

MICROARRAY DATA ANALYSIS METHODS AND THEIR
APPLICATIONS TO GENE EXPRESSION DATA ANALYSIS
FOR *SACCHAROMYCES CEREVISIAE* UNDER OXIDATIVE STRESS

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**Dissertation submitted to the faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of**

**Doctor of Philosophy
in
Genetics, Bioinformatics and Computational Biology**

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May 12, 2006

Blacksburg, Virginia

**Keywords: Microarray Data Analysis, Oxidative Stress, *Yap1*, Cumene
Hydroperoxide**

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Data Analysis for *Saccharomyces cerevisiae* under Oxidative Stress**

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Genetics, Bioinformatics and Computational Biology

(ABSTRACT)

Oxidative stress is a harmful condition in a cell, tissue, or organ, caused by an imbalance between reactive oxygen species or other oxidants and the capacity of antioxidant defense systems to remove them. These oxidants cause wide-ranging damage to macromolecules, including proteins, lipids, DNA and carbohydrates. Oxidative stress is an important pathophysiologic component of a number of diseases, such as Alzheimer's disease, diabetes and certain cancers. Cells contain effective defense mechanisms to respond to oxidative stress. Despite much accumulated knowledge about these responses, their kinetics, especially the kinetics of early responses is still not clearly understood.

The Yap1 transcription factor is crucial for the normal response to a variety of stress conditions including oxidative stress. Previous studies on Yap1 regulation started to measure gene expression profile at least 20 minutes after the induction of oxidative stress. Genes and pathways regulated by Yap1 in early oxidative stress response (within 20 minutes) were not identified in these studies.

Here we study the kinetics of early oxidative stress response induced by the cumene hydroperoxide (CHP) in *Saccharomyces cerevisiae* wild type and *yap1Δ* mutant. Gene expression profiles after exposure to CHP were obtained in controlled conditions using Affymetrix Yeast Genome S98 arrays. The oxidative stress response was measured at 8 time points along 120 minutes after the addition of CHP, with the earliest time point at 3 minute after the exposure. Statistical analysis methods, including ANOVA, *k*-means clustering analysis, and pathway analysis were used to analyze the data. The results from

this study provide a dynamic resolution of the oxidative stress responses in *S. cerevisiae*, and contribute to a much richer understanding of the antioxidant defense systems. It also provides a global view of the roles that Yap1 plays under normal and oxidative stress conditions.

ACKNOWLEDGEMENT

First, I would like to express my sincere gratitude to my advisor, Dr. Pedro Mendes, for his guidance and support throughout this project, and I greatly appreciate the opportunity that he gave me to pursue this degree. Pedro has guided me into the exciting new field of bioinformatics. It has been an amazing learning experience. I appreciate his patience and wisdom at key points in the process, and his insightful suggestions and instructions on my research.

I am grateful to my committee member Dr. John Tyson. Dr. Tyson was my co-advisor for my Master's study in Biology. When I started my doctoral study, Dr. Tyson kindly agreed to be my co-advisor till the establishment of the GBCB program. I am very grateful that I was able to have my Ph.D. study in such an exciting field. Dr. Tyson allowed for this to happen.

I am grateful to my committee member and good friend Dr. Jill Sible. Jill was my advisor for my Master's study. I have learned so much from her over these years, not only in academics but also to be a loving and caring person.

I would like to thank my committee member Dr. Keying Ye. Dr. Ye has always been there to answer statistical questions. He has given me so much detailed guidance in statistical analysis. His input to this project is invaluable.

My thanks also go to Dr. Cynthia Gibas, committee member for both my Master's and Ph.D. studies. I would like to thank her for sharing her knowledge and providing valuable comments to my research.

This dissertation was written during the last a few months while I was working as a research scientist in Dr. Vladimir Shulaev's group at Virginia Bioinformatics Institute. I am grateful to Dr. Shulaev for his patience and understanding when I needed time for thesis writing, and I feel excited about my continued postdoctoral research in his group.

I would like to extend my warm thanks to the fellow members of the Biochemical Networks Modeling Group and the Yeast Systems Biology Group, including Dr. Bharat

Mehrotra, Dr. Stefan Hoops, Xing Jing Li, Aejaaz Kamal, Kimberly Heard, Hui Cheng, Revonda Pokrzywa, Alberto de la Fuente, Diogo Camacho, Autumn Clapp, Elena Dimitrova, Leepika Tuli, Paola Vera Licon, Dr. Dustin Potter, Dr. Brandy Stigler, Dr. Pedro Mendes, Dr. Vladimir Shulaev and Dr. Reinhard Laubenbacher. I would like to thank all of you for your help during my Ph.D. study, for being wonderful colleagues and friends, and for the very pleasant time that we spent together.

In particular, I would like to thank Dr. Ana Martins. Ana did most of the experiments to generate the yeast microarray data. Many time series figures showing in this dissertation were generated with her help. She is a know-all expert in yeast oxidative stress. I have received so much help and learned a lot from her.

I would like to thank my family and friends who have always supported and encouraged me. My parents receive my deepest gratitude and love for their endless love and encouragement. I would never make it so far without their love and support.

Special thanks go to my husband and best friend, Aixi, for his love, patience and positive attitude have helped me all the way through my graduate study.

Finally I gratefully acknowledge the National Institute of General Medical Sciences (grant R01 GM068947-01) and the National Science Foundation (grant DBI-0109732) for their generous support to this research.

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Nomenclature

- Wild Type genes are capitalized and italicized. For example, *YAP1*
- The deleted gene name is in lower case followed by ' Δ ' and is italicized. For example *yap1 Δ*
- Proteins have the same name as the gene but are not italicized. The first letter of protein name is capitalized and the remaining letters are in lower case. For example, Yap1. Sometimes, a "p" is added at the end to clearly designate a protein. For example Yap1p

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Chapter 1. Microarray data analysis methods

1.1. Introduction

Oxidative stress is a harmful condition in a cell, tissue, or organ, caused by an imbalance between reactive oxygen species (ROS) or other oxidants and the capacity of antioxidants and repair systems. These oxidants interact with certain biomolecules and start a chain reaction of damage among important cellular components, such as membranes and DNA. Oxidative stress is an important pathophysiologic component of a number of diseases, such as Alzheimer's disease (Christen, 2000), diabetes (Maritim, et al., 2003) and cancers (Klaunig and Kamendulis, 2004). Cells contain effective defense mechanisms to respond to oxidative stress. Despite much accumulated knowledge about these responses, their kinetics, especially the kinetics of early responses is still not clearly understood.

Yap1 is a member of the basic leucine zipper (bZIP) family of transcription factors, and is a functional homologue of mammalian AP-1 (Harshman, et al., 1988; Moye-Rowley, et al., 1989). The Yap1 is crucial for the normal response to a variety of stress conditions including oxidative stress. Genes and pathways regulated by Yap1 in early oxidative stress response had not been identified in previous studies.

The objective of this project is to study the kinetics of early oxidative stress response induced by the cumene hydroperoxide (CHP) in wild type and *yap1Δ* mutant *Saccharomyces cerevisiae*. Gene expression profiles after exposure to CHP were obtained using Affymetrix Yeast Genome S98 arrays (Affymetrix CA, USA). The oxidative stress response was measured at 8 time points within 120 minutes after the addition of CHP, with the earliest time point at 3 minute after the exposure.

These microarray experiments generate large amounts of data. The following statistical analysis methods were used to extract biological information from this data set. Robust Multi-array Average (RMA) (Bolstad, et al., 2003; Irizarry, et al., 2003) was used for microarray data normalization and summarization. ANalysis Of VAriance (ANOVA) model was used to detect genes that were significantly differentially expressed across different experimental conditions or different genotypes. Clustering analysis was used to

detect genes that have similar expression patterns. Pathway analysis was used to find pathways that were significantly affected at the level of transcription by oxidative stress.

The algorithms of these methods and a simulation study for method selection will be discussed in this chapter. The application of these methods to study the oxidative stress response in wild type yeast will be discussed in Chapter 2. The application of these methods to study the oxidative stress response in *yap1Δ* mutant yeast will be discussed in Chapter 3.

1.2. Affymetrix GeneChip™ technology

Microarray technology utilizes nucleic acid hybridization techniques and advanced computational data analysis methods to evaluate the mRNA expression profile of thousands of genes within a single experiment.

The Affymetrix GeneChip™ Yeast S98 Array was used in this project to study yeast oxidative stress response. Each Affymetrix GeneChip™ array is hybridized to one mRNA sample. Affymetrix arrays are composed of 25-mer oligonucleotide probes which are directly synthesized on the array by photolithography (Affymetrix) (Lockhart, et al., 1996). The Affymetrix Yeast S98 Array uses a set of 16 probe pairs to estimate the expression level of a single gene, which allows the representation of multiple regions of that gene's coding region, thus provides robustness of detection. Each probe pair consists of a perfect match (PM) probe and a mismatch (MM) probe. Affymetrix expects that the MM probe should not hybridize to the target sequence, but should hybridize to most of the sequences that cross-hybridize to the PM probe, thus MM can be used as controls for non-specific cross-hybridization. However, Naef *et al.* found that MM also binds to the target gene (Naef, et al., 2001), and they suggested not to use MM as controls for non-specific binding.

1.3. Microarray data analysis methods

1.3.1. Data normalization and summarization

As mentioned above, the Affymetrix Yeast S98 Array uses a set of 16 probe pairs to estimate the expression level of a single gene. The raw data generated from Affymetrix GeneChip are probe level data, which need to be summarized to gene level data, and the data need to be normalized for downstream analysis. Affymetrix GeneChip Operating Software (GCOS) (Affymetrix CA, USA), and Robust Multi-array Average (RMA) (Bolstad, et al., 2003; Irizarry, et al., 2003) are two widely used methods for microarray data normalization and summarization.

1.3.1.1. Affymetrix GeneChip Operating Software (GCOS)

a) Data summarization

In GCOS, the signal log value for each gene is defined as:

$$SLV_k = Biweight\{\log_2(PM_{i,k} - CT_{i,k}) : i = 1, \dots, n\} \quad (1.1)$$

where k represents the probe set for gene k ; n represents the number of probe pairs in each probe set (Affymetrix Microarray Suite User Guide version 5). When PM-MM is a positive value, CT equals to MM. When PM-MM is zero or a negative value, CT equals to a value defined by the algorithm, and this value is defined slightly smaller than PM. Biweight represents the tukey biweight algorithm, which assigns high weights to the probe pair that is close to the center of the data, thus the SLV is less affected by outliers.

b) Data normalization

GCOS provides two kinds of normalization: single array expression analysis and comparison array expression analysis (Affymetrix GCOS version 1.1 user's guide). For both normalizations, the normalized signal log values are calculated as follows:

$$normalized\ signal\ log\ value(i) = nf \times sf \times 2^{(SignalLogValue_i)} \quad (1.2)$$

The scaling factor sf is calculated as:

$$sf = \frac{Sc}{TrimMean(2^{SignalLogValue_i}, 0.02, 0.98)} \quad (1.3)$$

where Sc is the target signal (default $Sc=500$); the $TrimMean$ function takes the average value of all observations after removing the values in the lowest 2% and the highest 2% of observations on each array.

In single array analysis, nf equals to 1. In comparison analysis, one array is defined as baseline, and nf is calculated as:

$$nf = \frac{TrimMean(SPVB_i, 0.02, 0.98)}{TrimMean(SPVE_i, 0.02, 0.98)} \quad (1.4)$$

where $SPVB_i$ is the baseline signal, and $SPVE_i$ is the experiment signal.

1.3.1.2. Robust Multi-array Average (RMA)

a) Data normalization

The RMA method first computes background-corrected perfect match intensities for each perfect match value on every array. After background correction, RMA calculates the base-2 logarithm of each background-corrected perfect match intensity. These background-corrected and log-transformed perfect-match intensities are normalized using the quantile normalization method developed by Bolstad and coworkers (Bolstad, et al., 2003). In the quantile normalization method, the highest perfect-match intensity on each array is determined. The average of the highest perfect-match on each array is calculated, and then the highest perfect-match value on each array is replaced by the average. This process is repeated with what were originally the second highest perfect-match intensities on each array, the third highest, etc. until each perfect-match on the array is replaced by the corresponding average value. The purpose of quantile normalization is to give each array the same data distribution.

b) Data summarization

As mentioned above, in the Affymetrix Yeast S98 array, each gene is represented by a probe set that contains 16 probe pairs. Thus, there are 16 normalized perfect match values for each gene (probe set). To summarize these perfect match values and obtain one expression value for each gene on each array, a linear model is used to fit to the normalized data. The linear model for a particular probe set (gene) is as follows,

$$Y_{ij} = m_i + a_j + e_{ij} \quad (1.5)$$

where Y_{ij} denotes the normalized perfect match value corresponding to the i th array and the j th probe within the probe set, m_i denotes the expression value for the probe set on the i th array, a_j denotes the probe affinity effect for the j th probe within the probe set, and e_{ij} denotes random error. Tukey's median polish (Tukey, 1977) is used to obtain estimates of the m_i values, *i.e.* estimated array-specific probe set values. These m_i values would be reported as the RMA measures of expression for this gene (probe set).

1.3.1.3. Comparing GCOS and RMA

To compare GCOS and RMA, we used GCOS and RMA to normalize CHP treated wild type yeast data. The detailed information about this data set will be discussed in section 2.2. The GCOS normalized data and the RMA normalized data were then analyzed separately by the same ANOVA model. The detailed the information about the ANOVA model will be discussed in section 2.3. As shown in Table 1.1, the analysis results from the GCOS data and from the RMA data are different. For example, when we compared the sample collected at 20 min with the sample collected at 0 min. ANOVA result from GCOS data suggested that 1933 genes differentially expressed; ANOVA result from RMA data suggested 2597 genes differentially expressed. Among them, 1699 genes were common in both analyses. We found that the genes with the most significant changes in gene expression (*i.e.* lower p -value) seem to be detected by both methods. Genes with less significant changes, especially genes that are on the border line of the p -value cutoff

are those for which the two methods disagree. We cannot evaluate the performance of these two methods, though, because the real mRNA levels in the samples are not known.

Bolstad and coworkers compared the performance of RMA and MAS5 (GCOS) by using dilution data and spike-in data where the mRNA concentrations in the samples are known (Bolstad, et al., 2003). Their results suggested that RMA performed favorably in terms of speed, variance, and bias criteria, and they suggested that RMA should be used in preference to the other methods. Thus, the data normalized from RMA were chosen for the downstream analysis discussed in Chapter 2 and 3.

Table 1.1. Differentially expressed genes detected by ANOVA by using GCOS or RMA normalized data. *P*-value cutoff = 0.05.

	Number of genes detected from GCOS data	Number of genes detected from RMA data	Number of genes detected by both methods	Number of genes detected by both methods / Number of genes detected from GCOS data	Number of genes detected by both methods / Number of genes detected from RMA data
3 min vs. 0 min	66	83	21	0.318181818	0.253012048
6 min vs. 0 min	420	556	257	0.611904762	0.462230216
12 min vs. 0 min	1287	1848	1058	0.822066822	0.572510823
20 min vs. 0 min	1933	2597	1699	0.878944646	0.654216404
40 min vs. 0 min	2002	2829	1715	0.856643357	0.60622128
70 min vs. 0 min	2318	3387	1905	0.821829163	0.562444641
120 min vs. 0 min	3531	4573	2644	0.748796375	0.578176252

1.3.2. Differential expression analysis

1.3.2.1. Introduction to differential expression analysis methods

Identifying differentially expressed genes between two phenotypes and/or genotypes is one of the most important goals in microarray data analysis. Many statistical methods are available for this purpose. In the early days of microarrays, the method of fold changes was used. It determines differential expression by simply computing the ratio of sample means of the two states being compared. Change is assumed when the “fold change” is above (or below) a predetermined threshold, which is often taken as 2x (and 0.5x). This method is very crude and likely to produce a large number of false positives. When the

signal-to-noise ratio is low, higher than threshold fold changes can occur in many genes by chance. It is also not clear why 2x should be considered a significant change, and whether this should be the same for all genes.

Since then, many more sophisticated statistical methods have been proposed (Ideker, et al., 2000; Kerr and Churchill, 2001; Kerr, et al., 2000; Pan, et al., 2003; Tusher, et al., 2001). The most widely used ones are Welch's t -test and ANalysis Of VAriance (ANOVA) models. Kerr was the first to apply ANOVA models in microarray data analysis (Kerr and Churchill, 2001; Kerr, et al., 2000).

The t -test assesses whether the means of two groups are statistically different from each other. The Welch t -test for gene i under experimental conditions 1 and 2, with n_1 and n_2 replicates, respectively, is calculated as:

$$t_i = \frac{\bar{x}_{2i} - \bar{x}_{1i}}{\sqrt{\frac{s_{1i}^2}{n_1} + \frac{s_{2i}^2}{n_2}}} \quad (1.6)$$

where s_{1i}^2 and s_{2i}^2 represent the variance of replicates for gene i for experimental condition 1 and 2, respectively. Thus, the t test determines if the difference between two gene expression levels is significant by comparing this difference with the variance of replicates, which represents the noise of the experiment. The bigger the t score, the more significant is the difference.

A disadvantage of the t test is that the standard error can be hard to estimate, because the number of replicates used in microarray studies is usually small (in this yeast project, $n_1=n_2=3$). In this case, small sample error can occur purely by chance, and this can cause large numbers of false positives in genes that have small sample error, and it can also cause many false negatives in genes that have big sample error.

The fundamental idea behind ANOVA is that, given an appropriate experimental design, variability in the quantity being measured (in microarray it is variability in gene expression) can be partitioned into various identifiable sources. The assumed sources of

variability will include the experimental factors and system error. ANOVA allows one to examine whether the measured variability due to a particular experimental factor is statistically significant by comparing it to the measured variability due to random sources. Thus, ANOVA can be used to examine whether the difference of gene expression is due to a certain experimental condition, for example oxidative stress, or due to random error. When there are multiple comparisons, the advantage of ANOVA over multiple t tests is that we can test more than two means at one time, and we can determine effects of more than one factor and any interactions between factors.

After t -test or ANOVA, a p -value is generated for each gene. The p -value is an estimate of the probability that this gene appears to be differentially expressed by chance. For example, if the p -value after t -test is 0.02, it means that there is a 2% chance that this gene is not differentially expressed but random effects make it look so. Genes with p -values smaller than a certain probability cutoff are deemed as genes that have indeed changed expression level significantly between the two states. The smaller the p -value, the less likely is that the gene expression level difference was caused by noise.

In microarray data analysis, tens of thousand statistical tests are performed at the same time. For example, in an experiment with 10,000 genes and two treatments, there will be 10,000 statistical tests, one for each gene. This generates a problem of multiple tests, because the false positive rate can increase sharply when the number of tests is large. When a cutoff of 0.05 is used in the comparison, it is expected that there will be $10,000 * 0.05 = 500$ false positives. Let's assume that statistical tests declare 800 genes to be significantly differentially expressed. The false identification among these 800 genes will be $500/800 = 62.5\%$, which is clearly not acceptable. To alleviate this problem, Bonferroni developed a correction method (Bonferroni, 1935) to adjust the p -values. In this method, the original p -value cutoff is divided by the total number of tests, and the new cutoff can then be used to detect differentially expressed genes. Bonferroni correction controls the family-wise error rate, which is the probability of having at least one false positive among all the tests. However, microarray data analysis does not require a protection of even a single false positive (Reiner, et al., 2003), which can be too stringent and leads to a large false negative rate. A more practical method is the False

Discovery Rate (FDR) controlling approach (Benjamini and Hochberg, 1995; Storey and Tibshirani, 2003). FDR equals the number of false positives divided by the number of genes declared to be significant. The FDR controlling procedure developed by Benjamini and Hochberg is as follows. Consider testing null hypotheses H_1, H_2, \dots, H_m based on the corresponding p -values P_1, P_2, \dots, P_m . Let $P_{(1)} \leq P_{(2)} \leq \dots \leq P_{(m)}$ be the ordered p -values, and denote by $H_{(i)}$ the null hypothesis corresponding to $P_{(i)}$.

Let k be the largest i for which $P_{(i)} \leq \frac{i}{m} q^*$ (1.7),

where q^* is the defined FDR cutoff (in this study, the FDR cutoff is 0.05), then reject all $H_{(i)}$ if $i \leq k$. Benjamini and Hochberg mathematically proved that this procedure can effectively control FDR below or equal to cutoff q^* .

1.3.2.2. Simulation study to select differential expression analysis method

To make an objective comparison of differential expression analysis methods discussed above, a simulation study was carried out. By using artificial gene networks (Mendes, et al., 2003), we simulated fifty cDNA microarray experiments. The simulated data were then analyzed by four differential expression analysis methods: a) Welch t -test, b) gene-by-gene fixed model, c) gene-by-gene mixed model, and d) global mixed model (Table 1.2).

P -values generated from each method were adjusted by the Bonferroni or FDR methods. Among these differential expression analysis methods, b, c and d are different ANOVA models. The global model uses all of the data from all the genes in the experiment to estimate the variance, thus it has the highest statistical power among all four methods. However, the global model can be computationally very demanding, particularly when the number of genes is very high. The gene-by-gene models (b and c) use all of the data for a specific gene to estimate its variance. The gene-by-gene models are more computationally efficient than the global model, but their statistical power is smaller due to the smaller sample sizes. The relative performance of these methods in terms of accuracy in detecting differentially expressed genes was evaluated.

Table 1.2. Methods for differential expression analysis. In gene-by-gene models, Y_{jkl} represents the measured gene expression level for a certain gene at the j th array, the k th treatment, and the l th replicate. μ represents the overall average signal of this gene; A_j represents the effect of the j th array; T_k represents the effect of the k th treatment; ε_{jkl} represents the random error. In global mixed model, Y_{ijkl} represents the measured gene expression level for the i th gene, j th array, the k th treatment, and the l th replicate. μ represents the overall average signal of the whole experiment; G_i represents the effect of the i th gene; A_j represents the effect of the j th array; T_k represents the effect of the k th treatment; AT_{jk} represents the interaction effect between array j and treatment k ; GA_{ij} represents the interaction effect between gene i and array j ; GT_{ik} represents the interaction effect between gene i and treatment k ; ε_{ijkl} represents the random error.

a) Welch t test	$t_i = \frac{\bar{x}_{it} - \bar{x}_{ic}}{\sqrt{\frac{s_{it}^2}{n_i} + \frac{s_{ic}^2}{n_c}}}$	(same as Equation 1.6)
b) gene by gene fixed model	$y_{jkl} = \mu + A_j + T_k + \varepsilon_{jkl}$	(A is fixed effect) (1.8)
c) gene by gene mixed model	$y_{jkl} = \mu + A_j + T_k + \varepsilon_{jkl}$	(A is random effect) (1.9)
d) global mixed model	$y_{ijkl} = \mu + G_i + A_j + T_k + (AT)_{jk} + (GA)_{ij} + (GT)_{ik} + \varepsilon_{ijkl}$	(A is random effect) (1.10)

Artificial gene networks are mathematical models that resemble gene networks and that allow the production of simulated gene expression data. Artificial gene networks enable the evaluation of various analysis methods, simply because the interactions between genes in these networks are completely known (Mendes, et al., 2003). These networks were generated following one of the three topologies, Erdős-Renyi random networks (Erdős and Renyi, 1959), Watts-Strogatz small-world networks (Watts and Strogatz, 1998), and Albert-Barabási scale-free networks (Barabasi and Albert, 1999). In the program Gepasi (Mendes, 1997), gene expression data were generated from these

networks. To mimic microarray experiments, noise arising from array, spot, and other sources was simulated and added to the synthetic gene expression data obtained from Gepasi. In this study, we simulated fifty cDNA microarray experiments in a reference design. In each simulated microarray experiment, there were 100 genes and six treatments. For each treatment there were three replicates.

Fifty simulated microarray experiments were analyzed by Welch *t*-test, gene-by-gene fixed model, gene-by-gene mixed model and global mixed model. *P*-values were adjusted by the Bonferroni or FDR methods to account for multiple testing.

The average false positive rate and average false negative rate of fifty experiments were calculated for each method (Figure 1.1). We found that ANOVA models are more sensitive than the *t* test in detecting differentially expressed genes. We did not find a big difference between the performance of gene-by-gene model and the performance of the global model. We also found that Bonferroni adjustment is too stringent in detecting differentially expressed genes, and resulted in high false negative rate.

These findings may provide some guidance on the selection of microarray differential expression methods. Although this is a simulation study for cDNA microarray, the results could also be useful for Affymetrix array. In the yeast oxidative stress study, we chose the gene-by-gene model, because it has higher statistical power than *t*-test, and it is computationally more efficient than the global model (which seems to be beyond practical use for the number of conditions and genes in these experiments). The details of the gene-by-gene models used in this yeast oxidative stress project will be discussed in sections 2.3 and 3.3.

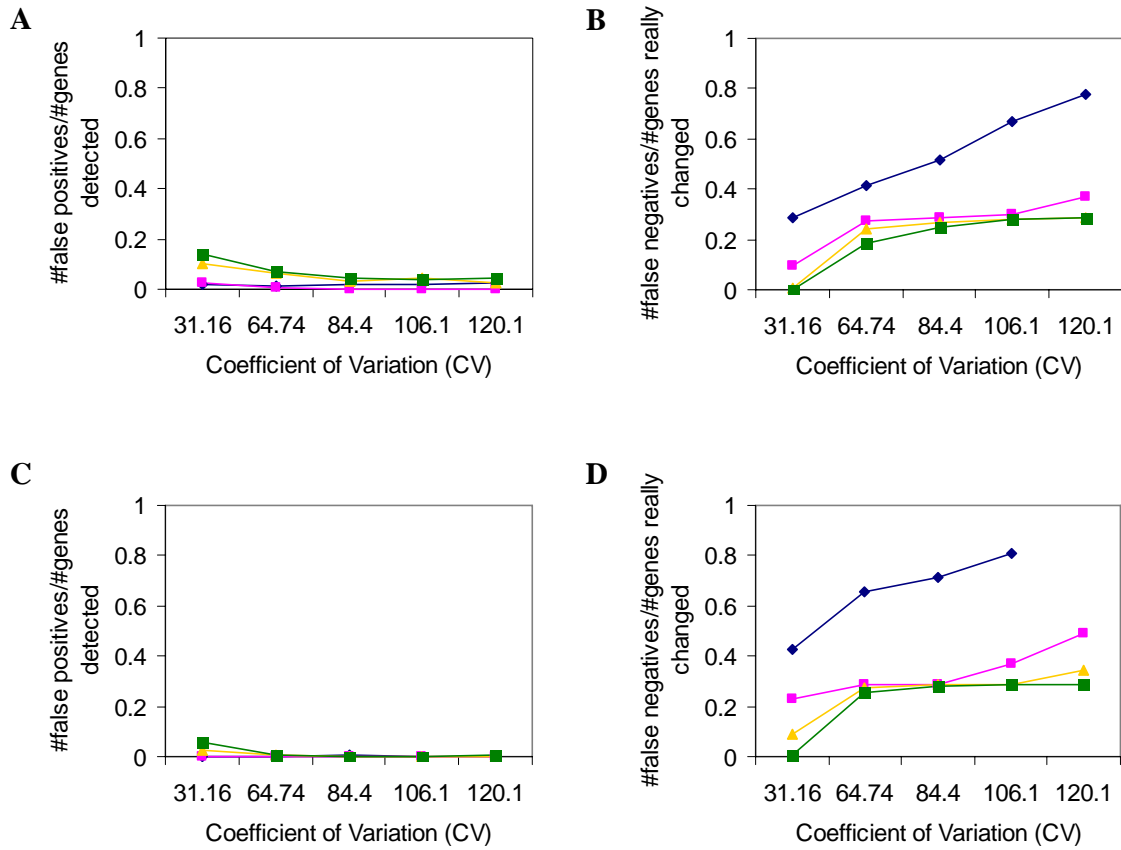


Figure 1.1. False positive rates and false negative rates from each method. A) False positive rates after FDR adjustment. B) False negative rates after FDR adjustment. C) False positive rates after Bonferroni adjustment. D) False negative rate after Bonferroni adjustment. Welch t-test is in blue. Gene-by-gene fixed model is in pink. Gene-by-gene mixed model is in yellow. Global mixed model is in green.

1.3.3. *k*-means Clustering analysis

The *k*-means clustering method (Tavazoie, et al., 1999) can be used to group genes with similar expression patterns. *K*-means clustering requires the analyst to choose *k*, the number of clusters, before the analysis. The algorithm then attempts to maximize inter-cluster variability and minimize intra-cluster variability by choosing appropriate cluster membership for each gene.

In *k*-means clustering, genes are divided into a fixed number of clusters. The centroid of each cluster is calculated. Each gene is then reassigned to its closest centroid. The centroid of each new group is recalculated based on new genes assigned to it. Each gene is reassigned again to its closest centroid, and the centroid of each new group is recalculated again. This process continues until genes have converged into immutable groups.

1.3.4. Pathway analysis

When analyzing gene expression changes, it may be useful to identify which biochemical pathways, or biological processes, have changed significantly, rather than reporting changes gene by gene. Pathway analysis detects pathways that are significantly affected by a certain experimental condition, in this case, oxidative stress. But pathway analysis requires that we start with a clear definition of pathways or biological processes, and there are several ways to do this. Here I used two such classifications: the Kyoto Encyclopedia of Genes and Genomes (KEGG) biochemical pathways (Ogata, et al., 1999) and the Gene Ontology Slim (GO Slim) classification provided by the Saccharomyces Genome Database (SGD, <http://www.yeastgenome.org>). Several bioinformatics tools were also used to estimate the pathways or processes that were significantly altered: High-Throughput GoMiner (<http://discover.nci.nih.gov/gominer/>) (Zeeberg, et al., 2003; Zeeberg, et al., 2005), Database for Annotation, Visualization and Integrated Discovery (DAVID) (<http://david.niaid.nih.gov/david/version2/index.htm>) (Dennis, et al., 2003), and the SGD GO Term Finder. These tools estimate whether a pathway or process is significantly represented in a group of genes of a particular interest, for example a group of genes that were significantly induced by oxidative stress. The

basic algorithm behind these tools to determine whether a pathway is significant is as follows.

Suppose that we have a total number of N genes on the array, in which M have a particular annotation. If we detect n genes to form a group of a particular interest, and among them x genes have that annotation, then we can calculate the probability of seeing x genes out of that annotation (pathway) by chance is, using the hypergeometric distribution:

$$p = \frac{\binom{M}{x} \binom{N-M}{n-x}}{\binom{N}{n}} \quad (1.11)$$

The probability of seeing x or more genes with a particular annotation (pathway) out of n by chance, given that in the population of N , M of those genes have that annotation, is:

$$p_value = \sum_{j=x}^n \frac{\binom{M}{j} \binom{N-M}{n-j}}{\binom{N}{n}} \quad (1.12)$$

Pathways with p -value smaller than a cutoff value are deemed to be pathways significantly represented in the group of genes of a particular interest, for example a group of genes induced by oxidative stress.

Despite similar formula for p -value calculation, these tools have different features. High-Throughput GoMiner allows the fast GO analysis for high-throughput data, but it can not perform KEGG pathways analysis. DAVID performs both GO and KEGG analysis, but the calculation is slower for high-through data than High-Throughput GoMiner. Thus High-Throughput GoMiner was chosen for GO analysis, and DAVID was chosen for KEGG analysis. The advantage of SGD GO Term Finder is that it generates a table which lists significant genes in each significant GO category, and there is a hyperlink associated

with each gene. Each hyperlink is linked to SGD for the annotation of a particular yeast gene, thus allowing a faster biological interpretation of these genes.

The results from GO analysis often return hundreds of significant GO categories. As well known, GO categories are organized in structures called Directed Acyclic Graphs (DAGs). As shown in Figure 1.2, a DAG is a graph composed of nodes and edges. It is very similar to hierarchical tree structure, with the upper-level nodes most generic and bottom-level nodes most specialized. Nevertheless, a DAG differs from a hierarchy in that a “child” node can have many “parent” nodes, and may have different types of relations with its different parents. Thus, the concept of “level” of a node is not unique. For example, the GO category “cell growth” has at least two parents, “Regulation of cell size” and “growth” (Figure 1.2). Thus, “cell growth” can be either assigned to level 6 or level 3, if “biological process” is counted as level 1 (Figure 1.2). To summarize hundreds of significant GO categories generated from GO analysis, higher level GO categories need to be selected. However, as discussed above, the level of each category cannot be easily determined. GO Slims are cut-down versions of the GO ontologies containing a subset of the terms in the whole GO. GO Slims terms are generally high level categories in GO. SGD GO Slim was created by SGD curators for the yeast community. SGD GO Slim was used to summarize the GO analysis results from this yeast oxidative stress project.

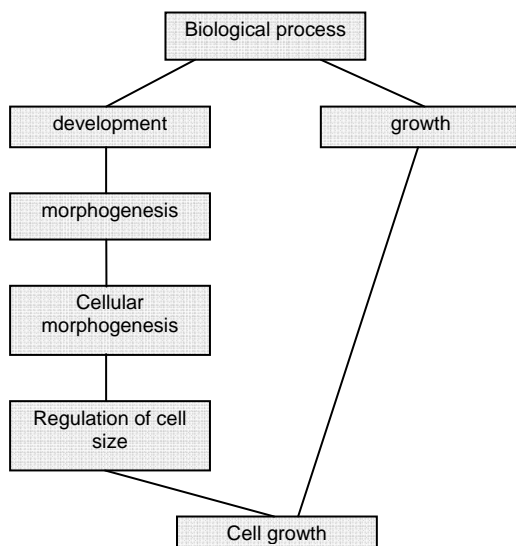


Figure 1.2 The DAG of the “cell growth” GO category.

Chapter 2. The genome-wide kinetics of *S. cerevisiae* response to oxidative stress

2.1. Introduction

The yeast *Saccharomyces cerevisiae*, also called brewer's or baker's yeast, is a single cell eukaryotic microorganism. It is one of the simplest eukaryotes, but many essential cellular processes are conserved between yeast and human. Many yeast proteins have been shown to be functionally interchangeable with their homologous human proteins. Yeast is easy to culture and manipulate genetically and biochemically, and there is a vast knowledge for this organism at the physiological, genetic, and molecular levels. Therefore, it is not surprising that the yeast *S. cerevisiae* is widely used as a model system to understand the molecular mechanisms of oxidative stress response.

Oxidative stress is a harmful condition in a cell, tissue or organ caused by reactive oxygen species (ROS) or other molecules with high oxidative potential. All aerobic organisms are exposed to ROS. ROS such as the superoxide anion, H₂O₂, and the hydroxyl radical are generated in the course of normal aerobic metabolism or after exposure to radical-generating compounds. ROS can cause wide-ranging damage to macromolecules, including proteins, lipids, DNA and carbohydrates. ROS have been recognized as important pathophysiologic components of a number of diseases, such as Alzheimer's disease (Christen, 2000), diabetes (Maritim, et al., 2003) and cancers (Klaunig and Kamendulis, 2004). To protect against oxidant damage, cells contain effective defense mechanisms including enzymes, such as catalase, superoxide dismutase and glutathione peroxidase, as well as low-molecular-weight compounds, such as glutathione, thioredoxin and vitamins C and E (Yu, 1994). Oxidative stress occurs when the formation of oxidants exceeds the ability of antioxidant systems to remove them. These ROS and the corresponding cellular defense systems have been studied extensively in the yeast *S. cerevisiae* (Jamieson, 1998).

In most previous transcriptomics studies of oxidative stress induced by ROS, time series started at 10 minutes or later (Causton, et al., 2001; Gasch, et al., 2000; Koerkamp, et al., 2002). Lucau-Danila and coworkers studied early oxidative stress response to chemical inducer benomyl with the first time point at 30 seconds (Lucau-Danila, et al., 2005). However, there is a difference between stress induced by benomyl and stress induced by

ROS. The details of the difference will be discussed in section 2.5.5. It is important to study the genome-wide kinetics of early oxidative stress response (within 10 minutes) induced by ROS, such as CHP.

It was reported that cells have distinct mechanisms to respond to different ROS (Thorpe, et al., 2004). Previous kinetic transcriptomics studies have been performed on H₂O₂, oleate and benomyl induced stresses (Causton, et al., 2001; Gasch, et al., 2000; Koerkamp, et al., 2002; Lucau-Danila, et al., 2005). The kinetics of oxidative stress response to organic peroxides such as cumene hydroperoxide (CHP) is not clearly understood.

CHP is an aromatic organic peroxide and thus is a free radical-generating compound. The chemical structure of CHP is shown in Figure 2.1. ROS generated by CHP cause membrane damage, cell lysis, organ necrosis, tumor promotion (Taffe, et al., 1987) and certain aspects of aging (Wendel and Feuerstein, 1981). It has been reported that this compound and/or a cytotoxic aldehyde derived from it inhibit protein synthesis in human skin fibroblasts (Poot, et al., 1988).

CHP is an attractive model oxidant to study oxidative stress response. It mimics natural organic compounds; it can be easily taken up by cells and is not metabolized by catalase (Koster and Slee, 1983). The lipophilicity of CHP allows its use as an initiator of lipid peroxidation (Onaran, et al., 1997), which in turn gives rise to diffusible products.

One advantage of using CHP as the oxidant perturbation, in contrast to H₂O₂, is that its degradation product can be easily monitored. H₂O₂ is reduced to H₂O, which cannot be easily measured in cells, whereas CHP is reduced to cumyl alcohol (COH) (Figure 2.1). COH is easily measurable, thus the progress of the redox reaction can be easily monitored.

This project studies the kinetics of the *S. cerevisiae* response to oxidative stress induced by CHP. Yeast cultures were exposed to single application of CHP and its effects on gene expression (mRNA levels) were monitored over time. A logarithmic sample collection schedule was designed, with the first sample collection at 3 minute after exposure to CHP. mRNA was extracted from the collected samples and gene expression levels at

each time point were measured by Affymetrix Yeast Genome S98 arrays (Affymetrix CA, USA).

This set of experiments was planned by Ana Martins, Pedro Mendes and Vladimir Shulaev. The experiments were performed by Ana Martins and Autumn Clapp, with the help of several people from the Shulaev, Mendes and Laubenbacher's research groups at VBI: Alberto de la Fuente, Beth Henry, Bharat Mehrotra, Brandylin Stigler, Diego Cortes, Diogo Camacho, Dustin Potter, Erica Mason, Hope Gruszewski, Jianghong Qian, Joel Shuman, Jiming Chia, Nigel Deighton, Wei Sha, Pedro Mendes, Reinhard Laubenbacher and Vladimir Shulaev.

The microarray data will be submitted to Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>).

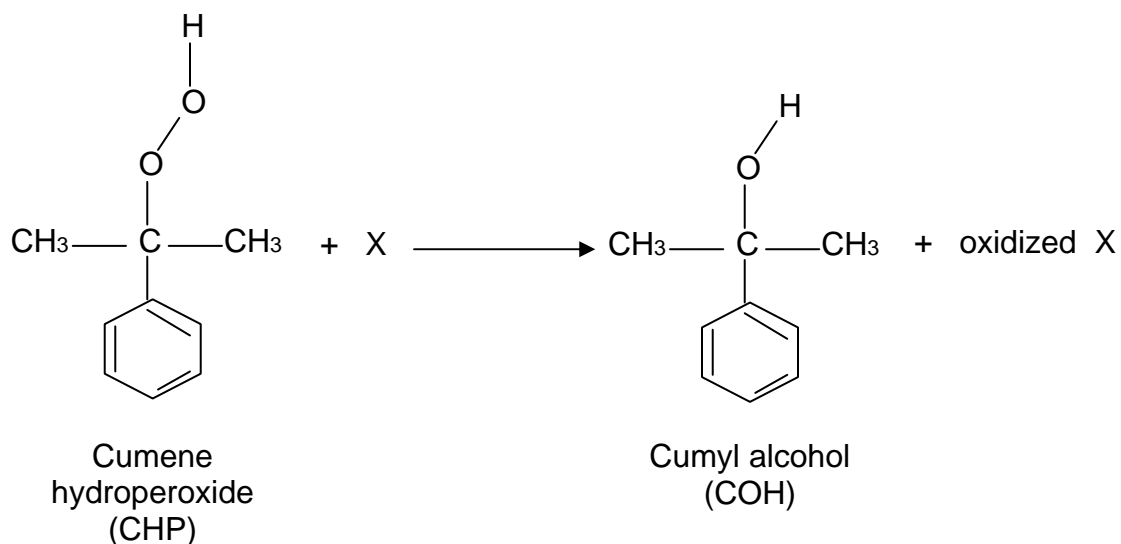


Figure 2.1. Cumene hydroperoxide (CHP) is reduced to cumyl alcohol (COH). X represents a molecule oxidized by CHP.

2.2. Experimental design

The yeast cell culture was divided into six fermentors. CHP was added to three fermentors to make treated samples. Ethanol (EtOH) was added to the other three fermentors to make control samples. EtOH was the solvent for CHP in CHP treated samples. Samples were collected immediately (0 minutes) before the addition of CHP (or EtOH) and at the following time points: 3, 6, 12, 20, 40, 70 and 120 minute. mRNA extracted from the samples collected at each time point and fermentor was hybridized to Affymetrix Yeast Genome S98 arrays (Affymetrix CA, USA) for the measure of transcriptome. Experiment design is shown in Figure 2.2.

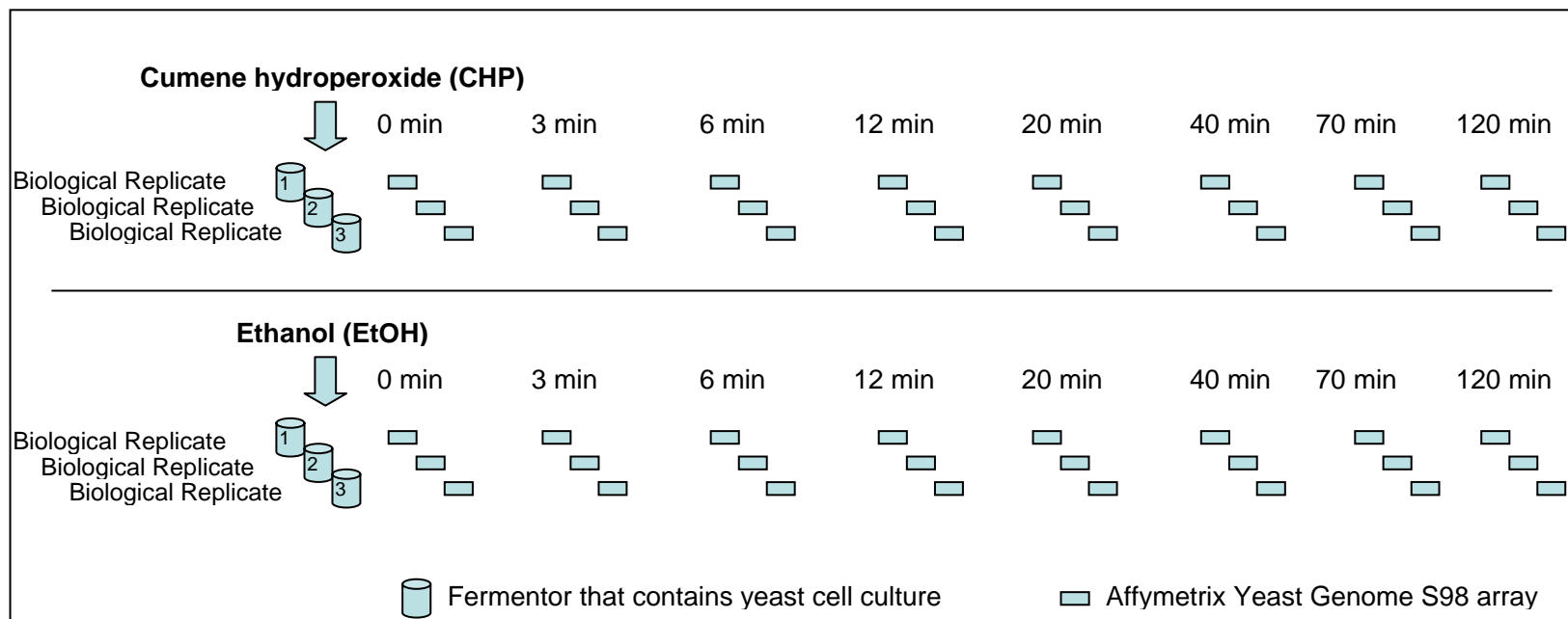


Figure 2.2. Experimental design.

2.3. Data analysis methods

Robust Multichip Average (RMA) (Bolstad, et al., 2003; Irizarry, et al., 2003) was used for microarray data summarization and normalization.

To assess the significance of differences between transcripts across two different experimental conditions, we built a 2-way ANOVA gene-by-gene model in SAS version 9 (SAS Institute Inc., Cary, NC, USA):

$$y_{ijk} = \mu + T_i + V_j + (TV)_{ij} + \varepsilon_{ijk} \quad (2.1)$$

where y_{ijk} is the intensity measured on the array for time i ($i=0,2,\dots,7$), treatment j (in this case, control or CHP) and replicate k ; μ is the overall mean intensity of this gene; T_i is the effect of the i th time point; V_j is the effect of the j th treatment; $(TV)_{ij}$ is the interaction effect between time i and treatment j ; ε_{ijk} is the residual for time i , treatment j and replicate k . The positive False Discovery Rate (pFDR, cutoff 0.05) multiple-testing adjustment (Storey and Tibshirani, 2003) was applied to correct p values for multiple testing.

Genes with similar expression patterns were discovered by k -means clustering by using TIGR Multiexperiment Viewer (MeV) Version 3.1 (<http://www.tm4.org/mev.html>) (Saeed, et al., 2003).

To discover KEGG pathways that were significantly affected by CHP, pathway analysis was performed in Database for Annotation, Visualization and Integrated Discovery (DAVID) version 2 (<http://david.niaid.nih.gov/david/version2/index.htm>) (Dennis, et al., 2003). High-Throughput GoMiner (<http://discover.nci.nih.gov/gominer/>) was used to determine gene ontology categories that were significantly affected by CHP (Zeeberg, et al., 2003; Zeeberg, et al., 2005). pFDR was used as multiple-comparison correction method in these analyses (Storey and Tibshirani, 2003). Heat maps based on pFDR were built to visualize the responses of pathways that were significantly affected by CHP.

2.4. Problem in control samples

According to the results of ANOVA, the control samples were stable until 40 minutes (Table 2.1). Some artifacts affected the stability of control samples after 20 minutes.

Table 2.1. The number of genes that significantly differentially expressed when control sample at each time point was compared with control sample at time 0.

comparisons	significantly changed genes (p<0.01)	up-regulated genes	down-regulated genes
Cont_3min vs. Cont_0min	1	0	1
Cont_6min vs. Cont_0min	4	2	2
Cont_12min vs. Cont_0min	2	1	1
Cont_20min vs. Cont_0min	18	12	6
Cont_40min vs. Cont_0min	1054	571	483
Cont_70min vs. Cont_0min	2709	1343	1366
Cont_120min vs. Cont_0min	2829	1344	1485

There could be many reasons for the observed changes in gene expression levels, which are quite pronounced (1/2 of the genome was affected). We suspect that it might be due to amino acid starvation in the cell culture after 20 minutes. The *S. cerevisiae* strain used in this work was BY4743 ([4741/4742] *MATa/MAT α his3 Δ 1/his3 Δ 1 leu2 Δ 0/leu2 Δ 0 lys2 Δ 0/+ met15 Δ 0/+ ura3 Δ 0/ura3 Δ 0*, ATCC # 201390). This strain, purchased from American Type Culture Collection (ATCC), was originally constructed by the Yeast Deletion Consortium (Giaever, et al., 2002; Winzeler, et al., 1999). BY4743 is not able to synthesize leucine, histidine and uracil (which were used as genetic markers for selection). L-leucine, L-histidine and uracil were added to the media of yeast cell culture. We suspect that the concentration of these supplements in the medium may have become limiting for the cell culture after the 20 minute time point (which is slightly after mid-exponential phase). This hypothesis is supported by a few observations. First, we are sure that changes in mRNA level were not due to carbon limitation, since the substrate, sucrose, was still abundant at the end of the experiment (i.e. 2 hours after the perturbation). Secondly, the gene expression patterns of genes that are documented to

respond to amino acid starvation were induced/repressed accordingly. Gcn4 is a master regulator of gene expression during amino acid starvation in yeast (Natarajan, et al., 2001). Among 290 genes that are documented to be regulated by Gcn4, 231 of them (80%) significantly changed their gene expression levels in control samples after 20 minutes. We also checked genes controlled by Msn2 and Msn4, which are the transcription factors responding to general stress. 75% of genes controlled by Msn2 and Msn4 significantly changed their gene expression levels in control samples after 20 minutes. Our hypothesis is that control samples were under some form of stress after 20 minutes, and that this stress is perhaps amino acid starvation. Many genes controlled by Gcn4, Msn2 and Msn4 also changed in the CHP treated sample.

Due to this artifact, I believe that only the data up to 20 minutes can be used to study the oxidative stress response caused by the addition of CHP. In the rest of this dissertation, the conclusions about oxidative stress will be drawn according to the results from the first five time points, 0 minutes, 3 minutes, 6 minutes, 12 minutes and 20 minutes. While some genes were not altered in the control samples after 20 minutes but were in the CHP treated samples, I chose not to draw any conclusions from these data points.

This problem observed in the control samples demonstrates the need for carrying out gene expression experiments with proper controls. Other time-course gene expression studies in oxidative stress were carried out without controls, and comparisons were made only to the time zero sample. Such studies assume that no changes of gene expression happen without the perturbation, but it is impossible to verify that. Thus conclusions from such studies should be taken with skepticism.

2.5. Results and discussions

2.5.1. The transformation of CHP to COH by the yeast cell culture

As shown in Figure 2.3A., the yeast cell culture successfully transformed almost all of the CHP to COH within only 20 minutes after the addition of CHP. In medium without yeast cells, CHP was stable for at least 48 hours (Figure 2.3B). Cultures of the *yap1Δ* mutant were not able to carry out a successful reduction (Figure 3.2 in chapter 3). Thus, a healthy antioxidant defense system in the wild-type yeast seems to be essential for the reduction of CHP to COH.

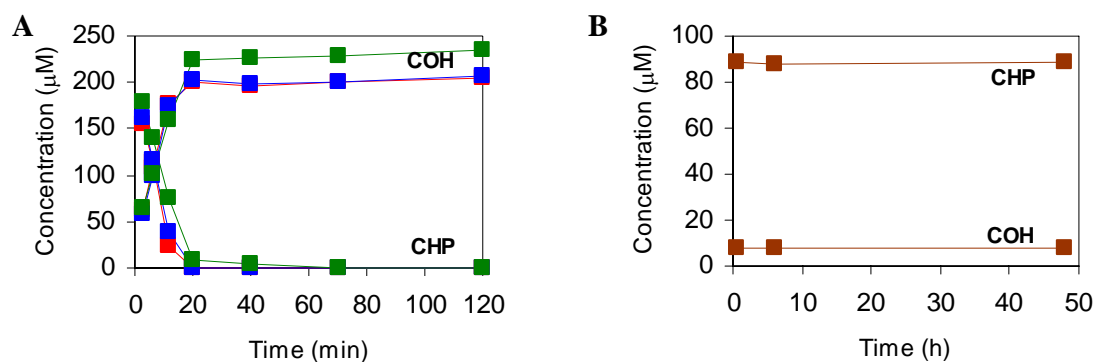


Figure 2.3. CHP and COH progress curves. (A) The concentration of CHP and COH in wild type yeast cell culture within 120 minutes after the addition of CHP. Three biological replicates are labeled in green, blue and red. (B) The concentrations of CHP and COH in culture medium within 48 hours after the addition of CHP. (Data courtesy of Ana Martins).

2.5.2. Antioxidant defense systems

2.5.2.1. Glutathione/glutaredoxin system

The tripeptide glutathione (GSH) and the small proteins glutaredoxins are among the most important intracellular antioxidants. The glutathione/glutaredoxin system is maintained by a complex series of reactions. Figure 2.4 depicts a model for the glutathione/glutaredoxin system based on the current knowledge.

The tripeptide γ -glutamyl-cysteinyl-glycine GSH is synthesized by the action of γ -glutamylcysteine synthetase (Gsh1) and glutathione synthetase (Gsh2) (Grant and Dawes, 1996). Once oxidized, GSH is dimerized to form the disulfide glutathione (GSSG). The ratio of GSH to GSSG can be used as an indicator of the redox status of the cell. The ratio between GSH and GSSG is sustained by glutathione reductase (GLR) and glutathione peroxidase (GPX). It was reported that yeast glutaredoxins have glutathione peroxidase activity (Collinson, et al., 2002).

Apart from serving as an electron donor in redox reactions, GSH is also used by glutathione-S-transferase (GST) (Hayes, et al., 2005). There are two GST genes in yeast, *GTT1* and *GTT2*. GST catalyses the conjugation of GSH to a wide variety of electrophiles such as lipid hydroperoxides and their alkenal breakdown products, in order to remove these toxic products from the cytosol (Raftogianis, et al., 2000). A large proportion of the human population displays a homozygous deletion in two GST genes (*GSTM1* and *GSTT1*) and this null phenotype is associated with malignancies and inflammatory bowel disease (Chernajovsky, et al., 2002), which underlines the importance of the GST-centered antioxidant defense. It was reported that glutaredoxins are also active as GSTs (Collinson and Grant, 2003).

The expression levels of *GSH*, *GLR*, *GPX* and *GST* genes are shown in Figure 2.5. At a *p*-value cutoff of 0.05, all of these genes were detected as significantly induced by CHP. At a more stringent *p*-value cutoff of 0.01, all of them, except *GPX1*, were still significantly induced by CHP. *S. cerevisiae* has three *GPX* genes. The regulations of these genes are different. Inoue and co-workers reported that *GPX1* is induced by glucose

starvation, and *GPX2* is induced by oxidative stress in a Yap1p-dependent manner, whereas *GPX3* was not induced by any of the stresses tested in their study, including oxidative stress induced by CHP (0.2mM) (Inoue, et al., 1999). Our results, on the other hand, showed that both *GPX2* and *GPX3* significantly responded to CHP. The maximum induction of *GPX2* occurred at 20 minutes, 11.29x higher expression level than before adding CHP. The maximum induction of *GPX3* also occurred at 20 minutes, but was only 1.38x higher than before the CHP induction. In agreement with Inoue's study, we observed that the basal mRNA level of *GPX3* was constitutively high (about 10 times higher) than *GPX1* and *GPX2*, as shown in Figure 2.5. Thus, a 1.38 fold change for *GPX3* is considered a big change in terms of total mRNA concentration.

Glutaredoxins are small heat-stable proteins that act as glutathione-dependent disulfide oxidoreductases. Yeast have five glutaredoxin genes. They were detected in the complete *S. cerevisiae* genome sequence on the basis of sequence homology to glutaredoxins in other organisms. Two of the glutaredoxin genes encode glutaredoxin proteins (also called thioltransferases) with two cysteines at the active site (*GRX1* and *GRX2/TTR1*) and three others encode glutaredoxins with one cysteine at the active site (*GRX3*, *GRX4*, and *GRX5*) (Holmgren and Aslund, 1995). It was previously reported that the expression of both *GRX1* and *GRX2* was induced by various stress conditions, including exposure to H₂O₂ and menadione, as well as osmotic shock and heat shock, however expression levels of *GRX3*, *GRX4* and *GRX5* were not altered by any of these stresses (Carmel-Harel and Storz, 2000). Our results show that *GRX1*, *GRX2* and *GRX5* were significantly induced with maximum changes of 1.77x, 3.08x and 1.55x, respectively. *GRX3* and *GRX4* were not significantly induced within 20 minutes (Figure 2.6).

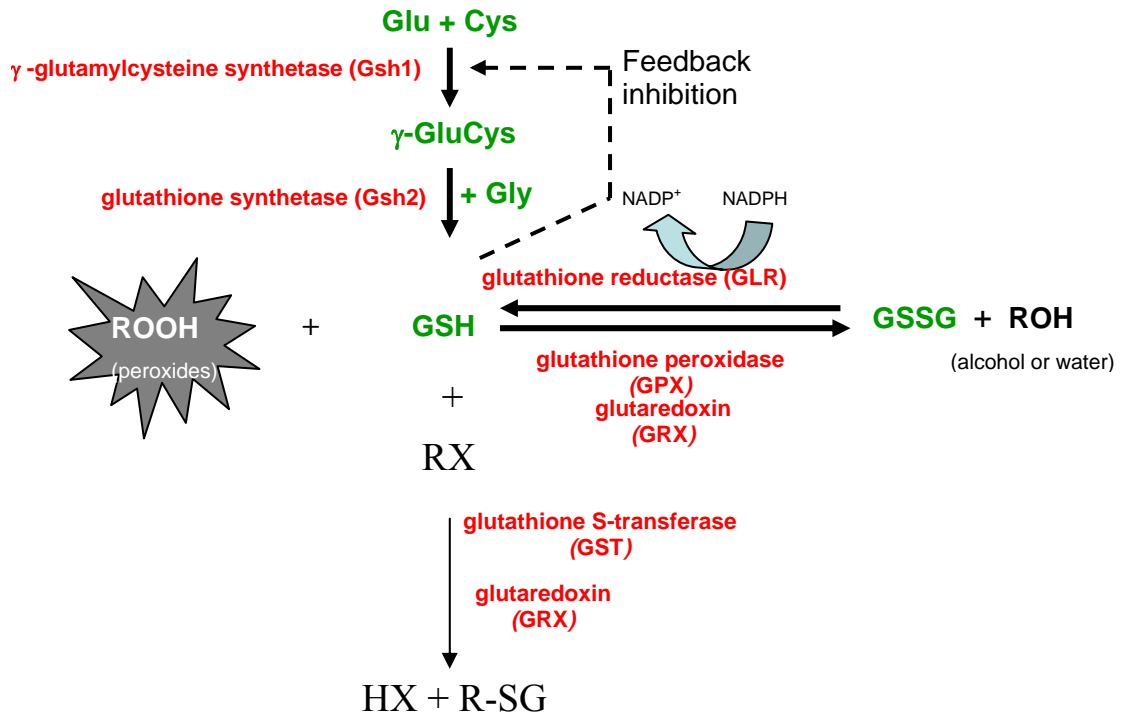


Figure 2.4. Glutathione/glutaredoxin system.

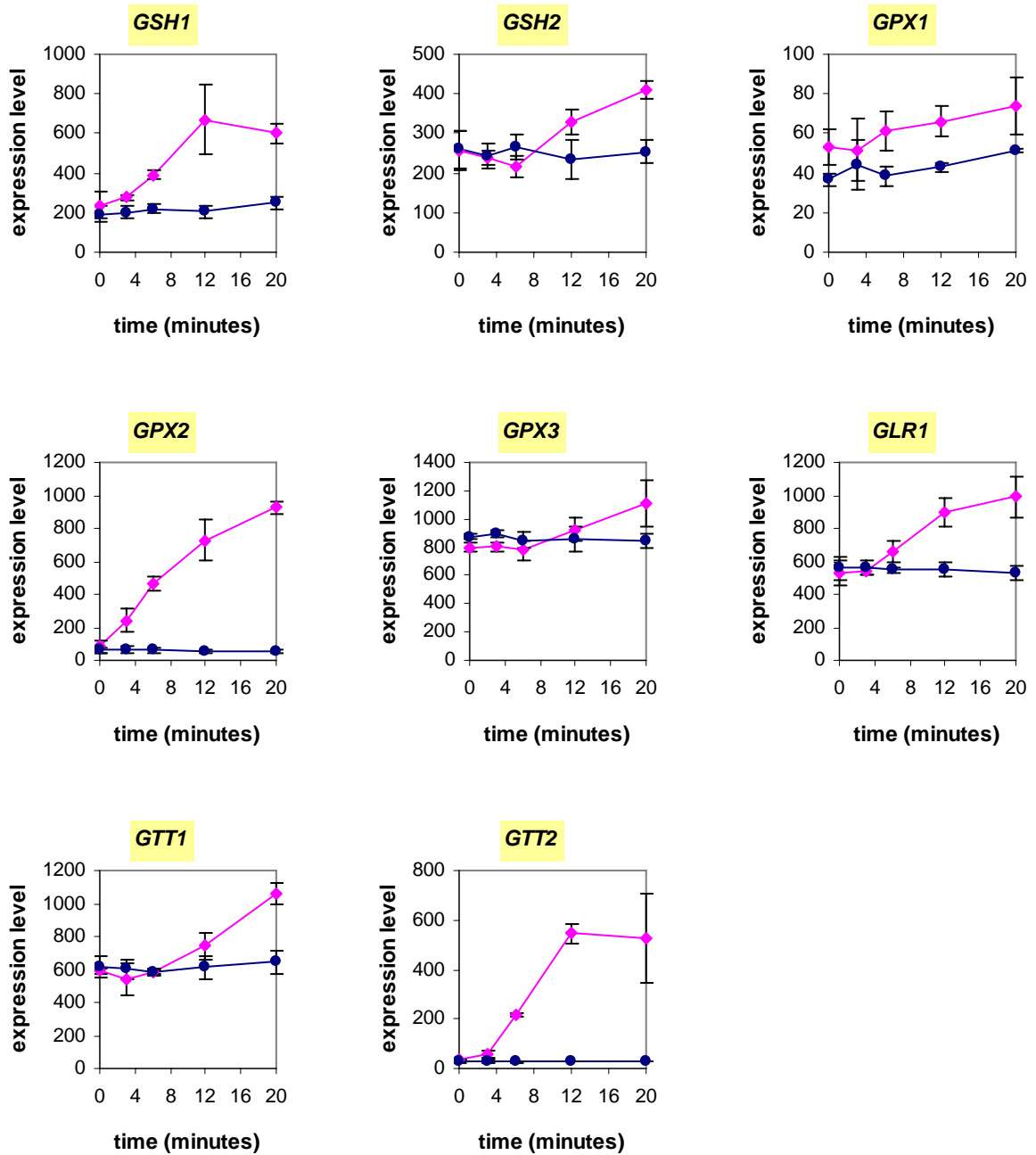


Figure 2.5. Time series of *GSH*, *GPX*, *GLR* and *GST* genes. The time series for CHP treated sample is labeled in pink. The time series for control sample is labeled in blue. Genes that were significantly differentially expressed ($p < 0.05$) by comparing to time 0 are labeled with yellow shades.

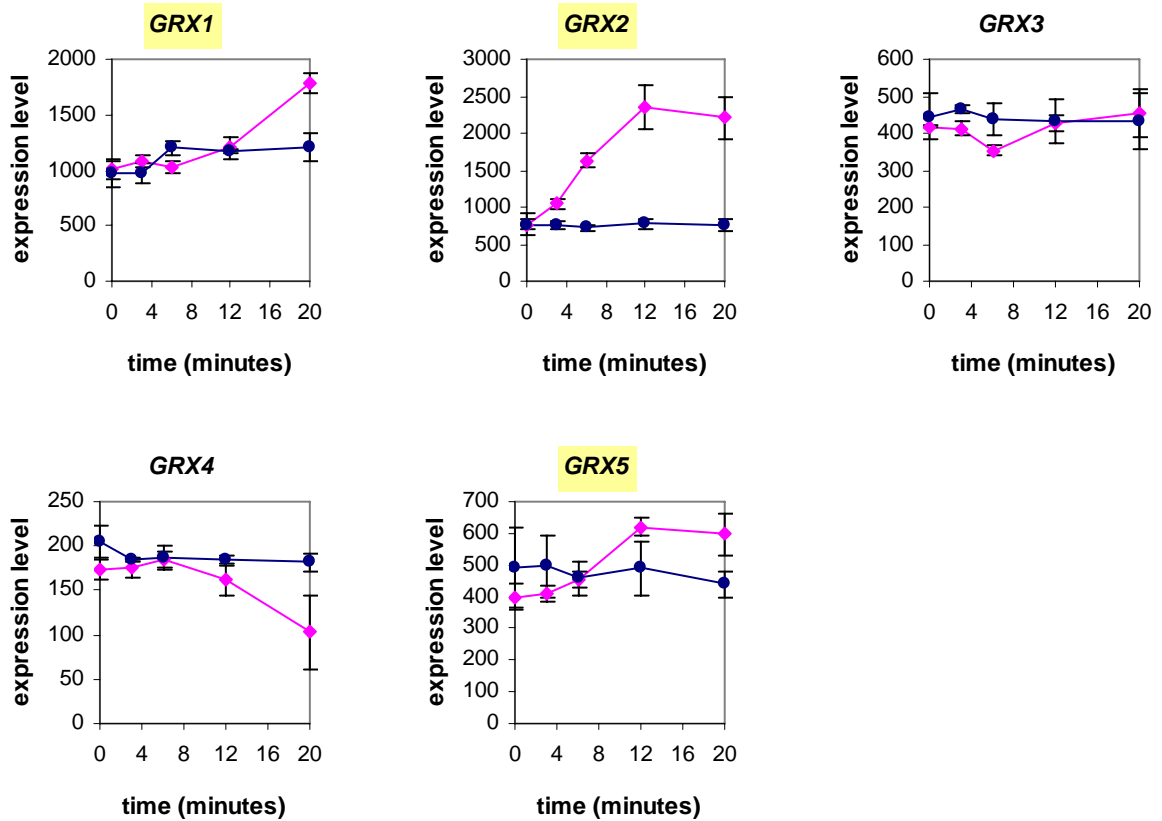


Figure 2.6. Time series of *GRX* genes. The time series for CHP treated sample is labeled in pink. The time series for control sample is labeled in blue. Genes that were significantly differentially expressed ($p < 0.05$) by comparing to time 0 are labeled with yellow shades.

2.5.2.2. Thioredoxin system

Thioredoxin (TRX) is a small protein with two active cysteines within the conserved active site. Thioredoxin has the capacity to reduce oxidized proteins at the expense of the oxidation of its own cysteine residues. This reaction is catalyzed by thioredoxin peroxidase (TPX). The oxidized TRX (TRX-S₂) is then reduced by thioredoxin reductase (TRR). A model of thioredoxin system based on the current knowledge is shown in Figure 2.7.

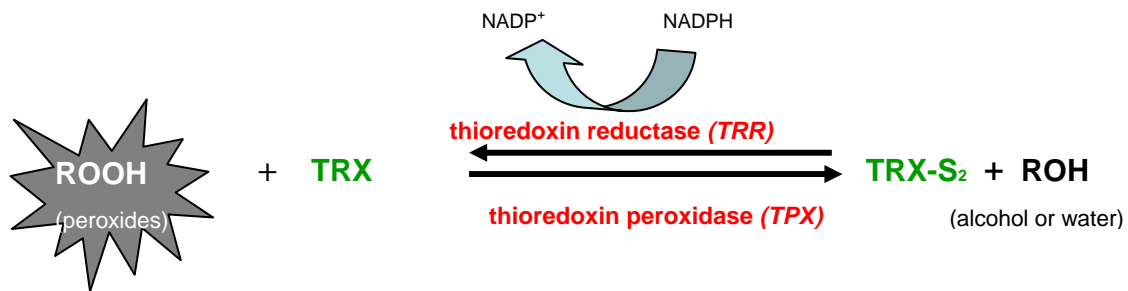


Figure 2.7. Thioredoxin system.

S. cerevisiae has three thioredoxin genes: *TRX1*, *TRX2* and *TRX3*. Among them, *TRX1* and *TRX2* encode cytoplasmic thioredoxins and *TRX3* encodes a mitochondrial one. Thioredoxins are required for protection against ROS and provide reducing power for thioredoxin peroxidase (Park, et al., 2000).

Yeast has two thioredoxin reductases: cytoplasmic thioredoxin reductase (*TRR1*) and mitochondrial thioredoxin reductase (*TRR2*). The enzymatic mechanism of thioredoxin reductases involves the transfer of reducing equivalents from NADPH to a disulfide bond via FAD (Holmgren and Bjornstedt, 1995).

Yeast has five thioredoxin peroxidases, including cytoplasmic (*TSA1*, *TSA2* and *AHP1*) mitochondrial (*PRX1*) and nuclear (*DOT5*) species (Park, et al., 2000). Different from glutathione peroxidase, which has an active site of selenocysteine, thioredoxin peroxidases have only one conserved cysteine within the primary site of catalysis.

Yeast, like most eukaryotes, contains a complete mitochondrial thioredoxin system including a thioredoxin (*TRX3*), thioredoxin reductase (*TRR2*) and thioredoxin peroxidase (*PRX1*), which is thought to function in protection against oxidative stress generated during respiratory metabolism (Pedrajas, et al., 1999).

Our results show that *TRX2*, *TRR1*, *TSA2*, *DOT5* and *PRX1* were significantly induced by CHP within 20 minutes (Figure 2.8).

Comparing our data with the data generated from oxidative stress induced by oleate and H₂O₂ (Causton, et al., 2001; Gasch, et al., 2000; Koerkamp, et al., 2002), a general difference is that genes involved in glutathione/glutaredoxin system and genes involved in thioredoxin system responded faster in stress induced by CHP than in stress induced by oleate or H₂O₂. One possible explanation could be that in CHP induced stress, the product COH is toxic and needs to be removed through the conjugation of GSH, thus CHP may deplete GSH faster than oleate and H₂O₂. This hypothesis is also supported by the fact that we observe an induction of the GSH biosynthetic genes, including genes involved in cysteine synthesis, a precursor for GSH.

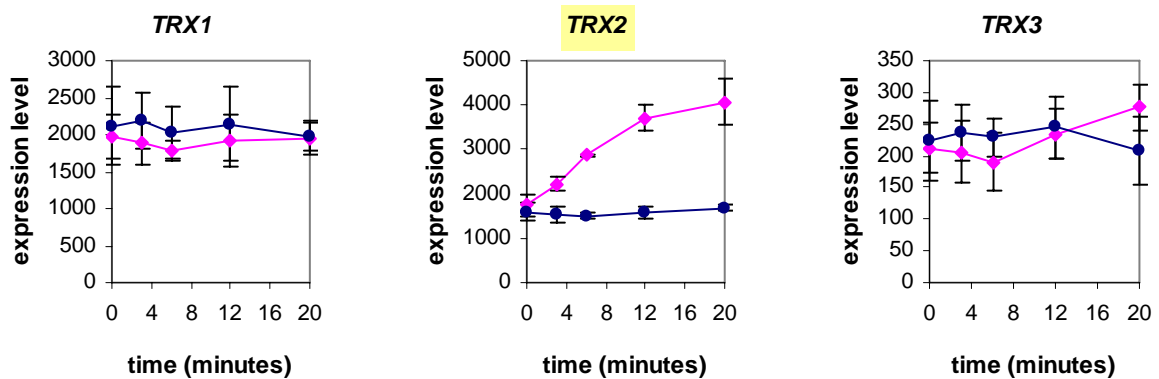


Figure 2.8. Time series of genes documented or potentially involved in thioredoxin system. The time series for CHP treated sample is labeled in pink. The time series for control sample is labeled in blue. Genes that were significantly differentially expressed ($p < 0.05$) by comparing to time0 are labeled with yellow shades.

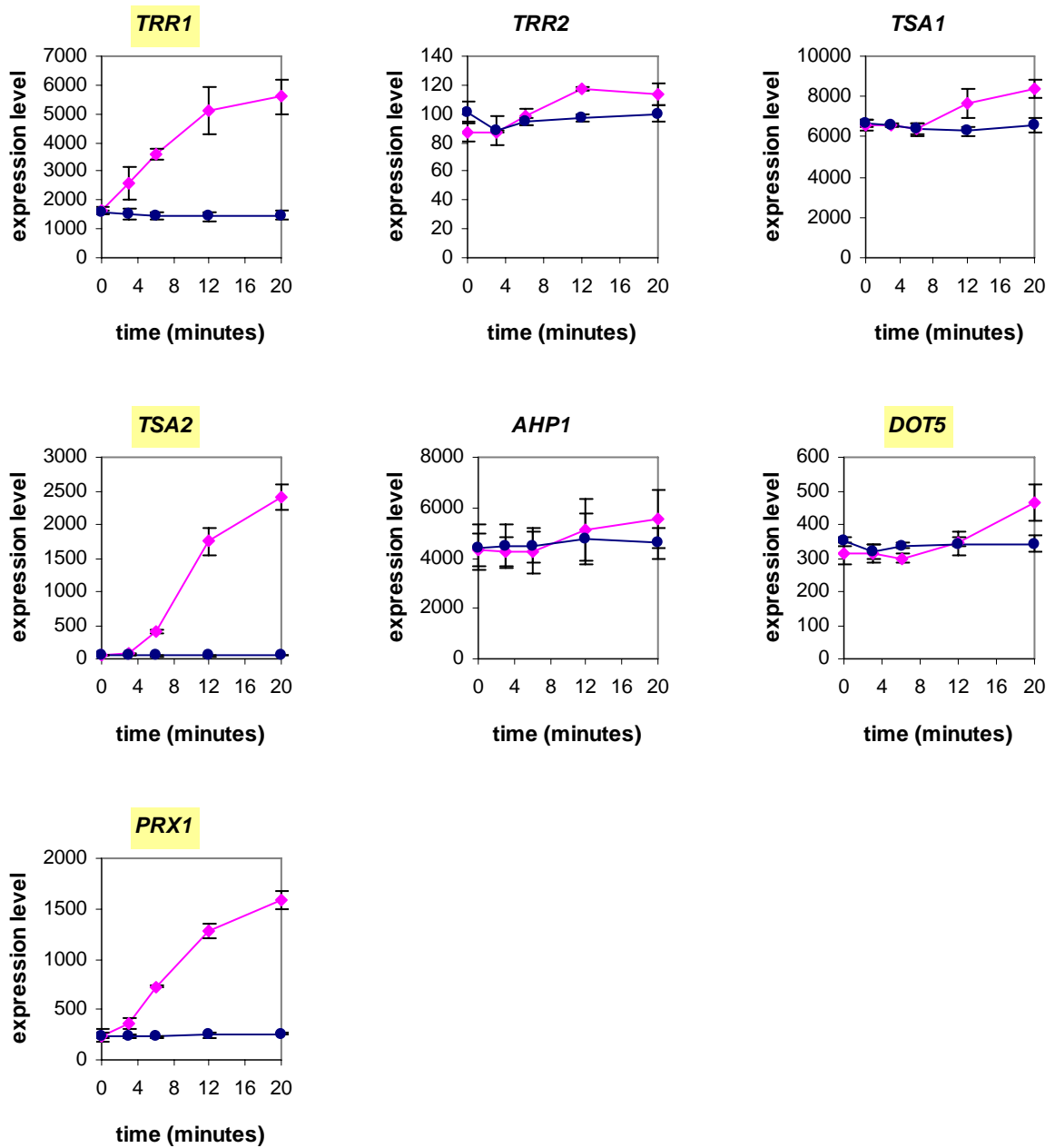


Figure 2.8. (Continued) Time series of genes documented or potentially involved in thioredoxin system. The time series for CHP treated sample is labeled in pink. The time series for control sample is labeled in blue. Genes that were significantly differentially expressed ($p < 0.05$) by comparing to time0 are labeled with yellow shades.

2.5.3. The *YAP* family of genes

Eukaryotic organisms, from yeast to human, contain AP-1 transcription factors that stimulate the expression of specific classes of genes in response to a wide variety of extracellular stimuli (Angel and Karin, 1991; Karin, 1995; Ransone and Verma, 1990). AP-1 proteins contain a conserved basic leucine zipper (bZIP) DNA-binding domain (Landschulz, et al., 1988).

In yeast, there are eight genes classified in the *AP-1* family: *YAP1*, *YAP2*, *YAP3*, *YAP4*, *YAP5*, *YAP6*, *YAP7* and *YAP8* were identified. Among them, *YAP1*, *YAP2*, *YAP3*, *YAP4*, *YAP5*, *YAP7* were found significantly induced by CHP in this study. The expression time series of these genes are shown in Figure 2.9A.

The regulation of *YAP1* has been described at the level of its location. Essentially Yap1p is distributed across the nucleus and cytoplasm, but under oxidative stress conditions, it becomes more concentrated in the nucleus, thus triggering effects on the genes it regulates (Rodrigues-Pousada, et al., 2004). However, it was already clear that mutants exhibiting a constitutive nuclear localization of Yap1p do not show greater resistance to H₂O₂ than normal strains (Kuge, et al., 1997), suggesting that mechanisms other than simply increasing nuclear concentration may be important in regulation by Yap1p. In our study, we observed an increase in the expression level of *YAP1*, from 6 to 20 min, with a maximum of 1.92x at 20 minutes.

Genes documented to be controlled by Yap1 were induced more or less at the same time as *YAP1* itself (Figure 2.9B). This then suggests that, under oxidative stress, Yap1p is first regulated at the level of translocation, becoming highly concentrated in the nucleus and then activating the genes under its control. Our results show that *YAP1* itself is also induced, which is observable already at 6 minutes after adding CHP - *YAP1* is thus also regulated at the transcription level (auto-induction). By searching the upstream sequence of *YAP1*, I found the promoter sequence binding site (TKACAAA) for Yap1p itself. It is then highly likely that Yap1p activates its own expression by binding to the promoter of its own gene (*YAP1*).

Yap2 has also been described as a regulator of gene expression under oxidative conditions induced by cadmium (Rodrigues-Pousada, et al., 2004). In the present study, I observe that the induction of *YAP2* started at 12 min, reaching a maximum of 2.34x at 20 min.

In previous transcriptomics studies, *YAP3* showed no response to any environmental stress (Rodrigues-Pousada, et al., 2004). However, here *YAP3* was significantly induced ($p=0.015$) by 1.26x at 20 minutes.

YAP4, *YAP5* and *YAP7* were also induced in our study. *Yap4* has been previously reported to be involved in the response to osmotic stress. Cells subjected to stress induced by H_2O_2 also displayed increase of *YAP4* expression (Gasch, et al., 2000). In our study, *YAP4* was significantly induced by 5.76x at 12 minutes. *YAP5* has been shown to be strongly induced under amino acid starvation, nitrogen depletion and stationary and diauxic phases (Rodrigues-Pousada, et al., 2004). In our study *YAP5* expression was 1.47x higher at 6 min after CHP addition. To our knowledge, this is the first time that *YAP5* is reported to respond to oxidative stress; most likely because it peaks quite early and previous studies have not monitored early changes. *YAP7* shows a significant early induction, from 3 to 12 min, with a maximum of 2.62x at 6 minutes. This gene was previously shown to be repressed under nitrogen depletion and in stationary phase (Rodrigues-Pousada, et al., 2004), but not by oxidative stress; here the same explanation related to early induction may be responsible for the lack of prior evidence for this.

In summary, six genes from the *YAP* family – *YAP1*, *YAP2*, *YAP3*, *YAP4*, *YAP5* and *YAP7* – seem to be involved in the response to oxidative stress induced by CHP and the involvement of *YAP3*, *YAP5* and *YAP7* in this response had not been previously described.

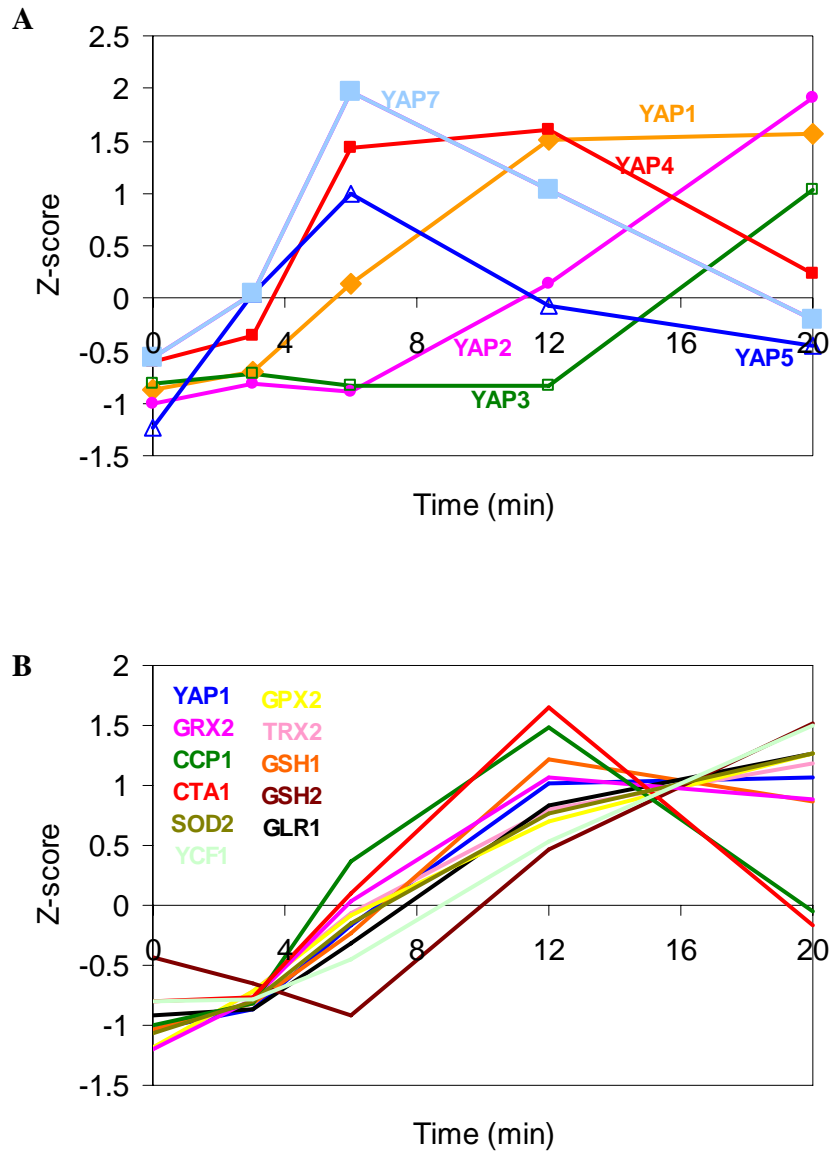


Figure 2.9 Gene expression time series of the *YAP* family and genes regulated by Yap1. (A) The gene expression time series of the *YAP* family of genes. (B) The gene expression time series of *YAP1* and antioxidant genes documented to be regulated by Yap1.

2.5.4. Pathway analysis

We identified GO Slim categories and KEGG pathways that significantly responded to oxidative stress induced by CHP. To the best of my knowledge, this is the first statistical and global identification of pathways responding to oxidative stress.

2.5.4.1. Significantly up-regulated pathways

Significantly up-regulated pathways are GO Slim categories or KEGG pathways that are significantly represented in the group of genes that were significantly induced by CHP. The up-regulated GO Slim categories are shown in Table 2.2. The up-regulated KEGG pathways are shown in Table 2.3.

Table 2.2. GO Slim categories that were significantly up-regulated in CHP treated samples. These categories were selected by the criteria that they have p -value < 0.001 at at least one time point within 20 minutes. P -values below 0.001 are framed in red. P -values between 0.001 and 0.05 are framed in orange. P -values greater than 0.05 are framed in gray.

GO Slim term	3min	6min	12min	20min
response to stress	>0.1	0.016645	0	0
carbohydrate metabolism	>0.1	>0.1	0	0
Sporulation	>0.1	>0.1	0.02106	0.000644
protein catabolism	>0.1	>0.1	0.030576	0
signal transduction	>0.1	>0.1	0.032308	0.000449

Table 2.3. KEGG categories that were significantly up-regulated in CHP treated samples. These categories were selected by the criteria that they have p -value < 0.01 at at least one time point within 20 minutes. P -values below 0.01 are framed in red. P -values between 0.01 and 0.05 are framed in orange. P -values greater than 0.05 are framed in gray.

KEGG term	3min	6min	12min	20min
glutathione metabolism	>0.1	0.002443	0.013898	5.4E-05
glycerolipid metabolism	>0.1	0.01106	0.00064	0.020554
starch and sucrose metabolism	>0.1	>0.1	0.000485	0.000364
fructose and mannose metabolism	>0.1	>0.1	0.006715	0.028673
Proteasome	>0.1	>0.1	>0.1	8.81E-15
ubiquitin mediated proteolysis	>0.1	>0.1	>0.1	0.004418

a) Glutathione metabolism and response to stress

The significant induction of glutathione metabolism started from 6 minutes (Table 2.3). The induction reached a peak at 20 minutes (Table 2.3 and Figure 2.10). The expression pattern of each gene involved in glutathione metabolism is shown in Figure 2.5. The detailed discussion of glutathione metabolism has been detailed above, in section 2.5.2.1.

b) Proteasome and ubiquitin mediated proteolysis

Proteins are susceptible to oxidative damage. Oxidatively modified proteins often lose their functions. Substantial evidence strongly suggests that proteolysis is responsible for degrading oxidized proteins in the cytoplasm, nucleus, and endoplasmic reticulum of eukaryotic cells to avoid excessive accumulation of these oxidized non-functional proteins (Grune, et al., 2003). Many studies indicate that mildly oxidized proteins are readily degraded, whereas severe oxidation stabilizes proteins by inducing highly aggregated and cross-linked proteins, which are poor substrates for proteases (Grune, et al., 1995; Shringarpure, et al., 2000; Ullrich, et al., 1999). Thus, the removal of mildly oxidized proteins before they are severely cross-linked is essential for maintaining cellular homeostasis by preventing the accumulation of non-degradable proteins. The inability to degrade extensively oxidized proteins may contribute to certain diseases, including Alzheimer's disease (Nunomura, et al., 2001) and Parkinson's disease (Sidell, et al., 2001), and could also play a significant role in the aging process (Merker and Grune, 2000). In yeast, the ubiquitin-dependent pathway is required to withstand oxidative stress (Cheng, et al., 1994). In our study, the proteasome and proteolysis were significantly induced by CHP (Figure 2.11, 2.12, 2.13). Godon and co-workers observed twelve *S. cerevisiae* proteasome subunits induced by H₂O₂ (Godon, et al., 1998) in their proteomics study. We found many more of the subunits induced by CHP at the transcript level (Figure 2.11, 2.12). Figure 2.12 shows that *RPN4*, encoding the transcription activator of proteasome subunits, was significantly induced before the induction of the proteasome subunits under its control. These data strongly confirm that *RPN4* is regulated at the transcription level and that it is indeed the transcriptional activator of the proteasome subunit genes.

c) **Glycerolipid metabolism**

Based on my knowledge, the induction of glycerolipid metabolism under oxidative stress was not reported in previous literature. It has been shown that the primary action of CHP occurs at the cell plasma membrane (Vroegop, et al., 1995). Plasma membranes contain both polyunsaturated and monounsaturated lipids, which are susceptible to the action of ROS. Lipid peroxidation in plasma membrane in turn provides a steady supply of free radicals such as alkyl and peroxy radicals, which attack cellular components such as lipids, proteins and DNA. Oxidative modification of the membrane lipids also leads to alterations in structure and fluidity of the membrane, affecting cell function and can ultimately lead to loss of integrity (Carr, et al., 1997; Halliwell and Gutteridge, 1990). Glycerophospholipids are the main component of biological membranes, and are good substrates for lipid peroxidation. Lipid peroxidation, along with glycerophospholipid deficiency, plays an important role in the pathophysiology of Alzheimer's disease (Farooqui, et al., 1997). The induction of glycerolipid metabolism could be part of the membrane repair response.

d) **Starch and sucrose metabolism and galactose metabolism**

The induction of starch and sucrose metabolism genes could be due to a need for producing high concentrations of trehalose. As shown in Figure 2.14, genes that encode enzymes involved in the production of trehalose were significantly induced. Trehalose is essential for cell survival during oxidative stress (Benaroudj, et al., 2001). Trehalose accumulation reduces oxidative damage to cellular proteins (Benaroudj, et al., 2001). Trehalose has been shown to protect protein against thermal inactivation (Hottiger, et al., 1994). It was demonstrated that trehalose reduces protein aggregation and maintains polypeptide chains in a partially folded state, thus facilitating their refolding by cellular chaperones (Singer and Lindquist, 1998). Benaroudj and co-workers suggested that trehalose enhances resistance to oxidative stress by quenching oxygen radicals (Benaroudj, et al., 2001). Expression of the trehalose-6-phosphate phosphatase (*TPS2*), which catalyzes the second step in trehalose biosynthesis, is controlled by the stress response transcription factor Yap1p (Gounalaki and Thireos, 1994). Enhanced survival of

yeast under oxidative stress was also observed when the sugar alcohol mannitol or the monosaccharide galactose was added to the medium, although trehalose seemed more potent in protecting cells (Benaroudj, et al., 2001). Both mannitol and galactose have been shown to scavenge hydroxyl radicals *in vitro* (Litchfield and Wells, 1978; Tauber and Babior, 1977). In our data, enzymes involved in trehalose metabolism were significantly up-regulated as shown in the starch and sucrose metabolism chart (Figure 2.14). Galactose metabolism was also up-regulated, the *p*-value of galactose metabolism at 6, 12 and 20 minutes are 0.039, 0.014 and 0.014, respectively.

e) **Sporulation**

Sporulation has been linked to carbon and nitrogen starvation, and to my knowledge has not been linked to oxidative stress in literature. Upon starvation of both carbon and nitrogen sources, diploid yeast cells enter meiosis to generate four haploid spores. These spores are formed within the old cell, and are surrounded by their own ascospore walls. Different from walls of vegetatively growing cells, ascospore walls are designed to resist severe environmental stresses (Smits, et al., 2001). The inner layer of the spore wall is composed mainly of glucan and mannoproteins, similar to vegetative cell walls. The inner layer is surrounded by a layer of chitosan. On the outside of the chitosan layer is a proteinaceous layer which is made highly insoluble through dityrosine cross-links. Together, these layers render the spores resistant to, amongst others, glucanase and zymolyase, heat stress and ether (Smits, et al., 2001). Our hypothesis is that sporulation could be a protection that yeast use to resist oxidative stress.

f) **Carbohydrate metabolism**

Carbohydrate metabolism was significantly up-regulated from 12 to 20 minutes (Table 2.2). Carbohydrate metabolism includes starch and sucrose metabolism and galactose metabolism which were discussed in section 2.5.4.1d.

In our CHP treated samples, it was observed that most of glycolysis and TCA cycle genes were either down-regulated or unchanged. However, the gene encoding the glycolysis enzyme glucokinase (*GLK1*) was up-regulated in stress conditions. Glucokinase catalyzes

the formation of glucose-6-phosphate (G6P) from glucose, and G6P is also the first compound in the pentose phosphate pathway (PPP), which is the main pathway involved in regenerating reducing equivalents in the form of NADPH. This pathway is well known to be important in oxidative stress responses (Izawa, et al., 1998; Juhnke, et al., 1996; Minard and McAlister-Henn, 2001). Genes encoding enzymes of the PPP were almost all up-regulated. It is interesting to notice that four enzymes of the PPP are encoded by a pair of genes, but only one gene of each pair was up-regulated in response to CHP (*SOLA*, *GND2*, *TKL2*, *YGR043c*). Because the PPP is also a major source of biosynthetic precursors (e.g. ribose for nucleic acid synthesis), it may be that these particular isoenzymes that were induced are better adapted to the main role of regenerating NADPH, while the other isoenzymes may be better adapted for synthesis of biosynthetic precursors.

G6P is also involved in trehalose metabolism. Genes involved in trehalose metabolism were all up-regulated in CHP treated sample, as described in 2.5.4.1d.

Therefore, under oxidative stress conditions, glucose seems to be diverted from energy production (glycolysis, TCA cycle) to NADPH production in the PPP and to the production of trehalose, through its conversion to G6P in the reaction catalyzed by glucokinase. This is in agreement with the arrest of growth in the culture, which would be a major energy consumer.

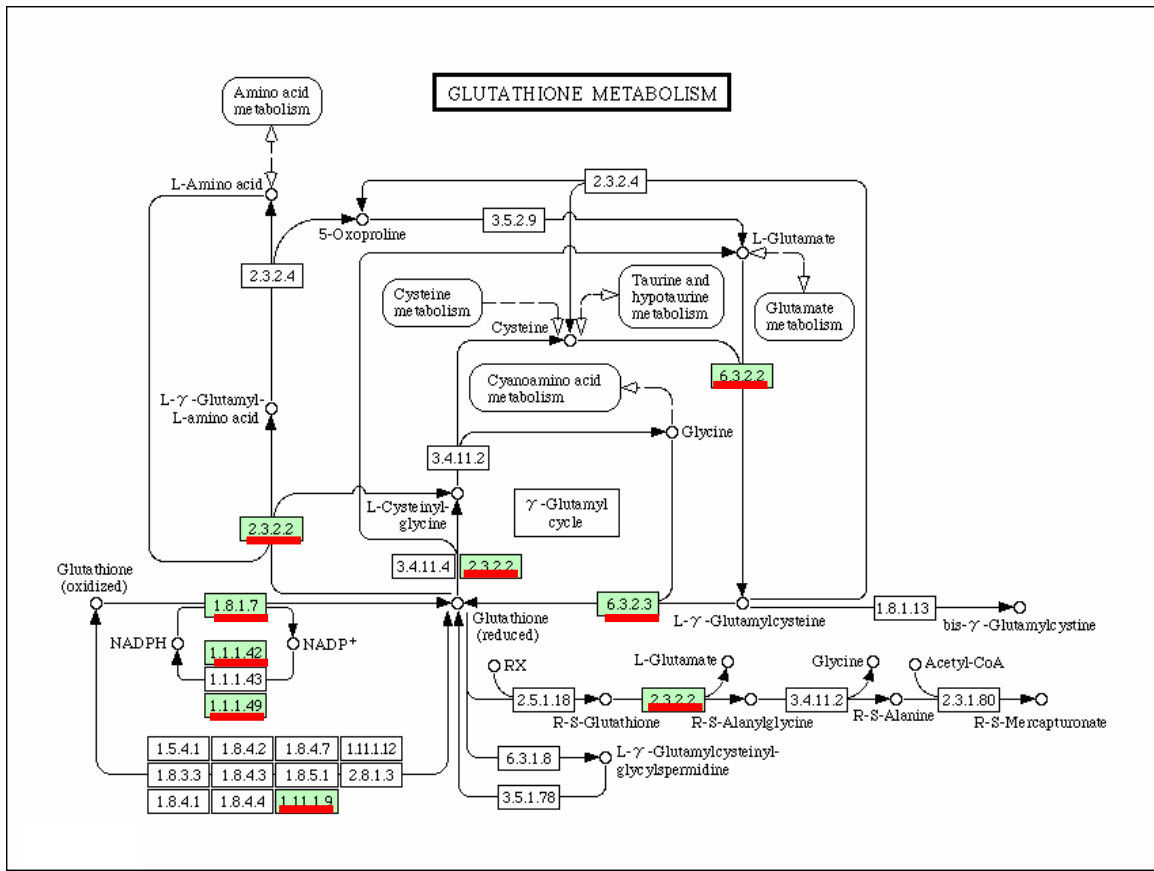


Figure 2.10. Glutathione metabolism (from KEGG) at 20 minutes. Green boxes indicate genes identified in the yeast genome. Red underlines indicate genes that were significantly induced by CHP at 20 minutes by comparing to time 0.

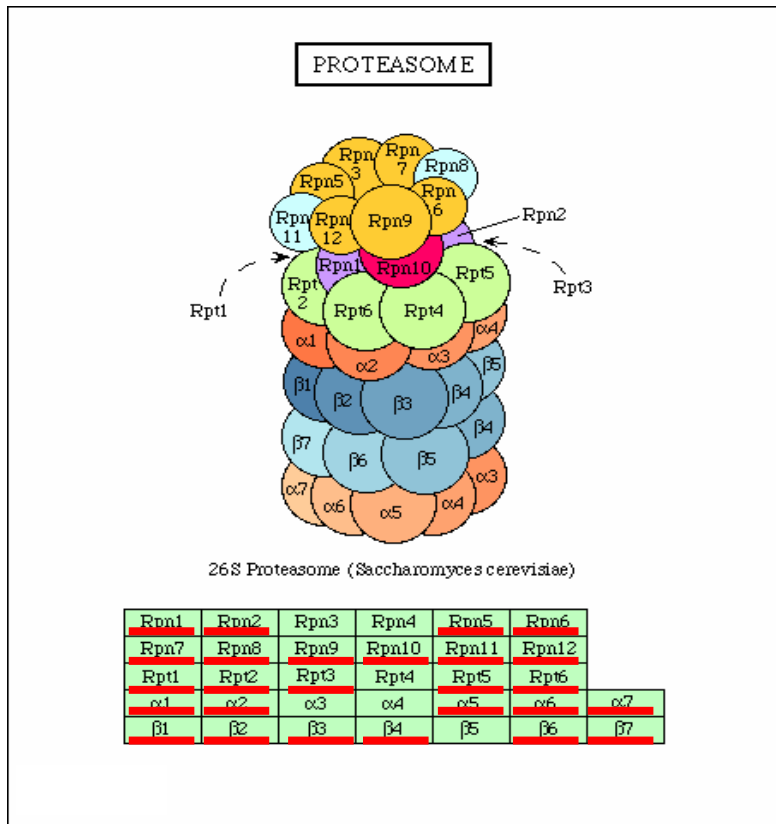


Figure 2.11. Proteasome (from KEGG) at 20 minutes. Green boxes indicate genes identified in the yeast genome. Red underlines indicate genes that were significantly induced by CHP at 20 minutes by comparing to time 0.

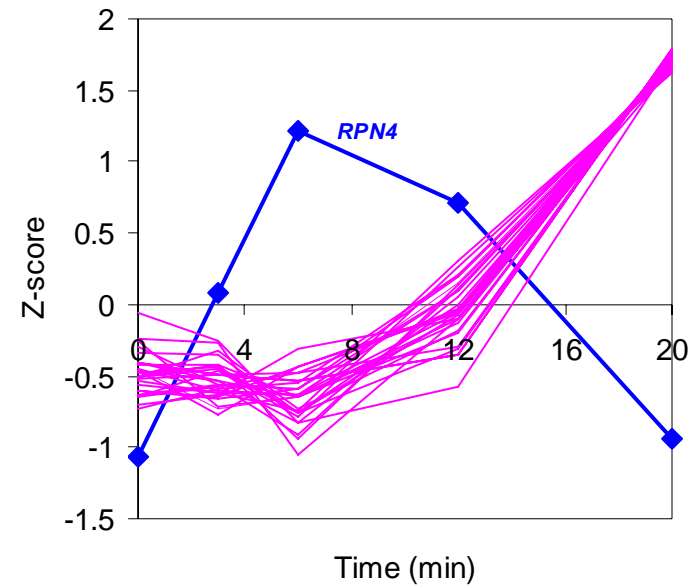


Figure 2.12. Gene expression time series for proteasome subunits that were significantly induced at 20 minutes, which are the genes labeled with red underlines in Figure 2.11. RPN4 is the transcription factor that regulates genes encoding proteasome subunits.

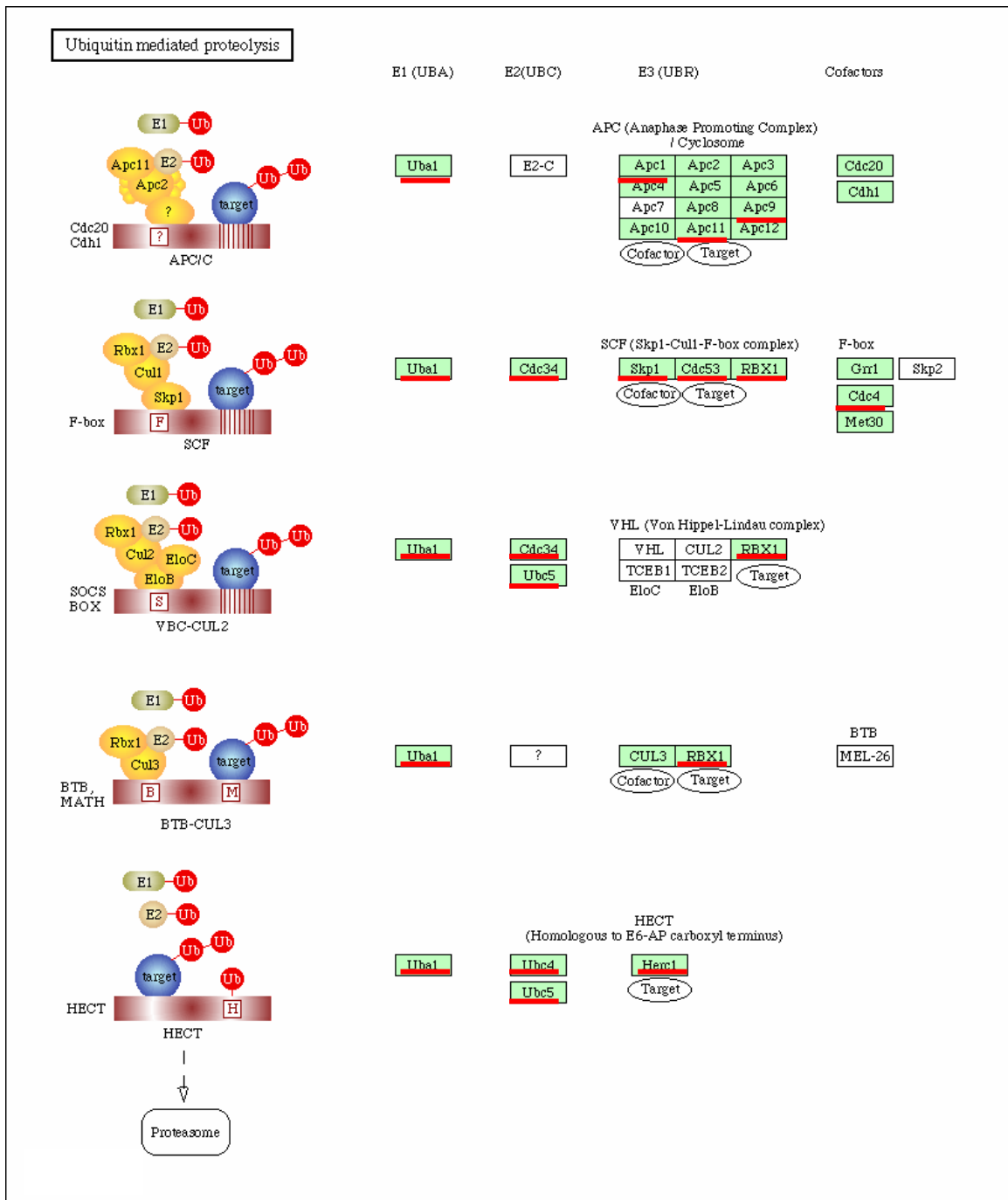


Figure 2.13. Ubiquitin mediated proteolysis (from KEGG) at 20 minutes. Green boxes indicate genes identified in the yeast genome. Red underlines indicate genes that were significantly induced by CHP at 20 minutes by comparing to time 0.

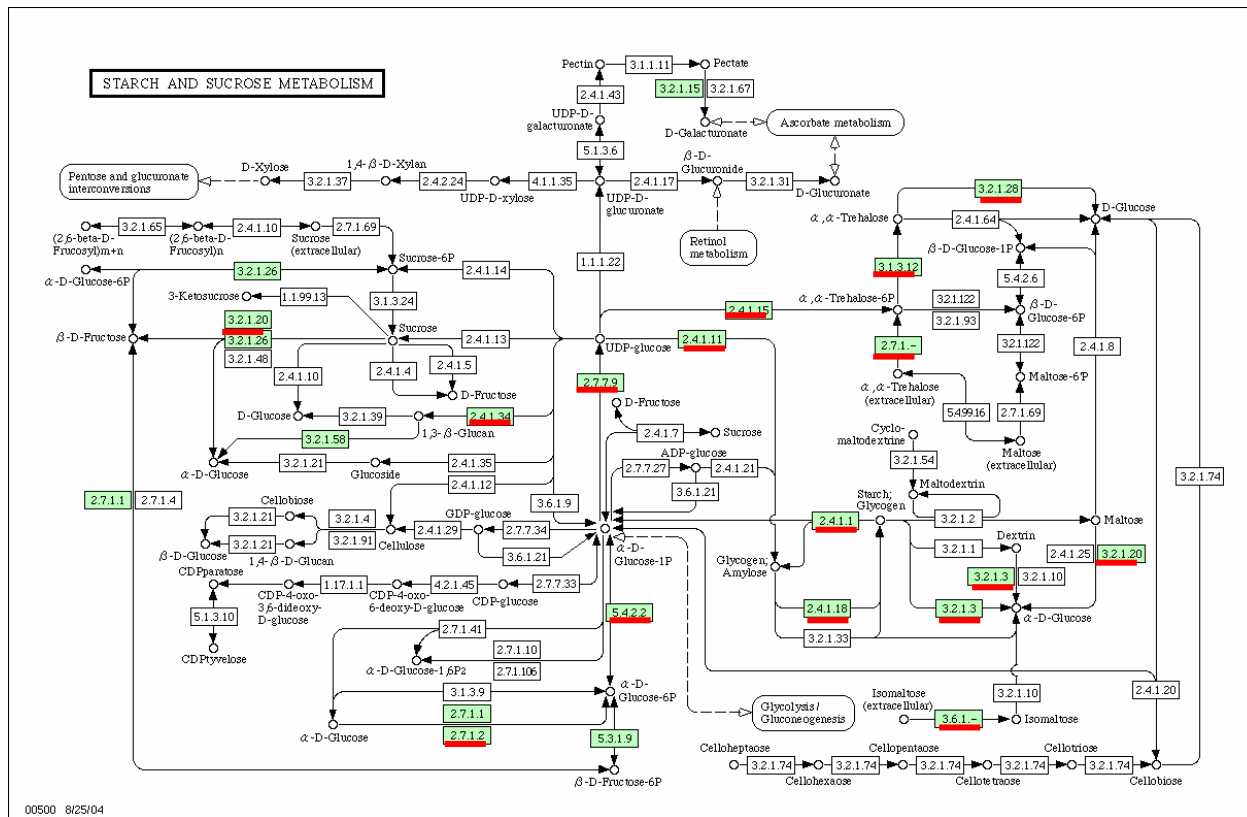


Figure 2.14. Starch and sucrose metabolism (from KEGG) at 20 minutes. Green boxes indicate genes identified in the yeast genome. Red underlines indicate genes that were significantly induced by CHP at 20 minutes by comparing to time 0.

2.5.4.2. Significantly down-regulated pathways

As shown in Table 2.4 and 2.5, a significant number of genes involved in categories related to cell cycle, transcription, translation and nucleotide metabolism were down-regulated.

Table 2.4 GO Slim categories that were significantly down-regulated in CHP treated sample. These categories were selected by the criteria that they have p-value < 0.001 at at least one time point within 20 minutes. *P*-values below 0.001 are framed in darker green. *P*-values between 0.001 and 0.05 are framed in lighter green. *P*-values greater than 0.05 are framed in gray.

GO Slim term	3min	6min	12min	20min
nuclear organization and biogenesis	>0.1	0.022552	0.000632	0.036472
ribosome biogenesis and assembly	>0.1	0.093905	0	0
organelle organization and biogenesis	>0.1	>0.1	0	0
RNA metabolism	>0.1	>0.1	0	0
cell cycle	>0.1	>0.1	0.00014	0.036506
Cytokinesis	>0.1	>0.1	0	0
electron transport	>0.1	>0.1	>0.1	0

Table 2.5 KEGG categories that were significantly down-regulated in CHP treated sample. These categories were selected by the criteria that they have p-value < 0.01 at at least one time point within 20 minutes. *P*-values below 0.01 are framed in darker green. *P*-values between 0.01 and 0.05 are framed in lighter green. *P*-values greater than 0.05 are framed in gray.

KEGG term	3min	6min	12min	20min
cell cycle	>0.1	0.006487	1.232E-07	0.001035
purine metabolism	>0.1	0.009725	5.656E-10	1.133E-09
RNA polymerase	>0.1	>0.1	5.396E-13	2.423E-09
pyrimidine metabolism	>0.1	>0.1	8.983E-11	6.318E-09

a) Cell cycle

Oxidative stress has a strong influence on the cell cycle. It was reported that H₂O₂ induces temporary growth arrest and lengthening of the cell cycle in several mammalian cell types (Wiese, et al., 1995). In the yeast *S. cerevisiae*, G₁ arrest in response to dioxygen stress was observed (Lee, et al., 1996). In our study, CHP has a clear inhibiting effect on cell division (Figure 2.15, 2.16). At 3 minutes, only three genes (*CLB6*, *VHS1* and *PCL8*) were significantly down-regulated, and all of them were cell cycle genes. Vhs1 and Pcl8 are documented to be involved in G₁-S transition (Measday, et al., 1997) (Munoz, et al., 2003). Clb6 is involved in DNA replication (Schwob and Nasmyth, 1993). The lengthening of G₁ phase and the slowdown of S phase allow time for the synthesis of enzymes that respond to oxidative stress and repair of damage. DNA damage by oxidants could cause irreversible mutations if it is not repaired before replication. In *S. cerevisiae*, DNA checkpoints inhibit the G₁-S transition (Siede, et al., 1993), and slow down S phase (Paulovich and Hartwell, 1995). DNA checkpoints also delay chromosome segregation (Weinert and Hartwell, 1988; Weinert and Hartwell, 1993; Weinert, et al., 1994). In addition, DNA checkpoints prevent mitosis when DNA replication is blocked. As shown in Figure 2.15, a significant number of genes involved in each phase of the cell cycle were repressed.

b) RNA polymerase and transcription

A recent report showed that oxidative stress does not significantly damage the transcription machinery (Berthiaume, et al., 2006). That study showed that the amount of RNA polymerase III in nuclear extracts prepared from H₂O₂-treated cells was similar to extracts prepared from untreated cells. Our results, however, show that 23 out of 29 genes encoding RNA polymerase I, II and III were down-regulated at the mRNA level at 12 minutes after the addition of CHP (Figure 2.17, 1.18). Our study suggests that oxidative stress has a significant repression effect on the making of transcription machinery.

c) Ribosome and translation

It has been reported that CHP and /or cytotoxic aldehydes derived from it inhibit protein synthesis in human skin fibroblastes (Poot, et al., 1988). Results from Ayala and co-workers showed that CHP treatment for 7 days produced a decrease in the rate of peptide chain elongation along with a higher state of polyribosome aggregation in rat liver. Their findings suggested that the elongation step of the translation is slowed down by CHP. We observed that a significant number of genes involved in ribosome biogenesis and assembly were down-regulated.

d) Purine and pyrimidine metabolism

Godon and co-workers showed in their proteomics study that five proteins Gua1, Ade3, Ade6, Ade 57 and Ura1, involved in purine and pyrimidine metabolism were repressed by H₂O₂ (Godon, et al., 1998). The mechanism of this repression was not studied in literature. Our study showed that 17 genes in purine metabolism and 13 genes in pyrimidine metabolism were significantly down-regulated at the transcription level. The repression of nucleotide metabolism could be due to the slowdown of cell cycle and the slowdown of transcription.

e) Electron transport and mitochondria

Mitochondria are the primary source of ATP under aerobic conditions via the process of oxidative phosphorylation. During respiration, mitochondria are also the primary source of ROS in the cell. Leakage of electrons from the respiratory chain can result in the reduction of oxygen which is the primary source of ROS that damage various macromolecules in the cell.

Our results show that a significant number of genes involved in electron transport were repressed. This phenomenon could be due to mitochondria damage by CHP and ROS derived from CHP. Oxidative stress may damage mitochondrial lipids, mitochondrial DNA, and mitochondrial proteins. Oxidative damage to mitochondrial genome can render mitochondria functionally defective, resulting in respiratory deficiency.

Publications about the role of mitochondria under oxidative stress have been somewhat conflicting. It was reported that mitochondrial function is required for resistance to oxidative stress in *S. cerevisiae* (Grant, et al., 1997). However, experiments performed by Davermann and co-workers showed that impaired mitochondrial function is a protection against oxidative stress (Davermann, et al., 2002). Cells lacking their entire mitochondrial genome were remarkably more resistant to oxidative stress than cells with functional mitochondria (Davermann, et al., 2002). It is difficult to explain the contradictory results from these two studies. Different oxidants were used in these studies, which may explain some of the differences. The first result can be explained by the requirement of ATP production. The repair of damaged proteins and DNA, and the detoxification of lipid peroxidation products requires energy. The transport of oxidized molecules from the cell or into the vacuole for subsequent breakdown may also require energy. The second result can be explained by the ROS generating nature of mitochondria. Cells that lose mitochondria function would reduce the production of ROS from mitochondria, thus have a selective advantage of survival. The common result of these two studies and our own study is that ROS do have a clear damage effect on mitochondria. It was previously suggested that mtDNA damage could be a useful biomarker for diseases associated with ROS (Yakes and Van Houten, 1997).

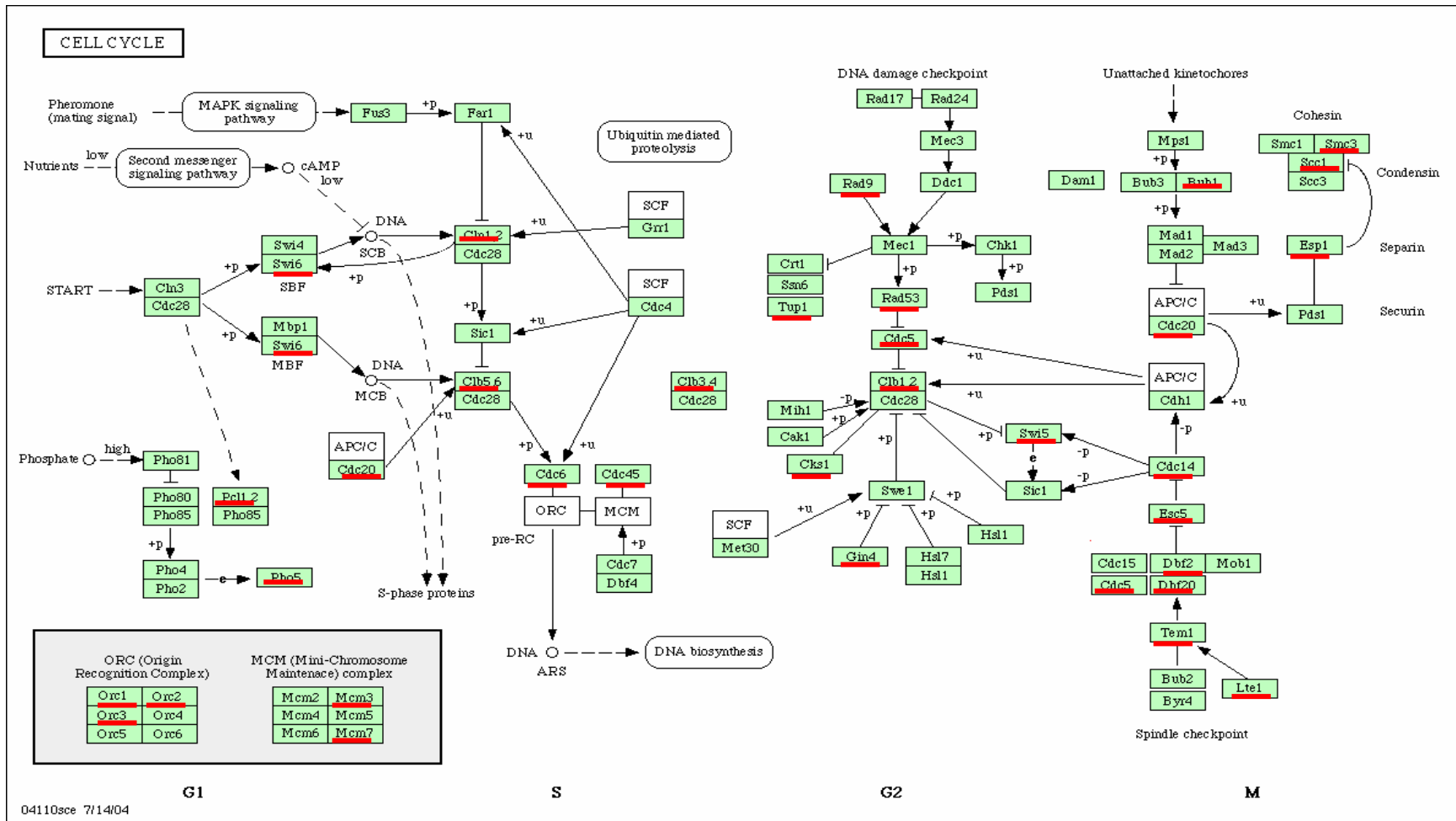


Figure 2.15. Cell cycle (from KEGG) at 12 minutes. Green boxes indicate genes identified in the yeast genome. Red underlines indicate genes that were significantly repressed by CHP at 12 minutes by comparing to Time 0.

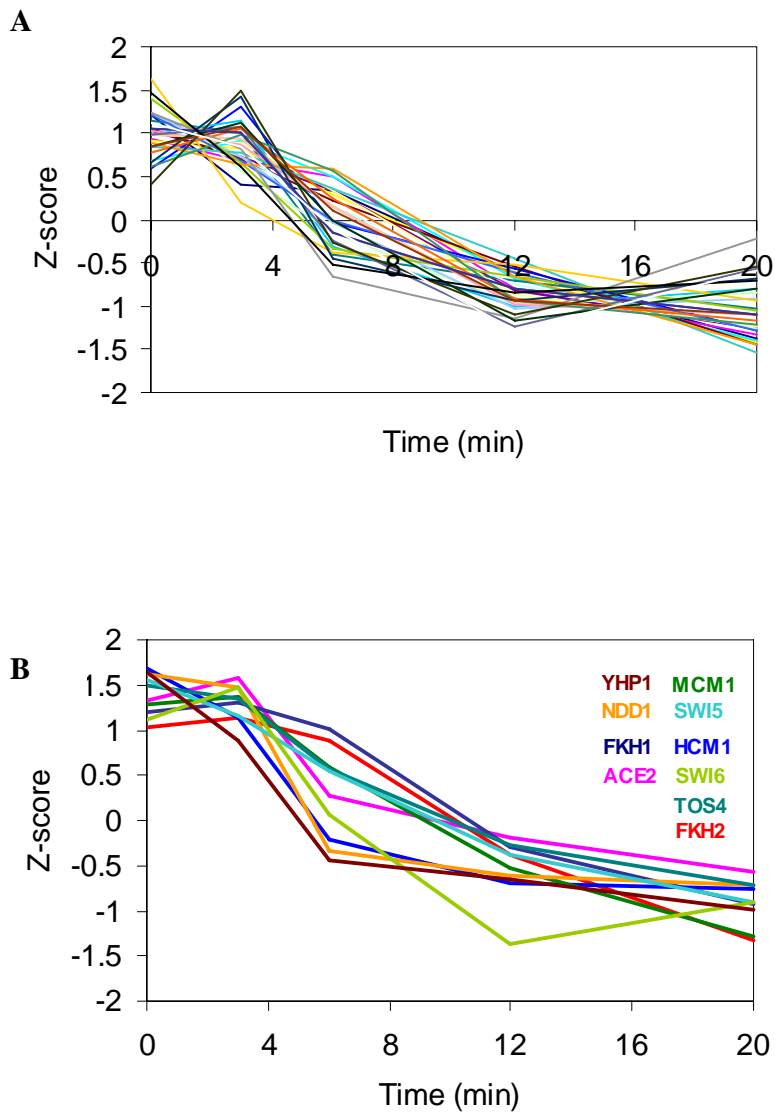


Figure 2.16. Gene expression time series of cell cycle genes. (A) Cell cycle genes that were significantly repressed by CHP at 12 minutes, which are the genes labeled with red underlines in Figure 2.15. (B) Transcription factors that regulate cell cycle genes and that were significantly repressed by CHP within 20 minutes.

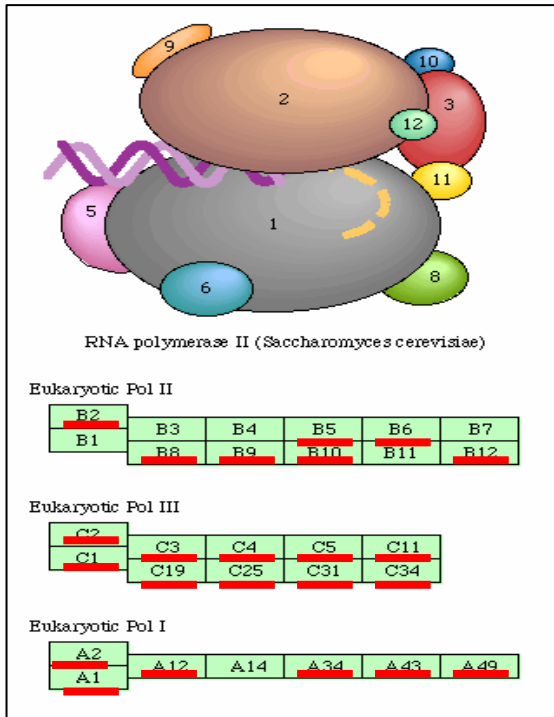


Figure 2.17. RNA polymerase (from KEGG) at 12 minutes. Green boxes indicate genes identified in the yeast genome. Red underlines indicate genes that were significantly repressed by CHP at 12 minutes by comparing to Time 0.

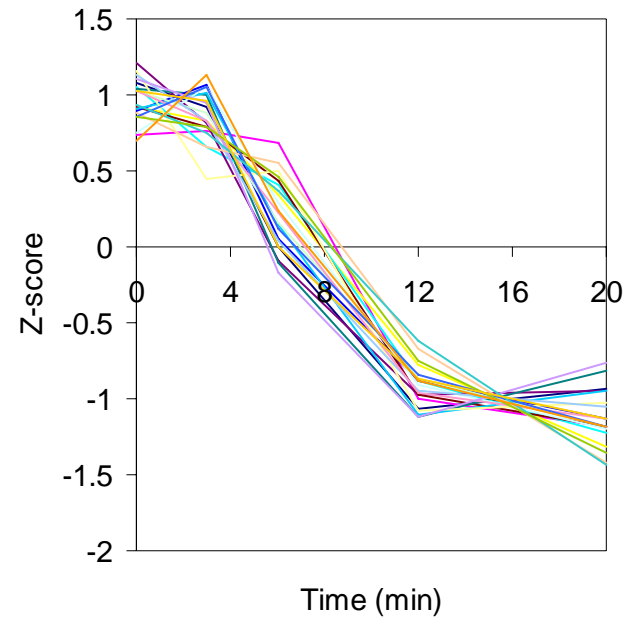


Figure 2.18. Gene expression time series of RNA polymerase genes that were significantly repressed by CHP at 12 minutes, which are the genes labeled with red underlines in Figure 2.17.

2.5.5. Clustering analysis

k-means clustering analysis was carried out to group yeast genes into 5 clusters according to their expression patterns under oxidative stress condition (Figure 2.19). Pathway analysis was then performed to find pathways that are significantly represented in each cluster.

Galactose metabolism ($p=0.0042$) and starch and sucrose metabolism ($p=0.0062$) are significantly represented in cluster 2 with a pattern of induction. Proteasome ($p=1.18E-16$), Ubiquitin mediated proteolysis ($p=0.0059$) and MAPK signaling pathway ($p=0.0081$) are significantly represented in cluster 4 with a pattern of induction, but later than the induction of carbohydrate metabolisms in cluster 2. Ribosome ($p=1.78E-14$), cell cycle ($p=1.27E-05$), RNA polymerase ($p=6.08E-05$), purine metabolism ($p=0.0001$), and pyrimidine metabolism ($p=0.0006$) are significantly represented in cluster 5 with a clear pattern of repression. Oxidative phosphorylation is in cluster 1 and ATP synthesis is in cluster 3; it is not clear why these two pathways have opposite patterns, since ATP is generated through oxidative phosphorylation.

Glutathione metabolism was not significantly represented in any of these clusters. As shown above in Figure 2.5, all of the genes in glutathione metabolism were significantly induced by CHP; however, these genes have different expression patterns, which could explain why they were not grouped together. It suggests that clustering analysis is good at detecting the pattern of a pathway only if genes on the pathway are regulated coordinately. For example, proteasome genes are regulated coordinately (Figure 2.12), thus these genes are grouped into the same cluster (cluster 4).

Most of the clustering analysis results are in agreement with the pathway analysis results discussed in section 2.5.4. However, the patterns of some pathways are only discovered by clustering analysis, for example the MAPK signaling pathway; while the patterns of other pathways are only discovered in pathway analysis discussed in section 2.5.4, for example glutathione metabolism. It suggests that the results from these two analyses are complementary to each other.

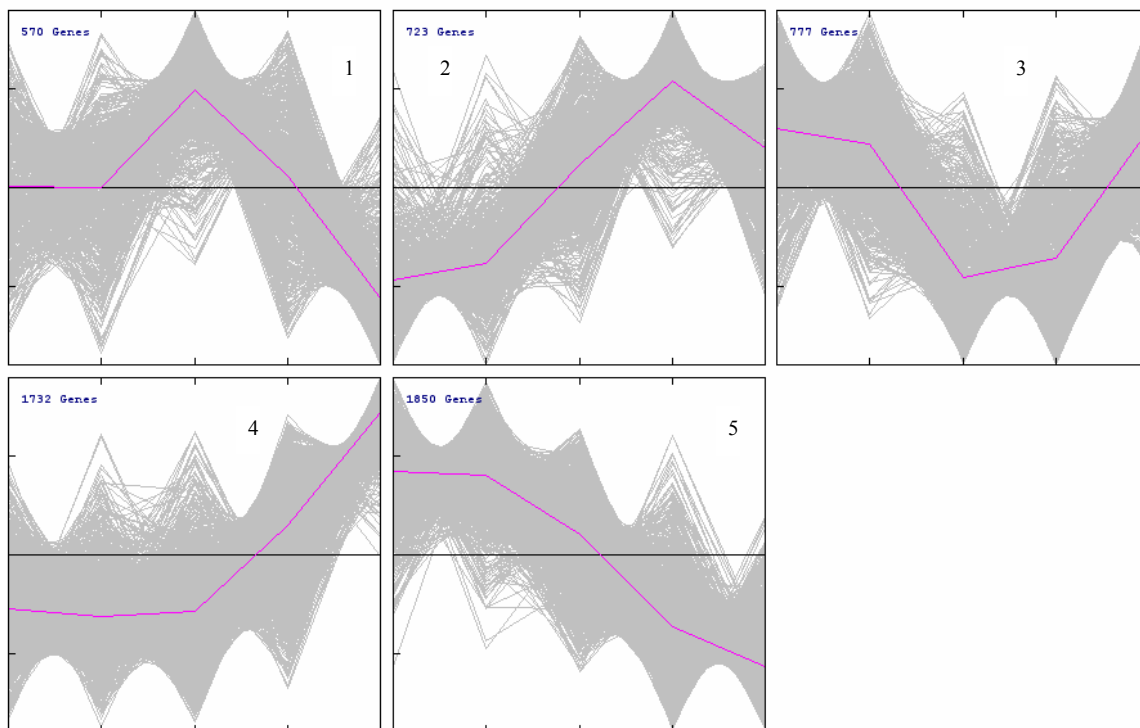


Figure 2.19. *k*-means clustering analysis results. The pink curve in each graph represents the average expression pattern of each cluster. Gray curves in each graph represent the expression time series of genes in each cluster. This analysis was performed in the software MeV. Scales are not shown in the results generated from MeV. The X axis in each graph represents time (0 minutes to 20 minutes). The Y axis in each graph represents Z score of expression value. The horizontal line in the middle of each graph is the line for Z score = 0 (*i.e.* the mean value of each curve).

2.5.6. Transient early response to oxidative stress

One of the main contributions of this study concerns the early oxidative stress responsive genes. Our time series started at 3 minutes after the addition of CHP. In previous microarray studies of oxidative stress, the earliest time point was taken at 10 minutes (Causton, et al., 2001; Gasch, et al., 2000; Koerkamp, et al., 2002). Lucau-Danila and co-workers did study early expression of yeast under chemical stress with the earliest time point at 30 seconds (Lucau-Danila, et al., 2005). The chemical stress in their study

mimics oxidative stress, because the chemical inducer used in their study was benomyl, which is known to activate Yap1p by assisting its nuclear localization. However, in oxidative stress, Yap1 is not just regulated by nuclear localization, as discussed in section 2.5.3. Other regulations, for example, the possible regulation at the transcription level cannot be reflected in benomyl study. Thus, there is a difference between Yap1 response activated by benomyl and Yap1 response activated by ROS such as CHP. Furthermore, oxidative stress responding genes which are not regulated by Yap1 cannot be identified in the benomyl study.

Since most microarray studies started time series at 10 minutes, there may be genes responding early that after 10 minutes have already resumed their basal expression levels, and so would never have been observed in those studies. These transient early responsive genes may play very important roles in oxidative stress, for example they may be involved with early (upstream) signaling events.

We found 46 transient induced genes that were significantly induced by CHP within 6 minutes, but which had already resumed their normal gene expression level after 6 minutes (Figure 2.20A, Table 2.6).

Met28 (Table 2.6A) is a transcription activator that regulates sulfur metabolism (Kuras, et al., 1996). *MET28* was transiently induced within 6 minutes. ANOVA results show that many other genes involved in sulfur metabolism, such as *MET1*, *MET8*, *MET12*, *MET16*, *MET22*, *CYS3*, *STR3* AND *MET30*, were also significantly induced within 20 minutes. Sulfur metabolism is required for the production of the sulfur amino acids, cysteine and methionine. The induction of sulfur metabolism under oxidative stress could be due to an increased need of cysteine for glutathione synthesis. Cysteine residues play ubiquitous roles to mediate cellular responses to oxidative stress through their ability to detect changes in redox status, and introducing changes in protein structure and function. Many cysteine-containing proteins such as glutaredoxin, thioredoxin and Yap1p require redox-active cysteine residues for their activity (Akerboom, et al., 1982; Holmgren, 1989; Kuge, et al., 1997).

YAP5 (Table 2.6A) has been shown to be strongly induced under amino acid starvation, nitrogen depletion, stationary and diauxic phases (Rodrigues-Pousada, et al., 2004). *YAP5* has not been documented to be induced by oxidative stress until the present observations. In our study, a significant induction of *YAP5* of 1.6 fold at 6 minutes was observed, though it was already back to normal levels at 12 minutes. ChIP-chip experiments showed that *YAP5* is regulated by Met28 (Lee, et al., 2002). According to our results, *MET28* was significantly induced at 3 minutes, and *YAP5* was significantly induced at 6 minutes right after the induction of *MET28*. It suggests that the regulation of Met28 and Yap5 could be at the level of transcription. The oxidative stress response of *YAP5* is novel and the role of this gene and its product in this response is not yet clear.

Mrk1p (Table 2.6B) was shown to activate Msn2p-dependent transcription of stress responsive genes in yeast. Msn2 is a transcription factor that regulates general stress response (Hirata, et al., 2003). It is not clear how Mrk1 regulates Msn2p. It is also not clear how Mrk1 itself is regulated. Our results show that *MRK1* was transiently induced within 6 minutes but quickly returned to its normal level. The regulation of Mrk1 appears to be at the level of transcription.

A few genes involved in cell wall and cytoskeleton also appeared to be transiently up-regulated in the early stages (Table 2.6C). As discussed in section 2.5.4.1c, the primary damage effect of CHP is at the plasma membrane (Vroegop, et al., 1995). The induction of genes involved in cell wall could be part of the membrane repair response. Changes in microtubule organization have been reported to take place in Alzheimer's disease (Hempen and Brion, 1996; Price, et al., 1986). Santa-Maria and co-workers suggested that oxidative stress in Alzheimer's disease results in tubulin modification and microtubule disassembly via reactive quinones (Santa-Maria, et al., 2005). The induction of genes involved in cytoskeleton could be a repair process.

There were 51 genes transiently repressed within 6 minutes (Figure 2.20B, Table 2.7). The repression of these genes would have been missed in previous microarray studies, where time series started at 10 minutes after the stress being applied. In agreement with the pathway analysis results (Table 2.4 and Table 2.5), genes involved in the cell cycle,

transcription, translation, and mitochondria were down-regulated (Table 2.7). It is interesting that genes involved in protein trafficking from the ER to the cell surface also appeared in Table 2.7E. The repression of genes involved in protein trafficking in response to oxidative stress was not reported in previous literature. Environmental stress is an important factor to cause erroneous protein biogenesis (Ellgaard and Helenius, 2003; Wickner, et al., 1999). Mazel and coworkers suggested that during transient stresses, the chaperones hold the newly synthesized proteins and fold them later when the stress condition is resolved (Mazel, et al., 2004). If the stress is not resolved rapidly, cells target these proteins for ER associated degradation (ERAD) to decrease the load of misfolded proteins that accumulate in the ER (Mazel, et al., 2004). The ERAD machinery is dependent on the up-regulation of ER-resident chaperones (Mazel, et al., 2004). The significant up-regulation of chaperone genes, such as *HSP82*, *SBA1*, *HSP78* and *HSP26*, was observed in this study. When ERAD is initiated, misfolded proteins are recognized, targeted and translocated to the ubiquitylation machinery located on the cytosolic face of the ER for modification. The molecules are then extracted from the membrane and finally degraded by proteasome (Sayeed and Ng, 2005). Our hypothesis is that oxidatively damaged proteins were held in ER for refolding or ERAD, which may slowdown the protein export from ER, thus lowering the traffic from ER to cell surface.

The exact roles of many genes in Table 2.6 and Table 2.7 in oxidative stress response are not clear. In both Tables, a category of unknown genes was found to have a transient early response to oxidative stress. BLAST analysis does not reveal good matches of the sequence of these genes to the sequence of any genes of known function, thus the functions of these genes remain completely unknown. Our study indicates that these genes seem to play a role in early oxidative stress response, and if nothing else, this type of annotation should be attributed to those unknown genes that are present in Tables 2.6 and 2.7.

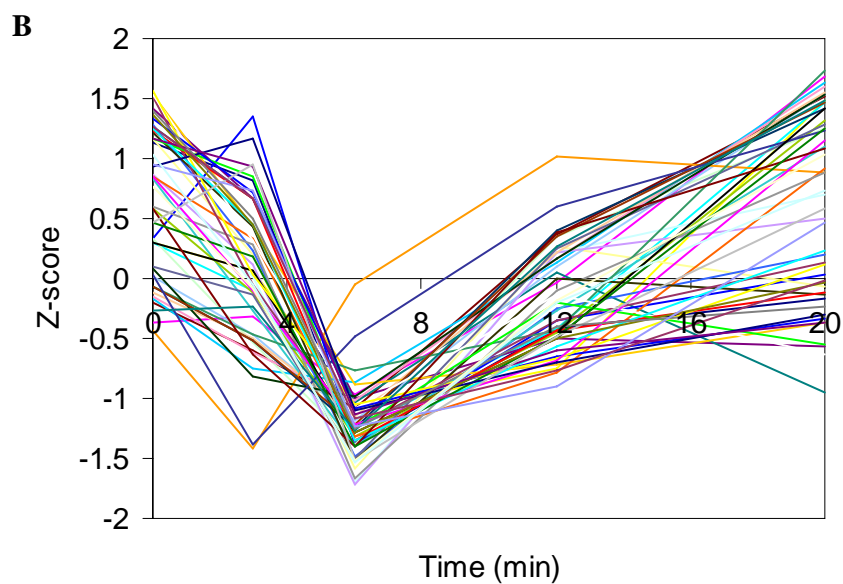
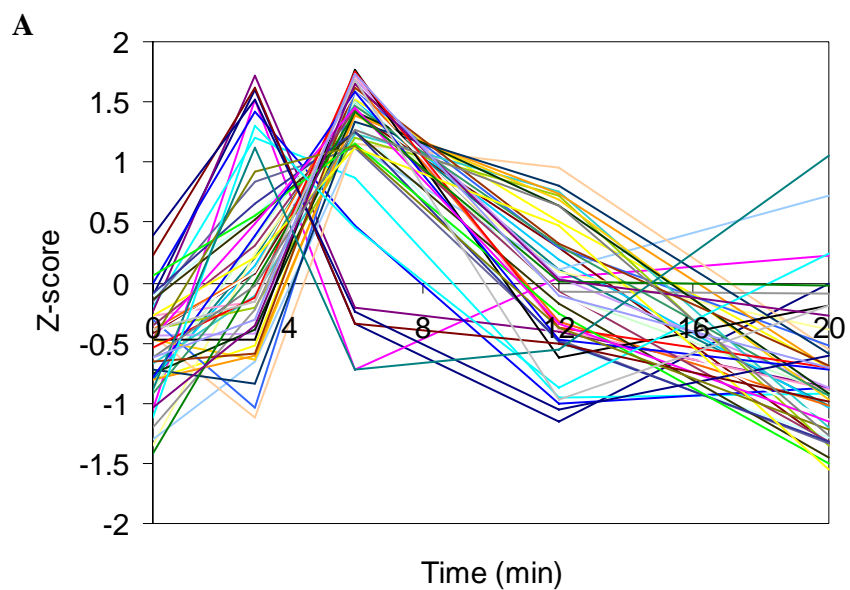


Figure 2.20. Genes that have transient early responses to oxidative stress. (A) Genes that were significantly induced by CHP within 6 minutes, but not after 6 minutes. The detailed information about these up-regulated genes is shown in Table 2.6. (B) Genes that were significantly repressed by CHP within 6 minutes, but not after 6 minutes. The detailed information about these down-regulated genes is shown in Table 2.7.

Table 2.6. Genes transiently induced by CHP within 6 minutes. These genes were significantly induced by CHP within 6 minutes, but not after 6 minutes.

A. Transcription factors

ORF	Gene	Description
YJR147W	<i>HMS2</i>	Protein with similarity to heat shock transcription factors
YIR017C	<i>MET28</i>	Transcriptional activator in the Cbf1p-Met4p-Met28p complex, participates in the regulation of sulfur metabolism
YIR018W	<i>YAP5</i>	Basic leucine zipper (bZIP) transcription factor
YPR196W	<i>MAL33</i>	nuclear protein (putative) /// MAL-activator protein, part of complex locus MAL3; nonfunctional in genomic reference strain S288C
YPR168W	<i>NUT2</i>	Component of the RNA polymerase II mediator complex, which is required for transcriptional activation and also has a role in basal transcription
YPR065W	<i>ROX1</i>	Heme-dependent repressor of hypoxic genes; contains an HMG domain that is responsible for DNA bending activity
YPR009W	<i>SUT2</i>	Putative transcription factor; multicopy suppressor of mutations that cause low activity of the cAMP/protein kinase A pathway; highly similar to Sut1p

B. Stress response or drug resistance

ORF	Gene	Description
YJL162C	<i>JJJ2</i>	Protein of unknown function, contains a J-domain, which is a region with homology to the E. coli DnaJ protein
YER038C	<i>KRE29</i>	Essential protein of unknown function; heterozygous mutant shows haploinsufficiency in K1 killer toxin resistance
YDL049C	<i>KNH1</i>	Protein with similarity to Kre9p, which is involved in cell wall beta 1,6-glucan synthesis; overproduction suppresses growth defects of a kre9 null mutant
YDL079C	<i>MRK1</i>	Glycogen synthase kinase 3 (GSK-3) homolog that function to activate Msn2p-dependent transcription of stress responsive genes and that function in protein degradation
YNL032W	<i>SIW14</i>	Tyrosine phosphatase that plays a role in actin filament organization and endocytosis; localized to the cytoplasm
YNL133C	<i>FYV6</i>	Protein of unknown function, required for survival upon exposure to K1 killer toxin; proposed to regulate double-strand break repair
YNL234W	---	Similar to globins and has a functional heme-binding domain; involved in glucose signaling or metabolism; regulated by Rgt1p
YIL171W	<i>HXT11</i> //	High-affinity hexose transporter
YGR035C	---	Protein of unknown function, potential Cdc28p substrate; transcription is activated by paralogous transcription factors Yrm1p and Yrr1p along with genes involved in multidrug resistance

Table 2.6. Genes transiently induced by CHP within 6 minutes. These genes were significantly induced by CHP within 6 minutes, but not after 6 minutes. (Continued)

C. Cell wall or actin cytoskeleton

ORF	Gene	Description
YHR101C	<i>BIG1</i>	Integral membrane protein of the endoplasmic reticulum, required for normal content of cell wall beta-1,6-glucan
YNL180C	<i>RHO5</i>	Non-essential small GTPase of the Rho/Rac subfamily of Ras-like proteins, likely involved in protein kinase C (Pkc1p)-dependent signal transduction pathway that controls cell integrity
YCL029C	<i>BIK1</i>	Microtubule-associated protein, component of the interface between microtubules and kinetochore, involved in sister chromatid separation; essential in polyploid cells but not in haploid or diploid cells
YOR284W	<i>HUA2</i>	Cytoplasmic protein of unknown function; computational analysis of large-scale protein-protein interaction data suggests a possible role in actin patch assembly
YPL250C	<i>ICY2</i>	Protein that interacts with the cytoskeleton and is involved in chromatin organization and nuclear transport, interacts genetically with TCP1 and ICY1; potential Cdc28p substrate

D. Sporulation

ORF	Gene	Description
YLL005C	<i>SPO75</i>	Meiosis-specific protein of unknown function, required for spore wall formation during sporulation; dispensable for both nuclear divisions during meiosis
YDR402C	<i>DIT2</i>	N-formyltyrosine oxidase, sporulation-specific microsomal enzyme required for spore wall maturation, involved in the production of a soluble LL-dityrosine-containing precursor of the spore wall, homologous to cytochrome P-450s
YNL210W	<i>MER1</i>	Protein with RNA-binding motifs required for meiosis-specific mRNA splicing; required for chromosome pairing and meiotic recombination

E. Ubiquitin

ORF	Gene	Description
YDL122W	<i>UBP1</i>	Ubiquitin-specific protease that removes ubiquitin from ubiquitinated proteins; cleaves at the C terminus of ubiquitin fusions irrespective of their size; capable of cleaving polyubiquitin chains
YPR169W	---	Nucleolar protein of unknown function, exhibits a physical interaction with Bre1p

Table 2.6. Genes transiently induced by CHP within 6 minutes. These genes were significantly induced by CHP within 6 minutes, but not after 6 minutes. (Continued)

F. Others

ORF	Gene	Description
YIL176C/// YGR294W	<i>PAU4</i> ///	Part of 23-member seripauperin multigene family encoded mainly in subtelomeric regions, active during alcoholic fermentation, regulated by anaerobiosis, negatively regulated by oxygen, repressed by heme
YEL046C	<i>GLY1</i>	Threonine aldolase, catalyzes the cleavage of L-allo-threonine and L-threonine to glycine; involved in glycine biosynthesis
YMR081C	<i>ISF1</i>	Serine-rich, hydrophilic protein with similarity to Mbr1p; overexpression suppresses growth defects of hap2, hap3, and hap4 mutants; expression is under glucose control; cotranscribed with NAM7 in a <i>cyp1</i> mutant
YMR177W	<i>MMT1</i>	Putative metal transporter involved in mitochondrial iron accumulation; closely related to Mmt2p
YOL010W	<i>RCL1</i>	RNA terminal phosphate cyclase-like protein involved in rRNA processing at sites A0, A1, and A2; does not possess detectable RNA cyclase activity
YHR049W	<i>FSH1</i>	Serine hydrolase that localizes to both the nucleus and cytoplasm; sequence is similar to Fsh2p and Fsh3p
YGR289C	<i>MAL11</i>	Maltose permease, inducible high-affinity maltose transporter (alpha-glucoside transporter); encoded in the MAL1 complex locus; member of the 12 transmembrane domain superfamily of sugar transporters
YKL198C	<i>PTK1</i>	Putative serine/threonine protein kinase that regulates spermine uptake; involved in polyamine transport; possible mitochondrial protein
YKL149C	<i>DBR1</i>	RNA lariat debranching enzyme, involved in intron turnover; required for efficient Ty1 transposition

G. Unknown function

ORF	Gene	Description
YLR287C	---	Hypothetical protein
YJR115W	---	Hypothetical protein
YJL218W	---	Hypothetical protein
YHR126C	---	Hypothetical protein
YGR031W	---	Hypothetical protein
YGL108C	---	Protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cell periphery
YDL183C	---	Hypothetical protein
YOR338W	---	Hypothetical protein
YOL015W	---	Hypothetical protein
YKR075C	---	Protein of unknown function; similar to YOR062Cp and Reg1p; expression regulated by glucose and Rgt1p
YIL166C	---	Hypothetical protein, member of the Dal5p subfamily of the major facilitator family

Table 2.7. Genes transiently repressed by CHP within 6 minutes. These genes were significantly repressed by CHP within 6 minutes, but not after 6 minutes.

A. DNA replication, cell growth and division

ORF	Gene	Description
YJR075W	<i>HOC1</i>	Alpha-1,6-mannosyltransferase involved in cell wall mannan biosynthesis; subunit of a Golgi-localized complex that also contains Anp1p, Mnn9p, Mnn11p, and Mnn10p; identified as a suppressor of a cell lysis sensitive <i>pkc1-371</i> allele
YKL092C	<i>BUD2</i>	GTPase activating factor for Rsr1p/Bud1p required for both axial and bipolar budding patterns; mutants exhibit random budding in all cell types
YAL040C	<i>CLN3</i>	G1 cyclin involved in cell cycle progression; activates Cdc28p kinase to promote the G1 to S phase transition; plays a role in regulating transcription of the other G1 cyclins, CLN1 and CLN2; regulated by phosphorylation and proteolysis
YDR247W	<i>VHS1</i>	Cytoplasmic serine/threonine protein kinase; identified as a high-copy suppressor of the synthetic lethality of a <i>sis2 sit4</i> double mutant, suggesting a role in G1/S phase progression; homolog of Sks1p
YPL219W	<i>PCL8</i>	Cyclin, interacts with Pho85p cyclin-dependent kinase (Cdk) to phosphorylate and regulate glycogen synthase, also activates Pho85p for Glc8p phosphorylation
YNL321W	---	Protein of unknown function, potential Cdc28p substrate
YJR065C	<i>ARP3</i>	Essential component of the Arp2/3 complex, which is a highly conserved actin nucleation center required for the motility and integrity of actin patches; involved in endocytosis and membrane growth and polarity
YOR039W	<i>CKB2</i>	protein kinase CK2, beta' subunit
YLR410W	<i>VIP1</i>	Protein of unknown function probably involved in the function of the cortical actin cytoskeleton; putative ortholog of <i>S. pombe asp1+</i>
YOL094C	<i>RFC4</i>	Subunit of heteropentameric Replication factor C (RF-C), which is a DNA binding protein and ATPase that acts as a clamp loader of the proliferating cell nuclear antigen (PCNA) processivity factor for DNA polymerases delta and epsilon

B. Transcription

ORF	Gene	Description
YER107C	<i>GLE2</i>	Component of the nuclear pore complex required for polyadenylated RNA export but not for protein import, homologous to <i>S. pombe Rae1p</i>
YDL070W	<i>BDF2</i>	Protein involved in transcription initiation at TATA-containing promoters; associates with the basal transcription factor TFIID; contains two bromodomains; corresponds to the C-terminal region of mammalian TAF1; redundant with Bdf1p
YNR023W	<i>SNF12</i>	73 kDa subunit of the 11-subunit SWI/SNF chromatin remodeling complex involved in transcriptional regulation; homolog of Rsc6p subunit of the RSC chromatin remodeling complex; deletion mutants are temperature-sensitive
YMR033W	<i>ARP9</i>	Actin-related protein involved in transcriptional regulation; subunit of the chromatin remodeling Snf/Swi complex

Table 2.7. Genes transiently repressed by CHP within 6 minutes. These genes were significantly repressed by CHP within 6 minutes, but not after 6 minutes. (continued)

C. Translation

ORF	Gene	Description
YJL138C	<i>TIF1</i> ///	Translation initiation factor eIF4A, identical to Tif2p; DEA(D/H)-box RNA helicase that couples ATPase activity to RNA binding and unwinding; forms a dumbbell structure of two compact domains connected by a linker; interacts with eIF4G /// Translation initiation factor eIF4A, identical to Tif1p; DEA(D/H)-box RNA helicase that couples ATPase activity to RNA binding and unwinding; forms a dumbbell structure of two compact domains connected by a linker; interacts with eIF4G
YNL306W	<i>MRPS18</i>	Mitochondrial ribosomal protein of the small subunit; essential for viability, unlike most other mitoribosomal proteins
YLR344W	<i>RPL26A</i> /	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl26Bp and has similarity to E. coli L24 and rat L26 ribosomal proteins; binds to 5.8S rRNA /// Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl26Ap and has similarity to E. coli L24 and rat L26 ribosomal proteins; binds to 5.8S rRNA
YMR242C	<i>RPL20A</i> /	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl20Bp and has similarity to rat L18a ribosomal protein /// Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl20Ap and has similarity to rat L18a ribosomal protein
YKL006W	<i>RPL14A</i> /	N-terminally acetylated protein component of the large (60S) ribosomal subunit, nearly identical to Rpl14Bp and has similarity to rat L14 ribosomal protein; rpl14a csh5 double null mutant exhibits synthetic slow growth /// Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl14Ap and has similarity to rat L14 ribosomal protein

D. Mitochondria

ORF	Gene	Description
YKL150W	<i>MCR1</i>	Mitochondrial NADH-cytochrome b5 reductase, involved in ergosterol biosynthesis
YJL003W	<i>COX16</i>	Mitochondrial inner membrane protein, required for assembly of cytochrome c oxidase
YCR028C-A	<i>RIM1</i>	Single-stranded DNA-binding protein that is essential for mitochondrial genome maintenance; Single-stranded zinc finger DNA-binding protein
YMR282C	<i>AEP2</i>	Mitochondrial protein, likely involved in translation of the mitochondrial OLI1 mRNA; exhibits genetic interaction with the OLI1 mRNA 5'-untranslated leader

Table 2.7. Genes transiently repressed by CHP within 6 minutes. These genes were significantly repressed by CHP within 6 minutes, but not after 6 minutes. (continued)

E. Vesicle trafficking

ORF	Gene	Description
YDR189W	<i>SLY1</i>	Hydrophilic protein involved in vesicle trafficking between the ER and Golgi; SM (Sec1/Munc-18) family protein that binds the tSNARE Sed5p and stimulates its assembly into a trans-SNARE membrane-protein complex
YKL176C	<i>LST4</i>	Protein possibly involved in a post-Golgi secretory pathway; required for the transport of nitrogen-regulated amino acid permease Gap1p from the Golgi to the cell surface
YAL042W	<i>ERV46</i>	Protein localized to COPII-coated vesicles, forms a complex with Erv41p; involved in the membrane fusion stage of transport

F. Unknown

ORF	Gene	Description
YLR225C	---	Hypothetical protein
YDR128W	---	Hypothetical protein
YDR063W	---	Hypothetical protein
YDL237W	---	Hypothetical protein
YPL184C	---	Hypothetical protein
YNR021W	---	Hypothetical protein
YMR247C	---	Hypothetical protein
YMR099C	---	Hypothetical protein
YLR094C	<i>G/S3</i>	Protein of unknown function

Table 2.7. Genes transiently repressed by CHP within 6 minutes. These genes were significantly repressed by CHP within 6 minutes, but not after 6 minutes. (continued)

G. Others

ORF	Gene	Description
YLR028C	<i>ADE16</i>	Enzyme of 'de novo' purine biosynthesis containing both 5-aminoimidazole-4-carboxamide ribonucleotide transformylase and inosine monophosphate cyclohydrolase activities, isozyme of Ade17p; ade16 ade17 mutants require adenine and histidine
YLR285W	<i>NNT1</i>	Putative nicotinamide N-methyltransferase, has a role in rDNA silencing and in lifespan determination
YKL039W	<i>PTM1</i>	Protein of unknown function
YKL057C	<i>NUP120</i>	Subunit of the Nup84p subcomplex of the nuclear pore complex (NPC), required for even distribution of NPCs around the nuclear envelope, involved in establishment of a normal nucleocytoplasmic concentration gradient of the GTPase Gsp1p
YKL126W	<i>YPK1</i>	Serine/threonine protein kinase required for receptor-mediated endocytosis; involved in sphingolipid-mediated and cell integrity signaling pathways; localized to the bud neck, cytosol and plasma membrane; homolog of mammalian kinase SGK
YKL152C	<i>GPM1</i>	Tetrameric phosphoglycerate mutase, mediates the conversion of 3-phosphoglycerate to 2-phosphoglycerate during glycolysis and the reverse reaction during gluconeogenesis
YDR155C	<i>CPR1</i>	Cytoplasmic peptidyl-prolyl cis-trans isomerase (cyclophilin), catalyzes the cis-trans isomerization of peptide bonds N-terminal to proline residues; binds the drug cyclosporin A
YBR199W	<i>KTR4</i>	Putative mannosyltransferase involved in protein glycosylation; member of the KRE2/MNT1 mannosyltransferase family
YNR041C	<i>COQ2</i>	Para hydroxybenzoate: polyprenyl transferase, catalyzes the second step in ubiquinone (coenzyme Q) biosynthesis
YNL091W	<i>NST1</i>	Protein of unknown function, mediates sensitivity to salt stress; interacts physically with the splicing factor Msl1p and also displays genetic interaction with MSL1
YNL085W	<i>MKT1</i>	Protein involved in propagation of M2 dsRNA satellite of L-A virus; forms a complex with Pbp1p that is involved in posttranscriptional regulation of HO endonuclease
YLR351C	<i>NIT3</i>	Nit protein, one of two proteins in <i>S. cerevisiae</i> with similarity to the Nit domain of NitFhit from fly and worm and to the mouse and human Nit protein which interacts with the Fhit tumor suppressor; nitrilase superfamily member
YLR354C	<i>TAL1</i>	Transaldolase, enzyme in the non-oxidative pentose phosphate pathway; converts sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate to erythrose 4-phosphate and fructose 6-phosphate
YOR086C	<i>TCB1</i>	Contains three calcium and lipid binding domains; green fluorescent protein (GFP)-fusion protein localizes to the cell periphery; C-terminal portion of Tcb1p, Tcb2p and Tcb3p interact
YJR126C	<i>VPS70</i>	Protein of unknown function involved in vacuolar protein sorting
YLR447C	<i>VMA6</i>	Subunit D of the five-subunit V0 integral membrane domain of vacuolar H ⁺ -ATPase (V-ATPase), an electrogenic proton pump found in the endomembrane system; stabilizes V0 subunits; required for V1 domain assembly on the vacuolar membrane

2.6. Conclusions

This study provided a wealth of information about the defense of the model eukaryote *S. cerevisiae* to the oxidative stress caused by CHP. In particular, I would like to stress the following novel observations: a) the dynamics of the antioxidant defense systems in response to CHP, b) a global identification of GO and KEGG pathways responding to oxidative stress, and c) the detection of genes that have a transient early response to oxidative stress.

The results of this study provide a dynamic resolution of the oxidative stress responses in *S. cerevisiae*, and contribute to a much richer understanding of the anti-oxidative defense system. It also raises many questions regarding the role and regulation of gene expression responses, and suggests hypotheses for the mechanisms the yeast *S. cerevisiae* employs to survive oxidative stress.

Many genes of unknown function were here identified to have roles in early oxidative stress response. A gene network study is being carried out for this data set. It may find possible correlations between these genes and genes with known functions. Future experiments are clearly needed to answer questions raised here, and to verify the functions of unknown genes detected in this study.

Proteomics data and metabolomics data are also being produced from this project. The combined analysis of these data with microarray data in the next step will provide an integrative view of cell physiology under oxidative stress.

Chapter 3. Oxidative stress response in *S. cerevisiae yap1Δ* mutant

3.1. Introduction

As discussed in section 2.5.2, yeast possesses diverse defense systems against oxidative stress. The main antioxidant systems of *Saccharomyces cerevisiae* are regulated at the transcription level (Jamieson and Storz, 1997). Yap1 is a member of the basic leucine zipper (bZIP) family of transcription factors, and is a functional homologue of mammalian AP-1 (Harshman, et al., 1988; Moyer-Rowley, et al., 1989). Yap1 is an important transcription factor that regulates antioxidant defense systems. The expression of many antioxidant genes is regulated by Yap1 triggered by oxidative stress conditions (Harbison, et al., 2004; Moyer-Rowley, 2003).

Yap1 activity is considered to be regulated mainly at the level of nuclear localization. Yap1 protein is normally distributed in both the cytoplasm and nucleus. It becomes highly concentrated in the nucleus under oxidative stress conditions (Kuge, et al., 1997). Yap1 has a nuclear export signal (NES) sequence in the C terminus. Crm1p exports Yap1p from the nucleus to the cytoplasm under normal conditions. Under oxidative stress conditions, ROS induce the formation of disulfide bonds between particular cysteine residues in Yap1p (Kuge, et al., 2001). The formation of these disulfide bonds results in a conformational change in Yap1p that masks its NES, as a consequence Yap1p is not easily transported from the nucleus to the cytosol, and becomes enriched in the nucleus. It is this elevated concentration of Yap1p that is believed to act as an inducer or repressor of its target genes (Kuge, et al., 1998; Yan, et al., 1998). As discussed in Chapter 2, the present study also revealed that *YAP1* is also regulated at the transcriptional level, becoming transiently up-regulated after addition of CHP.

In this study, we compared oxidative stress response in a *yap1Δ* mutant strain with the oxidative stress response in its corresponding genetic background strain (hereafter called “wild type”, even though it is not really a wild type yeast, but a common laboratory strain, BY4743). Detailed information about this strain was discussed in section 2.4 in chapter 2.

The purpose of this study is to identify 1) the *YAP1*-regulated component of the oxidative stress response, 2) the common genes and pathways that *yap1Δ* mutant and wild type use to respond to oxidative stress, and 3) genes and pathways that are only active in the *yap1Δ* mutant response to oxidative stress.

To the best of our knowledge, studies for the second and third purpose have never been reported previously. For the first point, several studies have already been reported, but as will be described, the present study provides additional information.

Harbison and coworkers carried out a Chromatin Immunoprecipitation chip (ChIP-chip) study to identify genes directly regulated by Yap1p in yeast under H₂O₂ induced oxidative stress (Harbison, et al., 2004). Essentially, this technique cross-links proteins to DNA, then subsequently the DNA is degraded, except the portions that had protein bound as it prevents DNases to cut; finally the proteins are hydrolyzed off the DNA and the remaining DNA sequences are hybridized against a chip that covers all of the genomic DNA sequence. This ChIP-chip technique is thus able to identify regions of genomic DNA that are transcription factor binding sites with direct physical evidence. In the Harbison et al. study samples were collected at 20 or 30 minutes after the addition of two different concentrations of H₂O₂. Because of the long incubation time, genes to which Yap1p was bound to transiently within 20 minutes will not have been detected in their study.

Cohen and coworkers used DNA microarrays to study the transcriptomics of *yap1Δ* and *yap2Δ* mutants under H₂O₂-induced oxidative stress (Cohen, et al., 2002). Their samples were collected 1 hour after the addition of H₂O₂. The early yap1p binding genes would also have been missed in their study. As reviewed by Moye-Rowley, the oxidative stress response is often rapid. Since the onset of oxidative stress can occur quickly, as during the oxidative burst (Babior, 2000), the response mechanisms of fungi must be similarly rapid (Moye-Rowley, 2003). The cell must be able to detect the altered redox balance, modulate appropriate transcription factors and then induce or repress the expression of relevant genes. As the earliest time point in our experiment was at 3 minutes after the

addition of CHP, our study allows a more complete identification of Yap1 regulated genes.

Harbison's and Cohen's studies (Cohen, et al., 2002; Harbison, et al., 2004) did not identify the genes regulated by Yap1 under normal growth conditions. Here I am able to identify many genes regulated by Yap1 under normal growth conditions by comparison with the wild type in the same condition, as well as those genes that are regulated by Yap1 under oxidative stress.

3.2. Experimental design

The design of the wild type experiment was discussed in section 2.2. The design of the *yap1Δ* experiment is exactly the same as for the wild type (Figure 2.2 in Chapter 2). The *yap1Δ* cell culture was divided into six fermentors; CHP was added to three fermentors to induce oxidative stress; ethanol (EtOH) was added to the other three fermentors that act as controls. EtOH was the solvent for CHP and that is why it is added to the controls. Samples were collected immediately (0 minutes) before the addition of CHP (or EtOH) and after it at the following time points: 3, 6, 12, 20, 40, 70 and 120 minutes. mRNA extracted from these samples was hybridized to Affymetrix Yeast Genome S98 arrays (Affymetrix CA, USA) for the measure of transcriptome.

This set of experiments was planned by Ana Martins, Pedro Mendes and Vladimir Shulaev. The experiments were performed by Ana Martins and Autumn Clapp, with the help of several people from the Shulaev, Mendes and Laubenbacher's research groups at VBI: Alberto de la Fuente, Beth Henry, Bharat Mehrotra, Brandylin Stigler, Diego Cortes, Diogo Camacho, Dustin Potter, Erica Mason, Hope Gruszewski, Jianghong Qian, Joel Shuman, Jiming Chia, Nigel Deighton, Wei Sha, Pedro Mendes, Reinhard Laubenbacher and Vladimir Shulaev.

The microarray data will be submitted to Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>).

3.3. Data analysis methods

I analyzed the wild type data and *yap1Δ* mutant data together in order to compare the oxidative stress response of wild type yeast with that of *yap1Δ* mutant yeast. In this combined data set, there are two genotypes (wild type and *yap1Δ*), two treatments (control and CHP), and five time points (0, 3, 6, 12 and 20 minutes). Only five of the eight time points were used, due to the artifacts in control samples discussed in section 2.4 in Chapter 2.

Robust Multichip Average (RMA) (Bolstad, et al., 2003; Irizarry, et al., 2003) was used for microarray data summarization and normalization. Note that this was carried out on the pool of 96 arrays that were used in all of the samples of the wild type and *yap1Δ*.

To assess the significance of the differences between transcripts from the same genotype across two different experimental conditions or between transcripts under the same experimental condition across two different genotypes, we built a 3-way ANOVA gene by gene model in SAS version 9 (SAS Institute Inc., Cary, NC, USA):

$$y_{ijkl} = \mu + T_i + V_j + M_k + (TV)_{ij} + (TM)_{ik} + (VM)_{jk} + (TVM)_{ijk} + \varepsilon_{ijkl} \quad (3.1)$$

where y_{ijk} is the intensity measured on the array for time i (in this case, $i=0, 2, \dots, 7$), treatment j (in this case, treatment is control or CHP), genotype k (in this case, genotype is wild type or *yap1Δ mutant*) and replicate l ; μ is the overall mean intensity of this gene; T_i is the effect of the i th time; V_j is the effect of the j th treatment; M_k is the effect of the k th genotype; $(TV)_{ij}$ is the interaction effect between time i and treatment j ; $(TM)_{ik}$ is the interaction effect between time i and genotype k ; $(VM)_{jk}$ is the interaction effect between treatment j and genotype k ; $(TVM)_{ijk}$ is the interaction effect between time i , treatment j and genotype k ; ε_{ijk} is the residual for time i , treatment j , genotype k and replicate l . The positive False Discovery Rate (pFDR, cutoff 0.05) multiple-testing adjustment (Storey and Tibshirani, 2003) was applied to correct p values.

To discover pathways that were significantly affected by CHP, pathway analysis was performed in Database for Annotation, Visualization and Integrated Discovery (DAVID) version 2 (<http://david.niaid.nih.gov/david/version2/index.htm>) (Dennis, et al., 2003), 2003). High-Throughput GoMiner (<http://discover.nci.nih.gov/gominer/>) was used to determine gene ontology categories that were significantly affected by CHP (Zeeberg, et al., 2003; Zeeberg, et al., 2005). pFDR was used as multiple-comparison correction method in these analyses (Storey and Tibshirani, 2003).

YEAST Search for Transcriptional Regulators And Consensus Tracking (YEASTRACT) (www.yeasttract.com) was used to find genes that are documented in the literature to be regulated by Yap1 and to find Yap1p binding sites in genes identified in this study (Teixeira, et al., 2006).

3.4. Results and discussions

3.4.1. *YAPI* was successfully knocked out in *yap1Δ* mutant yeast

As shown in Figure 3.1, the mRNA levels of *YAPI* in *yap1Δ* mutant control samples and *yap1Δ* mutant CHP treated samples were extremely low. This confirms that *YAPI* was successfully knocked out in the *yap1Δ* mutant strain used.

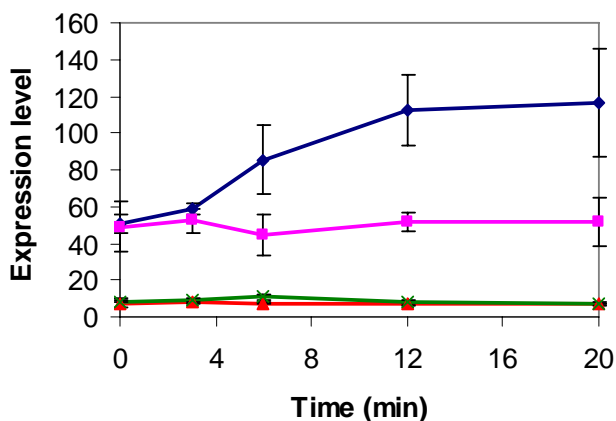


Figure 3.1. *YAPI* gene expression time series in wild type versus in *yap1Δ* mutant yeast. The *YAPI* gene expression time series for wild type control is in pink, for wild type CHP is in blue, for *yap1Δ* mutant control is in green, for *yap1Δ* mutant CHP is in red.

3.4.2. The transformation of CHP to COH in *yap1Δ* mutant yeast cell culture

Figure 3.2 illustrates that *yap1Δ* strain was not able to completely convert CHP to COH within 2 hours, as did the wild type strain (see Figure 2.3 in Chapter 2). This suggests that Yap1, or most likely its target genes, are important for a rapid reduction of CHP to COH. As discussed in section 3.1, the main antioxidant systems were documented to be regulated by Yap1. The deficient antioxidant systems in the *yap1Δ* strain could be responsible for the incomplete transformation of CHP to COH. One of the aims of this project is then to identify which components of the antioxidant system have been impaired by the knockout of this important (but not essential) transcription factor.

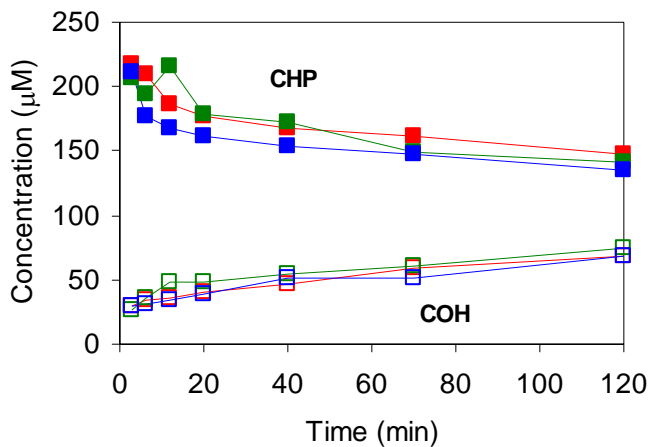


Figure 3.2. CHP/COH progress curve in *yap1Δ* mutant yeast cell culture. The concentration of CHP and COH in *yap1Δ* mutant yeast cell culture within 120 minutes after the addition of CHP. Three biological replicates are labeled in green, blue and red, respectively. These data were kindly supplied by Ana Martins.

3.4.3. Genes whose regulation by Yap1 is independent of stress

The first question that can be posed in terms of the effect of the knockout mutation of *YAP1* is which genes are affected under normal growth conditions (i.e. without oxidative stress)? I compared the data from the wild type control time series with those of the *yap1Δ* control at each corresponding time point. 37 genes were found that have higher

expression levels in the wild type than in *yap1Δ* mutant at least 4 out of the 5 time points (Table 3.1). This suggests that these genes are positively regulated by *YAP1* under unstressed conditions. 15 genes were also found to have lower expression levels in wild type control than in *yap1Δ* mutant control at least 4 of the 5 time points (Table 3.1). These genes are likely to be negatively regulated by Yap1 in normal conditions. It is not possible to infer from these data alone whether this regulation by Yap1 is directly by Yap1p or indirectly by a product of one of its target genes.

According to YEASTRACT (Teixeira, et al., 2006), which collects information on yeast transcription factors from the literature, 24 out of the 36 positively regulated genes identified here have never been documented to be regulated by Yap1 in previous publications. Among these 24 genes, 13 of them contain the Yap1p binding motif; 13 out of 15 negatively regulated genes were also not previously documented to be regulated by Yap1. Among them, 8 clearly have the Yap1p binding motif (Table 3.1).

Table 3.1. Genes whose regulation by Yap1 is independent of stress. Genes that were documented to be regulated by Yap1 are in green; genes that have the Yap1p binding motif but were not documented to be regulated by Yap1 are in blue.

	ORF/Genes
36 genes positively regulated by Yap1 under normal condition	<i>AAD10</i> , <i>AHP1</i> , <i>ARN2</i> , <i>BIO2</i> , <i>BIO3</i> , <i>BIO4</i> , <i>BIO5</i> , <i>BNA4</i> , <i>GPX2</i> , <i>GRE2</i> , <i>IZH2</i> , <i>IZH4</i> , <i>MRS4</i> , <i>NUP157</i> , <i>OLE1</i> , <i>PHO11</i> , <i>PHO5</i> , <i>PHO84</i> , <i>PHO89</i> , <i>PRD1</i> , <i>PRX1</i> , <i>ROX1</i> , <i>SPL2</i> , <i>SRO77</i> , <i>TRR1</i> , <i>TRX2</i> , <i>TSA1</i> , <i>VHT1</i> , <i>VTC2</i> , <i>VTC3</i> , <i>VTC4</i> , <i>YGL039W</i> , <i>YJR154W</i> , <i>YLR108C</i> , <i>YML131W</i> , <i>YNL134C</i>
15 genes negatively regulated by Yap1 under normal condition	<i>AAC3</i> , <i>ANB1</i> , <i>COX19</i> , <i>COX5B</i> , <i>FET4</i> , <i>FRT2</i> , <i>HEM13</i> , <i>HOR7</i> , <i>LAC1</i> , <i>MLH2</i> , <i>PRM9</i> , <i>WSC4</i> , <i>YLR407W</i> , <i>YML6</i> , <i>YMR278W</i>

Genes that were positively regulated by *YAP1* under normal growth conditions mainly belong to the following functional classes: oxidative stress response (*TRX2*, *GPX2*, *PRX1*, *TSA1*, *AHP1*, *GRE2*), biotin biosynthesis (*BIO2*, *BIO3*, *BIO4*, *BIO5*), phosphate transport (*PHO11*, *PHO5*, *PHO89*, *PHO84*), vacuole fusion (*VTC2*, *VTC3*, *VTC4*) and zinc ion homeostasis (*IZH2*, *IZH4*). Genes that were negatively regulated by Yap1 under

normal growth conditions mainly belong to cellular respiration (*COX5B*, *AAC3*), response to stress (*MLH2*, *HOR7*, *FRT2*, *WSC4*) and metal ion transport (*FET4*, *COX19*). Thus, Yap1 may repress as well as activate stress responding genes under unstressed conditions.

Another interpretation could be assigned to the fact that these genes have different expression levels in unstressed conditions in the wild type and *yap1Δ*. It could be that some of these genes are induced/repressed to compensate for the action of other genes that have been silenced/activated by the lack of Yap1 (pleiotropic effects).

3.4.4. Yap1 regulated oxidative stress responses

3.4.4.1. Genes and pathways positively regulated by Yap1 in yeast under oxidative stress

A comparison of the gene expression response induced by CHP in the wild type and *yap1Δ* strains reveals 619 genes that were significantly induced in the wild type but not in the *yap1Δ* mutant (within 20 minutes of the induction). Our study suggests that these genes may be positively regulated by Yap1, even though this could be either direct or indirect regulation. According to YEASTRACT (Teixeira, et al., 2006), 96 of these genes have already been documented to be regulated by Yap1 (Table 3.2); 308 of these genes have the Yap1p binding motif, which suggests direct regulation by Yap1p, but this regulation have not been documented (Table 3.2). The KEGG pathways that were significantly up-regulated in response to CHP in the wild type but not in *yap1Δ* are glutathione metabolism, the proteasome, starch and sucrose metabolism with $p < 0.01$ (Table 3.3A). Genes on these pathways could be positively regulated by Yap1. Galactose metabolism was also found to be positively regulated by Yap1, with p -value 0.021. An analysis based on the Gene Ontology (GO) classification is in agreement with the analysis based on the KEGG pathways (Table 3.3). GO analysis also indicates that some genes in signal transduction are regulated by Yap1 (Table 3.3B). These genes are likely downstream in signal transduction cascade triggered by Yap1 under oxidative stress.

Table 3.2. Genes positively regulated by Yap1 under oxidative stress condition. Among 619 genes that were identified in our study to be positively regulated by Yap1 under oxidative stress condition, 97 of them have been documented in the literature to be regulated by Yap1. 308 of them have not been explicitly documented to be regulated by Yap1, but yet have a recognizable Yap1 binding motif upstream their coding region. Genes belonging to glutathione metabolism are labeled in pink.

	ORF/Genes
96 genes were documented to be regulated by Yap1p	<i>AAD15, AAD16, AAD4, AAD6, AFG1, AGP3, AIF1, AVT6, BCH1, BRO1, CAF17, CCPI, CTA1, CTR2, CYC1, CYS3, CYT2, DCG1, DDII, DPL1, ECM4, EHD3, END3, FAR8, FLR1, FMP22, FRM2, FSP2, GDH2, GLR1, GPT2, GPX2, GRX2, GSH1, GSH2, GTT2, HOR2, HSP12, HSP82, HSV2, HXT9, MAK10, MDG1, MMT1, MPD1, NDE2, OCHI, OYE2, OYE3, PGM2, PRE8, PST2, RAD1, RAD4, ROM2, RPM2, RPN4, RTG2, SFAI, SOD1, SOD2, SPT20, SRX1, TAH18, TCB2, TRR1, TRR2, VID27, YBR285w, YCF1, YCR102c, YDR132c, YGR127w, YGR130c, YIL108w, YIL168w, YIL171w, YJL057c, YJL103c, YKL070w, YKL071w, YKL107w, YKL187c, YLL054c, YLR108c, YLR241w, YLR297w, YLR460c, YML131w, YMR196w, YNL176c, YNL274c, YPR1, YRR1, YTP1, ZRG8</i>
308 genes were not documented to be regulated by Yap1p, but have the Yap1 binding sites	<i>ABF2, ABZ1, ACF2, ACN9, ACP1, ADY3, AFRI, AHC1, ALD2, ALG13, ALO1, ARO80, ARO8, ARP2, AS11, ATG29, ATH1, AVO2, BLM10, BNA3, CAP2, CBP3, CDC34, CFD1, CIS1, CIT3, CK11, CMK1, COQ3, COQ4, COS4, COX14, COX15, COX16, CRT10, CTI6, CWH41, CYC3, CYR1, DAN4, DDP1, DFG5, DIG2, DNMI, DOA4, DOT5, EMI5, ERV1, ERV41, ETR1, FAD1, FAR11, FET5, FMP24, FMP27, FMP34, FMP39, FMP46, FRE1, FZFI, FZO1, GAC1, GDB1, GDE1, GDII, GIP2, GLC3, GND2, GPH1, GRH1, GRX5, GSP2, GSY2, GTT1, GUT1, HAL1, HMS2, HPA2, HRD1, HRD3, HRR25, HSF1, HULA, IML2, ISA2, ISN1, KIN1, KSS1, LAP3, LDB17, LDB18, LEE1, LGE1, LIP1, LSB3, LSB6, LYSS, MAD2, MAG1, MAM3, MDL2, MDM12, MET1, MET8, MIA40, MON2, MOT2, MRK1, MRL1, MRP17, MRP49, MRPL13, MSB3, MSF1, MSN5, MSS18, MXR1, NAS6, NCRI, NFS1, NGRI, NIT1, NMA2, NPL4, NPR1, NSG2, NTH2, OAF1, OSH2, OSH6, PAPI, PBS2, PCMI, PCP1, PDR1, PET8, PEX2, PEX30, PIMI, PPZ2, PRE3, PRE4, PRE5, PRP12, PRR2, PSK1, PTM1, PUP1, PUP2, RAD2, RAD52, RBK1, REG2, RIB1, RIM15, RNR2, RNY1, ROD1, ROM1, ROT1, RPN10, RPN7, RPN8, RPN9, RPT3, RPT6, RRD1, RRI2, RSB1, RVS167, SAC6, SDS22, SEC17, SEC9, SED4, SEM1, SEO1, SFH5, SHE10, SHP1, SHU1, SIP2, SLA2, SLF1, SLG1, SLM5, SNC1, SNC2, SNZ2, SRL3, SSL2, SUL1, SWC4, SWC5, SWRI, SYPI, TCM10, TGL2, THI6, TLG2, TMA17, TOS8, TPD3, TPKE1, TRE2, TVP23, UBA1, UBP6, UBR2, UBX4, UFD1, VAM6, VPS35, VPS55, VPS68, VPS70, WHI2, WWM1, XDJ1, XKS1, XYL2, YAL049c, YAP7, YBR014c, YBR053c, YBR225w, YBR284w, YCL042w, YCP4, YDL027c, YDL119c, YDL183c, YDL199c, YDL206w, YDR248c, YEL020c, YER066w, YER067w, YER093c-a, YFR042w, YGL114w, YGL250w, YGR201c, YGR266w, YHL010c, YHR122w, YHR140w, YHR192w, YIMI, YIR016w, YIR043c, YJL016w, YJL068c, YJL163c, YJL185c, YJR085c, YJR107w, YKL091c, YKL100c, YKL121w, YKL171w, YKR018c, YKT6, YLL064c, YLR001c, YLR046c, YLR132c, YLR152c, YLR199c, YLR218c, YLR247c, YLR287c, YLR312c, YLR356w, YLR408c, YMR086w, YMR099c, YMR124w, YMR210w, YMR244c-a, YMR258c, YMR262w, YMR31, YMR325w, YNL213c, YNR014w, YOL029c, YOL053w, YOL159c, YOR022c, YOR059c, YOR097c, YOR111w, YORI, YOR227w, YOR285w, YOR352w, YPK2, YPL109c, YPL113c, YPL230w, YPL260w, YPL277c, YPR118w, YPR127w, YPR148c, YPR196w, YTA12</i>

Table 3.3. Pathways positively regulated by Yap1 under oxidative stress condition. These KEGG pathways and GO Slim categories are significantly represented in the group of genes that were significantly induced by CHP in wild type within 20 minutes, but not induced by CHP in *yap1Δ* mutant within 20 minutes. *P*-value cutoff was set at 0.01.

A. KEGG pathways

KEGG term	<i>p</i> -value
Proteasome	1.81E-17
Starch and sucrose metabolism	0.001252
Glutathione metabolism	0.002727

B. GO Slim categories

GO Slim term	<i>p</i> -value
Protein catabolism	3E-10
Carbohydrate metabolism	0.001109
Response to stress	0.005797
Signal transduction	0.008587

a) **Glutathione metabolism**

Our results show that all of the genes in glutathione metabolism are regulated by Yap1 directly or indirectly (Figure 3.3). Among them, *GSH1*, *GSH2*, *GLR1* and *GPX2* have been well known to be regulated by Yap1 (Grant, et al., 1996; Sugiyama, et al., 2000; Tsuzi, et al., 2004; Wu and Moye-Rowley, 1994). Our results are in agreement with these documentations (Table 3.2). Our results also show that the response of *GPX3* to oxidative stress is regulated by Yap1. There is no recognizable Yap1 binding motif within the first 1000 nucleotides upstream of the *GPX3* coding sequence. Perhaps the binding site is further away from the coding sequence or the regulation of this gene is not directly by Yap1p, but rather indirectly through a signaling cascade. The glutathione S-transferases (*GTT1* and *GTT2*), are also part of glutathione metabolism (as shown in Figure 2.4 of Chapter 2) but are not included in the KEGG glutathione metabolism pathway map. Our results show that both *GTT1* and *GTT2* are also regulated by Yap1. Yap1 regulation of *GTT2* was already reported in the work of DeRisi and collaborators (DeRisi, et al., 1997). *GTT1*, while having a Yap1p binding site, had not yet been explicitly shown to be regulated by Yap1.

b) **Proteasome**

Our results show that the expression of proteasome subunits is regulated by Yap1 (Table 3.3 and Figure 3.4). As discussed in Chapter 2, the ubiquitin-mediated proteasome

degradation pathway provides a mechanism to remove proteins damaged by oxidative stress to prevent adverse consequences to cell maintenance under oxidative stress. Rpn4 is the transcription activator of the proteasome subunit genes. Rpn4 is required for normal expression levels of proteasome subunits (Xie and Varshavsky, 2001). Rpn4 is an extremely short-lived protein, and itself is degraded by the 26S proteasome (Xie and Varshavsky, 2001). *RPN4* expression has been shown to be regulated by transcription factors Pdr1, Pdr3 and Yap1 (Owsianik, et al., 2002). Owsianik and coworkers showed that Yap1p response element is important in the trans-activation of *RPN4* by Yap1p (Owsianik, et al., 2002). Thus, it is not surprising that *RPN4*, and genes encoding proteasome subunits regulated by Rpn4p did not respond to oxidative stress in the *yap1Δ* mutant strain. Our results also show that *PDR1* is also regulated by Yap1, and indeed *PDR1* has the Yap1p binding motif. No direct evidence for the regulation of *PDR1* by Yap1 had yet been reported previously. *PDR3* were not differentially expressed in wild type and *yap1Δ* mutant, which suggests the regulation of Pdr3 under oxidative stress may not be at the transcriptional level.

c) **Starch, sucrose, and general carbohydrate metabolism**

Carbohydrates are a major category of compatible solutes. Compatible solutes are stabilizers of biological structures. They provide protection from extreme environmental conditions, including heat, UV radiation or dryness. These compatible solutes can accumulate to high levels without disturbing intracellular biochemistry (Bohnert and Jensen, 1996). Compatible solutes, such as hexoses (mostly fructose and glucose), disaccharides (sucrose, trehalose) and sugar alcohols (inositol, mannitol) have been earlier observed to accumulate during stress conditions (Bohnert and Jensen, 1996; Jouve, et al., 2004). An adaptive biochemical function of compatible solutes is the scavenging of ROS. It was suggested that trehalose enhances resistance to oxidative stress by quenching oxygen radicals (Benaroudj, et al., 2001). Mannitol and galactose were shown to scavenge hydroxyl radicals *in vitro* (Litchfield and Wells, 1978; Tauber and Babior, 1977). The present results show that starch and sucrose metabolism is regulated by Yap1 under oxidative stress (Table 3.3 and Figure 3.5). Galactose metabolism is also regulated by Yap1, though with a weaker *p*-value of 0.021. Gounalaki

and coworkers found that the expression of trehalose-6-phosphatase (*TPS2*), which catalyzes the second step of trehalose biosynthesis, requires Yap1 (Gounalaki and Thireos, 1994). However, in our experimental results the expression of *TPS2* was independent of Yap1 (i.e. it was similar in the wild type and mutant). Other genes encoding enzymes of trehalose metabolism were induced by CHP in both the wild type and *yap1Δ* mutant, suggesting that this pathway is independent of Yap1.

d) Response to stress

57 stress responding genes were identified in this study to be positively regulated by Yap1 under oxidative stress condition (Table 3.4).

Table 3.4. Stress responding genes positively regulated by Yap1. Documented regulation is indicated in green. Genes that have Yap1p binding motifs but had not yet been experimentally shown to be activated by Yap1 are in blue.

GO Slim category	Genes annotated to the category
response to stress	<p><i>VRP1</i>, <i>SSK1</i>, <i>CCP1</i>, <i>SRX1</i>, <i>SOD1</i>, <i>CYR1</i>, <i>PRE3</i>, <i>HYR1</i>, <i>XPB1</i>, <i>IMP2</i>, <i>RRD1</i>, <i>SSL2</i>, <i>TRR2</i>, <i>SOD2</i>, <i>SHU1</i>, <i>RAD2</i>, <i>PUP2</i>, <i>HSF1</i>, <i>SIP2</i>, <i>HSP12</i>, <i>RIM15</i>, <i>RAD4</i>, <i>MAG1</i>, <i>HOR2</i>, <i>MXR1</i>, <i>PRE1</i>, <i>RAD23</i>, <i>GRX2</i>, <i>RVS167</i>, <i>DPL1</i>, <i>AHA1</i>, <i>SAC6</i>, <i>MRK1</i>, <i>GPX2</i>, <i>UMP1</i>, <i>NTH2</i>, <i>PIM1</i>, <i>ATH1</i>, <i>LSP1</i>, <i>HAL1</i>, <i>RAD1</i>, <i>GRX5</i>, <i>GLR1</i>, <i>PEP4</i>, <i>HRR25</i>, <i>OXR1</i>, <i>HSP82</i>, <i>GAC1</i>, <i>WHI2</i>, <i>SLG1</i>, <i>AIF1</i>, <i>YIM1</i>, <i>AIPI</i>, <i>RAD52</i>, <i>UFO1</i>, <i>ALO1</i>, <i>ROM2</i></p>

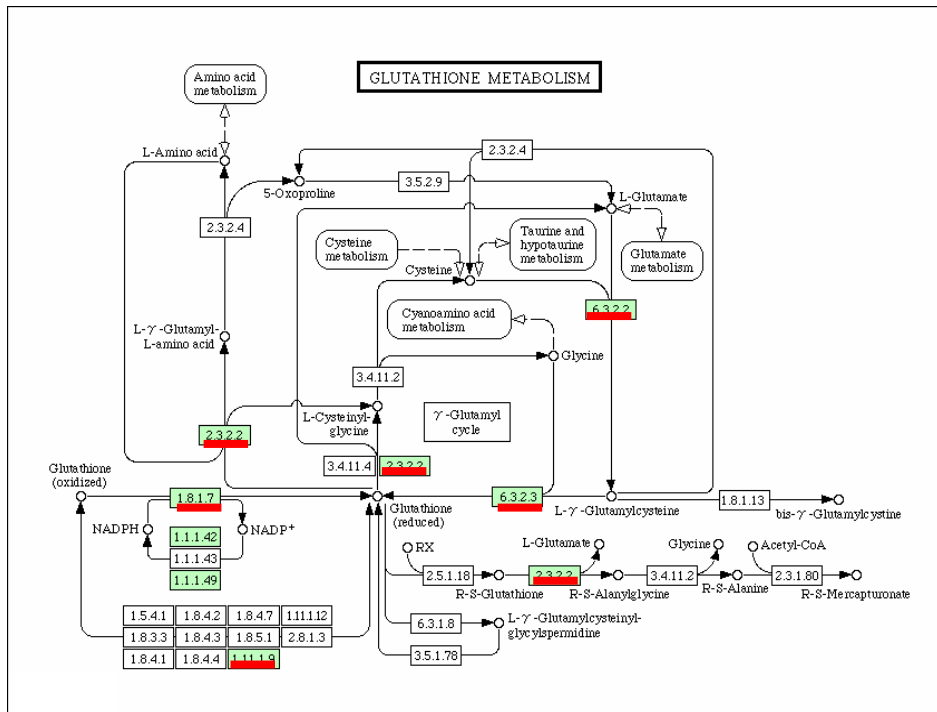


Figure 3.3. Genes in glutathione metabolism (from KEGG) positively regulated by Yap1. Green boxes indicate genes that were identified in yeast. Red underlining indicates genes that were significantly induced by CHP in wild type within 20 minutes, but not in the *yap1Δ* mutant within 20 minutes.

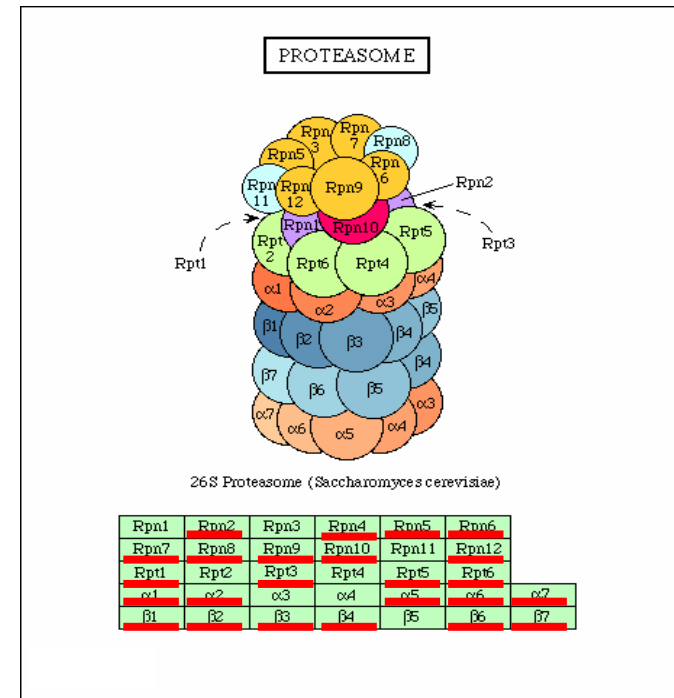


Figure 3.4. Proteasome (from KEGG) genes positively regulated by Yap1. Green boxes indicate genes that were identified in yeast. Red underlining indicates genes that were significantly induced by CHP in wild type within 20 minutes, but not in the *yap1Δ* mutant within 20 minutes.

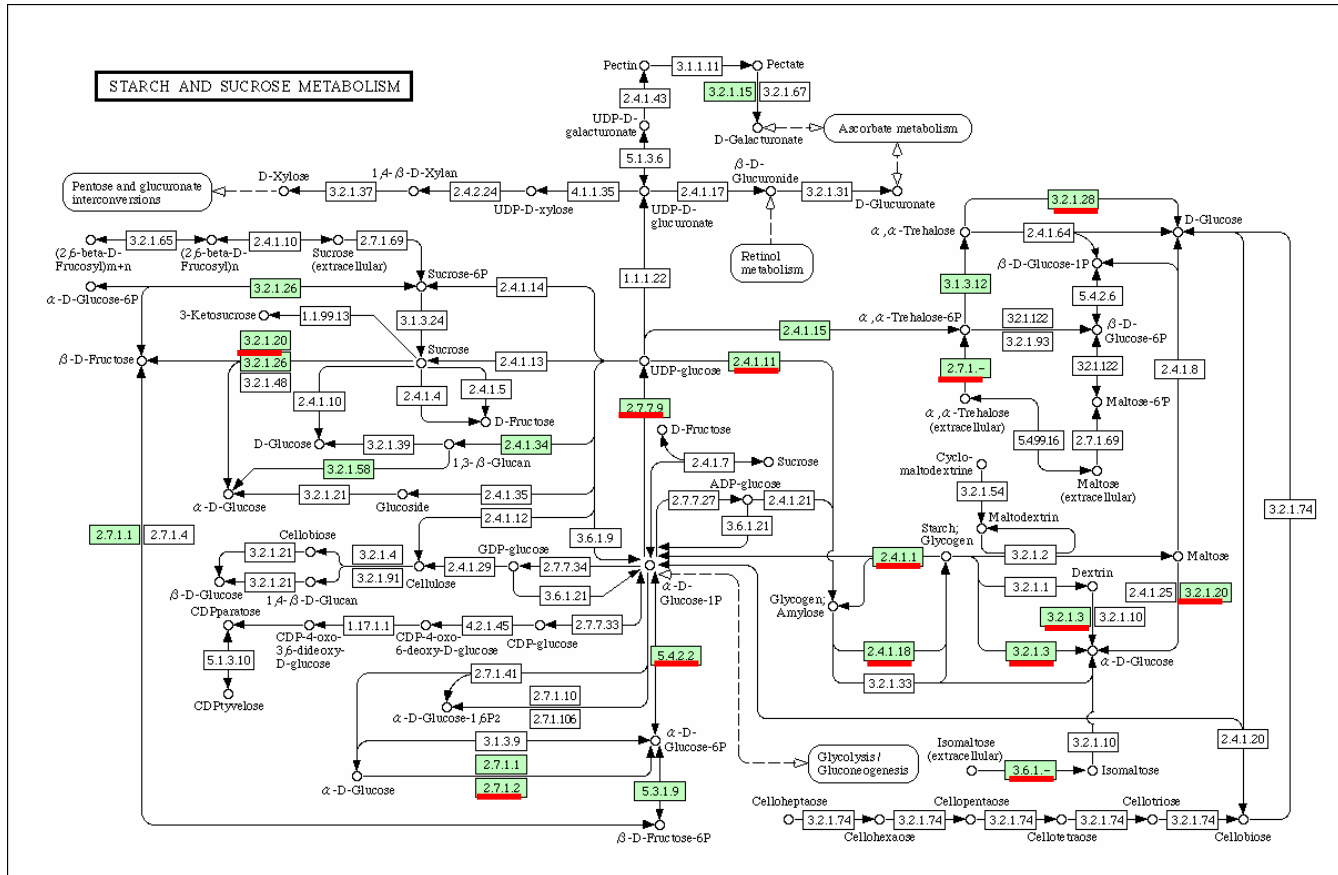


Figure 3.5. Genes in starch and sucrose metabolism (from KEGG) positively regulated by Yap1. Green boxes indicate genes that were identified in yeast. Red underlining indicates genes that were significantly induced by CHP in wild type within 20 minutes, but not in the *yap1Δ* mutant within 20 minutes.

3.4.4.2. Genes and pathways negatively regulated by Yap1 in yeast under oxidative stress

Our results suggest that Yap1 can also act as a negative regulator. 489 genes were found to be significantly repressed after exposure to CHP in wild type within 20 minutes, but not in the *yap1Δ* mutant. These genes are possibly negatively regulated by Yap1 under oxidative stress condition, and this regulation can be either directly through binding of Yap1p or indirectly by products of genes that are themselves regulated by Yap1. According to YEASTRACT (Teixeira, et al., 2006), 33 of these genes had already been documented in the literature to be regulated by Yap1 (Table 3.6). 269 of them contain Yap1p binding motifs but were not yet shown experimentally to be regulated by Yap1 (Table 3.6).

The ribosome and RNA polymerase genes are significantly represented in the group of genes that were found to be negatively regulated by Yap1 under oxidative stress condition (Table 3.5). The negative regulation of Yap1 on ribosome and RNA polymerase under oxidative stress condition had not been reported previously.

Table 3.5. Pathways negatively regulated by Yap1 under oxidative stress condition. These KEGG pathways and GO Slim categories are significantly represented in the group of genes that were significantly repressed by CHP in wild type within 20 minutes, but not repressed by CHP in *yap1Δ* mutant within 20 minutes. *P*-value cutoff was set at 0.01.

A. KEGG pathways

KEGG term	<i>p</i> -value
Ribosome	9.81E-06
RNA polymerase	0.00966

B. GO Slim categories

GO term	<i>p</i> -value
Ribosome biogenesis and assembly	9.62E-24
RNA metabolism	2.52E-11
Protein biosynthesis	0.001911

a) Ribosome

As shown in Figure 3.6, genes encoding 26 ribosomal proteins (RPs) were significantly repressed by CHP in wild type yeast, but not in *yap1Δ* mutant yeast. According to GO

analysis, 103 genes involved in ribosome biogenesis and assembly were repressed in wild type but not in *yap1Δ* mutant yeast.

In *S. cerevisiae*, the decision to commit to new ribosome synthesis is thought to be controlled primarily at the transcription level (Warner and Gorenstein, 1977). The cost of ribosome biosynthesis is very high both in terms of energy and building materials. In rapidly growing yeast cells, 50% of RNA polymerase II transcription and 90% of mRNA splicing are dedicated to ribosomal protein expression (Warner, 1999). To save resources under starvation or stress conditions, the regulation of rRNA genes and ribosomal protein genes is essential for the economy of the cell. In microorganisms, synthesis of ribosome has been linked to nutrient availability and stress-related signals (Warner, et al., 2001). A rapid and temporary repression of RP genes was observed in yeast under mild heat shock conditions (Eisen, et al., 1998; Warner and Gorenstein, 1977).

It has become clear in recent years that the TOR kinases play an important regulatory role in the induction of RP genes (Jacinto and Hall, 2003). Transcription factor Fhl1 in association with its coactivator Ifh1, and transcription factor Sfp1, interact with RP gene promoters to stimulate the expression of RP genes. TOR kinase acts to prevent escape of Sfp1 from the nucleus. In our data, the repression of *IFH1* was observed in both wild type and *yap1Δ* mutant. Other genes mentioned above on the TOR signaling network were not found to be repressed in either the wild type or *yap1Δ* mutant. Even though these results strongly suggest that Yap1 is needed for the RP response to oxidative stress, the interaction of Yap1 with the TOR signaling network remains to be explained. It seems that this interaction does not occur at the level of transcription, though.

b) RNA polymerase

It is not surprising to see that some genes encoding RNA polymerase were repressed when genes encoding RPs were repressed, since 50% of RNA polymerase II transcription is devoted to RPs (Warner, 1999), and new data also suggested that RNA polymerase I plays an important role in ribosome production (Jacinto and Hall, 2003). The role that Yap1 plays in the repression of RNA polymerase genes is not clear.

Table 3.6. Genes negatively regulated by Yap1 under oxidative stress condition. Among 489 genes that were identified in our study to be negatively regulated by Yap1 under oxidative stress conditions, 33 of them have been documented previously to be regulated by Yap1. 269 of them were not yet shown to be regulated by Yap1, but do have the Yap1 binding motif.

	ORF/Genes
33 genes were documented to be regulated by Yap1p	<i>ADE8, ASN1, BUD20, CAN1, CLB1, COX6, DBP2, FET3, FET4, HIS4, HXT4, ILV3, ILV5, LCB3, LEU9, MAE1, MCH5, MDH2, MET7, NNT1, NUP53, OPT2, RHO5, RIP1, ROX3, RPL17b, RPS26b, RSF2, SSU1, STP4, TAL1, YIP1, YMR247c</i>
269 genes were not documented to be regulated by Yap1p, but have the Yap1 binding sites	<i>AAC3, ADE12, AIR1, ALA1, ALT2, ARG3, ARO7, ASC1, ATC1, ATO3, ATP20, BCD1, BCPI1, BMS1, BUD21, BUD23, CBF1, CBF5, CIC1, CIN2, COR1, COX12, COX16, COX4, COX9, CPA1, CSL4, CTK2, DBP10, DBP3, DED1, DEG1, DFR1, DIM1, DIP5, DIS3, DMA2, DML1, DOM34, DPH5, DRS1, DSE1, DSE2, DSE3, DUS3, EBS1, ENP2, ERG2, FAF1, FAL1, FAP1, FAS1, FCY2, FKS1, FMP38, FUN30, FUS2, FYV7, GCD7, GCN3, GIM3, GIM5, GIS2, GLN1, GPD2, GPM1, GPM3, GRX4, GTT3, GUA1, HAS1, HEM1, HHT1, HIS1, HIS6, HIT1, HMS2, HOC1, HPR5, HTA2, IMP4, INM1, INO2, ISA1, JEM1, JIP5, JJJ3, KRE33, KRI1, KTI12, LCP5, LHP1, LSG1, LTV1, LYS21, MAK5, MED7, MIS1, MNN1, MPP10, MSH1, MTR3, NAF1, NHP2, NIT3, NMD3, NOB1, NOC2, NOP1, NOP2, NOP8, NOP9, NSA1, NSA2, NSP1, NUP145, NUP192, NUP1, NUP2, OAC1, OPI3, OPT1, PCL9, PEX21, PHO13, PIS1, PKR1, PMT5, PNO1, POP1, POP4, PPM2, PPT1, PRP24, PRS2, PRS3, PRS4, PUF6, PUS1, PWP1, PXR1, QCR7, RBG2, RER2, RFC4, RFC5, RIA1, RIO1, RKI1, RLP24, RMA1, RNH202, RNH203, RPA135, RPA190, RPB10, RPB5, RPC17, RPC37, RPC40, RPF1, RPG1, RPL14a, RPL16a, RPL21b, RPL31b, RPL33b, RPL40b, RPL7b, RPP1, RPS0b, RPS14b, RPS1a, RPS29a, RPS30b, RPS7a, RRB1, RRP12, RRP14, RRP15, RRP3, RRP42, RRP46, RRS1, RSA3, SAS10, SCP160, SDH3, SEC39, SFH1, SGD1, SHE2, SKI6, SLY41, SME1, SMF3, SMII, SPB4, SQT1, SRP21, SSF1, SSZ1, STB6, SXM1, SYG1, SYS1, TAD3, TEA1, THI80, THS1, TIF3, TIF4631, TMA16, TMA46, TPD3, TPO3, TRM112, TRM8, TSRI, URA4, URB2, USA1, UTP10, UTP11, UTP14, UTP20, UTP22, UTP5, WRS1, YAH1, YBL028c, YBR141c, YCL002c, YDL121c, YDR222w, YDR341c, YDR444w, YEF3, YGR093w, YHR020w, YHR032w, YHR100c, YIL083c, YIL110w, YJR061w, YJR119c, YJR129c, YLR051c, YLR243w, YLR278c, YLR407w, YLR419w, YMC2, YML096w, YML108w, YMR099c, YMR269w, YNL034w, YNL050c, YNL056w, YNR018w, YOL125w, YOR021c, YOR051c, YOR166c, YOR246c, YOR302w, YPR174c, ZRT1</i>

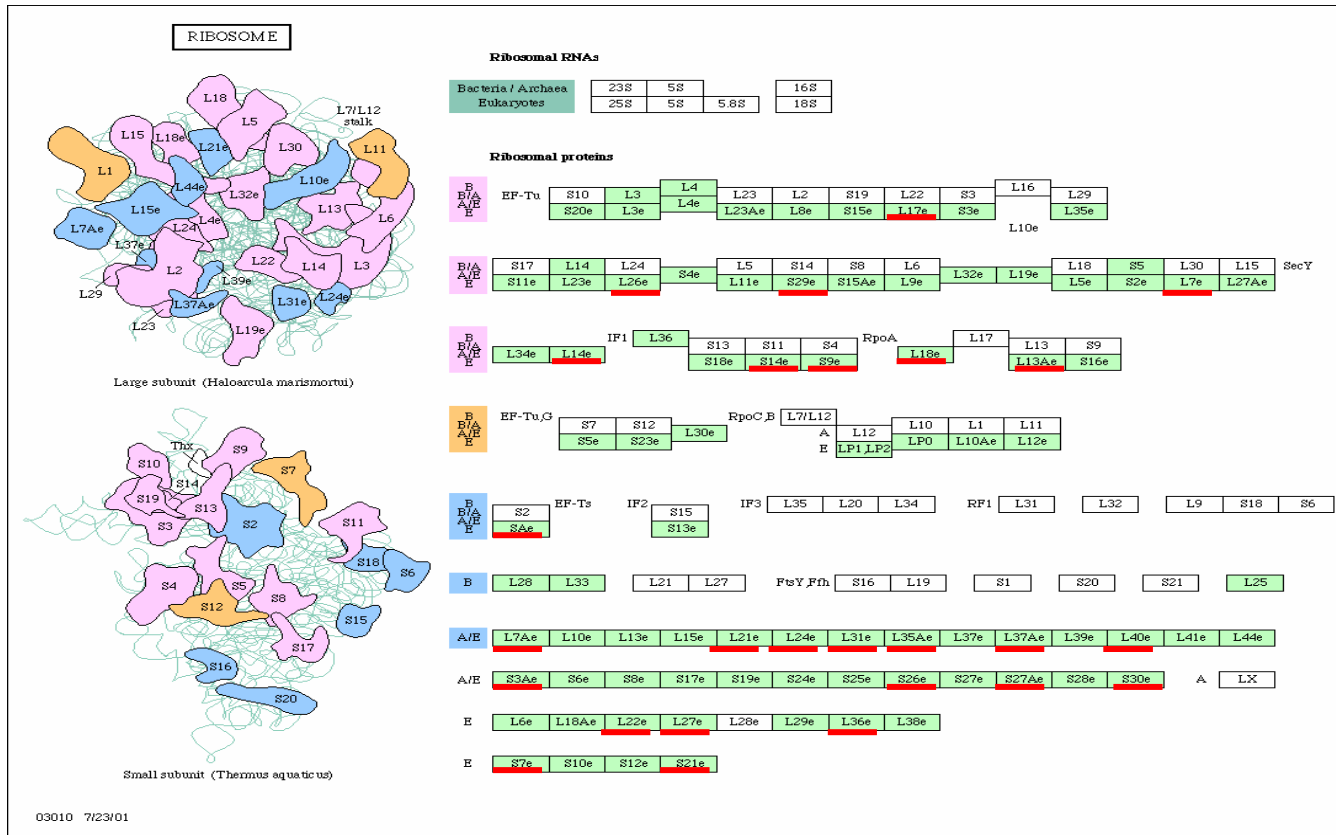


Figure 3.6. Ribosome (from KEGG) genes negatively regulated by Yap1 under oxidative stress condition. Green boxes indicate genes that were identified in yeast. Red underlining indicates genes that were significantly repressed by CHP in wild type within 20 minutes, but not in the *yap1Δ* mutant within 20 minutes.

3.4.5. Common oxidative stress responding genes in wild type and *yap1Δ* mutant yeast

596 genes were found to be significantly induced after exposure to CHP both in the wild type and *yap1Δ* mutant with similar kinetics. 622 genes were also found to be repressed in both cases with similar kinetics. This means that the regulation of these genes is independent of Yap1 and therefore yeast has other mechanisms to respond to oxidative stress.

In the group of genes that were commonly induced by CHP in wild type and *yap1Δ* mutant, the only GO Slim category that is significantly represented is entitled *response to stress* (Table 3.7A). It demonstrates that the most significant transcriptomics change induced by CHP in both wild type and *yap1Δ* mutant is stress response. 64 genes in this category were able to function independent of Yap1 (Table 3.7A). According to YEASTRACT (Teixeira, et al., 2006), among these stress responding genes, 20 of them have already been documented in the literature to be under Yap1 control (Table 3.7A). Our results contradict with these observations as here they responded to CHP in the wild type and *yap1Δ* mutant with the same kinetics.

In the group of genes that were commonly repressed in wild type and *yap1Δ* mutant, GO Slim categories related to transcription, translation, cell cycle and lipid metabolism are significantly represented (Table 3.7B). Genes in Table 3.7B were repressed in Yap1 independent manner.

It is interesting to see that ribosome biogenesis is significantly represented in the Yap1-independent genes group (Table 3.7B), but also significantly represented in the Yap1-dependent genes group (Table 3.5B). It means that many genes in this category function in a Yap1 independent manner, and many other genes require Yap1 to respond to oxidative stress. Thus, precautions should be used to interpret the results. When a certain pathway has a significant *p*-value, it means that this pathway is significantly represented in the group of genes that we are interested in, for example, the group of genes that were able to function in Yap1 independent manner. One cannot draw a conclusion that the

whole pathway is independent of Yap1, because some genes on this pathway could be Yap1-dependent.

Table 3.7. GO Slim categories significantly represented in common responding genes between wild type and *yap1Δ* mutant yeast under oxidative stress condition.

A. GO Slim categories are significantly represented ($p < 0.001$) in the group of genes that were significantly induced by CHP in *yap1Δ* mutant yeast at the same time point as they were significantly induced by CHP in wild type yeast. The induction of these genes is independent of Yap1. Genes that have previously been documented to be regulated by Yap1 are in red.

GO Slim category	P-value	Common responding genes annotated to the category
<u>Response to stress</u>	4.33e-07	<i>YBR016W</i> , <i>ORM2</i> , <i>MID2</i> , <i>UBI4</i> , <i>DOA1</i> , <i>YJL144W</i> , <i>FRT2</i> , <i>COS8</i> , <i>CTT1</i> , <i>DAK2</i> , <i>PRB1</i> , <i>HSP42</i> , <i>SNO2</i> , <i>GPD1</i> , <i>HSP30</i> , <i>TDPI</i> , <i>PHR1</i> , <i>GRE2</i> , <i>NCE103</i> , <i>HOR7</i> , <i>DDR48</i> , <i>SIP5</i> , <i>HSP104</i> , <i>GPX1</i> , <i>MYO3</i> , <i>RAD7</i> , <i>NTG1</i> , <i>BCY1</i> , <i>UBA4</i> , <i>GRE3</i> , <i>TRX2</i> , <i>PIL1</i> , <i>STF2</i> , <i>MMS2</i> , <i>SOH1</i> , <i>INO80</i> , <i>HAC1</i> , <i>HSP78</i> , <i>TPS2</i> , <i>UBC5</i> , <i>NTH1</i> , <i>RVS161</i> , <i>GRX1</i> , <i>SMP1</i> , <i>TPS1</i> , <i>AGP2</i> , <i>RAD16</i> , <i>HSP26</i> , <i>YDC1</i> , <i>AFT2</i> , <i>GCY1</i> , <i>CIN5</i> , <i>YGP1</i> , <i>RAD50</i> , <i>ZWF1</i> , <i>YKU70</i> , <i>TPS3</i> , <i>GAD1</i> , <i>TPP1</i> , <i>ALD3</i> , <i>RIM11</i> , <i>DAK1</i> , <i>TSL1</i> , <i>RAD10</i>

B. GO Slim categories are significantly represented ($p < 0.001$) in the group of genes that were significantly repressed by CHP in *yap1Δ* mutant yeast at the same time point as they were significantly repressed by CHP in wild type yeast. The repression of these genes is independent of Yap1.

GO Slim category	P-value	Common responding genes annotated to the category
<u>Ribosome biogenesis and assembly</u>	1.24e-17	<i>DBP9</i> , <i>UTP13</i> , <i>IFH1</i> , <i>EMG1</i> , <i>DIP2</i> , <i>SOF1</i> , <i>NOC3</i> , <i>RIX7</i> , <i>RPF2</i> , <i>UTP30</i> , <i>DBP7</i> , <i>DHR2</i> , <i>HCA4</i> , <i>MTR4</i> , <i>UTP18</i> , <i>MAK16</i> , <i>ECM1</i> , <i>UTP9</i> , <i>RIX1</i> , <i>DBP8</i> , <i>IMP3</i> , <i>IPI1</i> , <i>YGR272C</i> , <i>NSR1</i> , <i>UTP8</i> , <i>NOP7</i> , <i>POP6</i> , <i>ARB1</i> , <i>SNU13</i> , <i>RPL12A</i> , <i>UTP6</i> , <i>UTP4</i> , <i>RLI1</i> , <i>ARX1</i> , <i>RRP8</i> , <i>MAK21</i> , <i>NOP14</i> , <i>RSA4</i> , <i>PWP2</i> , <i>SPB1</i> , <i>ENP1</i> , <i>POP8</i> , <i>NOC4</i> , <i>RRP9</i> , <i>MRD1</i> , <i>TIF5</i> , <i>NOP4</i> , <i>NOG1</i> , <i>NOP53</i> , <i>NAN1</i> , <i>NIP7</i> , <i>NOP58</i> , <i>RAT1</i> , <i>RCL1</i> , <i>RRP6</i> , <i>YOR004W</i> , <i>REX4</i> , <i>RRP40</i> , <i>NOG2</i> , <i>ESF2</i> , <i>DBP6</i> , <i>IPI3</i> , <i>RIO2</i> , <i>POP3</i> , <i>RNT1</i> , <i>UTP15</i> , <i>ERB1</i> , <i>TSR2</i> , <i>UTP21</i>
<u>Organelle organization and biogenesis</u>	1.82e-12	<i>ACO1</i> , <i>SPH1</i> , <i>DBP9</i> , <i>YCS4</i> , <i>TUB4</i> , <i>UTP13</i> , <i>IFH1</i> , <i>EMG1</i> , <i>DIP2</i> , <i>STU2</i> , <i>SOF1</i> , <i>NOC3</i> , <i>RIX7</i> , <i>HIF1</i> , <i>SPA2</i> , <i>RPF2</i> , <i>NUP133</i> , <i>UTP30</i> , <i>DBP7</i> , <i>DHR2</i> , <i>LIA1</i> , <i>GEA1</i> , <i>HCA4</i> , <i>VTC4</i> , <i>MTR4</i> , <i>UTP18</i> , <i>MDV1</i> , <i>NUP60</i> , <i>BUD14</i> , <i>MYO4</i> ,

		<u>MAK16</u> , <u>ECM1</u> , <u>FKH1</u> , <u>SIM1</u> , <u>UTP9</u> , <u>RIX1</u> , <u>DBP8</u> , <u>SPC97</u> , <u>IMP3</u> , <u>TRAI</u> , <u>IPI1</u> , <u>RRM3</u> , <u>BRL1</u> , <u>YGR272C</u> , <u>NSR1</u> , <u>UTP8</u> , <u>CBF2</u> , <u>TEL2</u> , <u>NOP7</u> , <u>POP6</u> , <u>RPB9</u> , <u>SEH1</u> , <u>CDC20</u> , <u>NIC96</u> , <u>VTCL</u> , <u>ARB1</u> , <u>SNU13</u> , <u>CIN8</u> , <u>RPL12A</u> , <u>PAC11</u> , <u>UTP6</u> , <u>UTP4</u> , <u>YCG1</u> , <u>MNN10</u> , <u>SAN1</u> , <u>RLI1</u> , <u>ARX1</u> , <u>FOB1</u> , <u>RRP8</u> , <u>MAK21</u> , <u>MCD1</u> , <u>KCS1</u> , <u>SIR2</u> , <u>UBC9</u> , <u>NOP14</u> , <u>HCM1</u> , <u>RSA4</u> , <u>TUP1</u> , <u>PWP2</u> , <u>VAC17</u> , <u>SPB1</u> , <u>ENP1</u> , <u>RIM2</u> , <u>MSI1</u> , <u>ORC2</u> , <u>POP8</u> , <u>STU1</u> , <u>HEK2</u> , <u>KAR3</u> , <u>NOC4</u> , <u>RRP9</u> , <u>MRD1</u> , <u>TIF5</u> , <u>MAK3</u> , <u>VTC3</u> , <u>SWI1</u> , <u>NOP4</u> , <u>NOG1</u> , <u>HOS3</u> , <u>PEX25</u> , <u>NOP53</u> , <u>YPL144W</u> , <u>NAN1</u> , <u>KIP2</u> , <u>NIP7</u> , <u>VIK1</u> , <u>IOG1</u> , <u>MYO2</u> , <u>NOP58</u> , <u>SLK19</u> , <u>ELG1</u> , <u>RAT1</u> , <u>HST3</u> , <u>HTZ1</u> , <u>RCL1</u> , <u>RRP6</u> , <u>YOR004W</u> , <u>TAT2</u> , <u>REX4</u> , <u>PEX11</u> , <u>RRP40</u> , <u>NOG2</u> , <u>ESF2</u> , <u>DBP6</u> , <u>SUN4</u> , <u>TOP2</u> , <u>SPC98</u> , <u>UBP10</u> , <u>IPI3</u> , <u>RIO2</u> , <u>RAP1</u> , <u>POL2</u> , <u>POP3</u> , <u>YNL313C</u> , <u>RNT1</u> , <u>YHM2</u> , <u>SPT21</u> , <u>STO1</u> , <u>SAS2</u> , <u>UTP15</u> , <u>PDS5</u> , <u>ERB1</u> , <u>SPT5</u> , <u>NDC1</u> , <u>PIF1</u> , <u>OGG1</u> , <u>MDM1</u> , <u>CAC2</u> , <u>ZDS2</u> , <u>TSR2</u> , <u>UTP21</u> , <u>VIPI</u> , <u>SUR4</u>
<u>RNA metabolism</u>	1.19e-08	<u>DBP9</u> , <u>UTP13</u> , <u>IFH1</u> , <u>EMG1</u> , <u>DIP2</u> , <u>SEN2</u> , <u>FRS1</u> , <u>SOFL</u> , <u>NOC3</u> , <u>PRP16</u> , <u>TRZ1</u> , <u>RPF2</u> , <u>TRM2</u> , <u>UTP30</u> , <u>DBP7</u> , <u>HCA4</u> , <u>MTR4</u> , <u>TAD2</u> , <u>UTP18</u> , <u>GCD14</u> , <u>CBP1</u> , <u>SEN34</u> , <u>UTP9</u> , <u>RIX1</u> , <u>DBP8</u> , <u>IMP3</u> , <u>IPI1</u> , <u>YGR272C</u> , <u>NSR1</u> , <u>UTP8</u> , <u>NOP7</u> , <u>POP6</u> , <u>PRP43</u> , <u>FRS2</u> , <u>FIR1</u> , <u>SNU13</u> , <u>UTP6</u> , <u>UTP4</u> , <u>TRM82</u> , <u>TRM1</u> , <u>RRP8</u> , <u>TRM3</u> , <u>NOP14</u> , <u>CWC2</u> , <u>BUD31</u> , <u>PWP2</u> , <u>SPB1</u> , <u>ENP1</u> , <u>TRM7</u> , <u>POP8</u> , <u>NOC4</u> , <u>RRP9</u> , <u>MRD1</u> , <u>NOP4</u> , <u>NOP53</u> , <u>NAN1</u> , <u>NIP7</u> , <u>MRS2</u> , <u>NOP58</u> , <u>PUS7</u> , <u>GLN4</u> , <u>RAT1</u> , <u>RCL1</u> , <u>RRP6</u> , <u>YOR004W</u> , <u>REX4</u> , <u>TRM10</u> , <u>TRM11</u> , <u>DCP1</u> , <u>RRP40</u> , <u>ESF2</u> , <u>DBP6</u> , <u>PRP2</u> , <u>GCD10</u> , <u>IPI3</u> , <u>RIO2</u> , <u>PUS4</u> , <u>POP3</u> , <u>RNT1</u> , <u>STO1</u> , <u>UTP15</u> , <u>ERB1</u> , <u>TRM9</u> , <u>DUS1</u> , <u>TSR2</u> , <u>UTP21</u> , <u>DUS4</u>
<u>Cell cycle</u>	3.33e-07	<u>YCS4</u> , <u>CLB4</u> , <u>TUB4</u> , <u>SWI6</u> , <u>TOS4</u> , <u>ACE2</u> , <u>STU2</u> , <u>SSL1</u> , <u>ELM1</u> , <u>SMC3</u> , <u>SAP185</u> , <u>SWE1</u> , <u>LTE1</u> , <u>KCC4</u> , <u>FKH1</u> , <u>GIC1</u> , <u>MSC7</u> , <u>ZPR1</u> , <u>BUB1</u> , <u>CBF2</u> , <u>ESP1</u> , <u>CLB6</u> , <u>SCM4</u> , <u>ALK1</u> , <u>CDC20</u> , <u>CDC14</u> , <u>RAD3</u> , <u>CIN8</u> , <u>GIN4</u> , <u>YHP1</u> , <u>YCG1</u> , <u>SWI5</u> , <u>PDS1</u> , <u>MCD1</u> , <u>UBC9</u> , <u>HCM1</u> , <u>REI1</u> , <u>AMN1</u> , <u>HSL7</u> , <u>CKS1</u> , <u>YBL009W</u> , <u>KAR3</u> , <u>CTF4</u> , <u>CLB2</u> , <u>CLB5</u> , <u>TFB2</u> , <u>KIP2</u> , <u>CLN2</u> , <u>VIK1</u> , <u>NDD1</u> , <u>KIN4</u> , <u>SLK19</u> , <u>SGO1</u> , <u>BUB3</u> , <u>FKH2</u> , <u>TOP2</u> , <u>PMS1</u> , <u>SPC98</u> , <u>IBD2</u> , <u>TOF1</u> , <u>PCL1</u> , <u>CLN1</u> , <u>PDS5</u> , <u>CTF18</u> , <u>CSM3</u> , <u>YOX1</u>
<u>Lipid metabolism</u>	6.80e-07	<u>ERG3</u> , <u>IZH3</u> , <u>YEH1</u> , <u>GPI13</u> , <u>AUR1</u> , <u>LAC1</u> , <u>YPK1</u> , <u>MCRI</u> , <u>MCD4</u> , <u>GPI14</u> , <u>ELO1</u> , <u>FAA3</u> , <u>ERG7</u> , <u>NCPI</u> , <u>ERG11</u> , <u>ERG1</u> , <u>ATF2</u> , <u>ERG25</u> , <u>ERG4</u> , <u>VRG4</u> , <u>ERG28</u> , <u>IZH1</u> , <u>SUR2</u> , <u>GPI11</u> , <u>EKI1</u> , <u>SLC1</u> , <u>FEN1</u> , <u>TSC10</u> , <u>CDS1</u> , <u>TGL5</u> , <u>HST3</u> , <u>IZH2</u> , <u>PEX11</u> , <u>CYB5</u> , <u>NMA111</u> , <u>ERG24</u> , <u>SCS7</u> , <u>SEC14</u> , <u>PLB2</u> , <u>ERG5</u> , <u>NTE1</u> , <u>HMG1</u> , <u>ERG13</u> , <u>SUR4</u>
<u>Transcription</u>	1.54e-05	<u>YCS4</u> , <u>IFH1</u> , <u>SWI6</u> , <u>TOS4</u> , <u>ACE2</u> , <u>PPR1</u> , <u>SSL1</u> , <u>HIF1</u> , <u>RRN3</u> , <u>RPC25</u> , <u>RPA12</u> , <u>RRN7</u> , <u>RPA34</u> , <u>BUD14</u> , <u>FKH1</u> , <u>RPC10</u> , <u>TRAI</u> , <u>ELP2</u> , <u>RPB9</u> , <u>SUT1</u> , <u>RAD3</u> , <u>YER064C</u> , <u>POL5</u> , <u>YHP1</u> , <u>SWI5</u> , <u>FOB1</u> , <u>PDC2</u> , <u>RPC11</u> , <u>SIR2</u> , <u>RPC53</u> , <u>HCM1</u> , <u>TUP1</u> , <u>MSI1</u> , <u>CKS1</u> , <u>ORC2</u> , <u>RPO26</u> , <u>RPC82</u> , <u>GCR1</u> , <u>MED1</u> , <u>SUT2</u> , <u>ELP3</u> , <u>MOT1</u> , <u>TFB2</u> , <u>NDD1</u> , <u>SFG1</u> , <u>RPB8</u> , <u>RET1</u> , <u>RPB2</u> , <u>HIR2</u> , <u>STD1</u> , <u>RAT1</u> , <u>HST3</u> , <u>HTZ1</u> , <u>RPC34</u> , <u>FKH2</u> , <u>RPC31</u> , <u>UBP10</u> , <u>RAP1</u> , <u>NRD1</u> , <u>RPA49</u> , <u>POL2</u> , <u>SPT21</u> , <u>SAS2</u> , <u>SPT5</u> , <u>RRN11</u> , <u>YOX1</u> , <u>CAC2</u> , <u>DAT1</u> , <u>ZDS2</u> , <u>IKI3</u>
<u>Cytokinesis</u>	2.91E-05	<u>SPH1</u> , <u>RAX2</u> , <u>SPA2</u> , <u>ELM1</u> , <u>BUD4</u> , <u>KCC4</u> , <u>AXL2</u> , <u>GIC1</u> , <u>BUD27</u> , <u>GIN4</u> , <u>CYK3</u> , <u>BUD31</u> , <u>PWP2</u> , <u>SUP45</u> , <u>CHS2</u> , <u>IQG1</u> , <u>DSE4</u> , <u>BNI5</u> , <u>BNI4</u> , <u>EGT2</u> , <u>HOF1</u> , <u>BUD22</u> , <u>BUD8</u>

3.4.6. Alternative oxidative stress responding genes and pathways in *yap1Δ* mutant

Many genes responded to oxidative stress in the *yap1Δ* mutant but show no response to the stimulus in the wild type. 406 genes were found to be significantly induced by CHP in *yap1Δ* mutant within 20 minutes but not significantly induced in the wild type under the same conditions. 711 genes were found to be significantly repressed by CHP in the *yap1Δ* mutant but not in the wild type. Many of these genes are likely to be compensating for functions that were lost with the mutation (e.g. genes that are controlled by Yap1 and no longer respond in the mutant).

3.4.6.1. KEGG pathways alternatively induced in *yap1Δ* mutant

At a *p*-value cutoff of 0.01, none of the KEGG pathways were detected as significantly represented in the 406 alternatively induced genes. At cutoff 0.05, two pathways were identified: 1) phenylalanine, tyrosine and tryptophan biosynthesis ($p=0.0356$) and 2) glycolysis/gluconeogenesis ($p=0.0429$).

Phenylalanine, tyrosine and tryptophan biosynthesis

The aromatic amino acid residues of proteins are prime targets for oxidation by various forms of ROS (Stadtman and Levine, 2003). Phenylalanine residues are oxidized to ortho- and meta-tyrosine derivatives (Maskos, et al., 1992; Wells-Knecht, et al., 1993). Tyrosine residues are oxidized to the 3,4-dihydroxy (dopa) derivative (Davies, et al., 1987; Dean, et al., 1993; Maskos, et al., 1992) and to bi-tyrosine cross-linked derivatives (Heinecke, et al., 1993; Huggins, et al., 1993; Wells-Knecht, et al., 1993). Tryptophan residues are oxidized to hydroxyl derivatives and to N-formylkynurenine and kynurenine (Armstrong and Swallow, 1969; Kikugawa, et al., 1994; Winchester and Lynn, 1970). In the Yap1 mutant, the oxidative damage of aromatic amino acids is likely to be more severe than in the wild type due to the lower expression of many antioxidant genes. The induction of phenylalanine, tyrosine and tryptophan biosynthesis could be needed for increased protein repair processes.

Glycolysis

As discussed in Chapter 2, in wild type yeast under oxidative stress condition, glucose seems to be diverted from energy production (glycolysis, TCA cycle) to NADPH regeneration in the pentose phosphate pathway (PPP) and to production of trehalose. Different from the wild type, glycolysis was induced in *yap1Δ* mutant under oxidative stress. It suggests that the repression of glycolysis under oxidative stress is regulated by Yap1. Three genes (*ZWF1*, *SOLA* and *YGR043c*) on the oxidative branch in pentose phosphate pathway were induced, which suggests the regulation of these genes is independent of Yap1. *GND2* and *TKL2* on the oxidative branch were induced in wild type but not in *yap1Δ* mutant, thus these two genes could be regulated by Yap1. Trehalose metabolism is independent of Yap1, since genes encoding enzymes involved in trehalose metabolism were also induced by CHP in *yap1Δ* mutant.

3.4.6.2. KEGG pathways alternatively repressed in *yap1Δ* mutant

The only pathway that is significantly represented ($p < 0.01$) in the 711 alternatively repressed genes in *yap1Δ* mutant yeast is the biosynthesis of N-glycans ($p = 0.000241$). 14 out of 21 enzymes of this pathway were significantly repressed at in *yap1Δ* mutant. This repression was not observed in the wild type.

N-glycan biosynthesis

N-glycan biosynthesis is a pathway in which monosaccharides are added to a lipid carrier, dolichol pyrophosphate, by monosaccharyl-transferases in the ER membrane (Burda and Aebi, 1999; Kornfeld and Kornfeld, 1985). Synthesis occurs on both sides of the ER membrane. N-linked glycans are involved in the N-linked protein glycosylation.

Dolichol is the main component of the lipid carrier, dolichol pyrophosphate. It has been suggested that dolichol is an essential part of the antioxidant machinery of cell membranes, together with polyunsaturated fatty acids (PUFA) and vitamin E (Bergamini, 2003). It was suggested by Bergamini that PUFA and dolichol might act as a unit to trap free radicals and keep them from damaging membrane proteins. “Dolichol might be the

sink where electrons are stored to give vitamin E the time to shuttle back and forth to scavenge the radicals and conduct them to the hydrophilic compartment of the cell” (Bergamini, 2003). Dolichol may then undergo peroxidative decomposition, and may be re-synthesized by the mevalonate pathway (Bergamini, 2003). A possible explanation for the repression of N-glycans biosynthesis in *yap1Δ* mutant (but not in wild type) is that dolichol might be diverted to scavenging ROS in *yap1Δ* mutant due to the deficiency of other antioxidant systems (e.g. glutathione system). This could lead to lower levels of dolichol available to function as the main component of lipid carrier, where the repression of N-glycan biosynthesis would then be justified.

Ubiquitin-mediated proteolysis was also highly represented ($p=0.045$) in the alternatively repressed genes group. Ten proteolysis genes were repressed in *yap1Δ* mutant. In contrast to the *yap1Δ* mutant, proteolysis was significantly induced by oxidative stress in the wild type. It suggests that ubiquitin-mediated proteolysis is positively regulated by Yap1.

3.4.6.3. Alternative stress responding genes

Among genes that were alternatively induced in *yap1Δ* mutant, 23 genes were classified in the GO Slim category of *response to stress* (Table 3.8). At the transcriptional level, this group of genes did not respond to CHP in wild type yeast, but significantly responded to CHP in *yap1Δ* mutant. These genes could be alternative stress responding genes in *yap1Δ* mutant.

Table 3.8. Alternative stress responding genes in *yap1Δ* mutant.

GO Slim category	Genes annotated to the category
response to stress	<u><i>ASP3-1</i></u> , <u><i>NHA1</i></u> , <u><i>PSR1</i></u> , <u><i>SIS2</i></u> , <u><i>SPT23</i></u> , <u><i>HAC1</i></u> , <u><i>RHR2</i></u> , <u><i>ASK10</i></u> , <u><i>RAD6</i></u> , <u><i>LIF1</i></u> , <u><i>RPH1</i></u> , <u><i>SSA4</i></u> , <u><i>ATC1</i></u> , <u><i>NBP2</i></u> , <u><i>HEX3</i></u> , <u><i>SLX1</i></u> , <u><i>DNL4</i></u> , <u><i>NTG2</i></u> , <u><i>MSN1</i></u> , <u><i>SIW14</i></u> , <u><i>YNL234W</i></u> , <u><i>CSL4</i></u> , <u><i>MSN2</i></u>

Five of these genes are transcription factors, *MSN1*, *MSN2*, *RPH1*, *SPT23* and *HAC1*. Hac1 is a bZIP transcription factor for the unfolded protein-response pathway (Cox and Walter, 1996; Nojima, et al., 1994). Rph1 is transcriptional repressor of *PHR1*, which is a

photolyase induced by DNA damage (Jang, et al., 1999). *MSN1* and *MSN2* encode transcriptional activators for stress response (Martinez-Pastor, et al., 1996; Rep, et al., 1999). *Msn1* and *Msn2* could activate stress responding genes in *yap1Δ* mutant partly substituting those that were silenced by the mutation.

3.5. Conclusions

This study identified 1) genes whose regulation by Yap1 is independent of oxidative stress 2) genes and pathways regulated by Yap1 under oxidative stress condition 3) alternative genes and pathways that may compensate the oxidative defense provided by genes silenced by the *yap1Δ* mutation 4) common oxidative stress responding genes and pathways in wild type and *yap1Δ* mutant, which are therefore not regulated by Yap1.

This study provides a global view of the roles that Yap1 plays in yeast under normal and CHP-induced oxidative stress. Most of the genes and some pathways identified here to be regulated by Yap1 have not been documented in prior literature, at least not experimentally. I have proposed hypotheses to explain the possible regulatory roles of Yap1 in these genes and pathways. The exact regulatory mechanisms of genes and pathways identified in this study remain unknown. This study opens avenues for future research in the transcriptional response to oxidative stress in yeast.

This study does not discriminate between direct and indirect regulation by Yap1. Genes with Yap1 binding sites are likely to be directly regulated by Yap1. A ChIP-chip study with similar experimental design (i.e. a time course) would be helpful to confirm genes that are indeed directly regulated by Yap1.

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