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Fzf1p Regulates an Inducible Response to Nitrosative Stress in *Saccharomyces cerevisiae*

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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 dipropylenetriamine 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, whereas overexpression 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 (Brown, 1997; Stevanin *et al.*, 2000; Chenais *et al.*, 2002), altered iron metabolism (Chenais *et al.*, 2002), (Martinez *et al.*, 2001; D'Autreaux *et al.*, 2002), direct inhibition of enzymes (Gardner *et al.*, 1997), and DNA damage (Martinez *et al.*, 2001; Kow, 2002). ˙NO has been strongly implicated as a component in higher eukaryotic nonspecific immune response to parasites, fungi, bacteria, and viruses (Fang, 1997). Host inducible NOS 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 *Escherichia 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 (de Jesus-Berrios *et al.*, 2003; Ullmann *et al.*, 2004).

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 re-

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Abbreviations used: APD, *N*-(3-aminopropyl)1,3-propane diamine; DPTA NONOate, dipropylenetriamine NONOate; ˙NO, nitric oxide; RNI, reactive nitrogen intermediate.

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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 (Huntsville, AL)
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

sponse. 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 that *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 (Ann Arbor, MI). *N*-(3-Aminopropyl)1,3-propane diamine (APD) was purchased from Aldrich Chemical (Milwaukee, WI). DPTA NONOate was dissolved in 10 mM NaOH solution, and the pH was modified by the addition of 100 mM Tris buffer, pH 7.0, immediately before 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 liters of media in 2.8-liter Erlenmeyer flasks with mechanical agitation in a 30°C room. On reaching an OD_{600} of 0.2, the cultures were exposed to treatment. Time points were harvested at 0, 10, 20, 40, and 80 min by vacuum filtration and snap frozen in liquid nitrogen before RNA isolation.

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

A single time-point microarray experiment compared a DBY7283 culture exposed to chemically generated nitric oxide (5 ml of gas per 200 ml of culture) for 120 min to an untreated DBY7283 culture (Figure 1I).

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 min 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 Biosciences (Piscataway, NJ). Hybridization to cDNA microarrays representing the yeast genome was conducted as described previously (DeRisi *et al.*, 1997).

For each set of time courses, Cy5-labeled cDNA derived from each time point was compared with 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 0-h time point.

Microarray data were 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 SD 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 are in supplemental Table 4, and all raw microarray data from this study are freely available at the National Center for Biotechnology Information 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-h time points, and transcript levels were required to change greater than 5.6-fold ($2^{2.5}$) in two or more time points of the eight time courses shown.

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

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 100 μM DPTA NONOate for 3 h. Cells were then harvested and a standard

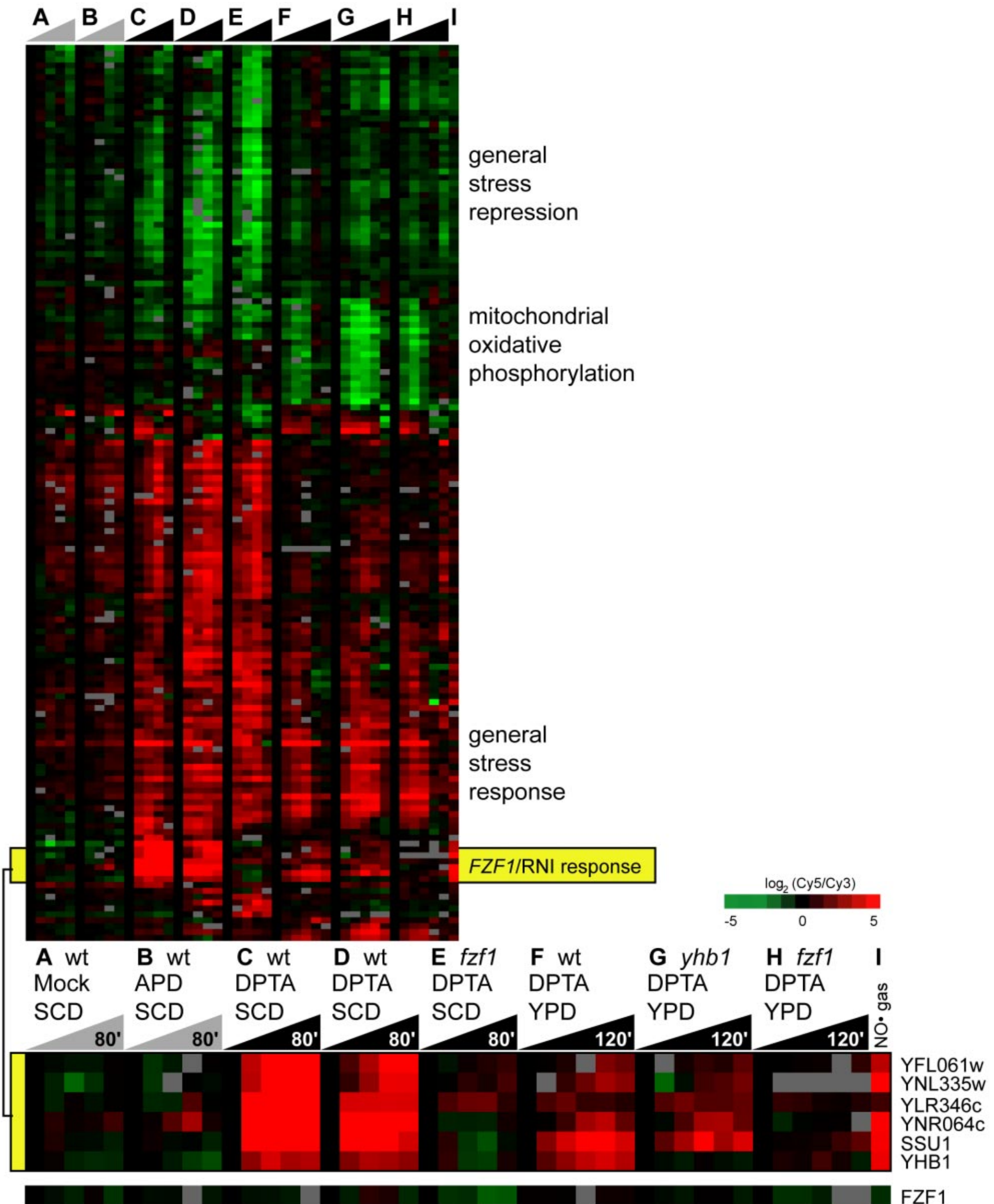

Figure 1. DNA microarray analyses. Data from eight independent time-course response experiments were analyzed using the Cluster Program. The lower region is a detailed view of the RNI responsive *FZF1*-dependent cluster. Although not part of this cluster, the expression profile for *FZF1* is also shown. The triangles indicate the passage of time and the column letter indicates experiment performed as follows: DBY7283 (wt) exposed to a mock treatment (A), DBY7283 (wt) exposed to APD (100 μM) (B), DBY7283 (wt) exposed to DPTA NONOate (100 μM) (C), *YHB1*-GFP exposed to DPTA NONOate (100 μM) (D), *YHB1*-GFP *fzf1*Δ exposed to DPTA NONOate (100 μM) (E), DBY7283 exposed to DPTA NONOate (1 mM) (F), diploid *yhb1*Δ/*yhb1*Δ exposed to DPTA NONOate (1 mM) (G), diploid *fzf1*Δ/*fzf1*Δ exposed to DPTA NONOate (1 mM) (H), and DBY7283 single time-point experiment after exposure to •NO gas (I). 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.

Table 2. Pearson correlations between time-course microarray data sets

	DBY7283	DBY7283	DBY7283	*YHB1*-GFP	*YHB1*-GFP *fzf1*Δ	DBY7283	Diploid *yhb1*Δ	Diploid *fzf1*Δ
	Mock	APD	100 μM DPTA	100 μM DPTA	100 μM DPTA	1 mM DPTA	1 mM DPTA	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 fzf1*Δ 100 μM DPTA					1	0.46	0.59	0.55
DBY7283 1 mM DPTA						1	0.80	0.72
diploid *yhb1*Δ/*yhb1*Δ 1 mM DPTA							1	0.75
diploid *fzf1*Δ/*fzf1*Δ 1 mM DPTA								1

bead beating protocol was used to generate whole cell extract for Western blots (Ausubel *et al.*, 2004). Protein concentration was measured using a Bradford assay (Bio-Rad, Hercules, CA) 15 μg of 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 Tris-buffered saline/Tween 20 with primary rabbit anti-CBP antibody (Bethyl, Montgomery, TX) diluted 1:2500 and the secondary goat anti-rabbit horseradish peroxidase-conjugated antibody (Bio-Rad) was used at a 1:5000 dilution. Western blots were visualized using Supersignal West Pico Chemiluminescence Substrate (Pierce Chemical, 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 were 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 after treatment with DPTA NONOate was compared with 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 h after 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 h, final cell density was monitored by OD_{600} and compared with untreated cell density.

LacZ Experiments

LacZ experiments were conducted as described previously (Russell *et al.*, 1986), with the following modification: Yeast Protein Extraction Reagent (Pierce Chemical) was used for the protein extractions from samples collected 3 h after exposure to the presence or absence of 100 μM DPTA NONOate. All LacZ experiment 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, *EcoR1* and *Xho*I sites were introduced into the promoter region –568 to −45 of the *SSU1* locus by PCR amplification using the primers 5′-GGAATTCATGTGGAAAAA-GAAGGGGTGG and 5′-ATACCGCTCGAGAATTGCGTATTGTCTGAG with genomic DNA as template, and cloned into placZi. This plasmid was then linearized with *Apa*I and integrated into the ura3 locus in YM4271. AS102, AS103, AS106, and AS108 were constructed similarly, using the underlined restriction sites (Table 3).

To construct AS104, a *SSU1* promoter reporter strain –568 to −45 with the region –389 to –370 removed, 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′-AATTCCTGCAAACTAT-CATTTTTT 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′-TCGAAGCGCAGACTAT-CATTTTCAG were used.

To construct the *fzf1*Δ strains AS201–209, the *FZF1* gene was deleted in AS101–109 and *YHB1*-GFP using a PCR product constructed using primers 5′-TACGCTGGTGTGCACAAGTGGTACCAGAATACGTGGCCAAAACAA-TCGGATCCCCGGGTTAATTAA and 5′-ATAGTTCGAATCACATGAGTA-GAGGACGGAAATTGCTCTTCTATGGCGTG AATTCGAGCTCGTTTAAAC, with a Pringle kanamycin resistance plasmid as template (Longtine *et al.*, 1998). This PCR product also was 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-base pair coding sequence for the *FZF1* gene using the primers 5′-ACAATGACGGATATAGGGAGAACCA and 5′-TCAGTATTCGAATAAATCCCAGACGCT, which was cloned into the pYES2.1 vector (Invitrogen, Carlsbad, CA), 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

Table 3. Strain construction PCR primers

Strain	Amplified region	Primer 1	Primer 2
AS101	SSU1 –568 to –45	GGAATTCATGTGGAAAAAGAAGGGGTGG	ATACCGCTCGAGAATTGCGTATTGTCTGA
AS102	SSU1–399 to –45	GGAATTCCTGATAAATT CCTGCAAACTATCAT	ATACCGCTCGAGAATTGCGTATTGTCTGA
AS103	SSU1 –369 to –45	GGGGTACCTTTTTTCATCCTTGTGCCGC	ATACCGCTCGAGAATTGCGTATTGTCTGAG
AS106	SSU1 –568 to –390	ATACCGAAGCTTCATGTGGAAAAAGAAGGGGTG	GGGGTACCAATTTATCAGTCAATTGATGGGAG
AS108	YHB1 –689 to –16	CGAATTCAAGCTTCGTATAATTGCCAA	ATACCGCTCGAGTGTGTGGTTTGTTGAAAATGG

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 ~3 h (Mooradian *et al*., 1995). After the release of two molar equivalents of ˙NO, DPTA NONOate degrades into 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 after treatment with 100 μM DPTA NONOate in SCD media. The response was reproducible: an independent biological replicate experiment showed a very similar genome-wide transcriptional response (correlation 0.77). After 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 1, A–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 open reading frames (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 transmembrane 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 α/β hydrolase superfamily 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 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 after DPTA NONOate treatment revealed that the majority belong 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*. (2000). The highest correlation was seen with the response to the thiol oxidant diamide (correlation 0.67) and a strong positive correlation also was found with the response to heat shock (correlation 0.61). However, *YHB1*, *SSU1*, YNR064c, and YNL335w/YFL061w transcript levels did not change significantly after 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 after DPTA NONOate treatment also would occur after 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 min. 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 1 mM DPTA NONOate. Increased concentrations of DPTA NONOate were used for the YPD experiments relative to the SCD experiments due to an ~10-fold increase in wild-type strain sensitivity in rich versus synthetic media. After 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 after DPTA NONOate treatment, also to a lesser degree than in SCD media despite the increased concentration of DPTA NONOate used. Although 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 1 mM DPTA NONOate (Figure 1F). Treatment with the DPTA NONOate breakdown product 1 mM APD did not elicit this response (our unpublished data).

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 *fzf1*Δ strain to 100 μM DPTA NONOate (Figure 1E) and found that the response of the mutant strain, compared with wild-type, revealed a comparable general stress response after DPTA NONOate exposure (correlation 0.79). However, the RNI-responsive cluster failed to be induced in the strain lacking *FZF1* (Figure 1E). 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 after DPTA NONOate treatment.

To confirm that *FZF1* was also necessary for the induction of *YHB1*, *SSU1*, and the ORFs observed in YPD media, we conducted a time course of transcriptional response after treatment with 1 mM DPTA NONOate 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 after 1 mM DPTA NONOate 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 after 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 FZF1-dependent Gene Set Results in Corresponding Increases in Protein Abundance

To confirm that increases in relative mRNA abundance after DPTA NONOate treatment also lead 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 100 μM DPTA NONOate. Consistent with changes in the levels of mRNA transcripts, DPTA NONOate induced expression of Yhb1p-TAP compared with an untreated control (Figure 2A). Ssu1p-TAP was undetectable before DPTA NONOate exposure, but a band was clearly visible after 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; John Newman, personal communication) was exposed to bolus addition of 100 μl of ˙NO gas generated by acidification of sodium nitrite and monitored by flow cytometry. After exposure to ˙NO gas for 180 min, median fluorescence intensity of the cell population increased by 7.5-fold (Figure 2B). To quantify relative Yhb1p 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 min after 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*-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).

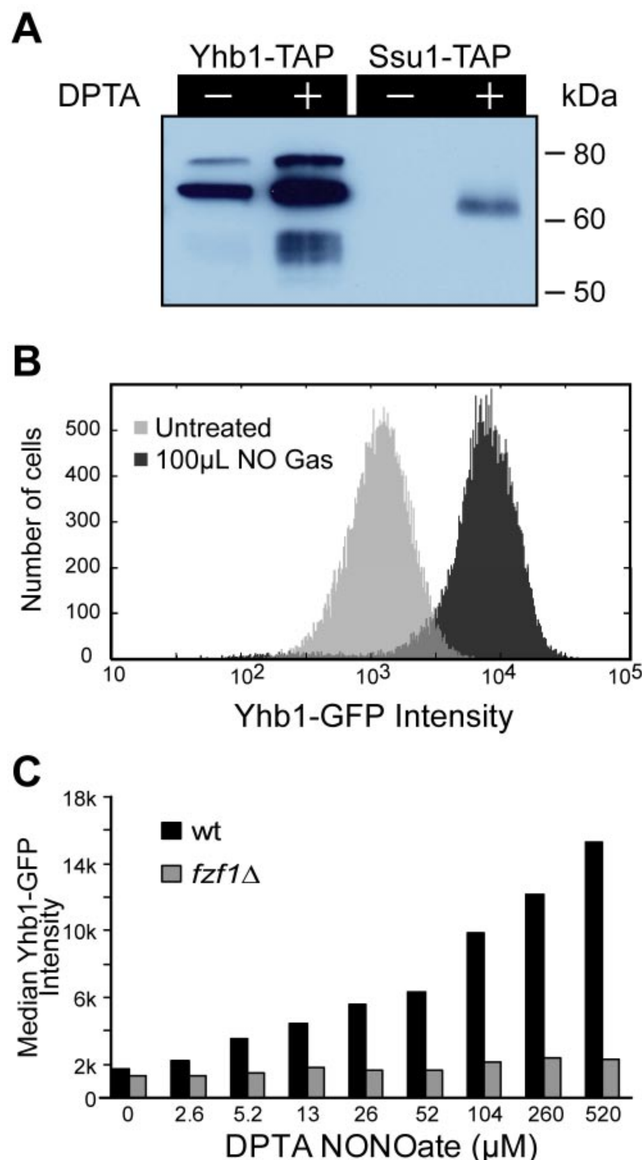

Figure 2. Protein levels after exposure to DPTA NONOate and ˙NO gas. (A) Western blot using rabbit anti-CBP antibody of TAP-tagged Yhb1p and Ssu1p in the presence or absence of DPTA NONOate (100 μM). (B) Flow cytometry of Yhb1p-GFP of fluorescein isothiocyanate (FITC) median intensity in the presence or absence of ˙NO gas for 180 min. (C) Dose-response curves 180 min 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.

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 after overexpression of the *FZF1* gene. Whereas the majority of the expression differences were 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 threefold (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 using sequence data from closely related yeast species. Such comparisons have been used to precisely identify several regulatory motifs (Cliften *et al.*, 2003; Kellis *et al.*, 2003). Multiple alignment of the *SSU1* promoter from *S. cerevisiae*, *Saccharomyces paradoxus*, *Saccharomyces mikatae*, and *Saccharomyces 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 seems

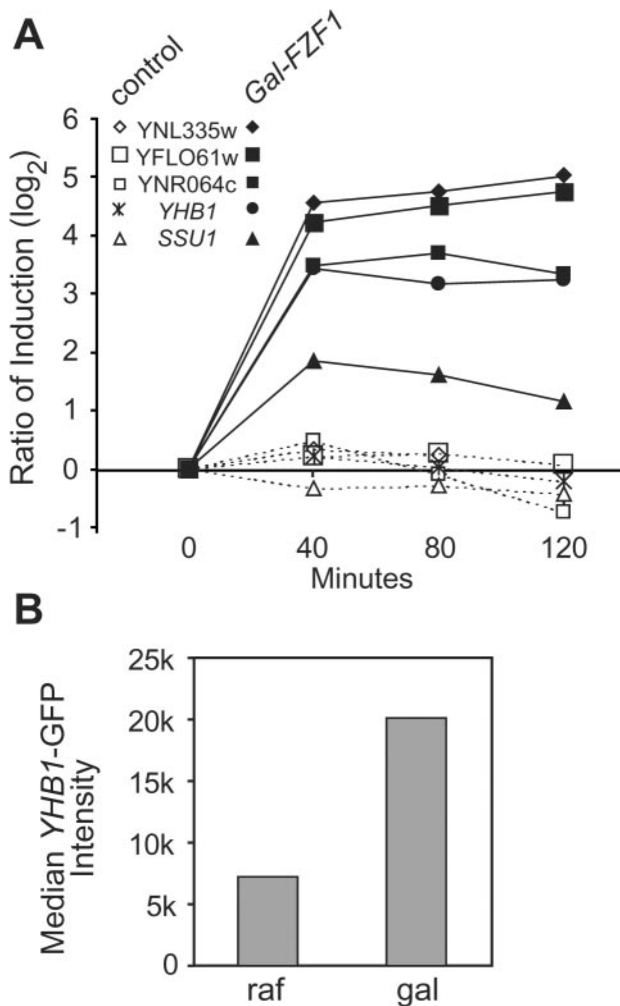

Figure 3. Overexpression of *FZF1* results in induction of the RNI-responsive gene cluster. (A) Transcript levels of *FZF1*-dependent RNI-inducible transcripts after overexpression of either Fzf1p or lacZ (control). For the Fzf1p time course, transcript levels are represented by filled in symbols and 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 intensity measured by flow cytometry after induction of Fzf1p. The *y*-axis indicates fluorescent (FITC) median intensity and the *x*-axis indicates status of Fzf1p induction.

unique to the *SSU1* promoter. Multiple alignment of the *YHB1* upstream sequences identified extensive conservation (our unpublished data), 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 base pairs upstream of each member of the *FZF1*-dependent gene set. A consensus motif (5′ YGSMNMM**C-TATCA**Y**TT**YY) was found in each promoter sequence that also coincided with a second island of sequence conservation (CS2) in the *SSU1* promoter (Figure 4, A and B). 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 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 *FZF1*-mediated response to DPTA NONOate, we constructed a reporter strain corresponding to the −399 to −45 region of the *SSU1* promoter (AS102). Although this construct still responded to DPTA NONOate treatment in an *FZF1*-dependent manner, the response was fourfold lower than that observed with the full-length *SSU1* reporter (Figure 5B). A further deletion that encompassed both the CS1 and CS2 region (AS103) abolished the ability of the reporter to respond to DPTA NONOate treatment, whereas 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). 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- and 8.8-fold, respectively) to DPTA NONOate treatment (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 h later relative to untreated controls. Although growth of wild-type yeast in YPD media were 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 (our unpublished data).

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 seems reversed in SCD

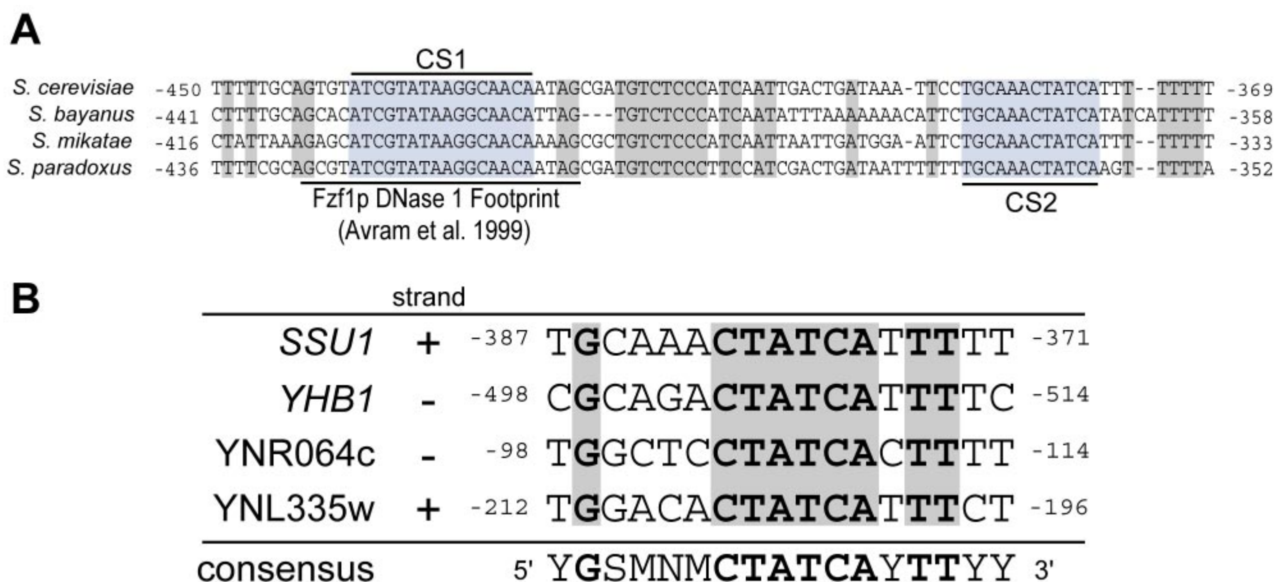

Figure 4. Comparison of upstream sequences reveals conserved motifs. (A) Multiple alignment of the promoter region of the *SSU1* gene for closely conserved *Saccharomyces* species. Conserved sequences in the four species are shaded. (B) Sequence motif found in promoter regions of *FZF1*-dependent RNI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

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 range of 0.5 to 1.5 mM DPTA NONOate, whereas deletion of *FZF1* in the *LacZ* control strain resulted in hypersensitivity to DPTA NONOate (Figure 6C).

DISCUSSION

S. cerevisiae uses 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 $^{\bullet}$NO-mediated nitrosative stress. Although treatment with DPTA NONOate led to a general stress response common to other perturbation experiments, DPTA NONOate and $^{\bullet}$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 $^{\bullet}$NO with oxygen to create nitrate, limiting the exposure of the cell to $^{\bullet}$NO and thus confers a growth advantage after $^{\bullet}$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 $^{\bullet}$NO exposure. This discrepancy might be due to insufficient $^{\bullet}$NO availability, cytotoxic $^{\bullet}$NO effects, media effects, or differences in strain background. In addition, we found that the deletion of the *YHB1* gene seems to lead to an increase in the general stress response after DPTA NONOate treatment yet does not affect the induction of *SSU1* and the other ORFs in the RNI-responsive gene cluster.

Ssu1p 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 after nitrosative stress. Furthermore, the presence of the *SSU1* gene conferred a growth advantage after exposure to DPTA NONOate in SCD media. We speculate that in addition to transporting sulfite, *SSU1* also may transport $^{\bullet}$NO metabolites out of the cell.

The predicted ORFs YNR064c and YNL335w/YFL061c also were induced after nitrosative stress. The presence of these genes did not confer significant growth advantages (our unpublished data). The function of these genes with regard to nitrosative stress remains unclear. It is possible that these ORFs may have roles in $^{\bullet}$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 after 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. Deletion of *FZF1* 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 after 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 posttranslational modification leads to a differential subcellular localization. The situation could be analogous in that the overexpression

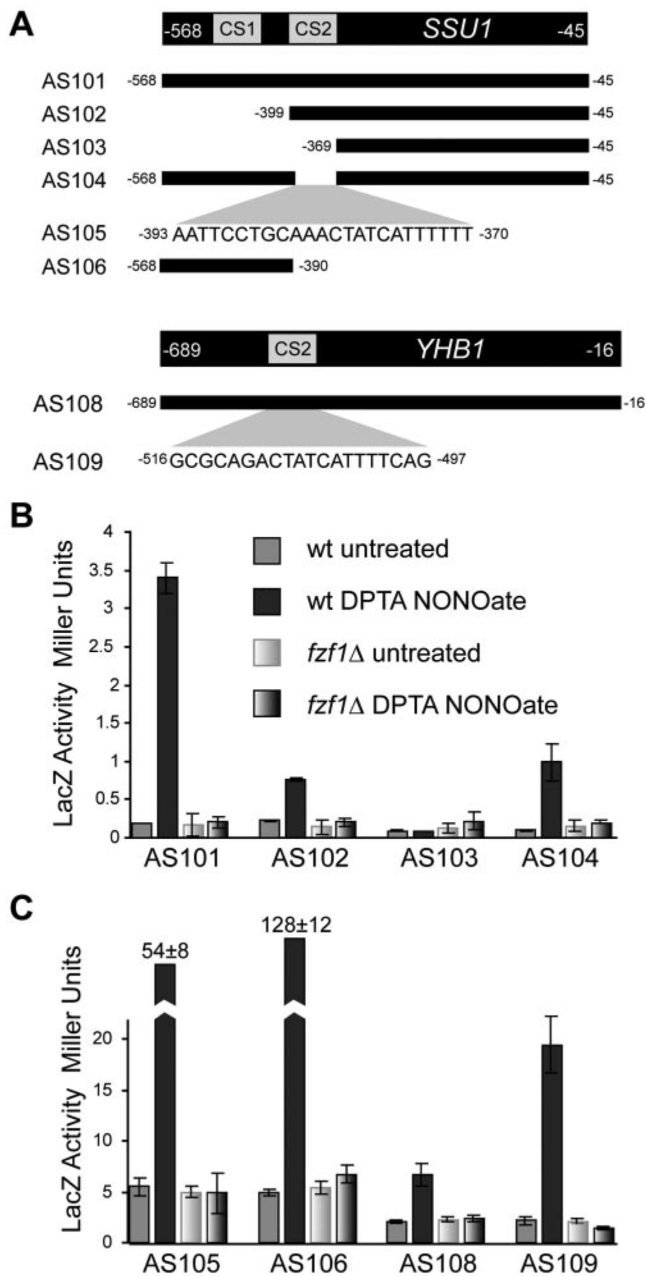

Figure 5. Two separate conserved sequence motifs are sufficient for DPTA NONOate-dependent reporter activation. (A) Representation of *SSU1* and *YHB1* promoter constructs cloned into the minimal *CYC* promoter driving lacZ expression. (B and C) lacZ activity in Miller units in the presence or absence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

of Fzf1p may stoichiometrically outcompete a regulatory mechanism that would ultimately result in transcriptional activation.

Although it may be that additional components are required for sensing $^{\bullet}$NO or $^{\bullet}$NO-derived metabolites before activation of Fzf1p, it also may 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 after treatment with the thiol oxidant

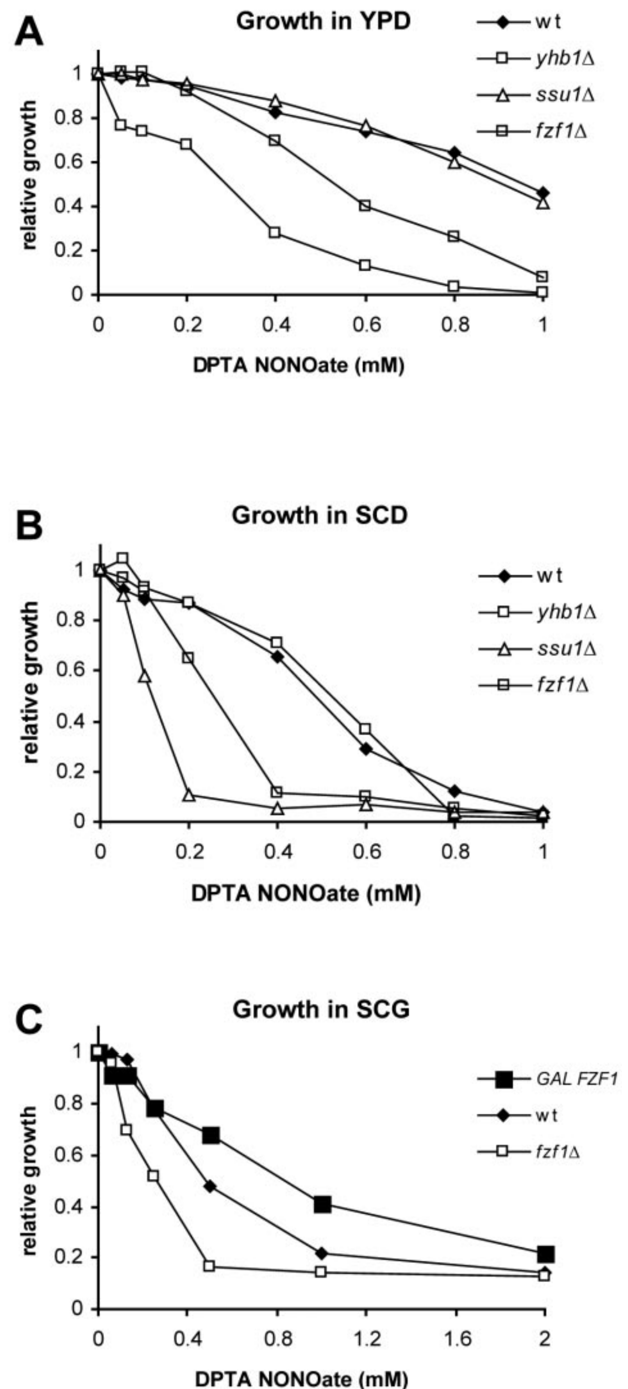

Figure 6. Genotype-dependent sensitivity and resistance after DPTA NONOate treatment. (A) Hypersensitivity to growth inhibition in response to DPTA NONOate (0–1 mM) in YPD media for *yhb1*Δ, *fzf1*Δ and *ssu1*Δ strains compared with wild type. The *x*-axis represents drug concentration and the *y*-axis represents growth relative to isogenic untreated culture 12 h after 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 after treatment with DPTA NONOate (0–2 mM) in SCG media for an Fzf1p-overexpressing strain compared with a wild-type lacZ overexpression strain and an *fzf1*Δ lacZ overexpression. The *x*-axis represents drug concentration and the *y*-axis represents growth relative to isogenic untreated culture 12 h after the start of the experiment.

diamide, heat shock, or in other oxidative stresses (Gasch *et al.*, 2000).

Previous studies have shown that after treatment with methyl methane sulfonate (MMS), *SSU1*, YNR064c, and YNL335w/YFL061c are induced raising the possibility that DNA damage also may activate the *FZF1*-dependent gene set. However, *YHB1* mRNA was not significantly induced after 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 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. Because we have not shown a direct biochemical interaction between CS2 and Fzf1p, it is formally 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 seems 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 compared with 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 use 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 (Bethann 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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