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Transcriptional response to mitochondrial NADH kinase deficiency in *Saccharomyces cerevisiae*

Gregory R. Stuart [a], Margaret M. Humble [a], Micheline K. Strand [a,b], William C. Copeland [a,*]

[a] *Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA*
[b] *Life Sciences Division, US Army Research Office, Research Triangle Park, NC 27709, USA*

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ABSTRACT

Yeast cells lacking the mitochondrial NADH kinase encoded by *POS5* display increased sensitivity to hydrogen peroxide, a slow-growth phenotype, reduced mitochondrial function and increased levels of mitochondrial protein oxidation and mtDNA mutations. Here we examined gene expression in *pos5*Δ cells, comparing these data to those from cells containing deletions of superoxide dismutase-encoding genes *SOD1* or *SOD2*. Surprisingly, stress–response genes were down-regulated in *pos5*Δ, *sod1*Δ and *sod2*Δ cells, implying that cells infer stress levels from mitochondrial activity rather than sensing reactive oxygen species directly. Additionally, *pos5*Δ, but not *sod1* or *sod2*, cells displayed an anaerobic expression profile, indicating a defect in oxygen sensing that is specific to *pos5*, and is not a general stress–response. Finally, the *pos5*Δ expression profile is quite similar to the *hap1*Δ expression profile previously reported, which may indicate a shared mechanism.

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1. Introduction

The *POS5* gene in *Saccharomyces cerevisiae* was originally identified by Krems and colleagues in a screen for peroxide-sensitive alleles (Krems et al., 1995). During a systematic screen of gene disruptions affecting mitochondrial function, we discovered that disruption of the *POS5* gene in *S. cerevisiae* resulted in a 50-fold elevation in the mutational rate in the mitochondrial genome (mtDNA), measured by reversion of a frameshift mutation using the Arg8^{m} assay (Strand and Copeland, 2002). *POS5* encodes the mitochondrial NADH kinase, the sole source of NADPH in this organelle (Outten and Culotta, 2003; Strand et al., 2003). Moreover, a 6-fold increase in petite frequency, a 28-fold increase in mitochondrial protein oxidation, and increased sensitivity to hydrogen peroxide and copper ions in *pos5* cells was also observed (Strand et al., 2003). These results suggested a critical role of POS5 in oxidative stress–response and mtDNA maintenance.

Glutathione peroxidase and thioredoxin peroxidase, which facilitate the elimination of hydrogen peroxide in the mitochondrion, are dependent on the reduced cofactors glutathione and thioredoxin. Pos5 plays a critical role in the detoxification of reactive oxygen species, as evident in *pos5* mutant cells which have reduced activity of glutathione reductase and thioredoxin reductase, both of which are dependent on NADPH for the regeneration of their reduced states, and lead to elevated levels of oxidized proteins and mitochondrial mutations (Strand et al., 2003). Whereas most multicellular eukaryotic genomes have one NADH kinase gene, the yeast genome contains three distinct genes encoding NAD/H kinase activity: *POS5, UTR1* and *YEF1* (Shi et al., 2005). Despite compartmentalization of the mitochondrial Pos5 and cytoplasmic Utr1 proteins, the *pos5*Δ *utr1*Δ double mutant is synthetically lethal, demonstrating an overlapping function related to NAD/H kinase activity that is essential for eukaryotic viability, and the interdependence of mitochondrial and cytoplasmic processes (Shianna et al., 2006).

HAP1 encodes the heme activator protein, a transcription factor involved in the complex regulation of gene expression in response to levels of heme and oxygen (Zitomer and Lowry, 1992; Zhang and Hach, 1999; Lombardía et al., 2000; Becerra et al., 2002; Ter Linde and Steensma, 2002; Emerling and Chandel, 2005; Hon et al., 2005). Many *S. cerevisiae* laboratory strains, including S288c and derivatives including BY4741 (Mortimer and Johnston, 1986; Brachmann et al., 1998), contain a Ty1 transposon insertion in *HAP1* (Gaisne et al., 1999). While the mutant protein encoded by *hap1*::Ty1 is believed to retain some activity (Gaisne et al., 1999), the effect of the *hap1*::Ty1 mutation on gene expression compared to isogenic strains containing *HAP1* is unknown. *S. cerevisiae* strain YPH925 was used previously for our studies involving *POS5* (Strand et al., 2003), as well as the microarray analyses presented here. PCR and DNA sequencing analyses of the 3′-region of the *HAP1* locus

Abbreviations: Mt, mitochondria; NADH, nicotinamide adenine dinucleotide.

* Corresponding author. Tel.: +1 919 541 4792; fax: +1 919 541 7613.
E-mail address: copelan1@niehs.nih.gov (W.C. Copeland).

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(data not shown) indicated that strain YPH925 encodes the wild-type *HAP1* gene, while confirming the presence of the *hap1*::Ty1 insertion near the 3′-end of *HAP1* in strain S288c (Gaisne et al., 1999). Hence, the current work also provides a comparison of our previous *pos5*-related microarray data in S288c cells (Shianna et al., 2006) with those in YPH925 cells.

To better understand how *POS5* affects cellular function, here we examined changes in gene expression in *pos5*Δ cells, comparing the results to those induced by deletions of the genes encoding the cytosolic and mitochondrial forms of superoxide dismutase (*sod1*Δ and *sod2*Δ, respectively). Superoxide dismutases catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. Since hydrogen peroxide is further detoxified in the mitochondria by glutathione reductase, which uses NADPH as a cofactor, we sought to determine the relationship of gene expression changes of *pos5*Δ with *sod1*Δ and *sod2*Δ. To facilitate these analyses, we constructed a comprehensive yeast interaction network (Stuart et al., 2009) for use in the bioinformatic data analysis and visualization tool Cytoscape (Shannon et al., 2003). We report that *pos5*Δ, *sod1*Δ and *sod2*Δ cells down-regulate a common set of genes, including those associated with the response to oxidative stress. Our earlier finding (Shianna et al., 2006) that *pos5*Δ cells display an anaerobic expression profile, which we demonstrate is not present in *sod1*Δ or *sod2*Δ cells, is confirmed and is thus not a general oxidative stress–response. The anaerobic transcriptional responses observed in aerobically grown *pos5*Δ cells, but not *sod1*Δ or *sod2*Δ cells, closely mirrors those described in *hap1*Δ cells (Ter Linde and Steensma, 2002), suggesting that a common mechanism is responsible for this response in *pos5* and *hap1* cells.

2. Materials and methods

2.1. Strains

The haploid *S. cerevisiae* strain YPH925 (*ade2-101 cyh2 his3-Δ200 kar1-Δ15 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*) (Spencer et al., 1994) was used for all experiments, and for convenience is referred to as being wild-type. The *POS5*, *SOD1* and *SOD2* open reading frames were precisely deleted using the delitto perfetto site-directed mutagenesis procedure (Storici et al., 2001). Duplicate sets of each deletion strain were independently constructed and used in each experiment, as were duplicate isolates of the wild-type parent strain. Strain identities were verified at each step of the construction by checking genetic markers and mutant-associated phenotypes, as well as the deletional status of the *POS5, SOD1* and *SOD2* loci by PCR analyses. Strain identities were also confirmed before and following isolation of total RNA. The wild-type strain is phenotypically Arg^+ Met^+, as is the *sod2*Δ strain. The *pos5*Δ strain (Met^+), while genetically *ARG*, is phenotypically Arg^- due to disruption of the mitochondrial portion of the arginine biosynthetic pathway in *pos5* cells.

To determine the status of the *HAP1* locus in the YPH925 yeast strain used in this study, we characterized the 3′-terminal region of the *HAP1* gene in each of the YPH925 derivatives used in this study, as well as the S288c and S288c *pos5*Δ::kanMX strains used by Shianna et al. (2006). PCR primers were chosen based on the *HAP1* sequence provided by Creusot et al. (1988) and the *hap1*::Ty1 sequence from the "reference" S288c strain at the *Saccharomyces* Genome Database (http://www.yeastgenome.org). The PCR/sequencing primers were hap1-fpcr (5′-atg tca aga atg ctg cta ttt caa-3′), hap1-wt-rpcr (5′-ggg tcc ctt gac aat cct aat-3′), and hap1-ty1-rpcr (5′-tct ttg acc cag gta ggt agg aat-3′). Primers hap1-fpcr and hap1-wt-rpcr amplify a *HAP1* fragment of 1652 bp; primer set hap1-fpcr and hap1-ty1-rpcr amplify a *hap1*::Ty1 fragment of 2121 bp. PCR and DNA sequencing analyses of the 3′-region of the *HAP1* locus (data not shown) indicated that YPH925 encodes the wild-type *HAP1* gene, while confirming the presence of the *hap1*::Ty1 insertion near the 3′-end of *HAP1* in strain S288c (Gaisne et al., 1999).

2.2. Growth of strains

Standard media and methods were used for the growth and handling of the *S. cerevisiae* strains (Sherman, 2002). The duplicate sets of strains (wild-type; *pos5*Δ; *sod1*Δ; *sod2*Δ) were grown individually in 10 ml of YPD (glucose) in 18 mm × 150 mm glass tubes with vigorous shaking (∼200 rpm) at 30 °C until the cells approached the mid-log phase of growth: on average, $\sim 1.7 OD_{600\ nm}$, corresponding to $\sim 5 \times 10^7$ cells/ml. The cells were then harvested by centrifugation (1000*g*, 5 min) and the supernatants were discarded. To investigate changes in expression in cells dependent on oxidative phosphorylation for continued growth, *pos5*Δ and wild-type cells were grown as described above to the mid-log phase of growth, harvested by centrifugation, resuspended in YPG, shaken for 2 h at 30 °C, and harvested by centrifugation.

2.3. Isolation of total RNA and microarray analyses

Immediately following the growth of the strains and harvesting the cells, total RNA was isolated using an RNeasy Midi Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. RNA from duplicate samples was pooled, quantitated by UV spectrophotometry, and stored at −80 °C. RNA from untreated wild-type cells (three preparations) was pooled and used as the reference (control) RNA for each of the experiments. These RNA were submitted to the Microarray Group at the National Institute of Environmental Health Sciences for microarray analysis (hybridizations and data collection). The concentration, purity and overall quality of each RNA preparation were verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA was reverse transcribed/labeled with the Cy3 and Cy5 dyes and hybridized to the *S. cerevisiae* DNA microarray chips (Yeast Oligo Microarray G4140A, Agilent Technologies, Palo Alto, CA). The chips are 1 in. × 3 in. glass slides each containing two identical oligonucleotide microarrays, consisting of 60-mer oligonucleotides probes representing 6256 known open reading frames from the S288c strain of *S. cerevisiae*, sourced from the May 25, 2002 open reading frame list at the *Saccharomyces* Genome Database (http://www.yeastgenome.org). In each experiment a duplicate aliquot of RNA was reversed-labeled with the Cy3/Cy5 dyes ("dye swap"), and hybridized to the duplicate array on each chip.

2.4. Bioinformatics

Microarray fluorescence data were imported into Rosetta Resolver (Rosetta Biosoftware, Seattle, WA) for the estimation of random error by application of an error model that calculated the confidence limits (*p*-values) for the expression values. The Agilent GeneSpring Analysis Platform (Agilent Technologies, Palo Alto, CA) was used for LOWESS data normalization, data analyses (including application of a cross-gene error model), and visualization (e.g. clustering analyses) of the microarray data. Microsoft Excel (Microsoft Corporation, Redmond, WA) was used to display and sort selected subsets of the microarray expression data.

To aid the visualization and the analysis of the expression data, in April 2008 we constructed a *S. cerevisiae* protein–protein and protein–DNA interaction network from the interaction data at the *Saccharomyces* Genome Database (http://www.yeastgenome.org) plus the transcription factors and their documented target genes at YEASTRACT (http://www.yeastract.com) (Teixeira et al., 2006), giving an interactome consisting of 6188 "nodes" (genes) and 109,179 "edges" (interactions) (Stuart et al., 2009).

Expression data from each experiment were then mapped onto this interactome using Cytoscape (http://www.cytoscape.org), a data analysis and visualization tool (Shannon et al., 2003). Genes associated with the highest-scoring subnetworks in each experiment were identified using the Cytoscape jActiveModules plugin, which identifies connected subnetworks within the interaction network whose genes show significant coordinated changes in mRNA expression over particular experimental conditions (Shannon et al., 2003). The algorithm iteratively reduces network complexity by pinpointing regions whose states are perturbed by the conditions of interest, while removing false-positive interactions and interactions not involved in the perturbation response.

Each of the five top-scoring jActiveModules-derived subnetworks from *pos5*Δ cells (containing 165, 188, 153, 187 and 83 genes) was scored by jActiveModules as being highly significant ($p < 10^{-4}$). jActiveModules is particularly useful for identifying the specific transcription factors that may themselves display small or no change in expression, but which significantly influence gene expression in each of these subnetworks.

2.5. Quantitative real-time PCR

To evaluate the overall quality of the microarray data, we employed real-time PCR to examine the expression of a subset of genes in *pos5*Δ cells, selected on the basis of function relative to the microarray data and the study aims. First strand cDNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), in a 20 μl reaction containing 1 μg of total RNA and 40 units of RNaseOUT (Invitrogen, Carlsbad, CA). The remaining protocols were performed as per the manufacturer's recommendations. The reaction was incubated in a thermocycler at 25 °C for 10 min, 37 °C for 90 min, 85 °C for 5 s, then held at 4 °C, until further use. Real-time PCR reactions were done in triplicate using an ABI Prism 7900 Sequence Detection system (Applied Biosystems, Foster City, CA). The primers (listed in a footnote to Table 2) were synthesized by Invitrogen (Carlsbad, CA). The real-time PCR reactions used 100 ng of cDNA in a 25 μl reaction using a SYBR Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA), according to the protocol provided by the manufacturer. Expression patterns of each gene determined by real-time PCR were normalized to the expression of the house-keeping gene, *ACT1* (encoding the structural protein actin). The fold-changes reported are relative to expression of the corresponding wild-type control.

3. Results

3.1. Overview of the expression data

In order to compare changes in gene expression in cells subject to increased oxidative stress arising from internal and external sources, deletions of the *POS5*, *SOD1* and *SOD2* genes were introduced into *S. cerevisiae* laboratory strain YPH925, used previously for our studies involving *POS5* (Strand et al., 2003). To explore changes in transcription associated with endogenous sources of oxidative stress, total RNA was isolated from wild-type YPH925, *pos5*Δ, *sod1*Δ and *sod2*Δ cells grown in YPD or shifted to YPG to obligate mitochondrial function, and subjected to microarray analysis. Preliminary analysis of the microarray data using Rosetta Resolver indicated that 944 genes were differentially expressed in *pos5*Δ cells as compared to wild-type cells, 1236 genes were differentially expressed in *sod1*Δ cells, and 612 genes were differentially expressed in *sod2*Δ cells at a significance level of $p < 10^{-4}$. At this level of stringency with 6256 genes represented on the yeast chip, ~0.6 false-positives were expected per experiment. Examining these data (e.g. Table 1), it was noted that *POS5* appeared to be down-regulated only 4.51-fold ($p = 8.83 \times 10^{-4}$) in the *pos5*Δ strain, whereas *SOD1* and *SOD2* expression were reduced 83.9-fold ($p < 1.4 \times 10^{-45}$) and 63.4-fold ($p < 1.4 \times 10^{-45}$), respectively. Each of these deletions was confirmed during strain construction, and prior to the RNA isolations, by PCR analysis and DNA sequencing. Additionally, real-time PCR (below) verified the absence of *POS5* gene expression in the *pos5*Δ strain, compared to the wild type strain. Hence, the reason for the higher than expected level of expression for *POS5* in *pos5*Δ cells remains unidentified.

3.2. Validation of selected genes by real-time PCR

Overall, the microarray data were shown to be very reproducible based on comparisons of the replicate inter-chip (dye-swapped) expression values, as well as inter-experiment (inter-chip) values for genes not under selective pressure. Nevertheless, we also evaluated the expression levels of selected genes-of-interest in *pos5*Δ cells using quantitative real-time PCR (Fig. 1; Table 2). These genes included those associated with oxidative stress (*OGG1*; *POS5*; *SOD1*; *SOD2*; *TSA2*), anaerobic gene expression (*ANB1*; *CYB2*; *DAN1*; *YLR256W*/*HAP1*; *HES1*), cellular redox (*NDE2*) and mitochondrial gene expression (mtCOX1). The real-time PCR data confirmed the disruption of *POS5* in *pos5*Δ cells, by a 1879-fold decrease in expression, and the up-regulation of genes associated with hypoxia and anaerobic gene expression (*ANB1*; *DAN1*; *HES1*; Fig. 1; Table 2) as well as the decrease in mtDNA gene expression (mtCOX1). Overall, there was a 75% concordance of the real-time PCR data with that of microarray data of the genes analysed.

3.3. Bioinformatic analyses

Extensive analyses of the expression data using various tools – including GeneSpring (not shown), Cytoscape, and GoMiner (Zeeberg et al., 2005) (http://discover.nci.nih.gov/gominer/; not shown), and the resources at the *Saccharomyces* Genome Database (e.g. GO Slim Mapper) indicated that while cellular metabolism was broadly affected in *pos5*Δ, *sod1*Δ and *sod2*Δ cells, overall no single biological pathway was dramatically altered, transcriptionally, in any experiment. Rather, the transcriptional changes observed were distributed among virtually all cellular biological processes and cellular functions (Table 3). While these tools were useful for this dataset, populated by small changes of expression affecting hundreds of genes, we found that simply applying fold-cutoffs, then examining relationships among these genes and their associated transcription factors (identified using the Cytoscape jActiveModules plugin) provided the most facile and meaningful interpretation of the data with highly significant *p*-values ($p < 10^{-4}$), as did visualization of the data in Cytoscape.

3.4. Transcriptional changes in *pos5Δ* cells

As mentioned, the *pos5*Δ mutation exerted pleiotropic effects on gene expression. Compared to their expression in wild-type control cells, genes associated with numerous biological processes (Table 3) were down-regulated in *pos5*Δ cells (these genes were also down-regulated in *sod1*Δ and *sod2*Δ cells), including amino acid metabolism, carbohydrate metabolism, RNA and DNA metabolism, transport and protein folding. Interestingly, all of the stress–response genes that were most affected (Table 1) were nearly identically down-regulated in *pos5*Δ, *sod1*Δ and *sod2*Δ cells (Tables 1 and 3), and up-regulated in *pos5*Δ and wild-type cells shifted to growth in glycerol (Table 1). These stress–response genes included those associated with the response to oxidative stress (Table 1: *AAD6*, *ALD6*, *BTN2*, *GAD1*, *HOR2*, *HSP12*, *NCE103*, *PUT4*).

Table 1
Comparison of the expression of differentially expressed genes in *pos5*Δ cells to their expression levels in *sod1*Δ and *sod2*Δ cells.[a]

Systematic name[b]	Common name[b]	Exp A *pos5*Δ	Exp B *sod1*Δ	Exp C *sod2*Δ	Exp D *pos5*Δ YPG shift	Exp E WT YPG shift Exp	Description[c]
YGR243W	FMP43	-20.03	-24.06	-21.00	5.10	7.02	The authentic, non-tagged protein was localized to mitochondria
YGR142W	BTN2	-11.79	-13.05	-16.09	7.54	1.52	Cytosolic protein; arginine uptake; pH homeostasis; protein trafficking
YDR536W	STL1	-10.21	-12.30	-11.49	16.02	21.12	Plasma membrane glycerol proton symporter; inactivated by glucose
YOR348C	PUT4	-9.29	-12.04	-4.39	3.81	6.60	Proline permease; repressed in ammonia-grown cells; antioxidant
YMR169C	ALD3	-8.93	-7.62	-8.87	9.35	3.38	Aldehyde dehydrogenase; β-alanine synthesis; induced by stress
YFL014W	HSP12	-7.13	-2.82	-2.11	16.03	18.36	Plasma membrane protein induced by oxidative stress
YOL084W	PHM7	-6.73	-6.46	-6.96	3.45	4.16	Protein of unknown function; expression is regulated by phosphate levels
YBR072W	HSP26	-6.66	-1.93	-6.53	31.96	9.48	Small heat shock protein with chaperone activity; response to stress
YJL144W	YJL144W	-6.35	-4.25	-7.12	4.11	2.15	Cytoplasmic hydrophilin of unknown function; response to stress
YGR043C	NQM1	-5.65	-2.71	-5.14	15.65	16.42	Unknown function; repressed by MOT1, induced by diauxic shift
YGR052W	FMP48	-5.48	-4.82	-3.53	1.18	1.21	The authentic, non-tagged protein was localized to the mitochondria
YDR171W	HSP42	-5.32	-5.56	-5.73	11.01	3.01	Small cytosolic stress-induced chaperone
YGR088W	CTT1	-5.18	-5.36	-3.84	6.85	7.18	Cytosolic catalase T; oxidative damage protection; $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$
YNL077W	APJ1	-4.84	-4.29	-4.63	1.30	1.50	Putative chaperone of the HSP40 (DNAJ) family
YHR033W	YHR033W	-4.79	-3.55	-4.00	2.83	4.83	Putative protein of unknown function; localizes to the cytoplasm
YHR096C	HXT5	-4.66	-7.47	-6.81	63.29	96.27	Hexose transporter; accumulation of carbohydrates during stress?
YAL061W	BDH2	-4.58	-3.64	-3.55	16.23	4.83	Putative polyol dehydrogenase
YDL022W	GPD1	-4.53	-5.08	-4.74	2.67	4.08	Glycerol-3-phosphate dehydrogenase; response to osmotic stress
YML128C	MSC1	-4.48	-3.56	-3.69	9.63	9.13	Mutant is defective in meiotic recombination of homologous chromatids
YFL030W	AGX1	-4.46	1.25	-2.03	5.38	7.59	One of three glycine biosynthesis pathways in yeast
YGR256W	GND2	-4.36	-1.65	-3.80	1.51	3.34	Catalyzes an NADPH-regenerating reaction in the PPP
YPL240C	HSP82	-4.28	-3.25	-5.05	3.13	1.40	Chaperone; regulates Hsf1; docks with Tom70; response to stress
YER103W	SSA4	-4.23	-4.36	-4.42	9.23	1.53	Heat shock protein that is highly induced upon stress
YML054C	CYB2	-4.20	-1.93	-1.94	11.78	19.79	Cytochrome *b2;* lactate use; repressed by glucose, anaerobic conditions
YDL085W	NDE2	-3.99	-3.11	-2.66	8.80	18.07	With Nde1p, provides cytosolic NADH to mitochondrial respiratory chain
YML123C	PHO84	-3.97	1.47	-1.17	-7.05	-1.55	High-affinity inorganic phosphate and low-affinity manganese transporter
YER062C	HOR2	-3.86	-1.34	-2.63	1.92	3.04	Glycerol biosynthesis; hyperosmosis, oxidative stress; diauxic transition
YLL026W	HSP104	-3.86	-3.15	-3.95	4.60	2.92	Stress response; refolds, reactivates denatured, aggregated proteins
YLR327C	TMA10	-3.85	-3.04	-3.57	34.04	12.62	Protein of unknown function that associates with ribosomes
YDL214C	PRR2	-3.82	-3.75	-4.02	15.16	11.13	Protein kinase; possible role in MAP kinase pheromone response signaling
YOR062C	YOR29-13	-3.70	-3.69	-3.52	1.28	1.82	Protein of unknown function; expression regulated by glucose and Rgt1p
YMR316W	DIA1	-3.58	-1.91	-2.15	-1.15	1.10	Protein of unknown function involved in invasive & pseudohyphal growth
YNL036W	NCE103	-3.40	-2.77	-2.34	4.17	3.62	Carbonic anhydrase; poorly transcribed under aerobic conditions
YKR069W	MET1	-3.27	-2.23	-2.67	1.76	1.81	Sulfate assimilation, methionine metabolism, and siroheme biosynthesis
YPL171C	OYE3	-3.25	3.77	-1.13	1.56	-2.38	Conserved NADPH oxidoreductase; may be involved in sterol metabolism
YJR010W	MET3	-3.24	-1.82	-2.52	1.05	1.21	Reduction of sulfate to sulfide; involved in methionine metabolism
YBR117C	TKL2	-3.23	-4.06	-4.16	9.50	19.33	PPP transketolase; needed for synthesis of

Table 1 (*continued*)

Systematic name[b]	Common name[b]	Exp A *pos5*Δ	Exp B *sod1*Δ	Exp C *sod2*Δ	Exp D *pos5*Δ YPG shift	Exp E WT YPG shift Exp	Description[c]
							aromatic amino acids
YGR248W	SOL4	-3.17	-2.15	-2.66	4.77	6.74	6-phosphogluconolactonase with similarity to Sol3p
YPR151C	SUE1	-3.17	-1.76	-1.45	5.52	5.45	Mitochondrial protein; degradation of unstable forms of cytochrome *c*
YDR258C	HSP78	-3.16	-2.88	-3.12	2.20	2.53	Mitochondrial matrix chaperone; misfolded proteins; proteolysis
YER037W	PHM8	-3.13	-1.38	-2.25	3.29	5.27	Protein of unknown function, induced by low phosphate levels
YNL277W	MET2	-3.07	-2.09	-3.13	1.79	1.67	Catalyzes the first step of the methionine biosynthetic pathway
YER150W	SPI1	-3.03	-2.44	-2.63	**13.32**	5.12	Cell wall; induced by stress, diauxic shift; regulated by Msn2/Msn4
YOR374W	ALD4	-3.02	-1.86	-2.43	**10.16**	**13.59**	Mitochondrial aldehyde dehydrogenase; repressed by growth on glucose
YHR087W	RTC3	-3.00	-4.25	-3.57	6.61	3.25	Protein involved in RNA metabolism
YMR250W	**GAD1**	-2.94	-2.28	-2.48	3.05	3.11	Converts glutamate into GABA; involved in response to oxidative stress
YDL204W	RTN2	-2.88	-2.37	-3.02	**10.46**	**12.36**	Protein of unknown function; similarity to mammalian reticulon proteins
YHR139C	SPS100	-2.82	-4.38	-3.80	1.82	2.49	Protein required for spore wall maturation; expressed during sporulation
YIL136W	OM45	-2.79	-2.26	-2.46	**12.73**	**17.60**	Unknown function, major constituent of mitochondrial outer membrane
YBR116C	YBR116C	-2.76	-2.27	-2.71	9.21	**14.90**	Hypothetical protein
YLR205C	HMX1	-2.73	-1.06	-1.17	1.02	1.45	ER-localized, heme-binding peroxidase involved in degradation of heme
YJL108C	PRM10	-2.68	-2.05	-2.99	3.18	4.19	Pheromone-regulated protein, predicted to have transmembrane segments
YKL071W	YKL071W	-2.68	5.80	-1.03	-4.95	**-14.01**	Putative protein of unknown function; localizes to the cytoplasm
Q0065	AI4	-2.67	-1.49	-1.23	-1.47	-1.23	Endonuclease I-SceII, encoded within the mitochondrial COX1 gene
YHR136C	SPL2	-2.67	1.03	-1.92	**15.92**	3.89	Protein with similarity to cyclin-dependent kinase inhibitors
YPL061W	**ALD6**	-2.64	-1.47	-1.77	1.84	1.68	Cytosolic catalysis of acetaldehyde to acetate; constitutively expressed
YGL052W	YGL052W	-2.61	-3.24	-2.85	1.97	2.48	Hypothetical protein
YOR338W	YOR338W	-2.61	1.60	-1.94	2.49	2.94	Hypothetical protein
YGR066C	YGR066C	-2.58	-3.41	-3.46	1.88	1.01	Putative protein of unknown function
YPL111W	CAR1	-2.54	-3.80	-2.14	2.83	2.82	Arginine degradation; induced by arginine, nitrogen catabolite repression
YLR149C	YLR149C	-2.53	-2.17	-2.52	5.81	5.20	Putative protein of unknown function; YLR149C is not an essential gene
YFL056C	**AAD6**	-2.51	3.25	1.14	-4.37	-5.55	Aryl-alcohol dehydrogenase?; oxidative stress response
(*etc.*)	(*etc.*)	(*etc.*)	(*etc.*)	(*etc.*)	(*etc.*	(*etc.*	(*etc.*)
YJL105W	SET4	2.58	1.17	1.03	1.52	1.08	Protein of unknown function, contains a SET domain
YEL065W	SIT1	2.62	-1.69	1.36	3.44	4.54	Siderophore-iron transporter induced by iron deprivation and diauxic shift
YMR317W	YMR317W	2.66	-1.18	-1.14	1.95	1.73	Hypothetical protein
YOR382W	FIT2	2.68	1.21	1.72	3.99	5.01	Cell wall mannoprotein involved in retention of cell wall siderophore-iron
YHL047C	ARN2	2.95	-1.13	1.84	3.09	2.91	Transporter that specifically recognizes siderophore-iron chelates
YLR355C	ILV5	3.39	-1.35	1.13	-2.76	-2.35	Mitochondrial branched-chain amino acid synthesis; mtDNA maintenance
YOR009W	TIR4	4.33	1.11	1.05	3.06	1.05	Cell wall mannoprotein expressed under anaerobic conditions
YOR383C	FIT3	4.74	-1.22	3.03	5.87	4.07	Cell wall mannoprotein involved in retention of cell wall siderophore-iron
YOR237W	HES1	5.28	-1.19	-1.69	1.89	-1.25	Protein implicated in the regulation of ergosterol biosynthesis
YJR047C	ANB1	7.94	-1.10	-1.02	2.11	-1.78	Translation initiation factor eIF-5A, expressed under anaerobic conditions
YJR150C	DAN1	**12.74**	1.00	1.11	3.69	-1.11	Cell wall mannoprotein expressed under anaerobic conditions

[a] The genes shown in this table are derived from a combined list of all genes showing ⩾2.5-fold-changes in expression in *pos5*Δ cells, with the corresponding expression values provided also for *sod1*Δ and *sod2*Δ cells. Cells are color-coded according to their expression values, provided that the associated *p*-value was $\leqslant 10^{-4}$.

[b] Genes encoding mitochondrial-associated proteins are shown with their systematic names in blue bold text. Genes associated with the response to oxidative stress are shown with their common names in red bold text.

[c] Abbreviated gene descriptions, from the full descriptions provided at the *Saccharomyces* Genome Database (www.yeastgenome.org). GABA, γ-aminobutyric acid; PPP, pentose phosphate pathway.

Table 2
Comparison of the fold-changes in expression of selected genes in *pos5*Δ cells by microarray and real-time PCR.

Gene		Microarray	RT-PCR[a]	Description[b]
Oxidative stress				
YGR088W	CTT1	−5.18	1.76	Cytosolic catalase T, has a role in protection from oxidative damage by hydrogen peroxide
YML060W	OGG1	−1.01	1.25	Mitochondrial 7,8-dihydro-8-oxoguanine glycosylase/lyase; repairs oxidative damage to mtDNA
YPL188W	POS5	−4.51	−1879.82	Mitochondrial NADH kinase, phosphorylates NAD^+, NADH; oxidative stress–response
YJR104C	SOD1	−1.15	1.07	Cu, Zn superoxide dismutase present in cytoplasm and mitochondrial intermembrane space
YHR008C	SOD2	−1.96	−1.09	Manganese-containing superoxide dismutase; protects cells against oxygen toxicity
YDR453C	TSA2	−1.26	7.98	Stress-inducible thioredoxin peroxidase; removal of reactive oxygen, nitrogen, sulfur species
Anaerobic expression				
YJR047C	ANB1	7.94	134.40	Translation initiation factor eIF-5A; expressed under anaerobic conditions
YML054C	CYB2	−4.20	1.11	Mitochondrial cytochrome *b2*; required for lactate utilization; repressed by glucose, anaerobic conditions
YJR150C	DAN1	12.74	2214.79	Cell wall mannoprotein expressed under anaerobic conditions; repressed during aerobic growth
YLR256W	HAP1	1.04	−3.51	Transcription factor involved in regulation of gene expression in response to levels of heme and oxygen
YOR237W	HES1	5.28	38.01	Protein implicated in the regulation of ergosterol biosynthesis
Cellular redox				
YDL085W	NDE2	−3.99	2.28	NADH dehydrogenase; with Nde1p, provides cytosolic NADH to mitochondrial respiratory chain
mtDNA gene expression				
Q0045	COX1	−1.61	−9.87	Reverse transcriptase encoded by a mobile group II intron within the mitochondrial COX1 gene

[a] The primers employed for real-time PCR were ACT1 (forward: 5′-ttg gcc ggt aga gat ttg ac; reverse: 5′-cag cag tgg tgg aga aag agt a-3′), COX1 (forward: 5′-gtg cgt ata ttt cgt tga tgc gt-3′; reverse: 5′-ttc aca ctg cct gtg cta tct aa-3′), ANB1 (forward: 5′-ggc acc aga agg tga att gg; reverse: 5′-tca ccc atg gca gaa atg ata g-3′), CTT1 (forward: 5′-tgg gag cca act atc agc aat; reverse: 5′-ggg aat cac ctt tgg agt atg ga-3′), CYB2 (forward: 5′-ttc gtc gtg gta cag atg tc; reverse: 5′-gag ttc gca tac aag aat ggt cta-3′), DAN1 (forward: 5′-gtg ccg cca ctg agt ctt; reverse: 5′-tgg gtg gaa gca gcg ttt-3′), GND2 (forward: 5′-tgc cgg ttc tgg tca cta tg; reverse: 5′-cgt aag cct cgc aaa tca ac-3′), HAP1 (forward: 5′-gct ggt gac aaa gac caa tta cag-3′; reverse: 5′-tgc atc gga gca gaa cct-3′), HES1 (forward: 5′-cgt cca aac tgc agc ttt ctg; reverse: 5′-caa tgc aat gcg gtt gaa aca tc-3′), NDE2 (forward: 5′-gcc aaa caa aca cgg ata tcg a; reverse: 5′-tgc tcc ggt att cta ctc atc ag-3′), OGG1 (forward: 5′-gcc tac cat tcg ttt cct aca ag; reverse: 5′-agc cca gct cac gca act-3′), POS5 (forward: 5′-tcc cag gat ttc aaa tct c-3′; reverse: 5′-tgt tca gga cca gta taa ag-3′), SOD1 (forward: 5′-gct ggt cct cac ttc aat cct t; reverse: 5′-ccg tct tta cgt tac cca tgt ca-3′), SOD2 (forward: 5′-gct gga cgt tgt tca aac cta; reverse: 5′-gcg tca atg gca act aga gga a-3′), SOL4 (forward: 5′-gaa gag atg gac cgc aag gtt t; reverse: 5′-gcc tcg gct act act gct aa-3′), TKL2 (forward: 5′-gct ctg gca aca aga aag aca; reverse: 5′-cca ccg atc aat tca ggc aaa-3′), TSA2 (forward: 5′-gcc tcc acc gac tct gaa; reverse: 5′-ctg gac cta atc cac cgt ctt-3′).
[b] Abbreviated gene descriptions, from the full descriptions provided at the *Saccharomyces* Genome Database (www.yeastgenome.org).

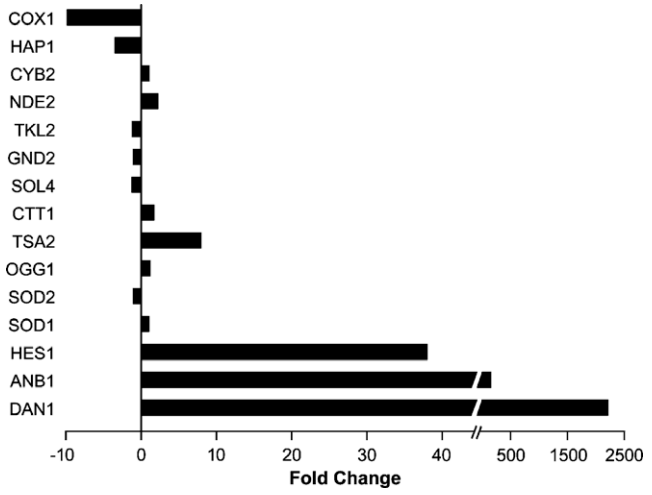

Fig. 1. Real-time PCR results from *pos5*Δ cells. Changes in expression are normalized to the expression of *ACT1* in wild-type cells.

To evaluate changes in gene expression in *pos5*Δ cells, we identified the genes most significantly affected in the five top-scoring subnetworks using the Cytoscape jActiveModules plugin, resulting in a combined list of 386 genes including their associated transcription factors. To reduce the complexity of this list, we next selected those genes that were ⩾1.5-fold up-regulated and ⩾3.0-fold down-regulated, plus their associated transcription factors, giving a list of 91 genes. To further simplify the display, transcriptional activator Msn4p was removed, and sterol regulatory element binding protein Upc2p was added giving the sub-network shown in Fig. 2.

All of the stress–response genes shown in Fig. 2 as nodes with exaggerated blue borders and the node names in blue text, were down-regulated including *ALD3*, *GPD1*, *GPX2*, *HOR2*, *HSP104*, *HSP12*, *HSP26*, *HSP42*, *HSP78*, *HSP82*, *HXT5*, *MSN2*, *OYE3*, *PUT4*, *ROX1*, *RPN4*, *SPI1*, *SSA4* and *YJL144W*. This is analogous to a similar finding among the genes with the greatest fold-changes in expression in *pos5*Δ, *sod1*Δ and *sod2*Δ cells (Table 1). Also evident in Fig. 2 are the numerous genes associated with anaerobic/hypoxic growth (Lombardía et al., 2000; Abramova et al., 2001; Becerra et al., 2002; Kwast et al., 2002; Ter Linde and Steensma, 2002; Lai et al., 2005; 2006). *ANB1*, encoding an anaerobically-induced transcription factor (Sertil et al., 2003), was up-regulated 7.9-fold in *pos5*Δ cells while cell wall mannoprotein gene *DAN1* was up-regulated 12.7-fold and *HES1*, associated with the regulation of ergosterol biosynthesis, was up-regulated 5.3-fold (Table 1). Also up-regulated in *pos5*Δ cells (Fig. 2) were anaerobic-associated cell wall mannoprotein genes *DAN3* (1.8-fold up-regulated), *TIR3* (2.2-fold) and *TIR4* (4.3-fold), the sterol uptake/transporter encoded by *AUS1* (2.4-fold), the mitochondrial inner membrane ADP/ATP translocator encoded by *AAC3* (2.2-fold), and seripauperin genes *PAU1* (1.6-fold), *PAU5* (1.7-fold) and *PAU6* (1.5-fold) (Rachidi et al., 2000). Although not previously reported as being associated with anaerobiosis, cell wall mannoprotein gene *FIT3* (Table 1) was up-regulated 4.7-fold in *pos5*Δ cells. Anaerobic-associated genes reported here for the first time in *pos5*Δ cells included up-regulated genes *AAC3*, *ANB1*, *AUS1*, *DAN1*, *HEM13* (2.2-fold up-regulated), *UPC2* (1.5-fold) and *YML083C* (1.9-fold).

Genes present in Fig. 2 known to be down-regulated during anaerobic growth included *CYB2*, encoding cytochrome *b2* (4.2-fold) and *NCE103*, encoding carbonic anhydrase (3.4-fold down-regulated). Hypoxic gene *CYC7*, encoding cytochrome *c* isoform 2, was also down-regulated in *pos5*Δ cells (2.0-fold; not shown). However, unlike the up-regulated anaerobic genes, that were only affected in *pos5*Δ but not *sod1*Δ or *sod2*Δ cells, *CYB2*, *NCE103* (Table 1) and *CYC7* (data not shown) were also down-regulated in *sod1*Δ and *sod2*Δ cells indicating that the changes in expression for these genes in *pos5*Δ cells were associated with a general stress–response not related to oxygen sensing.

Table 3
GO Slim Biological Processes associated with differentially expressed genes in *pos5*Δ cells.

GO ID	GO slim biological process[a]	Frequency (%)	Genes[b]
8150	Biological process unknown	20 of 73 genes, 27.4	APJ1, BDH2, FMP43, FMP48, NQM1, OM45, PHM8, **SET4**, RTN2, TMA10, YBR116C, YGL052W, YGR066C, YHR033W, YKL071W, YLR149C, **YMR317W**, PHM7, **TIR4**, YOR062C
6950	Response to stress	14 of 73 genes, 19.2	ALD3, CTT1, GAD1, GPD1, HOR2, HSP12, HSP104, HSP26, HSP42, HSP78, HSP82, NCE103, SSA4, YJL144W
6810	Transport	13 of 73 genes, 17.8	**ARN2**, BTN2, **DAN1**, **FIT2**, **FIT3**, **HES1**, HXT5, HSP78, PHO84, PUT4, **SIT1**, SSA4, STL1
42221	Response to chemical stimulus	9 of 73 genes, 12.3	AAD6, CTT1, GAD1, HSP12, NCE103, OYE3, PRM10, PRR2, SPI1
6519	Cellular amino acid and derivative metabolic process	9 of 73 genes, 12.3	ALD3, AGX1, CAR1, GAD1, **ILV5**, MET1, MET2, MET3, PUT4
51186	Cofactor metabolic process	8 of 73 genes, 11	**ARN2**, GND2, GPD1, HMX1, MET1, NDE2, SOL4, TKL2
9653	Anatomical structure morphogenesis	7 of 73 genes, 9.6	**ANB1**, **ILV5**, **HES1**, HSP42, HSP78, HSP82, SPS100
6457	Protein folding	5 of 73 genes, 6.8	HSP104, HSP26, HSP78, HSP82, SSA4
6766	Vitamin metabolic process	5 of 73 genes, 6.8	GND2, GPD1, NDE2, SOL4, TKL2
5975	Carbohydrate metabolic process	4 of 73 genes, 5.5	GND2, HOR2, SOL4, TKL2
16070	RNA metabolic process	4 of 73 genes, 5.5	AI4, PRR2, RTC3, YOR338W
6996	Organelle organization	4 of 73 genes, 5.5	**ILV5**, HSP42, HSP78, HSP82
19725	Cellular homeostasis	4 of 73 genes, 5.5	**ARN2**, GPD1, HMX1, **SIT1**
6259	DNA metabolic process	3 of 73 genes, 4.1	AI4, HSP82, MSC1
6091	Generation of precursor metabolites and energy	2 of 73 genes, 2.7	HOR2, NDE2
6350	Transcription	2 of 73 genes, 2.7	PRR2, YOR338W
746	Conjugation	2 of 73 genes, 2.7	PRM10, PRR2
46483	Heterocycle metabolic process	2 of 73 genes, 2.7	HMX1, MET1
30435	Sporulation resulting in formation of a cellular spore	2 of 73 genes, 2.7	SPS100, YOR338W
16192	Vesicle-mediated transport	2 of 73 genes, 2.7	BTN2, **HES1**
7124	Pseudohyphal growth	1 of 73 genes, 1.4	DIA1
7010	Cytoskeleton organization	1 of 73 genes, 1.4	HSP42
30163	Protein catabolic process	1 of 73 genes, 1.4	SUE1
6412	Translation	1 of 73 genes, 1.4	**ANB1**
7047	Cell wall organization	1 of 73 genes, 1.4	SPS100
7049	Cell cycle	1 of 73 genes, 1.4	MSC1
7126	Meiosis	1 of 73 genes, 1.4	MSC1
16044	Membrane organization	1 of 73 genes, 1.4	**HES1**
	Other	4 of 73 genes, 5.5	ALD4, ALD6, CYB2, SPL2

[a] The differentially expressed genes in *pos5* cells (Table 1) were parsed through the *Saccharomyces* Genome Database Gene Ontology Slim Mapper at http://www.db.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl.
[b] Genes shown in bold text are up-regulated in pos5Δ cells; the remaining genes are down-regulated in *pos5*Δ cells.

3.5. Complex inter-regulation among transcription factors

Equally apparent from Fig. 2 is the extraordinarily complex nature of gene regulation among even this selected group of genes. In addition to regulating large numbers of genes, transcription factors in *S. cerevisiae* appear to cross-regulate one another, with a high degree of interconnectivity. To clarify these interactions, the transcription factors present in Fig. 2 are shown separately in Fig. 3. Clearly, transcription factors regulate the expression of one another, while co-regulating the expression of their shared and unique target genes.

As indicated above, the "anaerobic" transcriptional profile previously reported by Shianna et al. (2006) in *pos5*Δ cells was confirmed and extended here, and demonstrated to be unique to *pos5*Δ but not *sod1*Δ or *sod2*Δ cells. To examine the regulation of expression of genes associated with oxygen metabolism and oxygen-dependent cellular processes, genes annotated at the *Saccharomyces* Genome Database as being associated with hypoxia or

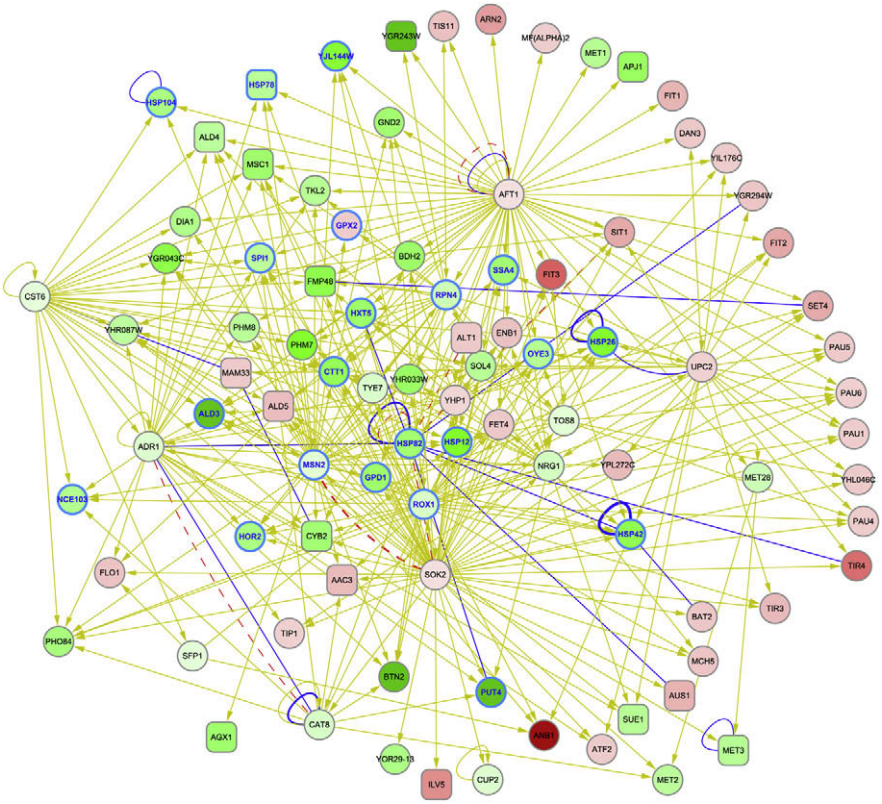

Fig. 2. A Cytoscape sub-network displaying the 75 genes from the Cytoscape interactome (Stuart et al., 2009) that were ⩾1.5-fold up-regulated and ⩾3.0-fold down-regulated in *pos5*Δ cells present among the five top-scoring jActiveModules subnetworks, plus their associated transcription factors. For clarity, transcription factor Msn4p plus its associated edges were deleted from this subinteractome, since Msn4p is transcriptionally equivalent to Msn2p. Lastly, transcription factor Upc2, involved in sterol biosynthesis and the anaerobic response, was specifically included, resulting in the 91-node sub-network shown in the figure. In each of these Cytoscape images, up-regulated genes (nodes) are colored red, while down-regulated genes are colored green, with the level of shading proportional to the level of expression (darker shades being more up- or down-regulated than lighter shades). Mitochondrial-associated nodes are shown as rectangles, and nodes associated with the response to stress are drawn with emphasized, blue borders with the gene names also displayed in blue text. Node–node interactions (edges) are color-coded with blue edges indicating protein–protein interactions, gold edges indicating transcription factors with the arrow pointing from the transcription factor toward the regulated gene, and broken red edges indicating genetic (rather than physical) interactions, e.g. synthetic lethality - the loss of viability when both alleles are inactivated. Nodes in which the blue edge loops back on itself indicate self-regulated genes. The thickness of the edges represent weights, i.e. heavier edges indicate more (multiple) interactions, of the indicated type, between two nodes, as described by Stuart et al. (2009). Each of Cytoscape images displayed here are also available as scalable, high-quality figures (Adobe PDF documents) from the authors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

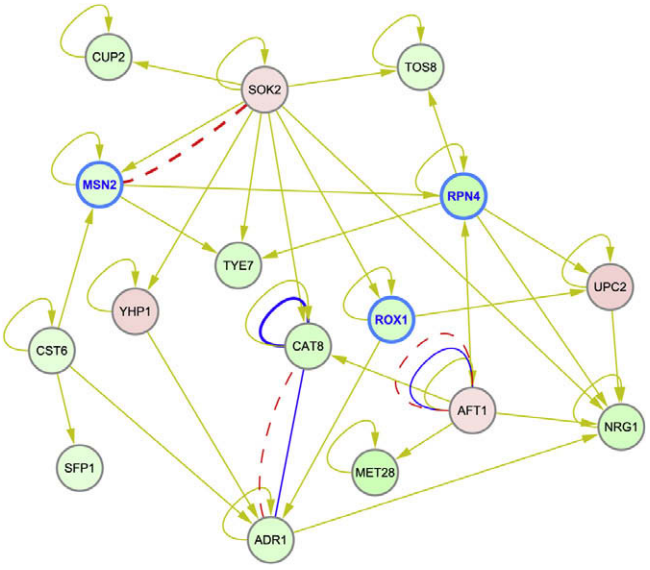

Fig. 3. Cross-regulation of transcription factors present in the top five-scoring Cytoscape ActiveModules subnetworks in *pos5*Δ cells. To simplify the display, Msn4p and its edges were deleted (Msn4p is transcriptionally equivalent to Msn2p, shown), and sterol regulatory element binding protein Upc2p was added, due to its relevance with respect to sterol biosynthesis and anaerobic gene expression.

anaerobiosis including cell wall mannoproteins, heme and sterol biosynthesis (both dependent on molecular oxygen), plus their associated transcription factors, were collated in a gene list and selected in Cytoscape, along with their neighboring nodes ("first neighbors"), giving 1138 genes (1127 with expression data). This list was simplified by selecting genes that were ⩾2.0 up-regulated and ⩾3.0-fold down-regulated in *pos5*Δ cells, plus their ten associated transcription factors, giving the sub-network shown in Fig. 4. From this figure, we can easily visualize the transcription factors associated with the up- or down-regulation of this group of genes. For example, Upc2p and Ecm22p are associated with the down-regulation of the majority of the genes shown in this figure, while Rox1p both up-regulates and down-regulates numerous genes, in these cells. Upc2p is a sterol regulatory element binding protein that induces the transcription of sterol biosynthetic genes and *DAN*/*TIR* gene products. Ecm22p, a homolog of Upc2p, also regulates transcription of sterol biosynthetic genes, while Rox1p is a heme-dependent repressor of hypoxic genes. Mot3p is a nuclear transcription factor involved in the repression of a subset of hypoxic genes by Rox1p, repression of several *DAN*/*TIR* genes during aerobic growth, and repression of ergosterol biosynthetic genes (taken from *Saccharomyces* Genome Database gene descriptions). However, the true level of complexity (cross-regulation) is sure to be more complex than shown in this simplified (filtered) collection of genes.

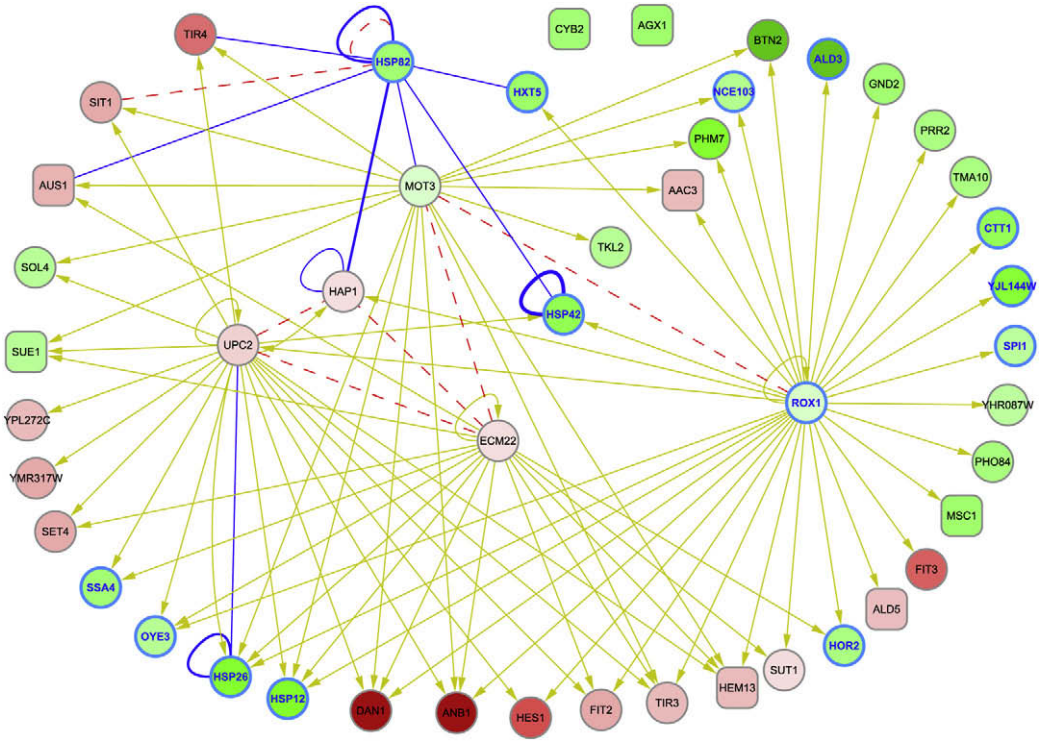

Fig. 4. Transcription factors associated with oxygen metabolism (hypoxia/anaerobic gene expression; cell wall mannoproteins; heme and sterol biosynthesis) based on their gene descriptions at the *Saccharomyces* Genome Database, plus their regulated genes, selected from the yeast interactome in Cytoscape. This selection resulted in 1138 genes; to simplify this analysis, genes that were $\geqslant$2.0 up-regulated and $\geqslant$3.0-fold down-regulated in *pos5*Δ cells were selected, plus their 10 associated transcription factors resulting in the 47 nodes (gene/proteins) shown in the figure, shaded according to their expression levels in *pos5*Δ cells.

4. Discussion

Energy demands in the facultative anaerobe *Saccharomyces cerevisiae* are met under different physiological states when cells are grown on fermentable carbon sources such as glucose, versus non-fermentable carbon sources such as ethanol or glycerol (Lagunas, 1976; Maris et al., 2001; Rosenfeld and Beauvoit, 2003). Fermentation and glycolysis supply the cell with energy through the breakdown of glucose and other simple fermentable sugars without requiring oxygen, generating ethanol as the major by-product (Maris et al., 2001). However, when the glucose concentration drops below ~0.2%, the cells stop growing for a few hours during the diauxic shift, during which they undergo transcriptional and translational changes including the biosynthesis of mitochondria. Cells then resume slower growth for a few generations by oxidative phosphorylation using the ethanol, glycerol and other by-products accumulated during the fermentative stage of growth, before entering the stationary phase of growth.

The expression of the genes that were the most down-regulated in *pos5*Δ cells was remarkably similar to that observed in *sod1*Δ and *sod2*Δ cells (Table 1). This similarity may reflect a general phenomenon in which cells grown in glucose, carrying genetic defects that affect mitochondrial function (*POS5*, *SOD1*, *SOD2*), redox balance (*POS5*) or the detoxification of reactive oxygen species (*POS5*, *SOD1*, *SOD2*) fine-tune their metabolism to minimize cellular stress and damage, consistent with the general down-regulation of genes associated with the response to stress, including oxidative stress (Fig. 2 and Table 1). It is possible that the complex interplay among transcription and their target genes, as indicated in Figs. 3 and 4, allows a similar transcriptional response despite the fact that different genes were deleted in each the *pos5*, *sod1* and *sod2* mutant strains.

In this study we examined changes in gene expression in *pos5*Δ, *sod1*Δ or *sod2*Δ YPH925 cells. Despite a 50-fold increase in the mutational rate in *pos5* cells (Strand and Copeland, 2002; Strand et al., 2003), we observed a *decrease* in the expression of genes associated with the response to stress in *pos5*Δ, *sod1*Δ and *sod2*Δ cells (Figs. 2 and 4; Table 2), and an increase in the levels of expression of stress genes in both *pos5*Δ and wild-type cells switched to growth in glycerol (Table 1). These results indicate that *pos5*Δ cells can respond to the increased oxidative stress that accompanies growth on glycerol, which is dependent on mitochondrial oxidative phosphorylation, but that *pos5*Δ, *sod1*Δ or *sod2*Δ cells appear to reduce their metabolism, relative to wild-type cells, minimizing the levels of cellular stress. The extensive interactions of transcription factors with one another and their target genes (Figs. 2–4) suggests that substantial redundancy exists among the regulation and utilization of metabolic pathways.

4.1. The anaerobic expression profile observed in *pos5Δ* cells

S. cerevisiae senses oxygen levels and regulates aerobic/hypoxic gene expression *via* mechanisms involving heme (Emerling and Chandel, 2005), or the mitochondrial respiratory chain (Kwast et al., 1999; David and Poyton, 2005; Emerling and Chandel, 2005) (Fig. 3). In the first mechanism, the presence of oxygen is sensed through its use in heme biosynthesis, which involves the mitochondrion. The second mechanism of oxygen sensing and gene regulation involves cytochrome *c* oxidase, which appears to be a hemoprotein sensor required for the induction of some hypoxic nuclear genes as yeast cells transition from normoxic to anoxic growth (Kwast et al., 1999; David and Poyton, 2005). Both of these mechanisms, which rely on mitochondrial components but not the sensing of reactive oxygen species, are consistent with

our data. The near-identity in the expression of stress–response genes in *pos5*Δ, *sod1*Δ and *sod2*Δ cells – conditions where cells preferentially derive their energy from fermentation and glycolysis rather than (mitochondrial) oxidative phosphorylation – indicates that these cells "sense" a reduction in cellular and oxidative stress, compared to wild-type cells. A second issue relates to our prior observations (Strand et al., 2003) that that *pos5*Δ cells also display a 6-fold increase in petite mutants, a 28-fold increase in the level of mitochondrial protein oxidation, and a 50-fold increase in the mutational reversion of a mitochondrial transgene ($arg8^{m} \rightarrow ARG8^{m}$). As *pos5* cells are clearly subjected to increased oxidative stress, collectively these data suggest that *pos5*Δ cells - and *sod1*Δ and *sod2*Δ cells, based on the similarity of their expression profiles to *pos5* cells - regulate the expression of their stress–response genes by sensing mitochondrial activity, rather than reactive oxygen species, directly.

As shown in this study, *pos5*Δ cells grown in glucose, and to a lesser extent *pos5*Δ cells grown in glucose and shifted to growth in glycerol (Table 1), up-regulated the expression of a number of genes that are normally expressed under anaerobic or hypoxic conditions, affecting cell wall mannoproteins, siderophore-iron transport, and sterol biosynthesis (Results; Table 1). Shianna et al. (2006) similarly reported the up-regulation of genes associated with anaerobiosis or hypoxia in their strain S288c-based *pos5*Δ::kanMX *hap1*::Ty1 double mutant cells. While transcriptional changes in gene expression associated with hypoxic or anaerobic cell growth are well-known (Lombardía et al., 2000; Abramova et al., 2001; Becerra et al., 2002; Kwast et al., 2002; Ter Linde and Steensma, 2002; Lai et al., 2005; 2006), we extended the list of hypoxia-related genes differentially expressed in *pos5*Δ cells to include *AAC3*, *ANB1*, *AUS1*, *DAN1* and *HEM13*, each of which were up-regulated in *pos5*Δ cells (Table 1). The changes in expression affecting iron sensing and transport genes described by Shianna et al. (2006) were not observed in the present study and are therefore more likely related to the *hap1*::Ty1 mutation and its effects on mitochondrial heme biosynthesis, than due to the *pos5*Δ::kanMX mutation.

The transcriptional responses observed in our *pos5*Δ (*HAP1*) cells that affected genes associated with anaerobic gene expression and oxygen-associated processes (the biosyntheses of cell wall mannoproteins, heme, cytochromes and sterols), and iron transport and metabolism, were virtually identical to those reported in *hap1*Δ cells (Ter Linde and Steensma, 2002) indicating that the *pos5* mimics the *hap1* mutation, at least with regard to oxygen sensing. Hap1p regulates the expression of *ROX1*, encoding a repressor of anaerobic-associated genes; hence *hap1* and *pos5* also mimic the *rox1* mutant phenotype (Kwast et al., 2002), affecting essentially the same set of genes and accounting for the "anaerobic" phenotype observed in *pos5* cells. The greater involvement of genes associated with iron transport and homeostasis observed by Shianna et al. (2006) in *pos5*Δ *hap1*::Ty1 cells than observed here (*pos5*Δ *HAP1* cells) or by Ter Linde and Steensma (2002) (*hap1*Δ and *rox1*Δ cells) probably reflects a contributing effect of the *hap1*::Ty1 mutation in S288c cells (Gaisne et al., 1999). Ferreira et al. (2007) list six genes (*ATM1*, *MET6*, *MNN1*, *NCP1*, *TDH2*, *YLR049C*) that are known to be up-regulated under anaerobic conditions. However, these genes either had no change in expression or were down-regulated in *pos5*Δ cells (data not shown), again indicating that the anaerobic gene expression profile observed in *pos5*Δ cells grown in a normoxic atmosphere is likely due to a defect in oxygen sensing, rather than *bona fide* anaerobiosis.

Since cells lacking Pos5p and Hap1p are similarly affected, transcriptionally, a common mechanism may be involved. Given the apparent defect in oxygen sensing in each of these strains, it seems probable that the mitochondrial dysfunction caused by *pos5*Δ (e.g. impaired detoxification of reactive oxygen species and depletion of NADPH, a reducing agent) and *hap1*Δ (impaired heme biosynthesis and oxygen sensing) overlap at the electron transport chain, with impaired transport of electrons to cytochrome *c* oxidase, a hemoprotein sensor required for the induction of some hypoxic nuclear genes as yeast cells transition from normoxic to anoxic growth (Kwast et al., 1999; David and Poyton, 2005). Aberrant oxygen metabolism would affect heme, cytochrome and sterol biosynthesis, as noted in *pos5* cells; additionally, cytochromes contain heme and copper-bearing prosthetic groups (Horn and Barrientos, 2008), that would support the involvement of iron transport (scavenging) processes (this study; Shianna et al., 2006) as well as the copper sensitivity of *pos5* cells (Strand et al., 2003). These processes could affect the transcriptional regulation of nuclear genes *via* mitochondrial retrograde signaling (Liu and Butow, 2006).

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