Title
Identification and characterization of the Fzf1-mediated response to nitrosative stress in Saccharomyces cerevisiae

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Identification and Characterization of the *FZF1*-mediated response to Nitrosative stress in *Saccharomyces cerevisiae*

by

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DISSERTATION

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This work is dedicated to
my wife Anne and my daughter Aurora.

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ABSTRACT

The mechanisms by which microorganisms sense and detoxify nitric oxide (NO·) are of particular interest due to the central role this molecule plays in innate immunity. I used DNA microarrays to investigate the genome-wide transcriptional response to exogenously supplied NO· in the model organism *S. cerevisiae*. Exposure to NO· generating compounds resulted in both a general stress response as well as a specific NO· detoxification response. This was characterized by the induction of a small set of genes, including the yeast flavohemoglobin *YHB1*, *SSU1*, and three additional uncharacterized open reading frames. The *YHB1* gene encodes a NO· dioxygenase gene that converts NO· to nitrate. Induction of *YHB1* in response to NO· is consistent with a specific NO· detoxification mechanism. Transcriptional induction of *SSU1*, which encodes a putative sulfite transporter, has previously been shown to require the zinc finger transcription factor Fzf1p. I discovered that deletion of Fzf1p eliminated the nitrosative stress-specific transcriptional response; while overexpression of Fzf1p recapitulated this response in the absence of exogenously supplied NO·. In addition to discovering the response and determining a necessary transcription factor, I used a combination of bioinformatic, phylogenetic, and experimental approaches to discover a *cis*-acting sequence unique to the promoter regions of Fzf1p-dependent, NO·-responsive genes. This sequence was found to be sufficient to activate reporter gene activity in an NO·- and Fzf1p-dependent manner. I also verified that the RNA transcript level increases seen by array led to increases in the Yhb1p and Ssu1p protein levels using both Western blotting and flow cytometry. My results suggest that the presence of NO· or NO· derivatives activate

Fzf1p, which leads to a physiologically relevant response that protects the cell from NO·-mediated stress. My results have already led to the characterization of a similar response in the pathogenic fungi *C. Albicans*.

CHAPTER 1

INTRODUCTION TO NITRIC OXIDE RESEARCH

Nitric Oxide (NO·) is a simple, odorless gas composed of a nitrogen atom bound to an oxygen atom through a double bond. NO· is referred to as a free radical because an unpaired electron is found on the nitrogen atom. Unlike most free radicals, NO· is not charged and is able to easily diffuse through biological membranes. As a result of its unique chemistry, NO· is capable of reacting in a concentration- and environment-dependent manner. This leads to a whole host of biological responses, and makes research on the biological effects of NO· a fascinating field.

Prior to 1987, the important biological roles of NO· were unknown. At that time, it was known that NO· and higher nitrogen oxides are produced within combustion engines and industrial processes via the high temperature combination of N_2 with O_2 (Dimitriades, 1967). Modern automobiles are fitted with catalytic converters to prevent this from occurring because NO· reacts with O_2 to form nitrogen dioxide, which is strongly implicated as a causative agent of smog. It has also been common knowledge for some time that NO· in high concentrations is toxic. In 1800, Sir Humphry David, well known for his discovery of the analgesic effects of nitrous oxide, nearly died following the inhalation of chemically generated NO· (Sprigge, 2002).

The search for the endothelial relaxation factor (EDRF), an unknown substance produced by the endothelium of blood vessels that regulates the tone of blood vessels, led to the discovery of the first biological roles of NO· (Furchgott and Zawadzki, 1980). It was initially proposed in 1987 that EDRF could be NO· based on the chemical reactivity of EDRF (Ignarro et al., 1987). This was then proven by two key experiments that

showed first that endothelial cells produced NO· following stimulation and second that chemically-produced NO· resulted in endothelial relaxation. (Palmer et al., 1987).

Robert Furchgott, Ferid Murad, and Louis Ignarro were awarded the Nobel Prize in 1998 for their research on the effects of NO· on blood vessels. It is now known that production of NO· is necessary for even basal blood vessel relaxation and thus required for normal blood flow through the cardiovascular system. NO· production also decreases platelet aggregation and keeps clotting from interfering with blood flow (Yamamoto and Bing, 2000). A number of commonly used drugs utilize the NO·-mediated endothelial relaxation pathway as their mechanism of action. Nitroglycerin releases NO·, leading to vascular relaxation which reduces the pain of angina (Marsh and Marsh, 2000). Perhaps the most well-known drug to utilize the NO·-mediated endothelial relaxation pathway is sildenafil citrate (Viagra). A secondary result of NO· production is the increased synthesis of cyclic GMP (cGMP), which leads to blood vessel relaxation and then erection. Viagra inhibits the enzyme PDE5 which normally breaks down cGMP, leading to higher levels of cGMP and amplifying the effect of NO·, resulting in increased blood flow to the penis (Koppiker, 2002). The discovery of a second distinct role for NO·, an integral part of the non-specific immune response, began as an initial observation that a research subject on a low-nitrate diet produced an extremely large amount of nitrate while concurrently infected with an intestinal disease. This was shown by the increased production of nitrate in rats exposed to *E. coli* liposaccharide (Wagner et al., 1983). Exploration of the enzymology responsible for this response led to the eventual discovery that NO· is the intermediate agent responsible for the increased nitrate levels produced by macrophages following pathogen stimulation (Marletta et al., 1988). Since this initial

discovery it has been shown that NO· produced by macrophages aids in the direct killing of engulfed pathogens and that NO· production is also important to the control of extracellular pathogens (Elahi et al., 2001). This response must be tightly regulated because overproduction of NO· by the immune system can lead to septic shock (Nava et al., 1991).

Surprisingly, NO· also has a number of other biological roles. It is used by the human body as a neurotransmitter (Kiss and Vizi, 2001) and as a signal that the sperm and egg have fused during fertilization (Francavilla et al., 2000). In addition it has a role in firefly flashing (Trimmer et al., 2001). NO· is also produced by plants in response to pathogen infection (Zeidler et al., 2004). While NO· has a very simple form, it has a very complex biology associated with it.

NO· synthase (NOS) proteins catalyze the five electron oxidation of arginine to NO· and citrulline utilizing the conversion of NADPH to NADP, tetrahydrobiopterin and molecular O_2 (Marletta, 1993). Higher eukaryote genomes contain three versions of the NOS gene. Endothelial NOS (eNOS) produces NO· for use in endothelial relaxation. Neuronal NOS (nNOS) produces NO· for use as a neurotransmitter. These first two isoforms are constitutively expressed. The third form, inducible NOS (iNOS), is expressed in macrophages and other cells following exposure to pathogens or pathogen oligosaccharides (Wang and Marsden, 1995). Inhibitors have been developed for the NOS family of enzymes although development of specific drugs for the specific isoforms remains a difficult problem. The eNOS gene has been knocked out in mice and results in increased pulmonary hypertension (Miller et al., 2005). The iNOS gene has been knocked out and results in mice that are less resistant to pathogenic infection

(MacMicking et al., 1995) (Wei et al., 1995). The nNOS gene has also been knocked out in mice, resulting in decreased cognitive performance in stressful situations (Weitzdoerfer et al., 2004).

In addition to production by the NOS enzymes, NO· is also produced from a number of other sources. Symbiotic bacteria, living in the human throat, convert nitrates into nitrites. The acidic gastric environment generates NO· from this nitrite, which then serves to kill bacteria infecting our food (Duncan et al., 1995). NO· is also produced by denitrifying bacteria as a part of their metabolism (Xu and Verstraete, 2001).

NO·, once produced, has been reported to react directly with its targets, as well as act in a secondary fashion through other molecules. These reactions occur in a concentration- and environmental-dependent manner. NO· will react vigorously with the superoxide (O_2^-) free radical to form peroxynitrite ($ONOO^-$) which is a strongly reactive molecule (Beckman et al., 1990).

In the biological matrix NO· has been reported to change the functional activity of proteins by direct binding, by nitrosylation of cysteine residues, and by disruption of iron-sulfur centers. NO· has been shown to bind proteins which directly leads to allosteric changes in activity. The canonical example of this type of activation mechanism is the binding of NO· to the heme domain of soluble guanylyl cyclase, leading to an allosteric change in the active site and activation of guanylyl cyclase activity (Stone and Marletta, 1994). NO· has also been shown to react with oxygen to nitrosylate cysteine residues leading to modifications of protein activity or binding. A canonical example of this type of mechanism is the nitrosylation of the ryanodine receptor (Sun et al., 2001). NO· has also been shown to disrupt iron-sulfur centers

modifying the activity of the protein. A classic example of this functionality is the treatment of aconitase with NO·, which disrupts the iron-sulfur center leading to activation of the iron response element binding functionality of the protein, which is completely unrelated to the aconitase enzymatic activity (Bouton and Drapier, 2003).

Nitrosative stress is a term describing the condition that occurs following exposure to NO·. Despite our understanding of how and why higher eukaryotes produce NO·, little is known about how NO· is detoxified. What is known is that at concentrations higher than signaling levels NO· is cytostatic, and at higher concentrations NO· is cytotoxic. Microorganisms have developed responses to cope with exposure to NO·. The flavohemoglobin protein has been shown to convert NO· to nitrate utilizing molecular oxygen NADPH and FAD (Gardner et al., 1998). In response to NO· exposure, bacteria induce higher expression levels of flavohemoglobin proteins. (Crawford and Goldberg, 1998; Stevanin et al., 2000).

Saccharomyces cerevisiae is an extremely effective model organism for several reasons. First off, protocols for fast and effective genetic manipulation are well worked out. *S. cerevisiae* was the first eukaryotic organism sequenced, and consequently the first eukaryotic organism that genome-wide experimental techniques were applied to. Powerful tools such as DNA microarrays (DeRisi et al., 1997), genome-wide deletion libraries (Winzeler et al., 1999) and tagged libraries of strains (Huh et al., 2003) (Ghaemmaghami et al., 2003) are available. Another advantage of working with the *S. cerevisiae* model organism is that closely related yeast strains have been sequenced and these sequences reveal functionally conserved DNA and protein sequences (Cliften et al., 2003) (Kellis et al., 2003). In addition to these genomic tools, there is a vast literature

describing genetic work in *S. cerevisiae*, allowing for significantly advanced hypothesis generation, which is cataloged in freely available databases such as SGD (http://www.yeastgenome.org/). Most of what we know about eukaryotic responses to stresses such as DNA damage (Bentley and Carr, 1997) and oxidative stress (Costa and Moradas-Ferreira, 2001) has been derived from research originally conducted in *S. cerevisiae*.

Preliminary studies were previously undertaken to determine how NO· interacts in the internal *S. cerevisiae* environment. In the Fukuto lab, it was shown that the presence of exogenous NO· could inhibit the copper-dependent transcriptional induction of the *cup1* gene by Ace1p (Shinyashiki et al., 2000). They later showed that oxygen was necessary for this inhibition and proposed that this was because of the reaction of oxygen with NO· to form higher nitrogen oxides and not due to the actions of molecular NO· (Shinyashiki et al., 2001).

Yeast hemoglobin (*YHB1*) was first identified in the 1950s due to the unique absorption spectra of its heme domain. Although there was speculation about the role of the *YHB1* gene in the oxidative stress response (Zhao et al., 1996), *YHB1* was shown to not be directly responsive to oxidative stress (Buisson and Labbe-Bois, 1998). Definitive work by Liu *et al.* showed that *YHB1* was necessary for detoxification of NO, had homology to bacterial NO· dioxygenase enzymes, and strains with deletions in *YHB1* were hypersensitive to NO·(Liu et al., 2000).

SSU1 and *FZF1* were originally identified during screens for sulfite-sensitive and sulfite-resistant strains. Screens were conducted following the observation that yeast strains differed in their ability to survive following exposure to sulfites, which are

commonly used in the process of wine fermentation. This work also identified a mutant of the *GRR1* gene, which was hypersensitive to sulfite. An additional mutant was identified that these authors were not able to complement (Avram and Bakalinsky, 1996). Follow up work identified Sensitivity Sulfite 1 (*SSU1*) as a sulfite efflux transporter localized to the outer membrane of the cell (Avram and Bakalinsky, 1997). The resistant strain was mapped to the zinc finger containing Resistant Sulfite 1 gene (*RSU1*), which was later shown to be isogenic to the Five Zinc Fingers 1 gene (*FZF1*). The first zinc finger was shown to bind a specific region within the promoter of the *SSU1* gene (Avram et al., 1999). This transcriptional activation was shown to occur basally and the authors of this study wondered what the true role of this network would turn out to be (Park and Bakalinsky, 2000).

INTRODUCTION TO GENOME-WIDE EXPRESSION PROFILING

Transcriptional programs are hardwired into the genetic programming of adaptive responsive organisms. The output of these programs is tightly controlled and organized transcriptional responses has been refined by millennia of successful adaptation. Changes in mRNA transcript levels are one of the simplest ways for an organism to remodel its internal physiology in response to stimulus. The analysis of mRNA transcript levels in biological organisms is an important and popular tool in molecular biology.

Following the general acceptance that DNA is transcribed into RNA, which is in turn translated into proteins, techniques for the determination of mRNA transcript level have been constantly improving. The first determination of RNA transcript levels occurred by utilizing the hybridization of a radiolabeled DNA probe to a gel-separated and electro-blotted RNA sample in a procedure commonly referred to as Northern blotting. This determined both the size of the transcript as well as the comparative level of the mRNA transcript between samples.

Improvements on this technology occurred following the revolutionary discovery and harnessing of reverse transcriptase (RT) and the polymerase chain reaction (PCR) (O'Driscoll et al., 1993). The RT reaction had allowed the formation of recombinant DNA (cDNA) by transcribing back the RNA. With the development of PCR, the concentration of specific RNA transcripts could then be calculated utilizing the measurement of the number of PCR cycles to achieve measurable amplification using transcript-specific primers, compared to a standard housekeeping gene such as *HPRT* and with other appropriate controls. This technique, commonly called Real-Time

Quantitative PCR (qPCR) has a number of advantages over Northern Blotting. Recombinant DNA (cDNA) is more stable then mRNA and is less likely to degrade over the course of the experiment. It is also possible to examine the levels of significantly more transcripts from a single sample utilizing RT-PCR then with Northern blotting. A final advantage is that this method does not utilize radioactive labels.

A major advance in the determination of mRNA transcript levels is the technology commonly referred to as microarray technology (DeRisi et al., 1997). The binding event is reversed from what occurs in a Northern blot. The probe cDNAs, in a massively parallel fashion, are fixed to a solid support. Labeled cDNA representative of the mRNA in a sample is then hybridized onto the bound probe and the concentration of DNA bound to the probe is then determined. In this manner the parallel observations for multiple thousands of spots are determined, limited only by the spacing and delivery of each spot on the solid surface as well as the sensitivity of the detection mechanism.

In the DeRisi lab, two different samples, labeled with either cy5 or cy3 fluorescent dye, are both competitively hybridized to a spotted DNA array on a polylysine glass slide. Using this strategy, the relative concentrations can be calculated by normalization and comparison of the fluorescence of the two dyes directly.

In addition to the advantages that microarray technology shares with RT-PCR over Northern blotting, microarrays also have a number of significant advantages over RT-PCR. First of all, the DNA microarray technology allows massively parallel observations of RNA transcript levels for as many transcripts as there are probes on the array. Microarrays also improve upon RT-PCR methods by allowing large numbers of parallel observations to be observed on the same sample using fluorescent labels instead

of radioactivity. Another advantage is that only one enzymatic step is required, while RT-PCR requires two enzymatic steps. A potential disadvantage is that relatively large quantities of mRNA (μgs) are necessary for satisfactory results.

While multiple technologies exist for the production of microarrays, the DeRisi lab has settled on a method we feel allows for an acceptable balance between price, reliability, and quality of data. The probes themselves consist of PCR products produced from primers designed to individually amplify every predicted open reading frame (ORF) from the yeast genome. While the effort and cost involved in the original amplification is significant, once accomplished enough material is produced for 10-100,000 arrays.

The array itself is manufactured on glass microscope slides coated with poly-lysine. Each probe is then deposited on multiple slides using a custom-built robotic spotter which uses either 16 or 32 print tips to deposit a small volume of PCR product upon each slide in a serial fashion (http://cmgm.stanford.edu/pbrown/mguide/index.html). Currently, in the course of one print run up to 265 identical arrays are produced. These arrays are then processed to prevent the labeled cDNA from sticking nonspecifically to the polylysine coating by the reaction of succinic anhydride in dimethylpyrilidone with the free lysine residues (see Appendix, Protocol #1). This technique can also work utilizing single synthetic 70-mer DNA molecules designed to have similar binding energies. This methodology allow for the low cost production of arrays that can be used to produce large high-quality data sets (Bozdech et al., 2003). We have found this method to be both cost efficient and highly reliable, allowing us to avoid using commercial products where the technology can be cost prohibitive for academic laboratories doing replicate experiments or experiments that

include enough time-points to get an accurate picture of a genome-wide transcriptional response.

There are several protocols currently being used by different laboratories for RNA isolation, sample labeling and hybridization. The extraction protocol used in the work I describe is based on the standard hot-acid phenol Guthry protocol with a few minor modifications (see Appendix, Protocol #2). The extraction protocol used for poly-A isolation procedure was also modified from the standard protocol (see Appendix, Protocol #3).

Poly-A mRNA was used as a template for reverse transcription to create cDNA with amino-allyl incorporation following slightly modified standard protocols (see Appendix, Protocol #5). For example, purification was carried out using DNA spin columns as opposed to filter columns.

Hybridization strategies affect the final quality of the data. Typically for high-quality data each experimental time-point is compared to an internal reference pool for each set of experimental conditions. Changes are defined as the relative ratio of change from a common starting state that is defined as the beginning time-point.

cDNA is labeled with either Cy3 or Cy5 dye by coupling to the amino allyl dUTP introduced during the RT synthesis. This product is purified and immediately hybridized at 65°C for 16 hours. Arrays are washed (see Appendix, Protocol #6) and scanned immediately in small batches of 5 due to the potential for cyanine dye degradation. This degradation has been reported to be due to exposure to environmental ozone (Fare et al., 2003). Each step of the isolation as well as cDNA synthesis is monitored by agarose gel analysis to gauge the success or failure of the procedure.

This experimental procedure is attractive because identical mRNA transcripts theoretically will have identical rates of cDNA formation and thus identical hybridization energies. Implicit in this entire procedure is that similar concentrations of RNA and cDNA are produced and recovered across an experimental set. Degradation of RNA, differential recovery of RNA, or differential rates of RT synthesis will undercut the theoretical premise of this experimental set u. It is therefore very important to be as consistent as possible within a specific set of data. Another important feature of the dual-probe approach versus the single-probe approach is that variations between different slides will affect both probes similarly in the dual probe approach, while utilizing a single-probe strategy, differential hybridization may present a serious flaw in the overall quality of the data.

Arrays are scanned on an axon 2000B scanner following excitation at 530 and 635 nm. Genepix software was used to convert the fluorescent, scanned images to numerical data point spreadsheet files. These files were then loaded into the NOMAD database for storage and retrieval (http://ucsf-nomad.sourceforge.net/). Once a dataset is created using NOMAD it is analyzed by the cluster program. The cluster program groups data with common characteristics (Eisen et al., 1998). It is used to either group genes with similar expressions, arrays with similar expression patterns, or both. The result is then visualized using Treeview which provides a color-based overview of the transcriptional response (Page, 1996) (Saldanha, 2004).

Following the initial publication of the *S. cerevisiae* cDNA microarray that documented yeast's response to the diauxic shift (DeRisi et al., 1997) a large number of responses were profiled. These include the transcriptional program of the cell cycle

(Spellman et al., 1998), sporulation (Chu et al., 1998), and responses to a large number of stresses (Gasch et al., 2001; Gasch et al., 2000), as well as many other drugs and biological compounds. Deletion and mutant strains have also been profiled to determine how mutants respond to specific stresses (Marton et al., 1998). Collectively, these as well as many other experiments have led to a wealth of data that are now available for researchers to explore the intricacies of transcription.

Chapter 2 of this dissertation describes my early work, which served to validate the power of DNA microarrays. During a limited amount of work I rediscovered a significant amount of knowledge about the *S. cerevisiae* response to amino acid limitation as well as how this response is controlled.

Chapter 3 is a reprint from my work published in Molecular Biology of the Cell which describes the discovery of a specific transcriptional response to NO· (Sarver and DeRisi, 2005). The *YHB1*, *SSU1 YNR064c* and *YFL061c/YNL335w* genes were shown to respond to NO· exposure and the role of *FZF1* in this response is described. This work was featured in the monthly periodical feature Incytes published by the American Society for Cell Biology and was nominated for their Paper of the Year.

Chapter 4 describes additional work undertaken to identify the mechanism of activation of *FZF1* in response to nitrosative stress. The body of work presented in Chapter 3 and followed up in Chapter 4 to our knowledge, represents the first published results of the genome-wide *S. cerevisiae* response to nitrosative stress.

Chapter 5 describes future directions others may choose to pursue to follow up on this project.

The studies in this dissertation have identified a novel response which fungal organisms utilize to respond to the presence of NO. The signaling mechanism necessary for this response requires the *FZF1* transcription factor whose role has been defined within this work. A novel response element was discovered to only be present upstream of *FZF1*-dependent responsive genes. The mechanism of activation of *FZF1* was shown to be independent of transcription, translation and *GRR1*-mediated degradation. Furthermore, this activation was shown to require oxygen, likely utilizing a novel post-translational modification. This work may eventually lead to the complete elucidation of an entirely novel yeast signaling pathway, components of which are likely to be conserved throughout higher eukaryotes. Directly stemming from this work, a similar response has been identified in the pathogen *Candida Albicans* and shown to be involved in host-pathogen interaction (Hromatka et al., 2005).

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CHAPTER 2

EXPLORATION OF THE CAPABILITIES OF MICROARRAY TECHNOLOGY

ABSTRACT

Saccharomyces cerevisiae is constantly changing its internal physiology to fit its environment. It has been reported that the transcriptional components of these responses can be accurately monitored on a genome-wide scale utilizing cDNA microarray technology. Applying this technology, the transcriptional responses of *S. cerevisiae* to amino acid deprivation were monitored by cDNA microarray experiments. Results were easily interpretable and had a high level of signal relative to noise. The transcriptional profiles observed following amino acid deprivation experiments recapitulated what was found in the literature and identified new components when analyzing the response to lysine deprivation in particular. In addition, a search of the promoters of both the canonical and novel lysine-responsive genes revealed a conserved response element. This element was important for transcriptional activation following amino acid deprivation and was present in all the promoters. Therefore, a mechanism for transcriptional induction following lysine deprivation was identified.

INTRODUCTION

Microarray technology has helped revolutionize how modern research science is conducted. Instead of labor-intensive screens and traditional single-gene genetic analyses, genome-wide transcriptional levels and responses can now be accurately analyzed and can provide a starting point for hypothesis-driven research. This chapter of my dissertation provides a record of some of my early work to establish my expertise with the techniques of microarray analyses and provide some insight into my rationale for working on the questions that I ended up addressing in the rest of this dissertation.

A vast amount of research has been published regarding how organisms respond to nutrient deprivation. *Saccharomyces cerevisiae* is capable of biosynthesizing the amino acids and nucleotides that are necessary to carry out the processes of life (Braus, 1991; Denis-Duphil, 1989; Kohlhaw, 2003). Following the limitation of amino acid or nucleotide availability, biosynthetic pathways are activated to provide a means for *de novo* biosynthesis of these essential building blocks. In the literature, two different types of regulation have been reported for the transcriptional control of this response. The first utilizes specific effector molecules to turn on and off specific transcriptional activators or repressors (usually by causing a conformational change or change in phosphorylation state upon binding), which leads to specific control of a single pathway (Bhattacharjee, 1985). The second type of amino acid biosynthesis regulation involves the sensing of excess uncharged tRNA by the cell, which then activates the kinase activity of General Control Nonderepressible 2 (*GCN2*), leading to a series of events that result in the increased translation of *GCN4*, which in turn leads to the transcriptional activation of

GCN4 target genes, resulting in the activation of a large number of biosynthetic pathways (Hinnebusch, 1993).

I chose to explore the genome-wide transcriptional response to deprivation of all supplementary amino acids and nucleotides, as well as deprivation of only lysine or only adenosine, within the growth medium of *S. cerevisiae* because there are specific transcriptional responses that are known to occur under conditions of limited availability. In undertaking these experiments I had several goals. First, I wondered what level of noise existed within genome-wide transcript level data from identically treated cultures. I also asked if a biologically meaningful response would be visible within the background noise. I was also interested in determining whether the response to the limitation of specific amino acids or nucleotides would fit directly within the larger response to all supplementary amino acids. Taking a hint from the work of Ogawa et al. on the phosphate metabolism (PHO) pathway utilizing expression profiling (Ogawa et al., 2000), I was interested in determining what new information I could uncover about well-studied metabolic responses using genomic methods. With the availability of the entire genome sequence, I additionally wanted to determine if meaningful promoter sequences could be deduced from examination of the sequence within the promoter region of transcriptionally-responsive genes. To sum it all up, prior to starting my doctoral work I wanted to assure myself that I could make the DNA microarray system work in my own hands and that I could interpret the data in a meaningful manner.

As a result of this work I was able to identify new genes responsive to lysine deprivation, identify a phenotype associated with the deletion of one of these genes,

rediscover a response element in the promoter of lysine-responsive genes, and identify new instances of this response element.

MATERIALS AND METHODS

Yeast strains and media

All microarray experiments were conducted using the DBY7283 mat alpha strain in either synthetic complete (SC), synthetic minimal (SM), synthetic complete without lysine (SCL), or synthetic complete adenosine (SCA) medias. All cultures were harvested at OD_{600} 1.0 by vacuum filtration following minimally 8 hours of logarithmic growth in the specific media. All deletion strains were obtained from the Research Genetics Deletion Library. Yeast strains and sources are found in TABLE 4-1.

Microarray production

Microarrays were produced using ~6000 PCR products representing the predicted open reading frames (ORFs) of *S. cerevisiae* (DeRisi et al., 1997). The PCR products were individually spotted in defined locations on poly-lysine coated glass slides utilizing a custom built spotting robot (http://cmgm.stanford.edu/pbrown/mguide/index.html).

Microarray experiments

Microarray experiments were conducted with modifications of existing lab protocols. Modified protocols for RNA isolation, mRNA isolation, reverse transcription, post-processing, labeling and hybridization are found in the Appendix. Arrays were scanned on a Genepix 4000B scanner (Axon instruments).

Data analyses

Cy3 and Cy5 plots were created using Genepix 3.0 software from Axon Instruments. Genepix 3.0 result files were submitted to the NOMAD database (http://dest-nomad.sourceforge.net). Unflagged spots were included for cluster analysis only if the sum of the median intensities was greater than the median background plus 2 times the standard deviation of the background. Following extraction of the data, results were analyzed using the cluster program (Eisen et al., 1998) and visualized using treeview (Page, 1996). In order for inclusion in Figure 2 genes needed to be induced greater then 2-fold in the dropout media. The MEME algorithm was used to search the promoter regions for likely response elements (Bailey and Elkan, 1994).

RESULTS

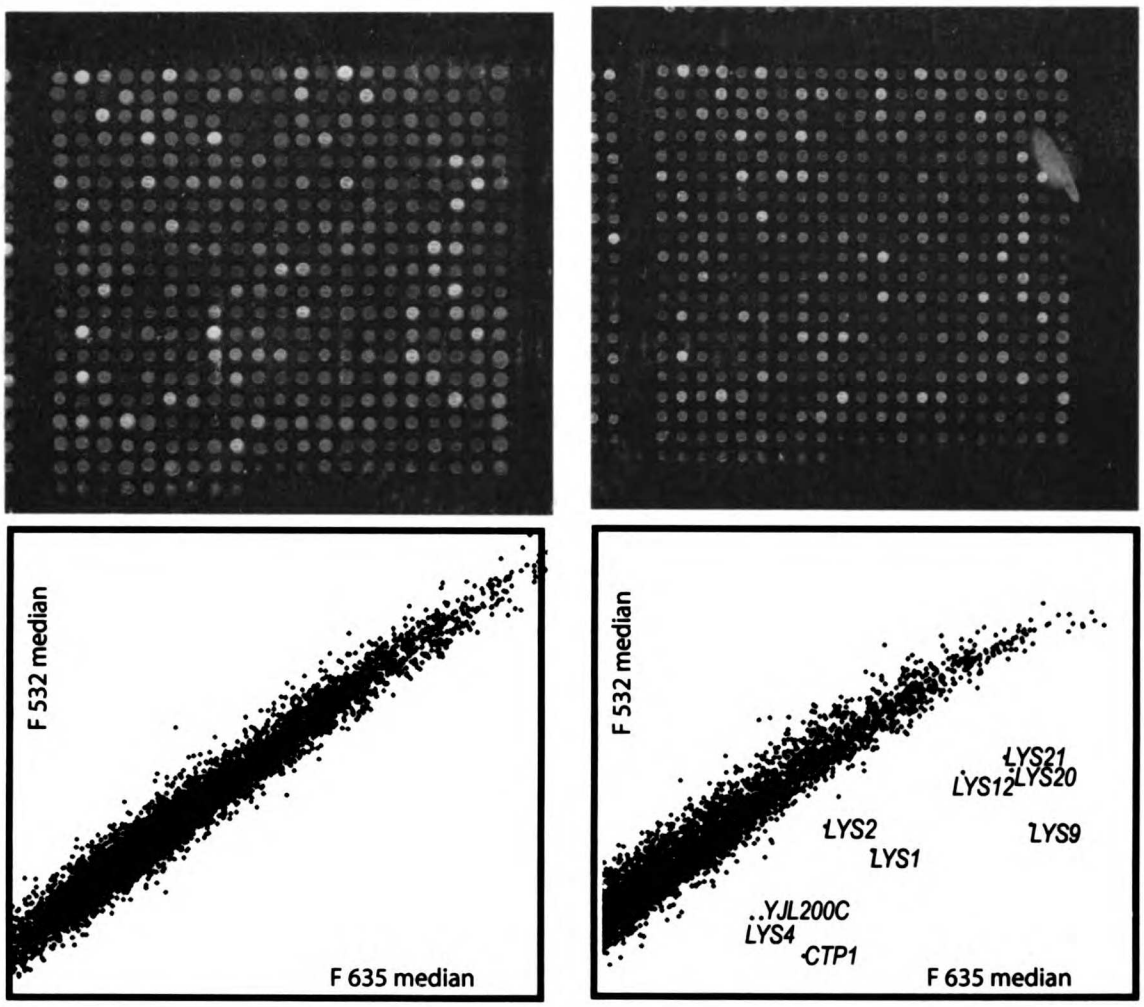
Prior to profiling the transcriptional response to diverse environmental conditions I needed to determine the level of noise that exists between two yeast cultures growing under identical conditions. To determine this level of noise by microarray experiments, mRNA was isolated and reverse transcribed from independently grown and isolated yeast cultures in SC media. The resulting cDNA was labeled with either Cy3 or Cy5 fluorescent dye and hybridized on spotted cDNA arrays. An overlaid image of the two-color fluorescent hybridization result is shown in Figure 1A. A graph of the Cy3 median ratio compared to the Cy5 median ratio is shown in the lower panel. In this biological-replicate hybridization no gene is induced greater than 2-fold in either the raw data or following normalization.

Conditional transcriptional responses

Figure 2-1

A. SC media (635) vs SC media (532)

B SCL media (635) vs SC media (532)

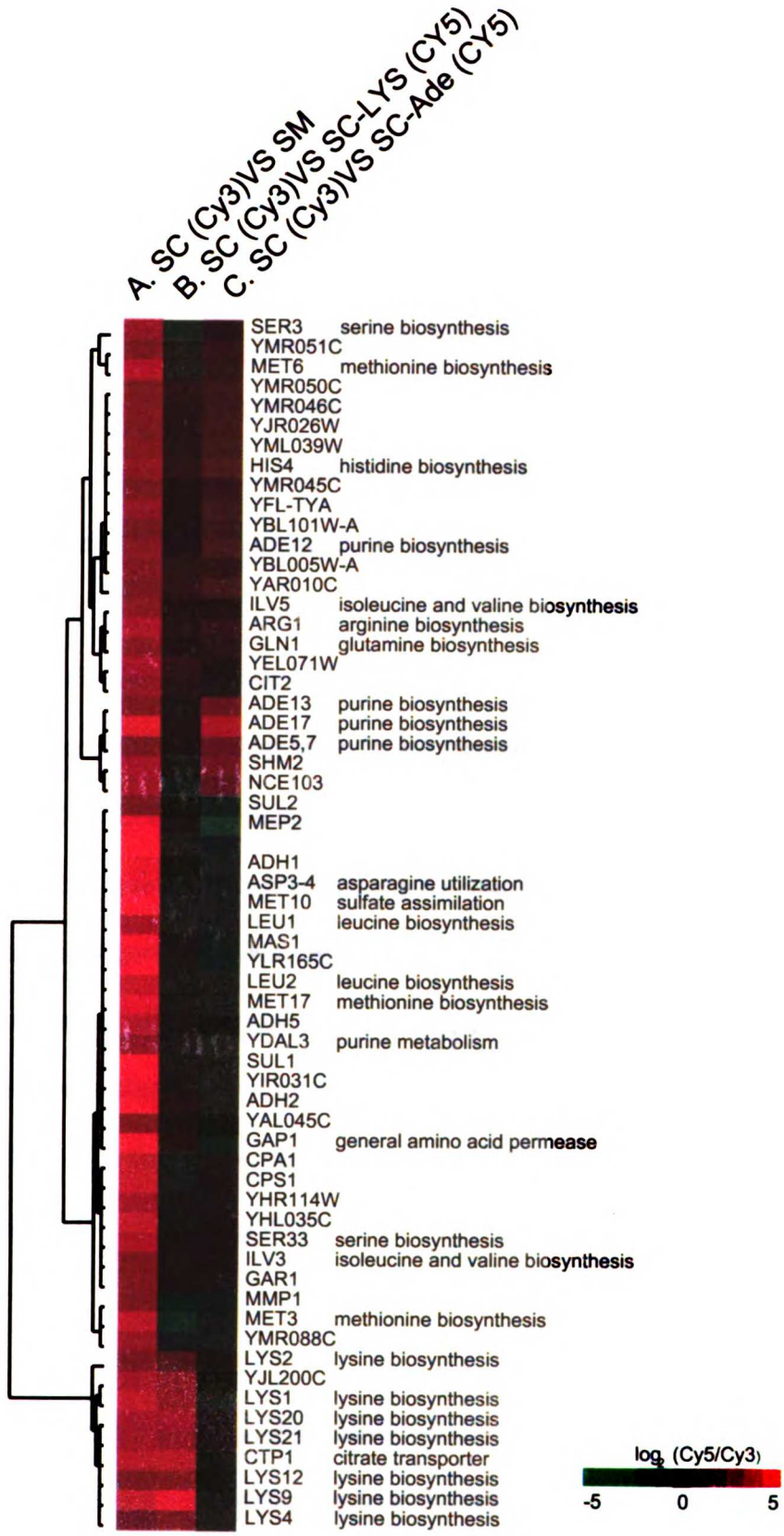


An experiment was carried out to compare the RNA transcript levels from yeast grown in SC media and SCL media. In the SCL media the yeast must biosynthesize the lysine they require for growth. DNA microarrays were used to examine the genome-wide transcriptional levels of these two different states. The cDNA library obtained from the SC media was labeled with Cy3 dye and the cDNA from the SCL media was labeled with Cy5 dye. An overlaid image of the resulting two-color hybridization is shown in Figure 1B. A graph of the Cy5 versus Cy3 ratio is again shown in the lower panel. Quantification of the Cy5- versus Cy3-fluorescent signal between the two samples revealed that 9 genes were induced by more than 2-fold in the SCL media compared to the SC media. Induced genes in the yeast grown in SCL media in descending order are *LYS9, CTP1, LYS20, LYS21, LYS1, LYS12, YJL200C, LYS14*, and *LYS4*. Seven out of these 9 genes have known roles in lysine biosynthesis. The results are also shown in treeview format in Figure 2B.

A second experiment was also conducted comparing yeast grown in SC media to yeast grown in SCA media. In the SCA media the yeast must synthesize their own adenosine. Genes induced greater then 2-fold in the yeast grown in ADE-delete media in descending order are *Ade17, SHM2, HTB2, ADE13, ADE5-7, TEF2* and *ADE2*. Six out of these 7 genes have known roles in adenosine biosynthesis (Figure 2C).

A third experiment was conducted comparing yeast grown in SC versus yeast grown in SM media. In this restricted media yeast must synthesize all amino acids that they need to grow *de novo*. Genes that were induced following deprivation of lysine and adenosine were also induced in the minimal media. In addition, genes involved in the biosynthesis of many other amino and nucleic acids were induced, as expected. The

Figure 2-2


A. SC (Cy3)VS SM
B. SC (Cy3)VS SC-LYS (CY5)
C. SC (Cy3)VS SC-Ade (CY5)
SER3 serine biosynthesis
YMR051C
MET6 methionine biosynthesis
YMR050C
YMR046C
YJR026W
YML039W
HIS4 histidine biosynthesis
YMR045C
YFL-TYA
YBL101W-A
ADE12 purine biosynthesis
YBL005W-A
YAR010C
ILV5 isoleucine and valine biosynthesis
ARG1 arginine biosynthesis
GLN1 glutamine biosynthesis
YEL071W
CIT2
ADE13 purine biosynthesis
ADE17 purine biosynthesis
ADE5,7 purine biosynthesis
SHM2
NCE103
SUL2
MEP2
ADH1
ASP3-4 asparagine utilization
MET10 sulfate assimilation
LEU1 leucine biosynthesis
MAS1
YLR165C
LEU2 leucine biosynthesis
MET17 methionine biosynthesis
ADH5
YDAL3 purine metabolism
SUL1
YIR031C
ADH2
YAL045C
GAP1 general amino acid permease
CPA1
CPS1
YHR114W
YHL035C
SER33 serine biosynthesis
ILV3 isoleucine and valine biosynthesis
GAR1
MMP1
MET3 methionine biosynthesis
YMR088C
LYS2 lysine biosynthesis
YJL200C
LYS1 lysine biosynthesis
LYS20 lysine biosynthesis
LYS21 lysine biosynthesis
CTP1 citrate transporter
LYS12 lysine biosynthesis
LYS9 lysine biosynthesis
LYS4 lysine biosynthesis
log2 (Cy5/Cy3)
-5
0
5

majority of the induced genes have known roles in the biosynthesis of amino acids and nucleic acids (Figure 2A).

YJL200c deletion phenotype

Because *YJl200c* was induced along with known lysine biosynthetic genes, I hypothesized that a yeast strain with a deletion in *YJL200c* would show a hypersensitivity phenotype in media lacking lysine. On complete media plates, the *YJL200c* deletion strain grew at a growth rate indistinguishable relative to wild type. On plates that did not contain lysine, the *YJL200c* strain showed a slow-growth phenotype relative to wild type. On the plates without lysine, strains with either the *LYS12* or *LYS5* genes deleted did not grow (data not shown).

Conserved sequence found in lysine-responsive genes

To determine if there was a physical basis for the transcriptional response to the absence of lysine, the 600-bp DNA sequences upstream from the start codon of the promoter regions of the nine lysine-responsive genes were searched using the Meme Sequence Similarity search algorithm. This search revealed that a conserved sequence was in the promoter regions of all nine lysine-responsive genes. Examination of this sequence revealed that it was composed of two regions of highly conserved bases with a less constrained middle region flanked by an AT sequence. The consensus sequence for this is WWWTCCVBNGGVAWW (Figure 3).

DISCUSSION

As a result of this work it became clear that DNA microarray technology can adequately represent the transcriptional state of the cell in a biologically meaningful

Figure 2-3 LYS response element

YJL200c	AAATTCCACTGGAAAA
LYS1	AAATTCCGCTGGAAAA
LYS9	AAATTCCGTTGGAAAA
LYS21	AAATTCCGCTGGAAAT
LYS2	GAATTCCGCTGGCAAA
LYS20	AAATTCCGCCGGAATA
LYS4	AAAATCCCGGGGGAAA
CTP1	AAATTCCGCAGGAATT
LYS12	TTTTTCCACGGGGAAA
consensus	wwwTCCVBNGGVAww
UASLYS	TCCRNYGGA

manner, with low levels of noise relative to the transcriptional responses visualized. In addition, differences between two biologically distinct transcriptional states can be determined, and these changes can easily be directly separated from the noise of well-designed and well-executed experiments. This transcriptional response data can then be used to further design hypothesis-driven research, which can define gene functions, define physical sequence basis for transcriptional activation and lead to a better understanding of signaling response networks.

During this work genes were identified as being induced in response to deprivation of lysine, adenosine and all supplementary amino acids and nucleotides. Comparison to the literature for these types of experiments reveals that the DNA microarrays were accurate and thorough in identifying responsive mRNA transcripts. All previously reported lysine-responsive genes were 'rediscovered' in this work (Bhattacharjee, 1985). In a manuscript published after the completion of this work, genome-wide studies of the lysine response further verified that *YJL200c* and *CTP1* are responsive to lysine depletion (Breitling et al., 2002). Previously reported adenosine-responsive genes were also 'rediscovered' and previously reported amino acid- and nucleotide-responsive genes were also 'rediscovered'.

This work also showed that transcriptional co-regulation can define gene function. In a sufficiently well-designed experimental system, novel roles of specific genes can be hypothesized and these suppositions can be tested. In the course of this research the *YJL200c* gene was shown to have a previously unknown role in lysine biosynthesis.

The genome-wide transcriptional responses of *S. cerevisiae* can be determined using DNA microarrays and the co-regulation discovered should have a physical basis.

The responsive gene promoter sequence can be analyzed to determine activation sequences unique to the specific response. In the course of this research the lysine activation sequence was identified in the promoters of lysine-responsive genes and additional copies of this response element were discovered in the promoters of the *YJL200c* and *CTP1* genes. The element found within these studies overlaps with the LYSUAS, an element that Lys14p has been shown to shift (Becker et al., 1998) and is necessary for transcriptional induction in the absence of lysine (Ramos et al., 1988). The LYSUAS is TCCYNRGGA in comparison to our discovered sequence WWWTCCVBNGGVAWW. The modest increased specificity of our sequence is likely due to the examination of all relevant promoters while the UAS was derived with incomplete genomic information.

Multiple overlapping conditions can better define and narrow down the transcriptional state compared to individual experiments. To put it another way, the more high-quality experiments or data you have, the better information you have to formulate a model about what biology is probably occurring. During the course of this research, genes that were responsive to either lysine or adenosine deprivation separately were also found to be responsive to deprivation of all amino acids and nucleotides (as would be expected).

During this time, I also used DNA microarrays to profile the response of *S. cerevisiae* to a number of anti-fungal and anti-fungal-related drugs (data not shown). The results of these experiments were difficult to interpret and reinforced in my mind the potential complexity of biological networks. It is unlikely that yeast were exposed to, and thus developed specific adaptations to, these artificially created anti-fungals as a part

of their evolutionary history. The response to these compounds is probably not a specific adaptation, but rather a non-specific response to a monkey wrench in the gears of the transcriptional program. The difficulty in interpreting these results led me to look for novel environmental perturbations that *S. cerevisiae* may have been exposed to, and thus adapted to respond to, during the course of evolutionary history. The end result of this train of thought, spurred by previous work (Sarver et al., 2001), led me to the following exploration of how yeast respond to the presence of exogenously supplied nitric oxide.

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Table 2-1 *S. cerevisiae* strains utilized

Strains	*Genotype*	*Origin*
DBY7283	MATa, ura3-52	Botstein (FY22)
Diploid yjl200cΔ	BY4743 yjl200cΔ/ yjl200cΔ + KAN	(Resgen)
Diploid lys5Δ	BY4743 lys5Δ/ lys5Δ + KAN	(Resgen)
Diploid lys5Δ	BY4743 lys12Δ/ lys12Δ + KAN	(Resgen)

Figure 1. Microarray images and CY5/CY3 ratios from hybridizations of A) identical growth conditions and separate isolations, and B) growth in synthetic complete media lacking lysine media (CY5) compared to growth in synthetic complete media (CY3).

Figure 2. DNA microarray analyses. Data from three separate array hybridizations were analyzed using the Cluster Program. All three experimental conditions were hybridized to a sample obtained following growth in synthetic complete media. A) Growth in synthetic minimal media supplemented with uracil. B) Growth in synthetic complete media lacking lysine. C) Growth in synthetic complete media lacking adenosine. The color saturation indicates the magnitude of the expression ratio as indicated by the scale in the lower left hand corner.

Figure 3 Response element 'Discovered' in the promoters of lysine responsive genes compared to the UASLYS

Fzf1p regulates an inducible response to nitrosative stress in *Saccharomyces cerevisiae*

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Running Title: *FZF1* nitric oxide response

Key Words: *YHB1 SSU1 FZF1* nitric oxide cerevisiae microarray

Abbreviations: NO·, RNI, DPTA NONOate, APD

ABSTRACT

The mechanisms by which microorganisms sense and detoxify nitric oxide (NO·) are of particular interest due to the central role this molecule plays in innate immunity. We investigated the genetic basis of inducible nitric oxide (NO·) detoxification in *Saccharomyces cerevisiae* by characterizing the genome-wide transcriptional response to exogenously supplied NO·. Exposure to the NO· generating compound DPTA NONOate resulted in both a general stress response as well as a specific response characterized by the induction of a small set of genes including the yeast flavohemoglobin *YHB1*, *SSU1*, and three additional uncharacterized open reading frames. Transcriptional induction of *SSU1*, which encodes a putative sulfite transporter, has previously been shown to require the zinc finger transcription factor Fzf1p. Deletion of Fzf1p eliminated the nitrosative stress-specific transcriptional response while over expression of Fzf1p recapitulated this response in the absence of exogenously supplied NO·. A *cis*-acting sequence unique to the promoter regions of Fzf1p-dependent genes was found to be sufficient to activate reporter gene activity in an NO·- and Fzf1p-dependent manner. Our results suggest that the presence of NO· or NO· derivatives activates Fzf1p leading to transcriptional induction of a discrete set of target genes that function to protect the cell from NO·-mediated stress.

INTRODUCTION

Saccharomyces cerevisiae responds to a wide array of environmental signals through a variety of mechanisms including induction and repression of specific transcriptional programs. For example, the response to oxidative stress is well characterized and involves the induction of antioxidant species such as superoxide dismutase, catalase, and peroxidase, all of which improve the ability of the cell to survive exposure to reactive oxygen species (Costa and Moradas-Ferreira, 2001). In this work, we sought to identify components of the pathway by which *S. cerevisiae* senses and detoxifies exogenously supplied nitric oxide (NO·).

NO· is a membrane-permeable free radical that is biologically produced by the nitric oxide synthase (NOS) family of enzymes (Sessa, 1994) and by denitrifying bacteria (Xu and Verstraete, 2001). NO· reacts in a concentration and environmental dependent manner leading to the formation of reactive nitrogen intermediates (RNIs). NO· and resulting RNIs have been shown to have both cytostatic and cytotoxic activity due to the inhibition of ATP production ((Stevanin *et al.*, 2000) (Brown, 1997) (Chenais *et al.*, 2002), altered iron metabolism (Chenais *et al.*, 2002), (D'Autreaux *et al.*, 2002), (Martinez *et al.*, 2001)), direct inhibition of enzymes (Gardner *et al.*, 1997), and DNA damage ((Kow, 2002), (Martinez *et al.*, 2001)). NO· has been strongly implicated as a component in higher eukaryote non-specific immune response to parasites, fungi, bacteria and viruses (Fang, 1997). Host inducible nitric oxide synthase (iNOS) expression and endogenous NO· levels increase in response to infection by a wide range of pathogens including *Plasmodium falciparum* (Kun, 2003), *Leishmania major* (Bogdan *et al.*, 2000),

Candida albicans (Elahi *et al.*, 2001), *Borrelia burgdorferi* (Harter *et al.*, 1999) and cytomegalovirus (Tanaka and Noda, 2001). For these pathogenic organisms, the ability to detoxify NO· may be important for their survival, proliferation, and virulence.

Microorganisms have developed mechanisms to detoxify NO· including the conversion of NO· to nitrate. This reaction is catalyzed by the *Escherichia coli* flavohemoglobin protein *hmp* (Gardner *et al.*, 1998). Homologues of *hmp* are present in many bacteria and yeast species and are induced by reactive nitrogen species in *E. coli* (Poole *et al.*, 1996), *Salmonella typhimurium* (Crawford and Goldberg, 1998), and *C. albicans* (Ullmann *et al.*, 2004). Deletion of flavohemoglobin in the human pathogens *C. albicans* and *Cryptococcus neoformans* leads to a decrease in virulence in mouse models of infection (Ullmann *et al.*, 2004) (de Jesus-Berrios *et al.*, 2003).

The *hmp* ortholog in *S. cerevisiae* is Yhb1p (Zhu and Riggs, 1992), which is required for metabolism of NO· (Liu *et al.*, 2000). *yhb1*Δ strains are hypersensitive to exogenous NO·, implying an important detoxification role for Yhb1p (Liu *et al.*, 2000).

We hypothesized that *S. cerevisiae* might possess mechanisms capable of responding to exogenous NO· or other forms of nitrosative stress. To test this hypothesis, we used cDNA microarrays to track changes in *S. cerevisiae* RNA transcript abundance levels after exposure to exogenously supplied NO·. From this genome-wide survey, we were able to identify and characterize a specific nitrosative stress response. This response includes the induction of the *YHB1* and *SSU1* genes as well as three other uncharacterized open reading frames. We also show that the transcription factor Fzf1p is necessary for this response and further characterize the *cis*-acting determinants sufficient

for Fzf1p-dependent transcriptional activation. Finally, we show both *YHB1* and *SSU1* contribute to nitrosative stress resistance, depending on growth conditions and that absence of *FZF1* results in hypersensitivity to nitrosative stress.

MATERIALS AND METHODS

Strains and media

Yeast strains and sources are listed in Table 1. All experiments were conducted in YPD, synthetic complete, or –URA media with galactose, dextrose or raffinose as a carbon source at 30°C.

Nitric oxide sources

DiPropyleneTriAmine NONOate (DPTA NONOate) was purchased from Cayman Chemical Company (Ann Arbor,). N-(3-Aminopropyl)1,3-Propane Diamine (APD) was purchased from Aldrich (Milwaukee, WI). DPTA NONOate was dissolved in NaOH Solution (10 mM), and the pH was modified by the addition of Tris buffer (100 mM, pH 7.0), immediately prior to addition to the cultures.

NO· gas was generated by the reaction of sodium nitrite with hydrochloric acid (Poole *et al.*, 1996).

Growth conditions

Strains were grown in 1.2 L of media in 2.8 L Erlenmeyer flasks with mechanical agitation in a 30°C room. Upon reaching an OD_{600} of 0.2, the cultures were exposed to

treatment. Time-points were harvested at 0, 10, 20, 40, and 80 minutes by vacuum filtration and snap frozen in liquid nitrogen prior to RNA isolation.

Time course response experiments were conducted using strain DBY7283 in SCD media following mock exposure, exposure to APD (100 μM), or exposure to DPTA NONOate (100 μM)(Figure 1 A-C). Similarly, time course response experiments were conducted using *YHB1-GFP* and *YHB1-GFP fzf1Δ* strains following exposure to DPTA NONOate (Figure 1D-E). Additionally, time course response experiments were conducted in YPD media utilizing DBY7283, diploid *yhb1Δ/ yhb1Δ*, and diploid *fzf1Δ/ fzf1Δ* strains starting at OD 1.0 following exposure to DPTA NONOate (1 mM). In addition to the first 5 time-points, cells were also harvested at 120 minutes (Figure 1F-H).

A single-timepoint microarray experiment compared a DBY7283 culture exposed to chemically generated nitric oxide (5 ml gas per 200 ml of culture) for 120 minutes to an untreated DBY7283 culture (Figure1I).

For the Fzf1p overexpression profiling experiments, DBY7283 + pLacZ, and DBY7283 + pFzf1p strains were both grown to OD_{600} 0.4 in Synthetic Complete media without uracil containing 2% raffinose. Galactose was added to a final volume of 2% and fractions were collected 0, 40, 80, and 120 minutes after addition for each time course.

RNA isolation and microarray analyses

Total RNA was isolated using hot acid phenol chloroform extraction and mRNA was then purified using oligo-dT resin. Poly(A) + mRNA was reverse transcribed, using a 1:1 mixture of oligo-dT and random hexamers to incorporate aminoallyl-dUTP into the

resulting cDNA. cDNA was differentially labeled with Cy3 and Cy5 dyes purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Hybridization to cDNA microarrays representing the yeast genome was conducted as previously described (DeRisi *et al.*, 1997).

For each set of time courses, Cy5 labeled cDNA derived from each time-point was compared to a Cy3 labeled reference pool representative of the time-points within each set of time courses. The time course profile was then obtained by dividing the ratios for each time-point by the corresponding ratio for the zero hour time-point.

Microarray data was stored and extracted using the NOMAD database (http://ucsf-nomad.sourceforge.net/). Unflagged spots were included for cluster analysis only if the sum of the median intensities was greater than the median background plus 2 times the standard deviation of the background. The Cluster program (Eisen *et al.*, 1998) was used for data analyses and Treeview (Saldanha, 2004) was used for data visualization. Microarray data used to generate Figure 1 is in supplementary Table 4, and all raw microarray data from this study is freely available at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). To be included in the nitrosative stress response overview (Figure 1), data needed to be present in all 0 hour timepoints and transcript levels were required to change greater than 5.6-fold ($2^{2.5}$) in 2 or more time-points of the 8 time courses shown.

To calculate the correlations to other environmental stress responses, data (Gasch *et al.*, 2000) was merged with the DPTA NONOate response data and genes with a greater than 5.6-fold ($2^{2.5}$) response in 2 or more arrays were selected to calculate Pearson

correlations.

Western blot analyses

TAP-tagged yeast strains *YHB1-TAP* and *SSU1-TAP* (Ghaemmaghami *et al.*, 2003), were grown to mid-logarithmic phase in SCD media and treated with DPTA NONOate (100 μM) for 3 hours. Cells were then harvested and a standard bead beating protocol was used to generate whole cell extract for Western blots (Ausebel FM, 2004). Protein concentration was measured using a Bradford assay (BIO-RAD) 15 μg protein was loaded into each well and proteins were resolved on a 10% SDS-PAGE gel followed by transfer onto nitrocellulose membrane using 25 mM Tris and 192 mM glycine in 20% methanol. Immunoblotting was conducted in 2.5% milk in TBST with primary rabbit anti-CBP antibody (Bethyl, Montgomery, TX) diluted 1:2500 and the secondary goat anti-rabbit HRP conjugated antibody (Bio-Rad) was used at a 1:5000 dilution. Western blots were visualized using Supersignal West Pico Chemiluminescence Substrate (Pierce, Rockford, IL).

Flow cytometry analyses

YHB1-GFP (Huh *et al.*, 2003) and *YHB1-GFP fzf1*Δ strains were grown to OD_{600} 0.2 and exposed to DPTA NONOate (2.6 - 500 μM) in SCD diluted as described above. NO· gas was removed from the generation apparatus by a syringe and bubbled directly into media containing growing yeast. Flow cytometry data was obtained on a BD Biosciences LSR II flow cytometer (San Jose, CA).

Growth inhibition and resistance

To determine genotype-dependent growth inhibition and resistance, growth following treatment with DPTA NONOate was compared to untreated growth. Exponentially growing haploid wild type (wt), *yhb1*Δ, *ssu1*Δ, and *fzf1*Δ strains at OD_{600} in both SCD and YPD were exposed to a wide range of DPTA NONOate concentrations (0-1 mM) and final cell density was monitored by OD_{600} 12 hours following treatment.

Exponentially growing DBY7283 pFzf1p, DBY7283 pLacZ, and *fzf1*Δ pLacZ strains were exposed to DPTA NONOate (0-2 mM) in SG-URA media. After 12 hours, final cell density was monitored by OD_{600} and compared to untreated cell density.

LacZ experiments

LacZ experiments were conducted as previously described (Russell *et al.*, 1986), with the following modification: Yeast Protein Extraction Reagent (Pierce) was used for the protein extractions from samples collected 180 minutes after exposure to the presence or absence of DPTA NONOate (100 uM). All lacZ experimental results are shown in Miller Units.

Strain and plasmid construction

All yeast transformations were done by the lithium acetate method (Gietz and Schiestl, 1991), and targeted integrations were verified by PCR.

To construct the *SSU1* promoter reporter strain AS101, *Eco*R1 and *Xho*1 sites were introduced into the promoter region –568 to –45 of the *SSU1* locus by PCR amplification using the primers 5'-GGAATTCATGTGGAAAAAGAAGGGGTGG and 5'-ATACCGCTCGAGAATTGCGTATTGTCTGAG with genomic DNA as template,

and cloned into placZi. This plasmid was then linearized with *Apa*1 and integrated into the ura3 locus in YM4271. AS102, AS103, AS106, and AS108 were constructed similarly, using the underlined restriction sites (Table 2).

To construct AS104, a *SSU1* promoter reporter strain –568 to –45 with the region –389 to –370 was removed, and the PCR product used to create AS106 was cloned into the plasmid created for the construction of AS103.

The *SSU1* CS2 reporter strain AS105 was constructed by annealing the oligos encoding the CS2 with sticky ends 5'- AATTCCTGCAAACTATCATTTTTT and 5'- TCGAAAAAATGATAGTTTGCAGG into the multiple cloning site of pLacZi, which was integrated as described above.

The *YHB1* CS2 reporter strain AS109 was constructed as described above for AS105 except oligos encoding the *YHB1* CS2 sequence with sticky ends 5'- AATTCTGAAAATGATAGTCTGCGCT and 5'- TCGAAGCGCAGACTATCATTTTCAG were used.

To construct the *fzf1Δ* strains AS201-209, the *FZF1* gene was deleted in AS101-109 and *YHB1*-GFP using a PCR product constructed using primers 5'- TACGCTGGTGTGCACAAGTGGTACCAGAATACGTGGCCAAAACAATCGGATC CCCGGGTTAATTAA and 5'- ATAGTTCGAATCACATGAGTAGAGGACGGAAATTGCTCTTCTATGGCGTG AATTCGAGCTCGTTTAAAC, with a Pringle kanamycin resistance plasmid as template (Longtine *et al.*, 1998). This PCR product was also used to remove the *FZF1* gene from

the *YHB1*-GFP strain (Huh *et al.*, 2003).

The DBY7283 +pFzf1p galactose-mediated Fzf1p overexpression strain, was created by amplifying the complete 900 bp coding sequence for the *FZF1* gene using the primers 5'-ACAATGACGGATATAGGGAGAACCA and 5'-TCAGTATTCGAATAAATCCCAGACGCT which was cloned into the pYES2.1 vector (Invitrogen), and the resulting plasmid was transformed into DBY7283. To construct *YHB1*-GFP +pFzf1p, the pYES2.1 Fzf1p plasmid was transformed into *YHB1*-GFP. To construct DBY7283 +pLacZ, the pYES2.1 LacZ plasmid was transformed into the DBY7283 strain. To construct the DBY7283 pLacZ strain, the pYES2.1 LacZ (Invitrogen) plasmid was transformed into DBY7283. To construct the *fzf1*Δ pLacZ strain, the pYES2.1 LacZ (Invitrogen) plasmid was transformed into *fzf1*Δ.

RESULTS

Transcriptional response to DPTA NONOate in SCD media

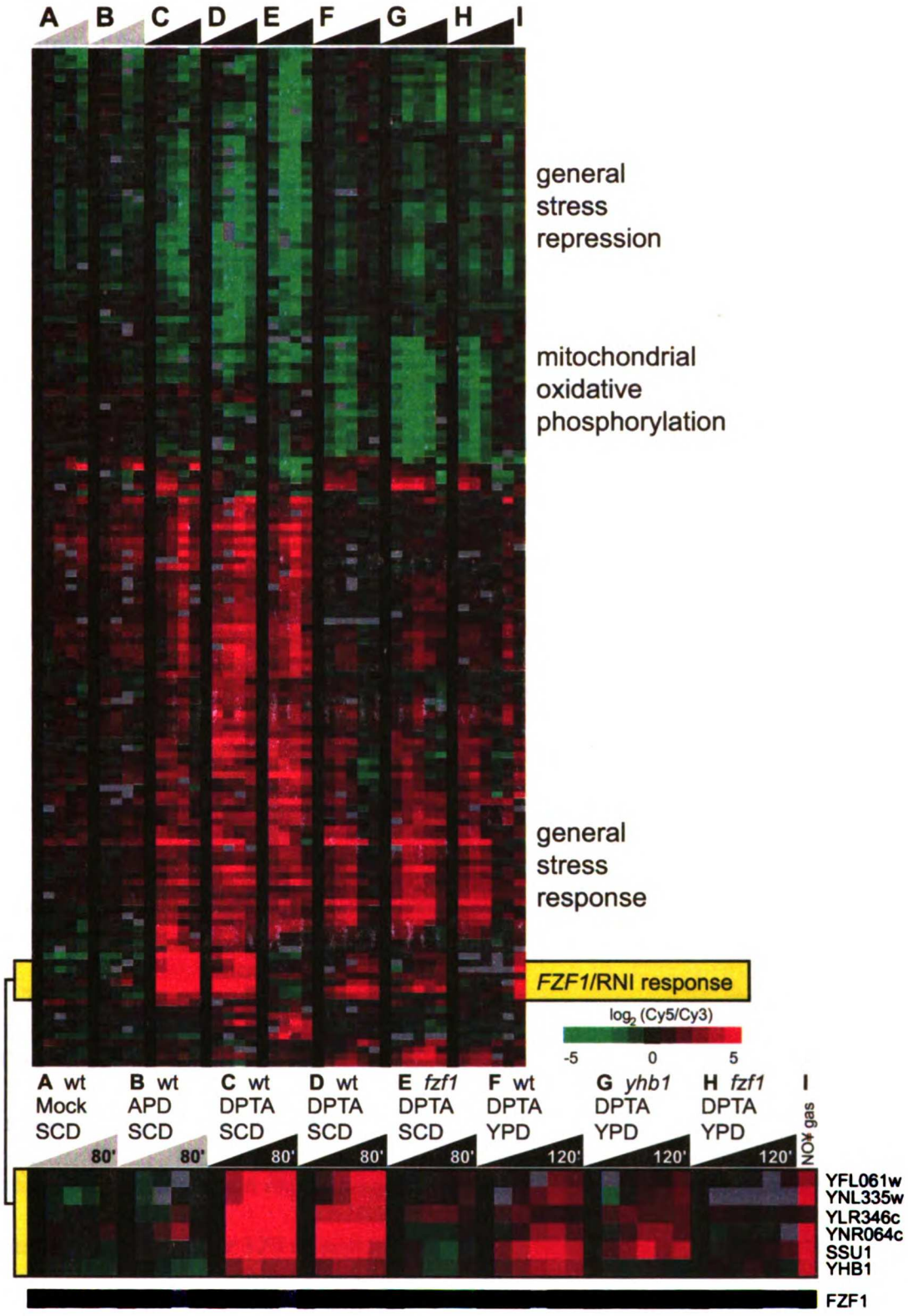
We used microarray expression analysis to identify the set of genes transcriptionally regulated by exposure to the NO· releasing compound DPTA NONOate. DPTA NONOate releases NO· in a time, temperature, and pH dependent manner. The half-life of DPTA NONOate for NO· production in aqueous solution at neutral pH is approximately 3 hours (Mooradian *et al.*, 1995). Following the release of two molar equivalents of NO·, DPTA NONOate degrades into N-(3-aminopropyl)-1,3-propane diamine (APD). Production of NO· in the presence of oxygen may generate additional reactive nitrogen species (RNIs), including peroxynitrite, NO_2, and N_2O_3. It is important

to note that these experiments do not attempt to distinguish between the direct effects of NO· and RNIs.

A distinct transcriptional response occurred following treatment with DPTA NONOate (100 μM) in SCD media. The response was reproducible: an independent biological replicate experiment showed a very similar genome-wide transcriptional response (correlation 0.77). Following mock treatment or treatment with the DPTA NONOate breakdown product APD, expression profiles differed substantially from those produced after DPTA NONOate addition (correlation Mock to DPTA1 0.35 and DPTA2 0.37) (correlation APD to DPTA1 0.45 and DPTA2 0.46). The mock and APD time courses were very similar to each other (correlation 0.80) and showed very little response overall (Figure 1A-D).

Examination of the response to DPTA NONOate treatment revealed that the strongest and most lasting induction occurred in a cluster which contained *YHB1*, *SSU1*, and three uncharacterized ORFs: YFL061w, YNL335w, and YNR064c. Yhb1p catalyzes the reaction of NO· with oxygen to create nitrate, limiting exposure of the cell to NO· (Liu *et al.*, 2000). The *SSU1* gene encodes a trans-membrane sulfite efflux transporter that confers resistance to sulfite when present in multiple copies, and when deleted produces sulfite hypersensitivity (Park and Bakalinsky, 2000). YNR064c is a conserved member of the alpha/beta hydrolase super-family with orthologues in *Homo sapiens* (25% identical) ($1 \times e^{-9}$), *Arabidopsis thaliana* (24% identical) ($1 \times e^{-10}$) and *Mycobacterium tuberculosis* (24% identical) ($1 \times e^{-14}$). YNL335w and YFL061w are homologous open reading frames, which differ by only one silent mutation. They are

Figure 3-1


A B C D E F G H I
general stress repression
mitochondrial oxidative phosphorylation
general stress response
FZF1/RNI response
log2 (Cy5/Cy3)
-5 0 5
A wt Mock SCD 80'
B wt APD SCD 80'
C wt DPTA SCD 80'
D wt DPTA SCD 80'
E fzf1 DPTA SCD 80'
F wt DPTA YPD 120'
G yhb1 DPTA YPD 120'
H fzf1 DPTA YPD 120'
I NO gas
YFL061w
YNL335w
YLR346c
YNR064c
SSU1
YHB1
FZF1

located within a 15 kb duplication in the telomeric regions of chromosomes 6 and 14. The function of these two genes is unknown although they have homology to a cyanimide hydratase from *Myrothecium verrucaria* (36% identical) ($2 \times e^{-24}$).

Examination of other genes with altered expression profiles following DPTA NONOate treatment revealed that the majority belongs to a large set of genes associated with the yeast general stress response (Gasch *et al.*, 2000). We calculated correlations between the DBY7283 response to DPTA NONOate and other stresses as measured by Gasch *et al.* (Gasch *et al.*, 2000). The highest correlation was seen with the response to the thiol oxidant diamide (correlation 0.67) and a strong positive correlation was also found with the response to heat shock (correlation 0.61). However, *YHB1*, *SSU1*, YNR064c and YNL335w/YFL061w transcript levels did not change significantly following treatment with diamide, heat shock or other environmental stresses (Gasch *et al.*, 2000), indicating that they may be specific to NO·-mediated nitrosative stress. On the basis of this data, we labeled this cluster RNI-responsive.

Treatment with NO· gas induces the RNI-responsive cluster

To determine whether the transcriptional responses observed following DPTA NONOate treatment would also occur following treatment with chemically generated nitric oxide, NO· gas (5 ml) was injected directly into YPD media exposed to the air. A single time-point comparison experiment was conducted comparing RNA from treated and untreated cultures after 120 minutes. In response to NO· gas treatment, a general stress response was observed as well as induction of the RNI-responsive cluster (Figure 1I).

Transcriptional response to DPTA NONOate in YPD

To determine whether the nitrosative stress-mediated response occurred independent of media effects, we exposed DBY7283 grown in YPD media to DPTA NONOate (1 mM). Increased concentrations of DPTA NONOate were used for the YPD experiments relative to the SCD experiments due to a ~10 fold increase in wild type strain sensitivity in rich versus synthetic media. Following exposure to DPTA NONOate in YPD, microarray expression analyses revealed induction of the RNI responsive cluster, although to a lesser degree than in SCD media. Similarly, a general stress response occurred following DPTA NONOate treatment, also to a lesser degree than in SCD media despite the increased concentration of DPTA NONOate used. While the majority of the response was highly similar between cells grown in synthetic and rich media, we noted that cells grown in YPD exhibited a strong repression of mitochondrial genes involved in oxidative phosphorylation upon treatment with 1mM DPTA NONOate (Figure 1F). Treatment with the DPTA NONOate breakdown product APD (1mM) did not elicit this response (data not shown).

FZF1 is necessary for the DPTA NONOate- mediated induction of the RNI-responsive cluster

The transcriptional activator Fzf1p has previously been identified as both necessary and sufficient for *SSU1* transcription (Avram *et al.*, 1999). We reasoned that *FZF1* might have a role in the NO·-mediated induction of the RNI-responsive cluster. We examined the transcriptional response of an *fzf1Δ* strain to DPTA NONOate (100 μM) (Figure 1E) and found that the response of the mutant strain, when compared to wild

type, revealed a comparable general stress response following DPTA NONOate exposure (correlation 0.79). However, the RNI-responsive cluster failed to be induced in the strain lacking *FZF1* (Figure1E). Because the RNI-responsive cluster was dependent upon Fzf1p, this cluster was relabeled as the *FZF1*-dependent gene set. Examination of the wild type transcriptional response to DPTA NONOAte revealed that *FZF1* mRNA levels did not significantly increase following DPTA NONOate treatment.

To confirm that *FZF1* was also necessary for the induction of *YHB1*, *SSU1*, and the ORF's observed in YPD media, we conducted a time course of transcriptional response following treatment with DPTA NONOate (1 mM) in a diploid *fzf1*Δ strain. Similarly to the response observed in SCD media, transcript profiles of a diploid *fzf1*Δ strain were comparable to wild type (r = 0.72), except that the *FZF1*-dependent gene set was not induced (Figure 1H).

Because Yhb1p has been implicated in the metabolism of NO·, we wished to determine whether the presence of Yhb1p was necessary for induction of the *FZF1*-dependent gene set. We conducted a time course experiment to measure the transcriptional response following DPTA NONOate (1 mM) treatment in a diploid *yhb1*Δ strain. Expression profiles in YPD were similar to wild type (r = 0.80), with the exception that the *YHB1* gene was silent because the gene had been deleted. Aside from *YHB1*, relative mRNA transcript abundance level increases were observed for the *FZF1*-dependent gene set (Figure 1G). We noted that the stress response following exposure to DPTA NONOate was qualitatively more pronounced in the *yhb1*Δ strain than in wild type. These results indicate that DPTA NONOate-mediated induction of the *FZF1*-

dependent gene set occurs independently of the *YHB1* gene.

DPTA NONOate-mediated transcriptional induction of the FZF- dependent gene set results in corresponding increases in protein abundance

To confirm that increases in relative mRNA abundance following DPTA NONOate treatment also led to an actual increase in protein abundance, tagged Yhb1p-TAP and Ssu1p-TAP (Ghaemmaghami *et al.*, 2003) protein levels were examined by Western blot after treatment with DPTA NONOate (100 μM). Consistent with changes in the levels of mRNA transcripts, DPTA NONOate induced expression of Yhb1p-TAP when compared to an untreated control (Figure 2A). *SSU1*-TAP was undetectable prior to DPTA NONOate exposure, but a band was clearly visible following treatment (Figure 2A). These results confirm that NO·-mediated induction of *YHB1* and *SSU1* mRNAs results in a corresponding increase in protein levels.

To quantitatively measure the relative abundance change for Yhb1p induction, a *YHB1*-GFP strain (Huh *et al.*, 2003) was exposed to bolus addition of 100 μL of NO· gas generated by acidification of sodium nitrite and monitored by flow cytometry. Following exposure to NO· gas for 180 minutes, median fluorescence intensity of the cell population increased by 7.5 fold (Figure 2B). To quantify relative Yhb1p protein levels as a function of DPTA NONOate concentration, the *YHB1*-GFP strain was exposed to increasing amounts of DPTA NONOate (2.5- 520 μM) and observed by flow cytometry 180 minutes following treatment. *YHB1*-GFP fluorescence increased in a dose-dependent manner and at the highest concentration (520 μM) we measured a 10-fold increase in *YHB1*-GFP fluorescence (Figure 2C). In a *YHB1*-GFP *fzf1*Δ strain, an increase in *YHB1*-

Figure 3-2

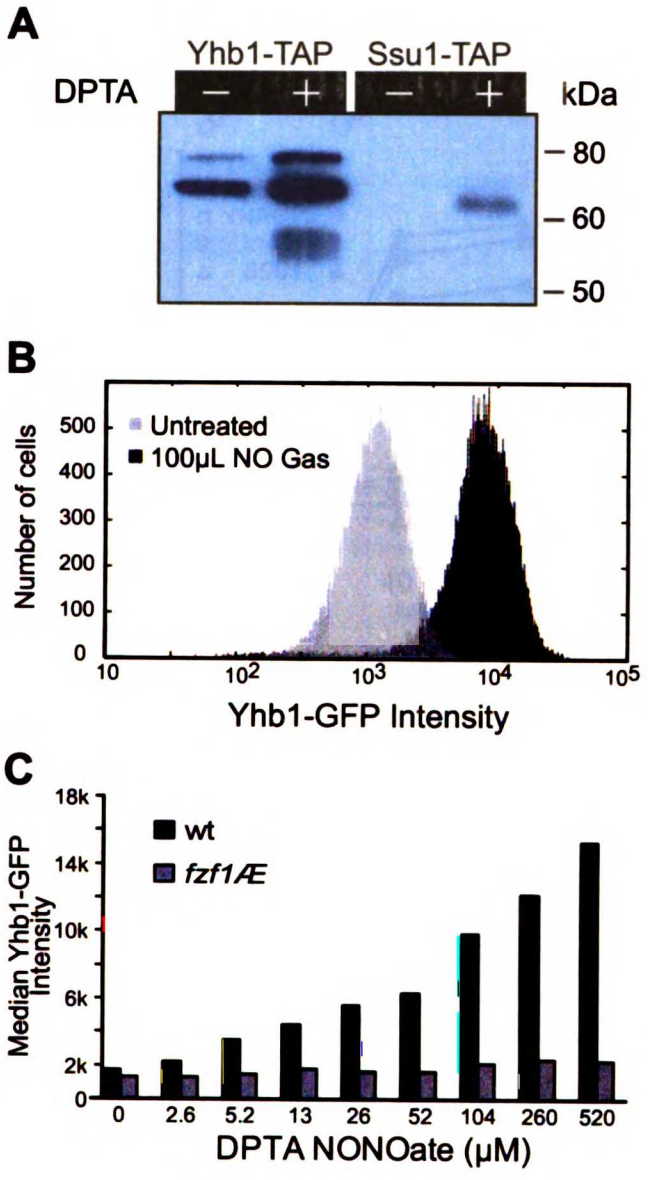
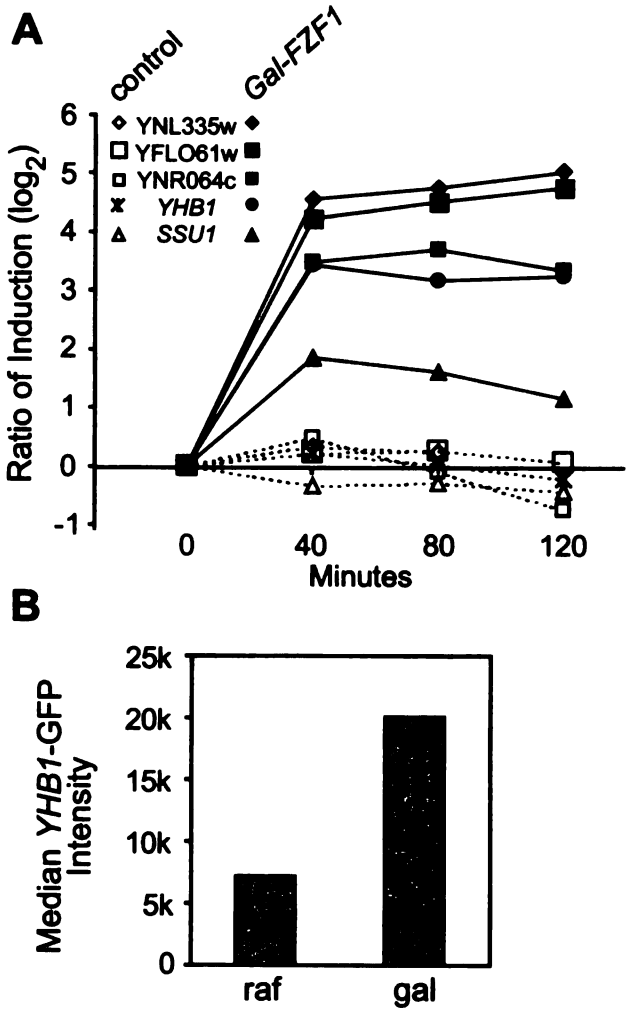

A
Yhb1-TAP
Ssu1-TAP
DPTA
–
+
–
+
kDa
80
60
50
B
Untreated
100µL NO Gas
Number of cells
500
400
300
200
100
0
10
10²
10³
10⁴
10⁵
Yhb1-GFP Intensity
C
wt
fzf1Æ
Median Yhb1-GFP Intensity
18k
14k
10k
6k
2k
0
0
2.6
5.2
13
26
52
104
260
520
DPTA NONOate (µM)

Figure 3-3


A
control
Gal-FZF1
YNL335w
YFLO61w
YNR064c
YHB1
SSU1
Ratio of Induction (log2)
6
5
4
3
2
1
0
-1
0
40
80
120
Minutes
B
Median YHB1-GFP Intensity
25k
20k
15k
10k
5k
0
raf
gal

GFP fluorescence was not observed at any concentration of DPTA NONOate, indicating that *FZF1* was required for NO·-mediated increases in Yhb1p expression (Figure 2C).

Fzf1p overexpression is sufficient to induce the FZF1-dependent gene set

Overexpression of Fzf1p has been previously shown to increase mRNA levels of *SSU1* (Avram *et al.*, 1999). To examine Fzf1p's role in the induction of genes other than *SSU1*, Fzf1p overexpression was monitored by microarray expression analysis in the absence of nitrosative stress. Parallel time courses were collected for the DBY7283 strain carrying a plasmid with the *GAL1-10* promoter driving *FZF1* or *LacZ*. Overexpression of FZF1 RNA was evident, ~16-fold increase was seen in the *FZF1* mRNA following overexpression of the *FZF1* gene. While the vast majority of the expression differences was related to galactose utilization, we observed that *FZF1* overexpression resulted in the specific induction of all previously identified members of the *FZF1*-dependent gene set (Figure 3A).

To determine whether overexpression of Fzf1p also affected levels of Yhb1p, the same *GAL*-driven *FZF1* plasmid was introduced into the *YHB1*-GFP strain. One hour after galactose induction, *YHB1*-GFP fluorescence increased by 3-fold (Figure 3B). In the absence of the Fzf1p overexpression plasmid, this induction was not observed.

Sequence analysis reveals the presence of a conserved promoter element

We sought to define whether *cis*-acting sequences necessary for NO· mediated transcriptional induction might be present in the promoter regions of the *FZF1*-dependent

gene set by utilizing sequence data from closely related yeast species. Such comparisons have been used to precisely identify several regulatory motifs (Kellis *et al.*, 2003) (Cliften *et al.*, 2003). Multiple alignment of the *SSU1* promoter from *S. cerevisiae*, *S. paradoxus, S. mikatae,* and *S. bayanus* revealed several conspicuous islands of conservation (Figure 4A). The first of these islands, Conserved Sequence 1 (CS1), overlaps with the region reported to be protected by Fzf1p in an *in vitro* DNAse I footprinting assay (Avram *et al.*, 1999). Interestingly, this sequence appears unique to the *SSU1* promoter. Multiple alignment of the *YHB1* upstream sequences identified extensive conservation (data not shown), yet a sequence homologous to CS1 could not be located. Furthermore, examination of promoters from the other members of the *FZF1*-dependent gene set did not reveal the presence of a homologous CS1 sequence.

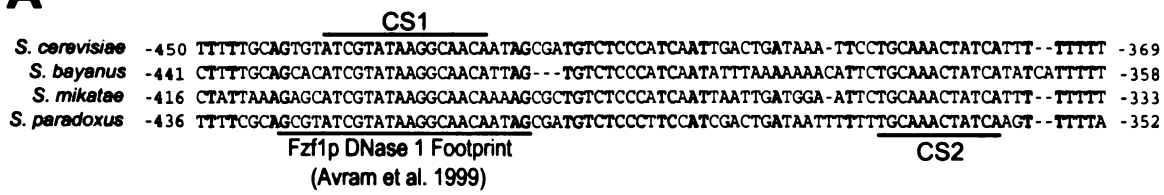
To investigate the possibility of a second regulatory motif, we used the MEME analysis tool (Bailey and Elkan, 1994) to probe 600 bp upstream of each member of the *FZF1*-dependent gene set. A consensus motif (5' YGSMNM**CTATCAYTTYY**) was found in each promoter sequence that also coincided with a second island of sequence conservation (CS2) in the *SSU1* promoter (Figure 4A & 4B). Strikingly, a search for the CS2 consensus sequence in the promoter regions of the entire *S. cerevisiae* genome revealed that this motif is only found upstream of the five *FZF1*-dependent genes.

Both CS1 and CS2 are sufficient for* FZF1*-dependent response to DPTA NONOate

To experimentally validate *cis*-acting elements required for NO·-mediated induction, *LacZ* reporter strains featuring various portions of the *SSU1* promoter region were constructed (Figure 5A). In agreement with microarray and Western analyses, the

Figure 3-4

A



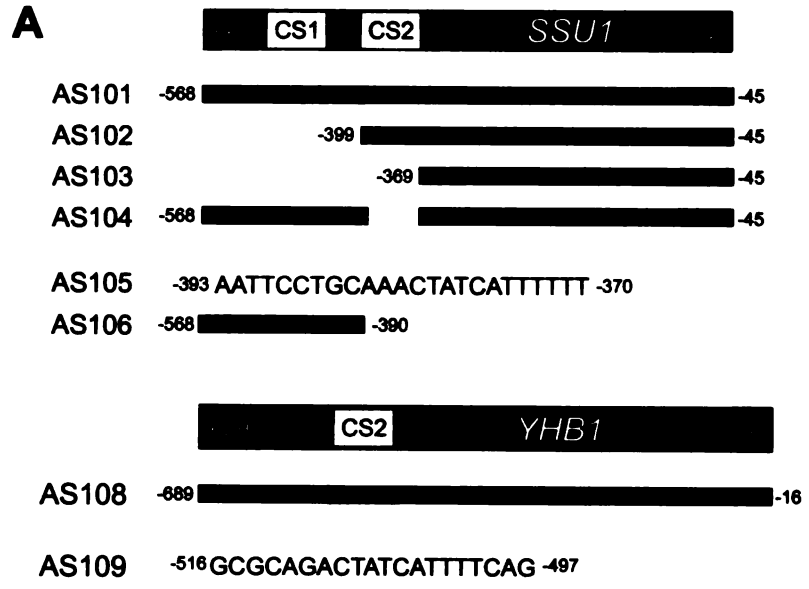
B

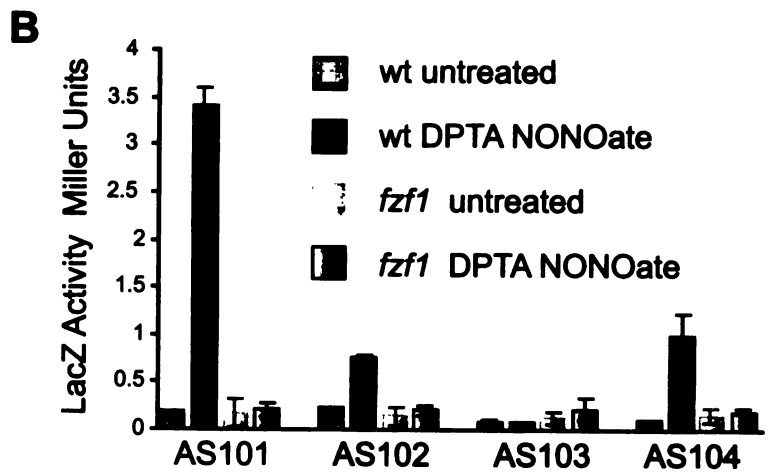
	strand		
SSU1	+	-387	TGCAAACTATCATTTTT -371
YHB1	-	-498	CGCAGACTATCATTTTC -514
YNR064c	-	-98	TGGCTCCTATCACTTTT -114
YNL335w	+	-212	TGGACACTATCATTTCT -196
consensus		5'	YGSMNMCTATCAYTTYY 3'

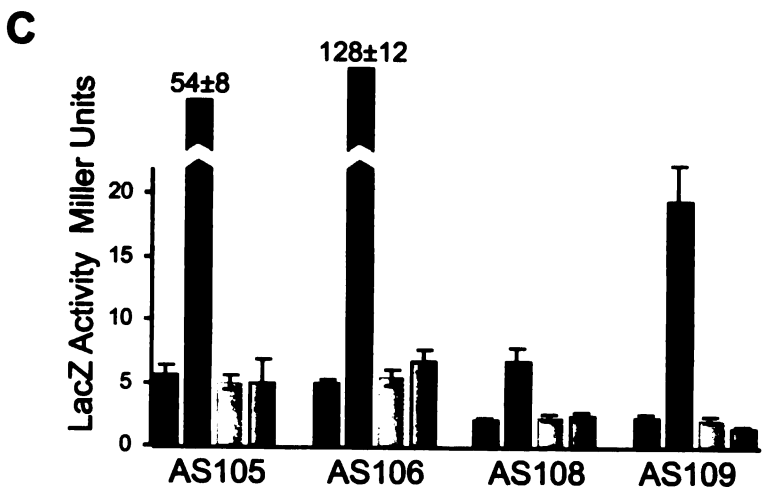
full-length promoter region (AS101) activated the *LacZ* reporter 17-fold in response to treatment with 100 μM DPTA NONOate (Figure 5B) in an *FZF1*-dependent manner. To determine whether the CS1 element was necessary for a *FZF1*-mediated response to DPTA NONOate, we constructed a reporter strain corresponding to the –399 to –45 region of the *SSU1* promoter (AS102). While this construct still responded to DPTA NONOate treatment in an *FZF1*-dependent manner, the response was 4-fold lower than that observed with the full length *SSU1* reporter (Figure 5B). A further deletion which encompassed both the CS1 and CS2 region (AS103) abolished the ability of the reporter to respond to DPTA NONOate treatment, while removal of only the CS2 motif (AS104) reduced responsiveness. A reporter construct bearing the CS2 motif (AS105), or the region containing CS1 (AS106), conferred strong DPTA NONOate responsiveness in an *FZF1*-dependent manner (Figure 5C). Taken together, CS1 and CS2 are independently capable of mediating *FZF1*-dependent transcriptional induction, but in the genomic context of the *SSU1* promoter, both elements are necessary for wild type levels of induction. Removed from this context, both elements are sufficient to mediate a robust *FZF1*-dependent response to DPTA NONOate treatment.

As noted above, the other members of the *FZF1*-dependent gene set, including *YHB1,* do not contain a recognizable CS1 motif. To test whether the CS2 motif found in *YHB1* was sufficient to drive the *FZF1*-dependent gene set, *LacZ* reporters were constructed using either the full length (AS108) upstream sequence, or the *YHB1*-derived CS2 motif (AS109) (Figure 5A). Consistent with the results for *SSU1*, the wild type upstream sequence and the *YHB1* CS2 sequence were capable of mediating an *FZF1*-dependent response (3.4-fold and 8.8-fold, respectively) to DPTA NONOate treatment

Figure 3-5


A
CS1
CS2
SSU1
AS101
-568
-45
AS102
-399
-45
AS103
-369
-45
AS104
-568
-45
AS105
-393 AATTCCTGCAAACTATCATTTTTT -370
AS106
-568
-390
CS2
YHB1
AS108
-689
-16
AS109
-516 GCGCAGACTATCATTTTCAG -497


B
LacZ Activity Miller Units
4
3.5
3
2.5
2
1.5
1
0.5
0
wt untreated
wt DPTA NONOate
fzf1 untreated
fzf1 DPTA NONOate
AS101
AS102
AS103
AS104


C
LacZ Activity Miller Units
54±8
128±12
20
15
10
5
0
AS105
AS106
AS108
AS109

(Figure 5C).

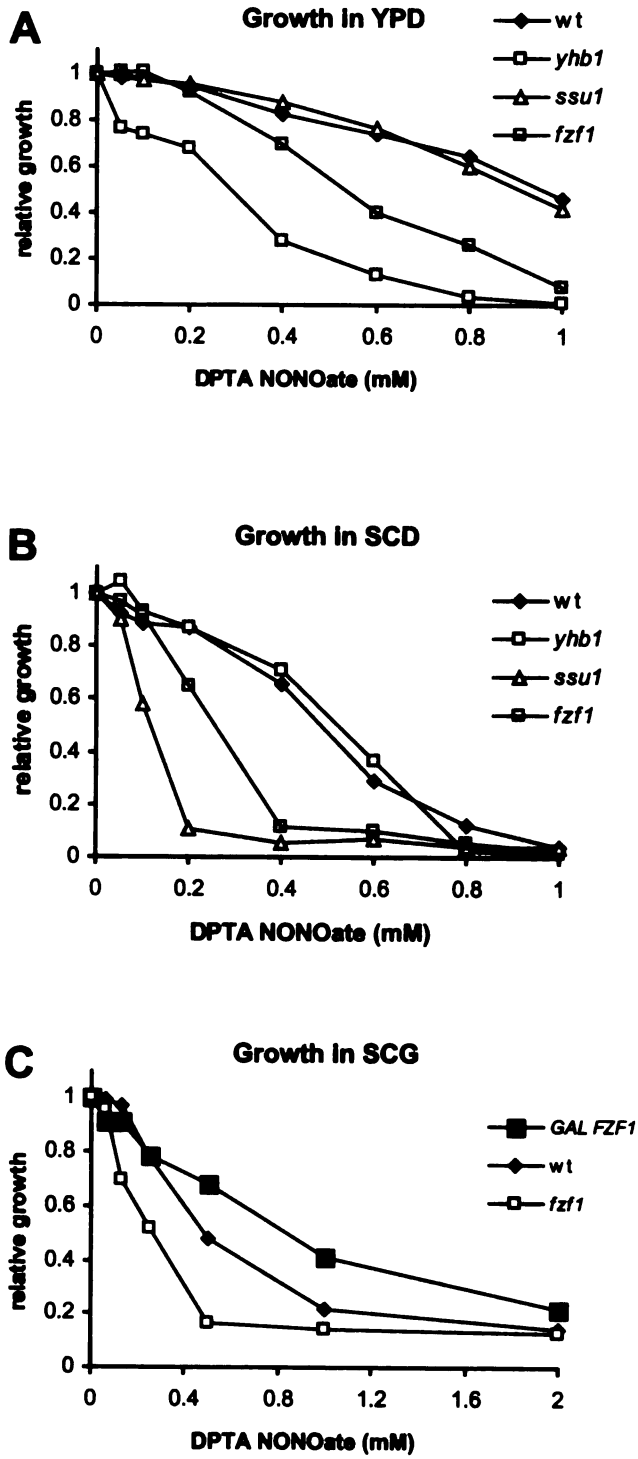
FZF1, *SSU1*, and *YHB1* confer a growth advantage under nitrosative stress

To further understand the physiological relevance of the *FZF1*-dependent response to nitrosative stress, we examined the growth inhibition of wild type, *yhb1*Δ, *ssu1*Δ, and *fzf1*Δ strains after treatment with DPTA NONOate (0.1 – 1 mM) in YPD media. Exponentially growing strains were treated and then monitored for growth 12 hours later relative to untreated controls. While growth of wild type yeast in YPD media was mildly inhibited by DPTA NONOate, strains lacking *FZF1* were found to have enhanced sensitivity (Figure 6A). Consistent with previous reports, strains lacking *YHB1* were highly sensitive to DPTA NONOate-mediated nitrosative stress (Liu *et al.*, 2000). A strain lacking *SSU1* behaved similarly to wild type (Figure 6A), as did *ynr064c*Δ and *ynl3335w*Δ strains (data not shown).

In SCD media, both wild type and *fzf1*Δ strain growth was more severely affected by DPTA NONOate treatment. Interestingly, a *yhb1*Δ mutant strain revealed a growth inhibition that was indistinguishable from wild type, yet deletion of *SSU1* resulted in hypersensitivity. Although the relative sensitivity of *yhb1*Δ and *ssu1*Δ strains appears reversed in SCD media, deletion of *FZF1* led to an intermediate phenotype similar to what was observed in YPD media (Figure 6B).

To determine whether overexpression of Fzf1p would lead to a physiologically relevant response, we compared a *GAL1-10*-driven *FZF1* strain to a control *GAL1-10*-driven *LacZ* strain after exposure to DPTA NONOate (0.1-2 mM). Overexpression of Fzf1p resulted in a protective advantage relative to wild type in a range of 0.5 mM to 1.5

Figure 3-6


A
Growth in YPD
wt
yhb1
ssu1
fzf1
relative growth
DPTA NONOate (mM)
B
Growth in SCD
wt
yhb1
ssu1
fzf1
relative growth
DPTA NONOate (mM)
C
Growth in SCG
GAL FZF1
wt
fzf1
relative growth
DPTA NONOate (mM)

mM DPTA NONOate while deletion of *FZF1* in the *LacZ* control strain resulted in hypersensitivity to DPTA NONOate (Figure 6C).

DISCUSSION

S. cerevisiae utilizes a complex network of signaling systems and transcriptional regulons to recognize and respond to environmental pressures. We report the transcriptional response of *S. cerevisiae* to NO·-mediated nitrosative stress. While treatment with DPTA NONOate led to a general stress response common to other perturbation experiments, DPTA NONOate and NO· gas also induced a genetically separable, physiologically relevant set of genes. This set of genes is comprised of *YHB1*, *SSU1*, YNR064c, and YNL335w/YFL061w.

Yhb1p catalyzes the reaction of NO· with oxygen to create nitrate, limiting the exposure of the cell to NO· and thus confers a growth advantage following NO· treatment in YPD media (Liu *et al.*, 2000). In contrast to previous studies in which *S. cerevisiae* was exposed to DETA NONOate (Liu *et al.*, 2000), nitrosoglutathione (Ullmann *et al.*, 2004), or sodium nitrite (Ullmann *et al.*, 2004), we found that both mRNA and protein levels of *YHB1* were responsive to NO· exposure. This discrepancy might be due to insufficient NO· availability, cytotoxic NO· effects, media effects, or differences in strain background. In addition, we found that the deletion of the *YHB1* gene appears to lead to an increase in the general stress response following DPTA NONOate treatment yet does not effect the induction of *SSU1* and the other ORFs in the RNI-responsive gene cluster.

SSU1 has been reported to be a sulfite efflux transporter and to be located at the outer membrane (Park and Bakalinsky, 2000). We found that the *SSU1* transcript and

protein levels increased following nitrosative stress. Furthermore, the presence of the *SSU1* gene conferred a growth advantage following exposure to DPTA NONOate in SCD media. We speculate that in addition to transporting sulfite, *SSU1* may also transport NO· metabolites out of the cell.

The predicted ORFs YNR064c and YNL335w/YFL061c were also induced following nitrosative stress. The presence of these genes did not confer significant growth advantages (data not shown). The function of these genes with regard to nitrosative stress remains unclear. It is possible that these ORFs may have roles in NO· detoxification that were not revealed by the laboratory conditions used for the growth advantage assay.

FZF1 encodes a Zn-finger DNA binding transcription factor necessary for *SSU1* transcription, and until this study, *SSU1* was the only known target. We found that the *FZF1* gene was required for the induction of *YHB1*, *SSU1* and the other ORFs following nitrosative stress. Furthermore, overexpression led to induction of *YHB1*, *SSU1*, and the other uncharacterized ORFs of the RNI-responsive gene cluster. Importantly, the Fzf1p overexpressing strain exhibited a growth advantage relative to wild type after DPTA NONOate treatment. Absence of Fzf1p resulted in a growth disadvantage that was less profound than the growth disadvantage caused by deletion of *YHB1* in YPD media, or deletion of *SSU1* in SCD media. This is likely due to *FZF1*-independent basal transcription of *YHB1* and *SSU1*.

Overexpression of Fzf1p resulted in increased transcription of the target genes, despite *FZF1* mRNA levels remaining constant following DPTA treatment. The

mechanistic explanation for these observations may be similar to the regulation of Pho4p and other transcription factors for which phosphorylation or some other post-translational modification leads to a differential subcellular localization. The situation could be analogous in that the overexpression of Fzf1p may stoichiometrically out-compete a regulatory mechanism which would ultimately result in transcriptional activation.

While it may be that additional components are required for sensing NO· or NO·-derived metabolites prior to activation of Fzf1p, it may also be the case that these capabilities are inherent to Fzf1p alone. It is plausible that nitrosylation of Fzf1p could lead to its modulation as an activator. Interestingly, induction of the *FZF1*-dependent gene set did not occur following treatment with the thiol oxidant diamide, heat shock, or other oxidative stresses (Gasch *et al.*, 2000).

Previous studies have shown that following treatment with methyl methane sulfonate (MMS), *SSU1*, YNR064c, and YNL335w/YFL061c are induced, raising the possibility that DNA damage may also activate the *FZF1*-dependent gene set. However, *YHB1* mRNA was not significantly induced following this treatment (Gasch *et al.*, 2001) and a genome-wide screen of the deletion library for MMS hypersensitivity did not find that *ssu1*Δ, *yhb1*Δ or *fzf1*Δ strains were sensitive (Chang *et al.*, 2002).

It has previously been reported that Fzf1p specifically binds the *SSU1* promoter *in vitro* (Avram *et al.*, 1999). The conserved sequence motif CS1 is contained within the region protected from DNAse I cleavage, yet this sequence could not be located in the promoter regions of the other NO·-responsive genes. Sequence comparisons revealed a second conserved sequence (CS2) in the promoters of the *FZF1*-dependent gene set that

was sufficient for DPTA NONOate-mediated, *FZF1*-dependent induction. These data imply that Fzf1p possesses the ability to interact with at least two distinct consensus binding sequences, given that CS1 and CS2 have no obvious similarity. Since we have not shown a direct biochemical interaction between CS2 and Fzf1p, it is possible that induction via CS2 is an indirect effect of Fzf1p action. Further *in vitro* and *in vivo* DNA binding studies will directly address this issue.

The growth inhibition effect of NO·-mediated stress appears to be partially dependent on environmental factors and understanding the effect of growth conditions is essential for proper interpretation of these assays. In minimal media, 10- to15-fold less DPTA NONOate or NO· gas was necessary to induce a specific response when compared to experiments conducted in YPD. An obvious difference between the composition of YPD and SCD includes higher thiol concentrations, which may account for the differential sensitivity of yeast in these two media.

S. cerevisiae is a relevant model organism for studying the response to NO· because pathogenic fungi *C. albicans* and *C. neoformans* are likely to utilize similar molecular signaling mechanisms to induce Yhb1p levels in response to NO·. The orthologous flavohemoglobin in *C. albicans* has already been shown to respond to NO· (Ullmann *et al.*, 2004) and the *C. albicans* genome-wide transcriptional response bares significant similarity to the response we observe in *S. cerevisiae*, although the corresponding transcription factor has not been identified (B. Hromatka, *personal communication*). Further dissection of the mechanism by which *S. cerevisiae* senses and responds to NO· may shed light on this important molecule.

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Table 1 *S. cerevisiae* strains used in this study

Strain	Genotype	Origin
DBY7283	MATα, ura3-52	(Botstein FY22)
YHB1-GFP	(S288C) MATa *YHB1::GFP*	(Huh *et al.*, 2003)
YHB1-GFP fzf1Δ	(S288C) MATa *YHB1::GFP fzf1Δ* + KAN	This study
diploid *fzf1Δ*	BY4743 *fzf1Δ/ fzf1Δ* + KAN	(Resgen)
diploid *yhb1Δ*	BY4743 *yhb1Δ/ yhb1Δ* + KAN	(Resgen)
YHB1-TAP	S288C *YHB1-TAP*	(Ghaemmaghami *et al.*, 2003)
SSU1-TAP	S288C *SSU1-TAP*	(Ghaemmaghami *et al.*, 2003)
DBY7283 +placZ	pyes2.1 lacZ + URA	This study
DBY7283 +pFzf1p	pyes2.1 Fzf1p + URA	This study
YHB1-GFP +pFzf1p	(S288C) MATa *YHB1::GFP* pyes2.1 Fzf1p + URA	This study
AS101-AS109	YM4271 MATa *ura3-52*	(Invitrogen)
AS101	*SSU1*(-568 to -45)::lacZ + URA	This study
AS102	*SSU1*(-399 to -45)::lacZ + URA	This study
AS103	*SSU1*(-369 to -45)::lacZ + URA	This study
AS104	*SSU1*(-568 to -45)::lacZ *cs2Δ* (-389 to -370) + URA	This study
AS105	*SSU1* CS2 (-393 to –370) CS2::lacZ + URA	This study
AS106	*SSU1*(-568 to -390)::lacZ + URA	This study
AS108	*YHB1*(-689 to -16):: lacz + URA	This study
AS109	*YHB1* CS2 (-516 to –497)::lacZ + URA	This study
AS 201-AS209	AS101-109 *fzf1Δ* +KAN	This study
WT (*his3Δ)*	BY4741 MATa *his3Δ* + KAN	Resgen
yhb1Δ	BY4741 MATa *yhb1Δ* + KAN	Resgen
ssu1Δ	BY4741 MATa *ssu1Δ* + KAN	Resgen
fzf1Δ	BY4741 MATa *fzf1Δ* + KAN	Resgen
fzf1Δ +pLacZ	*fzf1Δ* pyes2.1 lacZ + URA	This study

Table 2. Strain construction PCR primers

strain	amplified region	primer 1	prime r 2
AS101	SSU1 –568 to –45	GGAATTCATGTGGAAAAAG AAGGGGTGG	ATACCGCTCGAGAATTGC GTATTGTCTGA
AS102	SSU1–399 to –45	GGAATTCCTGATAAATT CCTGCAAACTATCAT	ATACCGCTCGAGAATTGC GTATTGTCTGA
AS103	SSU1 –369 to –45	GGGGTACCTTTTTTCATCCT TGTGCCGC	ATACCGCTCGAGAATTGC GTATTGTCTGAG
AS106	SSU1 –568 to –390	ATACCGAAGCTTCATGTGGA AAAAGAAGGGGTG	GGGGTACCAATTTATCAGTCA ATTGATGGGAG
AS108	YHB1 – 689 to –16	CGAATTCAAGCTTCGTATAA TTGCCAA	ATACCGCTCGAGTGTGTGGTTT GTTGAAAATGG

Table 3. Pearson correlations between time course microarray datasets

	DBY7283 Mock	DBY7283 APD	DBY7283 100 µM DPTA	*YHB1-GFP* 100 µM DPTA	*YHB1-GFP fzf1Δ* 100 µM DPTA	DBY7283 1 mM DPTA	diploid *yhb1Δ* 1 mM DPTA	diploid *fzf1Δ* 1 mM DPTA
DBY7283 mock	1	0.80	0.35	0.37	0.43	0.11	0.23	0.24
DBY7283 APD		1	0.45	0.46	0.47	0.13	0.26	0.20
DBY7283 100 µM DPTA			1	0.77	0.58	0.60	0.56	0.48
YHB1-GFP 100 µM DPTA				1	0.79	0.51	0.58	0.51
YHB1-GFP fzf1Δ 100 µM DPTA					1	0.46	0.59	0.55
DBY72831 mM DPTA						1	0.80	0.72
diploid *yhb1Δ/Δ yhb1Δ* 1 mM DPTA							1	0.75
diploid *fzf1Δ/fzf1Δ* 1 mM DPTA								1

Figure 1. DNA microarray analyses. Data from eight independent time course response experiments were analyzed using the Cluster Program. The lower region is a detailed view of the RNI-responsive *FZF1*-dependent cluster. Although not part of this cluster, the expression profile for *FZF1* is also shown. The triangles indicate the passage of time and the column letter indicates experiment performed as follows: A) DBY7283 (wt) exposed to a mock treatment, B) DBY7283 (wt) exposed to APD (100 μM), C) DBY7283 (wt) exposed to DPTA NONOate (100 μM), D*)* *YHB1*-GFP exposed to DPTA NONOate (100 μM), E) *YHB1*-GFP *fzf1*Δ exposed to DPTA NONOate (100 μM), F) DBY7283 exposed to DPTA NONOate (1 mM), G) diploid *yhb1*Δ/*yhb1*Δ exposed to DPTA NONOate (1 mM), H) diploid *fzf1*Δ/*fzf1*Δ exposed to DPTA NONOate (1 mM), I) DBY7283 single time-point experiment following exposure to NO• gas. Experiments A-E were conducted in SCD media and F-I were conducted in YPD media. Genes showing greater than 5.6-fold ($2^{2.5}$) response in two or more arrays were included. The color saturation indicates the magnitude of the expression ratio as indicated by the scale in the lower left hand corner.

Figure 2. Protein levels following exposure to DPTA NONOate and NO• gas. A) Western blot using rabbit anti-CBP antibody of TAP-tagged Yhb1p and Ssu1p in the presence or absence of DPTA NONOate (100 μM). B) Flow cytometry of *YHB1*-GFP of FITC median intensity in the presence or absence of NO· gas for 180 minutes. C) Dose response curves 180 minutes after treatment with DPTA NONOate between 0 and 520 μM DPTA NONOate in synthetic complete media for both wild type *YHB1*-GFP and

YHB1-GFP *fzf1*Δ strains. The Y-axis indicates FITC median intensity of 20,000 cells and the X-axis indicates drug concentrations.

Figure 3. Overexpression of *FZF1* results in induction of the RNI-responsive gene cluster. A) Transcript levels of *FZF1*-dependent RNI-inducible transcripts following overexpression of either Fzf1p or lacZ (control). For the Fzf1p time course, transcript levels are represented by filled-in symbols, and for the control time course transcript levels are indicated by open symbols. Symbols for each gene are indicated in the figure legend. Time after addition of 2% galactose to culture growing in raffinose is presented on the X-axis and log base 2 of induction is on the Y-axis. B) *YHB1*-GFP GFP intensity measured by flow cytometry following induction of Fzf1p. The Y-axis indicates FITC median intensity and the X-axis indicates status of Fzf1p induction.

Figure 4. Comparison of upstream sequences reveals conserved motifs. A) Multiple alignment of the promoter region of the *SSU1* gene for closely conserved *Saccharomyces* species. Conserved sequences in the four species are shaded. B) Sequence motif found in promoter regions of *FZF1*-dependent RNI-responsive genes.

Figure 5. Two separate conserved sequence motifs are sufficient for DPTA NONOate-dependent reporter activation. A) Representation of *SSU1* and *YHB1* promoter constructs cloned into the minimal *CYC* promoter driving lacZ expression. B) and C) lacZ activity in Miller Units in the presence or absence of DPTA NONOate (100 μM) for the indicated strain, both *FZF1* wild type and *fzf1*Δ as indicated in the figure legend.

Figure 6. Genotype-dependent sensitivity and resistance following DPTA NONOate treatment. A) Hypersensitivity to growth inhibition in response to DPTA NONOate (0-1 mM) in YPD media for *yhb1*Δ, *fzf1*Δ, and *ssu1*Δ strains compared to wild type. The X-axis represents drug concentration and the Y-axis represents growth relative to isogenic untreated culture 12 hours following addition of DPTA NONOate. B) Same strains and treatment as experiment A except conducted in SCD media instead of YPD media. C) Resistance and hypersensitivity following treatment with DPTA NONOate (0-2 mM) in SCG media for an Fzf1p over-expression strain compared to a wild type lacZ overexpression strain and a *fzf1*Δ lacZ overexpression. The X-axis represents drug concentration and the Y-axis represents growth relative to isogenic untreated culture 12 hours following the start of the experiment.

Nitric oxide sensing by *S. cerevisiae* occurs by a translation-independent mechanism that requires oxygen

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ABSTRACT

Experiments were undertaken to elucidate the mechanism of activation of Fzf1p by nitrosative stress in the model organism *Saccharomyces cerevisiae*. To determine whether new protein translation was necessary for this activation, translation was halted by cycloheximide, and the genome-wide transcriptional response to the nitric oxide (NO·) releasing compound DPTA NONOate was characterized by DNA microarray. The mechanism was further investigated by characterizing the genome-wide transcriptional response to DPTA NONOate in a nitrogen environment. Additionally, the genome-wide response of a strain with the *GRR1* gene deleted was profiled following exposure to DPTA NONOate. The results presented indicate that activation of Fzf1p by NO· occurs by an event independent of translation, which requires the presence of oxygen. These results are important to understanding the mechanism by which *S. cerevisiae* senses and responds to NO· and a model for the activation of Fzf1p is proposed.

INTRODUCTION

A large variety of sensory mechanisms have been shown to exist for biological adaptation to environmental cues. Such mechanisms are constantly utilized by *Saccharomyces cerevisiae* to respond constructively to environmental signals. In this work we sought to clarify mechanisms leading to the activation of Fzf1p-mediated transcription in response to nitric oxide (NO·).

In response to nitrosative stress, fungal organisms *S. cerevisiae* and *C. albicans* induce both *YHB1* and *SSU1* (Sarver and DeRisi, 2005) (Hromatka *et al.*, 2005). *YHB1* has been shown to act as an NO· dioxygenase (Liu *et al.*, 2000). *SSU1* has been shown to act as a sulfite efflux transporter (Park and Bakalinsky, 2000). These two genes are important in coping with the presence of exogenously-supplied NO·. In *S. cerevisiae,* the *FZF1* gene is necessary for the transcriptional induction of *SSU1* and *YHB1* in response to NO·. Additionally, overexpression of Fzf1p is sufficient to activate transcription in the absence of NO· and this response provides a protective affect following exposure to exogenously-supplied NO· (Sarver and DeRisi, 2005).

Upstream mechanisms for the activation of transcription factors in response to stimuli can be analyzed by examining whether the transcriptional activation requires active protein translation. Biological response mechanisms can be arbitrarily divided into two groups, those that require active translation as a necessary component prior to the transcriptional response and those that occur independently of active translation of new proteins. An example of a transcriptional activation mechanism that requires translation is the *HAC1*-mediated activation of the unfolded protein response. *HAC1* mRNA must be

alternatively spliced following the recognition of unfolded protein and then translated into Hac1p in order for transcriptional response to unfolded-protein-response stimulus to occur (Ruegsegger *et al.*, 2001). Therefore, translational machinery is required to activate the unfolded protein response.

An example of a transcriptional activation mechanism that can occur independently from translation would be activation of Yap1p in response to oxidative stress. Prior to oxidative stress, Yap1p shuttles into and out of the nucleus. Following oxidative stress, cysteine residues are oxidized, leading to an allosteric change that occludes the Yap1p nuclear export signal, consequently increasing the Yap1p concentration in the nucleus thereby leading to the activation of transcription (Wood et al., 2004).

Cycloheximide, a ribosomal translation inhibitor, has previously been used to analyze the role of translation in response to specific signaling (Popolo *et al.*, 1982; Fass *et al.*, 2003; Lemire *et al.*, 1985). Cycloheximide exposure should block the unfolded protein response from occurring following activation, because no new Hac1p protein will be translated following the initial response activation. In the second example, no new protein is necessary for the response to occur and following cycloheximide treatment Yap1p-mediated transcriptional response to oxidative stress would still occur. I utilized cycloheximide translational blockage to determine whether translation was necessary for the Fzf1p-mediated transcriptional response to NO·.

Molecular responses to NO· have been reported to occur by two distinctly different mechanisms. The first more canonical mechanism includes the allosteric shift of a heme sensor leading to an activated enzymatic form. Upon binding of NO· to the

heme domain, the soluble guanylyl cyclase enzyme converts from a catalytically inactive state to a catalytically active state (Stone and Marletta, 1994). A second mechanism reported for the molecular response to NO· relies on the post-translational modification of thiol groups in the presence of NO·. Much of the potential chemistry of thiol modification is thought to depend on the presence of molecular oxygen (Shinyashiki *et al.*, 2000; Shinyashiki *et al.*, 2001). The first mechanism can occur in the absence of oxygen, while the second has been reported to have a requirement for oxygen. Experiments done in either the presence or absence of oxygen can lead to differentiation between these two general types of mechanisms.

GRR1 has been genetically linked to *FZF1* and *SSU1* sulfite sensitivity (Avram and Bakalinsky, 1997). GRR1, an F-box protein also referred to as DSG1/MDM30, is necessary for the specific proteosomal degradation of Gal4p. In turn, this degradation then plays a role in the translation of transcripts induced by Gal4p. In the absence of the *GRR1* gene, Gal4p-induced transcription of targets occurs but they are not translated (Muratani *et al.*, 2005). Because of the genetic connection reported by Avram and Bakalinsky (Avram and Bakalinsky, 1997), we hypothesized that the deletion of *GRR1* may affect the *FZF1*-mediated response to DPTA NONOate. Intriguingly, proteosomal degradation has been reported to be involved in NO· detoxification in *Mycobacterium tuberculosis* (Darwin *et al.*, 2003).

To better understand how Fzf1p activation by NO· occurs, I used DNA microarrays to follow the transcriptional response to the NO· donor DPTA NONOate in *S. cerevisiae* under the following experimental conditions: First, to determine whether translational machinery was necessary for activation by Fzf1p, I exposed yeast to

cycloheximide treatment followed by DPTA NONOate treatment. Second, to determine which of the two competing models for the NO·-mediated response *S. cerevisiae* may be using for activation of Fzf1p leading to transcriptional induction, I examined the response to DPTA NONOate in a nitrogen environment. Finally, to determine the role of *GRR1* in the *FZF1*-mediated response to NO·, I profiled the response to DPTA NONOate in a strain with a deletion in the *GRR1* gene.

MATERIALS AND METHODS

Strains and media

Yeast strains and sources are found in TABLE 4-1. All experiments were done in YPD or synthetic complete media, at 30°C.

Chemicals

DiPropyleneTriAmine NONOate was purchased from Cayman Chemical Company (Ann Arbor, MI). Cycloheximide was obtained from Sigma-Aldrich (St. Louis, MO).

Microarray growth conditions

For the array time-course experiments, cultures were grown in 2.8 liter Erlenmeyer flasks with mechanical agitation in a 30°C room. Each time-point was

obtained as an ~200 ml fraction of these larger cultures, which were harvested by vacuum filtration and frozen in liquid nitrogen prior to RNA isolation.

For the cycloheximide treatment profiling and flow cytometry experiments the *YHB1*-GFP strain starting at OD_{600} 0.2 was exposed to either DPTA NONOate (100 μM), cycloheximide (30 μg per ml), or both, and time-points were taken at 0, 40, and 80 minutes after the initial treatment. Flow cytometry analysis was also undertaken to monitor the fluorescein isothiocyanate (FITC) intensity at 0, 40 and 80 minutes after treatment. These experiments were done in synthetic complete media.

A time-course experiment was also conducted following the exposure of the DBY7283 strain at OD_{600} 1.0 to DPTA NONOate (1 mM) after switching to an environment composed of pure nitrogen for 120 minutes. Samples for array profiling were taken at 0, 10, 20, 40, and 80 minutes following treatment. The response in a nitrogen environment was compared to the response in normal air conditions (Sarver and DeRisi, 2005). These experiments were done in YPD media.

Single time-point experiments were also conducted following 40 minutes of treatment in both a wild type and a *GRR1* delete strain. These experiments were also compared to experiments previously undertaken (Sarver and DeRisi, 2005).

RNA isolation and microarray analyses

Total RNA was isolated using hot-acid phenol-chloroform extraction and mRNA was then purified using oligo-dT resin. Poly(A) + mRNA was reverse-transcribed, using a 1:1 mixture of oligo-dT and random hexamers to incorporate amino-allyl-dUTP into the

resulting cDNA. cDNA was differentially labeled with Cy3 and Cy5 dyes purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Hybridization to cDNA microarrays representing the yeast genome was conducted as previously described (DeRisi *et al.*, 1997).

For each set of time courses, Cy5-labeled cDNA derived from each time-point was compared to a Cy3-labeled reference pool representative of the time-points within each set of time courses. The time course profile was then obtained by dividing the ratios for each time-point by the corresponding ratio for the zero-hour time-point.

All microarray data were stored using the NOMAD database (http://ucsf-nomad.sourceforge.net/). Unflagged spots were included for cluster analysis only if the sum of the median intensities was greater than the median background plus 2 times the standard deviation of the background. The Cluster program (Eisen *et al.*, 1998) was used for data analyses and Treeview (Saldanha, 2004) was used for data visualization. To be included in Figure 1 and Figure 2, data needed to be present in all 0-hour time-points and transcript levels were required to change greater than 5.6-fold ($2^{2.5}$) in 2 or more time-points of the time courses shown.

Flow cytometry analyses

Fluorescence intensities from each time-point of the YHB1-GFP NO· treatment, cycloheximide treatment, and cycloheximide plus DPTA NONOate treatment, were obtained on a BD Biosciences LSR II Flow cytometer. FITC intensities are given as the average signal for 50,000 events

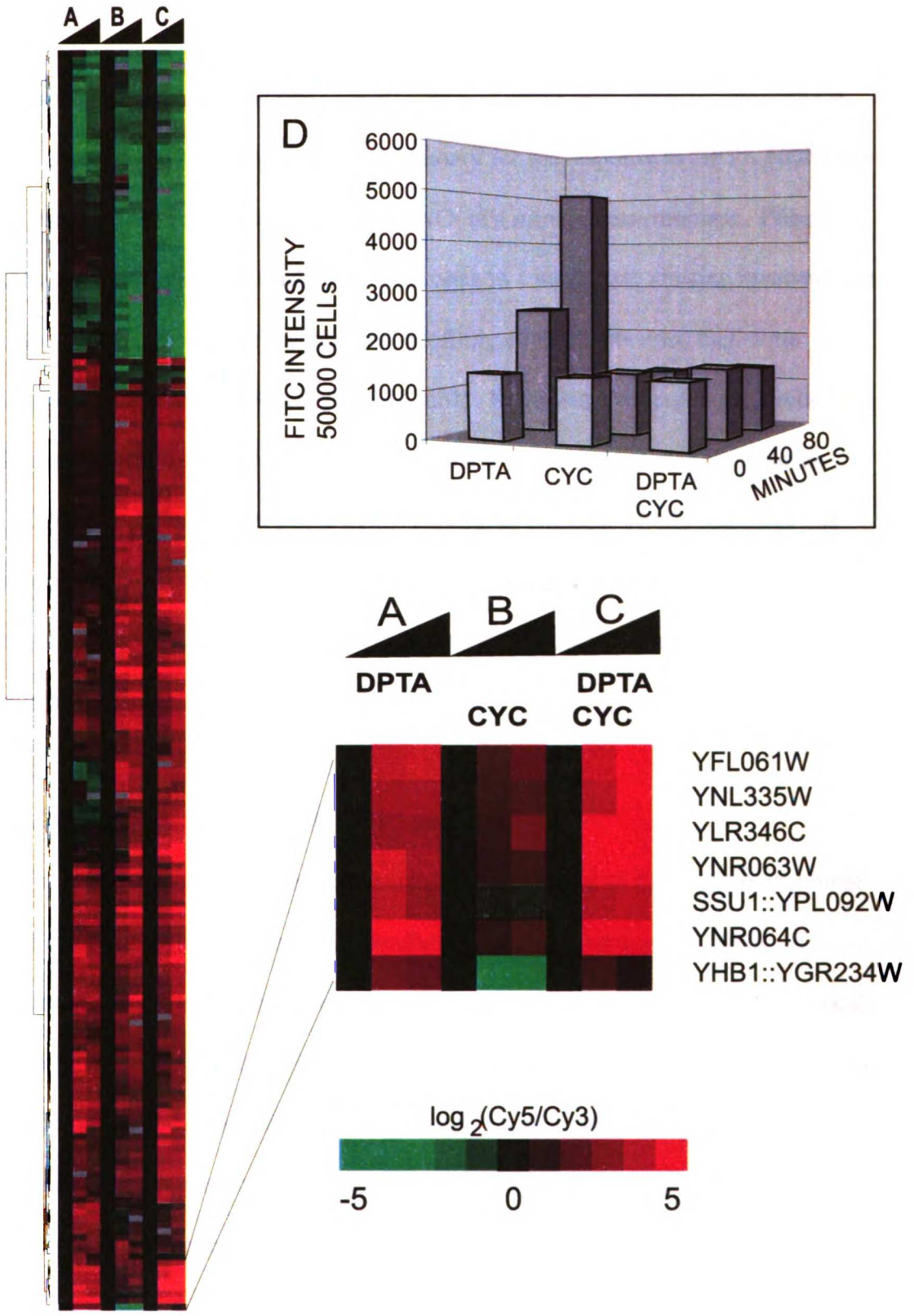
RESULTS

Treatment with cycloheximide inhibits translation ofYhb1p-gfp following DPTA NONOate treatment

To determine whether translation was necessary for activation of the Fzf1p-dependent response to DPTA NONOate, we examined the response in the presence of the translational inhibitor cycloheximide. To ensure that cycloheximide had effectively stalled translation we did these experiments in the *YHB1-GFP S. cerevisiae*. This strain has the *GFP* gene fused to the *YHB1* locus driven by the endogenous *YHB1* promoter. Flow cytometry has been used to monitor the fluorescence of this strain in response to DPTA NONOate (Sarver and DeRisi, 2005). Cultures were treated with DPTA NONOate (100 mM), cycloheximide (30 μg/ml), or both. Cultures were monitored by flow cytometry analysis for FITC intensity at 0, 40 and 80 minutes following initial drug exposure. As each time-point was analyzed by flow cytometry, a sample was also taken for transcriptional profiling. Prior to exposure to DPTA NONOate, *YHB1*-GFP has significant GFP (FITC) signal (1320) relative to a strain without any GFP (~200). Following exposure to DPTA NONOate alone, GFP (FITC) signal increased 1.9-fold (2548) after 40 minutes and 3.8-fold (5032) after 80 minutes, indicating that new protein was being translated in response to DPTA NONOate (100 mM). Following exposure to cycloheximide, the GFP signal slightly decreased after 40 (1267) and 80 (1168) minutes. Following exposure to DPTA NONOate in the presence of cycloheximide, GFP signal did not significantly increase after 40 (1417) or 80 (1317) minutes, signifying that translation was effectively stopped.

To determine whether the cycloheximide-mediated block in GFP signal increase following DPTA NONOate treatment was occurring due to a translational block of transcriptional activation or due to activation of transcription followed by a translational block, transcriptional profiling experiments were undertaken on the samples collected as described for the flow cytometry experiments. DPTA NONOate (100 mM) treated, cycloheximide (30 μg/ml) treated, and both DPTA NONOate plus cycloheximide treated strains were all profiled and compared to the starting culture following 40 and 80 minutes of treatment. Following exposure to DPTA NONOate the transcriptional profiles occurred as previously reported (Sarver and DeRisi, 2005). Briefly, induction of *FZF1*-dependent genes *YHB1*, *SSU1*, *YNR064c* and *YNL335w/YFL061c*, as well as a general stress response, occurred as previously reported. Following exposure to cycloheximide alone, a robust transcriptional response occurred and responsive genes were similar to previous reports (Hughes *et al.*, 2000). Following exposure to DPTA NONOate and cycloheximide together, both the response to cycloheximide and the transcriptional response to DPTA NONOate occurred, indicating that translation is not necessary for activation of NO·. The transcriptional levels of *SSU1* and the ORF's mRNA increased in response to DPTA NONOate and DPTA NONOate plus cycloheximide, but did not respond to cycloheximide alone. Interestingly *YHB1* mRNA levels increased in response to DPTA, decreased in response to cycloheximide, and increased in response to cycloheximde plus DPTA, although not as much as with only DPTA treatment. A rational explanation for this effect is that the increases seen following DPTA treatment are offset by the decreases seen following cycloheximide treatment in the combined treatment. These results indicate that all the protein components necessary for sensing

Figure 4-1


A
B
C
D
FITC INTENSITY
50000 CELLs
6000
5000
4000
3000
2000
1000
0
DPTA
CYC
DPTA
CYC
0
40
80
MINUTES
A
B
C
DPTA
CYC
DPTA
CYC
YFL061W
YNL335W
YLR346C
YNR063W
SSU1::YPL092W
YNR064C
YHB1::YGR234W
log 2(Cy5/Cy3)
-5
0
5

and responding to NO· through the Fzf1p pathway are present within *S. cerevisiae* prior to exposure to DPTA NONOate.

Fzf1p response to DPTA NONOate (1 mM) does not occur in a nitrogen atmosphere

To determine whether oxygen was necessary for the response to DPTA NONOate to occur, the DBY7283 strain was exposed to NO· in a nitrogen environment. Prior to NO· exposure, the culture was flushed with nitrogen in a water bath shaking incubator for 120 minutes in YPD media. Transcriptional profiling experiments were then done following treatment with DPTA NONOate (1 mM). Following this exposure, a robust general stress response was seen with strong similarities to the normoxic response to DPTA NONOate. Examination of the *FZF1*-dependent response cluster indicates that this response did not occur following DPTA NONOate exposure in a nitrogen environment (Figure 2). These results also indicate that oxygen is necessary for the Fzf1p-mediated response to DPTA NONOate.

Nitrosylation sequence present in the FZF1 gene.

It has been suggested that certain residues are important to the local environment for the efficient nitrosylation of cysteine residues by NO· (Ascenzi *et al.*, 2000). A search of the *FZF1* sequence for the known cysteine-modification sequence motif reveals that this sequence motif is present in the *FZF1* gene surrounding cysteine 44 (Figure 3A). Interestingly, in the *S. bayanus* and *S. mikatae* yeast species, which were shown to have a conserved CS2 sequence in Fzf1p dependent NO· responsive genes, the nitrosylation sequence motif is conserved. Additionally in *S. castelli* and *S. kluyveri,* where the CS2

Figure 4-2

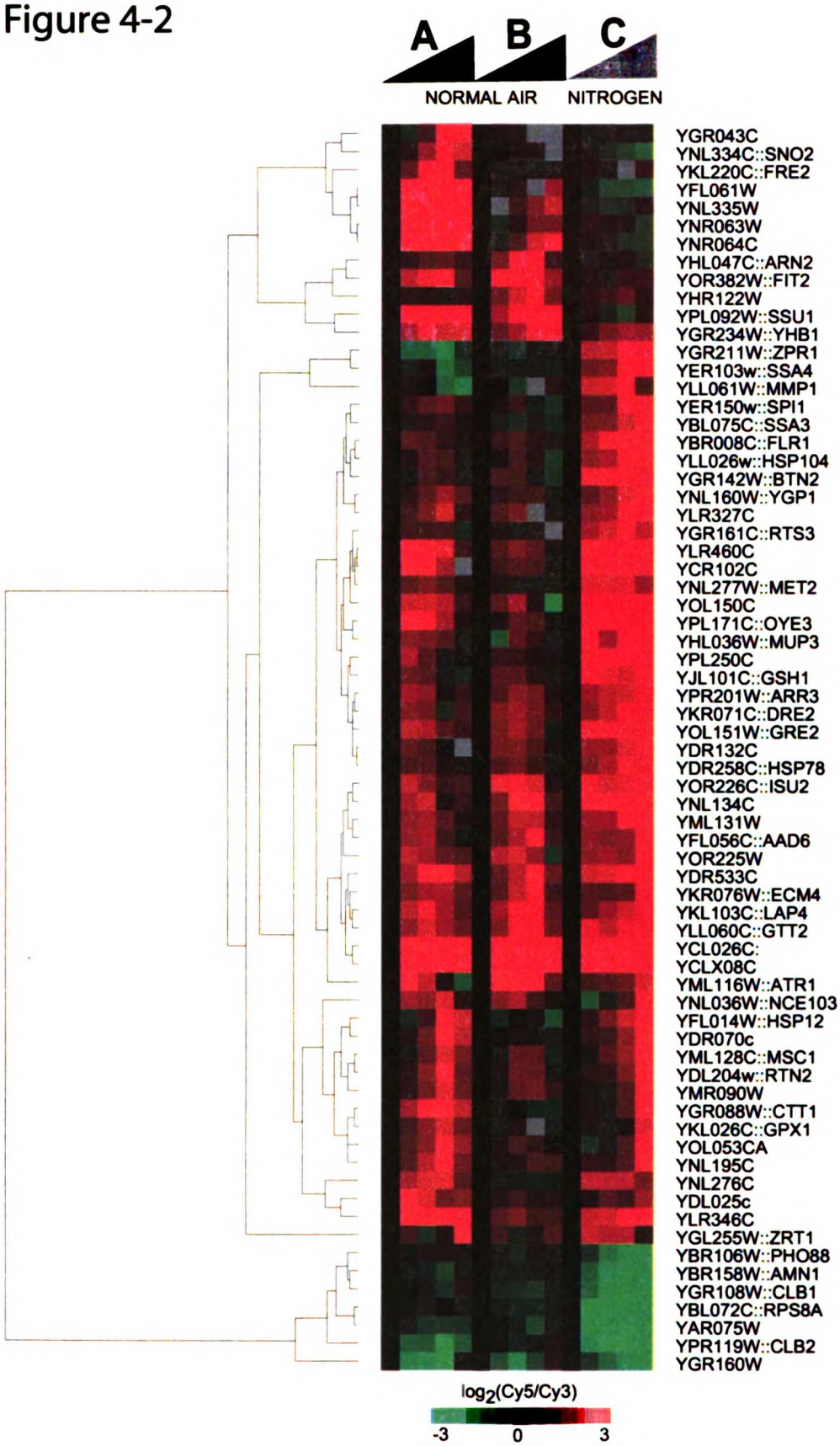

A
B
C
NORMAL AIR
NITROGEN
YGR043C
YNL334C::SNO2
YKL220C::FRE2
YFL061W
YNL335W
YNR063W
YNR064C
YHL047C::ARN2
YOR382W::FIT2
YHR122W
YPL092W::SSU1
YGR234W::YHB1
YGR211W::ZPR1
YER103w::SSA4
YLL061W::MMP1
YER150w::SPI1
YBL075C::SSA3
YBR008C::FLR1
YLL026w::HSP104
YGR142W::BTN2
YNL160W::YGP1
YLR327C
YGR161C::RTS3
YLR460C
YCR102C
YNL277W::MET2
YOL150C
YPL171C::OYE3
YHL036W::MUP3
YPL250C
YJL101C::GSH1
YPR201W::ARR3
YKR071C::DRE2
YOL151W::GRE2
YDR132C
YDR258C::HSP78
YOR226C::ISU2
YNL134C
YML131W
YFL056C::AAD6
YOR225W
YDR533C
YKR076W::ECM4
YKL103C::LAP4
YLL060C::GTT2
YCL026C:
YCLX08C
YML116W::ATR1
YNL036W::NCE103
YFL014W::HSP12
YDR070c
YML128C::MSC1
YDL204w::RTN2
YMR090W
YGR088W::CTT1
YKL026C::GPX1
YOL053CA
YNL195C
YNL276C
YDL025c
YLR346C
YGL255W::ZRT1
YBR106W::PHO88
YBR158W::AMN1
YGR108W::CLB1
YBL072C::RPS8A
YAR075W
YPR119W::CLB2
YGR160W
log2(Cy5/Cy3)
-3
0
3

Figure 4-3 Fzf1p sequence

A.

```
 1   MTDIGRTKSR NYKCSFDGCE KVYNRPSLLQ QHQNSHTNQK PYHCDEPGCG
                          Zinc figer #1
51   KKFIRPCHLR VHKWTHSQIK PKACTLCQKR FVTNQQLRRH LNSHERKSKL
       Zinc figer #2              Zinc figer #3
101  ASRIDRKHEG VNANVKAELN GKEGGFDPKL PSGSPMCGEE FSQGHLPGYD

151  DMQVLQCPYK SCQKVTSFND DLINHMLQHH IASKLVVPSG DPSLKESLPT
                  Zinc figer #4
201  SEKSSSTDTT SIPQLSFSTT GTSSSESVDS TTAQTPTDPE SYWSDNRCKH

251  SDCQELSPFA SVFDLIDHYD HTHAFIPETL VKYSYIHLYK PSVWDLFEY
         Zinc figer #5
```

S-NO sequence [(G/S/T/C/Y/N/Q)(L/R/H/D/E)(C),(D/E)]

B.

```
S. cer(31) QHQNSHTNQK PYHCDEPGCG KKFIRPCHLR VHKWTHSQIK
S. bay     QHENSHFNQK PYLCDEPGCG KKFIRPCHLR VHKWTHSQIK
S. mik     QHQNSHTNRK PYHCDEPGCG KKFIRPCHLR VHKWTHSQIK
           ** ***  ** ** ******* ********** **********

                      ####
S. cas     QHRYSHTNER PYICDVEGCG KRFMRPCHLK VHKWTHSKVK
S. klu     SRRRSHTNER PFVCEEPGCG KGFLRASHLK VHKWSHSQVK
               ** *   *  *   *** * * *  **  **** **  *
```

sequence is not conserved, the nitrosylation sequence has not been conserved as well. (Fig 3B). . It is also important to note that the 3-dimensional environment may also be important for the process of NO· mediated cysteine nitrosylation (Ascenzi *et al.*, 2000).

GRR1-delete strains have a wild type response to DPTA NONOate

To determine the role of *GRR1* in the *FZF1*-dependent response, a *GRR1*-delete strain and a wild type strain were both profiled following exposure to DPTA NONOate (100 mM) in SCD media for 40 minutes. The wild type response occurred as expected. Examination of a strain with the *Grr1* gene deleted indicated that response occurred similarly to wild type (data not shown).

DISCUSSION

In this work we determined that active translation was not necessary for activation of Fzf1p by NO·. This indicates that a translation-independent mechanism is utilized by *S. cerevisiae* to respond to NO· through the actions of Fzf1p. As a follow-up, we also determined that *S. cerevisiae* is not capable of responding to DPTA NONOate in a nitrogen environment. We presume that this is due to the lack of molecular oxygen that would result in the inability to form higher nitrogen oxides, which have previously been implicated in the formation of nitroso-cysteine compounds.

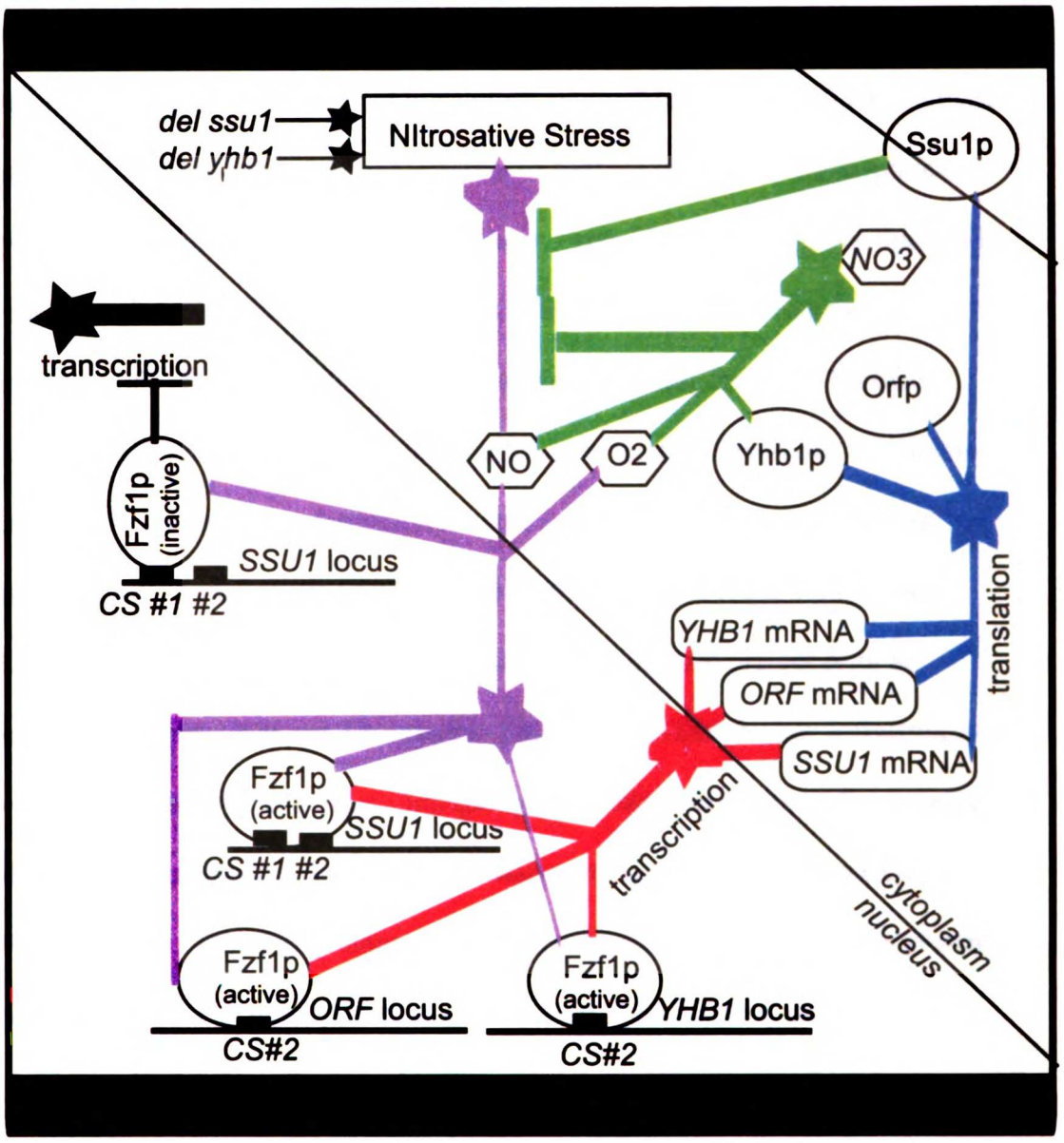
These two important new pieces of data indicate that a post-translational modification using a higher nitrogen oxide may be the mechanism of activation of the signaling network to specifically respond to the presence of NO·. It is unclear whether

this is a direct activation of Fzf1p or if this is due to removal of a repressive function of another element. This is in direct contrast to the NO· sensing that occurs in the canonical NO·-response network. A heme sensor in soluble guanylyl cyclase leads to an allosteric shift that leads to activation (Stone and Marletta, 1994). Bacterial homologues for this eukaryotic NO· sensor have been identified by homology and have been shown to be capable of binding NO· (Boon and Marletta, 2005). However, oxygen is not required for the activation of these prototypical NO· sensors. Since a homolog has not been found in *S. cerevisiae* and oxygen is required for activation of Fzf1p, it appears that *S. cerevisiae* must use a different and potentially novel mechanism to sense and respond to the presence of NO·.

A sulfite-resistant strain r*SSU1* has been identified and the resistance has been shown to be due to increased levels of SSU1p. The increase in SSU1p levels has been shown to be due to a repeat sequence present in the promoter region of *SSU1* (Perez-Ortin *et al.*, 2002). This induction has been shown to occur independently of the *FZF1* gene (Yuasa *et al.*, 2005). Important to this research, deletion of FZF1 in the r*SSU1* reporter strain results in an increase in lacZ activity (Yuasa *et al.*, 2005). One explanation for these results is that Fzf1p is present at the promoter prior to NO· treatment and functions as a repressor in the absence of NO·. Deletion of *FZF1* may lead to a situation where access to the r*SSU1* reporter promoter is no longer hindered by Fzf1p, leading to the increased activation observed.

The present model for Fzf1p-mediated activation in response to NO· is as follows. Fzf1p is present at the *SSU1* promoter in the absence of NO·. At this point it is in a closed conformation and does not activate transcription. In the r*SSU1* reporter strain

Figure 4-4



NO mediated event
protein increases

mRNA increases
remediation event

Fzf1p even represses access to the promoter by other transcriptional activators. Following exposure to NO·, a conformational change occurs in Fzf1p, which leads to an increase in binding between Fzf1p and transcriptional machinery, leading to an increase in transcription. I believe that the conformation of Fzf1p controls the transcriptional activity of Fzf1p. Following translation of nascent Fzf1p, Fzf1p is in a transcriptionally active conformation, as evidenced by increases in transcription following the overexpression of Fzf1p. Fzf1p then goes through a functional change to become inactive transcriptionally, but still capable of binding to the *SSU1* promoter. Following exposure to NO·, the active conformation is achieved leading to the transcriptional response observed. Achieving the active conformation does not require translation indicating that all necessary protein components for response are present prior to treatment. The response does require oxygen. This indicates that the key activating event may be a cysteine nitrosylation event. Intriguingly, a consensus sequence for cysteine nitrosylation has been reported based on acid base catalyses of nitrosylation (Ascenzi et al., 2000). A search of the Fzf1p amino acid sequence reveals that this motif is present surrounding Cys-44. The nitrosylation event important for activation may not be on Fzf1p, but may also be occurring on a binding partner that represses Fzf1p or at any point in an upstream signaling cascade. Results both published and unpublished agree with this model of activation. Future work will serve to either validate or refine this model.

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Table 4-1 *S. cerevisiae* strains utilized

Strain	*genotype*	*origin*
DBY7283	MATα, ura3-52	(Botstein FY22)
YHB1-GFP	(S288C) MATa *YHB1::GFP*	(Huh *et al.*, 2003)
grr1Δ	BY4741 MATa *grr1Δ* + KAN	Resgen

Figure 1. DNA microarray analyses. Data from three independent time course response experiments were analyzed using the Cluster Program. The blown up region is a detailed view of the RNI-responsive *FZF1*-dependent cluster. The triangles indicate the passage of time and the column letter indicates experiment performed as followed. All experiments were conducted using the YHB1-GFP strain in synthetic complete media following exposure to A) DPTA NONOate (100 μM); B) cycloheximide (30 μg/ml); C) DPTA NONOate (100 μM) and cycloheximide (30 μg/ml). Genes showing greater than 5.6-fold ($2^{2.5}$) response in 2 or more arrays were included. The color saturation indicates the magnitude of the expression ratio as indicated by the scale in the lower left hand corner. D) (INSET) Flow cytometry FITC median intensity of the average of 50,000 events corresponding to the time-points and conditions used for array profiling.

Figure 2. DNA microarray analyses. Data from 3 independent time course response experiments were analyzed using the Cluster Program. Experiments were conducted using the DBY7283 strain. The triangles indicate the passage of time and the column letter indicates experiment performed as followed A) exposed to DPTA NONOate (100 μM) in normal air; B) exposed to DPTA NONOate (1 mM) in normal air; C) exposed to

DPTA NONOate in a nitrogen environment. Experiment A was conducted in synthetic complete media, and experiments B and C were conducted in YPD media. Genes showing greater than 5.6-fold ($2^{2.5}$) response in 2 or more arrays were included. The color saturation indicates the magnitude of the expression ratio as indicated by the scale in the lower left hand corner.

Figure 3. Fzf1p sequence analyses

Figure 4. Model

CHAPTER 5

FUTURE DIRECTIONS

The discovery and characterization of the Fzf1p-mediated response to nitric oxide (NO·) has been a challenging and satisfying scientific experience. Very few people have had the opportunity to explore their own signaling response network with the tools and freedom that I have had available to me. I feel very fortunate for having had this opportunity as a part of my graduate career. I hope that these lines of research will be followed up in the future and become a canonical signaling pathway, adding something to the knowledge base and the illustrious history of yeast genetics.

Prior to embarking on a course of scientific research the motivation for the work must be examined. For this work the motivation was straightforward. NO· is produced by the immune system in response to pathogenic infection as a mechanism to defend against pathogenic organisms. The response of the pathogen to NO· will then determine whether the organism can survive to further infect the host. Thus, the ability to respond to the presence of NO· is an important pathogenic factor. Research into how *S. cerevisiae* responds to NO· will allow the better understanding of how microorganisms recognize and respond to NO·, which then leads to a better understanding of host-pathogen interactions. In addition, these results may shed light on novel eukaryotic responses to NO·, which may play important roles in human health due to the ubiquitous nature of NO· in modern biology.

The results presented in Chapters 3 and 4 of this dissertation brought to light a number of questions that future research in this area should attempt to answer. These

future projects can be grouped in a few main areas: The first and most obvious area is to test the model presented at the end of chapter 4. A second important area for this research is to fill in the blanks about the response system, as defined by the presented results. A third major area of research is to expand beyond what is known and determine the precise molecular mechanism for how activation of *FZF1* by NO· is occurring. These points will be discussed more thoroughly in the rest of this chapter.

A key tenet of the model proposed in Chapter 4 is that Fzf1p is located at the *SSU1* promoter prior to activation by NO·. This could be tested both directly and indirectly. To directly examine the location of Fzf1p prior to NO· treatment a one-hybrid experiment could be conducted. The Fzf1p binding sequence discovered in Chapter 3 could be inserted into a lacZ strain and an *FZF1-GAL4* activation domain fusion protein could be expressed in this strain. Comparison of the lacZ activity of the strain carrying the *FZF1-GAL4* fusion protein to a *GAL4* control strain should indicate whether Fzf1p is present at the promoters prior to NO·-mediated activation. This information could be further validated by examining nuclear and cytoplasmic fractions for the location of Fzf1p by Western blotting using either an antibody to Fzf1p or a tagged version of Fzf1p. The *in vivo* binding of Fzf1p could also be determined by chromatin IP experiments prior to and after NO· treatment. While these experiments were being undertaken one could also undertake similar experiments to determine if Fzf1p was found at the *YHB1* and *ORF* promoters. The results of these experiments may provide insight into the structural accessibility of the Zinc finger that is binding CS#2 prior to and following NO· treatment.

A second line of experiments designed to test the model presented in Chapter 4 is centered on determining the role of CYS-44 in the activation of Fzf1p using mutational

analyses. The CYS-44-GLY mutation could be constructed and its ability to respond to NO· examined. If this hypothesis is correct, this mutant would not be able to induce *SSU1* and *YHB1* in response to nitrosative stress, although it may be constitutively active. Other mutations that could be informative would be the Tyr-42-GLY, His-43-gly and ASP-45-Gly mutation. I n the event that the CYS-44-GLY was constitutively active and not viable, these mutants may trap the "off" form of Fzf1p because the sequence catalyzed nitrosylation event may be unable to occur.

A number of exciting findings were shown to occur but the exact mechanisms remain to be elucidated. The first experiment one could do to further this research is to determine whether *E. coli*-generated Fzf1p will directly interact with the element found in the promoter region of all NO·-responsive *FZF1*-dependent genes by a gel shift assay. As a follow-up to this research, if a shift is observed, the exact zinc finger of FZF1, which is binding the promoter element discovered as a part of this dissertation, could then be determined. The role of Fzf1p as a repressor could be further explored by inserting the Fzf1p-binding sequences into highly active lacZ reporter strains and then looking for Fzf1p-mediated repression of lacZ activity.

The minimal *FZF1* activation domain could also be characterized in a very linear series of experiments. Specific mutations in the minimal activation domain could be explored to help determine a mechanism of activation.

A more hypothesis-driven approach could also be followed. The results in Chapter 4 indicate that a post-translational modification utilizing molecular oxygen may be the activation mechanism. If the modification is occurring through the direct

modification of Fzf1p this may be seen through the *in vitro* reaction of Fzf1p with NO· in an oxygen environment. Identification of any resulting modification, either by mass spectrometry or mobility shifts, could then be assayed back in the organism by targeted mutation of the identified amino acid(s) as described above.

An unbiased proteomic approach to identifying targets of cysteine nitrosylation could also be undertaken. Recently, methodology has been developed to allow genome-wide identification of nitrosylated cysteine residues in complex mixtures of peptides by mass spectrometry (Hao et al., 2006). This methodology relies on adding biotin tags to nitrosylated cysteine residues, purification and identification of the site of nitrosylation based on the peptide sequence and specific attachment of the biotin. In addition to potentially identifying the specific nitrosylation of Fzf1 in an unbiased manner, identification of the *S. cerevisiae* cysteine nitrosylation proteome may provide important clues to identify a nitrosylation event that leads to the activation of Fzf1p by a more traditional signaling cascade. Identification of the cysteine nitrosylation proteome may also lead to a better understanding of how NO· exposure leads to cell stress, which may have important implications in a large number of NO·-related human diseases.

Further research into this field is likely to be extremely fruitful. In many fields of research the lack of a published result of a direct line of research is not in the literature not because the experiments have not been tried, but because the experiments did not work for either technical or personal reasons. The more obvious and high interest a research area it is the more likely the reasons tend towards technical. This starting point provides fertile ground for interesting and exciting research in an area of yeast biology with a high relevance to human health.

References

Hao, G., Derakhshan, B., Shi, L., Campagne, F., and Gross, S. S. (2006). SNOSID, a proteomic method for identification of cysteine S-nitrosylation sites in complex protein mixtures. Proc Natl Acad Sci U S A *103*, 1012-1017.

Appendix 1. DNA microarray post processing

Prior to starting, etch arrays on the back of slide above and below print area; spots will disappear following post processing. Pre-boil the water to be used in step 3 prior to starting step 3.

I. "Shampoo"

A. Soak for 5 minutes in 65°C solution of 3x SSC and 0.2% SDS.

B. Soak for 30 seconds in room temperature water followed by 30 seconds in room temperature 95% ethanol.

C. Spin in tabletop centrifuge at 400 rpm for 1 minute.

II. "Rehydrate" (Be careful not to splash liquids on or drop array into liquids)

A. Turn slide warmer on and let heat to 37°C with fresh 0.5x SSC in the hydration chamber. Also turn on heating block to 140°C.

B. Add arrays to hydration chamber and let hydrate for 2-6 minutes until a smooth polylysine coating has been achieved.

C. Snap dry each array on the heating block individually for 3 seconds.

III. "Post-Processing"

A. Add 335 ml of methyl pyrillidone to beaker containing 6.5 g succinic anhydride mix until all solids are dissolved.

B. Plunge arrays into the solution and shake for 30 seconds, then gently mix for 12 minutes.

C. 90 seconds in hot water (just under boiling).

D. 6 minutes back in succinic anhydride methyl pyrillidone.

E. Two, 30-second ethanol rinses.

F. Spin in table top centrifuge at 400 rpm for 1 minute.

Appendix 2. Hot acid phenol isolation of tRNA from *S. cerevisiae*

I followed this procedure with groups of 8 samples, had all components ready prior to beginning and tried to do all steps as fast as possible.

1. 200 mL of yeast at OD_{600} 1.0 are harvested by vacuum filtration onto filters and the filters are frozen in 50 ml Falcon tubes by submersion into liquid nitrogen and stored at -80°C. This step is to prevent temperature shock associated with centrifugation. Samples can be stored indefinitely prior to RNA isolation.

2. Cultures are re-suspended in 12 ml AE buffer by shaking and added to Oak Ridge tubes containing 12 ml phenol (acid pH 4.2) and 800 µl of 25%SDS. Vortex.

3. Incubate 10 minutes at 65°C in hood

4. Incubate 5 minutes on ice.

5. Centrifuge 15 minutes at 12,000 rpm in SS-34 rotor.

6. Carefully pour supernatant into a 50 ml phase lock tube (pre-spun in table top centrifuge at 1500 rpm after adding 15 ml of chloroform).

7. Centrifuge in tabletop 10 minutes at 3000 rpm.

8. Dump supernatant into clean 50 ml Oakridge tube containing 12 ml isopropanol with 1.2 ml 3 M sodium acetate pH 5.4.

9. Freeze in liquid nitrogen or place in –80°C overnight to increase yields.

10. Centrifuge 60 minutes at 14,000 rpm.

11. Carefully invert tube, remove cap, and pour off supernatant, retaining pelleted RNA. (Note: if pellet moves from bottom of tube during initial inversion just centrifuge for 5 more minutes.

12 Wash with 5 ml of 70% ethanol, centrifuge 15 minutes at 14,000 rpm.

13. Repeat step 11.

14 Dry for 10 minutes in speed vacuum at low heat. Store RNA at –80°C indefinitely prior to re-suspension.

Appendix 3. Oligo-dT resin isolation of mRNA from S. cerevisiae

I followed this procedure with up to 16 samples at a time, had all components ready prior to beginning and tried to do all steps as fast as possible. Works best with sample input >2 mg of total RNA. Should return 1-3% of input RNA. Oligo-dT can be reused indefinitely following the protocol in Appendix 4.

1. Prepare 75 mg of oligo-dT per sample by washing 3x with 1x NETS in a 50 ml Falcon tube.

2. Resuspend in 2x NETS (750ul X # of samples) and portion out 750 µl into 2 ml screw cap tube Eppendorf tubes.

3. Resuspend tRNA samples in 760 µl water in Oak Ridge tubes, heat at 65°C for 10 minutes and add 750 µl to Eppendorf tubes.

4. Rotate for 1 hour at room temperature.

5. Wash mini-columns with 750 µl 1X NETS.

6. Add RNA Oligo-dT to column by gentle pouring.

7. Wash column gently 2x with 1X NETS.

8. Elute with 650 µl of 65°C 1X ETS solution twice into two separate Eppendorf tubes.

9. Add 65 µl of 3 M sodium acetate pH 5.4 and 650 µl isopropanol.

10. Freeze at –80°C for at least 30 minutes .
(Note: this greatly increases yields)

11. Spin sample in microcentrifuge in cold room for 30 minutes.

12. Carefully aspirate off supernatant and wash pellet with 250 µl 70% ethanol, spin.

13. Carefully aspirate off supernatant and dry in speed vacuum for 30 seconds.

14. Quantify total RNA and polyadenylated mRNA by $OD_{260/280}$ ratio and run 1 µg on gel prior to carrying out RT.

NETS: 41.5 ml water, 6.0 ml 5.0 M NaCl. 1 ml 0.5 M EDTA, 600 µl 10% SDS
ETS: 47.5 ml water, 1 ml 0.5 M EDTA, 400 µl 10% SDS
Note: add SDS last otherwise it will precipitate.

Appendix 4 Oligo-dT resin regeneration protocol

I was able to reuse Oligo-dT supplied by Howard Hughes labs infinitely using this protocol. Columns can be stored at -20°C following elution to be regenerated at a future time.

1. Pool all Oligo-dT resin available and wash with 65°C ETS in 50 ml Falcon tubes.

2. Spin in tabletop centrifuge and remove supernatant.

3. Wash 3X with H_20 spin and remove supernatant after each wash.

4. For immediate use, resuspend in 1X NETS and begin mRNA isolation protocol. (Prepare resin by 3x rinses in 1X NETS)

5. For long term storage, wash 1X in 100% ethanol, spin and remove supernatant and dry thoroughly in speed vacuum and store in -20°C.

Appendix 5. Reverse transcription reaction with amino-allyl

1. To 2 μg polyA mRNA add 2 μg Oligo-dT (hexamer) and random oligo (ninemer) in 20 μl water total volume in 600 μl Eppendorf tubes.

2. Incubate at 70°C for 10 minutes.

3. Chill at 4°C for 10 minutes.

4. To the side of the test tube:
   Add 3 μl 10X RT buffer
   Add .6 μl 50X dATP, dCTP, dGTP, 3/5 amino-allyl dUTP 2/5 dTTP
   (final concentration 500 μM each dNTP)
   Add 3 μl 100 mM DTT
   And spin down.

5. Add 3 μl RT and pipette up and down to ensure good mixing.

6. Incubate 42°C for 2 hours (can leave this overnight).

7. Add 10 μl 0.5 M EDTA followed by 5 μl 1 N NAOH.

8. Incubate 65°C for 15 minutes.

9. Purify cDNA by ZYMO spin column eluting with 12 μl 100 mM sodium bicarbonate buffer.

10. Verify cDNA synthesis by running 2 μl on an agarose gel and observing smear.

Store at -20 indefinitely

If no product is observed and the positive control RT was successful, then further purify the remaining mRNA by chloroform extraction and reprecipitation. Reverse transcriptase is inhibited by something that can carry through the mRNA purification (possibly SDS).

Appendix6. Coupling, DNA microarray hybridization, and washing

Coupling

1. Resuspend Cy3 and Cy5 packet for one labeling reaction in 10 µl DMSO.

2. Add 1µL of dye to 10 µl cDNA in 100 mM sodium bicarbonate.
   Note: can speed vacuum remaining dye in 1 µl fractions for later use.

3. Let sit in dark at room temperature for one hour.

4. Purify labeled cDNA using Zymo spin column. Elute in 7.5 µl water.

Hybridization

1. Combine Cy3 and Cy5 labeled samples (15 µl total):
   Add 3 µl 20X ssc
   Add 1.5 µl PolyA (10 mg/ml)
   Add .5 µl 1 M HEPES pH 7.0
   Add .5 µl 10 % SDS

2. Boil probe for 2 min at 100°C, cool in microcentrifuge.

3. Carefully pipette sample under cover slip onto post-processed array prepared in hybridization chamber containing excess 3X SSC.

4. Hybridize at 65°C in water bath overnight.

Washing

1. Transfer arrays from hybridization chamber into wash solution 1.
   Wash solution 1: 330 µl water 20 µl 20X SSC 100 µl 10% SDS.

2. Individually transfer arrays from wash solution 1 to wash solution 2.
   Wash solution 2: 350 ml water 2 ml 20X SSC.

3. Remove array rack from wash solution 2 and spin in table top centrifuge at 400 rpm for one minute.

4. Scan.

Appendix 7

Array data source key to figures and database record on NOMAD2

Figure 2-2

Ya2s3p4-140
Ya2s3p4-141
Ya2s3p4-142

Figure 3-1

A mock 0	ya2s3p2_Nov2003-073
A mock 10	ya2s3p2_Nov2003-080
A mock 20	ya2s3p2_Nov2003-081
A mock 40	ya2s3p2_Nov2003-082
A mock 80	ya2s3p2_Nov2003-083
B APD 0	ya2s3p2_Nov2003-073
B APD 10	ya2s3p2_Nov2003-085
B APD 20	ya2s3p2_Nov2003-086
B APD 40	ya2s3p2_Nov2003-087
B APD 80	ya2s3p2_Nov2003-088
C DPTA 0	ya2s3p2_Nov2003-073
C DPTA 10	ya2s3p2-075
C DPTA 20	ya2s3p2-076
C DPTA 40	ya2s3p2-077
C DPTA 80	ya2s3p2_Nov2003-078
D DPTA 0	ya2s3p2_Nov2003-120
D DPTA 10	ya2s3p2_Nov2003-107
D DPTA 20	ya2s3p2_Nov2003-108
D DPTA 40	ya2s3p2_Nov2003-109
D DPTA 80	ya2s3p2_Nov2003-123
E DPTA 0 fzf1del	ya2s3p2_Nov2003-111
E DPTA 10 fzf1del	ya2s3p2_Nov2003-112
E DPTA 20 fzf1del	ya2s3p2_Nov2003-113
E DPTA 40 fzf1del	ya2s3p2_Nov2003-114
E DPTA 80 fzf1del	ya2s3p2_Nov2003-115
F DPTA 0	DPTA 0 tp
F DPTA 10	DPTA 10 tp
F DPTA 20	DPTA 20 tp

F DPTA 40	DPTA 40 tp
F DPTA 80	DPTA 80 tp
F DPTA 120	DPTA 120 tp
G DPTA 0 yhb1del	dpta del-yhb1 0
G DPTA 10 yhb1del	dpta del-yhb1 10
G DPTA 20 yhb1del	dpta del-yhb1 20
G DPTA 40 yhb1del	dpta del-yhb1 40
G DPTA 80 yhb1del	dpta del-yhb1 80
G DPTA 120 yhb1del	dpta del-yhb1 120
H DPTA 0 fzf1del	dpta del-fzf1 0
H DPTA 10 fzf1del	dpta del-fzf1 10
H DPTA 20 fzf11del	dpta del-fzf1 20
H DPTA 40 fzf1del	dpta del-fzf1 40
H DPTA 80 fzf1del	dpta del-fzf1 80
H DPTA 120 fzf1del	dpta del-fzf1 120
I NO gas 120	ya1s4p3-217

Figure 3-4

gal 0	ya2s3p2_Nov2003-100
gal 20	ya2s3p2_Nov2003-099
gal 40	ya2s3p2_Nov2003-098
gal 80	ya2s3p2_Nov2003-097
fzf1 0	ya2s3p2_Nov2003-105
fzf1 20	ya2s3p2_Nov2003-102
fzf1 40	ya2s3p2_Nov2003-104
fzf1 80	ya2s3p2_Nov2003-101

Figure 4-1

0 DPTA	ya2s3p2_Nov2003-120
40 DPTA	ya2s3p2_Nov2003-109
80 DPTA	ya2s3p2_Nov2003-123
0 CYC	ya2s3p2_Nov2003-120
40 CYC	ya2s3p2_Nov2003-116
80 CYC	ya2s3p2_Nov2003-118
0 CYC + DPTA	ya2s3p2_Nov2003-120
40 CYC + DPTA	ya2s3p2_Nov2003-117
80 CYC + DPTA	ya2s3p2_Nov2003-119

Figure 4-2

DPTA 0 (normal air)	ya2s3p2_Nov2003-073
DPTA 10 (normal air)	ya2s3p2-075
DPTA 20 (normal air)	ya2s3p2-076
DPTA 40 (normal air)	ya2s3p2-077
DPTA 80 (normal air)	ya2s3p2_Nov2003-078
DPTA 0 (normal air)	DPTA 0 tp
DPTA 10 (normal air)	DPTA 10 tp
DPTA 20 (normal air)	DPTA 20 tp
DPTA 40 (normal air)	DPTA 40 tp
DPTA 80 (normal air)	DPTA 80 tp
DPTA 0 (nitrogen)	NO6-0
DPTA 10 (nitrogen)	NO6-10
DPTA 20 (nitrogen)	NO6-20
DPTA 40 (nitrogen)	NO6-40 tp
DPTA 80 (nitrogen)	NO6-80 goog