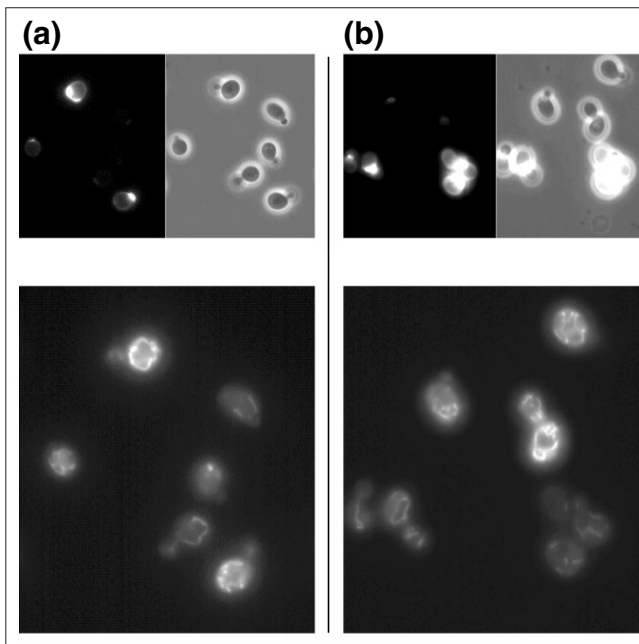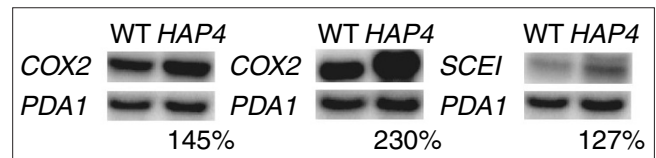

		%	Cyt $c + c_1$	Cyt b
I	WT	YPD	100	100
II	*HAP4*	YPD	166	151
III	WT	YPEG	193	211

Figure 2
HAP4-overexpressing cells contain more protein and more cytochromes compared to wild-type cells. **(a)** Five independent mitochondrial extracts were isolated from logarithmic-phase, glucose-grown wild-type and *HAP4* strains. The amount of mitochondrial protein per gram cellular wet weight was determined. **(b)** Absorption difference spectra of whole-cell suspensions from respiro-fermentative, glucose-repressed wild-type and *HAP4*-overexpressing cells (YPD) and respiratory grown wild-type cells (YPEG) were obtained using a split-beam spectrophotometer. The cellular content of cytochromes $c + c_1$, b and aa_3 was calculated from the difference in absorption between reduced versus oxidized forms of the cytochromes.

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 CalcoFluorWhite (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.

Figure 4
Transcription in mitochondria is increased as a result of overexpression of the nuclear *HAP4* gene. Northern analysis of wild-type and *HAP4*-overexpressing cells grown logarithmically in rich glucose medium. Transcripts from *COX1*, *COX2* and *SCEI* are expressed from the mitochondrial genome. *PDA1* mRNA is used as internal control. Transcriptional changes were quantified and indicated as a percentage of the wild-type transcript level.

fermenter. For each strain, two separate hybridizations were carried out and the $\log_2$-expression ratios or fold-changes were calculated for *HAP4* overexpression versus wild type.

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* overexpressor 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 significantl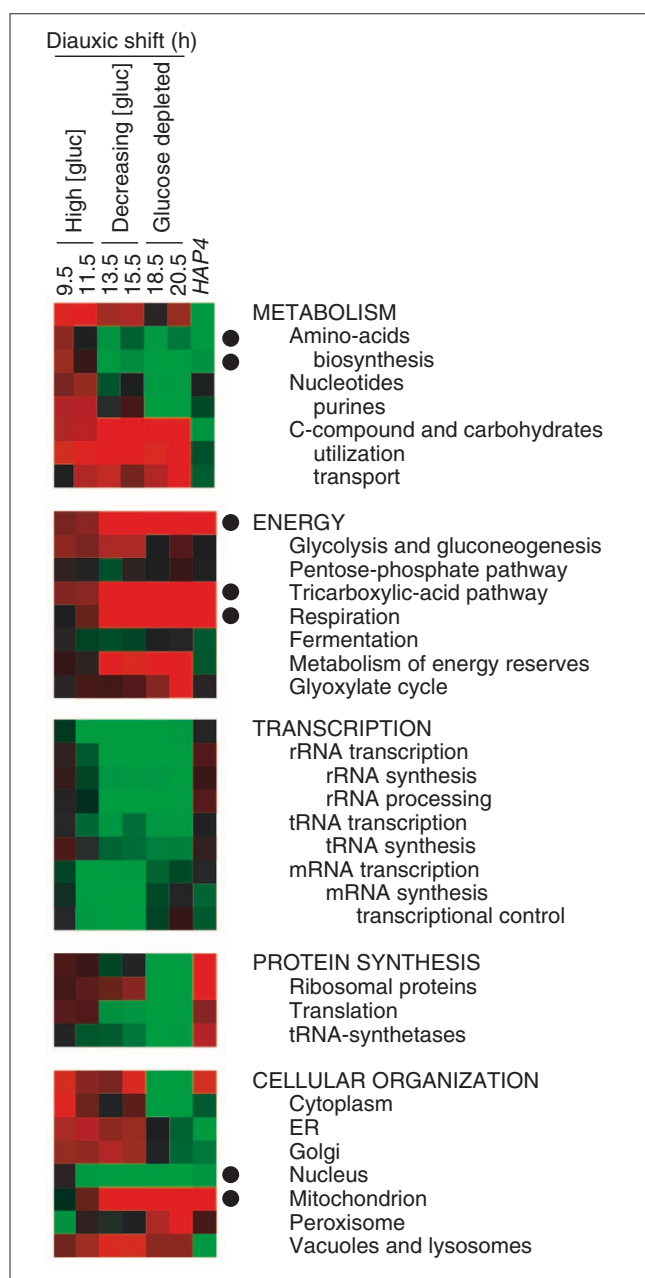y. Figure 5 shows all functional categories that were induced or repressed significantly in the *HAP4*-overexpressing strain or in one of the expression profiles from the diauxic shift.

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

Figure 5
Fingerprinting of the yeast regulatory activity network using Quontology (H.J.B. and R.L., unpublished work, see Materials and methods). Functional categories are induced (red) or repressed (green) by overexpression of *HAP4* or in the diauxic shift [1]. The whole-genome expression data of the diauxic shift are divided into three datasets that represent: glucose excess; a strongly decreasing glucose concentration; and glucose depletion. Using the quontology algorithm, Z-scores were calculated for the functional categories defined by MIPS [8]. Generally, all categories that have at least one Z-score larger than 4 or smaller than -4 are listed ($p < 0.1$). The hierarchy of the categories is preserved: the main categories in capitals, the subcategories indented and in lower case. A filled circle marks MIPS categories that show the same pattern of expression in more respiratory conditions (the five columns on the right). Interestingly, overexpression of *HAP4* results in specific induction of MIPS categories that are involved in mitochondrial function, causing the regulatory activity network to move to a state that is different from the diauxic shift.
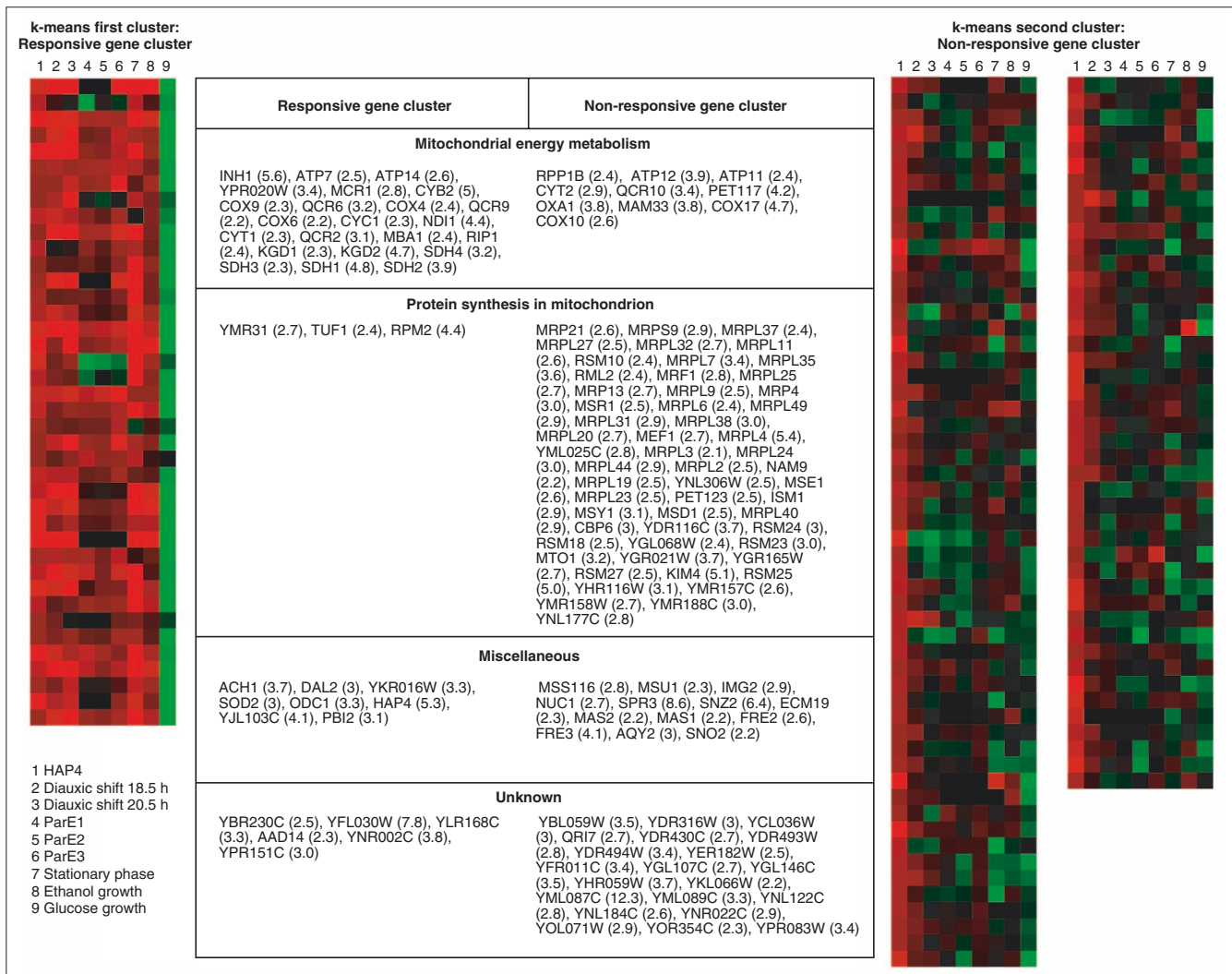
that there are a large number of genes that are strongly upregulated in the *HAP4* overproducer but not upregulated in respiring cells. As these genes also encode mitochondrial proteins, this suggests that overexpression of *HAP4* leads to a novel state in which the expression of mitochondrial proteins 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), ***ADH4*** (-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

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.

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
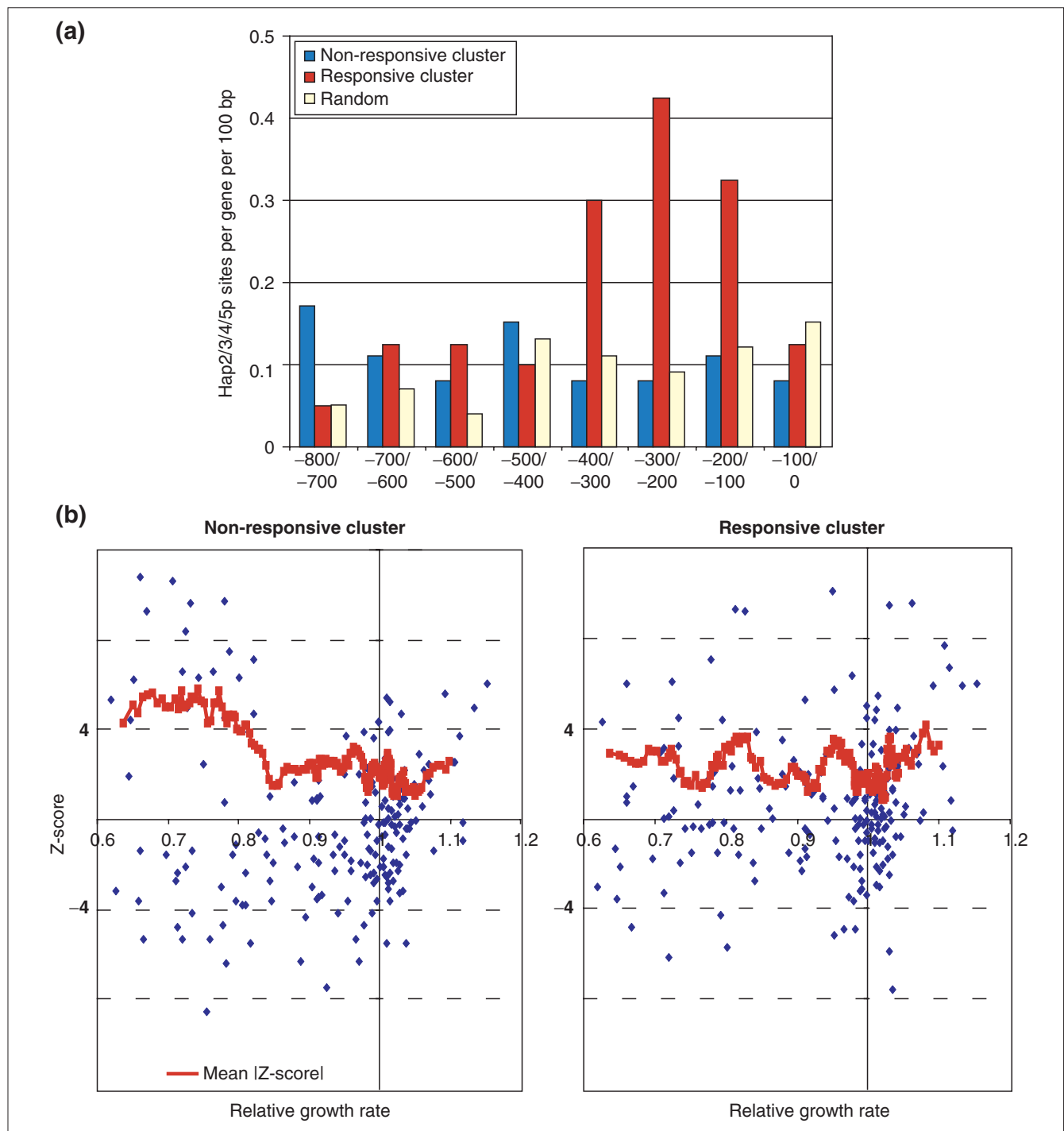
Figure 7
Non-responsive gene promoters rarely contain Hap2/3/4/5p regulatory elements and respond to signals that depend on growth rate. **(a)** Frequency distribution of potential Hap2/3/4/5p sites in the promoters of genes from the responsive gene cluster (red) and the non-responsive gene cluster (blue) from Figure 6. The frequency of occurrence of potential Hap2/3/4/5p sites as detected by a Hap2/3/4/5/p 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 *SCEI* and the loading control *PDA1* were obtained by [^{32}P]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 OD_{600} = 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 1 vvm (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 $q_{glucose}$ = 15.6 mmol/g/h and $q_{ethanol}$ = 19.8 mmol/g/h for the wild-type strain, to $q_{glucose}$ = 11.1 mmol/g/h and $q_{ethanol}$ = 15.6 mmol/g/h for the *HAP4*-overexpressing strain. The cultures were grown until OD_{600} = 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 $\log_2$ 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 ($p \leq 0.01$; 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 ($\log_2$) of HAP4 versus wild type, and one column containing the average log ratio of four data points ($\log_2$).

References

1. DeRisi JL, Iyer VR, Brown PO: **Exploring the metabolic and genetic control of gene expression on a genomic scale.** *Science* 1997, **278:**680-686.
2. Blom J, De Mattos MJ, Grivell LA: **Redirection of the respiro-fermentative flux distribution in *Saccharomyces cerevisiae* by overexpression of the transcription factor Hap4p.** *Appl Environ Microbiol* 2000, **66:**1970-1973.
3. Wagner A: **Robustness against mutations in genetic networks of yeast.** *Nat Genet* 2000, **24:**355-361.
4. Bussemaker HJ, Li H, Siggia ED: **Regulatory element detection using correlation with expression.** *Nat Genet* 2001, **27:**167-171.
5. Bereiter-Hahn J: **Dimethylaminostyrylmethylpyridiniumiodine (DASPMI) as a fluorescent probe for mitochondria *in situ*.** *Biochim Biophys Acta* 1976, **423:**1-14.
6. Pringle JR: **Staining of bud scars and other cell wall chitin with calcofluor.** *Methods Enzymol* 1991, **194:**732-735.
7. Mewes HW, Rafael J: **The 2-(dimethylaminostyryl)-1-methylpyridinium cation as indicator of the mitochondrial membrane potential.** *FEBS Lett* 1981, **131:**7-10.
8. Mewes HW, Albermann K, Heumann K, Liebl S, Pfeiffer F: **MIPS: a database for protein sequences, homology data and yeast genome information.** *Nucleic Acids Res* 1997, **25:**28-30.
9. Lyons TJ, Gasch AP, Gaither LA, Botstein D, Brown PO, Eide DJ: **Genome-wide characterization of the Zap1p zinc-responsive regulon in yeast.** *Proc Natl Acad Sci USA* 2000, **97:**7957-7962.
10. Zhao H, Butler E, Rodgers J, Spizzo T, Duesterhoeft S, Eide D: **Regulation of zinc homeostasis in yeast by binding of the ZAP1 transcriptional activator to zinc-responsive promoter elements.** *J Biol Chem* 1998, **273:**28713-28720.
11. Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO: **Genomic expression programs in the response of yeast cells to environmental changes.** *Mol Biol Cell* 2000, **11:**4241-4257.
12. Shamji AF, Kuruvilla FG, Schreiber SL: **Partitioning the transcriptional program induced by rapamycin among the effectors of the Tor proteins.** *Curr Biol* 2000, **10:**1574-1581.
13. Kang W, Matsushita Y, Grohmann L, Graack HR, Kitakawa M, Isono K: **Cloning and analysis of the nuclear gene for YmL33, a protein of the large subunit of the mitochondrial ribosome in *Saccharomyces cerevisiae*.** *J Bacteriol* 1991, **173:**4013-4020.

14. Partaledis JA, Mason TL: **Structure and regulation of a nuclear gene in *Saccharomyces cerevisiae* that specifies MRP13, a protein of the small subunit of the mitochondrial ribosome.** *Mol Cell Biol* 1988, **8:**3647-3660.

15. Hughes TR, Marton MJ, Jones AR, Roberts CJ, Stoughton R, Armour CD, Bennett HA, Coffey E, Dai H, He YD*, et al.*: **Functional discovery via a compendium of expression profiles.** *Cell* 2000, **102:**109-126.

16. van Maris AJA, Bakker BM, Brandt M, Boorsma A, De Mattos MJ, Grivell LA, Pronk JT, Blom J: **Modulating the distribution of fluxes among respiration and fermentation by overexpression of *HAP4* in *Saccharomyces cerevisiae*.** *FEMS Yeast Res* 2001, **1:**139-149.

17. Nijtmans LG, de Jong L, Artal Sanz M, Coates PJ, Berden JA, Back JW, Muijsers AO, van der Spek H, Grivell LA: **Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins.** *EMBO J* 2000, **19:**2444-2451.

18. Schagger H, von Jagow G: **Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form.** *Anal Biochem* 1991, **199:**223-231.

19. Hemrika W, Berden JA, Grivell LA: **A region of the C-terminal part of the 11-kDa subunit of ubiquinol-cytochrome-c oxidoreductase of the yeast *Saccharomyces cerevisiae* contributes to the structure of the Q_{out} reaction domain.** *Eur J Biochem* 1993, **215:**601-609.

20. Lascaris RF, Groot E, Hoen PB, Mager WH, Planta RJ: **Different roles for abf1p and a T-rich promoter element in nucleosome organization of the yeast *RPS28A* gene.** *Nucleic Acids Res* 2000, **28:**1390-1396.

21. **Stanford: functional genomics facility** [http://www.microarray.org]

22. Eisen MB, Spellman PT, Brown PO, Botstein D**: Cluster analysis and display of genome-wide expression patterns.** *Proc Natl Acad Sci USA* 1998, **95:**14863-14868.

23. Wingender E, Chen X, Hehl R, Karas H, Liebich I, Matys V, Meinhardt T, Pruss M, Reuter I, Schacherer F: **TRANSFAC: an integrated system for gene expression regulation.** *Nucleic Acids Res* 2000, **28:**316-319.

24. Quandt K, Frech K, Karas H, Wingender E, Werner T: **MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data.** *Nucleic Acids Res* 1995, **23:**4878-4884.

25. van Helden J, Andre B, Collado-Vides J: **A web site for the computational analysis of yeast regulatory sequences.** *Yeast* 2000, **16:**177-187.

26. Pavlidis P, Lewis DP, Noble WS: **Exploring gene expression data with class scores.** *Pac Symp Biocomput* 2002, 474-485.

27. Serrano R, Kielland-Brandt MC, Fink GR: **Yeast plasma membrane ATPase is essential for growth and has homology with (Na$^+$ + K$^+$), K$^+$- and Ca^{2+}-ATPases.** *Nature* 1986, **319:**689-693.

28. Ferea TL, Botstein D, Brown PO, Rosenzweig RF: **Systematic changes in gene expression patterns following adaptive evolution in yeast.** *Proc Natl Acad Sci USA* 1999, **96:**9721-9726.